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Dual *flaA1 flaB1* Mutant of *Serpulina hyodysenteriae* Expressing Periplasmic Flagella Is Severely Attenuated in a Murine Model of Swine Dysentery

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The motility imparted by the periplasmic flagella (PF) of *Serpulina hyodysenteriae* is thought to play a pivotal role in the enteropathogenicity of this spirochete. The complex PF are composed of multiple class A and class B polypeptides. Isogenic strains containing specifically disrupted *flaA1* or *flaB1* alleles remain capable of expressing PF, although such mutants display aberrant motility in vitro. To further examine the role that these proteins play in the maintenance of periplasmic flagellar structural integrity, motility, and fitness for intestinal colonization, we constructed a novel strain of *S. hyodysenteriae* which is deficient in both FlaA1 and FlaB1. To facilitate construction of this strain, a chloramphenicol gene cassette, with general application as a selectable marker in prokaryotes, was developed. The cloned *flaA1* and *flaB1* genes were disrupted by replacement of internal fragments with chloramphenicol and kanamycin gene cassettes, respectively. The inactivated flagellar genes were introduced into *S. hyodysenteriae*, and allelic exchange at the targeted chromosomal *flaA1* and *flaB1* loci was verified by PCR analysis. Immunoblots of cell lysates with antiserum raised against purified FlaA or FlaB confirmed the absence of the corresponding sheath and core proteins in this dual flagellar mutant. These mutations selectively abolished the expression of the targeted genes without affecting the synthesis of other immunologically related FlaB proteins. The resulting *flaA1 flaB1* mutant exhibited altered motility in vitro. Surprisingly, it was capable of assembling periplasmic flagella that were morphologically normal as evidenced by electron microscopy. The virulence of this strain was assessed in a murine model of swine dysentery by determining the incidence of cecal lesions and the persistence of *S. hyodysenteriae* in the gut. Mice challenged with the wild-type strain or a passage control strain showed a dose-related response to the challenge organism. The dual flagellar mutant was severely attenuated in murine challenge experiments, suggesting that the FlaA1 and FlaB1 proteins are dispensable for flagellar assembly but critical for normal flagellar function and colonization of mucosal surfaces of the gastrointestinal tract. This strain represents the first spirochete engineered to contain specifically defined mutations in more than one genetic locus.

Serpulina hyodysenteriae is an anaerobic spirochete that preferentially colonizes the cecal and colonic mucosal surfaces of the swine intestine (28) and causes a highly contagious mucohemorrhagic diarrheal disease of growing and finishing pigs (17, 18). Like gram-negative bacteria, it possesses a cytoplasmic membrane, peptidoglycan, and an outer membrane. *S. hyodysenteriae* also expresses flagellar filaments that, in contrast to the simple external flagella produced by many rod-shaped bacteria, wrap around the protoplasmic cylinder and are referred to as periplasmic flagella (PF) (21). The unique spiral morphology of the bacterium and the corkscrew-like motility imparted by the PF are thought to play a pivotal role in the enteropathogenicity of this spirochete by facilitating association with intestinal mucosal surfaces (28). Underscoring this hypothesis is the finding that *S. hyodysenteriae* is unable to attach directly or indirectly to the epithelium (28). Moreover, *S. hyodysenteriae* is chemotactic toward mucin (29, 39) and is highly motile in intestinal mucus from infected pigs (28). Such directed motility by *S. hyodysenteriae* results in its penetration of the viscous mucus gel and subsequent colonization of the relatively stagnant underlying regions, close to the epithelium and within the mucus-filled crypts of Lieberkühn (28). Mucosal association in the absence of direct or indirect adhesion to the epithelium proper would necessitate active motility in vivo because the mucus layer quickly eliminates unattached organisms from the gastrointestinal tract (4). Therefore, motility represents an important virulence factor as a mechanism by which *S. hyodysenteriae* may escape physical clearance from the intestine (16).

The motility apparatus of *S. hyodysenteriae* includes two bundles of periplasmic flagella which are attached subterminally to the ends of the protoplasmic cylinder and overlap in the middle of the cell (21). Unlike most bacterial flagella, which are composed of a single flagellar protein (flagellin), the PF of *S. hyodysenteriae* contain multiple protein species. The flagella are composed of at least three FlaB-related core proteins (FlaB1, FlaB2, and FlaB3) which share extensive immunological and N-terminal sequence homology with each other and with other spirochete flagellar core proteins (7, 9, 10, 32, 33). A sheath structure, unique to the spirochete genera *Leptospira*, *Treponema*, *Spirochaeta*, and *Serpulina* (9, 52), surrounds the flagellar core. The sheath appears to be composed of two class A proteins (FlaA1 and FlaA2) which are transported to the periplasmic space, where they subsequently polymerize around the core (33, 34). A model of PF structure and assembly based on similarities between *S. hyodysenteriae* and those of other spirochetes has been proposed (9).

To examine the role of the class A and class B polypeptides in flagellar synthesis and to gain insight into the importance of motility in pathogenesis, we previously constructed and char-

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acterized isogenic PF mutants of *S. hyodysenteriae* B204 which contained specifically disrupted *flaA1* or *flaB1* alleles (45). These singular mutants displayed an altered yet motile phenotype in vitro (45), were compromised in murine cecal colonization (27), and were rendered avirulent for swine (26, 46). Although these mutants no longer synthesized the corresponding flagellar FlaA1 or FlaB1 proteins, they remained capable of assembling PF that appeared morphologically normal by electron microscopy (45). To extend these observations and further examine the role of FlaA1 and FlaB1 peptides in the maintenance of PF structural integrity and fitness for intestinal colonization, we generated a single isogenic strain containing specifically targeted *flaA1* and *flaB1* mutations and assessed its virulence in a murine model of swine dysentery. The characterization of this dual *flaA1 flaB1* mutant and its virulence properties is reported here.

(A preliminary account of this work was reported at the 95th General Meeting of the American Society for Microbiology [47].)

MATERIALS AND METHODS

Bacterial strains, culture conditions, and inoculum preparation. *S. hyodysenteriae* A201 was reisolated from a pig exhibiting clinical signs of swine dysentery following challenge with *S. hyodysenteriae* B204 (ATCC 31212; serotype 2). This highly virulent strain was used to construct strains A205 (*flaB1*::*kan*) and A202 (isogenic passage control) as previously described (45). *S. hyodysenteriae* A205 and A202 were subsequently used to generate strain A206, a dual *flaA1*::*cat flaB1*::*kan* periplasmic flagellar mutant, and its appropriate isogenic passage control, strain A207, as described below.

