JOURNAL OF BACTERIOLOGY, July 1997, p. 4493–4500 Vol. 179, No. 14
0021-9193/97/$04.00+0

Cloning and Characterization of the Region III Flagellar Operons of the Four *Shigella* Subgroups: Genetic Defects That Cause Loss of Flagella of *Shigella boydii* and *Shigella sonnei*

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Received 6 January 1997/Accepted 17 May 1997

To detect genetic defects that might have caused loss of flagella in *Shigella boydii* and *Shigella sonnei*, the region III flagellar (*fli*) operons were cloned from certain strains and analyzed with reference to the restriction maps and genetic maps of *Escherichia coli fli* operons. *S. boydii* NCTC9733 (strain C5 in this paper) had the 988-bp internal deletion in the *fliF* gene that encodes a large substructural protein of the basal body. Two strains (C1 and C8) had deletions of the entire *fliF* operon, and the remaining three (C3, C4, and C9) differed in the size of the restriction fragments carrying the *fliF* and *fliL* operons. Loss of flagella in *S. boydii* appears to originate in some defect in the *fliF* operon. *S. sonnei* IID969 lacked the *fliD* gene and, in place of it, carried two IS*600* elements as inverted repeats. Genes downstream from *fliD* were not detected in the cloned fragment despite its large size but did appear elsewhere in the chromosome. The *fliD* gene encodes a cap protein of the flagellar filament, and its deletion results in overexpression of class 3 operons by the increased amount of FliA (σ^F) caused by the excess export of the anti-sigma factor FlgM. Three other strains also had the *fliD* deletion, and two of them had another deletion in the *fliF-fliG-fliH* region. The *fliD* deletion might be the primary cause of loss of flagella in *S. sonnei*. The lack of FliF or FliD in each subgroup is discussed in connection with the maintenance of virulence and bacterial growth. We also discuss the process of loss of flagella in relation to transposition of IS elements and alterations of the noncoding region, which were found to be common to at least three subgroups.

The genus *Shigella* is divided into four subgroups or species: *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei* (46). All are pathogens which cause diarrhea and dysentery in humans and certain other primates (37). Shigellae have been regarded as nonmotile organisms lacking flagella (15), and this characteristic has been used as one of the taxonomic and diagnostic criteria to distinguish shigellae from other enteric bacteria. However, it has been reported that some prototypical strains of the four subgroups and two fresh clinical isolates of *S. sonnei* are sparsely flagellate (19). We have not yet detected any motile strain among the four subgroups in the more than 20 laboratory strains we have examined so far; instead we have detected defective flagellar genes in some strains of these subgroups (4, 56). Therefore, we have been studying loss of motility by shigellae from the point of view that almost all shigellae are nonmotile because of a lack of flagellation.

Approximately 50 genes, composing at least 13 operons, are involved in the biogenesis and functioning of a flagellum of *Escherichia coli* and *Salmonella typhimurium* (33). These operons are clustered into three different regions on the chromosome. Region I operons mainly contain flagellar structural genes designated *flg*; region II contains a mixture of flagellar (*flh*) and motility-chemotaxis genes; and region III is subdivided into IIIa and IIIb. Genes in region III are designated *fli*. With respect to genetic regulation, these operons are grouped into three classes to construct a complex transcriptional hierarchy (28, 33, 52).

The region I operons from the four subgroups are almost identical and are very similar to those of *E. coli* (unpublished data). The *flhB* operon in region II is conserved well among the four subgroups (unpublished data). The *flhD* master operon (31) in region II suffers IS*1* insertion mutations in both *S. dysenteriae* and *S. flexneri* (4), and these defects might be the primary causes of loss of flagella in the two subgroups. However, the same operon of the two other subgroups is functional (4). In this paper we mainly report characterization of region III operons of *S. boydii* and *S. sonnei* to detect stable mutations that might have caused loss of flagella in these subgroups. Region III fragments were cloned from a certain strain of each subgroup and were examined in detail by restriction mapping, by gene mapping, and occasionally by sequencing. A genetic defect of interest was then examined by Southern blotting to discover whether it is common to other strains in the same subgroup. The results show that three of the six *S. boydii* strains examined have an internal deletion in the *fliF* gene or deletions of the entire *fliF* operon and that all four of the *S. sonnei* strains examined have deletions of the entire *fliD* gene. The three subgroups other than *S. sonnei* also had alterations in the noncoding region that lies between regions IIIa and IIIb. We discuss why and how a certain flagellar gene or operon has acquired large-scale defects and propose a hypothesis for the process.

MATERIALS AND METHODS

Strains, plasmids, and media. Strains of the four *Shigella* subgroups and the main strains of *E. coli* are shown in Table 1. Almost all *E. coli fli* mutants (29) were supplied by the National Institute of Health, Japan. These mutants were transduced with Δ(*recA-srl*)*306 srl*::Tn*10* of strain CSH126 (38) and were used for complementation tests. Plasmids pUC118 (60) and pMW119 (8, 62) were the cloning vectors. TLY broth, nutrient agar, nutrient semisolid agar for the motility test, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) medium were as described previously (14, 55). Tetracycline (Sigma Chemical Co.) and ampicillin (Sigma) were used at final concentrations of 25 and 50 mg/liter, respectively.

