Development of a Novel Chloramphenicol Resistance Expression Plasmid Used for Genetic Complementation of a fliG Deletion Mutant in Treponema denticola

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A new expression plasmid containing the fla operon promoter and a staphylococcal chloramphenicol resistance gene, was constructed to help assess the role of fliG in Treponema denticola motility. Deletion of fliG resulted in a nonmotile mutant with a markedly decreased number of flagellar filaments. Wild-type fliG genes from T. denticola and from Treponema pallidum were cloned into this expression plasmid. In both cases, the gene restored the ability of the mutant to gyrate its cell ends and enabled colony spreading in agarose. This shuttle plasmid enables high-level expression of genes in T. denticola and possesses an efficient selectable marker that provides a new tool for treponemal genomics.

Treponema denticola is an anaerobic, motile spirochete bacterium that is associated with periodontal disease (21, 29). T. denticola possesses four flagellar filaments, two of which are inserted at each end of the cell (2, 7). These complex filaments are composed of three FlaB core proteins and one FlaA sheath protein (26). Their unique periplasmic location allows the spirochete to locomote in high-viscosity environments, which is an important factor in pathogenesis (1, 12–14, 16, 27, 30).

Research on T. denticola has been hampered by a paucity of genetic tools and efficient selectable markers. An erythromycin resistance cassette has been used to generate a number of insertional mutants of T. denticola (15). More recently, a coumermycin-based shuttle plasmid was developed for complementation analysis (4). However, coumermycin-based selection suffers from a high background level of spontaneously resistant cells. Thus, there is a need to develop additional selectable markers for use in creating T. denticola shuttle plasmids that can be used for expression and complementation studies. Development of an efficient expression plasmid would enable the use of T. denticola as a surrogate system (3) to study homologous genes from Treponema pallidum, a pathogenic spirochete that is not cultivable in cell-free medium.

A motility-related operon in T. denticola is transcribed from a sigma 28-like promoter and encodes seven polypeptides that are homologous with components of the Salmonella flagellar basal body (FlgB, FlgC, FlfE, FlfF, and FlgI), as well as three putative flagellar export proteins (FlfH, FlfI, and FlgI) (5, 6, 22–24). T. denticola FlgI has a 31% amino acid sequence identity with Salmonella enterica serovar Typhimurium FlgI, which is part of the flagellar switch complex (10, 22, 23, 32). In S. enterica serovar Typhimurium, FlgI is involved in torque generation, directional rotation of the flagellar filament (8, 19, 20), and flagellar filament structure (8, 11). The role of FlgI in T. denticola is unknown. Because the organization and regulation of spirochete motility genes are different from those of enteric bacteria, analysis of T. denticola fliG will enable us to gain a better understanding of the role of this protein in spirochete motility.

Strains and media. T. denticola ATCC 33520 was grown at 36°C in New Oral Spirochete (NOS) broth with 10% rabbit serum or was plated on NOS medium containing 0.5% agarose (9, 18).

Construction and analysis of a fliG deletion mutant. A suicide plasmid, pFEH1, was constructed (Fig. 1A) and was electroporated into T. denticola, using 5 to 10 μg of DNA as previously described (18). Plating was done on NOS–0.5% agarose plates containing 100-μg/ml erythromycin to select for double-crossover recombinants (18). After 1 week of incubation, small dense colonies grew on the surface of NOS-agarose plates. After growth in liquid medium, cells of this fliG deletion mutant, TDWΔflig, showed no movement when observed by dark-field microscopy.

Electron microscopic analysis revealed that 39% of TDWΔ Flig cells did not possess any flagella, and 94% of cells for which both ends could be observed lacked a flagellar filament on at least one end. The flagellar filaments of TDWΔflig were usually shorter in length than those of the wild type. Cell ends lacking flagellar filaments also lacked hook and basal body structures. The phenotypic analysis of TDWΔflig is summarized in Table 1.

Quantitation of RNA expression in TDWΔflig by RT-PCR. RNA was isolated from logarithmic-phase T. denticola cells with the Masterpure Complete DNA and RNA purification kit (Epicentre Technologies, Madison, Wis.), with an additional DNase step employing a manganese buffer to eliminate residual DNA (31).

Primers specific for flgB, flgC, flfH, and flgI (Table 2) were designed with the Primer Express software (Applied Biosys-
tems, Foster City, Calif.). For reverse transcription-PCR (RT-PCR), 90 ng of RNA was used in an RT-PCR according to the manufacturer’s instructions (Applied Biosystems). T. denticola 16S rRNA was used as a reference target for quantitation (see Table 2 for primer sequences).

