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Genetic and Transcriptional Analysis of *flgB* Flagellar Operon Constituents in the Oral Spirochete *Treponema denticola* and Their Heterologous Expression in Enteric Bacteria

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Oral spirochetes possess many potential virulence factors, including the capacity for tissue invasion and persistence despite a vigorous host immune response. In an attempt to identify treponemal immunoreactive components, sera derived from individuals with advanced periodontal disease were used as a reagent to isolate recombinant bacteriophage lambda clones expressing antigens of the oral spirochete *Treponema denticola* ATCC 35405. Nucleotide sequence analysis of a clone expressing three immunoreactive products has revealed seven *T. denticola* genes which appear to encode homologs of flagellar basal body constituents, FlgB, FlgC, FliE, and FliF, a flagellar switch component, FliG, and the putative flagellar export proteins, FliH and FliI, initially characterized in *Salmonella typhimurium*. Also identified was a gene resembling *fliJ*. Primer extension analysis identified a transcriptional start site 5′ to the treponemal *flgB* gene. Appropriately spaced with respect to this start site was a σ^{28} binding motif. The absence of additional identifiable sigma factor binding motifs within the treponemal sequence and the proximity of adjacent genes suggested operonic arrangement, and reverse transcriptase PCR provided evidence of cotranscription. Supporting the identification of these genes as flagellar components, heterologous expression in enteric bacteria of the putative switch basal body genes from *T. denticola* interfered with motility. Specifically, the presence of a plasmid expressing treponemal *fliG* reduced swarming motility in *S. typhimurium*, while in *Escherichia coli*, this plasmid conferred a nonmotile phenotype and a reduction in flagellar number. Thus, while spirochetal flagella are subject to unique synthetic and functional constraints, the organization of flagellar genes and the presence of σ^{28}-like elements are reminiscent of the flagellar systems of other bacteria, and there appears to be sufficient conservation of constituent proteins to allow interaction between *T. denticola* switch-basal body proteins and the flagellar machinery of gram-negative bacteria.

The oral spirochete *Treponema denticola* is strongly associated with diseases of the tooth-supporting structures, or periodontium, in which it has been shown to breach the epithelial barrier, entering connective tissue (14). While the elaboration of degradative enzymes (47, 63) is thought to facilitate penetration of the lamina propria, spirochetal motility is also an important factor. Spirochetes are distinguished from other motile bacteria by the localization of their flagella to the periplasmic space and enhanced motility in highly viscous environments (39), traits which are thought to contribute to the virulence of such spirochetal pathogens as *Treponema pallidum*, the causative agent of syphilis (8), and *Borrelia burgdorferi*, the Lyme disease spirochete (51). In addition to the mechanical advantage afforded to the invading spirochete, localization of the flagella within the outer membrane shields immunoreactive flagellar components from contact with host antibody, thereby avoiding immobilization of the spirochete within infected tissues (60) and rendering these antibodies ineffective for opsonization (54).

The majority of our knowledge of bacterial motility and chemotaxis has been derived from genetic and structural analyses of *Salmonella typhimurium* (36), with the gram-positive organisms *Bacillus subtilis* and *Caulobacter crescentus* diverging from the *Salmonella* paradigm in terms of components (5), gene organization (1), and control of flagellar biogenesis (23). It is only recently that investigators have focused on the molecular basis for motility in spirochetes, obtaining some insight into the structure of the spirochetal flagellum and the mechanisms by which it is assembled and controlled.

Electron micrographs of spirochetal flagella reveal ultrastructural elements (Fig. 1) common to well-described species, such as the flagellar filament, the adapter-like hook and rod, and the basal body, which contains the force-generating structures and anchors the flagellum to the cell membrane (31). Distinguishing structural features include the treponemal flagellar filaments, which exhibit a sheath and core structure (10) and are wound around the body of the spirochete, contrasting with the homogeneous filaments, unconfined by the outer membrane, seen in *S. typhimurium* (36). Differences are also readily apparent in the ring structures of the basal body. In *S. typhimurium*, two distinct rings are associated with the peptidoglycan layer and the outer membrane (37), while an additional double-ring structure, the MS ring, traverses the cytoplasmic membrane and is a multimer of a single subunit, FliF (62). Treponemal basal bodies, by contrast, display a total of one to two rings, a conformation more closely resembling that of gram-positive organisms (31). This difference may be attributed to the spirochetal flagellum passing through only the cytoplasmic membrane and suggests that the MS ring would be the predominant ring structure visible in electron micrographs of spirochetes.

Spirochetal flagella rotate, as do the flagella of other bacterial species (9), and their coordination is a presumptive requirement for effective translation through the medium. Each spirochetal flagellum is anchored at one end at a subterminal

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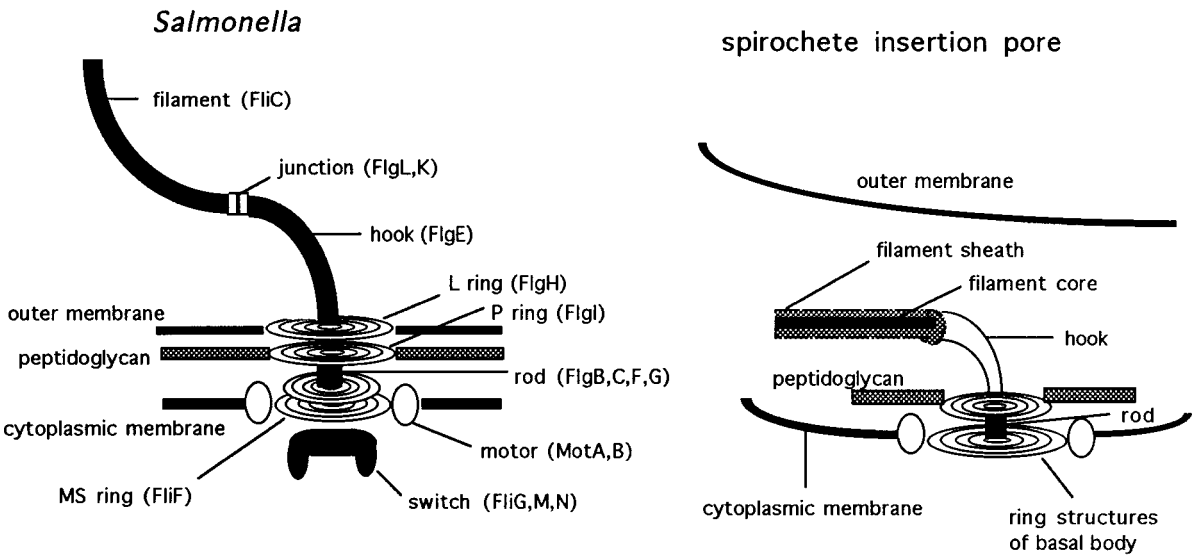

FIG. 1. Diagram of the ultrastructural features and component proteins observed in the *S. typhimurium* flagellum (18) and ultrastructural features of the flagellar insertion pore in cultivable treponemes (31).

insertion pore (6, 31), and in order for translation to occur, the filaments originating at one pole must either not rotate or rotate in the opposite sense to filaments arising at the other pole of the bacterium (4). In *S. typhimurium*, by contrast, the rotational sense of a majority of flagellar motors determines whether the cell will swim (counterclockwise) or tumble (clockwise) (36). The rotational sense of the individual flagellar motor is determined at the flagellar switch, where chemical and mechanical influences are integrated to alter the rotational bias of the motor. Three presumptive components of the spirochetal flagellar switch, FliG, FliM, and FliY, have been identified, the *fliG* gene from *T. denticola* in our laboratory (28) and the *fliM* and *fliY* genes from *T. pallidum* (26). These flagellar switch proteins, particularly FliG, exhibit substantial conservation with their counterparts in other bacteria, perhaps as a result of functional constraints. Studies of *S. typhimurium* have shown that FliG is central to interactions within the switch motor complex (18, 19). Readily observable phenotypic effects, including inability to assemble flagella, nonmotile flagella, and an abnormal rotational bias of the flagellar motor, result from mutations in the switch proteins, with FliG and FliN being most involved in motor rotation and FliM seeming to function primarily in the determination of the direction of flagellar rotation (35).

