JOURNAL OF BACTERIOLOGY, Apr. 2004, p. 2319–2327 Vol. 186, No. 8
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.8.2319–2327.2004


# A 12-Base-Pair Deletion in the Flagellar Master Control Gene *flhC* Causes Nonmotility of the Pathogenic German Sorbitol-Fermenting *Escherichia coli* O157:H$^-$ Strains

Steven R. Monday,[1] Scott A. Minnich,[2] and Peter C. H. Feng[1]*

*Division of Microbiological Studies, Food and Drug Administration, College Park, Maryland,[1] and Department of Microbiology, Molecular Biology, and Biochemistry, University of Idaho, Moscow, Idaho[2]*

Received 28 October 2003/Accepted 7 January 2004

**An atypical, Stx2-producing, pathogenic *Escherichia coli* O157:H$^-$ strain has been isolated with increasing frequency from hemolytic uremic syndrome patients in Germany. The lack of the H7 antigen coupled with the strain's ability to ferment sorbitol and express β-glucuronidase have complicated its detection and identification. In this study, we have determined that the loss of motility in these German sorbitol-fermenting (SF) O157 strains is due to a 12-bp in-frame deletion in *flhC* that is required for transcriptional activation of genes involved in flagellum biosynthesis. Either complementation with a functional *flhC* or repair of this mutation restored H7 antigen expression and motility. PCR analysis of several nonmotile *E. coli* O157 strains from various geographical sources confirmed that the 12-bp *flhC* deletion is found only in the cluster of German SF O157 strains, providing a potentially useful marker by which these atypical strains can be identified. The loss of motility via mutations in the *flhDC* operon that we observed in the German SF O157 strains is consistent with a similar phenomenon currently observed in a significant subset of other important gram-negative pathogens.**

Enterohemorrhagic *Escherichia coli* (EHEC) of serotype O157:H7 is routinely identified by its inability to ferment sorbitol, its lack of β-glucuronidase activity, and the presence of somatic (O)157 and flagellar (H)7 antigens. There are many O157:H7 phenotypic variants, but the Shiga toxin 2 (Stx2)-producing, sorbitol-fermenting (SF) O157:H$^-$ strains originally isolated from hemolytic uremic syndrome (HUS) patients in Germany (17) have emerged as important human pathogens in Europe (4, 7, 8, 20). The German SF O157 strains, which have β-glucuronidase activity and are nonmotile (NM) (21), have been found in up to 40% of HUS cases (20). These strains are not detected by assays routinely used for O157:H7 because of their atypical phenotype, namely, the ability to ferment sorbitol, express β-glucuronidase activity, and are NM. As a consequence, German SF O157 strains are only identified by testing first for the *stx* gene or Stx, with those Stx-positive isolates being further characterized for the presence of the other O157:H7-associated phenotypes and virulence markers. Despite the many phenotypic differences, however, the German SF O157 strains are a unique clone that is closely related genetically to O157:H7 (13, 21) and is postulated to have diverged from O157:H7, in part by the loss of motility (26).

Motility in *E. coli* is under complex genetic control and can be induced or repressed physiologically by environmental factors, including glucose (catabolite repression), temperature, and high-salt conditions (24). Most O157:NM strains that produce Stx also possess other O157:H7 trait markers and, in some cases, can be induced to express the H7 antigen that restores motility, suggesting that these strains are NM due to environmental factors (12). The German SF O157 strains, however, seem to be an exception, as motility cannot be restored by passages in motility medium (12).

Flagellum biosynthesis in *E. coli* requires ca. 40 genes (3), 30 of which encode structural components of the basal body, motor, and filament. These flagellar genes are under a three-class regulatory system and are sequentially expressed in the order of assembly. Class I genes consist of *flhDC*, the master control operon whose products are required to activate class II genes that encode the structural proteins for the basal body and hook, as well as FliA and FlgM, two regulators of class III genes. Class III genes encode the motor (Mot), chemotaxis (Che), and filament (FliC) proteins (3).

The German SF O157 strains, although NM, carry the entire *fliC* gene that exhibits the restriction fragment length polymorphism profile characteristic of strains in the O157:H7 group (16). Previously, the *fliC* gene of 493-89, a German SF O157 strain, was reported to contain two single-site nucleotide insertions that produced a frameshift mutation that could, presumably, account for the loss of motility (30). In this study, we examined the motility-related regulatory and structural genes to identify the genetic defect(s) that caused the NM phenotype in the German variant.

## MATERIALS AND METHODS

**Bacterial strains and characterization.** The bacterial strains used in this study are listed in Table 1. All strains were tested by five-product PCR (14), which confirmed the presence of genes encoding Shiga toxin (*stx*$_1$, *stx*$_2$), γ-intimin (*eae*), enterohemolysin (*ehxA*), and the +92 *uidA* mutation that is unique to O157:H7 and its Stx-producing NM variants (Fig. 1 and Table 1). All strains, before and

* Corresponding author. Mailing address: HFS-516, FDA, 5100 Paint Branch Pkwy., College Park, MD 20740-3835. Phone: (301) 436-1650. Fax: (301) 436-2644. E-mail: pfeng@cfsan.fda.gov.

TABLE 1. *E. coli* strains used in the study

| Strain | Serotype[a] | Mot[b] | $stx_1$[c] | $stx_2$[d] | *uidA*[e] | *eae*[f] | *ehxA*[g] | Δ*flhC*[h] | Source[j] |
|---|---|---|---|---|---|---|---|---|---|
| 35150 | O157:H7 | + | + | + | + | + | + | − | ATCC |
| 43890 | O157:H7 | + | + | − | + | + | + | − | ATCC |
| 493–89 | O157:H$^-$ | − | − | + | + | + | + | + | Karch, Germany |
| CB569 | O157:H$^-$ | − | − | + | + | + | + | + | Karch, Germany |
| CB1009 | O157:H$^-$ | − | − | − | + | + | + | + | Karch, Germany |
| 5412 | O157:H$^-$ | − | − | + | + | + | + | + | Karch, Germany |
| 514–91 | O157:H$^-$ | − | − | + | + | + | + | + | Karch, Germany |
| 210–89 | O157:H$^-$ | − | − | − | + | + | + | + | Karch, Germany |
| TT7 | O157:H$^-$ | − | + | − | + | + | + | − | Takeda, Japan |
| 3204–92 | O157:NM | − | − | + | + | + | + | − | CDC |
| H0482 | O157:NM | − | − | + | + | + | + | − | CDC |
| 7123 | O157:NM | − | − | − | − | − | − | − | USDA |
| DEC5A | O55:H7 | + | − | − | − | + | − | − | Whittam, MSU |
| DEC5D | O55:H7 | + | − | − | − | + | − | − | Whittam, MSU |
| DH5α | | − | − | − | − | − | − | ND[i] | |

[a] Designated H$^-$ or NM by original source.
[b] Motile phenotype.
[c] Shiga toxin 1 gene.
[d] Shiga toxin 2 gene.
[e] +92 mutation in glucuronidase gene.
[f] γ-Intimin gene.
[g] Enterohemolysin gene.
[h] Presence of 12-bp *flhC* deletion.
[i] ND, not done.
[j] ATCC, American Type Culture Collection; CDC, Centers for Disease Control and Prevention; USDA, U.S. Department of Agriculture; MSU, Michigan State University.

after genetic manipulations, were serologically typed for the O157 and H7 antigens using the RIM O157:H7 test (REMEL, Lenexa, Kans.). Motility was determined by microscopic examination of wet mounts of cell suspensions for the presence or absence of motility. NM strains were passed in motility agar (BD Diagnostic Systems, Franklin Lakes, N.J.) several times and reexamined for motility.

**Plasmid constructs used in complementation studies.** The plasmid constructs used in this study are shown in Table 2, and the primer sequences used to amplify the respective genes are shown in Table 3. Constructs were made as described below.

