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Molecular Genetic Analysis of a Class B Periplasmic-Flagellum Gene of *Treponema phagedenis*

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Treponema phagedenis **is a host-associated spirochete with multiple polypeptides making up its periplasmic flagella (PFs). Each PF has a 39-kDa polypeptide making up the sheath (class A PF polypeptide) and two to four antigenically similar 33- to 34-kDa polypeptide species making up the core (class B PF polypeptides). A genetic analysis of the PF-deficient mutants T-40 and T-55 has shown that the PFs are involved in motility. To better understand the synthesis and assembly of these complex organelles and to compare the PF genes with those of other spirochetes, we cloned and characterized the *T. phagedenis flaB2* gene, which encodes one class B polypeptide. The *flaB2* gene consists of an open reading frame of 858 nucleotides capable of encoding a protein of 31.5 kDa. The predicted amino acid sequence of the FlaB2 polypeptide was 92% identical to that of *T. pallidum* FlaB2, with a 76% identity at the nucleotide level. These results confirm previous immunological and N-terminal-sequence analyses which suggested that the PF genes are well conserved in the spirochetes. Primer extension analysis of *T. phagedenis flaB2* indicated that the start site of transcription was 127 nucleotides upstream from the ATG initiation codon. Preceding the start site is a DNA sequence similar to the σ^{28} consensus promoter sequence commonly found associated with motility genes. Northern (RNA) blots probed with a segment of *flaB2* DNA revealed a 1,000-nucleotide monocistronic transcript in the wild type and in PF-deficient mutants T-40 and T-55. DNA sequencing of most of the *flaB2* gene of the mutants revealed no differences from the wild-type gene. Because the mutants fail to synthesize detectable class B PF polypeptides yet synthesize extensive amounts of *flaB2* mRNA, PF synthesis in *T. phagedenis* is likely to involve regulation at the translational level.**

Treponema phagedenis is a nonpathogenic host-associated spirochete that is morphologically and antigenically related to *Treponema pallidum*, the causative agent of syphilis (49; for a review of treponemal antigens, see reference 50). Although the two have similar morphologies, there is only about 5% DNA-DNA hybridization between the two species (31). *T. phagedenis* consists of a cell wall and inner membrane that encloses the cytoplasmic contents; a periplasmic space, wherein lie the periplasmic flagella (PFs; also referred to as axial fibrils, axial filaments, and endoflagella); and an outer membrane surrounding the cell (15, 17). Cells are right-handed helices except at the ends, which are typically bent and left-handed (9). *T. phagedenis* has from four to six short, left-handed PFs (9) that are inserted subterminally at each end of the cell cylinder, but these PFs can protrude under certain conditions (8).

PFs are important organelles for *T. phagedenis* and other spirochetes. Genetic evidence has shown that PFs are involved in motility of *Leptospira* (5), *Treponema* (24), *Spirochaeta* (4, 39), and *Borrelia* (44) species, and PFs are essential elements of the current models of spirochete motility (2, 13). Recently, direct evidence has shown that the PFs actually rotate, a key element in both models (8). Among the pathogenic spirochetes, evidence suggests that motility is likely to be an important virulence factor (44, 51); these organisms can swim in highly viscous gellike environments such as connective tissue, where other organisms fail to penetrate (6, 20). Finally, because PFs are major immunogens in syphilis (3) and Lyme disease (28), seroreactivity to PF antigens has been used in disease diagnosis (34, 41, 52).

We have been using *T. phagedenis* as a model system for analyzing spirochete structure and motility. We find, for example, that the bent ends are in part dictated by the shape of the PFs. Motility mutants T-40 and T-55, which fail to synthesize PFs, also fail to form bent ends, whereas all revertants to motility regain the PFs and the bent-end morphology. These results suggest that PFs are involved in motility and the bent-end characteristic (9, 24).

Biochemical studies have shown that the PFs of *T. phagedenis* consist of multiple polypeptides (see reference 50 for a review) and that at least two different polypeptides reside together on a given PF (24). The PFs consist of three major proteins: FlaB1 (formerly Tphg34), FlaB2 (formerly Tphg33), and FlaA (formerly Tphg39), with masses of 34, 33, and 39 kDa, respectively (35). Purified PFs also produce two minor bands of 33 and 34 kDa, and in some preparations, they produce minor bands of 30 and 37 kDa (35). Immunoprecipitation, immunoelectron microscopy, epitope cross-bridging, and analogy to other spirochete PFs indicate that the FlaA polypeptide represents the sheath protein, whereas the FlaB polypeptides represent the core (50). The PFs of *T. phagedenis* are antigenically and structurally related to the PFs of other spirochetes. The FlaB polypeptides of *T. phagedenis* cross-react immunologically with those of other treponemes, i.e., *Serpulina* spp. and *Spirochaeta aurantia* (4, 25, 35). In addition, the FlaA polypeptides of *T. phagedenis* and *T. pallidum* show weak antigenic similarity (35). At the molecular level, N-terminal amino acid sequence