Manipulation of DNA. Genomic DNA was isolated and purified according to the method described previously (10). Isolation of plasmid DNA, cloning, trans-

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TABLE 1. Strains used

Strain	Serotype[a] or genotype	Reference or source
S. dysenteriae		
16		51
NCTC9716	A2	National Institute of Health, Japan
S. flexneri IID642	2a	54, 55
S. boydii		
NCTC9733	C5	National Institute of Health, Japan
CDC2064-59	C1	National Institute of Health, Japan
CDC1050-50	C3	National Institute of Health, Japan
NCTC9770	C4	National Institute of Health, Japan
NCTC9354	C8	National Institute of Health, Japan
NCTC9356	C9	National Institute of Health, Japan
S. sonnei		
IID969		54, 55
506II		National Institute of Health, Japan
3831		National Institute of Health, Japan
D12[b]		54, 55
E. coli		
W3110	Wild type	30
DH5α	Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1*	21
EJ2060	*fliA4181 recA56*	This laboratory
EJ2081	Δ*fliC recA56*	42
EJ2061	*fliD1101 recA56*	This laboratory
EJ2258	*fliE4193 recA56*	This laboratory
EJ2256	*fliF4126 recA56*	This laboratory
EJ2926	*fliI4127* Δ(*recA-srl*)	This study
EJ2927	*fliJ1862* Δ(*recA-srl*)	This study
EJ2752	*fliK694 recA56*	This laboratory
EJ2255[c]	*fliLM4160 recA56*	This laboratory
EJ2929	*fliN4117* Δ(*recA-srl*)	This study
EJ2930	*fliP4150* Δ(*recA-srl*)	This study
EJ2931	*fliQ4120* Δ(*recA-srl*)	This study
EJ2751	*fliR4144 recA56*	This laboratory

[a] In this study, serotype numbers prefixed by the subgroup symbols A for *S. dysenteriae* and C for *S. boydii* were used as strain designations for convenience.
[b] This strain was called C12 in previous papers (54, 55) but was renamed D12 to avoid confusion with *S. boydii* (group C) strains.
[c] This mutant was formerly named *flaA4160* (29), and the *flaA* gene was subdivided and renamed as the *fliL* and *fliM* genes; it is unknown whether this mutation is in *fliL* or *fliM*.

formation, and restriction analysis of cloned fragments were all carried out by standard procedures (47). Restriction enzymes, calf intestine alkaline phosphatase, T4 DNA ligase, Klenow fragment, exonuclease III, and mung bean nuclease were obtained from Takara Shuzo Co.

Cloning of the region III operons of *E. coli*. Most of the *E. coli* region III operons are cloned, and their restriction maps are reported (6, 25, 34, 39, 40, 44). We have also isolated these operons as three clones, pAZY1, pECF181, and pEHR274 (Fig. 1), which were constructed by ligating *Eco*RI fragments of W3110 DNA to the P_{lac} promoter of pMW119 and which were able to confer motility on the *E. coli fliA*, *fliC*, and *fliK* mutants (Table 2), respectively. Each plasmid was mapped for various restriction sites, and *fli* genes were assigned to the map by making reference to the published data (6, 25, 34, 40, 44). Loci of *fli* genes were then confirmed by complementation with *E. coli fli* mutants after being subcloned. Some loci (*fliZY*, *fliST*, *fliG*, and *fliO*) were determined only by comparing restriction sites with the sequence data (25, 34, 40, 45) because corresponding *E. coli* mutants were not available for complementation tests.

DNA probes and hybridization. Nineteen *E. coli* probes that cover all the region III operons (Fig. 1) were prepared from the above-mentioned three clones. At first each clone was subcloned as three or four fragments into the polylinker of pMW119 or pUC118. When the gene(s) could not be excised as one probe with one or two restriction enzymes, a subclone was further divided into small fragments or deleted for its internal segment to yield one probe with one or two enzymes. A restriction fragment for a probe was labeled by the random-primed method (16) with a nonradioactive DNA labeling kit (Boehringer Mannheim). Various insertion (IS) elements used as probes were as described previously (4). Hybridization was performed by standard methods (47, 53), and hybrid bands were detected by the Dig-enzyme-linked immunosorbent assay method (16) with a kit. To detect *Shigella* region III clones, white colonies among transformants of strain DH5α were reisolated and blotted by the method described previously (16, 22, 47). To locate *fli* genes and IS elements in *Shigella* region III clones, cloned fragments were subcloned into pUC118 as small fragments, and each subclone was further digested with one restriction enzyme or two enzymes combined and then electrophoresed in separate lanes. Bands were checked to verify the restriction maps and blotted with either *E. coli* probes or IS probes. Hybrid bands in each lane were joined to adjacent restriction fragments, and *fli* genes or IS elements were positioned on the restriction maps with reference to the corresponding maps of *E. coli*. Sometimes the same filter was used two or three times for blotting with different probes.

DNA sequencing. For sequencing the *S. boydii* noncoding (NC)-*fliF-fliG* region, the 2.8-kb *Bam*HI-*Sal*I fragment of plasmid pBFG56 (Fig. 2A) was used after construction of a series of deletion plasmids (55). For sequencing the *E. coli fliF* gene, the 1.3-kb *Sma*I-*Bgl*II (5′ *fliF*) and 0.6-kb *Bgl*II (3′ *fliF*) fragments of pECF181 (Fig. 1B) were subcloned and the former was used to prepare deletion plasmids while the latter was directly used for sequencing. Sequences were determined by a dye terminator cycle-sequencing ready-reaction kit (Applied Biosystems) and analyzed by the GENETYX information processing software (Software Development Co., Tokyo, Japan) and the National Center for Biotechnology Information BLAST network server.

Nucleotide sequence accession number. The nucleotide sequence data of the *E. coli fliF* gene and the *S. boydii* NC-*fliE-fliF* region will appear in the DDBJ, EMBL, and GenBank databases under accession no. D89826 and D89825, respectively.

