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Organization, Transcription, and Expression of the 5′ Region of the *fla* Operon of *Treponema phagedenis* and *Treponema pallidum*

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A locus encoding polypeptides associated with flagellar structure and function was identified, sequenced, and characterized in *Treponema phagedenis* and *Treponema pallidum*. This locus includes homologs of the FlgD, FlgE, MotA, MotB, FliL, and FliM polypeptides found in *Salmonella typhimurium* and *Bacillus subtilis*. These polypeptides are extensively conserved between the two treponemes. Several additional polypeptides of unknown function, including Tap1, located upstream of FlgD, and ORF4, located between FlgE and MotA, were also identified. Transcription analysis using RNA PCR indicated that these genes are likely transcribed as part of a single operon and comprise the 5′ region of the treponemal *fla* operon. Primer extension analysis identified a putative promoter, P_{fla}, preceding *T. phagedenis tap1* in a region of divergent transcription. P_{fla} resembles the class II or class III motility-related promoters of *S. typhimurium*. FlgE and Tap1 were further characterized. Western blotting (immunoblotting) indicated that *T. pallidum* FlgE exhibited an unusual polypeptide ladder that was similar but not identical to that of *T. phagedenis*. Triton X-114 phase partitioning of *T. phagedenis* cells coupled with Western blotting revealed that Tap1 was located in the aqueous phase. Computer analysis indicated that Tap1 had no significant membrane spanning regions, suggesting that it resides primarily in the cytoplasm. The organization and expression of this operon are similar in both treponemes but different from those of previously described motility-related operons. These results indicate that despite extensive amino acid sequence conservation, the expression of spirochete flagellar polypeptides is different from that in other bacteria.

Treponema phagedenis is a nonpathogenic host-associated spirochete; in contrast, *Treponema pallidum* is a pathogenic spirochete and the etiologic agent of syphilis (43). There is minimal DNA-DNA homology between these spirochetes (33), and they have markedly different genome sizes (26, 46). In contrast to functional and genetic differences, these spirochetes have similar features related to motility. For example, the flagellar filaments are located in the periplasm and are surrounded by the outer membrane; they are not in contact with the external environment except for the occasional protruding filaments (4, 6, 7). In addition, the treponemal flagellar polypeptides have extensive amino acid sequence homology (35). However, limited information regarding the flagellar hook and basal body of the treponemes is available. For example, the *T. phagedenis* hook polypeptide consists of an unusual ladder of bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27), but the *T. pallidum* hook has not been characterized. Motility of spirochetes is a likely factor in pathogenesis (41, 45), and analysis of the genes involved in the motility of a nonpathogenic and a pathogenic spirochete may help to understand their genetic relationship and polypeptides important for structure, function, or pathogenesis.

There are approximately 50 known genes encoding structural and functional polypeptides involved in the motility of enteric bacteria (29, 30). Extensive genetic analysis has indicated that motility genes are often conserved and frequently associated in large operons (1, 10–12, 14, 15, 30). Regulation of these operons in enteric bacteria involves the temporal expression of a cascade of bacterial motility genes that are transcriptionally regulated through different sigma factors that recognize specific promoter elements (23, 30). Genes encoding polypeptides required earlier in flagellum synthesis, such as the hook, are expressed from class II promoters. The gene encoding the flagellar filament is expressed later from a class III promoter (23). These two classes of promoters have similar −10 DNA sequences, but class II promoters lack a consensus −35 region (23).

In contrast to the well-characterized motility of enteric bacteria, little is known about the genetics of *T. phagedenis* motility, particularly the genes encoding the flagellar hook and basal body. Previous work has identified two contiguous genes: *flgE*, which encodes the flagellar hook, followed by *motA*, which encodes a polypeptide essential for functioning of the flagellar motor (2, 27). The lack of putative promoter sequences together with an open reading frame (ORF) located immediately upstream of *flgE* suggested that *flgE* may be part of an operon (27). In addition, the extensive region between *flgE* and *motA* suggested that *flgE* may be the final gene of the operon. However, the identity, regulation, and expression of these and nearby genes were unknown. In this report, the organization of genes at this locus was identified, and transcription studies determined that *T. phagedenis flgE* was part of an extensive operon of motility-associated genes controlled by a class II- or class III-like promoter. In addition, this locus was sequenced in *T. pallidum*, a pathogenic treponeme that has not been extensively characterized at the genetic level. In contrast to the poor DNA-DNA homology and different G+C contents previously noted for *T. pallidum* and *T. phagedenis* (33, 43), we found similar gene arrangements and extensive homology of

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this operon in these treponemes. This work shows that treponemes share some homologous motility-associated polypeptides with other bacteria, but their organization and expression in this operon are unique in the spirochetes.

MATERIALS AND METHODS

Strains and reagents. The culture conditions for *T. phagedenis* Kazan 5 and the basic materials used for molecular studies have been previously described (24). *T. pallidum* subsp. *pallidum* was kindly provided by K. Wicher (Wadsworth Center, Albany, N.Y.), and DNA was isolated by standard methods (32). Lambda ZAP Express cloning kits, pBluescript II SK DNA, and pBK-CMV DNA were obtained from Stratagene (La Jolla, Calif.). The primer extension system was obtained from Promega Corp. (Madison, Wis.). *Taq* polymerase and GeneAmp RNA PCR materials were purchased from PE Express (Norwalk, Conn.). Oligonucleotides were synthesized and automated DNA sequencing was done by the Molecular Genetics Core Facility of the Wadsworth Center, using a DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Div. of PE, Foster City, Calif.) in a PE 9600 ThermalCycler. DNA probes were prepared by amplification and labeled with digoxigenin by using the Genius system (Boehringer Mannheim Corp., Indianapolis, Ind.).

Identification of *flgE* and flanking DNA of *T. phagedenis* and *T. pallidum*. *T. phagedenis flgE* was previously sequenced and characterized (27). To obtain additional DNA sequence flanking *flgE*, a *T. phagedenis* genomic library in Lambda ZAP Express was screened by using a plaque hybridization assay with digoxigenin-labeled DNA probes located adjacent to *flgE* (27, 42).