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analysis reveals a striking homology among the FlaB polypeptides of *T. phagedenis*, *T. pallidum*, and *S. aurantia* (35, 37). DNA sequence analysis of the entire *flaB* genes of *T. pallidum* and *Leptospira borgpetersenii* and the PF filament gene of *Borrelia burgdorferi* also indicate that PF genes are well conserved among the spirochetes (7, 33, 36, 53).

We are extending our previous studies of *T. phagedenis* to include a characterization of the PF genes of wild type and of PF-deficient motility mutants. In contrast to *T. pallidum*, *T. phagedenis* is readily cultivable in vitro (49). Accordingly, regulatory elements involved in transcription of PF genes can be directly identified. Also, the PF-deficient mutants T-40 and T-55 have been well characterized (9, 24). These mutants lack not only PF structures but also detectable levels of FlaA or FlaB polypeptides (24, 35). The nature of the mutations in T-40 and T-55 remains unknown, but these mutants are a useful starting point in studying the regulation of expression of PF genes.

In this communication, we report the cloning and characterization of the gene encoding FlaB2 of *T. phagedenis*. We find that the deduced amino acid sequence of FlaB2 of *T. phagedenis* is strikingly similar to the FlaB2 protein of *T. pallidum*. In addition, primer extension and DNA sequence analyses indicate that a σ^{28} promoter is involved in *flaB2* transcription. Finally, a genetic analysis of the PF-deficient mutants T-40 and T-55 suggests that translational control may be involved in regulating the synthesis of PF proteins.

MATERIALS AND METHODS

Strains, media, and reagents. *T. phagedenis* Kazan 5 and PF-deficient mutants T-40 and T-55 have been previously described (9, 24, 35). Cultures were grown at 37°C in PYG-RS broth (24). Both the wild type and the mutants were routinely recloned on PYG-RS agar. *T. pallidum* subsp. *pallidum* was kindly provided by K. Wicher (Wadsworth Center for Laboratories and Research, Albany, N.Y.). *Escherichia coli* ER1578 [F^- Δ(*lac*)*U169 lon-100 hsdR araD139 rpsL*(Str^r) *supF mcrA trp*$^+$ *zjj-202*::Tn*10*(Tet^r) *hsdR2*(r_K^- m_K^-) *mcrB1*) contains the plasmid pMC9 and was obtained from New England BioLabs, Beverly, Mass. *E. coli* JM109 and JM101 were used for M13 transformations (54). All *E. coli* strains were grown in L broth and plated on L-agarose plates at 37°C (32). Restriction enzymes and bacteriophage M13 DNA were obtained from New England BioLabs. Radioisotopes were purchased from Amersham Corp, Arlington Heights, Ill. Oligonucleotides were synthesized at the Molecular Genetics Core Facility of the Wadsworth Center for Laboratories and Research and at the West Virginia University Recombinant DNA facility.

Construction of DNA probes for *flaB2*. A probe for the gene encoding FlaB2 was constructed by using the polymerase chain reaction (PCR; 10, 45). Primer 1, a mixed oligonucleotide with the sequence 5′-CTCGGATCCAAYCAYAAY ATG-3′ and containing the *Bam*HI restriction endonuclease recognition sequence, was synthesized on the basis of the previously determined N-terminal amino acid sequence of FlaB2 (35). Primer 2, a mixed oligonucleotide with the sequence 5′-GGGTCGACTTNGCYTGNGC-3′ and containing a *Sal*I restriction endonuclease recognition sequence, was selected on the basis of the conserved amino acid sequence AQAN near the carboxy terminus of several PF and flagellar polypeptides (11, 19, 22, 30, 36). Approximately 100 ng of *T. phagedenis* DNA was amplified by using the GeneAmp kit (PE Express, Norwalk, Conn.) according to the manufacturer's recommendations. The reaction conditions were 93°C for 1 min, 50°C for 1 min, and 72°C for 1 min for a total of 30 cycles. The DNA product was gel purified, digested with *Bam*HI and *Sal*I, and ligated into M13mp18 DNA by standard methods (29, 54). The recombinant clones were partially sequenced to confirm that the amplified product was a PF gene. On the basis of this DNA sequence, two additional primer pairs (F1 and F2) having the sequences 5′-CCGCTGCAACGTTTACGCCG-3′ and 5′-GTGGTTCC ATATCGGGGCC-3′, respectively, were synthesized. These primers were used to amplify a 248-bp segment of PF DNA from *T. phagedenis* by using identical PCR conditions. The 248-bp DNA segment was purified on an agarose gel, and approximately 100 ng was radiolabeled with [^{32}P]dCTP and a random primer oligolabeling kit (Pharmacia Corp., Uppsala, Sweden).