RESULTS

Cloning of region III operons from *Shigella* subgroups. *E. coli* region III contains seven flagellar (*fli*) operons, which are separated into regions IIIa and IIIb by the large intervening segment that includes the *amyA* gene, a few open reading frames (ORF), and an NC region (Fig. 1B) (33, 43, 44). To detect possible genetic defects that cause loss of flagella in the two *Shigella* subgroups, *S. boydii* and *S. sonnei*, the region III

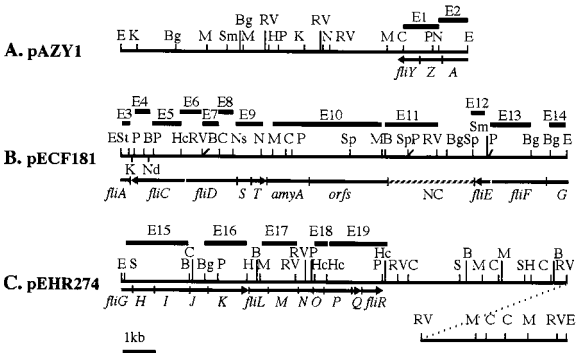

FIG. 1. *E. coli* region III operons. The three *Eco*RI fragments, carrying all of the region III operons, were cloned into pMW119. (A) Plasmid pAZY1 (10.8 kb). (B) Plasmid pECF181 (13.8 kb). (C) Plasmid pEHR274 (18.3 kb). Loci of *fli* genes are shown under the restriction maps. Each of the *fliA* and *fliG* genes is cut in two by the *Eco*RI sites. The NC region is shown by a hatched line. Arrows on gene symbols denote genes or operons and their transcriptional direction. Restriction fragments used as probes are shown above the restriction maps as thick lines. Restriction cleavage sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hc, *Hinc*II; K, *Kpn*I; M, *Mlu*I; N, *Nru*I; Nd, *Nde*I; Ns, *Nsp*V; P, *Pst*I; RV, *Eco*RV; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; and St, *Stu*I. *Sma*I sites were examined only for pAZY1 and pECF181. *Nde*I, *Nsp*V, *Sph*I, and *Stu*I sites of pECF181, *Nru*I sites of pAZY1 and pECF181, *Eco*RV sites of pEHR274, and *Hinc*II sites of pECF181 and pEHR274 were examined only for subclones.

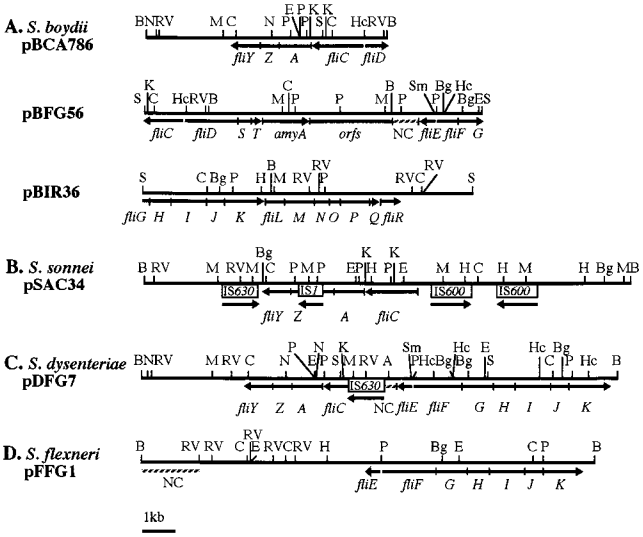

FIG. 2. Restriction and gene maps of the region III fragments cloned from the four *Shigella* subgroups. All fragments were cloned into pUC118. (A) The three fragments from *S. boydii* C5. Plasmids pBCA786 (7.5 kb), pBFG56 (10.5 kb), and pBIR36 (10.3 kb) carried all of the region III operons. (B) pSAC34 with the 15.1-kb *Bam*HI fragment from *S. sonnei* IID969. (C) pDFG7 with the 14.8-kb *Bam*HI fragment from *S. dysenteriae* 2A. (D) pFFG1 with the 14.1-kb *Bam*HI fragment from *S. flexneri* IID642. Insertion elements IS*1*, IS*600*, and IS*630* are depicted by open boxes, and the arrow under each box shows the 5′-to-3′ direction. Abbreviations for restriction sites are the same as for Fig. 1 plus A for *Acc*I. *Acc*I, *Hin*cII, *Nru*I, and *Sma*I sites were examined only for subclones from each parental plasmid.

operons were cloned and gene maps were compared with those of *E. coli*. Genomic DNA from *S. boydii* C5 was digested with *Sal*I, electrophoresed, and blotted with each of the three probes, E2 (part of *fliA*), E5 (part of *fliC*), and E15 (*fliHI*) (Fig. 1), which yielded single hybrid bands of approximately 22, 11, and 10 kb, respectively (data not shown). The fragments corresponding to the 10-kb and 11-kb bands were ligated to pUC118 and transformed into strain DH5α. Plasmids pBFG56 and pBIR36 were isolated by colony hybridization from the transformants which were positive to probes E5 and E15, respectively. The fragment of pBFG56 was shown by the mapping described below to carry the *fliC* gene at the extreme left end (Fig. 2A), and hence the operon downstream from *fliC* seemed to be included in the hybrid band probed with E2. However, the 22-kb band is too large to clone, so the *Bam*HI digest from the same strain was blotted with probe E2, yielding a 7.5-kb hybrid band. Plasmid pBCA786 was isolated by using this band. To clone the region III operons from *S. sonnei* IID969, the *Bam*HI digest was used for blotting with the three probes as described above: E2 and E5 each yielded one hybrid band of 15 kb, and E15 showed a very thin hybrid band about 19 kb long. Plasmid pSAC34 was isolated with the 15-kb band (Fig. 2B). This clone hybridized with probes E2 and E5 as expected. The fragment corresponding to the thin 19-kb band could not be cloned by repeated colony hybridization. This is attributable to the low efficiency of the hybridization between probe E15 and a cloned fragment. This suggests that the *fliH* and/or *fliI* genes of *S. sonnei* IID969, corresponding to probe E15, are partly missing.