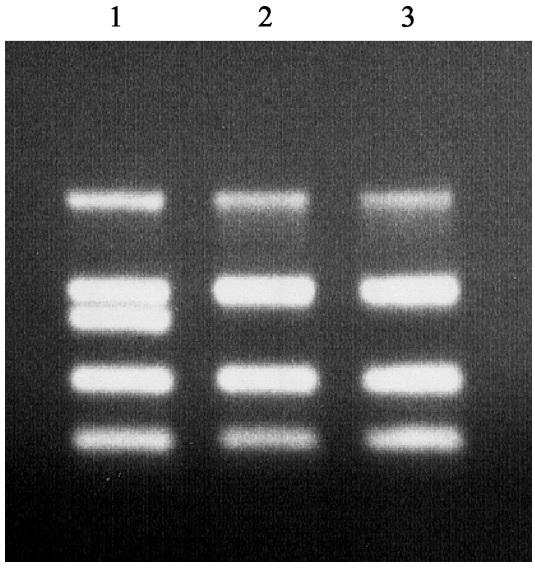


FIG. 1. Agarose gel electrophoresis of five-product PCR amplification products derived from representative strains used in this study. Lanes: 1, 35150; 2, 493-89; 3, 5412. The products (top to bottom) are $stx_2$ (584 bp), γ-*eae* (397 bp), $stx_1$ (348 bp), *uidA* (252 bp), and *ehxA* (166 bp).

***fliC* constructs.** The *fliC* gene from strains 35150, 493-89, and CB569 was amplified using the F-FLIC1 and R-FLIC2 primers (16), except the forward primer was modified to match the GenBank *fliC* sequences (accession numbers L07388 and AE005415) and also to introduce a NcoI restriction site at the 5′ terminus. The PCR mix contained 1× Thermopol buffer (New England Biolabs [NEB], Beverly, Mass.), 6 mM $MgSO_4$, 200 μM deoxynucleoside triphosphate (dNTP), 300 nM phosphorylated primers, and ~$1.0 \times 10^5$ bacterial cells as template. The reaction mix was heated at 95°C for 10 min, during which 0.5 U of *Vent* DNA polymerase (NEB) was added, followed by 32 successive cycles, each consisting of 95°C for 1 min, 55°C for 45 s, and 75°C for 2 min. The reaction was terminated with a 75°C, 7-min incubation. The ~1,750-bp product was isolated from a 0.8% Tris-borate-EDTA (TBE) agarose gel, ligated into an EcoRV-digested and phosphatase-treated pBluescript SK(−) vector (Stratagene, San Diego, Calif.), and transformed by electroporation into *E. coli* DH5α cells (31). Transformants were selected on tryptic soy agar (TSA) containing 100 μg of ampicillin/ml, and plasmids from those ampicillin-resistant (Amp$^r$) clones carrying the *fliC* gene from strains 35150, 493-89, and CB569 were designated pSM1, pSM2, and pSM3, respectively. Construct pSM4 was made by digesting pSM1 with NcoI and HindIII and ligating the resulting 1,750-bp fragment that contained *fliC* into a similarly digested pTrc99A plasmid expression vector (6).

***flhDC* constructs.** The *flhDC* operons from 35150 and 493-89 were amplified using *Vent* polymerase and the SRM16 and SRM11A primers. Amplification reactions were set up and performed as described above, except annealing was done at 56°C for 30 s and extension was at 75°C for 1.5 min. Properly sized amplicons were isolated, cloned into pBluescript SK(−), and transformed into DH5α. Several constructs were screened by SmaI restriction digestion to determine insert orientation. The pBluescript SK(−) constructs with the 35150 *flhDC* and 493-89 *flhDC* operons were designated pSM5 and pSM6, respectively (Table 2).

To study gene functionality, the *flhDC* operons from strains 35150, 493-89, and DEC5A (O55:H7) were also cloned into the pACYC184 vector (NEB). The operon from the respective strains was amplified with *Vent* polymerase and the SRM11A and SRM20 primer pair using conditions described above, except with a 58°C annealing temperature and for 35 cycles. Amplicons were isolated from agarose gels, ligated into EcoRV-digested, phosphatase-treated pACYC184, and transformed into DH5α. Transformants were selected on TSA containing 20 μg of chloramphenicol/ml. Plasmids from several chloramphenicol-resistant (Cm$^r$) clones were isolated and characterized by SphI restriction digestion to determine insert orientation. The construct pSM7 contained the 35150:*flhDC* operon with the native promoter oriented distal to the vector tetracycline resistance (Tc$^r$) promoter. Construct pSM8 had the DEC5A:*flhDC* operon with the native pro-

TABLE 2. Plasmid constructs engineered for this study

| Plasmid | Description | Primers used |
|---|---|---|
| pSM1 | pBluescript SK(−) containing the 35150 *fliC* at the vector EcoRV site | F-FLIC1/R-FLIC2 |
| pSM2 | pBluescript SK(−) containing the 493-89 *fliC* at the vector EcoRV site | F-FLIC1/R-FLIC2 |
| pSM3 | pBluescript SK(−) containing the CB569 *fliC* at the vector EcoRV site | F-FLIC1/R-FLIC2 |
| pSM4 | pTrc99A containing the 35150 *fliC* ORF for expression from the vector Trc promoter | NA[a] |
| pSM5 | pBluescript SK(−) containing the 35150 *flhDC* operon at the vector EcoRV site | SRM11A/SRM16 |
| pSM6 | pBluescript SK(−) containing the 493-89 *flhDC* operon at the vector EcoRV site | SRM11A/SRM16 |
| pSM7 | pACYC184 containing the 35150 *flhDC* operon at the vector EcoRV site; insert is oriented with the native *flhDC* promoter distal to the vector tetracycline ($Te^r$) promoter | SRM11A/SRM20 |
| pSM8 | pACYC184 containing the DEC5A *flhDC* operon and promoter at the vector EcoRV site; insert is oriented with the native promoter oriented adjacent to the $Tc^r$ promoter | SRM11A/SRM20 |
| pSM9 | pACYC184 containing the 493-89 *flhDC* operon and promoter at the vector EcoRV site; insert is oriented with the native promoter adjacent to the vector $Tc^r$ promoter | SRM11A/SRM20 |
| pSM10 | pACYC184 containing the 493-89 *flhDC* operon and promoter at the vector EcoRV site; insert is oriented with the native promoter distal to the vector $Tc^r$ promoter | SRM11A/SRM20 |
| pSM11 | pBluescript SK(−) containing the 493-89 *flhDC* operon with the 12-bp *flhC* deletion repaired by two-round PCR; operon is inserted into the vector at the EcoRV site | SRM11A/SRM20 and SRM51/SRM52 |
| pSM12 | pBluescript SK(−) containing the 35150 *flhDC* operon with the *flhC* F126A mutation; operon is inserted into the cloning vector at the EcoRV site. | SRM11A/SRM20 and SRM51/SRM59 |
| pSM13 | pBluescript SK(−) containing the 35150 *flhDC* operon with the *flhC* V127A mutation; operon is inserted into the cloning vector at the EcoRV site | SRM11A/SRM20 and SRM51/SRM60 |
| pSM14 | pBluescript SK(−) containing the 35150 *flhDC* operon with the *flhC* E128A mutation; operon is inserted into the cloning vector at the EcoRV site. | SRM11A/SRM20 and SRM51/SRM61 |
| pSM15 | pBluescript SK(−) containing the 35150 *flhDC* operon with the *flhC* S129A mutation; operon is inserted into the cloning vector at the EcoRV site. | SRM11A/SRM20 and SRM51/SRM62 |

[a] NA, not applicable.

moter adjacent to and transcribed in the same direction as the $Tc^r$ promoter, and pSM9 and pSM10 had the 493-89:*flhDC* operon inserted in opposite orientations, with pSM9 having the *flhDC* native promoter adjacent to and transcribed in the same direction as the $Tc^r$ promoter while pSM10 contained the insert in the opposite orientation.