Preparation and screening of a *T. phagedenis* genomic-DNA library. A genomic library of *T. phagedenis* Kazan 5 DNA was prepared in λgt11. Briefly, DNA was partially digested with *Rsa*I, *Alu*I, and *Hae*III to give an average fragment size of 2 to 5 kb. The purified fragments were ligated overnight at 16°C to *Eco*RI linkers (5′-CCGGAATTCCGG-3′; New England BioLabs), digested with *Eco*RI, and then purified by using a Sepharose CL-4B column (Pharmacia). This purified DNA was ligated for 18 h to λgt11 vector DNA (Promega Corp, Madison, Wis.). The ligated DNA was packaged by using the Gigapack kit (Stratagene, La Jolla, Calif.) and then amplified on *E. coli* ER1578 by standard methods (32).

The *T. phagedenis* DNA library was screened by using a plaque hybridization assay (47). Briefly, the library was diluted, plated on *E. coli* ER1578 on L-agarose plates containing 50 μg of ampicillin per ml, and transferred to nitrocellulose filters (Schleicher & Schuell, Keene, N.H.). After lysis and denaturation, the filters were prehybridized for 2 h and then hybridized overnight at 65°C. The filters were washed under high-stringency conditions and exposed to Kodak X-Omat AR film for 24 h. Positive plaques were picked, replated, and screened by hybridization twice more to ensure purity.

Subcloning, DNA sequencing, and blotting. DNA was isolated from purified bacteriophage, digested with *Eco*RI, and ligated into M13mp18 vector DNA (54). Single-stranded DNA was isolated by standard methods, and double-stranded DNA was purified on a plasmid isolation column (Qiagen, Chatsworth, Calif.) as directed by the manufacturer. DNA sequencing was done by the Sanger dideoxy method (46) using Sequenase (United States Biochemical Corp, Cleveland, Ohio) or the Amplitaq kit (PE Express) as directed by the manufacturers. Initial sequence information was obtained by using M13 primers (New England BioLabs), and then specific oligonucleotides were synthesized and used as primers for extending the sequence. DNA and amino acid sequences were assembled and analyzed by the programs available through the University of Wisconsin Genetics Computer Group (12).

Southern and Northern (RNA) transfers were performed according to standard methods (47, 55) using a Zeta-Probe membrane (Bio-Rad, Richmond, Calif.). The RNA was separated by using 1.4% agarose–1.5% formaldehyde gels as described previously (55). Hybridization and washings were done under stringent conditions as described earlier (55).

Primer extension mapping. The 5′ end of the RNA transcript was mapped with avian myeloblastosis virus reverse transcriptase and a primer extension system (Promega Corp.). Briefly, the oligonucleotide primer 5′-CTGTGCAA ACATAGCGC-3′ was end labeled with T4 polynucleotide kinase and hybridized to 1 to 5 μg of RNA for 20 min at 50°C.

The primer was extended with 10 U of avian myeloblastosis virus reverse transcriptase at 42°C for 40 min. The product was run on a 6% polyacrylamide sequencing gel adjacent to a ^{35}S-labeled DNA sequencing ladder that was produced by using the same oligonucleotide primer, DNA template, and Sanger dideoxy sequencing technique.

Amplification and sequencing of *flaB2* in the nonmotile mutants T-40 and T-55. The *flaB2* gene was amplified from the nonmotile mutants by PCR under the same conditions as described above but with primers F4 (5′-CTGCAAAAA GTTTAGCGC-3′) and F5 (5′-TTTAGAATTCGTTCCGTC TTCTGCGAGGG-3′). The F5 primer has an *Eco*RI site engineered at the 5′ end to facilitate cloning. The 3′ end of the amplified DNA product contains the authentic *Sal*I site of the gene. The amplified product was purified on an agarose gel, digested with *Eco*RI and *Sal*I, and purified on a Spin-Bind column (FMC Corp., Rockland, Maine). The DNA segment was ligated to M13mp19, transformed into JM109, and sequenced.