S. hyodysenteriae was cultured at 38°C in either brain heart infusion broth or Trypticase soy broth supplemented with 10% (vol/vol) fetal bovine serum (BHI-FBS or TSB-FBS; HyClone Laboratories, Inc., Logan, Utah). The spirochete was grown under an anaerobic atmosphere (5% H_2, 10% CO_2, 85% N_2) in broth cultures or on solid plate medium consisting of Trypticase soy agar supplemented with 5% sheep blood (TSAB). When appropriate, liquid media contained chloramphenicol (10 mg/liter) or kanamycin (200 mg/liter). Selection of *S. hyodysenteriae* A206 on TSAB agar was done in the presence of 10 mg of chloramphenicol per liter and 200 mg of kanamycin per liter. For murine challenge experiments, *S. hyodysenteriae* wild-type, passage control, or flagellar mutant strains were harvested from 24-h cultures grown in TSB-FBS. Following centrifugation, the cells were resuspended in sterile saline at a concentration of $\sim 2 \times 10^8$/ml. This cell suspension was either directly inoculated into mice or further diluted to the appropriate inoculum dose in sterile saline.

Electroporation-mediated allelic exchange. The suicide delivery plasmids pER141 and pER199, which contain specifically inactivated *flaB1*::*kan* and *flaA1*::*cat* alleles, respectively, are schematically illustrated (see Fig. 2) and have been described previously (45). For transformation of the *flaB1*::*kan* progenitor, plasmid pER199 was introduced into *S. hyodysenteriae* A205 by electroporation essentially as reported previously (45). Briefly, a stock of *S. hyodysenteriae* A205 from −80°C storage was inoculated 1/40 into BHI-FBS at 38°C. After 40 h of growth, the seed culture was diluted 1/25 into fresh BHI-FBS, grown for an additional 12 h, and processed for electroporation. The culture (optical density at 520 nm, 0.49) was harvested (9,600 × *g* at 5°C for 10 min), resuspended in 1/10 volume of chilled (4°C) 0.5 M sucrose, and incubated on ice for 30 min. The cells were then harvested as above, resuspended in 1/50 volume of chilled 0.5 M sucrose, and maintained on ice until use.

Samples were prepared for electroporation as described for the generation of single-knockout mutants (45), except that 500 ng of pER199 plasmid DNA was added to 100 μl of competent *S. hyodysenteriae* A205 cells prior to delivery of the electropulse. Treated cells were recovered by addition of 0.5 ml of BHI-FBS containing 200 mg of kanamycin per liter to the cuvette followed by transfer to a screw-cap tube (16 by 150 mm) fitted with a 3/8-in. (0.95-cm) stir bar. The cells were maintained with stirring during an 8-h outgrowth period. Chloramphenicol (10 μg/ml) was then added for selection of electroporants. Following an additional 19 h of incubation, the culture was divided in half and plated onto TSAB containing 10 mg of chloramphenicol per liter plus 200 mg of kanamycin per liter. After a further 7-day incubation period, colonies were inoculated into 5 ml of BHI-FBS containing the above antibiotics and grown for 46 h; glycerol (17% vol/vol) was then added, and the mixture was stored at −80°C. In an analogous approach, an in vitro-passaged control strain (A207) was generated from *S. hyodysenteriae* A202, except that input plasmid was omitted from the electroporation mixture and all subsequent growth took place without antibiotic selection.

DNA manipulations, PCR, and oligonucleotides. Recombinant plasmids constructed by standard techniques (49) were maintained in *E. coli* DH5αF′I^q (23), HB101 (5), or XL1Blue (6). Chromosomal DNA was isolated from wild-type and mutant *S. hyodysenteriae* strains for use in PCR by resuspension of harvested cells in PCR lysis buffers A and B (49) and treatment with proteinase K. Following heat inactivation of proteinase K, PCR DNA amplification was performed with VENT DNA polymerase (New England Biolabs, Beverly, Mass.) supplemented with 6 mM $MgSO_4$ as recommended by the manufacturer. Amplification was carried out at 95°C for 3 min and was followed by 30 cycles of denaturation (95°C for 1 min), annealing (50°C for 1.5 min), and extension (72°C for 2 min) and then by a final 72°C extension for 7 min. For synthesis of longer products (>1.8 kb), cycle extension times were increased to 3.5 min.

Several oligonucleotide primers capable of differentiating the precise nature of allele replacement were used for amplification of regions encompassing *flaA1* or *flaB1*. The binding sites for these primers are schematically illustrated in Fig. 2. Oligonucleotides ER10 (5′-GGGGATCCTATGAAAAAGTTATTCGTAGTATTAACTTCC-3′) and ER11 (5′-GGGGATCCTGCTCATCTATACATATATATAAAGTATCATCTTTATTGATCTAAGG-3′) flank the *flaA1* gene, while ER13 (5′-GGGAAGAATTCATACAAATAATTCATGG-3′) and ER14 (5′-GCCATATCTGTATCACGAATTCTGC-3′) border the *flaB1* locus. Primer annealing sites for oligonucleotides ER12 (5′-CGCGGCCTCGAGCAAGACG-3′) and ER16 (5′-GATTAAAGATCTCTTTTCTCTTCC-3′) are unique to the inserted kanamycin and chloramphenicol antibiotic markers, respectively. Amplifications with primers ER10, ER11, and ER16 allowed verification of the *flaA1*::*cat* allele present in *S. hyodysenteriae* A206, while ER12, ER13, and ER14 were used to confirm the integrity of the *flaB1*::*kan* background mutation in the newly constructed dual flagellar mutant.

SDS-PAGE and immunodetection of flagellar proteins. Total cellular protein was standardized by centrifugation of an *S. hyodysenteriae* culture volume equivalent to 0.2 optical density unit at 520 nm and resuspension in sodium dodecyl sulfate (SDS) sample lysis buffer before being boiled and resolved in a discontinuous buffer system (36) with a 5% polyacrylamide stacking gel and a 12.5% polyacrylamide running gel. Proteins were visualized by Coomassie brilliant blue R-250 staining or were detected with rabbit antisera generated against the species-specific 44-kDa periplasmic flagellar sheath (FlaA1) protein (37) or a common 39-kDa periplasmic flagellar core (FlaB) protein from *S. hyodysenteriae* FM 88-90 (serotype 8) (38) as described previously (45). Molecular mass markers were either unstained (Broad Range SDS-PAGE Marker; New England BioLabs) or prestained (SeeBlue; Novex, San Diego, Calif.) when used in immunoblotting techniques.

Electron microscopy and assessment of in vitro motility. *S. hyodysenteriae* cells were grown in BHI-FBS to the late logarithmic phase, harvested, and resuspended in phosphate-buffered saline (PBS)–0.2% Triton X-100 before being fixed in PBS-1% glutaraldehyde. Treatment with 0.2% Triton X-100 resulted in selective disruption of the cellular outer membrane, thereby allowing direct visualization of exposed periplasmic flagella. Fixed samples were applied to Formvar-coated grids and blotted dry. For negative staining, filtered 0.1% uranyl acetate (pH 4) was applied to the grids and blotted dry before they were viewed on a JEOL 1200EX transmission electron microscope.

S. hyodysenteriae motility was measured as described previously (45). Briefly, the motility of *S. hyodysenteriae* A206 was compared with that of the wild type by direct examination of cells by phase-contrast microscopy at ×400 magnification. The motility of cells grown in TSB-FBS to the mid- to late logarithmic phase of growth also was examined by use of a modified capillary assay (2, 45) which tested the ability of the wild-type and mutant strains to move through 0.9% NaCl. Motility was expressed as the percentage of cells from a washed bacterial suspension that had entered 10-μl capillary tubes (Accupette pipettes; Dade Diagnostics). Each motility assay was conducted in triplicate on two separate days.