For comparison, the region III operons of *S. dysenteriae* and *S. flexneri* were also cloned. At first, *S. dysenteriae* strain 16 was used as the DNA donor, but probe E15 did not show any hybrid band with the *Bam*HI digest. Moreover, probes E13 and E17 (Fig. 1) did not hybridize with the digest either, suggesting that in strain 16 most of the region IIIb operons are missing. Then the *Bam*HI digest from *S. dysenteriae* A2 was blotted with E15, and the 14.8-kb fragment was cloned as described above, yielding plasmid pDFG7 (Fig. 2C). Likewise, plasmid pFFG1 was isolated from the *Bam*HI digest of *S. flexneri* IID642 DNA by blotting with probe E15 (Fig. 2D).

Mapping of region III genes in each clone. The three fragments cloned from *S. boydii* C5 were analyzed with various restriction enzymes and mapped for loci of *fli* genes. The 10.5-kb *Sal*I fragment of pBFG56 contained the *Sal*I-*Bam*HI segment at the left end, which overlaps the right end of the 7.5-kb *Bam*HI fragment of pBCA786 (Fig. 2A). The two *Sal*I fragments of pBFG56 and pBIR36 seemed to be contiguous because there was no overlap. The *Sal*I fragment of pBFG56 was subcloned as three fragments, and each was digested with a restriction enzyme(s). These digests, electrophoresed in separate lanes, were examined with each of the *E. coli* probes. Nineteen probes were used (Fig. 1). pBFG56 included *fliC-fliD-fliS-fliT-amyA*-ORF just as in *E. coli*. However, the NC region and the *fliF* gene were smaller than those of *E. coli*, although the NC-*fliE-fliF-fliG* genes were detected in that order (Fig. 2A). The NC region of *E. coli* occupies 2.7 kb (Fig. 1B), but in this clone it was about 1 kb: the 3.1-kb *Bam*HI-*Sma*I fragment with the NC region and *fliE* of *E. coli* (Fig. 1B) was only 1.4 kb (Fig. 2A). This 1.4-kb fragment still hybridized with probes E11 (NC) and E12 (*fliE*) but lacked the *Sph*I and *Eco*RV sites that were in the *E. coli* NC region, suggesting that the short size of the NC-*fliE* segment is due to deletion in the NC region, but not in the *fliE* gene. The normal size (0.4 kb) of *fliE* was confirmed by sequencing, as will be described below. *fliF* was inferred to be about half the size of the *E. coli fliF* gene (1.7 kb), because the 1.4-kb *Pst*I-*Sal*I fragment on the extreme right of this clone hybridized with both E13 (*fliF*) and E14 (*fliG*), and the truncated *fliG* was inferred to be about 0.6 kb, the same size as the truncated *E. coli fliG* (Fig. 1B). Two other clones, pBCA786 and pBIR36 (Fig. 2A), from *S. boydii* C5 were analyzed in the same way. The pBCA786 fragment, examined with probes E1 to E8 after being divided into two subclones, contained the *fliA* operon, the *fliC* gene, and part of the *fliD* gene in the same order as those of *E. coli*. The restriction map of this clone showed high similarity to that of *E. coli*. The pBIR36 fragment, examined with six probes (E14 to E19), contained part of the *fliF* operon (from part of *fliG* to *fliK*) and the intact *fliL* and *fliR* operons as in *E. coli*. The restriction map was almost identical to that of *E. coli*. Thus, it was found that the *S. boydii* region III operons are the same in organization and gene arrangement in each operon as those of *E. coli*: the only difference was the small size of the *fliF* gene.

The 15.1-kb *Bam*HI fragment of pSAC34 from *S. sonnei* IID969 was subcloned into four fragments and examined in the same way. The *fliA* operon and *fliC* gene were located in the left half and the middle of the fragment, respectively (Fig. 2B); however, 14 probes, E6 to E19, did not hybridize with digests from the four subclones. The restriction map of this clone was quite different from that of *E. coli* even in the region containing the *fliA* operon and the *fliC* gene.

The pGFG7 fragment (14.8 kb) from *S. dysenteriae* A2 and the pFFG1 fragment (14.1 kb) from *S. flexneri* were also examined for gene loci. The A2 clone contained the full-size *fliA*, *fliE*, and *fliF* operons, but the region extending from the 5′ end of *fliC* to the ORF, corresponding to the probes from E5 to E10, was deleted (Fig. 2C). A part of the NC region remained downstream from *fliE*, as shown by the fact that probe E11 (NC) weakly hybridized with the short *Sma*I-*Eco*RV fragment in the middle of this clone. In the *S. flexneri* clone the *fliE* and *fliF* operons were conserved in the right half of the fragment

TABLE 2. Restoration of motility to *E. coli fli* mutants by *Shigella* region III clones[a]

Plasmid	Motility[b]												
	fliA	*fliC*	*fliD*	*fliE*	*fliF*	*fliI*	*fliJ*	*fliK*	*fliLM*	*fliN*	*fliP*	*fliQ*	*fliR*
S. boydii													
pBCA786M	−	+*	−	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
pBFG56M	−	−	+	−	−	NT	NT	NT	NT	NT	NT	NT	NT
pBIR36M	NT	NT	NT	−	−	+	+	+	+	+	+*	+	+
S. sonnei pSAC34M	+	+	−	−	NT	NT	NT	NT	NT	NT	NT	NT	NT
S. dysenteriae pDFG7M	−	−	−	+	−	−	+	+	−	NT	NT	NT	NT
S. flexneri pFFG1M	NT	NT	−	+	−	+*	+	+	−	NT	NT	NT	NT
E. coli													
pAZY1	+	−	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
pECF181	−	+	+	+	+	−	NT	NT	NT	NT	NT	NT	NT
pEHR274	NT	NT	NT	−	−	+	+	+	+	+	+	+	+
pMW119	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] Each of 13 *E. coli fli* mutants (Table 1) was transformed with an appropriate plasmid carrying a region III clone (Fig. 2) from each *Shigella* subgroup and was selected for ampicillin resistance. Five transformants in each experiment were restreaked onto the same selective medium, and single colonies were stabbed into nutrient semisolid medium with ampicillin and incubated at 30°C for 12 h. Single colonies from the five transformants showed the same phenotype. The three *E. coli* plasmids (Fig. 1) and the vector plasmid pMW119 were used as controls.