Immunoprecipitation. *T. phagedenis* Kazan 5 and PF-deficient mutants T-40 and T-55 were grown in 25 ml of PYG-RS broth to late logarithmic phase. Cultures were placed on ice and sonicated intermittently for 10 min. A 400-μl sample of the sonicate was mixed with 100 μl of 5× NETS buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40) and 10 μl of PF-specific antiserum (23) and incubated for 1 h at 25°C with gentle shaking. To immunoprecipitate PFs, 50 μl of protein G-agarose (Pierce, Rockland, Ill.) was added to the sonicate and incubated for 60 min at 25°C with gentle shaking. The immunoprecipitate was centrifuged at 12,000 × *g* for 1 min and washed four times (10 min each) with 500 μl of 1× NETS. After the last wash, the immunoprecipitate was suspended in 20 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer, and Western blots (immunoblots) were done as previously described with PF-specific antiserum (24).

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number for the *flaB2* gene of *T. phagedenis* is M94015.

```
                                                                *-35*
1    TAATTATGTAAAAACAAAAATACAATAAAAAAAGATAAAATATTGAAACTTTTTTCTTAA   60

                  **-10**      ↓
61   GTGTTTTTTCCGCCATCCGATACTAAATACAAGCACAGCGAAAACTGTGTTCCGTCTTCT  120
121  GCGAGGGTTTTCAGAGGATCCGGAATAAATATGACATATAGGCAGGGATGCCATGGCGGA  180
181  ATGAACCGCTAAAAATCGCCAAAGGAGTTAGCAATATGATCATTAATCACAACATGAGCG  240
                                    M  I  I  N  H  N  M  S

241  CTATGTTTGCACAGAGAACATTAGGAAACACAAACCTATCAGTTCAGAAAAACATGGAAA  300
     A  M  F  A  Q  R  T  L  G  N  T  N  L  S  V  Q  K  N  M  E

301  AGCTTTCATCGGGATTGCGCATTAATCGTGCCGGAGATGATGCTTCAGGTTTGGCTGTAT  360
     K  L  S  S  G  L  R  I  N  R  A  G  D  D  A  S  G  L  A  V

361  CGGAAAAAATGAGAAGCCAAATTCGCGGTTTGAACCAGGCAAGTACCAACGCACAAAACG  420
     S  E  K  M  R  S  Q  I  R  G  L  N  Q  A  S  T  N  A  Q  N

421  GTATTTCTTTTATTCAAGTTGCGGAGTCATATCTCCAGGAAACAACCGATGTTATTCAGC  480
     G  I  S  F  I  Q  V  A  E  S  Y  L  Q  E  T  T  D  V  I  Q

481  GCATTCGGGAATTGAGTGTCCAGTCTGCTAACGGAATTTATTCAGCAGAAGACCGCATGT  540
     R  I  R  E  L  S  V  Q  S  A  N  G  I  Y  S  A  E  D  R  M

541  ACATTCAGGTTGAAGTATCGCAACTCGTTGCGGAAATTGACCGAATTGCAAGCCATGCAC  600
     Y  I  Q  V  E  V  S  Q  L  V  A  E  I  D  R  I  A  S  H  A

601  AATTCAACGGCATGAATATGCTTACCGGAAGGTTCGCTCGAGAAACCGGAGAAAACACTG  660
     Q  F  N  G  M  N  M  L  T  G  R  F  A  R  E  T  G  E  N  T

661  TTACCGCTTCTATGTGGTTCCATATCGGGGCCAACATGGACCAGAGAACACGTGCATACA  720
     V  T  A  S  M  W  F  H  I  G  A  N  M  D  Q  R  T  R  A  Y

721  TCGGAACAATGACTGCCGCAGCACTCGGCGTTCGCGATGTTGGGGATGAATCAATTCTTA  780
     I  G  T  M  T  A  A  A  L  G  V  R  D  V  G  D  E  S  I  L

781  ATATTGATGATCCCGAAAAAGCAAACCGCGCAATCGGTACTCTTGATGAGGCCATCAAGA  840
     N  I  D  D  P  E  K  A  N  R  A  I  G  T  L  D  E  A  I  K

841  AGATTAACAAACAAAGAGCTGATCTCGGTGCATACCAGAACAGACTCGAGTACACTGTTA  900
     K  I  N  K  Q  R  A  D  L  G  A  Y  Q  N  R  L  E  Y  T  V

901  TCGGCGTAAACGTTGCAGCGGAAAACCTTCAAGCAGCCGAATCACGCATTCGTGATGTCG  960
     I  G  V  N  V  A  A  E  N  L  Q  A  A  E  S  R  I  R  D  V

961  ACATGGCAAAGGAAATGGTGGACTACACCAAAAATCAGATTCTCGTGCAATCAGGCACCG 1020
     D  M  A  K  E  M  V  D  Y  T  K  N  Q  I  L  V  Q  S  G  T

1021 CAATGCTTGCCCAAGCAAATCAGGCAACGCAAAGCGTCTTAAGCCTCTTGCGCTAAACTT 1080
     A  M  L  A  Q  A  N  Q  A  T  Q  S  V  L  S  L  L  R  *

1081 TTTGCAGTTGACGGACGACCGGCTCTTGAACTAAGAGCCGGTTTTTTTTATTTTACGATC 1140

1141 AAAATAATAATCAACGCAAAGTTAATCGATGTTTAATACGCCCTATTTGCAGAGAGGG   1198
