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Conversion of the *Salmonella* Phase 1 Flagellin Gene *fliC* to the Phase 2 Gene *fljB* on the *Escherichia coli* K-12 Chromosome

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The *Escherichia coli-Salmonella typhimurium-Salmonella abortus-equi* hybrid strain EJ1420 has the two *Salmonella* flagellin genes *fliC* (antigenic determinant *i*) and *fljB* (determinant *e,n,x*) at the same loci as in the *Salmonella* strains and constitutively expresses the *fliC* gene because of mutations in the genes mediating phase variation. Selection for motility in semisolid medium containing anti-i flagellum serum yielded 11 motile mutants, which had the active *fliC*(*e,n,x*) and silent *fljB*(*e,n,x*) genes. Genetic analysis and Southern hybridization indicated that they had mutations only in the *fliC* gene, not in the *fljB* gene or the control elements for phase variation. Nucleotide sequence analysis of the *fliC*(*e,n,x*) genes from four representative mutants showed that the minimum 38% (565 bp) and maximum 68% (1,013 bp) sequences of the *fliC*(*i*) gene are replaced with the corresponding sequences of the *fljB*(*e,n,x*) gene. One of the conversion endpoints between the two genes lies somewhere in the 204-bp homologous sequence in the 5′ constant region, and the other lies in the short homologous sequence of 6, 8, or 38 bp in the 3′ constant region. The conversions include the whole central variable region of the *fljB* gene, resulting in *fliC*(*e,n,x*) genes with the same number of nucleotides (1,503 bp) as the *fljB* gene. We discuss the mechanisms for gene conversion between the two genes and also some intriguing aspects of flagellar antigenic specificities in various *Salmonella* serovars from the viewpoint of gene conversion.

One of the specific aspects of the genus *Salmonella* is the presence of the two flagellin genes *fliC* and *fljB*, which are separately located on the chromosome (51), alternatively expressed by the phase variation system (26, 74), and determine the antigenic specificities of phase 1 and phase 2 flagella, respectively. A number of flagellar antigens, including complex ones, have been found in both phases, and combinations of these antigens have been used to assign a large number of *Salmonella* serovars or serotypes (more than 2,000 [16, 57]) to different groups, each specified by a combination of somatic lipopolysaccharide antigens (29). Antigenic polymorphism of flagella seems to have been generated by the accumulation of ordinary genetic events in flagellin genes, such as point mutations (28), deletions (8, 16, 46), and insertions (8), in addition to an evolutionarily conceivable big event in which the genetic material was laterally transferred, resulting in interspecific recombination between flagellin genes (56). Besides these events, interaction between the *fliC* and *fljB* genes is considered one of the causes generating antigenic diversity among *Salmonella* serovars, because this genus is characterized by the two flagellin genes. We report here experimental evidence that gene conversion, the nonreciprocal exchange of genetic information, occurs between the *fliC* and *fljB* genes, which lie at the same loci in the *Escherichia coli* chromosome as in *Salmonella typhimurium* (9).

Flagellin genes are useful as a model system for studying gene differentiation, because they are so elastic to various mutations that their mutation products are functional unless the 5′ and 3′ coding frames, which encode the regions important for secretion and polymerization of flagellin (25, 66) are changed (35). In the *E. coli fliC* gene ($fliC_E$) and the *Salmonella fliC* ($fliC_S$) and *fljB* genes, the 5′ and 3′ constant regions can be regarded as sequences of 519 bp (encoding the N-terminal 173 amino acid residues) and 270 bp (C-terminal 90 residues), respectively, and between them is a central variable region (27, 35, 57, 68). Different kinds of flagellin mutants can be isolated from *E. coli-S. typhimurium* hybrids, depending on the selection methods. In the gene conversion mutants reported here, the whole central variable region of the *fliC* gene has been replaced with the corresponding region of the *fljB* gene, resulting in an *fliC* gene with a determinant for the *e,n,x* antigen specific to the *fljB* product.

Gene conversion has been reported for various organisms from viruses (64) to primates (33) since the classical studies of meiotic segregation in *Saccharomyces* (38) and *Neurospora* (45) species. For *E. coli*, most experiments concerning gene conversion have been done with plasmids and phages with a view to clarifying the relationship between gene conversion and homologous recombination mechanisms (31, 48, 61, 71, 72) and in relation to mismatch repair mechanisms (2, 7, 14, 15). These experiments have been performed on the basis of various recombination models (24, 44, 60) under various backgrounds of homologous recombination systems and revealed the systems that promote gene conversion (14, 48, 70, 71). On the other hand, very few conversion events have been reported for chromosomal markers; these have been reported, so far as we know, only for *rrn* operons (20, 21), which are one of a few multigene families in *E. coli*. In eukaryotes, a number of multigene families are known, and gene conversion appears to play an important role in maintaining and evolving gene families (22, 30, 69). Conversely, the rare occurrence of gene conversion in *E. coli* K-12 seems to be ascribable to a few gene families in this strain; this idea might be endorsed by experimental evidence that the two

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flagellin genes *fliC* and *fliB*, an artificial gene family in *E. coli*, indeed undergo gene conversion in *E. coli*.

We have examined four gene conversion mutants by cloning and sequencing their flagellin genes and showing that their conversion endpoints lie in the 5′ and 3′ constant regions. One of the endpoints lies in a 6-bp homologous sequence, which seems to be too short to be a substrate for homologous recombination systems. We discuss some interesting features concerning the combination of phase 1 and phase 2 flagellar antigens in various *Salmonella* serovars from the viewpoint of gene conversion.