[b] +, confluent swarms; +*, dotted swarms; −, no swarms; NT, not tested. An arrow on the mutant *fli* genes shows that these genes are in one operon and transcribed in the direction of the arrow.

and the NC region was on the extreme left (Fig. 2D). An extra 5.2-kb segment was located between them, which did not hybridize with any of the 19 probes. The NC region (1.7 kb) was located only in this fragment, not in the adjacent fragment cloned (data not shown), and it was shorter than that of *E. coli* and lacked the *Pst*I and *Bgl*II sites that were in the *E. coli* NC region (Fig. 1B).

Restoration of motility to *E. coli fli* mutants by the cloned *Shigella* genes. To test whether *Shigella fli* genes in the cloned fragments were functional or mutated, each clone was transformed into *E. coli fli* mutants and the transformants were examined for restoration of motility. Prior to transformation, the originally cloned fragments were recloned into low-copy-number plasmid pMW119 to exclude the effect of gene dosage. Recloned fragments are under the control of the P_{lac} promoter. We used a total of 13 *fli* mutants (Table 1), which were stable nonflagellates and *recA* derivatives. The three *S. boydii* clones were able to complement a total of 10 mutations, excluding those in the *fliA*, *fliE*, and *fliF* genes (Table 2). The *fliA* and *fliE* genes of *S. boydii* have some mutations despite their normal size. The *S. boydii fliF* gene was nonfunctional as expected from its small size, but the *fliI*, *fliJ*, and *fliK* genes in the *fliF* operon complemented the respective *E. coli* mutations. The *S. sonnei* clone complemented only the *fliA* and *fliC* mutations (Table 2), which was in agreement with the result of gene mapping. The cloned fragments of *S. dysenteriae* A2 and *S. flexneri* IID642 were also examined by the complementation test. The results are summarized in Table 2.

Detection of insertion elements in the cloned fragments. The gene mapping and complementation test described above showed that *S. boydii* pBFG56 has genetic aberrations in the NC-*fliF* region and *S. sonnei* pSAC34 has undergone a large-scale gene rearrangement in the region upstream of the *fliC* gene. *S. dysenteriae* pDFG7 and *S. flexneri* pFFG1 also had deletions in the *fliC*-NC and NC regions, respectively. The four *Shigella* subgroups are known to harbor a large number of copies of several IS elements in their chromosomes (35, 36). To disclose the relationship between IS elements and the genetic defects in the cloned fragments, each clone was blotted with seven IS probes (4): IS*1*, iso-IS*2*, IS*3*, IS*600*, IS*629*, IS*630*, and IS*640*. No hybrid band was detected in the three clones from *S. boydii* C5. *S. sonnei* pSAC34 hybridized with the three probes IS*1*, IS*600*, and IS*630* (data not shown). To localize these elements on the restriction map, digests of subclones were blotted with each IS probe. Two copies of IS*600* were detected upstream of the *fliC* gene in the inverted orientation (Fig. 2B), which was judged by relative positions of the *Mlu*I and *Hin*dIII sites, each unique to IS*600* (36). IS*1* and IS*630* were located in the *fliZ* gene and downstream from the *fliY* gene, respectively (Fig. 2B). This IS*1* insertion is irrelevant to loss of flagella because the *fliZ* gene, as well as *fliY*, is not involved in flagellation (40). Thus, it is suggested that in this region the two IS*600* copies, capable of functioning as a transposon, were once involved in large-scale gene rearrangement, so the *fliD* operon and its adjacent large segment could not be detected despite the large size of the pSAC34 fragment.

S. flexneri pFFG1 did not have an IS element, but *S. dysenteriae* pDFG7 carried IS*630*, which was located in the 5′ coding region of the *fliC* gene (Fig. 2C). The deletion of the *fliC*-NC region in pDFG7 might be explained by the insertion of IS*630* into either *fliC* or the NC region followed by deletion of the adjacent region (27).

Sequence analysis of the NC-*fliF* region of *S. boydii*. The pBFG56 clone from *S. boydii* C5 did not have an IS element, although the size of *fliF* and the NC region was markedly reduced by deletion. To confirm these deletions, the 2.8-kb fragment with the NC-*fliE-fliF-fliG* region of pBFG56 (Fig. 2A) was subcloned, sequenced, and then compared with the corresponding sequences of *E. coli*. The *E. coli fliF* gene was also sequenced because this sequence has not yet been deposited in data banks. The *fliF* gene in pBFG56 had a 988-bp deletion in comparison with the *E. coli fliF* sequence (1,659 bp) (Fig. 3). At the deletion junction, we detected the 8-bp sequence 5′ GTGGGCGA, which was also detected in the *E. coli* sequence as the direct repeats flanking the sequence that was deleted in *S. boydii*. This suggests that the *fliF* deletion in *S.*