**Constructs for site-directed mutagenesis repair.** The plasmid construct used to repair the 12-bp deletion in 493-89 *flhC* was made using a two-round PCR technique (25) that introduced site-specific mutations in target sequences. In the first-round PCR, ~150 ng of the pSM9 construct, carrying the 493-89 *flhDC*, was amplified by *Vent* polymerase in two separate reactions, one using the primer set SRM52 and SRM11A and the other using SRM20 and SRM51. Both 32-cycle PCRs were performed as described above using 54°C for 45 s for annealing, but the primer extensions at 75°C were for 25 s and 1.5 min for the SRM52 plus SRM11A and SRM20 plus SRM51 reactions, respectively. Products from each reaction mixture were purified from a 1% TBE agarose gel, mixed, and used as templates in the second-round PCR, which was performed using the SRM20 plus SRM11A primer pair and a 75°C for 1.5 min primer extension. The properly sized amplicon was isolated from a 1% agarose gel, ligated into an EcoRV-digested, phosphatase-treated pBluescript SK(−) vector, and transformed into DH5α. Plasmids from $Amp^r$ clones were screened by PCR for the presence of the insert using the vector-specific T3 and T7 promoter primers and for the absence of the 12-bp *flhC* deletion using deletion-specific PCR primers (see below). The construct in which the 12-bp deletion was repaired, as verified by sequencing, was designated pSM11.

The two-round PCR technique was also used to individually alter to the neutral amino acid alanine each of the four amino acids (phenylalanine 126, valine 127, glutamate 128, and serine 129) in the 35150 *flhC* open reading frame (ORF) region that are affected by the 12-bp deletion in 493-89. In the first-round

TABLE 3. Primer sequences

| Primer | Sequence (5′ → 3′) | Gene | Nucleotides |
|---|---|---|---|
| F-FLIC1 | CCA TGG CAC AAG TCA TTA ATA CCA AC | *fliC* | +1–24 |
| R-FLIC2 | CTA ACC CTG CAG CAG AGA CA | *fliC* | +1758–1739 |
| FliC2 | CGT CAT TCG CAC CAA CCT G | *fliC* | +457–439 |
| SRM11A | ACT GTA CCG AGA ACA ACC AGG | *flhDC* | 167–147 downstream of *flhC* stop |
| SRM16 | GTT GTA TGT GCG TGT AGT GAC G | *flhDC* | −175–154 |
| SRM17 | TCA CCC TGG ATG CTG TAG | pACYC184 | 1621–1638 (X06403) |
| SRM18 | CGG TCG GAC AGT GCT C | pACYC184 | 1786–1771 (X06403) |
| SRM19 | TGG TGC GGT TTG TTG AAA G | *flhC* | +368–386 |
| SRM20 | GAT CTG CAT CAC GCA TTA TTG | *flhDC* | −270–250 |
| SRM51 | CAG CAA CCA AGA CTC TGA CCA TGA CAG GAT GTT CAG | *flhC* | 126–106 downstream of *flhC* stop |
| SRM52 | CCT GGA CAT TGG TGC GGT TTG TTG AAA GTG GAT TAC TGC AAC TTT CC | *flhC* | +359–394 |
| SRM59 | TGG TGC GGG CTG TTG AAA G | *flhC* | +368–386 |
| SRM60 | GTG CGG TTT GCT GAA AGT GG | *flhC* | +370–389 |
| SRM61 | CGG TTT GTT GCA AGT GGA TTA C | *flhC* | +373–394 |
| SRM62 | GGT TTG TTG AAG CTG GAT TAC TGC | *flhC* | +374–397 |
| SRM86 | GCT AGT TGC TAA CCT AAC GGC T | 16S rDNA | +232–253 (Z83205) |
| SRM87 | GTG GAC TAC CAG GGT ATC TAA TC | 16S rDNA | +793–771 (Z83205) |
| SRM145 | CTG CTG GCA TTA ACC CTG G | *flhC* | +340–358 |
| SRM146 | CTG CCA ACA GGC TGG TGA G | *flhC* | +461–443 |

PCR, pSM7 (~100 ng) was amplified with the primer pair SRM20 and SRM51 and also with the primer SRM11A in combination with each of the mutation-specific primers, SRM59 (F126A), SRM60 (V127A), SRM61 (E128A), and SRM62 (S129A). Reactions were set up as described above, except that a 51°C annealing temperature was used. All amplicons were gel purified, mixed, and used in the second-round PCR with primer pair SRM11A and SRM20. Amplicons were cloned into pBluescript SK(−), screened by PCR, and sequenced to confirm that the expected site-specific alteration had been introduced. These 35150 *flhC* constructs were designated pSM12 (FlhC F126A), pSM13 (FlhC V127A), pSM14 (FlhC E128A), and pSM15 (FlhC S129A).

**DNA sequencing.** All DNA sequencing was done double-stranded by Amplicon Express (Pullman, Wash.), using target-specific primers and the Big Dye chemistry with an Applied Biosystems 377 automated sequencer.

**PCR analysis for the 12-bp deletion in *flhC*.** The presence or absence of the 12-bp *flhC* deletion in various strains was examined by PCR. The SRM19 primer, whose 3′ terminus is complementary to the 12 nucleotides deleted in 493-89, with SRM11A generates a 379-bp amplicon only from templates that do not contain the *flhC* deletion. The 50-μl reaction mixture contained 1× *Taq* polymerase buffer, 2.5 mM $MgCl_2$, 200 μM dNTP, and a 300 nM concentration of the SRM11A and SRM19 primers, as well as a 100 nM concentration of each of the 16S ribosomal DNA gene-specific primers (SRM86 and SRM87) and ~500 ng of DNA template. The reaction mixture was heated at 95°C for 10 min, during which 2.5 U of *Taq* DNA polymerase was added. Amplification was done with 32 cycles, each consisting of 95°C for 1 min, 60°C for 45 s, and 72°C for 1 min, and was terminated with a 72°C 7-min incubation. Those strains that generated the 562-bp amplicon from the 16S ribosomal DNA but did not yield the 379-bp *flhC* amplicon presumably carried the 12-bp *flhC* deletion.

The strains that tentatively carried the deletion were further tested by PCR with primers SRM145 and SRM146, which flank the 12-bp *flhC* deletion, to ascertain the size of the deletion. The reaction mix contained 1× *Taq* PCR buffer (Qiagen), 2 mM $MgCl_2$, 200 μM dNTP, a 300 nM concentration of each primer, and 0.5 U of HotStar *Taq* DNA polymerase (Qiagen). After 15 min of enzyme activation at 95°C, amplification was done for 30 cycles, each consisting of 95°C for 30 s, 59°C for 30 s, and 72°C for 15 s, and terminated with a 72°C, 7-min incubation. PCR products were examined on a 5% TBE NuSieve (FMC, Rockland, Maine) agarose gel. The expected sizes of amplicons from templates containing and not containing the deletion were 122 and 110 bp, respectively.

**Western blotting.** To verify flagellin protein expression, isolates were examined by Western blotting with the 15D8 monoclonal antibody that is specific for enteric flagella (15) and with anti-H7 polyclonal sera (BD Diagnostic Systems) to confirm H7 antigen production. Cell suspensions from overnight cultures grown in TSB were extracted by boiling, fractionated by discontinuous (3% stack-10% separating) sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose paper, and probed with 15D8 (1:2,000) or anti-H7 antibody (1:1,500), as described previously (12).

**Electron microscopy.** The surfaces of NM and motile cells were examined for the presence of flagella by negative staining. Cells from TSA plates were suspended to a density of about $10^9$ cells/ml in 2% phosphotungstic acid (pH 6.8), and 15-μl aliquots were applied to the surface of a carbon-coated, 300-mesh copper grid and allowed to settle for 1 min. After removing the excess liquid, the specimen grids were examined at either ×37,000 or ×79,000 magnification using a Philips 400 transmission electron microscope at an accelerating voltage of 80 kV.