Thus far, from studies with the flagellar genes of *T. pallidum* and *B. burgdorferi*, it appears that genes encoding flagellar structural components and those of the assembly apparatus are arranged into large operons, a pattern closer to that described for *B. subtilis* than to the *S. typhimurium* paradigm (21, 26). Independent of gene arrangement, however, there is a common need to control and coordinate the production of the numerous flagellar components, and an alternative sigma factor of the σ^{28} family regulates flagellar gene transcription in numerous species (25, 36). In *S. typhimurium*, σ^{28} is employed in a regulatory cascade which ties production of proteins required at later stages in the flagellar assembly process to the proper assembly of preliminary structures, such as the flagellar basal body and export apparatus. The interaction between σ^{28} and the anti-sigma factor, FlgM, levels of which are modulated by transcriptional regulation and export of FlgM by the flagellar assembly apparatus (22, 33), is thought to influence flagellar length and number (33, 40). Since spirochetal flagella are confined by the outer membrane, restricting flagellar length, and their number and relative length are characteristic of the spirochetal species (7, 60), spirochetes may also employ this form of transcriptional regulation in flagellar biogenesis, and similarities to the consensus binding motif for the σ^{28} family (8, 10) have been noted 5′ to treponemal flagellar filament genes.

In this report, we describe the characterization of an apparent operon of *T. denticola* containing genes coding for spirochetal homologs of eight flagellar proteins, including the previously described switch gene *fliG* (28) and the basal body gene *fliF*, and demonstrate the effects of expression of these treponemal genes on flagellar synthesis and motility in *S. typhimurium* and *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *T. denticola* was grown in a modification of the serum-free medium PTY (47) [10 g of proteose peptone, 5 g of Trypticase peptone, 1 g of yeast extract, 2.5 g of KCl, and 2.5 g of cysteine per liter (pH 7.2), with filter-sterilized supplements of 5 ml of 10% sodium bicarbonate, 0.025 g of thiamine pyrophosphate, and 5 ml of volatile fatty acid mix, consisting of 0.5% isobuteric acid, 0.5% valeric acid, 0.5% isovaleric acid, 0.5% (*S*)-(+)-2-methylbutyric acid, 0.02% propionic acid, and 0.02% butyric acid (Sigma Chemical Co., St. Louis, Mo.), in 0.1 N KOH] in a Coy anaerobe chamber with an atmosphere of 5% H_2–10% CO_2–85% N_2 at 37°C. *E. coli* and *S. typhimurium* were cultured in L broth (5 g of yeast extract, 10 g of tryptone, and 10 g of NaCl per liter) (53) or on McConkey agar (Difco, Detroit, Mich.) plates. Swarming motility was examined on soft tryptone agar (57) (13 g of tryptone, 7 g of NaCl, and 3.5 g of agar per liter) (Difco). Antibiotics, when required, were included in the culture media at the following concentrations: ampicillin, 100 mg/liter; kanamycin, 50 mg/liter (Sigma).

Recombinant DNA and genetic techniques. Recombinant DNA manipulations were carried out by using standard procedures as described by Sambrook et al. (53). The genomic library was prepared in λEMBL3, from partial *Sau*3A1 digests of chromosomal DNA isolated from exponentially growing *T. denticola* ATCC 35405 (28), using an EMBL3-*Bam*HI cloning kit (Promega Inc., Madison, Wis.) as recommended by the supplier. Recombinant phage DNA was prepared by the method of Chisholm (11). For subcloning, restriction fragments were ligated into the vector pUC19 (68) or pGEM7 (Promega Inc.). Restriction enzymes and T4 ligase were obtained from Gibco BRL (Grand Island, N.Y.) and were used according to the manufacturer's instructions. Exonuclease III was used to generate nested deletions for sequencing using an Erase-A-Base kit (Promega) as recommended by the supplier. Plasmid DNA was isolated from *E. coli* by using

TABLE 1. Bacterial strains, bacteriophages, and plasmids used in this study

Strain, bacteriophage, or plasmid	Genotype	Source
Strains (relevant phenotype[s])		
T. denticola ATCC 35405	Type strain	American Type Culture Collection
E. coli DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*) *U169 deoR* [ϕ80 d*lac* Δ(*lacZ*) M15]	Gibco BRL
S. typhimurium SJW1611 (Fla)	*fliG* Δ499–512 frameshift	R. Macnab, Yale University
S. typhimurium SJW2835 (Che, tumbly)	*fliG* G553A	R. Macnab
S. typhimurium SJW1791 (Mot)	*fliG* Δ229–378 in frame	R. Macnab
S. typhimurium MY2214 (Che, smooth)	*fliG* A364C	R. Macnab
Bacteriophages		
λEMBL3		Promega
λHH-1	12.2-kbp insert	This study
λHH-2	15-kbp insert	This study
Plasmids		
pUC19		C. Yannish-Perron (67)
pGEM 7 zf(+)		Promega
pTDF5.2	ORF_T-partial $fliG_T$	This study
pTDF3.8	$flgB_T$-partial $fliG_T$	This study
pTDF7.4	Partial $fliG_T$-$fliJ_T$ + 4 kbp	This study
pTDF5.15	$flgB_T$-$fliH_T$	This study
pTDFP1300	$flgB_T$ promoter region in pGEM7	This study
pTDF2.9	Partial $fliG_T$-partial $fliI_T$	This study

miniprep spin columns (Qiagen, Chatsworth, Calif.) according to manufacturer's instructions or by using cesium chloride gradients (53). Transformations of *E. coli* and *S. typhimurium* were accomplished by electroporation using a Gene Pulser apparatus and a 0.2-cm electrode gap cuvette (Bio-Rad, Hercules, Calif.) under conditions described by Sixou et al. (55).

SDS-polyacrylamide gel electrophoresis and Western blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Laemmli (42), using a 6% acrylamide stacking gel and a 12% acrylamide separating gel. Cells were pelleted by centrifugation, resuspended in 1× sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 1 mM bromophenol blue), heated to 100°C for 5 min, and recentrifuged prior to loading. Separated proteins were electrophoretically transferred as described by Towbin et al. (61) to Immobilon-P membranes (Millipore Corp., Bedford, Mass.). Filters were immersed for 4 h in 5% skim milk in Tris-buffered saline (TBS; 10 mM Tris [pH 7.4], 0.9% NaCl), subjected five times to TBS washes of 7 min, and exposed to primary antibody (polyclonal antiserum raised to *E. coli* FliG, a kind gift from P. Matsumura [50], or human sera [28] diluted 1:500 in TBS), rewashed, and then exposed to horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G or goat anti-human immunoglobulin G (Gibco BRL). Immunoblots were developed by using diaminobenzidine (Sigma) (2).

DNA sequencing. DNA sequencing was performed by the method of Sanger et al. (53a), using a Sequenase version 2.1 kit (United States Biochemical, Cleveland, Ohio) on double-stranded plasmid templates. Subclones generated by exonuclease III digestion of restriction fragments cloned into the vectors pUC19 (67) and pGEM7 (Promega) were sequenced by using the M13 −40 primer (United States Biochemical) or universal reverse primer (Promega), as well as synthetic oligonucleotide primers (Gibco BRL) designed from previous nucleotide sequence data.

RNA isolation. Total RNA was isolated from *T. denticola* by the method of Coleman et al. (13). Pelleted cells were resuspended, vortexed for 1 min in hot lysis buffer (0.01 M Tris-HCl [pH 8.0], 0.35 M NaCl, 2% SDS, 1 mM EDTA, 7 M urea), and then immediately extracted twice with phenol-chloroform (1:1) and twice with chloroform alone. RNA was precipitated from the aqueous phase with 1/10 volume of 3 M sodium acetate and 3 volumes of ethanol, resuspended in DNase I buffer, and digested for 15 min with RNase-free DNase I (Gibco BRL). The RNA was again extracted with phenol-chloroform and precipitated with ethanol.