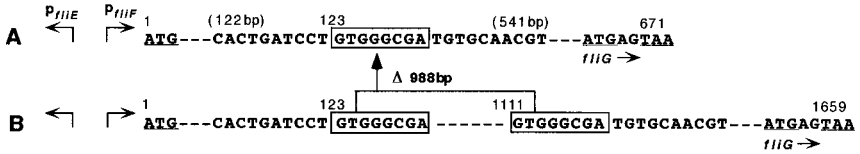

FIG. 3. Nucleotide sequence containing the deletion junction of the *S. boydii fliF* gene (A) and the corresponding sequence of *E. coli* (B). Numbering of the sequences starts with the initiation codon and ends at the stop codon. The 988-bp deletion was inferred to have occurred between the 8-bp direct repeats (boxes) that are still present in the corresponding *E. coli* sequence. We know that the two genes once had very high identity because the 122-bp sequences upstream of the 8-bp boxes are identical and the 541 bp downstream from the boxes differ only in 11 bp. In the upstream region of the two genes, two divergent σ^F-specific promoters were detected, one (position −39) for the *fliF* gene and another (position −59) for the *fliE* gene. The sequences containing these promoters are also identical in the two genes. The initiation and stop codons are underlined. The initiation codon of the *fliG* gene overlaps the 3′ coding region of the *fliF* gene for both strains.

boydii C5 occurred between these direct repeats. The *S. boydii* and *E. coli fliF* genes had 100% homology in the sequence upstream of the deletion and 98% homology in the downstream sequence. In the NC region, two large deletions of 603 and 1,223 bp were detected in the middle of the sequence (data not shown). The total deletion length was more than 60% of the *E. coli* NC region. The *fliE* sequence was identical to that of *E. coli* (39) except for two base substitutions at positions 35 (G to T) and 184 (G to A) in the numbering starting from the *fliE* initiation codon (data not shown). These mutations account for the failure of the pBFG56 clone to complement the *E. coli fliE* mutation (Table 2).

Genetic defects common to strains of the same subgroup. *S. boydii* C5 had the large deletion in the *fliF* gene and *S. sonnei* IID969 had the large-scale rearrangement upstream of the *fliC* gene. When the same kind of defect is detected in common in different strains of the same subgroup, such a defect may be regarded as the primary cause of loss of flagella in an ancestor of these strains. For this reason, six *S. boydii* strains (Table 1), including C5, were examined by blotting with *E. coli* probes. *Bam*HI and *Eco*RI digests of genomic DNA were examined with probes E13 (*fliF*), E16 (*fliK*), and E19 (*fliPQR*). The digests from strain C5 showed a thin hybrid band with probe E13 (Fig. 4A, gel b), which reflects the large internal deletion in *fliF*. When probes E16 and E19 were used, the digests showed thick hybrid bands (Fig. 4A, gels c and d) as expected from the normal sizes of the *fliK* and *fliPQR* genes (Fig. 2A). Strains C1 and C8 did not show a hybrid band in blotting with probes E13 and E16 (Fig. 4A, gels b and c), suggesting that these two strains have undergone deletion extending to the entire *fliF* operon (*fliF* to *fliK*). This was confirmed by blotting with other probes: probe E17, carrying *fliLM* next to the *fliF* operon (Fig. 1C), hybridized with the digests from these strains, whereas probe E14, carrying *fliG* in the *fliF* operon, did not (data not shown). The digests from three other strains, C3, C4, and C9, showed hybrid bands in every blotting; however, the size and intensity of the hybrid bands, and even their number in the C3 digest (Fig. 4A, gel d), were different when the probes from the *fliF* and *fliL* operons were used (Fig. 4A, gels b, c, and d). On the other hand, when the same digests were blotted with probes from other operons, the hybrid bands were uniform in size. One such experiment is shown in Fig. 4A, gel a.

Four *S. sonnei* strains (Table 1), including IID969, were examined in the same way. *Bam*HI and *Eco*RI digests from the strains IID969, 506II, 3831, and D12 did not hybridize with probe E6 (5′ *fliD*) (Fig. 4B, gel a). The *fliD* gene of IID969 was missing not only in the cloned fragment but also in the chromosome. To test whether the four strains have the entire *fliD* deletion, the *Bam*HI digests were blotted with probe E8 (3′ *fliD*) (Fig. 1B); no hybrid band was detected (data not shown). The four strains all lacked the *fliD* gene. Probes E9 (*fliST*) and E10 (*amyA* and ORF) hybridized with the digests from the four strains, and the sizes and intensities of hybrid bands in each blotting were almost identical for the four strains (data not shown). This result was unexpected because pSAC34 did not carry any genes upstream of the *fliC* gene, but it showed that the undetected genes, except *fliD*, are not deleted from the chromosome but lie elsewhere. Probe E13 (*fliF*) showed thin hybrid bands, and E15 (*fliHI*) showed much thinner hybrid bands, in the digests from strains 3831 and IID969 (Fig. 4B, gels b and c). The same digests were then tested with the adjacent probes, E14 (*fliG*) and E16 (*fliK*). The hybrid bands