A probe to identify *T. pallidum flgE* was constructed by amplification of *T. pallidum* chromosomal DNA, using the degenerate oligonucleotide primers tpall2 (5′-CTAAGGATCCATGCA[G,A]AA[C,T]CA[C,T]CA[G,A]AC-3′) and tpall4 (5′-TCAAGTCGAC[C,T]TC[G,A,C,T]CC[G,A]AA[G,A,C,T]GT[G,A]TC[G,A]T-3′). The oligonucleotides were synthesized on the basis of conserved N- and C-terminal amino acid sequences of *T. phagedenis* and *Salmonella typhimurium* FlgE (19, 27). The amplification reaction conditions were 93°C for 1 min, 45°C for 1 min, and 72°C for 1 min for a total of 30 cycles. The 615-bp DNA product was washed with a Centricon-100 unit (Amicon, Danvers, Mass.) and then digested with *Bam*HI and *Sal*I. The digested DNA was purified by using the Qiaex DNA purification resin (Qiagen Corp., Chatsworth, Calif.) and then ligated to M13mp18 DNA. DNA sequencing of appropriate recombinant clones revealed the sequence of a segment of *T. pallidum flgE*. A *T. pallidum* library of genomic DNA in Lambda ZAP Express was screened with this and other probes by using a plaque hybridization assay (42). Additional flanking DNA sequence was obtained by using similar methods and probes specific for the 5′ and 3′ ends of known DNA sequence.

DNA segments from purified bacteriophages isolated from the genomic library screenings were subcloned into various vectors, including M13mp18 and -mp19, pBluescript II SK, and pBK-CMV. Sequencing of the cloned DNA segments was accomplished by generating deletions with either exonuclease III (18) or the appropriate restriction endonucleases. Remaining gaps in the DNA sequence were filled in by using synthetic oligonucleotides prepared by the Molecular Genetics Core Facility. Sequences were analyzed by using programs available through the University of Wisconsin Genetics Computer Group (9), and membrane spanning regions were predicted by using TopPred II (8).

Total RNA isolation, RNA PCR, and primer extension assays. Approximately 10^9 *T. phagedenis* cells were washed twice with cold phosphate-buffered saline, and then RNA was isolated by using a Rapid RNA kit (Amresco, Solon, Ohio) according to the manufacturer's instructions. To remove contaminating DNA, the final nucleic acid pellet was resuspended in 90 μl of 1 mM dithiothreitol containing 40 U of RNase inhibitor (Promega) and 20 U of RNase-free DNase, and then 10 μl of 10× DNase buffer was added. After 30 min of incubation at 37°C, the RNA was extracted with phenol-chloroform and precipitated with sodium acetate and ethanol.

A reverse transcriptase reaction which was followed by PCR using a GeneAmp RNA PCR kit (PE Express) was used to detect RNA transcripts. Briefly, about 20 ng of RNA (usually 1 μl of a 1:30 dilution of the total RNA sample) was mixed with the appropriate oligonucleotide primer and the required reagents according to the manufacturer's instructions. After a 15-min incubation at 42°C, the reverse transcriptase was inactivated by a 5-min incubation at 99°C. The reaction mixture was incubated for 5 min at 4°C, required reagents and forward oligonucleotide primer were added, and the cDNA was then amplified for 30 cycles consisting of 1 min at 93°C, 1 min at 50°C, and 1 min at 72°C. DNA products were analyzed on agarose gels. RNA PCRs were also done on the same RNA templates without reverse transcriptase to serve as a control to detect any contaminating DNA.

Primer extensions were performed according to the manufacturer's instructions, using about 2 μg of *T. phagedenis* total RNA. Two different oligonucleotides were used as primers in separate extension reactions.

Expression and purification of *T. phagedenis* recombinant Tap1 polypeptide. The gene encoding Tap1 was amplified from *T. phagedenis* genomic DNA, and a maltose-binding protein system (New England Biolabs, Beverly, Mass.) was used to clone, express, and purify the recombinant polypeptides (13). The primers TPHM1END (5-TAATTGAATTCGGCGCGGAAATTCTG-3′), which contains a synthetic *Eco*RI site, and KC15 (5′-GCGCAACAAACTGTGTGTC-3′) were used to amplify *tap1* from *T. phagedenis* genomic DNA. A native *Bam*HI site is located downstream from the 3′ end of the *tap1* gene. After amplification, the PCR product was washed and concentrated in a Centricon-100 unit. The DNA was then digested with *Eco*RI and *Bam*HI and further purified by phenol-chloroform extraction and use of a QiaexII gel extraction kit. This DNA was ligated into pMal-c2 and transformed into *Escherichia coli* JM109. Clones that contained the proper insertion were transformed into JM101 for protein expression. Recombinant MBP-Tap1 fusion protein was expressed and purified by previously described methods (27). The purified fusion proteins were analyzed by