**Nucleotide sequence accession number.** The nucleotide sequence of the *flhDC* operon of 493-89 was determined from pSM6 and deposited in GenBank (accession no. AY225162).

## RESULTS

**Characterization of bacterial strains.** All of the O157:H7 strains and their NM variants used in this study were genetically characterized and confirmed to carry EHEC trait markers and virulence genes (Table 1). An exception was strain 7123, which had none of the markers. This strain had previously been found to cluster with an O157:H16 strain (13) and was suspected to be an O157 NM strain that has other than the H7 antigen (12). Two German SF O157 strains (CB1009 and 210-89) had lost the $stx_2$ gene but carried all the other EHEC markers (Table 1). Each strain was serologically typed, and the NM strains were confirmed to be NM after repeated passages on motility soft agar stabs.

**Sequencing of *fliC*.** The 5′ region of the *fliC* gene from strains 35150 (pSM1), 493-89 (pSM2), and CB569 (pSM3) were double-strand sequenced with primers F-FLIC1 and Flic2. The sequences for all three strains were consistent with that of the O157:H7 *fliC* sequence previously deposited in GenBank (accession no. AE005415), indicating that the frameshift mutation reported (30) to exist in this region of 493-89 *fliC* was not present.

**Complementation studies.** Analysis of strain 493-89 with a flagellin-specific monoclonal antibody showed that it did not produce flagellin (data not shown). In contrast, 493-89 transformed with pSM4, which carries the 35150 *fliC* gene expressed from a strong $\sigma^{70}$ vector promoter, produced flagellin as determined by Western blotting of cell lysates (data not shown), although it did not react with anti-H7 latex reagent and remained NM.

Previously, pBluescript KS:*Yersinia enterocolitica fliA* and pACYC184-*Y. enterocolitica flhDC* were shown to complement respective mutations in *Salmonella enterica* serovar Typhimurium. Given the relatedness of these loci in *E. coli* and *Salmonella* (3), we attempted to restore motility to 493-89 by complementation using these same constructs. Strain 493-89 transformed with the *fliA* construct failed to express flagellin or regain motility. But, when transformed with the *flhDC* construct, 493-89 produced flagellin, became motile, and reacted with anti-H7. Similarly, complementation with pSM7 and pSM8, which carried the *flhDC* operon of motile *E. coli* strains 35150 and DEC5A, respectively, also restored motility and H7 antigenicity to 493-89, and examination by electron microscopy confirmed that the cells produced flagella (Fig. 2C and D). In contrast, transformation of 493-89 with its own *flhDC* operon, cloned in either orientation (pSM9 or pSM10), did not restore motility.

**Sequencing of *flhDC*.** The DNA sequence of the 35150 *flhDC* operon obtained from pSM5 was identical to the annotated sequence for this region previously submitted to GenBank for O157:H7 (accession no. AE005411) and was ~99% homologous to that of the *E. coli* K-12 *flhDC* locus (accession no. D90831) (Fig. 3). Comparing the sequences of the individual genes in the operon showed that the 35150 *flhD* ORF sequence had several nucleotide substitutions that differed from that of K-12. However, all except one of these mutations were silent, and the single nucleotide difference that resulted in an amino acid change is probably insignificant in terms of motility, since both K-12 and 35150 are motile and the FlhD proteins of both of these strains shared greater than 99% amino acid homology. Similarly, although the 35150 *flhC* ORF had three nucleotides that differed from that of K-12, none resulted in any alteration of the amino acid sequence, and the two predicted protein sequences showed 100% identity.

The nucleotide sequence of the *flhDC* operon of 493-89 was determined from pSM6 (accession no. AY225162). The sequences of the 5′ untranslated region containing the native promoter for the *flhDC* operon were found to be identical in both 493-89 and 35150; however, there were two sites within the *flhDC* coding sequences where differences occurred. The first, located within the *flhD* ORF, was a single-nucleotide polymorphism at position +239 (assuming the GTG start

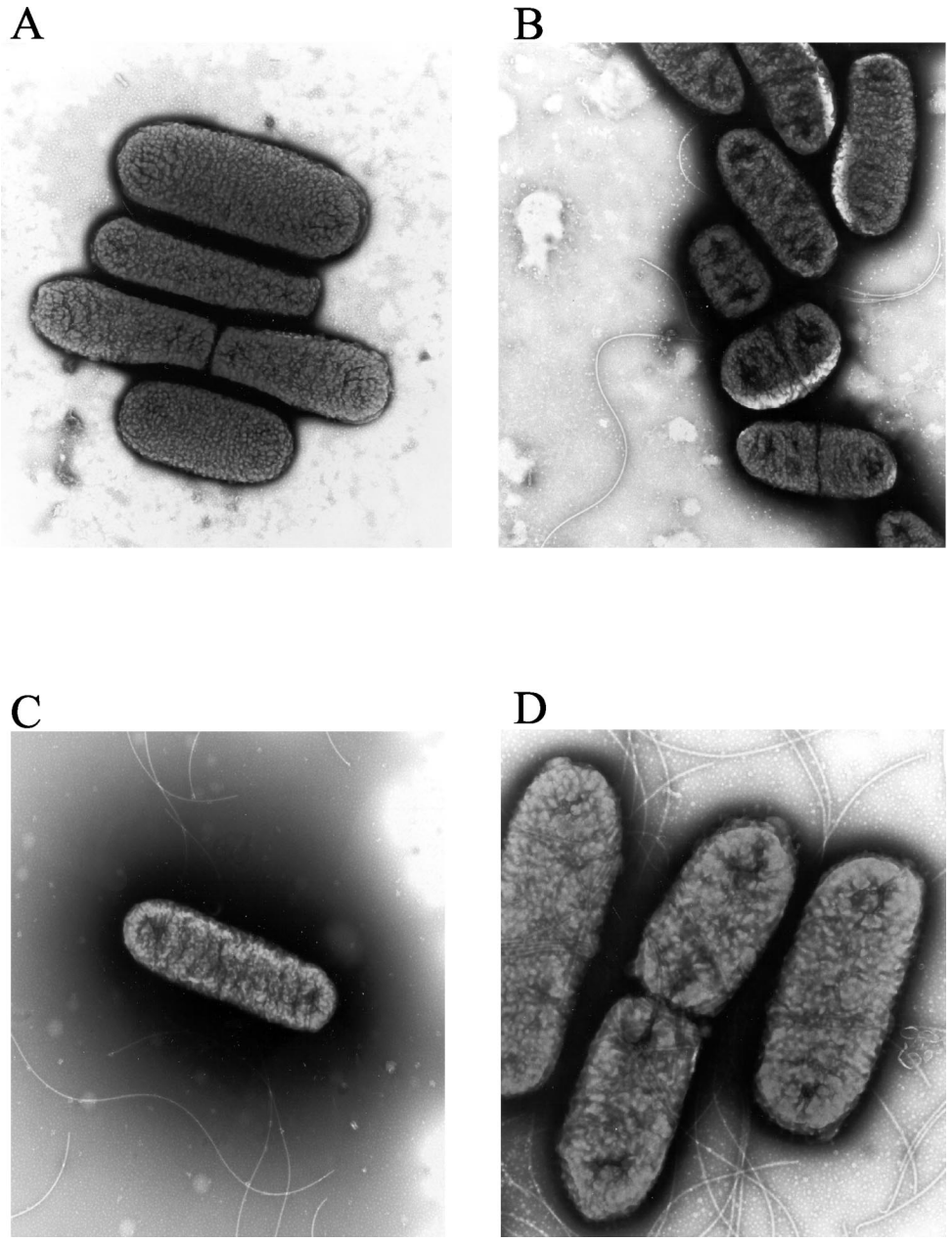


FIG. 2. Electron micrographs showing the presence or absence of flagellum synthesis. The strains shown are NM 493-89 (A), motile 35150 (B), 493-89 strain transformed with pSM7 (C), and 493-89 strain transformed with pSM8 (D).

codon as defined in GenBank accession no. AE005411) that caused an L80Q amino acid substitution in the 35150 FlhD. The second mutation site where the 493-89 sequence differed from that of 35150 was the presence of an in-frame, 12-bp deletion within the 493-89 *flhC* ORF at +374 to 385 that caused the loss of phenylalanine, valine, glutamate, and serine at positions 126 to 129, respectively.