Reverse transcriptase PCR (RT-PCR). Reverse transcription was accomplished using an avian myeloblastosis virus reverse transcriptase kit and random hexamers from Gibco BRL with 10 μg of total RNA per reaction as instructed by the manufacturer. Synthetic oligonucleotide PCR primers (Gibco BRL) were designed by using previously obtained nucleotide sequence data to give anticipated products of approximately 0.4 to 1.0 kbp which spanned two or more spirochetal genes (Table 2). PCR was performed by using *Taq* polymerase (Gibco BRL) and Perfect Match Enhancer (Stratagene, La Jolla, Calif.) for 40 cycles with an annealing temperature of 52°C and an extension temperature of 72°C. The resulting products were analyzed by Southern hybridization. Control reactions, in which reverse transcriptase was omitted, were included to eliminate the possibility that genomic DNA served as the template for the PCR.

Primer extension analysis. Synthetic oligonucleotide primers (Gibco BRL) were end labeled by using T4 polynucleotide kinase (United States Biochemical) according to the manufacturer's instructions, annealed with 10 μg of *T. denticola* RNA, and extended by using the avian myeloblastosis virus reverse transcriptase system (Gibco BRL). Products were electrophoresed on a denaturing 8% polyacrylamide gel.

RNase protection. An [α-^{32}P]CTP-labeled RNA probe was synthesized by using T7 RNA polymerase (United States Biochemical) from pTDFP1300 (Table 1) according to the manufacturer's instructions and hybridized with 10 μg of RNA from *T. denticola*. RNase digestion was accomplished as previously described (2), and the resulting fragments were sized on an 8% denaturing acrylamide gel.

Southern blotting. For Southern blot analysis, genomic and cloned DNAs, digested with *Eco*RV, were separated in 0.8% agarose gels, or ethanol-precipitated RT-PCR products were electrophoresed through 1.5% agarose gels. DNA was transferred to nitrocellulose by the method of Southern (59). Radiolabeled probe was generated by using a Rad Prime kit (Gibco BRL) according to manufacturer's instructions, using pTDF5150 and pTDF7.4 (Table 1) as templates. Filters were hybridized overnight in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS–5× Denhardt's solution–100 μg of denatured salmon sperm DNA per ml at 65°C and washed seven times in 0.1× SSC–0.01% SDS at 63°C.

TABLE 2. Oligonucleotide primer pairs used for RT-PCR and the resultant PCR products[a]

Lane in Fig. 5B	Primer pair	Expected product size (bp)	Genes amplified
A	1238, 1809	571	*flgB*, *flgC*
B	1238, 2268	1,030	*flgB*, *flgC*, *fliE*
C	1691, 2690	919	*flgC*, *fliE*, *FliF*
D	2360, 2971	689	*fliE*, *fliF*
E	3850, 4397	547	*fliF*, *fliG*
F	5069, 5881	812	*fliG*, *fliH*
G	5987, 6440	453	*fliH*, *FliI*
H	7305, 7685	380	*fliI*, *fliJ*

[a] Individual primers are listed by position of 5′ nucleotide on the sense strand, with primers in the first and second columns representing primers identical to the sense strand and the complementary strand, respectively.

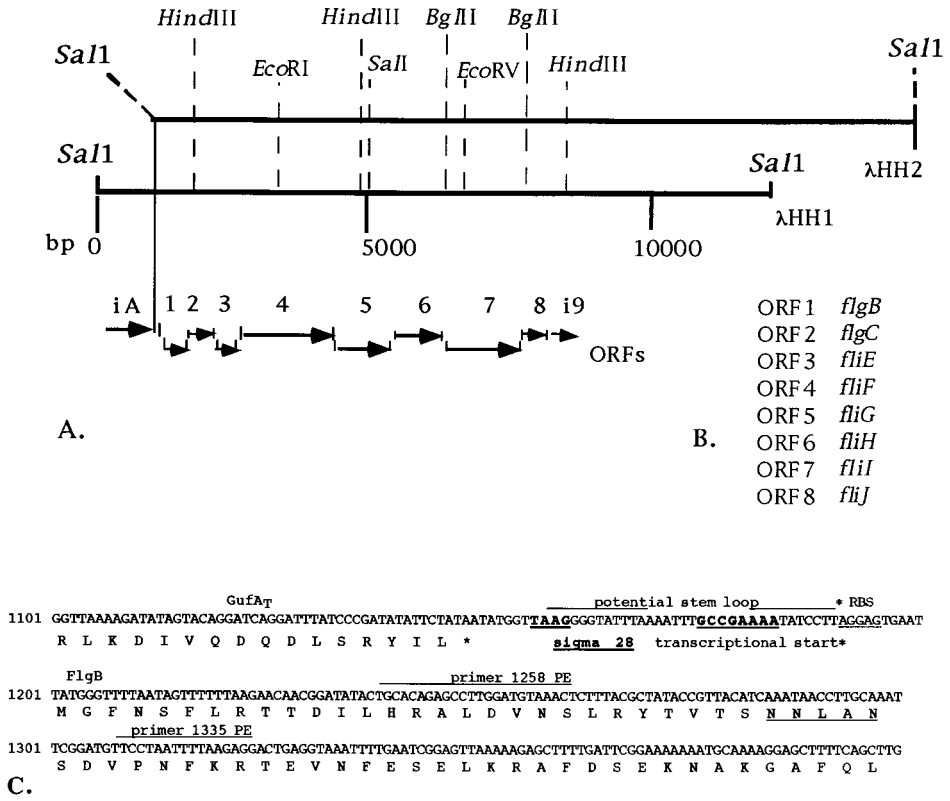

FIG. 2. Restriction and transcriptional maps and promoter sequences of recombinant bacteriophage inserts for λHH1 and λHH2. (A) Schematic diagram showing the inserted DNA in λHH1 and λHH2 and some restriction sites for several common enzymes. The terminal *Sal*I sites indicated in boldface are vector derived and used for subcloning. Below, arrows indicate the locations and directions of transcription for eight complete (1 to 8) and two incomplete (iA and i9) ORFs, designated above the arrows. (B) ORF1 to ORF8 correspond to flagellar genes previously identified in other organisms. (C) DNA sequence and deduced amino acid sequence of the *flgB* promoter region. The putative RBS is underlined. The transcriptional start site identified by primer extension in Fig. 4 is indicated above and below with asterisks, and the potential σ^{28} binding sequence is underlined and in boldface. Potential secondary structure and primers used in primer extension analysis (see text) are overlined, and the FlgB NLAN signature sequence is underlined.

Flagellar staining. Flagella were visualized by the wet mount technique of Heimbrook et al. (27). Bacteria lifted from the edge of a colony on soft tryptone agar were placed in a 20-μl drop of distilled water on the slide and covered with the coverslip for 15 min, allowing the bacteria to become fixed to the glass. A drop of the crystal violet-based Ryu stain was then allowed to diffuse beneath the coverslip by capillary action. Slides were examined and photographed at a magnification of ×1,000, using a BH2 phase-contrast microscope and the PM-10AK photomicrographic system (Olympus Optical Co., Tokyo, Japan) and TMX 100 film (Eastman Kodak, Rochester, N.Y.).

Nucleotide and amino acid sequence analysis. BLAST searches and computerized alignments were performed by using the University of Wisconsin Genetics Computer Group package. Initial alignment of nucleotide sequence data was accomplished and the predicted protein products were analyzed by using Assemblylign and MacVector programs (Eastman Kodak, Laboratory and Research Products, New Haven, Conn.).

Nucleotide sequence accession number. The nucleotide sequence data for the *T. denticola flgB* operon have been submitted to GenBank and assigned accession no. U78776.