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FIG. 1. Nucleotide and deduced amino acid sequences of *T. phagedenis flaB2*. Potential regulatory sequences are underlined and include the −10 and −35 regions of a σ^{28}-like promoter, a consensus ribosome-binding site at nt 203 to 207, and a 10-bp inverted repeat that is likely to be involved in transcription termination at nt 1098 to 1107 and 1113 to 1122. An arrow indicates the start site of transcription at nt 89 as determined by primer extension (Fig. 4).

RESULTS

Identification and subcloning of the gene encoding FlaB2. A probe for the gene encoding FlaB2 was prepared from *T. phagedenis* by PCR with mixed oligonucleotides that were synthesized on the basis of known N-terminal amino acid sequences of *T. phagedenis* FlaB2 and conserved amino acid sequences of PFs and flagella from other bacteria. After amplification and gel electrophoresis, two major DNA products of approximately 700 and 800 bp were noted. These products were gel purified, digested with *Bam*HI and *Sal*I, and ligated to M13mp18. We cloned the 700-bp product, and DNA sequencing revealed a segment of DNA that was closely related to the DNA sequence of the previously determined *flaB* PF genes of *T. pallidum* (7, 36).

To clone the entire gene, we used this DNA sequence information to synthesize two internal PF-specific primers for PCR amplification of *T. phagedenis* DNA. After amplification, a 248-bp PCR product was gel purified, radiolabeled, and used as a PF-specific probe in a plaque hybridization assay to screen a λgt11 library of *T. phagedenis* DNA. Approximately 50,000 plaques were screened, and nine of them were potential positives. Of these nine, three remained positive after purification and rescreening. All three recombinant phage had a 1.5-kb *Eco*RI insert, and one insert was selected for subcloning into M13 mp18.

DNA sequencing and identities. The partial DNA sequence of the 1.5-kb *Eco*RI clone was obtained by using M13 primers and primers F1 and F2. The remainder of the insert DNA was sequenced in both directions by using specific oligonucleotides that were synthesized on the basis of newly determined sequences. The DNA sequence of an open reading frame (ORF) and some flanking DNA is shown in Fig. 1. The sequence reveals a consensus ribosome-binding site followed by an ORF of 858 nucleotides (nt) capable of encoding a protein of 31.5 kDa. The sequence of the first 22 amino acids of this ORF is identical to the previously determined N-terminal amino acid sequence of FlaB2 of *T. phagedenis* (formerly Tphg33 [35]). In accordance with the current nomenclature and because of its similarity to *T. pallidum flaB2* (see below), this gene is designated *T. phagedenis flaB2*.

The nucleotide and deduced amino acid sequences of *flaB2* were compared with the sequences of the three class B PF polypeptides of *T. pallidum*. As shown in Table 1, *T. phagedenis flaB2* shared a high degree of homology at the nucleotide and amino acid levels with the *flaB* genes of *T.*

TABLE 1. Comparison of nucleotide and amino acid sequences of *T. phagedenis flaB2* with those of the class B PF proteins of *T. pallidum*

T. pallidum gene	% Correspondence to *T. phagedenis* gene		
	Nucleotides identical	Amino acids identical	Amino acids similar[a]
flaB1	68	77	89
flaB2	76	92	97
flaB3	66	69	80

[a] Identical plus strongly conserved amino acids.