**Site-directed mutagenic repair of *flhC* complements motility.** Transformation of strain 493-89 with pSM11, which carried the 493-89 *flhDC* operon with the 12-bp deletion in *flhC* repaired, restored motility and H7 antigenicity to 493-89.

To specifically characterize the effect of the 12-bp *flhC* deletion on motility, we prepared plasmid constructs in which the *flhC* sequences for the four amino acids encoded by the 12-bp region were individually replaced by the codon for the neutral amino acid alanine. Western blot analysis of 493-89 strains transformed with each of these constructs revealed that pSM13 (FlhC V127A), pSM14 (FlhC E128A), and pSM15 (FlhC S129A) restored H7 flagellin expression (Fig. 4, lanes 3, 4, and 5, respectively) and motility, as confirmed by both microscopic and motility plate assays. These strains also reacted with anti-H7 latex reagent, confirming that H7 antigen was produced. In contrast, 493-89 transformed with pSM12 (FlhC F126A) remained NM and had no flagellin expression (Fig. 4, lane 2),

```
          1                        -10                              50
35150     TATGTGCGTG TAGTGACGAG TACAGTTGCG TCGATTTAGG AAAAATCTTA
  K12     TATGTGCGTG TAGTGACGAG TACAGTTGCG TCGATTTAGG AAAAATCTTA
493-89    TATGTGCGTG TAGTGACGAG TACAGTTGCG TCGATTTAGG AAAAATCTTA

          51                                                  100
35150     GATAAGTGTA AAGACCCCCT TCTATTTGTA AGGACTTATT AAACCAAAAA
  K12     GATAAGTGTA AAGACCCATT TCTATTTGTA AGGACATATT AAACCAAAAA
493-89    GATAAGTGTA AAGACCCCCT TCTATTTGTA AGGACTTATT AAACCAAAAA

          101                                                 150
35150     GGTGGCTCTG CTTATTGCAG CTTATCGCAA CTATTCTAAT GCTAATTATT
  K12     GGTGGTTCTG CTTATTGCAG CTTATCGCAA CTATTCTAAT GCTAATTATT
493-89    GGTGGCTCTG CTTATTGCAG CTTATCGCAA CTATTCTAAT GCTAATTATT

          151                                                 200
35150     TTTTACCGGG GCTTCCCGGC GACATCACGG GGTGCGGTGA AACCGCATAA
  K12     TTTTACCGGG GCTTCCCGGC GACATCACGG GGTGCGGTGA AACCGCATAA
493-89    TTTTACCGGG GCTTCCCGGC GACATCACGG GGTGCGGTGA AACCGCATAA

          201                      flhD start                 250
35150     AAATAAAGTT GGTTATTCTG GGTGGGAATA ATGCATACCT CCGAGTTGCT
  K12     AAATAAAGTT GGTTATTCTG GGTGGGAATA ATGCATACCT CCGAGTTGCT
493-89    AAATAAAGTT GGTTATTCTG GGTGGGAATA ATGCATACCT CCGAGTTGCT

          251                                                 300
35150     GAAACACATT TATGACATCA ACTTGTCATA TTTACTACTT GCACAGCGTC
  K12     GAAACACATT TATGACATCA ACTTGTCATA TTTACTACTT GCACAGCGTT
493-89    GAAACACATT TATGACATCA ACTTGTCATA TTTACTACTT GCACAGCGTC

          301                                                 350
35150     TGATTGTTCA GGACAAAGCG TCCGCTATGT TTCGTCTCGG CATAAATGAA
  K12     TGATTGTTCA GGACAAAGCG TCCGCTATGT TTCGTCTCGG CATAAATGAA
493-89    TGATTGTTCA GGACAAAGCG TCCGCTATGT TTCGTCTCGG CATAAATGAA

          351                                                 400
35150     GAAATGGCGA CAACATTAGC GGCACTGACT CTTCCGCAAA TGGTTAAGCT
  K12     GAAATGGCGA CAACGTTAGC GGCACTGACT CTTCCGCAAA TGGTTAAGCT
493-89    GAAATGGCGA CAACATTAGC GGCACTGACT CTTCCGCAAA TGGTTAAGCT

          401                                                 450
35150     GGCAGAAACC AATCAGCTGG TTTGTCACTT CCGTTTTGAC AGCCACCAGA
  K12     GGCAGAAACC AATCAACTGG TTTGTCACTT CCGTTTTGAC AGCCACCAGA
493-89    GGCAGAAACC AATCAGCTGG TTTGTCACTT CCGTTTTGAC AGCCACCAGA

          451                                                 500
35150     CGATTACTCT GTTGACGCAA GATTCCCGCG TTGACGATCT CCAGCAAATT
  K12     CGATTACTCA GTTGACGCAA GATTCCCGCG TTGACGATCT CCAGCAAATT
493-89    CGATTACTCA GTTGACGCAA GATTCCCGCG TTGACGATCT CCAGCAAATT

          501                                                 550
35150     CATACCGGCA TCATGCTCTC AACACGCTTG CTGAATGATG TTAATCAGCC
  K12     CATACCGGCA TCATGCTCTC AACACGCTTG CTGAATGATG TTAATCAGCC
493-89    CATACCGGCA TCATGCTCTC AACACGCTTG CTGAATGATG TTAATCAGCC

          551                                    flhC start   600
35150     TGAAGAAGCG CTGCGCAAGA AAAGGGCCTG ATCATGAGTG AAAAAAGCAT
  K12     TGAAGAAGCG CTGCGCAAGA AAAGGGCCTG ATCATGAGTG AAAAAAGCAT
493-89    TGAAGAAGCG CTGCGCAAGA AAAGGGCCTG ATCATGAGTG AAAAAAGCAT
                                       flhD stop
          601                                                 650
35150     TGTTCAGGAA GCGCGGGATA TTCAGCTGGC AATGGAATTG ATCACCCTGG
  K12     TGTTCAGGAA GCGCGGGATA TTCAGCTGGC AATGGAATTG ATCACCCTGG
493-89    TGTTCAGGAA GCGCGGGATA TTCAGCTGGC AATGGAATTG ATCACCCTGG

          651                                                 700
35150     GCGCTCGTTT GCAGATGCTG GAAAGCGAAA CACAGTTAAG TCGCGGACGC
  K12     GCGCTCGTTT GCAGATGCTG GAAAGCGAAA CACAGTTAAG TCGCGGACGC
493-89    GCGCTCGTTT GCAGATGCTG GAAAGCGAAA CACAGTTAAG TCGCGGACGC

          701                                                 750
35150     CTGATAAAAC TTTATAAAGA ACTGCGCGGA AGCCCACCGC CGAAAGGCAT
  K12     CTGATAAAAC TTTATAAAGA ACTGCGCGGA AGCCCACCGC CGAAAGGCAT
493-89    CTGATAAAAC TTTATAAAGA ACTGCGCGGA AGCCCACCGC CGAAAGGCAT

          751                                                 800
35150     GCTGCCATTC TCAACCGACT GGTTTATGAC TTGGGAACAA AACGTTCATG
  K12     GCTGCCATTC TCAACCGACT GGTTTATGAC GTGGGAACAA AACGTTCATG
493-89    GCTGCCATTC TCAACCGACT GGTTTATGAC TTGGGAACAA AACGTTCATG

          801                                                 850
35150     CTTCGATGTT CTGTAATGCA TGGCAGTTTT TACTGAAAAC CGGTTTGTGT
  K12     CTTCGATGTT CTGTAATGCA TGGCAGTTTT TACTGAAAAC CGGTTTGTGT
493-89    CTTCGATGTT CTGTAATGCA TGGCAGTTTT TACTGAAAAC CGGTTTGTGT

          851                                                 900
35150     AATGGCGTCG ATGCGGTGAT CAAAGCCTAC CGTTTATACC TTGAACAGTG
  K12     AATGGCGTCG ATGCGGTGAT CAAAGCCTAC CGTTTATACC TTGAACAGTG
493-89    AATGGCGTCG ATGCGGTGAT CAAAGCCTAC CGTTTATACC TTGAACAGTG
```