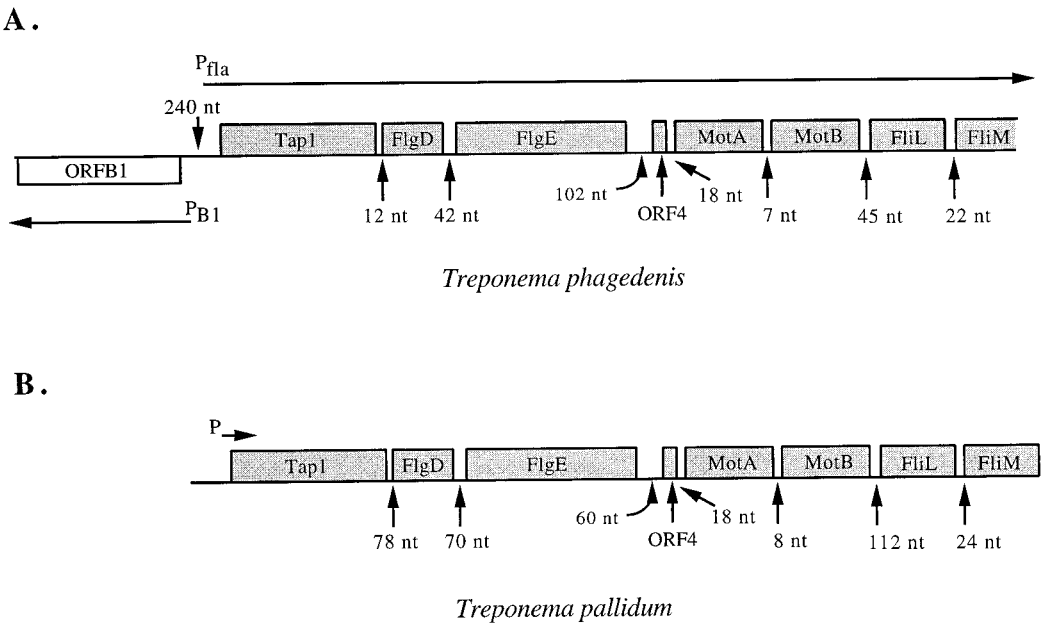

FIG. 1. The 5′ ends of the *fla* operons of *T. phagedenis* (A) and *T. pallidum* (B). Horizontal arrows in *T. phagedenis* indicate the extents and directions of RNA transcripts determined by RNA PCR. P indicates a putative promoter sequence. Vertical arrows indicate the lengths of intergenic regions in nucleotides (nt). Shaded boxes indicate polypeptides comprising the 5′ end of the *fla* operon. Lengths and identities of the genes are indicated in Table 1.

TABLE 1. Lengths and comparisons of polypeptides encoded by the *fla* operon of *T. phagedenis*

Protein	Length (amino acids)		% Identity[a]	
	T. phagedenis	*T. pallidum*	*T. pallidum*	*B. subtilis*[b]
Tap1	446	547	27	NA[c]
FlgD	163	153	55	31
FlgE	463	463	83	NA
ORF4	65	65	61	39
MotA	259	259	76	33
MotB	238	238	72	26
FliL	180	182	61	21
FliM[d]	NA	344	89	41

[a] Identical amino acids compared with *T. phagedenis.*
[b] From references 1, 47, and 34.
[c] NA, not available.
[d] *T. phagedenis* FliM is partially sequenced.

SDS-PAGE and stained with Coomassie blue R-250 (Bio-Rad Laboratories, Richmond, Calif.) (25).