MATERIALS AND METHODS

Strains, phages, and plasmids. Strain EJ1420 [*fliC*$_S$ H(−) *hin fljBA* (OFF) Δ*pin*], the parent of the gene conversion mutants, is an *E. coli* K-12 derivative which has received the phase 1 flagellin gene *fliC* (antigenic determinant *i*) from *S. typhimurium* LT2 (via strain EJ31 [12]) and the phase 2 flagellin gene *fljB* (antigenic determinant *e,n,x*) from *Salmonella abortus-equi* (via strain EJ44 [12]). The invertible H segment upstream of the *fljBA* operon contains a promoter for the operon and the invertase gene *hin* (75). In this strain, however, the promoter is nonfunctional because the H segment is fixed in the minus orientation (74) owing to mutations in the *hin* gene and another invertase gene, *pin* (10, 49), on element e14. As the result, this strain constitutively expresses i flagellin. The *fliC*$_S$ gene is homologous to *fliC*$_E$ of *E. coli* K-12 (12, 39), and the *fljBA* operon is integrated between the *tyrA* and *nalB* genes of *E. coli* (9) at the locus corresponding to that in *S. typhimurium* (51). Strain EJ1502 (=YK5016), used for genetic analysis of gene conversions, is a *fliC*$_E$::Tn*10* derivative of W3110 (11). EJ2081 (Δ*fliC*$_E$ *purB51* Δ*pin recA56*), a host for recombinant plasmids, was constructed from F′-cured JM101 (73) by successive transduction with *fliC*$_E$::Tn*10* (11), *purB51*-Δ*pin-zcg-2*::Tn*10* (10), and *recA56-srlA*::Tn*10* (6), with Tn*10* excision (40) after each transduction. Strain JM109 (73) was also used as a host for recombinant plasmids.

Phage P1 *vir* was used for transduction and for causing only one H segment inversion by the *cin* gene (34). Inversion was detected in one of the daughter cells from a cell infected with a *vir* phage that was preparing to divide. M13KO7 (65) was used as a helper for preparing single-stranded DNA from pUC118 and pUC119 (65).

Plasmids pAT101 (63) and pSI730 (62), derived from pBR322 (4) and pACYC177 (5), respectively, carry the *cin* gene from phage P1. pHI101 and pTY102 (18) carry the *fliC* and *fljB* gene, respectively, of strain EJ1420. pHI101 was constructed and examined previously, but its physical map and sequence have not yet been published. In brief, this plasmid was constructed by inserting *Hin*dIII fragments of DNA from EJ1420 into pBR322 and isolated as a recombinant that conferred motility on strain EJ2081. pHI101 carries a 4.5-kb insert containing the *fliC*(*i*) gene (see Fig. 2A). pTY102 (see Fig. 2B) is a pBR322 derivative that carries a 6.75-kb *Sal*I fragment containing the *fljBA* operon of *S. abortus-equi* origin, cloned from strain SL4266 (12), which is the *fljBA* parent of EJ1420. The nucleotide sequence of the *fljA* gene on pTY102 has been reported previously (18). pTY109 is a pTY102 derivative with the inverted H(−) segment and thus carries the *fljBA* operon with the same organization as in EJ1420. pHIF101 is a derivative of the mini-F plasmid pTN117 (47) and carries the same *fliC*(*i*) fragment as pHI101. Plasmid pSK131 (18) is an *fljA*-carrying derivative of pUC118 and has a 0.8-kb *Pst*I-*Hin*dIII fragment subcloned from pTY102.

Media and antiflagellum serum. TLY broth, nutrient agar, nutrient semisolid agar (NSS), and buffered saline have been described before (10). Chloramphenicol (Sigma Chemical Co.), kanamycin (Meiji Seika Co.), tetracycline (Sigma), and ampicillin (Sigma) were used at final concentrations of 12.5, 50, 25, and 50 mg/liter, respectively. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) plates were prepared as described before (32). Anti-i and anti-e,n,x sera were prepared in this laboratory by injecting *S. typhimurium* monophasic strains (derivatives of SL4013 [12]) into rabbits and used for *E. coli* cells producing *Salmonella* flagellar antigens at concentrations of 0.03 and 0.15%, respectively, without absorption by *Salmonella* somatic antigens.

Flagellin isolation and SDS-PAGE. Flagellin was prepared essentially by the same methods described before (1), and the rapid isolation method (19) was also used. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was done by the standard methods (36) slightly modified by the use of a 4.75% acrylamide stacking gel and a 9% acrylamide resolving gel.

DNA manipulation. Chromosomal DNA was prepared by the method described before (3, 62). Isolation of plasmid DNA, the cloning procedure, and restriction analysis of cloned fragments were done by standard methods (42). Transformation was done by the $CaCl_2$ procedure (41). Restriction endonucleases, T4 DNA ligase, Klenow fragment, exonuclease III, and mung bean nuclease were purchased from Takara Shuzo Co. and used as recommended. Nucleotide sequences were determined by the dideoxy chain termination method (52) with a sequencing kit (Takara) and [^{35}S]dCTP (Amersham International; 1,000 Ci/mmol).

Cloning of mutant flagellin genes and Southern hybridization. To clone the *fliC*(*e,n,x*) genes, a ligation mixture of pBR322 and mutant genomic DNA, digested with *Hin*dIII and *Sal*I, was transformed into strain EJ2081, and ampicillin-resistant swarms (motile colonies) were isolated on NSS medium. For cloning the silent *fljB* gene from the *fliC*(*e,n,x*) GC3 mutant, colony hybridization (17) was used. A ligation mixture of pUC118 and genomic DNA, digested with *Hin*dIII and *Sal*I, was transformed into strain JM109 and plated on X-Gal plates containing ampicillin. Colonies were transferred to nylon membrane filters (Amersham Hybond-N) and lysed with denaturing solution (1.5 M NaCl in 0.5 M NaOH). The membranes were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) after neutralization and used for hybridization with probe A (see Fig.2 B). The restriction fragments used for probes were electroeluted from gels, denatured by heat, and labeled by the random-primed method (13) with digoxigenin-labeled dUTP with a nonradioactive DNA labeling kit (Boehringer Mannheim). Hybridization was done by standard methods (42, 59), and hybrid bands were detected by the digoxigenin–enzyme-linked immunosorbent assay (Dig-ELISA) method (13) with the kit above.