Quantitative RT-PCR of regions upstream (\textit{flgB} and \textit{flgC}) and downstream (\textit{flgH} and \textit{flgI}) of the \textit{ermF-ermAM} cassette in the \textit{flG} deletion mutant showed expression levels similar to those of the wild type (Fig. 2). Since the presence of the erythromycin resistance cassette did not markedly affect tran-

FIG. 1. Plasmid constructions. (A) A suicide plasmid vector for generating a \textit{fltG} deletion in \textit{T. denticola} by allelic exchange. (B) A new expression plasmid, pBFC, that replicates and confers chloramphenicol resistance in \textit{T. denticola}. This shuttle vector was used for cloning of \textit{T. denticola} \textit{flG} as indicated for complementation analysis. NS, Nsil; P, PstI; M, MluI; K, KpnI; S, SpeI; S, SacI; N, NotI; A, ApaI; E, EcoRI; oriT.d., \textit{T. denticola} origin of replication.
scription of downstream genes, these results demonstrate that this cassette does not contain transcription terminators or promoters that are functional in *T. denticola*.

**FlaB expression analysis.** Immunoblots were incubated with a *T. phagedenis* FlaB antiserum (17) at a 1:3,000 dilution, followed by a peroxidase-conjugated goat anti-rabbit immunoglobulin G (H+L) antibody (Pierce) at a 1:250,000 dilution. The FlaB polypeptides were visualized with the Pierce Super Signal West Dura kit. FlaB polypeptides were detectable in TDW FLIG, although at a markedly reduced level, compared to the wild type (Fig. 3). A similar reduction was noted in levels of the FlaA polypeptide (not shown).

Quantitative RT-PCR using primers specific for flaB1, flaB2, and flaB3 (Table 2) revealed that RNA levels in TDW FLIG were comparable to each other and to those in the wild type (Fig. 2). The regulatory mechanisms involved in the generation of a filament-deficient phenotype despite adequate levels of flaB RNA are unknown. Previously, we and others have qualitatively demonstrated a similar phenotype in other spirochete motility mutants (1, 18, 27). It is unclear whether the FlaB polypeptide is synthesized but rapidly degraded or whether it is not made at all, due to posttranscriptional regulation. In *Borrelia burgdorferi*, a decrease of FlaA in a FlaB mutant was shown to be a posttranscriptional effect (25).

**Construction of a newly derived expression plasmid**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
<th>T. denticola gene target</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLGBRTF</td>
<td>GCAACGACTCATCCCTTTCAT</td>
<td>flaB</td>
</tr>
<tr>
<td>FLGBRTR</td>
<td>CCGTCTGCTTTTTCGGCAG</td>
<td>flaB</td>
</tr>
<tr>
<td>FIGCRTF</td>
<td>TTATGATCCTCGGACCACCTCT</td>
<td>flaC</td>
</tr>
<tr>
<td>FIGCRTR</td>
<td>GCTTTCCCTCATAGCCCGTTG</td>
<td>flaC</td>
</tr>
<tr>
<td>fliG1349</td>
<td>ACTCCTGCTGCACCTCTAAATG</td>
<td>fliG</td>
</tr>
<tr>
<td>fliG1449</td>
<td>TTTTACGGAGGACGTCGCTT</td>
<td>fliG</td>
</tr>
<tr>
<td>fliH424F</td>
<td>GTTCTTATACAGCGCGGCTGA</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>TGAAGATACGCCAAAATCTCT</td>
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</tr>
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</tr>
<tr>
<td>Flab160F</td>
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<td>flbB1</td>
</tr>
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<td>flbB2</td>
</tr>
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<td>flbB2</td>
</tr>
<tr>
<td>Flab338F</td>
<td>AGGCCGTTTTGCTCAAGATT</td>
<td>flbB3</td>
</tr>
<tr>
<td>Flab352R</td>
<td>TCCGCTTGGTGGTCCTCAT</td>
<td>flbB3</td>
</tr>
</tbody>
</table>

**TABLE 1. Motility-related characteristics of wild-type *T. denticola*, TDW FLIG, and TDW FLIG harboring fliG on a newly constructed plasmid**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Avg. no. of filaments/cell</th>
<th>Filament length (µm)</th>
<th>Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>33520 (wild type)</td>
<td>3.74</td>
<td>3.46</td>
<td>3.67</td>
</tr>
<tr>
<td>TDW FLIG</td>
<td>0.76</td>
<td>1.55</td>
<td>0.84</td>
</tr>
<tr>
<td>TDW FLIG (pBFCFliG)</td>
<td>1.05</td>
<td>3.36</td>
<td>3.08</td>
</tr>
</tbody>
</table>

* For *T. denticola*, *n* = 111; for TDW FLIG, *n* = 50; and for TDW FLIG(pBFCFliG), *n* = 72.