Expression and purification of *T. pallidum* recombinant FlgE. *T. pallidum* FlgE was cloned and expressed in *E. coli*, using DNA amplification and the maltose-binding protein system as described above. The primers used to amplify *flgE* were PtnA (5′-TTTAGAATTCACTACCGGTTTTAAGCGTGGG-3′), which contains a synthetic *Eco*RI restriction endonuclease cleavage site, and TpalHKC (5′-TATAAAGCTTCCCTCACCACATAGCAC-3′), which contains a synthetic *Hin*dIII site. After amplification, the DNA product was purified as described above and ligated into pMAL-c2, and cells harboring plasmids with the correct insert were identified. Expression and purification were carried out as described above.

```
  1 ..MQTLGIGAEILPLANTGDA..ELKEPSKKEAAEPVRKGDPFPAMLK..  44
      :..|    |  :.:|:.:.|   |  ..:.. ||.|.. ::.:.. ::
  1 MELRGLLARAIGIGVAESAPAAQETISVGTSCAAAPCSFAQSLHDHMRGA 50

 45 ..KMIAKAMEGENPQEKQTLAADFHIHKETGTDNGRDVIENKKLASIL..  90
      ..:. . .:...:|:   .||   ..:||.:...||:.:..|.::
 51 APQRVSLSPASAAVHERVREEAD...EQQTGSGTQQDVFTEESLSDVFLV 97

 91 ............KKDEKVLNFQKKDDKKGTFAFVDSKKNEPDVKNAKDKQ 128
                .: :.::: : .:. :. |   |....|:.  .. ..
 98 AYETGLRAAQEAASGVRPFDYVRAAQEASGGA...SWPSAPEFLVVERQA 144

129 ISPKKSSIKQNKNLNLHVGEA...EEFNIEKLQETIEINEPVLLFSSQKE 175
    .|....|: .:.  . . | |   .::. .  | . |....|  :::|..
145 TSVTPRSVVPEQPSSGERGRAARPAGLSRAYAQRVPETAPHVQDLCGQES 194

176 VEEKLESSG.....................................K 185
    .|.  ||.|                                      .
195 GEHLRESAGARGLRGGSLPSGAAPALLREQGSEATYAASSESEVFDVIFD 244

186 GKKKNKTNSATVVAEAAAKQDSLLAKLDSKLQKEEETAAVQKQSARTKKL 235
    :. :.:  .|.: :|:. . ::  ..:::| . ....||..  .: . .
245 AEVRQRLVAAAIRGEPVLQSGGAGSSFGGKAGASHASAAAGVSAGAAFQG 294

236 VFDVQDLRTGEAEQG.........TIHEA.....GSEVRVSTETA...PD 268
    . . .::| |:..|:          |: :|      |.:  ||...:   |.
295 ATAYGQIRHGDSPQELGSFSDARFTVLDARRHSRGAGSGVSSHAGAVLPA 344

269 ATLEFRADSTVEGAAQNK.............QGVADAK..PAQPQNV 300
    |  ::.|... .||.::              :|||.|:  .||.|.:
345 AQAQLSAADVDAGAEEHGTESLREGERAGVERAFEGVAHAREETAQSQGI 394

301 ......QSAFLQQIQSASTDIVQSGKIILRDNNQGLIRINLKPEQLGTVK 344
          |.|: .:|.. |.::.|||:::|||:..|.| |.||||:||.|.
395 RGFATTQAALSAHIRAHSAELAQSGRVVLRDHGRGYIDIALKPEHLGAVS 444

345 INLELSGDKKLTGRISVASKEAYEAFKESLHDLTVAFEQGGFETAGFDLQ 394
    |.|.||::|::.| | |||.||:|||.|.| ||. |||..||:.|.||:|
445 IRLALSENKRVVGTIHVASQEAFEAFQENLGDLARAFEANGFDAAQFDVQ 494

395 WFGESNAKAFSDENMFHS.LSAYESNSGLKS.GNEFTEKQIYSYGQAEA. 441
    |||   | | .::. : |  .||...  | : | | . .::...:|:.|
495 WFG...AGAHAEDGHLASQTAAYAAAQRLGGAGVEPVVQEVHWHAQGAAL 541

442 .VNILA  447     T. phagedenis Tap1
     ::::|
542 TIDVFA. 547     T. pallidum Tap1
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FIG. 2. Alignment of the amino acid sequences of Tap1 from *T. phagedenis* and *T. pallidum*, using the GAP program. Note that extensive homology is confined to the carboxyl terminus. | indicates identical amino acids; : and . indicate similar and weakly related amino acids, respectively.

TABLE 2. RNA PCR analysis of the *T. phagedenis fla* operon and upstream DNA[a]

Primer F	Primer R	RNA PCR product[b]
fla operon region		
IG1[c]	*tap1*	−
tap1	*tap1*	+
tap1	*flgD*	+
flgD	*flgE*	+
flgE	*motA*	+
motA	*motB*	+
motB	*fliL*	+
fliL	*fliM*	+
Upstream DNA region		
orfB1	*orfB1*	+
IG2[d]	*orfB1*	−

[a] Primer R is used for the reverse transcription reaction and anneals at the indicated region. Primer F is added to enable amplification after reverse transcription and anneals at the indicated region.
[b] Presence (+) or absence (−) of an RNA PCR product.
[c] DNA sequence is 5′-GAGATACAGAAGGGTTG-3′ and is located near P_{B1} (upper sequence, Fig. 4) in the noncoding region between ORFB1 and Tap1.
[d] DNA sequence is 5′-CGTTGCTTTTTCTTGACTCTTG-3′ and is located near P_{B1} (lower sequence, Fig. 4) in the noncoding region between ORFB1 and Tap1.

Western blots of *T. phagedenis* and *T. pallidum*. Antiserum was raised against *T. pallidum* FlgE and *T. phagedenis* Tap1 in BALB/c mice, using RIBI adjuvant (RIBI Immunochemical Corp., Hamilton, Mont.) as described previously (27). Western blotting (immunoblotting) using these antisera was performed as previously described (25), and blots were developed with alkaline phosphatase-conjugated goat anti-mouse antibody (Bio-Rad), using 5-bromo-1-chloro-3-indolyl phosphatase and nitroblue tetrazolium (Promega Corp.).

Triton X-114 phase partitioning of *T. phagedenis*. Extraction and Triton X-114 phase partitioning of *T. phagedenis* were done essentially as described by Radolf et al. (39), with minor modifications. Approximately 10^9 cells of *T. phagedenis* were washed three times with 1 ml of cold phosphate-buffered saline, and the final pellet was resuspended in 200 μl of cold phosphate-buffered saline. Cold Triton X-114 was added appropriately to bring the final concentration to 0.02, 0.1, 0.5, or 2.0%. The material was incubated for 20 min at 4°C with gentle agitation. Phase partitioning was then done exactly as previously described (39). The insoluble pellet and the detergent and aqueous phases for each concentration of Triton X-114 were analyzed by Western blotting with the Tap1 antiserum.

Nucleotide accession numbers. The GenBank nucleotide accession numbers for the *T. pallidum* DNA sequences are U42012 (*tap1* and *flgD*) and U28219 (*flgE*, ORF4, *motA*, *motB*, *fliL*, *fliM*, and *fliY*′). The accession numbers for the *T. phagedenis* DNA sequences are U32474 (ORFB1, *tap1*, and *flgD*) and U32475 (ORF4, *motA*, *motB*, *fliL*, and *fliM*′).

RESULTS

