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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 genetics.

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, FliE, FliF, and FliG), as well as three putative flagellar export proteins (FliH, FliI, and FliJ) (5, 6, 22–24). *T. denticola* FliG has a 31% amino acid sequence identity with *Salmonella enterica* serovar Typhimurium FliG, which is part of the flagellar switch complex (10, 22, 23, 32). In *S. enterica* serovar Typhimurium, FliG is involved in torque generation, directional rotation of the flagellar filament (8, 19, 20), and flagellar filament structure (8, 11). The role of *fliG* 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 TDWΔFLIG 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*, *fliH*, and *fliI* (Table 2) were designed with the Primer Express software (Applied Biosys-

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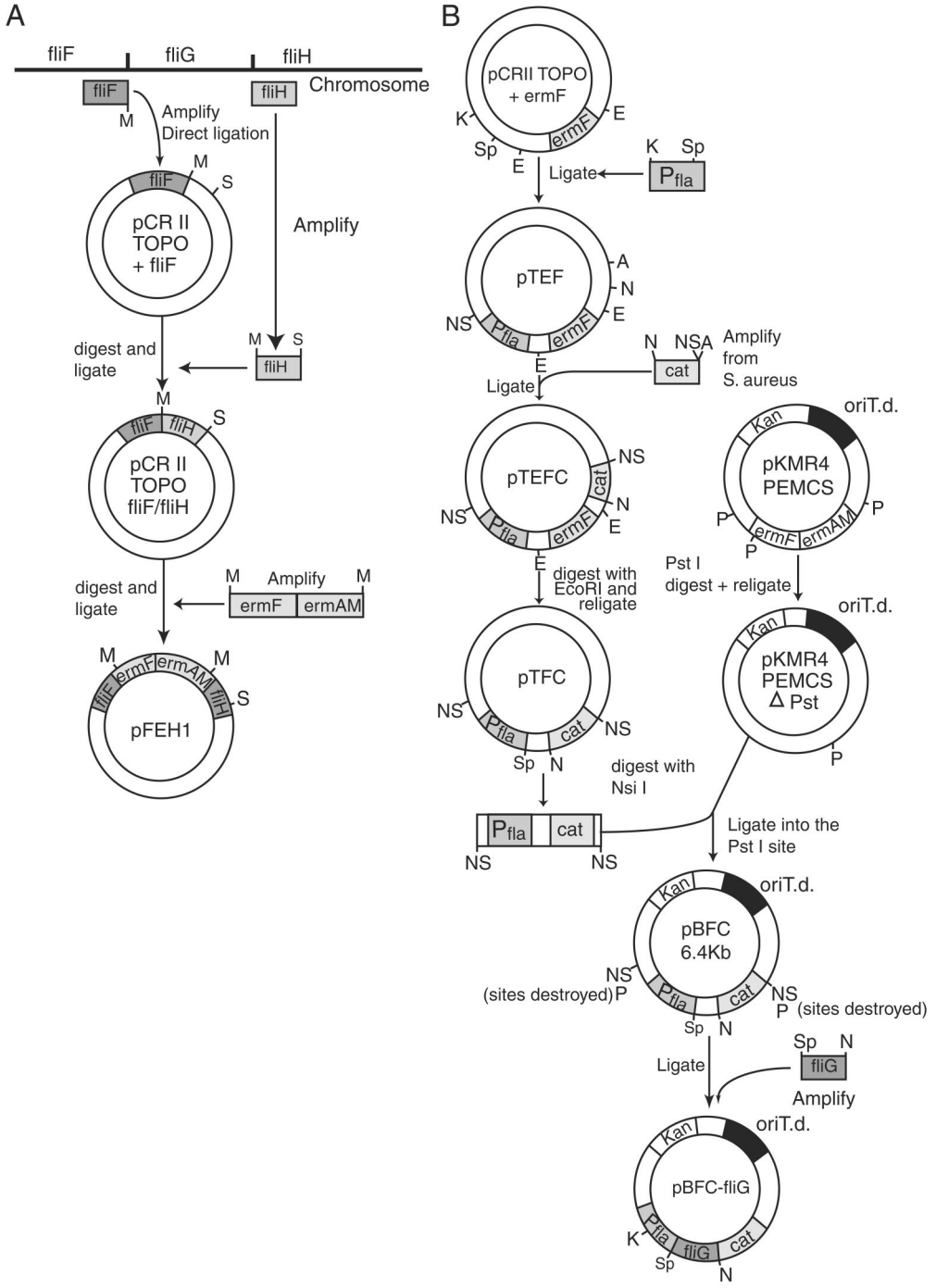

FIG. 1. Plasmid constructions. (A) A suicide plasmid vector for generating a *fliG* deletion in *T. denticola* by allelic exchange. (B) A new expression plasmid, pBFC, that replicates and confers chloramphenicol resistance in *T. denticola*. This shuttle vector was used for cloning of *T. denticola fliG* as indicated for complementation analysis. NS, NsiI; P, PstI; M, MluI; K, KpnI; Sp, SpeI; S, SacI; N, NotI; A, ApaI; E, EcoRI; oriT.d., *T. denticola* origin of replication.