pallidum; it showed the most homology to *T. pallidum flaB2*, with 76% identical nucleotides and 92% identical amino acids. The nonidentical amino acids were scattered throughout the length of the sequence. These results confirm the previous immunological and N-terminal-sequence studies, which indicated that the FlaB polypeptides of these species were similar (25, 35, 42). Significant homologies with the N termini of *S. aurantia* FlaB proteins were also noted (37). In addition, we noted that the *T. phagedenis* FlaB2 protein shared a 59% amino acid sequence identity with a FlaB protein of *L. borgpetersenii* (33) and homology with other PF and flagellin genes (11, 19, 22, 30, 53).

To determine whether the *T. phagedenis flaB2* gene would hybridize to *T. pallidum* DNA, Southern blotting with the 248-bp PCR-generated probe was performed. As shown in Fig. 2, the probe was specific for *T. phagedenis* DNA under high-stringency conditions and revealed a single band of 1.0 kb. No hybridization was evident with *T. pallidum* DNA. A control lane with *E. coli* DNA was negative (data not shown).

RNA transcription. To determine the size of the *flaB2* transcript in *T. phagedenis*, Northern blots were probed with the same radiolabeled DNA segment that was used for the library screening. After hybridization and washing, a 1.0-kb transcript was identified, as shown in Fig. 3. Because this size is consistent with a transcript encoding only FlaB2, we examined the DNA sequence for transcription termination and promoter elements. Analysis of the DNA sequence downstream from the 3′ end of *flaB2* reveals a potential stem-loop structure followed by a string of Ts similar to the

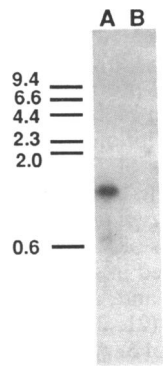

FIG. 2. Southern blot of *Hin*cII-digested chromosomal DNA probed with a ^{32}P-labeled segment of *T. phagedenis flaB2*. The blots were hybridized and washed under high-stringency conditions. Lane A, *T. phagedenis*; lane B, *T. pallidum*. Numbers on the left are molecular sizes in kilobase pairs.

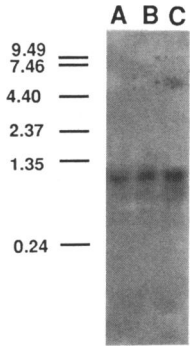

FIG. 3. Northern blot analysis of the *flaB2* RNA transcript. Each lane contains approximately 3 μg of total RNA. Blots were probed with the ^{32}P-labeled PF probe as for Fig. 2 and washed under stringent conditions. Lane A, *T. phagedenis* Kazan 5 (wild type); lane B, *T. phagedenis* nonmotile, PF-deficient mutant T-40; lane C, *T. phagedenis* nonmotile, PF-deficient mutant T-55. Numbers on the left are molecular sizes in kilobases.

sequence elements involved in a typical prokaryotic *rho*-independent transcription terminator (43; Fig. 1).

To determine the 5′ end of the transcript, an oligonucleotide primer complementary to the *flaB2* RNA was synthesized and used for primer extension. As shown in Fig. 4, a single band corresponding to a start site of transcription 127 nt upstream of the ATG initiation codon was noted (Fig. 1). Upstream from the start site is a sequence closely resembling that of the σ^{28} promoter often found in motility-associated genes of bacteria (1, 7, 14, 21, 36). The −10 and −35 regions of this promoter sequence were aligned with the putative *T. pallidum flaB2* promoter (36), and as shown in Fig. 5, these regions of the promoters are identical at 12 of 13 nt. In contrast, the DNA sequences between the −10 and

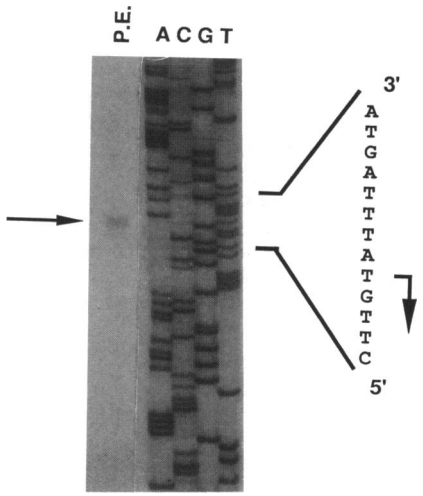

FIG. 4. Primer extension analysis of the *flaB2* transcript. The product (P.E.) was run on a DNA sequencing gel adjacent to a sequencing ladder (ACGT) produced by using the same primer. The lane containing the primer extension product was exposed to film for 1 week, whereas the sequencing-ladder lanes were developed after 18 h of exposure. The sequence shown is from the sequencing gel and therefore represents the complementary strand of the mRNA. Arrows indicate the primer extension product and the corresponding start site of transcription in the sequence.