RESULTS

Identification and sequence analysis of the *flg* operon of *T. denticola*. A recombinant λEMBL phage, λHH1, containing a cluster of *T. denticola* 35405 flagellar genes, was isolated as previously reported (28) on the basis of reactivity with antibody from individuals with advanced adult periodontitis. A second recombinant phage isolated in the same manner, λHH2, contained an overlapping fragment of *T. denticola* 35405 DNA (Fig. 2) and expressed, as did λHH1, a species reactive with human periodontal patient sera with an apparent molecular mass of 37 kDa. Restriction mapping and Southern blot analysis indicated that a 7.0-kbp fragment, derived from the *T. denticola* chromosome, was common to both λHH1 and λHH2 (data not shown). The *T. denticola* DNA insert from λHH1 was subcloned as two *Sal*I fragments into pUC19, and subsequent nucleotide sequence analysis of these plasmid clones revealed the existence of eight complete open reading frames (ORFs), oriented in the same direction, which display substantial similarity to known flagellar genes from *E. coli*, *S. typhimurium*, and *B. subtilis* (Fig. 2). The unified nomenclature for flagellar and motility genes (34) has been adopted to refer to these putative spirochetal flagellar homologs, with the subscript "*T*" denoting their treponemal origin. Similarly, the subscripts "*B*", "*S*" and "*E*" have been used to refer to genes and proteins from *B. subtilis*, *S. typhimurium*, and *E. coli*, respectively. The sequence, consisting of two partial and eight complete ORFs, is depicted schematically in Fig. 2A, and the complete nucleotide and deduced amino acid sequences can be accessed via GenBank (accession no. U78776). Characterization of these ORFs and their corresponding genes is described below; ORFs for which partial sequences are given have been prefixed with "i."

iORFA, *gufA*$_T$. λHH1 includes a partial ORF, located 5′ to the first putative flagellar gene, which does not display homology to previously described flagellar or chemotaxis genes. The deduced amino acid sequence displays similarity to proteins implicated in protein trafficking and degradation, such as the ClpX protease subunit from *E. coli* (30% identity) (24), LapA

from *Pasteurella haemolytica* (46% identity) (30), and the CodX protein (46% identity), which is located in the operon immediately upstream of the flagellar/chemotaxis operon in *B. subtilis* (56). Recent data from *B. burgdorferi* also locate a putative heat shock protein 46% identical to the *T. denticola* iORFA immediately 5′ to the first putative flagellar gene in an operon containing both heat shock and motility genes (21). This *Borrelia* protein, which has been designated HslU, shares with CodX and iORFA nearly identical Walker B-type ATP binding motifs (RVEQM**GIIFIDEID** in iORFA, with boldface type indicating conservation with HslU and CodX and underlines indicating conservation with the Walker B, R-X_{2-3}-G-X_3-(hydrophobic amino acid)$_4$-D (65), paradigm. In *T. denticola*, however, this partial ORF is separated from the flagellar gene cluster by a 54-bp intergenic region and, as in *B. subtilis*, appears to represent a separate transcriptional unit. Although nucleotide sequence analysis does not identify a strong factor-independent terminator sequence in the intergenic region, there is a potential stem-loop structure which may represent a termination signal. Further, primer extension experiments (see below) locate a transcriptional start site in this intergenic region. This gene has been temporarily designated *gufA*, for gene upstream of the *flgB* operon.

ORF1, *flgB*$_T$. ORF1 begins with the ATG codon at bp 1202 and is preceded by a potential ribosome binding site (RBS) (Fig. 2). ORF1 codes for a predicted 15.9-kDa protein of 140 amino acids, extending to a TAA codon at bp 1620. BLAST searches revealed alignment with the flagellar proximal rod gene, *flgB*, from *B. subtilis* (70). At the amino acid level, there is 27% identity between these two proteins and 22% identity between $FlgB_T$ and $FlgB_S$ (32), with regions of identity concentrated in the C-terminal third of these proteins and surrounding the conserved N-terminal signature sequence, N(I/L)AN, which is characteristic of flagellar rod proteins (32).

ORF2, *flgC*$_T$. ORF2 is separated from the stop codon of ORF1 by 23 bp incorporating a potential RBS. BLAST searches again revealed alignment with a gene coding for a flagellar component, here the proximal rod subunit, *flgC*, of *B. subtilis* (70). The predicted protein of 151 amino acids and 16.5 kDa is 38 and 32% identical to its counterparts in *B. subtilis* and *S. typhimurium* (32), respectively. $FlgC_T$ exhibits features common to FlgC proteins of several species, including an absence of cysteine residues, possession of the N(I/L)AN signature sequence, and a Gly-Tyr-Val triplet (32) at the C terminus, which is followed by a region displaying high levels of amino acid identity with the other two species.

ORF3, *fliE*$_T$. ORF3, which is separated from *flgC*$_T$ by 34 bp, including a potential RBS, extends from bp 2135 to the stop codon at bp 2500 and encodes a protein with a predicted molecular mass of 13.3 kDa. The highest-scoring amino acid alignment, at 19% identity, was with the *fliE* gene product of *E. coli* (48), which codes for a component of the flagellar basal body. $FliE_S$ has been hypothesized to act as an adapter structure between the MS ring and the flagellar rod (48). At 121 amino acids, $FliE_T$ is slightly larger than its counterparts, with an additional 6 amino acids at the N-terminal end of the molecule and a 10-amino-acid insertion 35 amino acids from the N terminus. An intergenic region of at least 63 bases, which includes a pair of inverted repeats, separates *fliE*$_T$ from the succeeding ORF.

ORF4, *fliF*$_T$. ORF4 has two potential start sites, the second of which has the most plausible RBS. ORF4 encodes a protein of 65.1 kDa, which resembles FliF proteins from several bacterial species, although the FliF protein from *T. denticola*, at 571 amino acids, is larger by about 30 amino acids. The levels of amino acid identity with FliF proteins from both *B. subtilis* (70) and *C. crescentus* (49) are not particularly striking, being approximately 24% (50% similarity); $FliF_T$ also displays only a modest 22% identity (44% similarity) with $FliF_S$ (62). Nevertheless, this degree of conservation is consistent with that observed between the FliF proteins described to date, and the similarity is distributed uniformly throughout the protein. Hydropathy plots of $FliF_T$ and $FliF_B$ indicate the potential for structural similarity between these proteins in the absence of strong conservation of primary amino acid sequence. Both $FliF_T$ and $FliF_B$ proteins are generally hydrophilic, except for the hydrophobic regions at the C and N termini, which appear to be characteristic of FliF proteins (70).

ORF5, *fliG*$_T$. ORF5 encodes a 39-kDa protein displaying high levels of identity with FliG proteins from various species and was the first gene from the hook-basal body complex to be described in *T. denticola* (28). FliG is a component of the flagellar switch involved in rotational flagellar assembly and torque generation, in addition to affecting the rotational bias of the flagellar motor (45).

ORF6, *fliH*$_T$. ORF6, which extends from bp 5339 to 6334, encodes a protein of 34.98 kDa. BLAST searches revealed 31% identity (53% similarity) with YscL, a protein required for the secretion of the *Yersinia enterolytica* virulence proteins known as YOPS (46), and also 24% identity (53% similarity) to FliH from *B. subtilis* (1). The positioning of ORF6 within a flagellar operon suggests that the encoded protein functions, like $FliH_S$ (64), as part of the flagellar export apparatus. The C-terminal region of $FliH_T$ aligns well with those of both YscL and $FliH_B$, and a 15-amino-acid segment within this region, beginning at residue 265 of the treponemal protein, is particularly well conserved between these proteins (Fig. 3), suggesting that it may be functionally important. It is clearly evident from this alignment, however, that $FliH_T$ is about 37% larger than either YscL or $FliH_B$ and that the size difference is attributable to the N-terminal third of the proteins. $FliH_T$ is similar in size to the FliH of *B. burgdorferi* (20) (Fig. 3), but these two spirochetal proteins display only a 32% amino acid identity overall, which decreases to 24% in the N-terminal third. The N-terminal region of $FliH_T$, which is much more hydrophilic than the C terminus, contains a number of glutamic acids and repetitive clusters of K, E, A, and D. As reported for *B. burgdorferi* FliH (20), this region has similarities to the β subunit of the membrane-associated F_0 portion of F_0F_1 ATPases from a variety of bacterial species.