Sequencing strategy. For sequencing the *fliC*(*i*) gene of strain EJ1420, a 2.2-kb *Eco*RI-*Sac*II fragment of pHI101 (see Fig. 2A), blunted at both ends with Klenow fragment, was inserted into the *Hin*cII site of the pUC119 polylinker in both orientations. A series of deletion plasmids were made from these two plasmids by cleaving the *Sac*I and *Xba*I sites on the polylinker and digesting from the *Xba*I site with exonuclease III and mung bean nuclease. Nine to 10 deletions were used for sequencing. For sequencing the wild-type *fljBA* operon, plasmid pTY107, a derivative of pTY102, was

digested with appropriate restriction enzymes, and short fragments within a 3.5-kb *Pvu*II-*Hin*dIII fragment (see Fig. 2C) were subcloned into the polylinker of pUC118 or pUC119 and used for sequencing. More than 38 fragments were sequenced and integrated into a complete sequence by aid of a computer. The *fljB* gene of mutant GC3, carrying *fliC(e,n,x)*, was sequenced by using a series of deletion plasmids from a pUC119 derivative carrying a 2.2-kb *Stu*I-*Pvu*II fragment of pHB322 in the same way as described above. Mutant *fliC(e,n,x)* genes were sequenced only for the region containing a conversion endpoint by using plasmids carrying a *Pst*I or a *Hin*cII fragment (see Fig. 4) subcloned from each of four *fliC(e,n,x)*-carrying plasmids. Nucleotide and amino acid sequences were analyzed with DNASIS sequence analysis software (Hitachi Software Engineering Co.).

Nucleotide sequence accession number. The nucleotide sequence data for the *fliC(i)* gene and the *fljB(e,n,x)* gene including the H segment will appear in the DDJB, EMBL, and GenBank data bases under accession numbers D13689 and D13690, respectively. The sequence of the *fljA* gene has been registered under accession number D12510.

RESULTS

Isolation of gene conversion mutants. The *E. coli* K-12 strain EJ1420, carrying the *Salmonella fliC(i)* and *fljB(e,n,x)* genes, constitutively expresses i flagellin and is motile. When broth cultures from single colonies of this strain were streaked as lines on NSS medium containing anti-i flagellum serum and incubated for 2 or 3 days at 30°C, mutant swarms (motile colonies) which were able to spread normally and thus seemed to escape serum inhibition almost completely appeared at the margins of the inocula. They were tested for motility on NSS containing anti-i, anti-e,n,x, or both sera and classified by their flagellar antigen(s) into three groups. Mutants of the first group failed to spread in the presence of anti-e,n,x serum but spread normally in the presence of anti-i serum, i.e., they express only antigen e,n,x. Mutants of the second group showed the same behavior as those of the first after overnight incubation, but after 2 or 3 days, they formed small swarms in the presence of anti-e,n,x serum, which indicated that they had changed to express the i antigen constitutively, probably by an additional mutation. Mutants of this group were confirmed, by use of purified flagellins, to express a normal amount of antigen e,n,x and a small amount of i simultaneously. Mutants of the third group spread in the presence of both sera, suggesting that they have new flagellar antigens. Subsequent studies showed that some mutants in the second group have an IS*2* or IS*5* element(s) in the H segment or its upstream region, and those of the third group have deletions of various extents in a middle part of the *fliC* gene (8). Mutants of the first group were first designated *pvh* mutants, since they seemed to have some defect in the phase variation mechanism, but they are reported here as gene conversion (GC) mutants with the symbol *fliC(e,n,x)*.

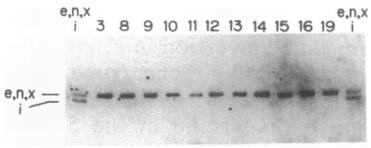

FIG. 1. SDS-PAGE of flagellins isolated from *fliC(e,n,x)* GC mutants. Lanes at the left and right ends show control flagellins e,n,x and i isolated from strain EJ1420 and its derivative expressing the *fljB(e,n,x)* gene. The GC mutant number is shown above each lane.

Eleven motile mutants, GC3 through GC19, were independently isolated from more than 200 single colonies. The frequency of mutations was estimated at roughly 10^{-9} per bacterium. All the mutants apparently produced flagella that carried only antigen e,n,x, as determined by agglutination tests with anti-e,n,x and anti-i sera and SDS-PAGE of purified flagellin (Fig. 1). The flagellins from all 11 mutants were indistinguishable in mobility from each other and from the control e,n,x flagellin. To test the ability of these mutants to undergo phase variation, they were transformed with plasmid pAT101 carrying the *cin* gene, which mediates H segment inversion, and tested for motility (Table 1). They did not swim in the presence of anti-e,n,x serum, suggesting that they had changed to express the *fljB* gene constitutively, because of unusual switching to phase 2 followed by a defect in the H segment, or that they had mutant *fliC* genes which are not expressed even in the phase 1 state, but these speculations were shown to be incorrect, as described below.