Experimental infection in mice. For virulence studies, 20- to 24-g female, specific-pathogen-free CF-1 mice (Harlan Sprague-Dawley, Madison, Wis.) were housed in wire cages with mesh bottoms and acclimated to environmentally controlled rearing facilities for 3 days before the initiation of each experiment. Mice were fed Purina mouse chow (item 5015) ad libitum and were treated with spectinomycin (dihydrochloride, 100 μg/ml; Sigma) in the water for 48 h before challenge to enhance their susceptibility to colonization. The mice were fasted by removal of mouse chow feed during an 18- to 24-h prechallenge and 1-h postchallenge period. Challenge was by gastric intubation and inoculation of 0.5 ml of a bacterial suspension in saline containing approximately 2×10^2, 2×10^4, 2×10^6, or 2×10^8 spirochetes per ml. Control mice were challenged with 0.5 ml of sterile saline. Spectinomycin-treated water was replaced with untreated water following the challenge.

For two separate experiments, mice ($n = 8$ per treatment dose) were necropsied at 1, 4, or 7 days postchallenge to evaluate gross cecal pathologic changes (gross lesions) and spirochetal colonization of the gut. The persistence of *S. hyodysenteriae* was assessed by culture of cecal swabs onto TSAB supplemented with spectinomycin (400 μg/ml), polymyxin B (25 μg/ml), and vancomycin (25 μg/ml) (TSAB-SPV). This medium is selective yet not inhibitory to wild-type *S. hyodysenteriae* (28). For recovery of strain A206, TSAB-SPV was further supplemented with 10 mg of chloramphenicol per liter and 200 mg of kanamycin per liter. Swabs of cecal contents from mice challenged with *S. hyodysenteriae* A206 were plated onto TSAB-SPV and TSAB-SPV containing 10 mg of chloramphenicol per liter and 200 mg of kanamycin per liter to verify the identity of recovered spirochetes. *S. hyodysenteriae* from mouse ceca was cultured in an anaerobic chamber (5% H_2, 10% CO_2, 85% N_2) for up to 5 days at 38°C. Ceca which did not support the recovery of spirochetes within this time were deemed culture negative and therefore not colonized by the *S. hyodysenteriae* challenge strain.

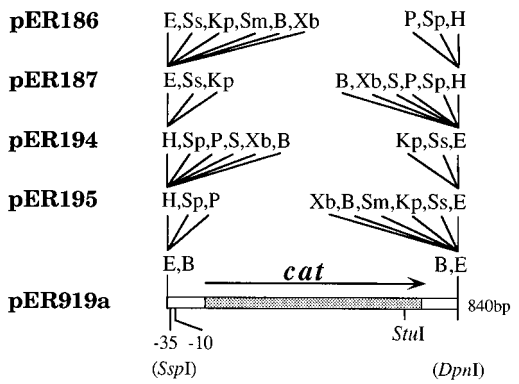

FIG. 1. Chloramphenicol resistance gene cassettes. The *cat* gene was obtained from the staphylococcal plasmid, pC194, and placed into pUC7, thereby generating symmetric restriction sites flanking the gene cassette in pER919a. Four additional derivatives were constructed by cloning into pUC18 (pER186 and pER187) and pUC19 (pER194 and pER195). The putative −35 and −10 regions of the native *cat* promoter are indicated. See the text for details. The *Ssp*I and *Dpn*I restriction enzyme sites were inactivated during cloning. Additional restriction sites are as follows: E, *Eco*RI; Ss, *Sst*I; Kp, *Kpn*I; Sm, *Sma*I; B, *Bam*HI; Xb, *Xba*I; S, *Sal*I; P, *Pst*I; Sp, *Sph*I; H, *Hin*dIII.

RESULTS

Chloramphenicol resistance cassettes of general utility in prokaryotes. We have recently identified and used a gene encoding chloramphenicol resistance to specifically inactivate the *flaA1* locus of *S. hyodysenteriae* B204 (45). The chloramphenicol acetyltransferase (*cat*) gene cassette was obtained from plasmid pER919a, which had been constructed (48) by insertion of an 840-bp *Ssp*I-*Dpn*I fragment (containing the chloramphenicol resistance determinant [nucleotides 1167 to 2006] of the staphylococcal plasmid, pC194 [22]) into the *Hin*cII restriction site of pUC7 (Fig. 1). Thus, this gene cassette is flanked by *Bam*HI and *Eco*RI restriction sites, is smaller than most antibiotic resistance cassettes, and can be readily utilized to specifically inactivate target loci in vitro. A unique *Stu*I restriction site located 25 bp from the carboxyl terminus of the *cat* open reading frame facilitates the verification of insert orientation during initial plasmid constructions. This gene cassette was further modified to improve its general utility for cloning by transfer of the 852-bp *Nla*IV fragment encoding chloramphenicol resistance from pER919a into the *Hin*cII and *Sma*I sites of pUC18 and pUC19 (Fig. 1).

Generation of a strain containing dual *flaA1* and *flaB1* mutations. The identification of a new antibiotic resistance marker of utility for gene disruption in *S. hyodysenteriae* made possible a directed approach to inactivation of two motility-associated genes in this spirochete. *S. hyodysenteriae* A206 was generated in a biphasic approach as outlined in Fig. 2. A progenitor, strain A205, in which the *flaB1* locus was inactivated by allelic exchange (45), thereby resulting in the replacement of an internal 589-bp *Bgl*II fragment with a *kan* gene cassette from pUC4K, was previously constructed. During construction of this strain, an additional in vitro passage control, strain A202, also was generated from the virulent parent by a parallel sham electroporation lacking input plasmid followed by growth without antibiotic selection (45).

S. hyodysenteriae A202 and A205 were further manipulated in the present study to yield a parallel in vitro passage control (strain A207) and a dual *flaA1 flaB1* flagellar mutant (strain A206), respectively. For construction of *S. hyodysenteriae* A206, the cloned *flaA1* gene was disrupted by replacement of an internal 169-bp *Nru*I-*Eco*RV fragment with the *cat* gene cassette from pER919a, resulting in pER199 (45). Plasmid pER199 was introduced into the *flaB1*::*kan* background strain; a period of recovery and phenotypic expression prior to selection of electrotransformants followed. Since a differentiating colonial morphology was not observed, candidate flagellar mutants which arose following growth on TSAB containing 10 mg