ORF7, *fliI*$_T$. Orf7 is a 1,413-bp ORF separated by 9 bp from the stop codon of *fliH*$_T$, coding for a protein of 51.3 kDa. The predicted protein displays 54% identity (71% similarity) to $FliI_B$ (1) and 45% identity to $FliI_S$ (16, 38), which has been postulated to provide energy for the export of the flagellar subunits. As noted for the other FliI proteins, there is a substantial resemblance to the β subunit of *E. coli* ATP synthetase. The conservation between FliI proteins from different species is most apparent in the central portion of the protein, where motifs associated with nucleotide binding can be identified (65). The amino acid sequence beginning at amino acid 160 of $FliI_T$, GRGQRLGIFAGTGIGKSTL, has many of the features described by Walker as the A motif, R-X_{4-5}-G-X_{3-4}-G-K-(T)-X_5-I, with a G-X-G-X-G loop, and is followed, at amino acid 244, by RDQGKDVMLLFD, which corresponds quite well with the R-X_{2-3}-G-X_3-(hydrophobic amino acid)$_4$-D Walker box B. Situated between these motifs are several glutamic acid residues, which, with their surrounding amino acids, have been implicated in the binding of magnesium-complexed nucleotides (1).

ORF8, *fliJ*$_T$. Three base pairs separate the ATG start codon of ORF8 from the termination codon of *fliI*$_T$. This ORF en-

```
B. burgdorferi FliH   MPKVLYKSSE VD.NLVKFEF VEIAKPV.FE SLEIKEKESK VYDIESQISN   50
  T. denticola FliH   MAKTIFRGFE VNKNNNDVVF LQLNKTFQEE PEEIIEEKVP VYE.GPTVED
   B. subtilis FliH   .......... .......... .......... .......... ..........
         YscL         .......... .......... .......... .......... ..........
    Conserved         M-K------E V--N-----F ----K----E --EI-E---- VY--------

B. burgdorferi FliH   LKEELQLLRD EKLQLEEELA KRQELAKEEV QIESKRLIEE AKAKANEVLE  100
  T. denticola FliH   LKKEAE.... ...DFKLEWE KQKEKMISDA KAEADKIIED AQNAAFDEVK
   B. subtilis FliH   .......... .......... .......... .......... ........MA
         YscL         .......... .......... .......... ...MSQTCQT GYAYMQPFVQ
    Conserved         LK-E------ -------E-- K--E------ --E----IE- A-A-A-----

B. burgdorferi FliH   AAKQEADLLQ KEAIYKKESI ETESNAEIER LAREYEEKLK TDLDIAIAKG  150
  T. denticola FliH   RQTDEAQVIA QNAKKDAEDI YSRSRAKSQR YIADSEKNKD SVNRDAYKEG
   B. subtilis FliH   RVKEEADRIS EQANSHIENI ....RRQIEQ EKNDWAAEKQ KLIEEAKAEG
         YscL         IIPSNLSLAC GLRILRAEDY ..QSSLTTEE LISAAKQDAE KILADA.QEV
    Conserved         R-K-EADLI- --AI--AEDI ---SRA-IER LI-D-E--K- K-L-DA-AEG

B. burgdorferi FliH   REEGYSKGYE SGFEDFDKVM RKLHAIIASL IAERKGILES SSGQIVSLVM  200
  T. denticola FliH   FNRGREEGFK EGNLEVQRLT DRLHTIINKT MDRRQEILSE TEQQIVDLVL
   B. subtilis FliH   FEQGVALGKA EAMKQYAELI GQANTITEMS RKAVEDKLED ANEEIVELAV
         YscL         YEQQKQLGWQ AGMDEARTLQ A...TLIHET QLQCQQFYRH VEQQMSEVVL
    Conserved         FEQG--LG-- EGM-E---L- --LHTII--T ---RQ-ILE- -EQQIVELVL

B. burgdorferi FliH   QIAIKVIKRI TDSQKDIVLE NVNEVLKRVK DKTQITIRVN LDDLDIVRHK  250
  T. denticola FliH   LMTRKVVKVI SENQRNVVVS NVVHALRKVK GRGDVVIRVN LADVKMTTEH
   B. subtilis FliH   ALAKKVWQQK SD.DKEAFLL LVQQVINEVK EYDDISIYVD PYYYETIFQQ
         YscL         LAVRKILNDY ..DQVDMTLQ VVREALALVS NQKQVVVRVN PDQAGTIREQ
    Conserved         L-ARKV-K-I SD-QKD-VL- NV-E-L--VK -----VIRVN -DD--TIREQ

B. burgdorferi FliH   KSDFISRFDI IENLEIIEDP NIGKGGCIIE TNFGEIDARI SSQLDKIEEK  300
  T. denticola FliH   IQNFISCRN. IKNITVVEDS TVDQGGCIIE TDFGAVDARI ASQLNELEQK
   B. subtilis FliH   KDEIQQLLYK ECRLGIYADE KAQKGTCYIE TPFGRVDASV DTQLMQLKDK
         YscL         IAKVHKDFPE ISYLEVTADA RLDQGGCILE TEVGIIDASI DGQIEALSRA
    Conserved         ----IS-F-- I-NLE---D- --D-GGCIIE T-FG--DA-I DSQL--LE-K

B. burgdorferi FliH   FKNFSLLS.. ......
  T. denticola FliH   ILEISPIKTK IKTGNI
   B. subtilis FliH   LLTALEAGAA E.....
         YscL         ISTTLGQMKV TEEE..
    Conserved         -LT------- ------
```

FIG. 3. Alignment of FliH proteins from *T. denticola*, *B. burgdorferi*, and *B. subtilis* with YscL from *Y. enterolytica*, using the Pileup program from the Genetics Computer Group. The regions of greatest identity are boxed.

codes a predicted protein of 153 amino acids displaying 42% similarity and 19% identity with FliJ from *B. subtilis*. The exact function of $FliJ_B$ is unknown, but it is required for basal body formation, and disruption of this gene interferes with chemotaxis (68). $FliJ_T$ is also 27% identical (50% similarity) with HrpJ5, which is involved in virulence protein export in *Pseudomonas syringae* (43), and a newly described protein, designated FlbA, from *B. burgdorferi* (21). One notable feature of $FliJ_T$ is the preponderance of four amino acids: E, L, I, and especially K. Charged residues (D, E, K, R, and H) account for 38% of amino acids, which is even higher than the 32% observed in $FliJ_S$, and K alone constitutes 15% of the protein. Like $FliJ_S$, $FliJ_T$ has a basic isoelectric point (pI = 8.7). $FliJ_T$ also contains 25.5% L and I, nearly double that found in $FliJ_B$ and $FliJ_S$ (64). The secondary structure of $FliJ_T$ was predicted to be predominantly alpha helical according to the algorithm of Chou and Fasman (12), and only the region between residues 24 and 39 is strongly hydrophobic.

iORF9. The next ATG codon is separated by 14 bp from ORF8 and is preceded by a potential RBS. Translation of the partial sequence available for this ORF did not reveal substantial similarity to the protein products of currently described flagellar or chemotaxis genes, including *fliK*, which succeeds *fliJ* in many other organisms (36), and *flbB*, which succeeds *flbA* in *B. burgdorferi* (21).

Promoter mapping. Primer extension was used to map the start site of the transcript containing *flgB*. A synthetic oligonucleotide primer (1258 PE), complementary to the *flgB* transcript, was extended to form a 68-nucleotide (nt) product, identifying the A residue at position 1190 as the transcriptional start site (Fig. 4A). This start site was confirmed using a primer at position 1335 (Fig. 4B). RNase protection using a probe complementary to nt 1121 to 1252 (Fig. 2) revealed a protected fragment of approximately 56 ribonucleotides, consistent with the primer extension results (data not shown). Examination of the nucleotide sequence immediately 5′ to this start site revealed a subsequence ($TAAGN_{15}GCCGAAAA$) identical at 9 of 12 residues to the σ^{28} consensus binding site ($TAAAN_{15}G$ CCGATAT; nt −10 is underlined) (25), appropriately spaced from the transcriptional start site.