Genetic analysis of GC mutants. To examine whether the *fliC* gene is stably repressed by FljA repressor or inactivated by its own mutation, the *fliC* gene of the GC3 mutant was transduced into the $fliC_E$::Tn*10* strain EJ1502, and motile transductants were selected. Transductants did appear, and they were all Tet^S (tetracycline sensitive) and expressed unexpected e,n,x flagellin. Reciprocal transduction from

TABLE 1. Motility of *fliC(e,n,x)* GC mutants and mutant GC3 derivatives in semisolid medium containing antiflagellum serum[a]

Strain(s)	Flagellin genes	Plasmid	Motility[b] with antiserum against flagellum:			
			i	e,n,x	i + e,n,x	No serum
GC mutants	*fliC(e,n,x) fljB(e,n,x)*(Off)	pAT101 (*cin*)	SW	CP	CP	SW
EJ1420	*fliC(i) fljB(e,n,x)*(Off)	pAT101 (*cin*)	SW	SW	CP	SW
EJ2521	$fliC_E$::Tn*10 fljB(e,n,x)*(Off)	None	NT	NT	NT	CP
Motile EJ2521	$fliC_E$::Tn*10 fljB(e,n,x)*(On)	None	SW	CP	CP	SW
		pHIF101[*fliC(i)*]	SW	CP	CP	SW
EJ2522	*fliC(e,n,x)*	None or pUC118	SW	CP	CP	SW
		pHIF101[*fliC(i)*]	CP	CP	CP	SW
		pSK131 (*fljA*)	NT	NT	NT	CP

[a] Eleven *fliC(e,n,x)* GC mutants (GC 3, 8, 10, 11, 12, 13, 14, 15, 16, and 19) showed the same phenotype. Strain EJ2521 is a GC3 derivative with gene $fliC_E$::Tn*10* transduced from EJ1502, and a motile derivative of EJ1521 in which the *fljB* gene is expressed was obtained after infection with P1 *vir*. Strain EJ2522 is a derivative of EJ1502 with gene $fliC_E$::Tn*10* replaced by *fliC(e,n,x)* from the GC3 mutant.

[b] SW, swarm; CP, compact growth; NT, not tested.

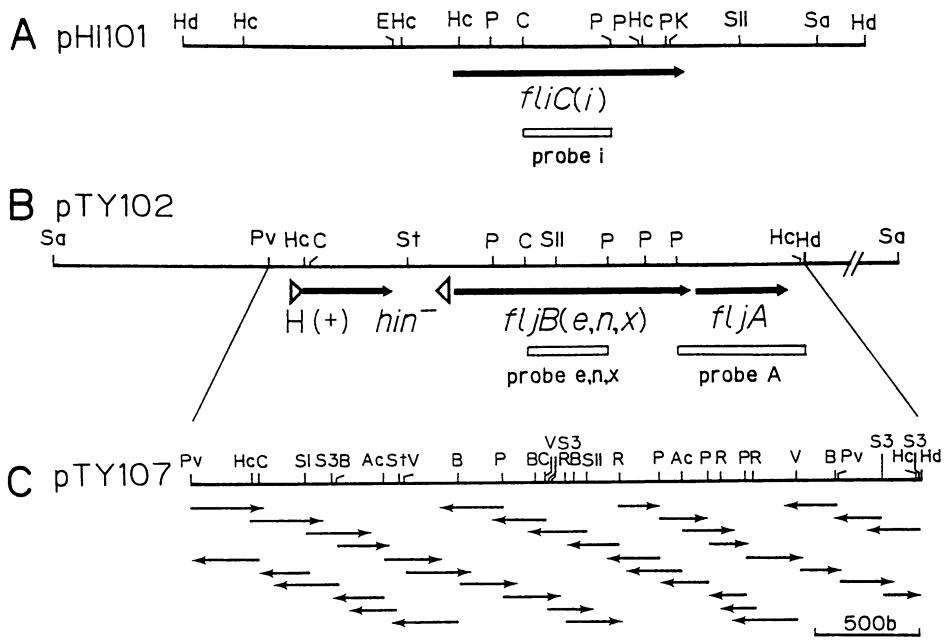

FIG. 2. Restriction maps of the fragments containing the flagellin genes from strain EJ1420. (A) 4.5-kb *Hin*dIII fragment carrying the *fliC(i)* gene (pHI101). (B) 6.75-kb *Sal*I fragment carrying the *fljBA* operon in the H(+) orientation (pTY102). Thick solid arrows indicate ORFs. The H segment is flanked by two open triangles. Open bars under the ORFs indicate probes used for Southern hybridization. (C) 3.4-kb *Pvu*II-*Hin*dIII fragment (pTY107) used for sequencing. The direction (5′ to 3′) and extent of sequencing are shown by thin arrows. Restriction cleavage sites: B, *Ban*I; C, *Cla*I; E, *Eco*RI; Hc, *Hin*cII; Hd, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; R, *Rsa*I; SI, *Sac*I; SII, *Sac*II; S3, *Sau*3AI; Sa, *Sal*I; St, *Stu*I; V, *Eco*RV.

strain EJ1502 to the GC3 mutant, with selection for Tet^r, yielded nonmotile transductants. These results indicate that it is not *fljB* but the *fliC* gene which is expressed in the GC3 mutant and that the *fliC* gene encodes the e,n,x flagellin. To test the antigenic specificity encoded on the *fljB* gene, one of the nonmotile Tet^r transductants of the GC3 mutant (EJ2521) was infected with P1 *vir* to invert the H segment and selected for motility. Motile bacteria were still Tet^r and expressed the e,n,x antigen. The GC3 mutant was found to have the gene constituent *fliC(e,n,x) fljB(e,n,x)*, in which the *fliC* gene is expressed and the invertible H segment upstream of the *fljB* gene remains intact.