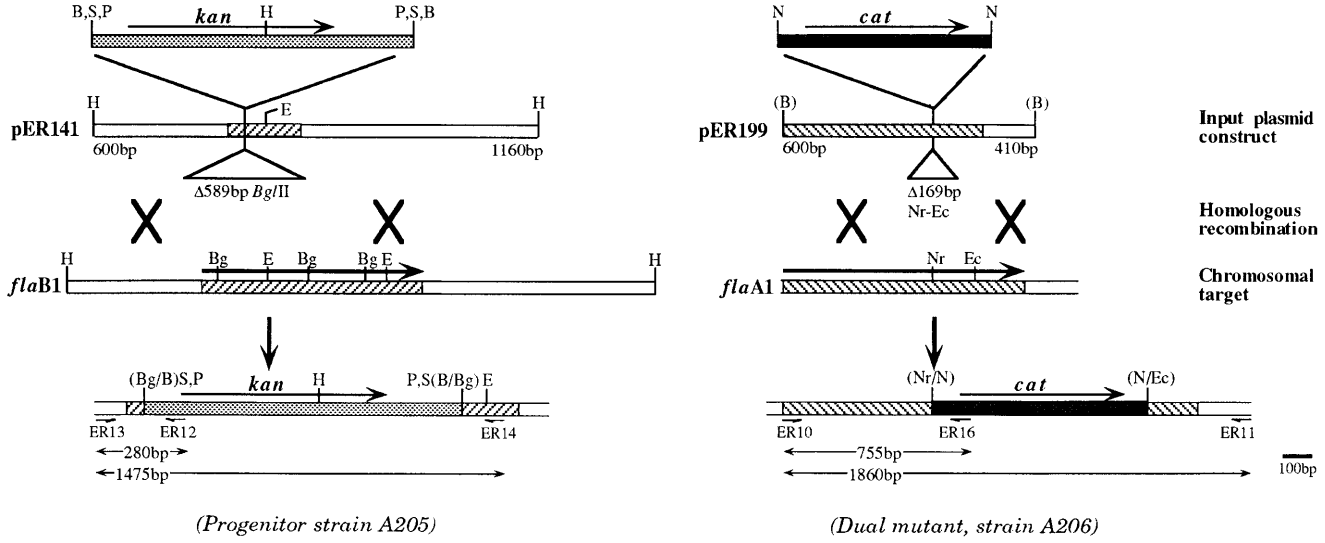

FIG. 2. Targeted disruption of *flaA1* and *flaB1* loci in *S. hyodysenteriae* A206. Electroporation-mediated allelic exchange involving plasmid pER141 resulted in generation of the *flaB1*::*kan* progenitor strain A205 as shown on the left (45). Regions depicted as chromosomal targets indicate the orientation and extent of the *flaA1* and *flaB1* open reading frames, which were targeted for disruption by homologous recombination (hatched bars). The kanamycin and chloramphenicol resistance gene cassettes (shaded bars) were inserted into the input suicide delivery plasmids, pER141 and pER199, respectively. Horizontal arrows denote the extent and orientation of antibiotic and flagellar genes. The locations of primer-annealing sites and the orientation of oligonucleotides used during PCR amplification are indicated. The predicted products (base pairs) resulting from amplification of *S. hyodysenteriae* A206 DNA are indicated below the *flaA1* and *flaB1* chromosomal alleles. Restriction endonuclease sites which have been introduced or disrupted during cloning are shown in parentheses. Restriction sites are as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Ec, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; N, *Nla*IV; Nr, *Nru*I; P, *Pst*I; S, *Sal*I.

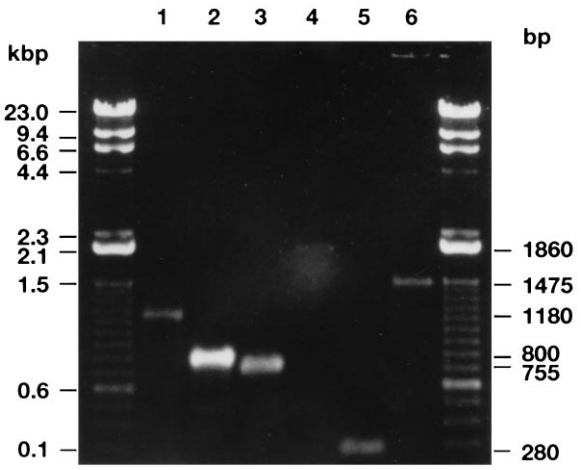

FIG. 3. Amplification of *S. hyodysenteriae* A206 chromosomal alleles. DNA template from the wild-type parent was used for amplification of the intact *flaA1* (lane 1; primer pair ER10/11) and *flaB1* (lane 2; ER13/14) genes. The status of the dual flagellar alleles in *S. hyodysenteriae* A206 was confirmed with amplimer sets for *flaA1*::*cat* (lane 3 [ER10/16] and lane 4 [ER10/11]) as well as the *flaB1*::*kan* allele (lane 5 [ER12/13] and lane 6 [ER13/14]). All reactions generated products of the predicted length for the wild-type *flaA1* (1,180-bp) and *flaB1* (800-bp) sequences, as well as for allelic exchange variants present within *S. hyodysenteriae* A206 (see the text). DNA size markers are indicated on the left, while sizes of amplified products are shown on the right.

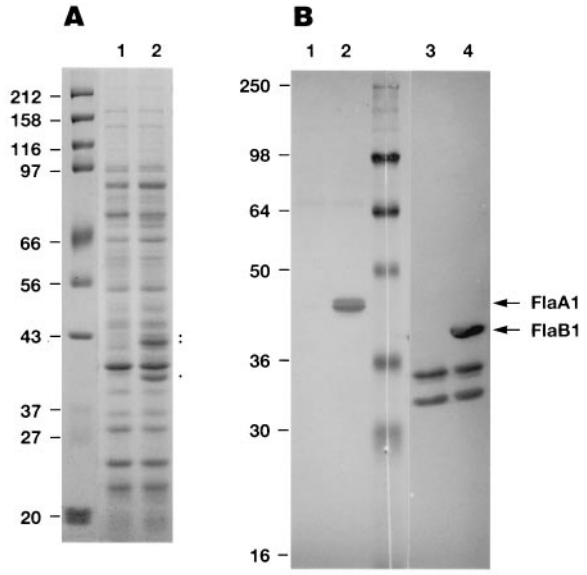

FIG. 4. SDS-PAGE and immunoblot analysis of *S. hyodysenteriae* PF proteins. Whole-cell extracts of wild-type or a *flaA1 flaB1* dual flagellar mutant were stained with Coomassie brilliant blue (A) or probed with antiserum against FlaA1 (B) (lanes 1 and 2) or FlaB (lanes 3 and 4) from FM 88-90. Equal amounts of *S. hyodysenteriae* A206 (lanes 1 and 3) and passage control strain A207 (lanes 2 and 4) were loaded in both panels A and B. Molecular size markers (in kilodaltons) are indicated on the left.

of chloramphenicol per liter plus 200 mg of kanamycin per liter were randomly chosen for further characterization. Chloramphenicol resistance imparted by recombination of homologous regions present within pER199 and chromosomal *flaA1* sequences allowed selection and clonal expansion of *S. hyodysenteriae* A206. An in vitro passage control, strain A207, was generated from the progenitor passage control by a second parallel sham electroporation followed by growth without antibiotic selection.