FIG. 3. Comparison of *flhDC* sequences from *E. coli* K-12, 35150 (O157:H7), and German SF O157 strain 493-89 (O157:H$^-$). Dissimilarities are boxed and indicated with arrows. The −10 promoter site and ORF start and stop codons are in boxes.

```
          901                                                 950
35150     CCCGCAAGCA GAAGAAGGAC CGCTGCTGGC ATTAACCCGT GCCTGGACAT
  K12     CCCACAAGCA GAAGAAGGAC CACTGCTGGC ATTAACCCGT GCCTGGACAT
493-89    CCCGCAAGCA GAAGAAGGAC CGCTGCTGGC ATTAACCCGT GCCTGGACAT

          951                                                1000
35150     TGGTGCGGTT TGTTGAAAGT GGATTACTGC AACTTTCCAG CTGCAACTGC
  K12     TGGTGCGGTT TGTTGAAAGT GGATTACTGC AACTTTCCAG CTGCAACTGC
493-89    TGGTGC.... ........GT GGATTACTGC AACTTTCCAG CTGCAACTGC

          1001                                               1050
35150     TGCGGCGGCA ATTTTATTAC CCACGCTCAC CAGCCTGTTG GCAGCTTTGC
  K12     TGCGGCGGCA ATTTTATTAC CCACGCTCAC CAGCCTGTTG GCAGCTTTGC
493-89    TGCGGCGGCA ATTTTATTAC CCACGCTCAC CAGCCTGTTG GCAGCTTTGC

          1051                                               1100
35150     CTGCAGCTTA TGTCAACCGC CATCCCGGGC AGTAAAAAGA CGTAAACTTT
  K12     CTGCAGCTTA TGTCAACCGC CATCCCGGGC AGTAAAAAGA CGTAAACTTT
493-89    CTGCAGCTTA TGTCAACCGC CATCCCGGGC AGTAAAAAGA CGTAAACTTT

          1101                                               1150
35150     CCCAGAATCC TGCCGATATT ATCCCACAAC TGCTGGATGA ACAGAGAGTA
  K12     CCCAGAATCC TGCCGATATT ATCCCACAAC TGCTGGATGA ACAGAGAGTA
493-89    CCCAGAATCC TGCCGATATT ATCCCACAAC TGCTGGATGA ACAGAGAGTA

          1151     1162
35150     CAGGCTGTTT AA
  K12     CAGGCTGTTT AA
493-89    CAGGCTGTTT AA
          flhC stop
```

indicating that phenylalanine 126 in the primary protein sequence is required for functional FlhC activity.

**The 12-bp deletion in *flhC* is clonal for the German SF O157 strains.** The distribution of the 12-bp *flhC* deletion was examined in other *E. coli* and O157:NM strains obtained from various geographic areas. The 379-bp PCR product was obtained from the two motile O157:H7 strains and a motile O55:H7 strain, as well as other O157:NM variants, indicating that none of these carried the 12-bp deletion in *flhC*. However, analysis of the other German SF O157 strains, CB569, CB1009, 5412, 514-91, and 210-89, demonstrated that none of them produced the 379-bp PCR product, indicating that these strains, like 493-89, also carried a defect in the *flhC* 12-bp deletion region that inhibited binding of the SRM19 primer (data not shown).

Subsequent PCR analysis with primers flanking the deletion site showed that each of the German SF O157 strains tested

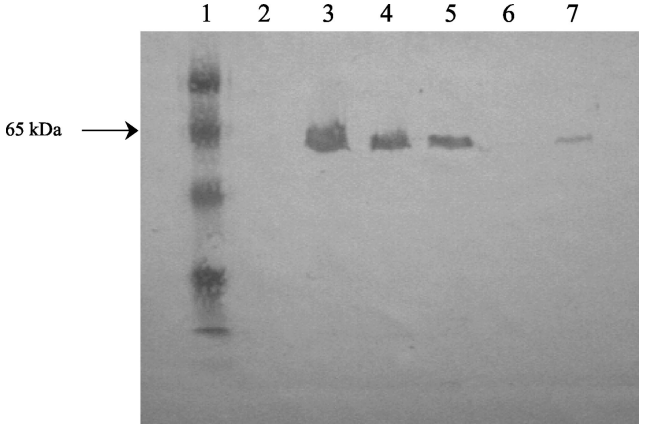


FIG. 4. Western blot analysis with anti-H7 polyclonal sera to examine H7 flagellin expression in 493-89 strains transformed with 35150 *flhDC* site-directed mutant constructs. Lanes: 1, low-molecular-mass standard (Bio-Rad); 2 to 6, 493-89 transformed with pSM12, pSM13, pSM14, pSM15, and untransformed, respectively; 7, 35150.

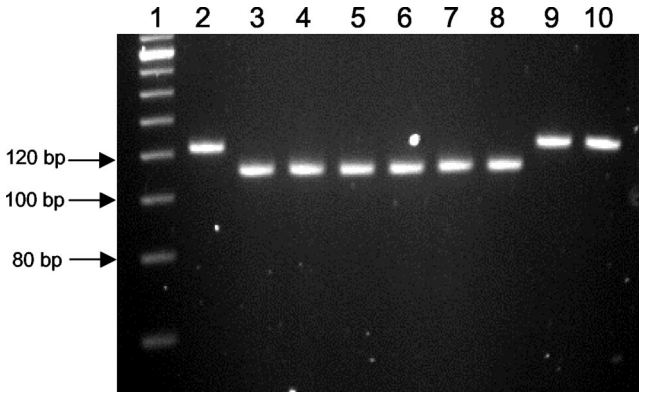


FIG. 5. Agarose gel electrophoresis of PCR products from primers SRM145 and SRM146 that flank the 12-bp *flhC* deletion. Strains generating the 122-bp amplicon do not contain the 493-89 *flhC* mutation, while those producing a 110-bp product do. Lanes: 1, 20-bp molecular-weight ladder; 2, 35150; 3, 493-89; 4, CB569; 5, CB1009; 6, 5412; 7, 514-91; 8, 210-89; 9, DEC5D; 10, 43890.

had the same size deletion as noted in 493-89 (Fig. 5, lanes 3 to 8), and sequence analysis confirmed the presence of the same 12-bp *flhC* deletion in each of the German SF O157 strains (data not shown). The results of the PCR deletion analysis are summarized in Table 1.

## DISCUSSION

The pathogenic German SF O157 strains are not easily identified, due to their atypical phenotypes and the lack of motility and H7 antigen expression. Previously, the nonmotility of the German SF O157 strain was postulated to be due to a frameshift mutation caused by two single-site nucleotide insertions at +73 and +78 of the *fliC* gene (30). We sequenced that region of the *fliC* ORF encompassing the putative mutations (from +1 to +458) of strain 493-89 and the other German SF O157 strains and found that, in all cases, the sequences in the purported frameshift region were identical to the *fliC* sequence derived from 35150, a motile O157:H7 strain, confirming that no insertion mutations were present. Consistent with this finding, 493-89 transformed with the functional 35150 *fliC* gene did not regain motility despite the fact that Western blot analysis of whole-cell lysates showed that the flagellin protein was made. The fact that flagellin production alone, being expressed from the vector Trc promoter, did not lead to flagellin secretion and assembly, as evidenced by the lack of serological H7 antigenicity, suggests that the factor causing nonmotility in 493-89 is epistatic to *fliC*.