To examine whether the repressor gene *fljA* is still linked to the *fljB* gene, an *fljB*-expressing derivative of strain EJ2521, isolated above, was transformed with the monocopy plasmid pHIF101, carrying the $fliC_S(i)$ gene. The transformants were motile in the presence of anti-i serum but not in the presence of anti-e,n,x serum, indicating that expression of the *fliC* gene is repressed by the product of *fljA* linked to the *fljB* gene; whereas one of the motile Tet^s *fliC(e,n,x)* transductants (EJ2522) from strain EJ1502, transformed with the same plasmid, was nonmotile in the presence of either anti-i or anti-e,n,x serum, showing that both genes are expressed (Table 1). The *fljA* gene is linked not to the *fliC* gene but to *fljB* and is functional, probably forming the *fljBA* operon, as in the parent EJ1420.

The *fliC(e,n,x)* gene was examined for the presence of its operator for FljA repressor. The motile *fliC(e,n,x)* strain EJ2522 transformed with plasmid pSK131, carrying the *fljA* gene, became nonmotile because of *fliC* repression, while the same strain with pUC118 remained motile (Table 1). An intact operator region is linked to the *fliC(e,n,x)* gene, as in the parent. These results indicate that in the GC3 mutant, the regulatory elements for phase variation lie in the same positions as in the parent, and only the antigenic determinant of the *fliC(i)* gene changes to that of the *fljB* gene. The remaining 10 GC mutants were also examined by replacing their *fliC* genes with $fliC_E$::Tn*10* by the procedure above and found to become nonmotile. These nonmotile derivatives were able to express the e,n,x antigen upon infection with P1 *vir*. Thus, the 10 GC mutants have the active *fliC(e,n,x)* and silent *fljB(e,n,x)* genes and are likely to have the same kind of mutation as the GC3 mutant.

Southern hybridization analysis of the flagellin genes of GC mutants. Genetic analysis showed that in the GC mutants, the *fliC* gene has lost its intrinsic determinant for antigenic specificity and instead acquired a new determinant whose product is immunologically indistinguishable from that of the *fljB* gene. To test this finding at the DNA level, Southern hybridization was performed with probes i and e,n,x (Fig. 2). Since a central region of the flagellin polypeptide is highly variable among serovars (27, 57, 68), a DNA fragment corresponding to this region can be used as a flagellin gene-specific probe. When a *Hin*dIII digest of chromosomal DNA from strain EJ1420 was examined, each probe hybridized to one band, whereas when DNAs from the 11 GC mutants were examined, probe i did not hybridize to any band; instead, probe e,n,x hybridized to two bands (Fig. 3), which corresponded in size to the fragments containing the *fliC* or the *fljB* gene of the parent. These results suggest that the 11 GC mutants have not undergone a large defect such as gene rearrangement but mutations only in the *fliC* gene, which is consistent with the genetic analysis mentioned above.

Cloning of the *fliC* and *fljB* genes from GC mutants and comparison with those from the parent. The *fliC* gene of the GC3 mutant was cloned into pBR322 as a 4.2-kb *Hin*dIII-*Sal*I fragment to yield pHB311, which conferred e,n,x fla-

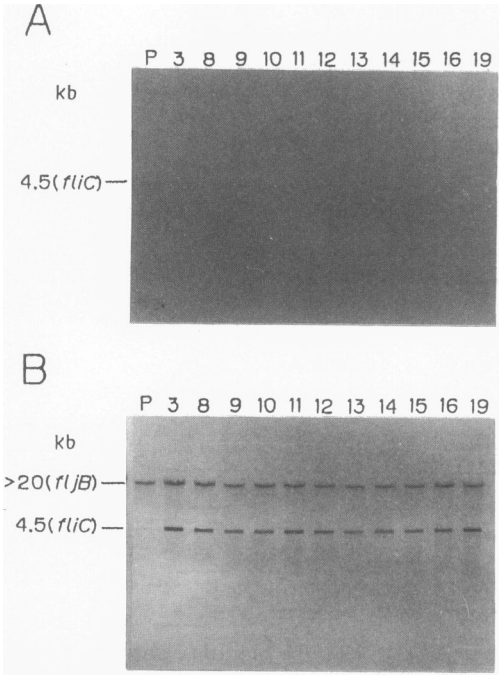

FIG. 3. Southern hybridization analysis of *fliC(e,n,x)* GC mutants. Genomic DNA was digested with *Hin*dIII and fractionated by electrophoresis on a 0.7% agarose gel. (A) Hybridization with probe i. (B) Hybridization with probe *e,n,x*. The probes used are shown in Fig. 2. The sizes of the hybrid bands are shown (in kilobases) at the left, and the GC mutant number is shown above each lane; P, parental strain EJ1420.

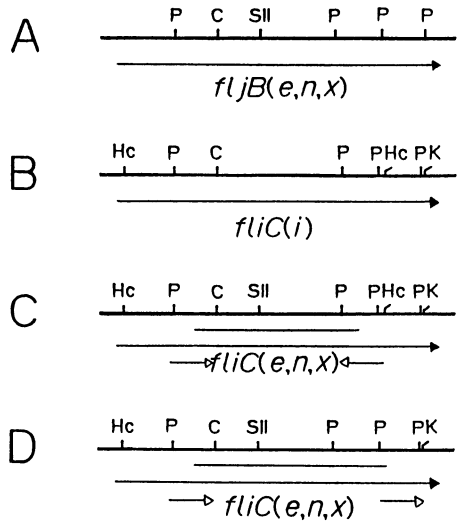

FIG. 4. Restriction maps of the wild-type and mutant flagellin genes. (A) *fljB(e,n,x)* gene from strain EJ1420. (B) *fliC(i)* gene from EJ1420. (C) *fliC(e,n,x)* genes from mutants GC3 and GC10. (D) *fliC(e,n,x)* genes from GC8 and GC9. Arrows with a solid arrowhead indicate ORFs of flagellin genes. The line under maps C and D indicates the region replaced by the *fljB(e,n,x)* gene (see Fig. 5). Arrows with an open arrowhead show the extent and direction (5′ to 3′) of sequencing. Abbreviations of restriction cleavage sites are the same as in Fig. 2.