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 (*flgB* and *flgC*) and downstream (*fliH* and *fliI*) of the *ermF-ermAM* cassette in the *fliG* 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-

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

Strain	Avg no. of filaments/cell	Filament length (μm)[a]			Motility	
		Mean	Median	Range	Gyration of cell ends	Agar spreading
33520 (wild type)	3.74	3.46	3.67	0.91–6.15	Yes	Yes
TDWΔFLIG	0.76	1.55	0.84	0.09–6.48	No	No
TDWΔFLIG (pBFCFliG)	1.03	3.36	3.08	0.88–10.46	Yes	Yes[b]

[a] For *T. denticola*, $n = 111$; for TDWΔFLIG, $n = 50$; and for TDWΔFLIG(pBFCFliG), $n = 72$.
[b] Colony diameter was less than that of the wild type.

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 TDWΔFLIG, 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 TDWΔFLIG 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 that 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_{fla}), 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 location of the promoter (L. Slivienski-Gebhardt and R. Limberger, unpublished observations), but they do not adversely affect cell growth or morphology.

Flagellated cells of TDWΔFLIG 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 level. 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).

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

Primer	Sequence (5′→3′)	*T. denticola* gene target
FLGBRTF	GCAACGACTCATCCCTTGCAT	*flgB*
FLGBRTR	CCGTTTGCTTTTTCGGCAG	*flgB*
FIGCRTF	TTTATGATCCGGACCCATCCT	*flgC*
FIGCRTR	GGTTTGCCTCATAAGCCCTTG	*flgC*
fliG1349	ACTGCCGTGCCTCCTAAAATG	*fliG*
fliG1449	TTTGTAGCAGGAAGCTCGCCT	*fliG*
FliH424F	GTTTTAATCGCGGCGGTGA	*fliH*
FliH424R	TGGCGCCTATCCATTGCTTT	*fliH*
FLIIRTF	CCTCCCGAAGAATTTGATGAAG	*fliI*
FliIRTR	GCAAGTCAATTTCTGCCTGATG	*fliI*
16SDENT1	TGAGATACGGCCCAAACTCCT	*16S*
16SDENT2	CAACCTTTCGGCCTTCTTCA	*16S*
Flab160F	ACAATCGAAACAGCCGATGC	*flaB1*
Flab172R	GACAACCGTCAACTCCATT	*flaB1*
Flab257F	CGAAAAAGCTAACCGCGCT	*flaB2*
Flab271R	GGAGGTTTTCTGCTGCGATA	*flaB2*
Flab338F	AGGCCGTTTTGCTCAAGATT	*flaB3*
Flab352R	TCCGCCTTGTTGTGCTCCTAT	*flaB3*

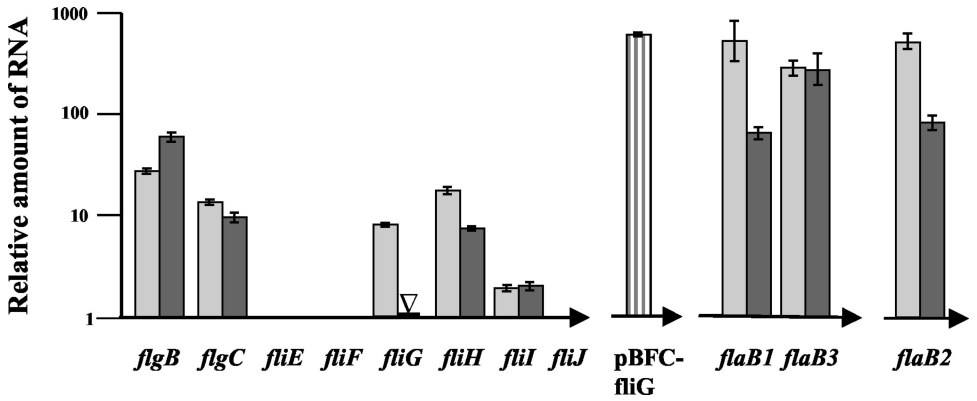

FIG. 2. Quantitative RT-PCR analysis of gene expression of wild-type *T. denticola* and TDWΔFLIG. The *y*-axis scale indicates the relative quantity of RNA, as compared to a 16S rRNA standard. The *x* axis indicates the genes comprising the noncontiguous operons, with the direction of transcription as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

Conceivably, the synthesis and assembly of flagellar structures require expression of *fliG* 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 *fliG* back into the *T. denticola* chromosome in order to attempt complementation from a chromosomal location.

T. pallidum fliG, cloned and expressed in pBFC, was also able to restore gyration of the cell ends of TDWΔFLIG, 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 *fliG* and partial sequences of *fliF* and *fliH* from *T. denticola* 33520 have been deposited in GenBank under accession no. AF343975.

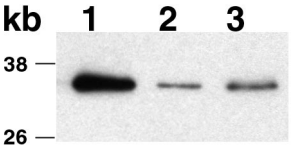

FIG. 3. Immunoblot with FlaB antiserum. The three FlaB polypeptides migrated as a single band under these gel running conditions. Lanes: 1, *T. denticola* wild type; 2, TDWΔFLIG; 3, TDWΔFLIG containing pBFCFliG. Note that the expression of flagellar filament polypeptides in TDWΔFLIG was only 17% that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

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