***S. hyodysenteriae* A206: a dual *flaA1 flaB1* periplasmic flagellar mutant. (i) Verification by PCR analysis.** *S. hyodysenteriae* A206, which was obtained following selection on antibiotic-containing solid media, was analyzed by PCR to verify that homologous recombination had occurred within the *flaA1* gene. Amplimer pairs specific for the 5′ and 3′ regions of *flaA1* or the 5′ region of *flaA1* and the 5′ region of the inserted *cat* gene cassette enabled verification of recombination between pER199 and the *S. hyodysenteriae* chromosome. During the primary screen, oligonucleotide ER10 was used in combination with oligonucleotide ER16. These primers bind to the 5′ regions of the *flaA1* and *cat* genes, respectively. As shown in Fig. 3, amplification of products of the predicted length (755 bp [lane 3]) suggested integration of the antibiotic marker within the targeted *flaA1* locus. A confirmatory screen with primer pairs ER10 and ER11 resulted in amplification of the entire antibiotic resistance marker as well as flanking regions of the targeted *flaA1* locus. This product, although faint in intensity, was of the predicted length (1,860 bp [lane 4]) expected for the desired double reciprocal recombination (allele exchange). The integrity of the chromosomal *flaB1*::*kan* allele also was verified in an analogous manner and resulted in amplification of 280- and 1,475-bp products (lanes 5 and 6) as predicted for this strain (also see Fig. 2). The PCR products amplified from *S. hyodysenteriae* A206 chromosomal DNA mirrored the results obtained during construction of single *flaA1*::*cat* and *flaB1*::*kan* flagellar mutants (45).

(ii) Abolition of FlaA1 and FlaB1 protein synthesis. PF null mutant strains may be expected to display a coordinate disappearance of the respective flagellar protein, because these proteins represent 5 to 10% of the total cellular protein in *S. hyodysenteriae* (34). Indeed, *flaA1*- or *flaB1*-null mutants were shown by Coomassie brilliant blue staining to be devoid of the predominately expressed 40- and 42-kDa doublet proteins and 38-kDa protein, respectively (45). The effect of targeted mutagenesis on the expression of *S. hyodysenteriae* flagellar proteins was assessed by SDS-PAGE and immunoblotting. As observed for the above single null mutants, *S. hyodysenteriae* A206, which harbors both *flaA1*::*cat* and *flaB1*::*kan* alleles, was deficient in 40- and 42-kDa doublet proteins as well as the 38-kDa protein (Fig. 4A). All of these proteins were expressed by the in vitro passage control strain A207.

The 40- and 42-kDa doublet and the 38-kDa protein were confirmed as FlaA1 and FlaB1, respectively, by probing whole-cell lysates with antisera generated against the species-specific flagellar sheath protein (FlaA1) or the 39-kDa flagellar core protein from *S. hyodysenteriae* FM 88-90 (37). The results of the Western blot analyses showed a corresponding disappearance of both the 40- and 42-kDa doublet FlaA1 protein and the 38-kDa FlaB1 protein in whole-cell lysates of the dual flagellar mutant (Fig. 4B; lanes 1 and 3). Expression of other immunologically related core proteins appeared unaffected by the dual mutations present in strain A206 (lanes 3 and 4). Similar yet singularly altered protein expression profiles were previously observed for strains containing specifically inactivated *flaA1* or *flaB1* loci (45). The results of PCR and Western blot analyses corresponded to the expected data for allelic exchange. Therefore, it was concluded that allelic exchange occurred in *S. hyodysenteriae* A206, thus generating a genetically defined, dual *flaA1*::*cat flaB1*::*kan* periplasmic flagellar mutant.

Assessment of PF. The intriguing observation that isogenic strains containing specifically disrupted *flaA1* or *flaB1* alleles were capable of assembling morphologically normal periplasmic flagella (45) led us to examine the effect of simultaneous inactivation of these genes on flagellar synthesis in *S. hyodysenteriae* A206. One may expect that the flagella of such cells