Except for the region between *fliE* and *fliF*, the intergenic regions within the flagellar cluster are substantially smaller than those usually associated with bacterial promoters. Primer extension experiments using a primer distal to the potential stem-loop between *fliE* and *fliF* revealed numerous high-molecular-weight products, presumably from mRNA 5′ to *fliE* (data not shown), suggesting that these genes are cotranscribed.

Operonic arrangement of the *flgB* cluster. Despite numerous attempts with different protocols, Northern blots of *T. denticola* RNA did not give a clear signal when probed with *T. denticola* flagellar gene probes, seemingly due to degradation of the potentially large *flgB* mRNA. Therefore, RT-PCR was used to explore the transcriptional organization. We selected oligonucleotide primer pairs which would amplify a region extending from the 3′ end of one flagellar gene through the 5′ end of a succeeding gene, should those genes be present on the same mRNA template fragment. Eight different primer pairs within the flagellar gene cluster were used to amplify products from randomly primed *T. denticola* RNA reverse transcription reactions (Table 2). In all cases, PCR products of the appropriate size were obtained. Control reactions, used to check for the presence of contaminating DNA, yielded no visible products after PCR. High-stringency Southern hybridization of

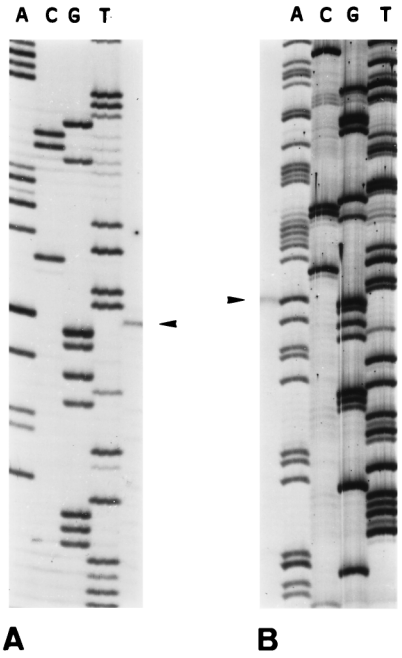

FIG. 4. Transcriptional mapping of the *flgB* gene by primer extension. (A) ACGT sequencing ladder and extension product from primer 1258. (B) ACGT sequencing ladder and extension product from primer 1335. Arrowheads indicate extension products.

probes synthesized from the cloned flagellar gene cluster to these PCR products indicated that these products were derived from the *flgB* gene cluster of *T. denticola* (Fig. 5). These results suggest that $flgB_T$, $flgC_T$, $fliE_T$, $fliF_T$, $fliG_T$, $fliI_T$, $fliH_T$, and $fliJ_T$ are part of the same transcriptional unit. No PCR products were evident in reactions using primer pairs from *gufA* and *flgB*, which suggests that these genes are transcribed separately. Definition of the 3′ end of this operon remains to be accomplished.

Expression of treponemal flagellar genes. To confirm the functional identity of these treponemal proteins, it would be desirable to demonstrate alterations in function associated with these genes. While gene transfer systems in spirochetes hold promise, the biochemically and genetically characterized flagellar systems of the gram-negative enteric species offer the advantage of a framework of knowledge in which to assess the function of treponemal proteins. Our DNA sequence analysis suggested that some treponemal components, $FliG_T$ in particular, might be capable of interacting with flagellar components from other organisms in a complementary fashion. To this end, several attempts, using four different host vector systems, were

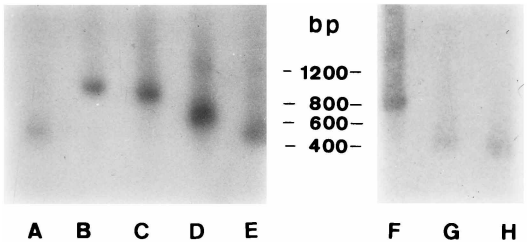

FIG. 5. Southern blot of RT-PCR products listed in Table 2, probed with a mixture of radiolabeled pTDF3.8 and pTDF7.4, showing that the amplified products from *T. denticola* hybridize with the cloned flagellar sequences.

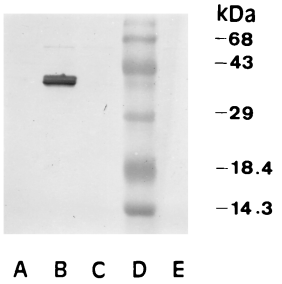

FIG. 6. Western blot analysis using anti-$FliG_E$ antibody of *S. typhimurium* SJW1611 containing various constructs. Lanes: A, pTDF7.4; B, pTDF5.15; C, pTDF3.8; D, protein standards; E, pUC19.

made to express $fliG_T$ independent of other treponemal flagellar components in *S. typhimurium* and *E. coli*. Independent expression of $fliG_T$ was not achieved, possibly because of lethal effects of expression in *E. coli*. It proved possible, however, to express $fliG_T$ from constructs which included other treponemal genes. Specifically, plasmid pTDF5.15 contains 5.15 kbp of the *flgB* operon, beginning 58 nt 5′ to the *flgB* start site and extending through the *fliH* gene, in the vector pUC19. When this plasmid was used to transform *S. typhimurium* SJW1611, a strain with a frameshift in *fliG* (Table 1), a protein with an apparent molecular mass of 36 kDa, reactive with antibody raised to *E. coli* FliG, was readily apparent on Western blots (Fig. 6). When pTDF5.15 was used to transform *E. coli* DH5α, the resulting strain was found to be essentially nonmotile on swarm plates (Fig. 7). These colonies were then stained for flagella, and nearly all organisms were found to be aflagellate (Fig. 8). This contrasts markedly with strains harboring pUC19, pTDF3.8, or pTDF7.4, all of which were motile on soft tryptone agar and in which individual bacteria often synthesized numerous flagella (Fig. 8). Since pTDF3.8 contains a truncated version of the pTDF5.15 insert, terminating at the *Sal*I site internal to *fliG*, and pTDF7.4 contains only the $fliG_T$ sequence distal to that same *Sal*I site but expresses an immunoreactive species tentatively identified as $fliH_T$, the phenotypic effects appear to require the synthesis of intact $FliG_T$.

Several strains of *S. typhimurium* with described mutations in *fliG* were also transformed with the same plasmids. Not surprisingly, Fla strains, such as SJW1611 (Table 1), remained aflagellate when the treponemal genes were introduced. Similarly, Mot strains, such as SJW1791 (Table 1), which were nonmotile but flagellate, displayed no motility on swarm plates after introduction of the treponemal genes. In contrast to the results for *E. coli*, however, introduction of $fliG_T$ into motile *S. typhimurium* SJW2835 and MY2414 (Table 1) produced

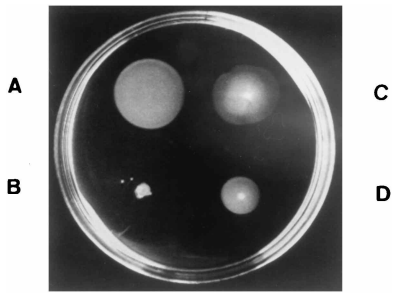

FIG. 7. Soft tryptone agar swarm plate. (A) *E. coli* DH5α harboring pTDF3.8; (B) *E. coli* DH5α harboring pTDF5.15; (C) *S. typhimurium* MY2214 harboring pTDF3.8; (D) *S. typhimurium* MY2214 harboring pTDF5.15.