Identification and characterization of polypeptides adjacent to FlgE of *T. phagedenis* and *T. pallidum*. Genomic libraries of *T. phagedenis* and *T. pallidum* were screened with DNA probes to identify *flgE* and to obtain a series of overlapping clones. A diagram of the encoded polypeptides and intergenic regions identified by DNA sequencing is shown in Fig. 1. ORFB1, Tap1, and ORF4 are newly identified polypeptides of unknown function, whereas the remainder have homology to *S. typhimurium*, *E. coli* or *Bacillus subtilis* flagellar polypeptides. Except for MotB, the amino acid sequence identities of the *T. phagedenis* polypeptides were greater to *B. subtilis* (21 to 41% [Table 1]) than to *S. typhimurium* or *E. coli* (21 to 28%, not shown) sequences. FlgD is involved in assembly of the flagellar hook in *S. typhimurium* (22, 36), MotA and MotB are components of the flagellar motor (2, 29, 30), the role of FliL is unclear (31, 40), and FliM is a component of the flagellar switch (21, 30, 47). A similar set of homologous polypeptides

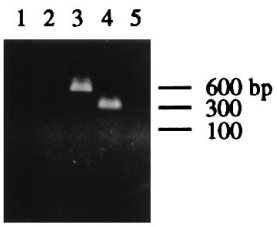

FIG. 3. RNA PCR products after electrophoresis on a 0.8% agarose gel and staining with ethidium bromide. These oligonucleotides were designed to localize promoter elements near the *T. phagedenis fla* operon. Lanes 1 to 3 use a primer pair that spans *tap1* and upstream intergenic DNA. Lane 1, test lane; lane 2, negative control with no reverse transcriptase; lane 3, positive control using a DNA template. Lanes 4 and 5 use a primer pair within *tap1*. Lane 4, test lane; lane 5, negative control with no reverse transcriptase. Relevant primer pairs are described in Table 2.

with extensive conservation of amino acid sequences was noted in *T. pallidum* (Fig. 1; Table 1). The DNA sequence of *T. pallidum fliM* reported here overlaps the *fliM′* sequence reported by Hardham et al. for the *T. pallidum fla* operon (14). In concordance with their nomenclature, we have named this region of the *T. phagedenis* chromosome the *fla* operon. Because of the extensive homology of most of the treponemal *fla* polypeptides to the *S. typhimurium* and *E. coli* homologs, we expect that they serve similar functions in the spirochetes. To this end, the amino acid sequence of MotA was analyzed by the TopPred II program (8), which predicted four hydrophobic membrane spanning regions which correlate closely with similar domains of *E. coli* MotA (data not shown) (3, 26).

ORFs of unknown function. ORF4 is a newly identified ORF of unknown function. Prior analysis of the region of DNA between *flgE* and *motA* of *T. phagedenis* did not reveal any likely ORFs (27). However, comparison of this region with *T. pallidum* revealed a similar, short ORF. In addition, homologous ORFs were noted in *Treponema vincentii* (26), *T. denticola* (26), *Borrelia burgdorferi* (10, 11), and *B. subtilis* as well (1).

Tap1 is also a unique polypeptide with no significant homologs detected in other bacteria. The amino acid sequence alignment of Tap1 from *T. phagedenis* and *T. pallidum* is shown in Fig. 2 and reveals minimal sequence conservation throughout most of the polypeptide, which abruptly changes to extensive identity near the carboxyl terminus. The deduced amino acid sequences of the treponemal Tap1 polypeptides showed no evidence of a typical signal peptide used in the SecA-mediated protein secretion pathway (37). Computer analysis using TopPred II indicated that Tap1 is hydrophilic and lacks significant predicted membrane spanning regions (not shown).

T. phagedenis ORFB1 was not homologous to any known polypeptides. It is not part of the *fla* operon and is transcribed in the opposite direction of the *T. phagedenis fla* operon, probably by a heat shock promoter (see below). There were insufficient sequence data available to determine whether *T. pallidum* contained a homolog of ORFB1.

Transcription analysis. RNA PCR was used to analyze transcription of the genes identified in *T. phagedenis*. Each of the oligonucleotides pairs used to span the intergenic regions downstream of Tap1 generated a DNA product after RNA PCR (Table 2). These results indicate that all of these genes are likely part of the same RNA transcript and therefore would constitute an operon of motility-associated genes. Primer pairs that span Tap1 and the upstream intergenic DNA produced no product after RNA PCR, indicating that the 5′ end of the operon is located in this region (Fig. 3; Table 2). Primer extension analysis (not shown) revealed the start site of transcription (Fig. 4). A putative promoter was identified upstream of the start site (Fig. 4) that was similar to the sequence of a class II or class III (σ^{28}) promoter element found in *S. typhimurium* (23). A similar promoter sequence was noted in *T. pallidum*, although the spacing between the −10 and −35 regions was 16 nucleotides (Fig. 5).

Upstream of *T. phagedenis* Tap1, the only significant ORF was ORFB1, which was directed in the opposite orientation of Tap1. RNA PCR confirmed that transcription of ORFB1 occurred in this direction (Table 2), and a putative σ^{32} (heat shock) promoter (17) was noted for this gene (Fig. 4). The two putative promoters, P_{fla} and P_{B1}, initiate transcription in opposite directions and are separated by 72 nucleotides.

Western blotting of *T. pallidum* and *T. phagedenis* with antiserum to FlgE. Whole cells of *T. phagedenis* and purified flagellar hooks produce identical ladders of polypeptide bands after Western blotting with FlgE antiserum (27). Such a ladder is not found in any other known bacterial hook polypeptide. To determine whether *T. pallidum* FlgE consisted of a ladder of polypeptide bands after SDS-PAGE, whole cells of *T. pallidum* and *T. phagedenis* were subjected to SDS-PAGE, transferred to nitrocellulose paper, and incubated with an antiserum generated against *T. pallidum* FlgE. Figure 6 shows that *T. pallidum* FlgE exhibits a polypeptide ladder similar but not identical to that described for *T. phagedenis*. As previously noted for *T. phagedenis*, the major polypeptide bands are approximate multimers of FlgE, but the exact sizes are difficult to measure since most are quite large polypeptides. These results