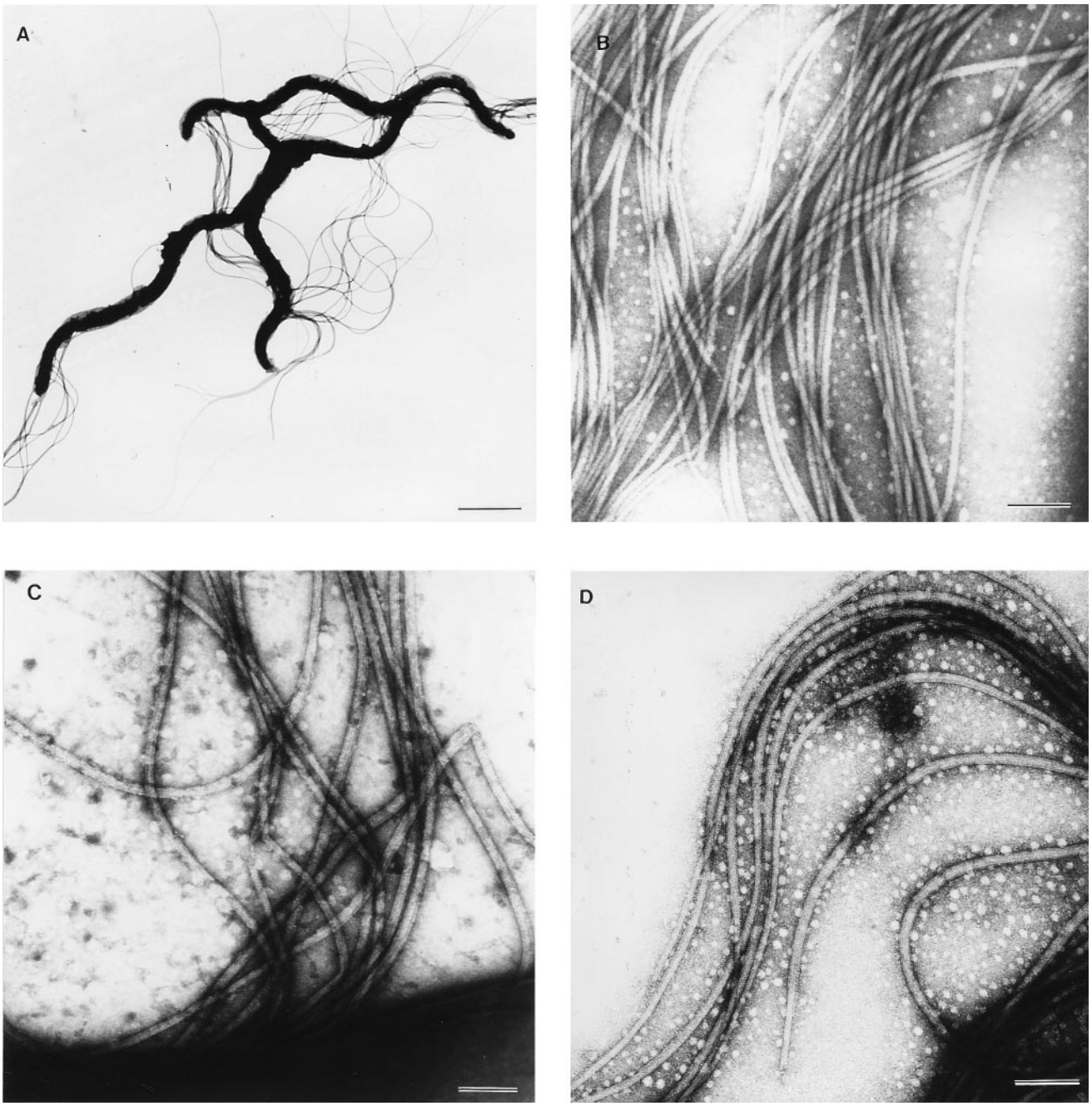

FIG. 5. *S. hyodysenteriae* PF. Representative electron photomicrographs show subterminal bundles of PF which have been exposed following treatment with 0.2% Triton X-100 to disrupt the cellular outer membrane. PF from wild-type (A201 [A and B]), dual *flaA1 flaB1* flagellar mutant (A206 [C]), and passage control (A207 [D]) strains appear morphologically similar. The dual mutant remained capable of expressing PF despite its deficiency in production of FlaA1 and FlaB1 polypeptides (C). Bars, 2 μm (A) and 0.1 μm (B to D).

(which lack two predominantly expressed flagellar proteins) would be significantly different from those of the wild type. As with *Treponema pallidum* (41), treatment with detergent (Triton X-100) released flagella from the periplasmic space of *S. hyodysenteriae*. Surprisingly, spirochete cells which are devoid of both FlaA1 and FlaB1 flagellar sheath and core proteins retained the ability to assemble periplasmic flagella (Fig. 5). The flagella of *S. hyodysenteriae* A206 were indistinguishable from wild-type flagella when examined by transmission electron microscopy. As observed for wild-type flagella, flagella of the dual-mutant strain were subterminally inserted and numbered 10 to 13 per bundle (Fig. 5C). The mean flagellum widths ($n = 10$) determined for wild-type (A201; mean width, 15.3 ± 0.81 nm), passage control (A207; 15.8 ± 0.73 nm), and dual-mutant (A206; 15.0 ± 1.34 nm) strains were not significantly different. The mean flagellum width determined by this analysis was similar to that observed for single *flaA1* or *flaB1* mutant strains (ca. 15.4 ± 1.0 nm) (45) and was within the estimate (15 to 22 nm) reported previously for other spirochetes (21).

Compromised in vitro motility of *S. hyodysenteriae* flagellar mutants. Previous microscopic observation of *flaA1* or *flaB1*

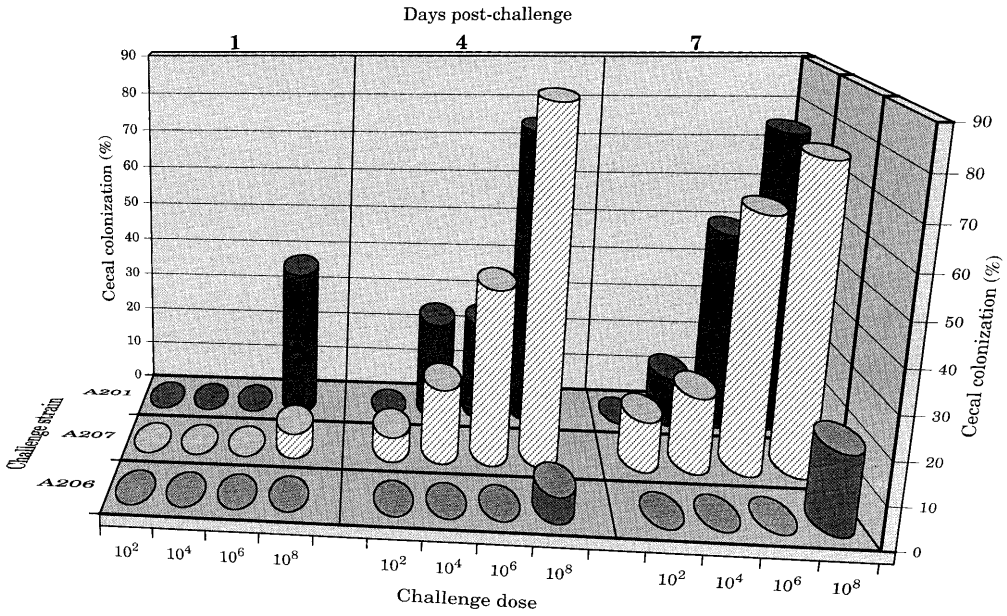

FIG. 6. Experimental challenge in mice. HSD:CF-1 female mice were challenged with the indicated dose of wild-type (A201), passage control (A207), or *flaA1 flaB1* flagellar mutant (A206) of *S. hyodysenteriae*. Mice (n = 8 per treatment group for A201; n = 16 per treatment group for A206 and A207) were necropsied at 1, 4, or 7 day postchallenge, and the cecum was cultured for *S. hyodysenteriae.*

mutant strains revealed that these variants displayed abnormal swimming behavior with respect to frequency of reversal and directed motility (45). Therefore, we wished to determine whether this aberrant motility was exacerbated by dual flagellar mutations. The motility of wild-type and passage control cells following suspension in 0.9% NaCl (29) was characterized by continuous straight or slightly curved runs of smooth swimming interrupted by either a short period of flexing or a reversal in swimming direction. The motility of *S. hyodysenteriae* A206 appeared similar to that observed for a *flaB1* mutant (45); the cells exhibited extended periods of flexing followed by short periods of erratic forward movement, with little or no change in the direction of running.

Quantitative differences in motility between the dual-mutant, passage, and wild-type strains were assessed by measuring the ability of suspended cells to randomly move into capillary tubes (25, 28, 45). The motility of either single *flaA1* or *flaB1* mutants or the dual-mutant strain A206 was considerably reduced. When tested in the above-described assay, approximately 9.6% of wild-type passage control cells randomly entered 10-μl capillary tubes. A sevenfold reduction was previously noted for strains containing a flagellar sheath (*flaA1*-null) mutation, while the ability to move into capillary tubes was reduced 27-fold (0.35% recovery from capillary tubes) for cells containing a flagellar core gene (*flaB1*) mutation (45). Analysis of *S. hyodysenteriae* A206 in the capillary assay indicated that this strain also displayed severely defective motility in vitro; it exhibited a 20-fold reduction (0.47%) in comparison with wild-type cells. No difference in random motility was observed between the wild type and the sham-electroporated, passage control strain. These data show that the effects of both flagellar mutations on motility of *S. hyodysenteriae* in vitro are significant. However, dual flagellar mutations did not lead to exacerbated reduction in random motility; the motility of strain A206 was not significantly different from that of the *flaB1* mutant strain reported previously (45).