In the three-tiered flagellar regulatory system, the class III *fliC* gene is regulated by class II genes, which include *flgM, fliA*, and the genes that encode the structural proteins for the basal body and hook (24). The *fliA* gene encodes the sigma F ($\sigma^F$) RNA polymerase subunit that is required for promoter recognition of class III genes, while FlgM (anti-$\sigma^F$) directly antagonizes FliA. In the normal sequence of events, when the basal body-hook structure is assembled, FlgM is secreted and its dilution from the cytosol releases FliA, enabling class III gene expression and flagellin production from *fliC*. In the absence of basal body-hook assembly, FlgM is not secreted and class III gene expression is repressed. We transformed 493-89 with a construct containing the *Y. enterocolitica fliA* gene, but the cells remained NM and did not express flagellin on Western blot analysis. Since the *fliA*-dependent expression of *fliC* did not occur in 493-89 transformed with a functional *Y. enterocolitica fliA* gene, we suspected that the defect was in another class II gene or, perhaps, in the class I *flhDC* operon, the transcriptional activator that serves as the master regulator of flagellum biosynthesis and regulates class II gene expression (10, 27, 28, 35).

Transformation of strain 493-89 with constructs carrying the *flhDC* operon from *Y. enterocolitica* or other motile *E. coli* strains (O157:H7 and O55:H7) restored motility to 493-89, confirming our earlier observation that this strain had a functional *fliC* gene. Furthermore, flagellum biosynthesis could be hyperexpressed when *flhDC* was cloned in the same transcriptional orientation as the vector $Tc^r$ promoter. Western blotting and examination by electron microscopy confirmed that flagella were synthesized by the motility-restored 493-89 strain, and serological typing confirmed that it was the H7 antigen. These results are consistent with the finding that the German SF O157 strains had the same H7 *fliC* restriction fragment length polymorphism profiles as the O157:H7 strains (16) and that these strains are closely related to the O157:H7 serotype (13, 21).

Comparison of the *flhDC* sequence cloned from strain 493-89 to the *flhDC* sequence from the motile O157:H7 strain (35150) showed no differences in the operon promoter region and only a single nucleotide change in the *flhD* ORF, which caused an L80Q substitution. However, a 12-bp deletion was found in the *flhC* ORF of 493-89 that was not present in the motile 35150 strain. Although the deletion was in frame, this 12-bp *flhC* deletion was confirmed to be responsible for the loss of motility, as 493-89 transformed with its own *flhDC* in which the 12-bp deletion had been repaired regained motility.

In the motile O157:H7 strain, the 12 bp covered in the *flhC* deletion of 493-89 encode phenylalanine, valine, glutamate, and serine. Complementation with constructs in which each of these amino acids was individually replaced with the codon specifying the neutral amino acid, alanine, restored motility and H7 antigen expression to 493-89, except for the one containing the F126A mutation. These results indicate that the phenylalanine at position 126 is essential for biological activity of FlhC. The *flhDC* operon encodes the FlhD and FlhC proteins, which initially associate to form $D_2$ and $C_2$ dimers that, subsequently, combine to form the $C_2D_2$ tetramer protein complex that regulates the class II genes (23). Whether phenylalanine 126 is essential for FlhC dimerization, the subsequent tetramerization with $FlhD_2$ or in DNA binding is currently unclear but should become more evident once the FlhDC crystal structure is resolved.

We looked at the distribution of the 12-bp *flhC* deletion among other O157:NM strains and found that it occurred only in the German SF O157 strains. The absence of the 12-bp *flhC* deletion in the other O157:NM strains indicates that nonmotility in these strains may be due to catabolite repression or other physiological mediators (24) or to other uncharacterized genetic defects. The finding that the 12-bp *flhC* deletion is specific to the German SF O157 strains coincides with the presence of plasmid-encoded fimbriae genes that are unique

only to this group (9), supporting previous reports that these strains are in a distinct clonal group (13, 21). The fact that the German SF O157 strains carry the H7 *fliC* gene (16) and can be manipulated to produce the H7 antigen also confirms their close genetic relation to O157:H7 (13, 21) and is in agreement with our evolution model that they diverged from O157:H7, in part, by the loss of motility (13, 26). As a consequence, the 12-bp *flhC* deletion provides a potentially useful marker with which strains in this clonal lineage may be identified and traced.

Regulation or loss of motility seems to be a common theme among gram-negative pathogens, as *Bordetella pertussis*, *Shigella* spp., and *Yersinia pestis* are all NM, yet each carries a full complement of flagellar genes (2, 11, 33). Interestingly, like our findings with the German SF O157 strains, these organisms also appear to be NM due to mutations in *flhDC* (2, 5, 32), which not only serves as the master flagellar control operon but also as a global regulator of numerous other operons (29). Other important pathogens, such as *Y. enterocolitica*, *Yersinia pseudotuberculosis*, *Bordetella bronchiseptica*, and *Legionella pneumophila* (18, 19, 22), show temperature-sensitive repression of flagellin expression, suggesting that there is a selection for motility repression in the host. The increase in the isolation of NM O157:H7 strains is consistent with this trend. All of these examples of pathogens require type III secretion systems (TTSS) for virulence. The process of flagellar assembly is also a subtype of TTSS (3), and there is evidence to suggest that the components of one TTSS are recognized by other TTSS in the cell (reference 35 and unpublished data). As a consequence, flagellar proteins may out-compete virulence type III proteins for export if both TTSS are simultaneously expressed. Consistent with this assumption, artificial expression of motility in *B. bronchiseptica* resulted in virulence attenuation (1), confirming the competitive interaction of the flagellar and virulence TTSS. Enteropathogenic *E. coli* and EHEC carry the locus for the enterocyte effacement pathogenicity island that encodes a TTSS that mediates the injection of virulence factors into the mammalian cell (34). These pathogens, therefore, also have multiple TTSS that can potentially interfere with one another and affect the expression of motility and/or virulence. As to whether the lack of motility in the German SF O157 strains offers any advantages in virulence or is a contributing factor to its increasing frequency in HUS infections remains to be determined.

In conclusion, nonmotility of the German SF O157 strains is due to a 12-bp deletion in the *flhC* gene of the *flhDC* master regulator operon. Specifically, phenylalanine 126 in FlhC appears to be the critical amino acid whose deletion resulted in an aberrant protein structure that could no longer function as a transcriptional activator for motility. The highly conserved nature of the 12-bp *flhC* deletion in the German SF O157 strains may be a useful marker for identifying these strains and also supports not only the clonality of the German SF O157 strains but also its postulated evolutionary divergence from O157:H7. Furthermore, this study has identified yet another pathogen that has selectively sustained a mutation that down regulates flagellar biosynthesis to, perhaps, eliminate competitive interactions between secretory pathways as well as the unnecessary expenditure of cellular energy, thereby resulting in a more virulent phenotype, a trend being more frequently observed among other bacterial pathogens.

## ACKNOWLEDGMENTS

We thank B. D. Tall and S. K. Curtis for assistance with electron microscopy. Special thanks go to Helge Karch for generously providing the strains used in this study. We also thank David Acheson for critical reading of the manuscript.

S. A. Minnich was supported by grant P20 RR164 54 from the BRIN Program from the National Center for Research Resources (National Institutes of Health).