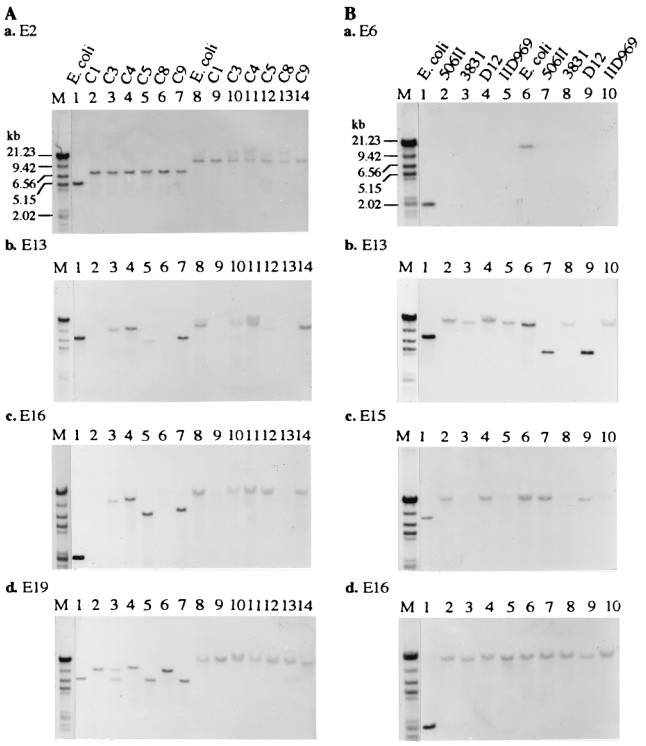

FIG. 4. Southern blots of DNA from *S. boydii* and *S. sonnei* strains with *E. coli* probes. (A) Blots of *Bam*HI digests (lanes 2 to 7) and *Eco*RI digests (lanes 9 to 14) of genomic DNA from the six *S. boydii* strains. The strain number is shown above each lane number of the top gel. Lanes 1 and 8 are *Bam*HI and *Eco*RI digests, respectively, of *E. coli* W3110 DNA. Lanes M contain DNA size markers, a mixture of the *Hin*dIII digest and the *Hin*dIII and *Eco*RI digests of λ DNA, and the sizes of major bands are shown on the left of the top gel. Gels and probes: a, E2 (*fliA*); b, E13 (*fliF*); c, E16 (*fliK*); d, E19 (*fliPQR*) (Fig. 1). (B) Blots of *Bam*HI digests (lanes 2 to 5) and *Eco*RI digests (lanes 7 to 10) of DNA from the four *S. sonnei* strains shown above the lane numbers of the top gel. Lanes 1 and 6 are *E. coli* DNA used as a control. Gels and probes: a, E6 (5′ *fliD*); b, E13 (*fliF*); c, E15 (*fliHI*); d, E16 (*fliK*).

disappeared in the blot with E14 (data not shown) but not with E16 (Fig. 4B, gel d). These results suggest that strains 3831 and IID969 have another deletion covering part of the *fliF* gene and the *fliGH* genes. Probes E17 (*fliLM*) and E19 (*fliPQR*) yielded hybrid bands with similar sizes and intensities in the digests from the four strains (data not shown).

DISCUSSION

The region III operons of *S. boydii* C5 had the *fliF* internal deletion in addition to the defective *fliA* and *fliE* genes but did not have an IS element. The *fliA* allele of this strain is unlikely to have a large defect like deletion, judging from its normal size (Fig. 2A and 4A, gel a). Therefore, the *fliA* and *fliE* alleles have the chance to revert to the wild types, and hence neither will be the primary cause of loss of flagella in this subgroup. The *fliF* deletion is the only large defect in this strain because the region II operons are intact and the region I operons are also well conserved. The deletion was inferred to have occurred between the 8-bp direct repeats. How was the deletion caused? Rates of deletion between direct repeats are enhanced when the distance between two repeats becomes short (9, 11, 32) and a palindromic or quasipalindromic sequence exists between them (3, 12, 20, 57). The 988-bp sequence is rather large to be deleted and contained no large palindromic sequence as far as we surveyed it by computer. Thus, we can speculate that at first an IS element had been inserted in the 988-bp sequence near one of the direct repeats and replicatively transposed in the opposite orientation near the other repeat, and then deletion occurred between the 8-bp direct repeats with the aid of a stable secondary structure between the two IS elements. An alternative speculation is that two IS elements were positioned in the same orientation and were deleted along with the sequence between them, resulting in the short distance between the 8-bp repeats, and then the existent deletion occurred between them.

The *fliF* gene of *E. coli* encodes the protein for the MS ring and a proximal rod (58, 59). This structure is the largest substructure of the basal body. Therefore, the deletion of this gene totally precludes construction of the basal body and assembly of subsequent structures. The *fliF*, *fliL*, and *fliR* operons are in class 2, and their expression depends on FlhDC, but not on FliA (σ^F). *S. boydii* C5 retains the functional *flhDC* genes (4) and intact *fliL* and *fliR* operons (Table 2) in addition to a normal P_{fliF} promoter (Fig. 3). Therefore, in this strain these class 2 operons can be expressed. It is intriguing that the gene product of *fliI* in the *fliF* operon, those of *fliO*, *P*, and *Q* in the *fliL* operon, and the product of *fliR* are partly homologous to proteins that are directly or indirectly involved in the virulence process of some enteric bacteria (7, 13, 17, 48). However, the function of these products in flagellar biogenesis is unknown. This strain economizes on energy for expression of class 3 operons by the *fliA* mutation and for construction of the basal body by the *fliF* deletion, but seems to expend it for some unknown proteins. Of the five other *S. boydii* strains, C1 and C8 lacked not only *fliF* but the entire *fliF* operon. The two strains seem to have the same pedigree because, in addition to the above-described deletion, the sizes of hybrid bands produced with probes from the *fliL* operon were identical in both of them (one such experiment is shown in Fig. 4A, gel d). Is there any relationship between this large deletion and the *fliF* internal deletion in strain C5? Supposing that the above speculation on deletion formation is plausible, the extent of deletion primarily depends on the site of replicative transposition of the first IS element inserted in the *fliF* gene. So the three strains C1, C5, and C8 might have been derived from a common ancestor with an IS element in *fliF*. The rest of the strains, C3, C4 and C9, showed hybrid bands different in size and intensity from one another only when the probes from the *fliF* and *fliL* operons were used (Fig. 4A), suggesting that these strains also have some kind of mutation in these operons. Taking these facts together, we can deduce that the six *S. boydii* strains examined have different kinds of genetic defects in the *fliF* operon and that these defects are causes of loss of flagella in these strains. There are so far no data to directly relate all of these defects to one another, but we cannot exclude the possibility that the defects have arisen from a common mutation like IS element insertion in the *fliF* gene.