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                                        ACAATATCATGTTCTTTAAACAAAAACAAGAAGTTTTTAAGTTTT
    AGTTTCGCCAAAAGTATATGTATGTTATAGTACAAGAAATTTGTTTTTGTTCTTCAAAAATTCAAAA
<----- L  A  T  K  M  Y  M [ORFB1]

    GAGATACAGAAGGGTTGATTTTATGCTTCAAGAGTCAAGAAAAAGCAACGCATTGCGGTTATTTCAA
    CTCTATGTCTTTCCCAACTAAAATACGAAGTTCTCAGTTCTTTTTCGTTGCGTAACGCCAATAAAGTT
             -10    <----PB1    -35
                                                -35      Pfla---->   -10
    GTAAATTTTTGTTAATCCGGTTGATTTTATAATAAAAATCTCAACAAAAGTTTTAACATGCCGAAAT
    CATTTAAAAACAATTAGGCCAACTAAAATATTATTTTTAGAGTTGTTTTCAAAATTGTACGGCTTTA


                                                                [Tap1]  M  Q  ----->
    TATTGTATAGAAGGCGTAGTTAATACACTTTGTATTTTATTAAAGAATTGGAGGTTCATATATGCAG
    ATAACATATCTTCCGCATCAATTATGTGAAACATAAAATAATTTCTTAACCTCCAAGTATA
            ↑
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FIG. 4. Nucleotide sequence of the intergenic region between ORFB1 and Tap1 showing the locations of the putative promoters of *T. phagedenis*. Promoter sequences are underlined and boldfaced, and the −10 and −35 regions are as indicated. P_{B1} and P_{fla} indicate the promoters transcribing the B1 gene and the *fla* operon, respectively. The vertical arrow indicates the start site of transcription of the *fla* operon as determined by primer extension. Horizontal arrows indicate the directions of transcription.

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-35              -10
CTCAA-caaaagttttaacatGCCGAAAT   T. phagedenis Tap1
CTAAGacgtgctgcgcagtgcGCCGAGAA   T. pallidum   Tap1
CTTAA-gtgttttttccgccaTCCGATAC   T. phagedenis FlaB2 (28)
CTCAA-gtgcacccggcgtatTCCGATAC   T. pallidum   FlaB2 (38)
CTCAA-gtccttttccgttttTCCGATTT   T. pallidum   FlaB1,3 (5)
CTAAA-nnnnnnnnnnnnnnnnCCGATAT   B. subtilis consensus (16,17)
NTAAA-nnnnnnnnnnnnnnnnGCCGATAA  E. coli/S. typhimurium (16,23)
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FIG. 5. Sequence alignment of the proposed P_{fla} promoters of *T. phagedenis* and *T. pallidum* with other motility-related treponemal promoters and the consensus σ^{28} promoters of *B. subtilis* and enteric bacteria.

also indicate that the treponemal FlgE polypeptides have extensive antigenic similarity.

Localization and Western blotting of *T. phagedenis* Tap1. The unique identity of Tap1 warranted further study to determine whether it was located in the cell membrane. Washed cells of *T. phagedenis* were solubilized with various concentrations of Triton X-114. As shown in Fig. 7, phase partitioning after 2% Triton X-114 treatment revealed that Tap1 remained with the aqueous phase. Similar results were noted when lower concentrations of Triton X-114 were used, although at these low levels there was a minor amount of Tap1 still associated with the insoluble pellet (not shown). Because computer analysis revealed no significant membrane spanning regions and no signal sequence, it is likely that it resides in the cytoplasm and was called Tap1 (for treponemal aqueous polypeptide 1). The *T. phagedenis* Tap1 antiserum showed no significant reactivity with whole cells of *T. pallidum* on Western blots (not shown).

DISCUSSION

This work has revealed an extensive locus of motility-associated genes in *T. phagedenis* and *T. pallidum* that are transcribed as an operon. In contrast to the overall poor DNA-DNA homology between *T. phagedenis* and *T. pallidum*, the extent of the homology in FlgD and downstream polypeptides is rather striking. Moreover, the organization of the motility genes in this treponemal *fla* operon is unique among the bacterial motility operons thus far characterized. For example, in *S. typhimurium*, *flgD* and *flgE* are expressed from a class II promoter and flanked by genes encoding components of the flagellar basal body. *motA* and *motB* are located in another operon (19, 20, 30) under control of a class III (σ^{28}) promoter. Moreover, in the *B. subtilis flaA* operon, the gene order includes *flgG*, ORF4 homolog, *fliL*, *fliM*, and *fliY* (1, 47). *B. subtilis motA* and *motB* exist as a bicistronic operon located elsewhere on the chromosome and are expressed from a specific σ^{28} promoter (17, 34). In contrast, the treponemal *motA* and *motB* are transcribed together with *fli* and *flg* genes under control of the same promoter. These results suggest that the treponemal Mot, Fli, and Flg polypeptides may be expressed differently from other bacteria. A gene arrangement similar to that of the treponemal *fla* operon was recently found in the spirochete *B. burgdorferi* (10, 11). Deduced amino acid sequences were similar to those of polypeptides of *T. phagedenis* from Tap1 to FliM (26). Unlike *T. phagedenis*, *B. burgdorferi* shows no evidence of divergent transcription upstream of Tap1; rather, upstream genes appear to be part of the *fla* operon as well. The available sequence of *T. pallidum* DNA upstream of Tap1 was not sufficient for comparison with *B. burgdorferi*. Recently, Hardham et al. (14) identified a number of motility-related genes in the *T. pallidum fla* operon that are contiguous with genes in this report. Together with the data reported here, these observations indicate that this *fla* locus comprises an extensive grouping of motility-related polypeptides.

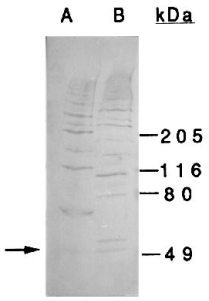

FIG. 6. Western blot of *T. pallidum* (lane A) and *T. phagedenis* (lane B) cells, using the *T. pallidum* FlgE antiserum. Numbers at the right indicate masses of molecular weight markers. The arrow indicates the faint band identifying the FlgE monomer.

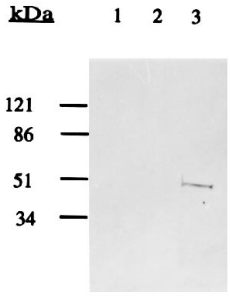

FIG. 7. Western blots of cell fractions after 2% Triton X-114 solubilization of *T. phagedenis* and phase partitioning. Blots were probed with Tap1 antiserum. Lane 1, insoluble pellet; lane 2, detergent phase; lane 3, aqueous phase.