gella on the nonmotile $\Delta fliC_E$ strain EJ2081 when transformed. A *Hin*dIII-*Sal*I fragment containing the silent *fljB* gene of the GC3 mutant was cloned into pUC118 to yield pHB321, which was isolated by colony hybridization with probe A (Fig. 2), which hybridizes only to the *fljBA* fragment and has a 4.8-kb insert. To evoke the silent *fljB* gene, plasmids pHB321 and pSI730 (*cin*) were cotransformed into strain EJ2081, and pHB321 was reisolated from a motile transformant. The pHB321 derivative that conferred motility on EJ2081 was designated pHB322 and expressed e,n,x flagellin.

For comparison the *fliC* and *fljB* genes from the parent EJ1420, which had previously been cloned and designated pHI101 and pTY102, respectively, were examined for restriction cleavage sites (Fig. 2). pTY102 carries the whole *fljBA* operon, including the H segment. The *fliC(e,n,x)* and *fljB* genes on plasmids pHB311 and pHB322 were then mapped and compared with those of the parent (Fig. 4). pHB322 also carried the whole *fljBA* operon, and its map was exactly the same as that of pTY102, as expected (Fig. 2B). For the *fliC(e,n,x)* gene on pHB311, a new *Sac*II site was found in the middle of the map, which is not present in the wild-type *fliC(i)* gene but is present in the *fljB(e,n,x)* gene. The *Cla*I and *Pst*I sites that flank the *Sac*II site mapped at the same positions as in the *fliC(i)* gene, suggesting that *fliC(e,n,x)* in the GC3 mutant is replaced by the *fljB* gene for the region flanked by the *Cla*I and *Pst*I sites.

The *fliC* genes of GC mutants 8, 9, and 10 were also cloned to yield plasmids pHB811, pHB911, and pHB1011, respectively. They were confirmed to encode e,n,x flagellin by motility testing of strains transformed with each plasmid. The fragments containing the *fliC(e,n,x)* gene were mapped and compared in the same way as above. The *fliC(e,n,x)* gene of pHB1011 showed the same restriction map as that of pHB311, and those of pHB811 and pHB911 had lost a *Hin*cII site on the 3′ side and instead gained the *Sac*II site in the middle, suggesting that in these two mutants, the region between the *Cla*I and *Hin*cII sites had undergone replacement by the *fljB* gene. These results indicate that the four mutants examined have *fliC(e,n,x)* genes in which the central variable region specific to *fliC(i)* has changed, to different extents, into the corresponding region of the *fljB(e,n,x)* gene and *fljB* itself has not undergone any change. Therefore, we can refer to these mutations as gene conversions.

Nucleotide sequences of the *fliC(e,n,x)* genes. Prior to sequence analysis of the *fliC(e,n,x)* genes, the sequences of the wild-type *fliC* and *fljB* genes from the parent EJ1420 were determined. The sequence of the *fliC(i)* gene from *S. typhimurium* has been reported previously by Joys (27) but did not agree with the sequence for the central region revealed by Smith and Selander (57). Our sequence was identical to that reported by Smith and Selander, not by Joys, in the central region and also differed from that of Joys in the constant regions at 5 nucleotides in the 5′ sequence and 2 nucleotides in the 3′ sequence (Fig. 5).

The entire *fljBA* operon, including the H segment, was sequenced with pTY107 by the strategy shown in Fig. 2C. Almost 3,000 nucleotides were sequenced, and they included, as expected, the three open reading frames (ORFs) for Hin invertase (190 amino acids), FljA repressor (179 amino acids), and e,n,x flagellin. The Hin invertase sequence was different in four amino acids from that reported for *S. abony* (54). The *fljB* ORF consists of 1,503 bp, encoding 501 amino acid residues (51.9 kDa), part of which is shown in Fig. 5. A σ^F promoter sequence (19, 23) for the *fljBA* operon lies in the H segment, and the rho-independent intercistronic