```
-35                  - 10
CTTAAgtgtttttttccgccaTCCGATAC  ----N132-ATG    T. phagedenis
CTCAAgtgcacccggcgtatTCCGATAC  ----N80 -ATG    T. pallidum
CTAAA        N16     CCGATAT                   B. subtilis
 TAAA        N15     GCCGATAA                  E. coli/ S.typhimurium
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FIG. 5. Alignment of the proposed promoter sequence of *T. phagedenis flaB2* with the putative promoter sequence of *T. pallidum flaB2* (36) and the consensus σ^{28} promoters of *B. subtilis* (14) and *E. coli* and *S. typhimurium* (14, 21). Note a difference of only 1 nt in the conserved −10 and −35 regions of *T. pallidum* and *T. phagedenis*.

−35 regions of the *flaB2* promoters of *T. phagedenis* and *T. pallidum* are identical at only 5 of 15 nucleotides. Likewise, the DNA sequences from the promoter to the ATG initiation codon (132 nt in *T. phagedenis* and 80 nt in *T. pallidum*) are not highly homologous.

Analysis of mutants T-40 and T-55. Mutants T-40 and T-55 fail to synthesize detectable PF structures and polypeptides (24). To determine whether these mutants released PF polypeptides in the culture medium in a manner analogous to the flagellar-hook-associated protein mutants described for *Salmonella typhimurium* (16), we assayed for these polypeptides in the culture medium. No PF polypeptides were detected in the sonicated culture after immunoprecipitation with PF-specific polyclonal antiserum (not shown). As a positive control, the 33-, 34-, and 39-kDa PF polypeptides were successfully immunoprecipitated from wild-type *T. phagedenis* by using the same antiserum (not shown). In an attempt to determine the genetic basis of these mutations in T-40 and T-55, the *flaB2* gene was amplified from both mutants by using PCR, and the DNA was cloned into M13mp19. We sequenced the greater part of *flaB2*, from nt 128 (Fig. 1, between the promoter and the ATG initiation codon) to nt 960 (114 nt before the end of the gene). The DNA sequences of these regions in the mutants exactly matched that from the wild type (not shown). Because previous studies have indicated that the mutations in T-40 and T-55 are pleiotropic (9, 24), these results suggest that a mutation in a regulatory gene may be responsible for the PF-minus phenotype.

PF-deficient strains of *B. burgdorferi* have previously been shown to synthesize extensive PF filament mRNA (44). To determine whether mutants T-40 and T-55 also synthesize *flaB2* mRNA, Northern blot analysis with these mutants was carried out. We found that both mutants synthesize a *flaB2* message with a transcript size similar to that of the wild type and in approximately the same amount (Fig. 3). These results suggest that the failure of T-40 and T-55 to synthesize FlaB2 polypeptides is not related to an inability to transcribe the *flab2* gene.

DISCUSSION

We report the cloning, DNA sequencing, and RNA transcription analysis of the gene encoding FlaB2, a class B core PF protein of *T. phagedenis*. The gene (858 nt) is predicted to encode a protein of 31.5 kDa, which is smaller than the molecular mass of 33 kDa estimated by SDS-PAGE. Simon et al. have noted that the structural conformation of the *Bacillus subtilis* flagellum filament can affect the migration of flagellar polypeptides in SDS-PAGE gels (48). Similar discrepancies have been noted for the PF polypeptides of *T. pallidum* also (7, 18, 36).

Previous serological studies and N-terminal amino acid sequencing have indicated that the core PF proteins of *T. phagedenis* and *T. pallidum* are related, but the extent of the homology was unknown. The predicted amino acid sequence of FlaB2 of *T. phagedenis* was compared with the sequences of the previously published *T. pallidum* FlaB PF proteins (7, 36). We expected to find conserved regions interspersed among nonconserved regions, which would enable the identification of potential *T. pallidum*-specific epitopes. However, the amino acid sequence of FlaB2 of *T. phagedenis* was 92% identical to the amino acid sequence of FlaB2 of *T. pallidum*. Thus, *T. pallidum* FlaB2 most likely possesses few if any pathogen-specific epitopes.