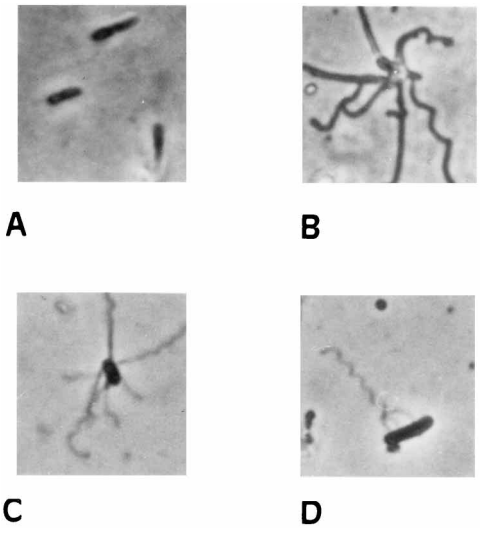

FIG. 8. Representative bacteria subjected to flagellar staining at a magnification of ×1,000. (A) *E. coli* DH5α harboring pTDF5.15; (B) *E. coli* DH5α harboring pTDF3.8; (C) *S. typhimurium* MY2214 harboring pTDF5.15; (D) *S. typhimurium* MY2214 harboring pTDF3.8.

colonies which did spread on swarm plates (Fig. 7), albeit with a reduced colony diameter compared to strains bearing plasmids not expressing $FliG_T$. After 24 h of incubation, colony diameters of *Salmonella* strains SJW2835 and MY2414 bearing pTDF5.15 were an average of 52% ± 4% of those of pUC19-, pTDF3.8-, or pTDF7.4-containing strains, consistent over three separate groups of transformants, each used on eight swarm plates containing four plasmid-bearing strains derived from the same parent.

Staining revealed that *Salmonella* strains expressing *fliG*$_T$ frequently displayed multiple flagella extending from the cell in many directions (Fig. 8), an appearance consistent with nonmotile flagella (64) but also with tumbling behavior (36). In tumbling, clockwise rotation of the flagellar motor alters the helical conformation of the individual flagellar filaments, facilitating their dispersal (36). Occasional bacteria also exhibited one or more flagellar bundles indicative of coordinated counterclockwise rotation. Microscopic examination revealed bacteria which were predominantly either slowly moving or nonmotile. Strains harboring pUC19, pTDF7.4, and pTDF3.8 exhibited greater numbers of vigorously motile cells, as might be expected from their behavior on swarm plates.

Reactivity with human antisera. The phages λHH1 and λHH2 were originally isolated on the basis of reactivity with human sera, with a 37-kDa antigen being the most prominent reactive species. Production of this immunoreactive species was associated with subclones of λHH1 bearing an intact *fliH*$_T$ gene, including pTDF7.4 and pTDF2.9, in which transcription appears to arise from vector promoter elements and AT-rich regions within the *fliG* coding sequence according to primer extension data from *E. coli*. Since the predicted molecular mass of $FliH_T$ is 35 kDa, very close to the apparent molecular mass of the immunoreactive species on Western blots with human sera, it is likely that $FliH_T$ is the source of the observed immunoreactivity. The remaining two immunoreactive species expressed from λHH1 appear to be independent of the flagellar operon and may represent treponemal proteins encoded by another operon (29).

DISCUSSION

The primary distinctive feature of spirochetes is the location of the flagella within the periplasmic space, which places significant constraints on the flagella in terms of architecture, synthesis, and coordination. The exceptional anatomical structure of the spirochete suggests that flagellar synthesis and function in spirochetes may deviate from current paradigms and thus provide additional insight into the process of flagellar biogenesis. The identification of the genes for components of spirochetal flagellar systems allows comparison with those from more intensely investigated species, providing a foundation for future studies in spirochetal motility and its relationship to virulence in spirochetal diseases.

Many of the flagellar genes isolated from *T. denticola* in this study encode proteins with substantial resemblance to proteins from *B. subtilis*, a gram-positive organism. As the spirochetal basal body needs to span only the cytoplasmic membrane, and ultrastructural observations are suggestive of the gram-positive basal body (31), it might be expected that the individual components would follow this pattern, and it is interesting that this is not uniformly the case. Of the *T. denticola* genes described here, five, *flgB*, *flgC*, *fliE*, *fliF*, and *fliJ*, have been associated with the basal body structure in other organisms. Of the products of these genes, only the proximal rod component, $FlgC_T$, displays a high level of amino acid identity (38%) with its counterpart in *B. subtilis*, and nothing currently known about the function of FlgC explains why this particular protein should be particularly well conserved between spirochetes and gram-positive organisms. The other four basal body proteins display less than 30% identity with their counterparts in *B. subtilis*, with $FliE_T$ exhibiting greater similarity to FliE from *E. coli*. $FliF_T$, the membrane-spanning structure, is larger than the FliF proteins of the other species and is almost equally divergent from *B. subtilis* and *S. typhimurium*. Such divergence may reflect the demands placed on the structure by the positioning of the flagella, serving to increase its stability to compensate for the substantial forces which are generated by the flagellar motor while the filament is wound around the body of the spirochete. The resemblance to gram-positive organisms is greatest not in the basal body structural proteins but in those associated in other organisms with flagellar synthesis and control, particularly FliG (28) and FliI.

In spirochetes, careful control of flagellar number and length can be inferred, given that flagellar number and degree of overlap are characteristic for individual species and that the subterminal flagellar anchorage suggests some specificity in the localization of flagellar synthesis (7, 60). While much remains unknown about the mechanics of flagellar synthesis, direct evidence in *Salmonella* has pointed to roles for three proteins, also encoded by this treponemal operon, in the assembly process: FliG, FliH, and FliI. FliG, located at the base of the basal body (18), may be in a position to affect access to the putative export channel, which is thought to pass through the center of the basal body, hook, and filament (36). FliI is postulated to function as the energizer of the flagellar export complex (1, 64), a suggestion based on its noted similarity to the β subunit of F_0F_1 ATPases. Both FliI and FliH are required for flagellar filament regrowth in *Salmonella* (64), and similar proteins are essential components of signal peptide-independent protein export systems (43, 46, 52, 66). Unlike FliG and FliI, however, $FliH_T$ differs substantially from its putative homolog in *B. subtilis*, both in size and in amino acid sequence, which may be reflective of differences in its interactions during the export-assembly process. Interestingly, $FliH_T$ and its counterpart in *B. burgdorferi* (20) have extended N termini bearing resemblance

to the β subunit of F_0F_1 ATPases. The highly charged, alpha-helical, cytoplasmic portion of the β subunit has been implicated in interaction with the F_1 portion of the enzyme, particularly the β subunit (17); $FliH_T$ resembles this cytoplasmic domain, suggesting the possibility of interaction with the F_1β-like $FliI_T$, which might merit further investigation. Given the essential involvement of FliG, FliH, and FliI in the flagellar protein export process, it is possible that the availability and interactions of these proteins contribute, in some capacity, to the control of flagellar number and length in spirochetes.

The expression of one of these proteins, $FliG_T$, in *S. typhimurium* and *E. coli* was correlated with a dominant negative effect on observed motility of the host strain, supporting the identification of $FliG_T$ as a protein involved with motility and as a possible homolog of $FliG_S$. Despite many attempts, $FliG_T$ could not be expressed independently of the other proteins encoded by the $flgB_T$ through $fliH_T$ construct, perhaps because limitation of the availability of $FliG_T$, through either regulation of synthesis or interaction with other treponemal proteins, such as FliF, was required to alleviate lethality. It was clear, however, that only pTDF5.15, which encoded an intact $FliG_T$, substantially affected the motility of the host strains. Neither pTDF3.8, encoding $FlgB_T$ through the N terminus of $FliG_T$, nor pTDF7.4, which appears to express $fliH_T$ from the *lacZ* promoter, elicited significant changes in observed motility. The effects observed are among those previously associated with mutations in $fliG_S$ (35), but curiously, although *S. typhimurium* and *E. coli* are very closely related and have FliG proteins which are 91.8% identical (50), expression of $fliG_T$ in *E. coli* results in the failure to assemble flagella whereas expression in *Salmonella* results, instead, in impaired motility. Finding the Fla phenotype in *E. coli* DH5α bearing pTDF5.15 is especially intriguing, because even substantial deletions in *S. typhimurium fliG* permit flagellar synthesis, almost one-third of $FliG_S$ being considered unimportant for flagellar assembly (35). The impaired motility observed in *S. typhimurium* expressing $FliG_T$ is a less profound defect, and even single base substitutions can give rise to this phenotype (35). Further work will be required to more fully delineate the interactions between the switch, motor, and chemotaxis components in both assembly and function before these observations can be fully explained.