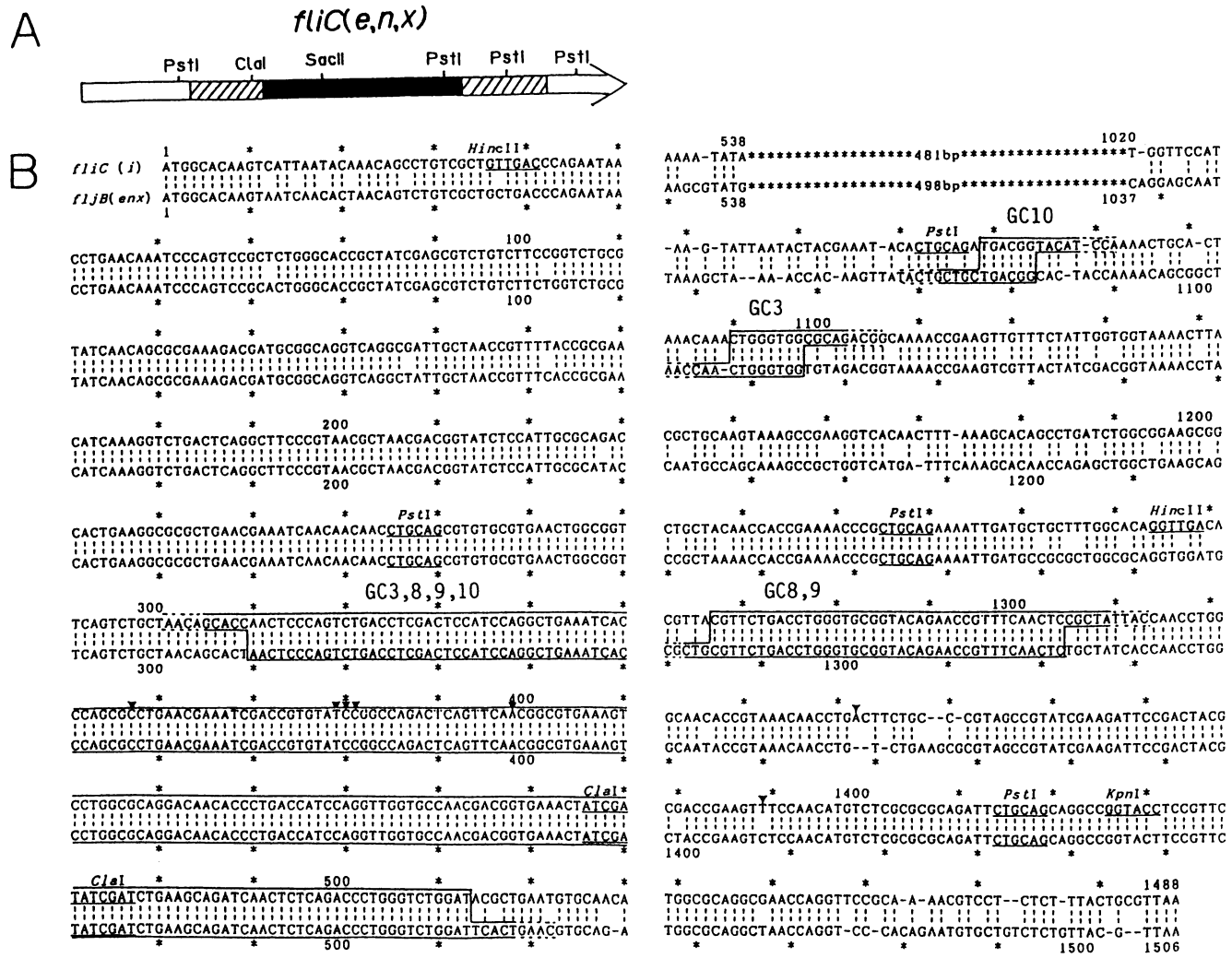

FIG. 5. Nucleotide sequences of the 5′ and 3′ constant regions of the wild-type *fliC*(*i*) and *fljB*(*e,n,x*) genes from strain EJ1420 and the conversion endpoints in *fliC*(*e,n,x*) from four GC mutants (GC 3, 8, 9, and 10). (A) Diagrammatic representation of the *fliC*(*e,n,x*) gene. The open regions at both ends are from the original *fliC*(*i*) gene, the central solid region is from *fljB*(*e,n,x*), and the hatched regions contain conversion endpoints. The arrow indicates transcriptional direction. (B) Sequences of the 5′ and 3′ constant regions of the *fliC*(*i*) (top) and *fljB*(*e,n,x*) (bottom) genes and the regions (boxed) contained conversion endpoints. The sequences are aligned for maximum homology. Numbering of the sequences starts with the initiation codon and ends at the termination codon. A solid triangle (▼) above the *fliC*(*i*) sequence indicates a nucleotide different from that reported previously (27). The sequences of the central variable regions of both genes have been deposited in nucleotide data bases (see Materials and Methods).

terminator (18, 50) lies between the *fljB* and *fljA* genes (18). The 5′ coding region of *fljB* is identical to the sequence reported for the *fljB*(*e,n,x*) gene from *S. abony* (73). The amino acid component deduced from the nucleotide sequence is similar to that of the e,n,x flagellin of *S. abortus-equi* SL23 (43). The *fljB* ORF of the GC3 mutant was also sequenced by using plasmid pHB322 to make sure that it is identical to the wild-type *fljB* gene; both sequences were exactly alike.

Comparison of the *fliC*(*i*) and *fljB*(*e,n,x*) sequences showed that the 5′ sequences are very much alike at nucleotide positions 1 to 513, and the 3′ sequence of the *fliC* gene from positions 1209 to 1488 is also very similar to the sequence of the *fljB* gene from positions 1227 to 1506 (Fig. 5); hence, these two sequences code for the constant regions of a flagellin molecule. The *fliC*(*e,n,x*) genes from four GC mutants were sequenced only for the regions that contain boundaries between the constant and variable regions, since the boundary regions are also likely to encompass switching points between the two genes (Fig. 4). The sequence of the mutant genes enables us to infer the sites where gene conversion starts and stops by comparison with those of the wild-type *fliC*(*i*) and *fljB*(*e,n,x*) genes. For the four mutants, the 5′ switching points for conversions were inferred to lie in the constant region between positions 310 and 513. Since the sequence of this region is identical in the two genes, switching points were not specified into narrower regions. The 3′ switching points of GC3 and GC10, however, were mapped within 8-bp and 6-bp sequences, respectively, and those of GC8 and GC9 were in the same sequence of 38 bp in the constant region. There is no common specificity in the sequences containing the 3′ switching points.

In conclusion, the four *fliC*(*e,n,x*) genes have switching points in the 5′ and 3′ constant regions and have undergone

conversion to the following lengths: GC3, 596 (minimum) to 806 (maximum) nucleotides; GC8 and GC9, 773 to 1,013 nucleotides; and GC10, 565 to 773 nucleotides. These lengths correspond to 38 to 68% of the *fliC(i)* sequence (1,485 bp). These genes, however, all consist of a 1,503-bp ORF, encoding the same number of amino acid residues as the wild-type *fljB(e,n,x)* gene. This leads to a rational explanation of the fact that the flagellins isolated from the 11 GC mutants showed the same mobility in SDS-PAGE gels as that from the *fljB*-expressing strain (Fig. 1).