On the basis of 16S rRNA sequencing, *T. phagedenis* and *T. pallidum* are known to have a close evolutionary relationship (40). In support of the close relationship, this report has revealed the extensive DNA sequence homology between the *flaB2* genes of both organisms. In contrast, the overall G+C content of *T. phagedenis* is much lower than that of *T. pallidum* (38 versus 53%) (49), and there is little DNA-DNA reassociation (31). However, the G+C content of the coding region of *flaB2* of *T. phagedenis* is closer to that of *T. pallidum flaB2* (46 versus 53%). In addition, the −10 and −35 regions of the *T. phagedenis flaB2* promoter were nearly identical to those of the putative *flaB2* promoter of *T. pallidum*. As with several other motility-associated genes in *S. typhimurium*, *E. coli*, *B. subtilis*, and *S. aurantia* (1, 14, 21, 22, 38), a σ^{28} promoter precedes the *flaB2* coding region. In contrast to the remarkable DNA sequence similarity between the *flaB2* promoters and ORFs of *T. phagedenis* and *T. pallidum*, DNA sequence differences were noted in flanking regions. Thus, in the promoters and ORFs of *T. phagedenis* and *T. pallidum*, we find highly conserved regions of DNA sequence separated by regions of lesser homology. The evolutionary significance of these data is unknown but may become clear as additional genes in both organisms are analyzed. However, these and other results (33, 37, 53) indicate that spirochete PF genes are highly conserved. Because of the complexity of treponemal PFs, conservation of amino acid sequences may be more strict than with other bacteria. Taken together, the results further support the proposition that the PFs play an important role in motility and thus survival of the cells.

There are at least two class B PF proteins of *T. phagedenis*, as determined by two-dimensional SDS-PAGE (35). Because the multiple protein species associated with the PF core appear to be products of three separate genes in *T. pallidum* (7, 36), we expect to find at least one additional *flaB* gene in *T. phagedenis*. Indeed, Southern blot analysis of *Not*I-digested *T. phagedenis* DNA separated by pulsed-field gel electrophoresis revealed two bands capable of hybridizing to the *flaB2* probe (26). Judging from this result, it is possible that the *flaB2* probe is also detecting *flaB1* in the Northern and Southern blots described in this report.

We analyzed transcription of *flaB2* in the wild type and in two nonmotile mutants of *T. phagedenis*. Our results were surprising and different from those for the previously described mutants of *S. typhimurium* (21, 27). Some flagellar-filament-minus mutants of *S. typhimurium* have mutations in the early genes involved in synthesis of the flagellar apparatus (such as the hook) and, as a result, do not transcribe the flagellin filament gene (21, 27). At present, regulation of the known motility genes of *S. typhimurium* and *E. coli* has been demonstrated only at the transcriptional level (21, 27). In contrast, we found that the two mutants of *T. phagedenis* generated RNAs that were similar in length and approximate amount to that of the wild type. These results were surprising because it was previously shown by Western blotting

that these mutants do not synthesize detectable levels of any of the class B or class A PF proteins (24, 35). Similar results have also been reported with PF-deficient mutants of *B. burgdorferi* (44).

What is the nature of the mutations in T-40 and T-55? One possibility is that there is a mutation in the *flaB2* gene which results in an altered polypeptide that is unable to assemble into an intact filament. However, no nucleotide changes were detected in the major part of the *flaB2* genes of the mutants, and PF polypeptides were not detected in sonicated cultures. These results indicate that these mutants are not directly analogous to the hook-associated polypeptide mutants described for *S. typhimurium* (16). Although a rapid degradation of PF proteins could also account for the inability to detect PF polypeptides by immunoprecipitation, a more likely alternative is that translational control or post-transcriptional factors are involved in the regulation of PF synthesis. Recent results with PF-deficient mutants of *B. burgdorferi* also suggest that PF synthesis may be regulated at the translational level (44). In vitro protein synthesis studies using wild-type and mutant cell extracts and purified *flaB2* RNA may help determine the nature of the translational control elements in *T. phagedenis*. Molecular cloning and analysis of transcription of other *T. phagedenis* motility genes may also help define the nature of the mutations in T-40 and T-55.

The cloning and sequencing of *flaB2* reveal the excellent conservation of amino acid sequences among the motility-related proteins of a wide variety of bacteria. Initial studies of spirochete PFs revealed a homology to the flagella of *Roseburia cecicola*, *B. subtilis*, *S. typhimurium*, and *E. coli*. As more PF sequences were determined, it became apparent that the amino acid sequences of the flagellar filaments of bacteria and the core PF proteins of spirochetes are conserved, particularly at the amino and carboxy termini. On the basis of this high level of conservation of motility-associated genes, we have identified a gene of *T. phagedenis* that is related to the hook genes of *S. typhimurium* and *E. coli* (26). Further delineation of the PF structural genes will be helpful in determining the molecular mechanisms involved in spirochete motility.

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