A putative promoter that likely regulates the genes comprising the *T. phagedenis fla* operon was identified by primer extension analysis. A similar promoter sequence was noted in *T. pallidum* as well. Our findings do not allow assignment of the treponemal *fla* promoter as either a class II or a class III element. Conserved nucleotide sequences reveal a greater similarity of P_{fla} to class III, but similar promoter sequences (P_{fliL}) have been designated class II in *E. coli* (23). Assignment of promoter class is premature because so little is known about regulation of spirochete motility genes, and it is not known whether they have a cascade of differential expression as in the enteric bacteria.

In a previous report we were intrigued by the large intergenic region between *flgE* and *motA* of *T. phagedenis* (27). On the basis of the extensive length of this intergenic region, it was initially postulated that *motA* could be the initial gene of an operon. However, the transcription analysis reported here show that *flgE* and *motA* are transcribed together as part of the same RNA segment. These results do not rule out the possibility of an additional promoter element located in this region. However, the location of ORF4 reduces the size of the noncoding intergenic region from 315 to 102 nucleotides.

An unusual result of earlier work on *T. phagedenis* flagellar hooks was the determination that purified hooks appeared as a ladder of polypeptide bands after SDS-PAGE (27). Indirect evidence has suggested that these polypeptides are cross-linked monomers of FlgE polypeptide. Because this was the first indication of the unusual composition of the hooks, we were interested in determining whether other spirochetes, particularly *T. pallidum*, shared this unusual feature. This report demonstrates that *T. pallidum* cells also share a similar ladder of FlgE polypeptides when analyzed by Western blotting. In-

terestingly, despite identical gene lengths, the ladder of FlgE polypeptides after Western blotting of *T. pallidum* was not identical to that in *T. phagedenis*. Expression of recombinant FlgE in *E. coli* did not result in a ladder but rather resulted in a single band of the predicted size (26), suggesting that perhaps only spirochetes possess the necessary elements to form a ladder of FlgE polypeptides. Other spirochetes, notably *T. vincentii* and *B. burgdorferi*, also possess a FlgE ladder (26).

Tap1 and ORFB1 possess no known homologs. Tap1 appears to be located primarily within the cytoplasm of *T. phagedenis*, there is no evidence of a typical signal sequence, and the function remains unknown. Other than its location in an operon associated with motility functions, there is no experimental evidence to suggest that Tap1 is involved in motility. Interestingly, the treponemal Tap1 polypeptides show only a short region of homology at the carboxyl terminus. ORFB1 was not characterized in detail. However, the presence of a promoter sequence similar to that of σ^{32} heat shock promoters indicates that it may be involved in the stress response. *T. phagedenis* is known to increase the production of several polypeptides after heat shock and is presumed to have a stress response system (44).

In summary, we have identified the 5′ end of the treponemal *fla* operon which is composed of a unique organization of motility-associated polypeptides that are expressed from a specific promoter. These results have also served to provide a better understanding of the genetic relationship of *T. pallidum* to *T. phagedenis.*

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REFERENCES

1. **Albertini, A. M., T. Caramori, W. D. Crabb, F. Scoffone, and A. Galizzi.** 1991. The *flaA* locus of *Bacillus subtilis* is part of a large operon coding for flagellar structures, motility functions, and an ATPase-like polypeptide. J. Bacteriol. **173:**3573–3579.
2. **Blair, D. F., and H. C. Berg.** 1990. The MotA Protein of *E. coli* is a proton-conducting component of the flagellar motor. Cell **60:**439–449.
3. **Blair, D. F., and H. C. Berg.** 1991. Mutations in the MotA protein of *Escherichia coli* reveal domains critical for proton conduction. J. Mol. Biol. **221:**1433–1442.
4. **Canale-Parola, E.** 1984. Motility and chemotaxis of spirochetes. Annu. Rev. Microbiol. **32:**69–99.
5. **Champion, C. I., J. N. Miller, M. A. Lovett, and D. R. Blanco.** 1990. Cloning, sequencing, and expression of two class B endoflagellar genes of *Treponema pallidum* subsp. *pallidum* encoding the 34.5- and 31.0-kilodalton proteins. Infect. Immun. **58:**1697–1704.
6. **Charon, N. C., S. F. Goldstein, K. Curci, and R. J. Limberger.** 1991. The bent-end morphology of *Treponema phagedenis* is associated with short, left-handed, periplasmic flagella. J. Bacteriol. **173:**4820–4826.
7. **Charon, N. W., S. F. Goldstein, S. M. Block, K. Kurci, J. D. Ruby, J. A. Kreiling, and R. J. Limberger.** 1992. Morphology and dynamics of protruding spirochete periplasmic flagella. J. Bacteriol. **174:**832–840.
8. **Claros, M. G., and G. von Heijne.** 1995. TopPred II: an improved software for membrane protein structure predictions. Comput. Appl. Biosci. **10:**685–686.
9. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
10. **Dunn, J. J., L. Butler-Loffredo, J. Kieleczawa, J. Medalle, and B. J. Luft.** 1995. Direct GenBank submission no. U43739.
11. **Ge, Y., and N. W. Charon.** 1996. Direct GenBank submission no. L75945.
12. **Ge, Y., I. Old, I. Saint Girons, D. B. Yelton, and N. W. Charon.** 1996. FliH and FliI of *Borrelia burgdorferi* are similar to flagellar and virulence factor export proteins of other bacteria. Gene **168:**73–75.
13. **Guan, C., P. Li, P. D. Riggs, and H. Inouye.** 1988. Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. Gene **67:**21–30.
14. **Hardham, J. M., J. G. Frye, and L. V. Stamm.** 1995. Identification and sequences of the *Treponema pallidum fliM′*, *fliY*, *fliP*, *fliQ*, *fliR*, and *flhB′* genes. Gene **166:**57–64.