Synthesis of a functional flagellum is dependent on the sequential synthesis and assembly of flagellar component proteins to form progressively more complex structures. Efficient flagellar synthesis implies regulation of the production of component proteins, particularly at the levels of transcription and translation. Recent studies examining flagellar genes and their transcriptional organization in spirochetes (26) suggest that spirochetes organize their motility and chemotaxis genes into relatively large operons, an organization more typical of *B. subtilis* (1, 70) than of the gram-negative species that serve as flagellar paradigms. The gene arrangement within the *T. denticola flgB* cluster, with intergenic regions smaller than those usually associated with bacterial promoters, is suggestive of organization into an operon of over 7 kbp, and the RT-PCR experiments support this conclusion. The presence of a DNA sequence strongly resembling a σ^{28} binding element, appropriately spaced with respect to the transcriptional start site identified 5′ to $flgB_T$, is consistent with previous reports of such elements in association with other spirochetal flagellar operons (8, 10, 26) and with the involvement of the σ^{28} family of sigma factors in flagellar synthesis in many other species, arguing that a σ^{28}-like factor is a very strong candidate for transcriptional control of these treponemal flagellar genes. Within the gene cluster, in neither coding nor intergenic regions were additional σ^{28}-like elements evident. Since few treponemal genes have been sequenced, there is a paucity of information about promoter elements in these species, and such observations do not preclude the use of different promoter elements. It is worth noting, however, that even between $fliE_T$ and $fliF_T$, where the intergenic region is relatively large, primer extension experiments were not indicative of an additional transcriptional start. It is likely, therefore, that the region 5′ to $flgB_T$ functions as the promoter for the entire gene cluster. Efforts to confirm the treponemal operonic structure by examining transcripts from pTDF5.15 were largely unsuccessful. The treponemal sequence is AT rich and appears to provide a number of sites which can be utilized as promoters by *E. coli*. In particular, primer extension analyses in *E. coli* indicated transcriptional starts 5′ to $flgB_T$, associated with σ^{70}-like consensus binding sequences, which were not evident in the *T. denticola* experiments (29). Transposon Tn*5* insertion in the *flgBC* region of pTDF5.15 was insufficient to relieve the effects on motility associated with expression of $fliG_T$ in *E. coli* (29), but as this observation could also result from transcription of the latter part of the operon from a promoter not used in the native spirochete, the results were considered inconclusive.

The data suggest, then, that the sequence described here contains at least eight ORFs, forming an operon in *T. denticola* beginning with the *flgB* gene and transcribed from a σ^{28}-like promoter. The gene organization of *gufA*, *flgB*, *flgC, fliE*, *fliF*, *fliG*, *fliH*, *fliI*, and *fliJ* in *T. denticola* displays significant similarity to that reported for *B. burgdorferi*. However, there is clearly a transcriptional start site prior to *flgB* in *T. denticola*, while in *B. burgdorferi*, the preceding gene is proposed to be in the same transcriptional unit under the control of a σ^{70}-like promoter element (21). Further, since iORFA displays nearly equivalent levels of identity with a number of traffic ATPases and proteases, it is by no means certain that *gufA* is truly homologous to *hslU* of *B. burgdorferi*. A similar situation is evident at the 3′ end of the operon, with $FliJ_T$ displaying nearly equivalent resemblance to *B. burgdorferi* and *P. syringae* proteins. Additionally, the partial ORF succeeding $fliJ_T$ does not exhibit much similarity to *flbB* from *Borrelia*, and we have no current evidence of a *flgE*-like ORF immediately 3′. Actually, since both λHH1 and λHH2 contain DNA sequences with substantial resemblance to glucohydrolases located 3′ to the flagellar sequence but independently expressed, this *T. denticola* flagellar operon does not appear to be as extensive as the *che/fla* operon of *B. subtilis*, which extends for approximately 26 kbp, or as large as that suggested by the recent data for *B. burgdorferi* (21). Current information from *T. pallidum* indicates that the large *fla* operon which begins with a gene designated *tap1*, located immediately 5′ to the *flgD* gene, and which also includes *flgE*, *motA*, *mot B*, and *fliM*, is associated with a σ^{28}-like promoter element (26, 44). Thus, treponemal species appear to organize flagellar genes in a manner distinct from *B. burgdorferi* as well as from more distantly related bacteria. Efforts are under way to better characterize the segment of *T. denticola* DNA distal to $fliJ_T$ and to define the 3′ end of this operon. Further work will also be necessary to determine the location of the *fliA* gene, which codes for the flagellum-related sigma factor, and to explore transcriptional control of the spirochetal flagellar genes and its impact on flagellar synthesis.

In addition to transcriptional control, one might expect other forms of regulation within the *flgB* operon because the genes within this operon encode proteins which compose very different structures within the basal body. The stoichiometry of the ring structure and rod structures, for example, has been examined in *Salmonella* (58), where FlgB and -C are thought to be present at about 6 units per basal body and FliF is thought

to be present at 26 units. FliE has also been estimated at nine subunits per basal body (48). While no estimates of treponemal stoichiometries have been made, it is nonetheless likely that differences exist in the quantities of treponemal basal body components required for flagellar assembly. If these genes are present in the same operon, differences in translation could compensate for the stoichiometric variation in the number of subunits required for flagellar synthesis. Differences in strength of RBSs, codon usage, and secondary structure are features which can influence levels of translation of different genes on the same RNA. Since the total number of *T. denticola* DNA sequences in the databases is relatively small, it is difficult to properly assess overall codon usage in this species at present, although the serine codons TCG and AGC seemed to be used at least three times as frequently in *flgB* and *fliE* as in other genes sequenced to date. There is also the potential for significant secondary structure within this operon, which may have regulatory implications. For example, an inverted repeat is evident between the *fliE* and *fliF* genes, which could conceivably affect ribosomal access to the Shine-Dalgarno sequence of *fliF* or alter the susceptibility of this mRNA to degradation by endoribonucleases, a phenomenon which has been postulated for other bacterial systems (3). In addition, the intergenic spacing of certain gene pairs is suggestive of translational coupling (15), with the RBSs of $fliG_T$ and $fliJ_T$ overlapping the coding sequences of $fliF_T$ and $fliI_T$, respectively.

Additional work will be necessary to more fully characterize the immunoreactive species on the basis of which these clones were isolated. The tentative identification of $FliH_T$, a cytoplasmic protein, as the 37-kDa species suggests that some of the immunoreactivity associated with *T. denticola* could be the result of antibodies cross-reactive with flagellar proteins from other organisms. Also, since $FliH_T$ is probably inaccessible to antibody in the intact spirochete, this reactivity would be of minimal significance in the elimination of the spirochete from the disease site.

Spirochetes have been associated with pathologies in many areas of the body, and their flagellum-based motility is thought to contribute to virulence in these organisms by advancing invasion and dissemination. Here, we have identified probable homologs of seven known flagellar genes in *T. denticola*, organized as an operon under the probable control of a σ^{28} family promoter, and shown that expression of one of them, $fliG_T$, is capable of affecting flagellar function in other organisms. Although spirochetes resemble other bacteria in many of their flagellar components, differences not only between larger groups of bacteria but between spirochetal species as well are beginning to be elucidated at the molecular level. The identification of these genes will allow us to better examine the assembly and function of the spirochetal flagellum when a gene transfer system for these organisms has been perfected. The identification of individual components immunoreactive with patient sera can also improve our understanding of the interaction between host and spirochete.

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