## REFERENCES

1. **Akerley, B. J., P. A. Cotter, and J. F. Miller.** 1995. Ectopic expression of the flagellar regulon alters development of the *Bordetella*-host interaction. Cell **80:**611–620.
2. **Akerley, B. J., D. M. Monack, S. Falkow, and J. F. Miller.** 1992. The *bvgAS* locus negatively controls motility and the synthesis of flagella in *Bordetella brochiseptica*. J. Bacteriol. **174:**980–990.
3. **Aldridge, P., and K. T. Hughes.** 2002. Regulation of flagellar assembly. Curr. Opin. Microbiol. **5:**160–165.
4. **Aleksic, S., H. Karch, and J. Bockemuhl.** 1992. A biotyping scheme for Shiga-like (Vero) toxin-producing *Escherichia coli* O157 and a list of serological cross-reactions between O157 and other gram-negative bacteria. Zentbl. Bakteriol. **276:**221–230.
5. **Al Mamun, A. A., A. Tominaga, and M. Enomoto.** 1996. Detection and characterization of the flagellar master operon in the four *Shigella* subgroups. J. Bacteriol. **178:**3722–3726.
6. **Amann, E., B. Ochs, and K.-J. Abel.** 1988. Tightly regulated *tac* promoter vector useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene **69:**301–315.
7. **Bielaszewska, M., H. Schmidt, M. Karmali, R. Khakhria, J. Janda, K. Bláhová, and H. Karch.** 1998. Isolation and characterization of sorbitol-fermenting Shiga toxin (verocytotoxin)-producing *Escherichia coli* O157:H$^-$ strains in the Czech Republic. J. Clin. Microbiol. **36:**2135–2137.
8. **Bitzan, M., K. Ludwig, M. Klemt, H. Konig, J. Buren, and D. E. Muller-Wiefel.** 1993. The role of *Escherichia coli* O157 infections in the classical (enteropathic) haemolytic uraemic syndrome: results of a central European, multicentre study. Epidemiol. Infect. **110:**183–196.
9. **Brunder, W., A. Salam Khan, J. Hacker, and H. Karch.** 2001. Novel type of fimbriae encoded by the large plasmid of sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157:H$^-$. Infect. Immun. **69:**4447–4457.
10. **Chilcott, G. S., and K. T. Hughes.** 2000. Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. Microbiol. Mol. Biol. Rev. **64:**694–708.
11. **Deng, W., V. Burland, G. Plunkett III, A. Boutin, G. F. Mayhew, P. Liss, N. T. Perna, D. J. Rose, B. Mau, S. Zhou, D. C. Schwartz, J. D. Fetherston, L. E. Lindler, R. R. Brubaker, G. V. Plano, S. C. Straley, K. A. McDonough, M. L. Nilles, J. S. Matson, F. R. Blattner, and R. D. Perry.** 2002. Genome sequence of *Yersinia pestis* KIM. J. Bacteriol. **184:**4601–4611.
12. **Feng, P., P. I. Fields, B. Swaminathan, and T. S. Whittam.** 1996. Characterization of nonmotile *Escherichia coli* O157 and other serotypes by using an anti-flagellin monoclonal antibody. J. Clin. Microbiol. **34:**2856–2859.
13. **Feng, P., K. A. Lampel, H. Karch, and T. S. Whittam.** 1998. Sequential genotypic and phenotypic changes in the emergence of *Escherichia coli* O157:H7. J. Infect. Dis. **177:**1750–1753.
14. **Feng, P., and S. R. Monday.** 2000. Multiplex PCR for detection of trait and virulence factors in enterohemorrhagic *E. coli* serotypes. Mol. Cell Probes **14:**333–337.
15. **Feng, P., R. J. Sugasawara, and A. Schantz.** 1990. Identification of a common enterobacterial flagellin epitope with a monoclonal antibody. J. Gen. Microbiol. **136:**337–342.
16. **Fields, P. I., K. Blom, H. J. Hughes, L. O. Helsel, P. Feng, and B. Swaminathan.** 1997. Molecular characterization of the gene encoding H antigen in *Escherichia coli* and development of a PCR-RFLP test for the identification of *E. coli* O157:H7 and O157:NM. J. Clin. Microbiol. **35:**1066–1070.
17. **Gunzer, F., H. Bohm, H. Russmann, M. Bitzan, S. Aleksic, and H. Karch.** 1992. Molecular detection of sorbitol-fermenting *Escherichia coli* O157 in patients with hemolytic uremic syndrome. J. Clin. Microbiol. **30:**1807–1810.
18. **Heuner, K., B. C. Brand, and J. Hacker.** 1999. The expression of the flagellum of *Legionella pneumophila* is modulated by different environmental factors. FEMS Microbiol. Lett. **175:**69–77.
19. **Kapatral, V., J. W. Olson, J. C. Pepe, V. I. Miller, and S. A. Minnich.** 1996. Temperature-dependent regulation of *Yersinia enterocolitica* class III flagellar genes. Mol. Microbiol. **19:**1061–1071.
20. **Karch, H., and M. Bielaszewska.** 2001. Sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H$^-$ strains: epidemiology, phenotypic and

molecular characteristics, and microbiological diagnosis. J. Clin. Microbiol. **39:**2043–2049.
21. **Karch, H., H. Bohm, H. Schmidt, F. Gunzer, S. Aleksic, and J. Heesemann.** 1993. Clonal structure and pathogenicity of Shiga-like toxin-producing, sorbitol-fermenting *Escherichia coli* O157:H⁻. J. Clin. Microbiol. **31:**1200–1205.
22. **Leigh, A. F., J. G. Coote, R. Parton, and C. J. Duggleby.** 1993. Chromosomal DNA from both flagellate and non-flagellate *Bordetella* species contains sequences homologous to the *Salmonella* H1 flagellin gene. FEMS Microbiol. Lett. **111:**225–231.
23. **Liu, X., and P. Matsumura.** 1994. The FlhD/FlhC complex, a transcriptional activator of *Escherichia coli* flagellar class II operons. J. Bacteriol. **176:**7345–7351.
24. **Macnab, R. M.** 1987. Flagella, p. 70–83. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. ASM Press, Washington, D.C.
25. **Mikaelian, I., and A. Sergeant.** 1992. A general and fast method to generate multiple site directed mutations. Nucleic Acids Res. **20:**376.
26. **Monday, S. R., T. S. Whittam, and P. C. H. Feng.** 2001. Genetic and evolutionary analysis of mutations in the *gusA* gene that cause the absence of β-glucuronidase activity in *Escherichia coli* O157:H7. J. Infect. Dis. **184:**918–921.
27. **Mytelka, D. S., and M. J. Chamberlin.** 1996. *Escherichia coli fliAZY* operon. J. Bacteriol. **178:**24–34.
28. **Pruss, B. M., and P. Matsumura.** 1997. Cell cycle regulation of flagellar genes. J. Bacteriol. **179:**5602–5604.
29. **Pruss, B. M., J. W. Campbell, T. K. van Dyk, C. Zhu, Y. Kogan, and P. Matsumura.** 2003. FlhD/FlhC is a regulator of anaerobic respiration and the Entner-Doudoroff pathway through induction of the methyl-accepting chemotaxis protein Aer. J. Bacteriol. **185:**534–543.
30. **Reid, S. D., R. K. Selander, and T. S. Whittam.** 1999. Sequence diversity of flagellin (*fliC*) alleles in pathogenic *Escherichia coli*. J. Bacteriol. **181:**153–160.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Smith, M. J.** 2000. Ph.D. dissertation thesis. University of Idaho, Moscow.
33. **Tominaga, A., M. A.-H. Mahmoud, T. Mukalhara, and M. Enomoto.** 1994. Molecular characterization of intact, but cryptic, flagellin genes in the genus *Shigella*. Mol. Microbiol. **12:**277–285.
34. **Yona-Nadler, C., T. Umanski, S.-I. Aizawa, D. Friedberg, and I. Rosenshine.** 2003. Integration host factor (IHF) mediates repression of flagella in enteropathogenic and enterohemorrhagic *Escherichia coli*. Microbiology **149:**877–884.
35. **Young, G. M., M. J. Smith, S. A. Minnich, and V. L. Miller.** 1999. The *Yersinia enterocolitica* motility master regulatory operon *flhDC* is required for flagellin production, swimming motility, and swarming motility. J. Bacteriol. **181:**2823–2833.