DISCUSSION

Eleven *fliC(e,n,x)* GC mutants, isolated from the *Escherichia coli-Salmonella* hybrid strain EJ1420 carrying the $fliC_S(i)$ and *fljB(e,n,x)* genes, were found to carry *fliC(e,n,x)* *fljB(e,n,x)*. They were also found to have mutations only in the *fliC* gene, not in the *fljB* gene, and to retain the control elements for phase variation in the same positions as in the parent. Nucleotide sequence analysis of the *fliC(e,n,x)* gene from four GC mutants showed that a minimum 38% (565 bp) and maximum 68% (1,013 bp) of the *fliC(i)* gene is replaced with the *fljB(e,n,x)* gene. One of the switching points between the two sequences lies somewhere in the 204-bp homologous sequence in the 5′ constant region through the four mutations, and the other lies in the short homologous sequence of 6, 8, or 38 bp in the 3′ constant region (Fig. 5). The sequence of the *fljB* gene from the GC3 mutant was confirmed to be the same as that from the parent. Thus, it is concluded that these mutants have undergone gene conversion, defined as unidirectional transfer of genetic information from one allele to another.

Transfer of the *fljB* information to the *fliC* gene can be explained by at least two models. One is double reciprocal recombination between the two genes when they are present on separate sister chromatids immediately after replication. The resulting daughter cells would have the two gene constituents *fliC(e,n,x)* *fljB(e,n,x)* and *fliC(i)* *fljB(i)*, and only the former can be detected as an apparent gene conversion, since cells carrying the latter constituents cannot spread in selection medium containing anti-i flagellum serum. In this case, recombination would be mediated by homologous recombination pathways, including RecA (55), and one recombination event occurs within the 204-bp homologous sequence and another, in the shortest case, occurs in the 6-bp homologous sequence between the two genes. However, homologous sequences of more than 20 bp are required for the homologous recombination pathway to work efficiently (53, 67), so the second recombination event within short regions seems unlikely to occur by the RecA-dependent pathway.

The other model is intra- or interchromosomal nonreciprocal exchange between the two genes. Two mechanisms are invoked to interpret this model. One is template switching or copy choice during replication. The *fliC* (42 min) and *fljB* (57 min) genes are replicated and transcribed in the same direction (9, 51). Thus, the *fljB* gene precedes the *fliC* gene in replication, and an *fliC* leading strand replicating the 5′ constant region migrates to make pairing with the complementary strand of *fljB*, proceeds to replication toward the 3′ constant region, and then switches the template back to *fliC* itself. The other mechanism requires a double-strand break and repair system (60) and could occur after replication. This mechanism has been examined by model recombination systems with plasmids in the λ Red pathway (61) and the *E. coli* RecE pathway (31, 48). A double-strand break in the variable region of the *fliC* gene followed by digestion with exonuclease (mainly with 5′ to 3′ exonuclease) creates a large gap with 3′ single-stranded tails, each carrying part of the 5′ or the 3′ constant region, which form pairs with the complementary strands of the *fljB* gene. The gap is repaired, replicating the variable region of *fljB*, resulting in the conversion of *fliC(i)* into the *fliC(e,n,x)* gene. To disclose the true mechanism responsible for this conversion event, a model recombination system making use of a plasmid with the *fliC* and *fljB* genes will be required. Such a system would enable us to rearrange the location and direction of the two genes in different ways with respect to a replication origin and to examine the mechanisms of conversion and its frequency in different backgrounds of recombination pathways. Such experiments are in progress.

Experimental evidence for gene conversion between the *fliC* and *fljB* genes might explain some aspects of flagellar antigenic specificities in various *Salmonella* serovars (29). Some serovars share part of complex antigens between the two genes; e.g., *S. chester* (*e,h:e,n,x*) and *S. sandiego* ($e,h:e,n,z_{15}$), where antigens are given as phase 1 (*fliC*): phase 2 (*fljB*). Most duplicate antigens might be explained by the presence of a third gene encoding a common antigen, as in the case of antigen d in the triphasic serovar *S. rubislaw* (*d/r: d,e,n,x*), in which the d antigen is encoded on a plasmid (58). However, the possibility remains that a common determinant, such as e in the above serovars, is transferred from the *fljB* gene to *fliC* by gene conversion. In some serovars, antigen e,n,x, intrinsic to the *fljB* gene, is encoded on the *fliC* gene; e.g., *S. makumira* (*e,n,x:1,7*) and *S. lindrick* (*e,n,x: 1,5,7), in which the 1,7* and *1,5,7* antigens also belong to the *fljB* gene by nature. These variations are explicable by a genetic event, such as interspecific transduction or transformation between two different alleles (12), but gene conversion of the *fliC* gene into *fliC(e,n,x)* followed by alteration of the original *fljB(e,n,x)* by an additional genetic event(s) might be considered as a means of diversification.

Many monophasic strains expressing either the *fliC* or the *fljB* gene are found in the Kauffmann-White scheme and some articles: e.g., *S. paratyphi* A (*a*:—), *S. paratyphi B* java ph2 (*1,2*:—), and *S. roggeveld* (—: *1,7*). Most might have arisen by mutations that cause some defect in the phase variation mechanism or defects in the flagellin structural genes; in fact, *S. abortus-equi* (—: *e,n,x*) had mutations in the *hin* gene, as described above. However, the *1,2* determinant of *S. paratyphi B*, which is intrinsic to phase 2, is encoded on the *fliC* gene (37), suggesting that it undergoes a genetic event similar to that reported here. Thus, this experiment proposes another possibility that in some monophasic strains, both the *fliC* and *fljB* genes encode the same antigenic specificity as the result of gene conversion.

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