The region III fragment (pSAC34) from *S. sonnei* IID969 carried only the functional *fliA* and *fliC* genes (Table 2) and harbored the four IS elements (Fig. 2B). The hybridization tests revealed that this strain has the two deletions, including Δ*fliD*, but retains other *fli* genes that contain *fliST*, the rest of the *fliD* operon. The same hybridization results were obtained from strain 3831. The two other strains, 506II and D12, had only Δ*fliD*. Thus, the *fliD* deletion is common to the four *S. sonnei* strains examined, and hence, this defect might be the primary cause of loss of flagella in this subgroup. In strain IID969 the two IS*600* elements were located upstream of the *fliC* gene, in the region where the *fliD* operon and its downstream genes should lie, and these elements formed inverted repeats capable of acting as a transposon. These elements probably mediated the deletion of *fliD* and the rearrangement of the large region including the *fliST* and region IIIb genes by multiple rounds of transposition and recombination events.

The *fliD* gene encodes a cap protein of the flagellar filament (24). The cap hinders the free release of transported flagellin molecules and facilitates their polymerization onto growing filament tips (23). FliD also negatively regulates the export of FlgM protein, which inhibits the flagellum-specific sigma factor FliA (18, 41, 63). Therefore, *fliD* mutants continue to excrete newly synthesized flagellin molecules into culture medium and excessively export the FlgM protein. As a result, functional FliA at a higher-than-normal level causes overexpression of class 3 genes that contain the flagellin (*fliC*) gene and idling of flagellin molecules continues. It has been shown that the FlgM-FliA regulatory pathway of *S. typhimurium* is involved in virulence in mice (49, 50). An *flgM* mutant shows the attenuated phenotype, which is restored to a wild-type level when a mutant *fliA* allele is introduced to the *flgM* mutant. The effect of the *fliA* allele is interpreted by the lack of *fliC* expression but not by restored expression of virulence genes (49), whose expression is presumed to be indirectly controlled by FliA in such a way that an inhibitory factor for virulence (genes) is positively regulated by FliA. It is finally proposed that the normal regulation of flagellum synthesis appears to be necessary for virulence (49). *fliD* mutants ought to show a phenotype similar to that of *flgM* mutants, except for the continuous excretion of flagellin molecules. The four *S. sonnei* strains have both of the *fliD* deletions and the silent *fliST* genes lacking a P_{fliD} promoter. It has been shown that FliD, FliS, and FliT are all involved in the export of FlgM (63). Supposing that in the two *S. sonnei* strains 506II and D12 the flagellar regulon is intact except for the *fliD* operon, a phenotype of these strains would be expected to be similar to that of the *flgM fliC* double mutant of *S. typhimurium* rather than that of the *flgM* mutant, since the excretion of flagellin molecules and the export of FlgM will be highly enhanced in these strains. If the mechanism for such virulence development is common to the two genera, the two *S. sonnei* strains will maintain virulence at a normal level. When flagellin and/or FlgM molecules themselves are toxic to the host, these strains will be more virulent. The two other strains,

IID969 and 3831, had another deletion covering part of the *fliF* gene and the *fliGH* genes, the three genes encoding the MS ring, motor switch, and export apparatus, respectively (26, 59, 61). Therefore, these strains cannot construct the basal body-hook structures through which FlgM and flagellin are exported. Therefore, the amount of functional FliA is lowered by excess flgM, resulting in a low level of expression of class 3 operons which include the *fliC* and many motility-chemotaxis genes. This may avoid futile expression of these genes and save energy for survival.

The present study and our previous studies show that the four *Shigella* subgroups have become nonmotile by undergoing different kinds of stable mutations in different flagellar genes. Moreover, the defects in the *S. boydii* strains may be suggestive of independent events of loss of flagella even in the strains of the same subgroup. The lack of flagellation, therefore, seems to be an evolutionarily recent event that happened separately after each subgroup had evolved from a common ancestor and was subdivided into several pedigrees. The important factor that triggered loss of flagella is considered to be the transposition of IS elements. In shigellae numerous copies of IS elements, in particular IS*1* and IS*600*, have been reported (35). This means that these elements repeated or have been repeating replicative transposition into various sites of the genome at high frequencies. Insertion mutations in essential genes are immediately eliminated from the bacterial population, but those in nonessential genes or in intergenic regions can survive unless they are deleterious. We noticed the large alterations in the NC region, which are manifested as different defects in each strain but which have occurred in common in the three subgroups other than *S. sonnei*, which has not been examined for this region. The NC region lies between regions IIIa and IIIb in the order of region I (24 min)-II (41 min)-IIIa (43 min)-IIIb (43 min) on the *E. coli* genetic map (5). Consequently, the *fliD* (region IIIa) and *fliF* (region IIIb) operons flank the NC region (Fig. 1B) and the *flhD* operon (region II) is located nearest the NC region among the *flh* operons (5). The *fliF* and *fliD* operons have undergone large-scale deletion as described above, and the *flhD* operon has undergone IS*1* insertion (4). Therefore, it is inferred that at first some IS element, which might have been a different kind in each subgroup, was inserted in the NC region without any deleterious effect on the cell and was then transposed into neighboring flagellar genes, causing deletion and/or rearrangement by subsequent transposition and recombination. This inference also provides a reason why the region I operons, which are most distant from the NC region, are well conserved in the four subgroups. The existent *Shigella* subgroups, survivors of such evolutionary events, are in general considered to avoid the high energy costs for flagellar biogenesis by maintaining stable mutations and to use the conserved energy for bacterial growth rather than for an increase in virulence. Nevertheless, the possibility cannot be excluded that a functional regulatory gene(s), such as *fliA* or *flhDC*, is related to the virulence expression of *S. boydii* and *S. sonnei*, as seen for *Bordetella bronchiseptica* (1, 2).

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