Reduced murine cecal colonization. The virulence of the dual mutant, A206, was compared with that of the wild type and an isogenic passage control (strain A207). Groups of CF-1 mice were challenged with *S. hyodysenteriae* and examined for the incidence of cecal lesions and the presence of the spirochete in the gut at various times after challenge. Mice challenged with the wild-type or passage control strain showed a dose-related colonization by the challenge organism (Fig. 6). The larger the inoculum size, the greater the percentage of mice colonized by *S. hyodysenteriae*. The presence of wild-type *S. hyodysenteriae* in the cecum was confirmed for 75% of mice challenged with the highest inoculum dose of strain A201 on days 4 and 7 postchallenge. Strain A207, a sham-electroporated control, was similarly fit for cecal colonization and lesion production, indicating that in vitro passage during construction of the strains described here did not adversely effect enteropathogenicity in mice. *S. hyodysenteriae* was not readily cultured during earlier stages of infection, because wild-type spirochetes could be recovered from the ceca of only 38% of mice 1 day following challenge with the highest challenge dose (10^8) tested.

In sharp contrast to wild-type and passage control strains, the dual *flaA1 flaB1* flagellar mutant was severely attenuated in murine challenge experiments (Fig. 6). Only 4 of 192 (2%) mice challenged with strain A206 were culture-positive for the spirochete. *S. hyodysenteriae* A206 was recovered on days 4 and 7 postchallenge only from mice which received the highest inoculum tested. The mutant was not recovered from the ceca of mice challenged with fewer than 10^8 organisms. In fact, when compared with its isogenic passage control, the colonization fitness of *S. hyodysenteriae* A206 in this murine model of swine dysentery was reduced four to six orders of magnitude on days 7 and 4 postchallenge, respectively (Fig. 6).

Although *S. hyodysenteriae* A206 is deficient in its ability to colonize the mouse cecum, successful colonization appeared to result in cecitis and induction of cecal lesions. Two of the four mice which were colonized by the dual flagellar mutant presented with typical cecal lesions on day 4 or 7 postchallenge. Colonization by wild-type *S. hyodysenteriae* resulted in an 87% lesion rate in this murine model of swine dysentery (data not

shown), which is similar to lesion rates observed following colonization by single flagellar mutant strains (27).

DISCUSSION

Previous work from our laboratory demonstrated that chemotactically regulated or motility-regulated mucus association appears to play a key role in establishing infection by *S. hyodysenteriae* (28, 29). In keeping with this hypothesis, isogenic flagellar mutant strains unable to express FlaA1 or FlaB1 were recently constructed and found to be significantly less motile and less able to colonize mice and pigs than was wild-type *S. hyodysenteriae* (26, 27, 45). To more precisely define the role that these flagellin subunits play in the maintenance of PF structural integrity, motility, and ability to colonize the intestinal tract of mice, we constructed and characterized a novel dual *flaA1 flaB1* flagellar mutant of *S. hyodysenteriae*. Construction of this double mutant was not a trivial undertaking, since spirochetes have been extremely recalcitrant to manipulation by molecular genetic approaches, largely because of a lack of reliable cultivation methods and a general paucity of natural genetic exchange mechanisms (45).

Nonetheless, some of these limitations have recently been overcome by the development of methods for electroporation in *S. hyodysenteriae* and the identification and development of appropriate selectable markers with utility for allelic exchange in this organism (see above and references 45 and 51). We have recently used a gene encoding chloramphenicol resistance to specifically inactivate the *flaA1* (45) and NADH oxidase (44) loci of *S. hyodysenteriae*. We report here several modifications of this gene cassette which facilitate its more general application as a selectable marker for gene inactivation. Selection of this marker has been demonstrated in *Escherichia coli* (48), *Staphylococcus aureus* (22), *Bacillus subtilis* (1, 53), and *S. hyodysenteriae* (45) at antibiotic concentrations ranging from 5 to 10 μg/ml. The chloramphenicol resistance gene cassettes contained in these derivatives may be of general utility in other prokaryotes as well.

A kanamycin gene cassette has been used for targeted disruption of *tlyA* (51), *flaA1* and *flaB1* (45), and, most recently, the gene encoding NADH oxidase (44) of *S. hyodysenteriae*. Accordingly, both kanamycin and chloramphenicol resistance markers were used to generate a *flaB1*::*kan* progenitor strain which was further modified by introduction of a *flaA1*::*cat* mutation by electroporation-mediated allelic exchange, thereby resulting in the construction of strain A206, a genetically defined *flaA1 flaB1* flagellar mutant.

Using this mutant, we found that dual mutations in the *flaA1* and *flaB1* genes significantly affect both in vitro motility and the ability of *S. hyodysenteriae* to colonize the mouse cecum, indicating that functional PF play a pivotal role in the virulence of this organism. This observation is similar to results obtained with single PF mutant strains (27), except that reduction in colonization and virulence in mice were greatly pronounced with the dual PF mutant. A significant reduction in virulence and ability to colonize the mouse cecum occurred with *flaA1* mutants, while specific inactivation of *flaB1* resulted in a more pronounced reduction in motility and ability to colonize mice (27). For example, when mice were challenged with 10^8 spirochetes, 100, 94, and 38% remained culture positive at 4 days for wild-type passage, *flaA1*, and *flaB1* strains, respectively. Cecal colonization by the above strains further decreased by day 7 postinoculation to 69, 31, and 0%, respectively. This apparent difference in single PF mutant strains may reflect the fact that the FlaB1 protein is localized to the core of the PF and therefore may play a key in maintaining the functional integrity of this complex organelle. Although both the *flaA1* and *flaB1* loci contain putative rho-independent terminator structures immediately downstream of their respective open reading frames, no information regarding the status of the transcribed mRNA exists. Therefore, it is conceivable that the altered motility and infectivity of the dual mutant may have resulted from inhibition of gene expression downstream of the introduced mutations.

All single-mutant strains were capable of establishing a modest transient infection in mice following a high-inoculum challenge (10^6 to 10^8 CFU) (27). In contrast, only very limited transient infection was observed in the present study for the dual mutant. These data indicate that although both FlaA1 and FlaB1 flagellin proteins are dispensable for flagellar assembly, they are critical for normal flagellar function in vivo. Taken together with the finding that the dual flagellar mutant strain was significantly less motile than its isogenic parent strain, such data further suggest that the major contribution of functional PF toward *S. hyodysenteriae* enteropathogenicity reflects their critical role in motility-regulated mucus association, which is necessary for evasion of physical clearance from the intestine in the absence of adhesive properties (4). These results parallel descriptions by others that both FlaA and FlaB flagellin subunits are necessary for full motility and the ability to persist and colonize the intestinal tract for certain *Helicobacter* and *Campylobacter* spp. (16, 24, 43).

It should be noted that the flagella of both *Helicobacter* and *Campylobacter* spp., in contrast to *S. hyodysenteriae*, exist as external polar and in some instances lateral structures. In addition to their obvious roles in motility, the flagella of these organisms also play important roles in adhesion to and invasion of eukaryotic cells (12, 15, 55). No similar adhesive or invasive properties have been identified for *S. hyodysenteriae*. This is probably because flagella of *S. hyodysenteriae* reside in the periplasmic space (33), and it further bolsters the argument that intact and functional PF are essential for full motility and subsequent colonization and pathogenesis by *S. hyodysenteriae* (26, 27, 45, 46).