**TABLE 2. Oligonucleotide sequences used in this study for RT-PCR**

**confers chloramphenicol resistance in *T. denticola*.** The *T. denticola* replicative shuttle plasmid pKMR4PEMCS (3) was used as the basis for construction of a newly derived selectable plasmid (Fig. 1B). The new plasmid, pBFC, contained the *T. denticola* fla operon promoter (*P*<sub>fla</sub>, located upstream of FliK/TapI (18), followed by the *Staphylococcus aureus* chloramphenicol resistance gene. The plasmid was electroporated into wild-type *T. denticola* and was selected on 10-µg/ml chloramphenicol.

The MIC of chloramphenicol for wild-type *T. denticola* is <1.0 µg/ml. *T. denticola* cells harboring pBFC grew well in 1.0 µg of chloramphenicol per ml, although they could grow in higher concentrations of this drug (up to 10 µg/ml). However, the growth rate slowed, and viability was reduced at the higher concentrations of chloramphenicol. For selection of transformants, 10 µg/ml was used in plated media to suppress background growth. The pBFC expression plasmid could be transformed and maintained in *Escherichia coli*, using kanamycin as a selectable marker, but it did not confer chloramphenicol resistance in *E. coli*.

The expression plasmid pBFC provides an important new tool for analysis of gene expression in *T. denticola* mutants. Use of chloramphenicol with this newly derived plasmid provides a method by which to obtain successfully transformed *T. denticola* cells with minimal or no background growth. It is likely, although not yet demonstrated, that this *cat* cassette will also be useful for generating chromosome-based gene interruptions.

**Complementation analysis using fliG from *T. denticola* and *T. pallidum*.** *T. denticola* fliG was cloned into pBFC to form pBFCFliG (Fig. 1B). Quantitative RT-PCR demonstrated that the amount of fliG RNA expressed from this plasmid was greater than the amount of wild-type fliG RNA expressed from the chromosomal location (Fig. 2). The higher expression levels of fliG RNA are likely the result of the plasmid-borne promoter of the *T. denticola* fli operon promoter (*P*<sub>fla</sub>). It is likely that the increased amount of fliG expression would also be useful for generating chromosome-based gene interruptions.

Flagellated cells of TDW FLIG harboring the plasmid pBFCFliG regained the ability to gyrate their cell ends in broth cultures, and the mean flagellar filament lengths were restored to the wild-type levels. Cells also regained the ability to form spreading colonies on agarose plates, although colony diameters were less than wild type. The number of flagellar filament structures and the amount of FlaB polypeptides were slightly increased, although not to wild-type levels (Fig. 3 and Table 1).
Conceivably, the synthesis and assembly of flagellar structures require expression of flgG at the lower wild-type levels, so as to ensure proper molecular stoichiometry. In *B. burgdorferi*, complementation of an flaB null mutant was achieved only after integration of flaB into the chromosome (28). Unfortunately, it is not yet technically feasible to insert flgG back into the *T. denticola* chromosome in order to attempt complementation from a chromosomal location.

*T. pallidum* flgG, cloned and expressed in pBFC, was also able to restore gyration of the cell ends of TDWFLIG, as well as colony spreading in agarose. This is the first report of a successful complementation of a *T. denticola* mutant by using a *T. pallidum* gene. *T. pallidum* FlIG shows an 84% amino acid sequence identity with *T. denticola* and a 90% similarity. These experiments demonstrate the potential utility of *T. denticola* as a surrogate system in which to analyze the function of *T. pallidum* polypeptides.

Future development and application of additional genetic tools will be critical in resolving the mechanisms involved in the expression, regulation, and function of spirochete motility genes.

**Nucleotide sequence accession number.** The entire sequence of flgG and partial sequences of flgF and flgH from *T. denticola* 33520 have been deposited in GenBank under accession no. AF343975.

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**REFERENCES**


FIG. 2. Quantitative RT-PCR analysis of gene expression of wild-type *T. denticola* and TDWFLIG. The y-axis scale indicates the relative amount of RNA, as compared to a 16S rRNA standard. The x-axis indicates the genes comprising the noncontiguous operons, with the direction of transcription as indicated by the horizontal arrows. Light gray bars are the *T. denticola* wild type, dark gray bars are TDWFLIG, and the striped bar indicates the expression of flgG from TDWFLIG harboring the plasmid pBFCflgG. The open triangle represents where the erythromycin resistance cassette is inserted in TDWFLIG and indicates that no flgG RNA was detected. The error bars for each gene represent 1 standard deviation derived from at least two independent assays.

![Image](http://iai.asm.org/)

FIG. 3. Immunoblot with FlaB antiserum. The three FlaB polypeptides migrated as a single band under these gel running conditions. Lanes: 1, *T. denticola* wild type; 2, TDWFLIG; 3, TDWFLIG containing pBFCflgG. Note that the expression of flagellar filament polypeptides in TDWFLIG was only 17% that of the wild type.

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