15. **Heinzerling, H. F., J. E. Penders, and R. A. Burne.** 1995. Identification of a *fliG* homologue in *Treponema denticola*. Gene **161:**69–73.
16. **Hellmann, J. D., and M. J. Chamberlin.** 1987. DNA sequence analysis suggests that expression of flagellar and chemotaxis genes in *Escherichia coli* and *Salmonella typhimurium* is controlled by an alternative σ factor. Proc. Natl. Acad. Sci. USA **84:**6422–6424.
17. **Helmann, J. D.** 1991. Alternative sigma factors and the regulation of flagellar gene expression. Mol. Microbiol. **5:**2875–2882.
18. **Henikoff, S.** 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. Methods Enzymol. **155:**156–165.
19. **Homma, M., D. J. DeRosier, and R. M. Macnab.** 1990. Flagellar hook and hook-associated proteins of *Salmonella typhimurium* and their relationship to other axial components of the flagellum. J. Mol. Biol. **213:**819–832.
20. **Homma, M., K. Kutsukake, M. Hasebe, I. Iino, and R. M. Macnab.** 1990. FlgB, FlgC, FlgF, and FlgG: a family of structurally related proteins in the flagellar basal body of *Salmonella typhimurium*. J. Mol. Biol. **211:**465–477.
21. **Kihara, M., M. Homma, K. Kutsukake, and R. M. Macnab.** 1989. Flagellar switch of *Salmonella typhimurium*: gene sequences and deduced protein sequences. J. Bacteriol. **171:**3247–3257.
22. **Kutsukake, K., and H. Doi.** 1994. Nucleotide sequence of the *flgD* gene of *Salmonella typhimurium* which is essential for flagellar hook formation. Biochim. Biophys. Acta **1218:**443–446.
23. **Kutsukake, K., Y. Ohya, and T. Iino.** 1990. Transcriptional analysis of the flagellar region of *Salmonella typhimurium*. J. Bacteriol. **172:**741–747.
24. **Limberger, R. J.** 1984. Periplasmic flagella of *Treponema phagedenis*. Ph.D. thesis. West Virginia University, Morgantown, W.Va.
25. **Limberger, R. J., and N. W. Charon.** 1986. *Treponema phagedenis* has at least two proteins residing together on its periplasmic flagella. J. Bacteriol. **166:** 105–112.
26. **Limberger, R. J., M. El-Afandi, L. Dantuono, and L. L. Slivienski.** 1995. Unpublished data.
27. **Limberger, R. J., L. L. Slivienski, and W. A. Samsonoff.** 1994. Genetic and biochemical analysis of the flagellar hook of *Treponema phagedenis*. J. Bacteriol. **176:**3631–3637.
28. **Limberger, R. J., L. L. Slivienski, D. B. Yelton, and N. W. Charon.** 1992. Molecular genetic analysis of a class B periplasmic flagellum gene of *Treponema phagedenis*. J. Bacteriol. **174:**6404–6410.
29. **Macnab, R. M.** 1987. Flagella, p. 70–83. *In* F. C. Neidhardt, J. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
30. **Macnab, R. M.** 1992. Genetics and biogenesis of bacterial flagella. Annu. Rev. Genet. **26:**131–158.
31. **Malakooti, J., Y. Komeda, and P. Matsumura.** 1989. DNA sequence analysis, gene product identification, and localization of flagellar motor components of *Escherichia coli*. J. Bacteriol. **171:**2728–2734.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Miao, R., and A. H. Fieldsteel.** 1978. Genetics of *Treponema*: relationship between *Treponema pallidum* and five cultivable treponemes. J. Bacteriol. **133:**101–107.
34. **Mirel, D. B., V. M. Lustre, and M. J. Chamberlin.** 1992. An operon of *Bacillus subtilis* motility genes transcribed by the σ^D form of RNA polymerase. J. Bacteriol. **174:**4197–4204.
35. **Norris, S. J., N. W. Charon, R. G. Cook, M. S. Fuentes, and R. J. Limberger.** 1988. Antigenic relatedness and N-terminal sequence homology define two classes of periplasmic flagellar proteins of *Treponema pallidum* subsp. *pallidum* and *Treponema phagedenis*. J. Bacteriol. **170:**4072–4082.
36. **Ohnishi, K., Y. Ohto, S. Aizawa, R. M. Macnab, and T. Iino.** 1994. FlgD is a scaffolding protein needed for flagellar hook assembly in *Salmonella typhimurium*. J. Bacteriol. **176:**2272–2281.
37. **Oliver, D.** 1985. Protein secretion in *Escherichia coli*. Annu. Rev. Microbiol. **39:**615–648.
38. **Palleson, L., and P. Hindersson.** 1989. Cloning and sequencing of a *Treponema pallidum* gene encoding a 31.3-kilodalton endoflagellar subunit (FlaB2). Infect. Immun. **57:**2166–2172.
39. **Radolf, J. D., N. R. Chamberlain, A. Clausell, and M. V. Norgard.** 1988. Identification and localization of integral membrane proteins of virulent *Treponema pallidum* subsp. *pallidum* by phase partitioning with the nonionic detergent Triton X-114. Infect. Immun. **56:**490–498.
40. **Raha, M., H. Sockett, and R. M. Macnab.** 1994. Characterization of the *fliL* gene in the flagellar regulon of *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **176:**2308–2311.
41. **Sadziene, A., D. D. Thomas, V. G. Bundoc, S. C. Holt, and A. G. Barbour.** 1991. A flagella-less mutant of *Borrelia burgdorferi*. Structural, molecular and in vitro functional characterization. J. Clin. Invest. **88:**82–92.

42. **Schleicher & Schuell, Inc.** 1987. Transfer and immobilization of nucleic acids to S&S solid supports. Schleicher & Schuell, Inc., Keene, N.H.
43. **Smibert, R. M.** 1984. Genus III. *Treponema* Schaudin 1905, 1728^{AL}, p. 49–57. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
44. **Stamm, L. V., F. C. Gherardini, E. A. Parrish, and C. R. Moomaw.** 1991. Heat shock response of spirochetes. J. Bacteriol. **59:**1572–1575.
45. **Thomas, D. D., M. Navab, D. A. Haake, A. M. Fogelman, J. N. Miller, and M. A. Lovett.** 1988. *Treponema pallidum* invades intracellular junctions of endothelial cell monolayers. Proc. Natl. Acad. Sci. USA **8:**3608–3612.
46. **Walker, E. M., J. K. Arnett, J. D. Heath, and S. J. Norris.** 1991. *Treponema pallidum* subsp. *pallidum* has a single, circular chromosome with a size of approximately 900 kilobase pairs. Infect. Immun. **59:**2476–2479.
47. **Zuberi, A. R., D. S. Bischoff, and G. W. Ordal.** 1991. Nucleotide sequence and characterization of a *Bacillus subtilis* gene encoding a flagellar switch protein. J. Bacteriol. **171:**3247–3257.