This also may help to explain the intriguing finding that single FlaA1 or FlaB1 mutant strains and the dual flagellar mutant described here appear capable of assembling morphologically normal PF and possess residual motility, despite the absence of these predominantly expressed proteins. The ability of the double mutant to assemble apparently normal PF was surprising, especially since FlaA1 and FlaB1 are indispensable for full motility. This is in contrast to the observation of Cockayne et al. (11), who noted that an isolate of *T. pallidum* was fully motile despite lacking FlaB1. Collectively, these data suggest that multiple PF class A and class B proteins either may be compensatory or are able to substitute for one another, making assembly of PF possible even in dual FlaA1- and FlaB1-deficient cells. This is consistent with the hypothesis that *S. hyodysenteriae* and other pathogenic spirochetes may have developed strategies to ensure the presence of PF during colonization or invasion of appropriate host tissues (45).

As with other spirochetes and spiral-shaped organisms (24, 30), *S. hyodysenteriae* must move through a viscous environment to initiate colonization and infection (28). This may require complex PF fortified by multiple flagellin subunits that are more rigid than simpler filaments consisting of a single flagellin (16). Perhaps such complex PF would be better able to withstand the strong mechanical force necessary to propel spiral-shaped bacteria through viscous environments (8, 16, 24, 30, 31). In agreement with this, flagellar filaments consisting of several different flagellin subunits have recently been reported for several spirochetes and spiral-shaped bacteria, such as

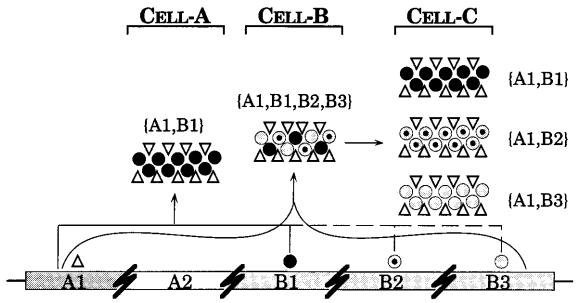

FIG. 7. Possible compensatory mechanisms for flagellar assembly. Chromosomal genes *flaA1* through *flaB3* are shown at the bottom. Expression of various flagellar genes may result in the flagellum structures depicted for individual cells A, B, and C as follows: cell A, single combination within cell, phase variation within population; cell B, stoichiometric incorporation of numerous flagellar proteins; cell C, individual flagella composed of limited flagellar proteins, yet multiple combinations within cell. Taken from reference 45.

Campylobacter spp. (at least two flagellins [3, 16, 43]), *Helicobacter* spp. (two flagellins [35, 50]), *Halobacterium halobium* (five flagellins [14]), *Treponema pallidum* subsp. *pallidum* (four flagellins [7, 40]), and *Leptospira interrogans* (seven flagellins [52]). Our present study demonstrated that in *S. hyodysenteriae*, both FlaA1 and FlaB1 are required for the assembly of fully functional PF.

It remains to be determined, however, whether single or multiple types of PF are produced within a single cell. For instance, it is not known whether all three FlaB-related proteins are present in the same flagellum (Fig. 7) or assembled into separate flagella composed of limited Fla proteins (e.g., cell A and cell C). Nearly equimolar amounts of the multiple flagellar polypeptides have been observed in whole-cell lysates (Fig. 4B) and purified flagella (33). Thus, in the absence of polypeptide-specific monoclonal antibodies, it is currently impossible to predict which expression pattern(s) (cell A, B, or C) is predominant in any given population of cells or whether an individual cell may express a single (cell A and cell B) or mixed (cell C) population of PF. It seems less likely, however, that a pure population of PF (e.g., cell A), each composed of only a single core and a single sheath protein, would be expressed. Such an expression pattern certainly would require a mechanism for flagellar phase variation within a population of cells. Moreover, the *flaB* genes in *S. hyodysenteriae* do not appear to be tandemly linked or part of a flagellar operon (13, 32), in contrast to the *flaB1* and *flaB3* genes from *T. pallidum* (7).

We hypothesize that *S. hyodysenteriae* can respond to certain as yet unknown environmental conditions (e.g., viscosity) to regulate the physicochemical properties of the PF and thus ensure optimal motility in different environments. This concept is supported by the fact that two distinct types of motility have been observed for *S. hyodysenteriae* (28) and is in keeping with observations for other spirochetes that colonize mucosal surfaces (8, 31).

Despite the availability of the *flaA1* (34), *flaB1* (13), and *flaB2* (32) gene sequences, very little is known about transcriptional regulation of flagellar genes in *S. hyodysenteriae*. The putative -10 and -35 sequences upstream of *flaB1* share homology with other flagellar genes and sigma-D (σ^{28}) catabolite-repressible promoters (20). This suggests that environmental conditions encountered by *S. hyodysenteriae* may indeed trigger a transcriptionally regulated cascade involving the synthesis of flagellar and chemotaxis proteins, as is the case with other bacteria (19). Similarly, the expression of the two flagellin genes in both *Campylobacter* spp. and *Helicobacter* spp. is regulated by different promoters (σ^{28} for the *flaA* genes and σ^{54} for the *flaB* genes [16, 42, 50, 54]) and environmental cues (3, 24, 54). Inactivation of single or both *flaA1* and *flaB1* genes in *S. hyodysenteriae* had no apparent effect on expression levels of other related FlaB proteins detected on immunoblots. Also, disruption of *flaB1* alone did not affect the expression of FlaA1 (45). These data suggest that if such a regulatory cascade involves feedback inhibition of flagellar synthesis and assembly, it must function independently of a requirement for the FlaA1 or FlaB1 proteins. Such a regulatory cascade may be circumvented as a result of the ability of the dual flagellar mutant to productively express PF composed of other cellular homologs.

Accordingly, the dual-mutant strain may represent a population of cells which are presumably capable of expressing and assembling alternative (FlaA2, FlaB2, or FlaB3) PF polypeptides. It is not known whether *S. hyodysenteriae* A206 assembles PF which are representative of those depicted by cell A, B, or C or is capable of producing PF of alternative composition. In light of the demonstration that both FlaA1 and FlaB1 proteins appear essential for full motility but not PF assembly, it may be informative to generate a single strain containing multiple *flaB*-related mutations to determine the dependence of flagellar assembly upon core protein synthesis. The gene sequences for both *flaB1* and *flaB2* and the existence of multiple antibiotic resistance gene cassettes now make such an approach feasible.

The profound effect of the *flaA1* and *flaB1* mutations in *S. hyodysenteriae* A206 on in vitro motility and murine cecal colonization strongly suggests that the remaining FlaA1- and FlaB1-deficient PF are significantly altered with respect to their normal function in vivo. A thorough understanding of the ultrastructure, composition, and assembly of spirochetal PF will require further analysis of specifically defined flagellar mutants. *S. hyodysenteriae* A206 and its singly mutated *flaA1* and *flaB1* counterparts should be amenable for such studies and may provide productive insight into this area of spirochete biology.

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