A Modified Shuttle Plasmid Facilitates Expression of a Flavin Mononucleotide-Based Fluorescent Protein in *Treponema denticola* ATCC 35405

Valentina Godovikova,^a M. Paula Goetting-Minesky,^a Jae M. Shin,^b Yvonne L. Kapila,^b Alexander H. Rickard,^c J. Christopher Fenno^a

Department of Biologic and Materials Sciences, University of Michigan School of Dentistry, Ann Arbor, Michigan, USA^a; Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor, Michigan, USA^b; Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, Michigan, USA^c

Oral pathogens, including *Treponema denticola*, initiate the dysregulation of tissue homeostasis that characterizes periodontitis. However, progress of research on the roles of *T. denticola* in microbe-host interactions and signaling, microbial communities, microbial physiology, and molecular evolution has been hampered by limitations in genetic methodologies. This is typified by an extremely low transformation efficiency and inability to transform the most widely studied *T. denticola* strain with shuttle plasmids. Previous studies have suggested that robust restriction-modification (R-M) systems in *T. denticola* contributed to these problems. To facilitate further molecular genetic analysis of *T. denticola* behavior, we optimized existing protocols such that shuttle plasmid transformation efficiency was increased by >100-fold over prior reports. Here, we report routine transformation of *T. denticola* ATCC 35405 with shuttle plasmids, independently of both plasmid methylation status and activity of the type II restriction endonuclease encoded by TDE0911. To validate the utility of this methodological advance, we demonstrated expression and activity in *T. denticola* of a flavin mononucleotide-based fluorescent protein (FbFP) that is active under anoxic conditions. Addition of routine plasmid-based fluorescence labeling to the *Treponema* toolset will enable more-rigorous and -detailed studies of the behavior of this organism.

In vitro studies of *Treponema denticola* and other oral spirochetes present unique challenges and opportunities in the field of molecular and cellular microbiology. As both commensal residents and pathogens of the subgingival oral mucosa, these nutritionally fastidious anaerobes are useful targets for research into microbe-host interactions and signaling, microbial communities, microbial physiology, and molecular evolution. Furthermore, due to both physiological similarities between *T. denticola* and *T. pallidum* and the absence of a workable *in vitro* system to culture *T. pallidum*, *T. denticola* is of high interest as a model for the study of the microbial physiology and biosynthetic pathways of *T. pallidum*.

Transformation of *T. denticola* ATCC 35405 by allelic replacement mutagenesis and by shuttle plasmids was first reported over 15 years ago (1, 2), but subsequent progress in this area continues to be hindered by extremely low transformation efficiency (1, 3). While *T. denticola* is somewhat tractable for simple gene knock-outs, basic methods such as complementation analysis and expression of heterologous genes are problematic. The available shuttle plasmid system has at best an extremely limited ability to replicate in the most widely studied *T. denticola* strain (3–5). Thus, while plasmid-based methodologies are straightforward in the study of many prokaryotes, such studies present major obstacles to the rigorous molecular genetic analysis of treponemes.

The *T. denticola* 35405 genome sequence reveals a robust DNA restriction-modification repertoire, including two complete type I restriction-modification loci and three type II restriction enzymes (6). In contrast, *T. pallidum* has no identified restriction systems (7). These restriction systems have been proposed as a major obstacle to genetic transformation of *T. denticola*, particularly strain 35405 (3, 8). One of the three *T. denticola* type II enzymes (*T. denticola* III [TdeIII], encoded by TDE0911) has been shown to

recognize the sequence 5'-GGCCC-3' and is reported to prevent transformation of strain 35405 with shuttle plasmid DNA (3). The others, encoded by TDE0227 and TDE1228, have not been characterized. In the present study, we demonstrated that significantly increased plasmid transformation efficiency in type strain 35405 is readily feasible independently of these DNA restriction-modification systems, thus facilitating both genetic complementation and expression of heterologous genes in *T. denticola*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *T. denticola* strains, including ATCC 33520 and ATCC 35405 as well as isogenic 35405 mutant 35405-ΔTDE0911 (3), were grown as previously described (9) under anoxic conditions in NOS broth or semisolid medium containing 0.8% Noble agar, supplemented with erythromycin (Em; 40 μg ml⁻¹) and chloramphenicol (Cm; 10 μg ml⁻¹) as appropriate. All growth media were incubated under anoxic conditions for at least 18 h prior to use (10). The purity of spirochete cultures was monitored by dark-field microscopy. *Escherichia coli* strains JM109 and JM110 (11), used as hosts for preparing methylated

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Address correspondence to J. Christopher Fenno, fenno@umich.edu.

V.G. and M.P.G.-M. contributed equally to this article.

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FIG 1 Shuttle plasmid constructs used in this study. *E. coli*-*T. denticola* shuttle plasmid pBFC was digested with Eco53kI and FspI and self-ligated to remove the documented TdeIII recognition site (GGCCC), yielding pCF693. Two other predicted TdeIII sites (GGTCC) are shown in parentheses. Plasmid pCF728 consists of shuttle plasmid pCF693 carrying the codon-optimized Bs2C gene under the transcriptional control of the *T. denticola* *tap1* promoter (Ptap1) inserted between the SpeI and EcoRV sites of pCF693. The REP gene from pTS1 and the *E. coli* origin of replication (*ori*) are labeled in red and yellow, respectively. Genes encoding resistance to kanamycin (KmR) in *E. coli* and chloramphenicol (CmR) in *T. denticola* are shown. Gene coding sequences cloned between SpeI and EcoRV were transcribed under the control of the *T. denticola* *tap1* promoter (Ptap1).

and unmethylated plasmid DNA, respectively, were grown in LB medium supplemented with kanamycin (Km; 50 $\mu\text{g ml}^{-1}$) and carbenicillin (Cb; 50 $\mu\text{g ml}^{-1}$) as appropriate for plasmid selection in *E. coli*.

Bioinformatics analysis. The presence and conservation of genes of interest in *Treponema* genomes were analyzed using BLAST search tools (blastn and tblastn) (12) on the NCBI, HOMD, and BioCyc websites. Operon prediction of the TDE0911 region was done using the DOOR algorithm for prokaryotic operon analysis (13). Transcription promoter prediction was done using the BPROM algorithm (14).

***E. coli*-*T. denticola* shuttle plasmids.** Available shuttle plasmids for *T. denticola* are all based on pTS1, a cryptic plasmid from an oral *Treponema* clinical isolate (2, 15). For these studies, we used pBFC (16), which encodes resistance to Km in *E. coli* and Cm in *T. denticola* (Fig. 1). Plasmid DNA was purified using an E.Z.N.A. plasmid DNA kit (Omega Bio-Tek, Norcross, GA). For some experiments, pBFC was modified to remove a documented recognition site (5'-GGCCC-3') for the TdeIII restriction enzyme encoded by TDE0911 (3). The resulting pCF693 plasmid (Fig. 1)

lacks a 146-bp FspI-*E. coli* Eco53kI fragment from an intergenic region of the *E. coli* replicon carried on pBFC (nucleotide positions 18 to 164).

For expression of a flavin mononucleotide-based fluorescent protein (FbFP) in *T. denticola*, the gene encoding Bs2C carried on pGlow-CK^{XN}-Bs2 (Jena Bioscience, Jena, Germany) was amplified using Taq-HF polymerase (Life Technologies, Grand Island, NY) and oligonucleotide primers CX984 (5'-GAACTAGTATGGGATCCGCAAGTTTTC-3') and CX985 (5'-TGATATCTTTATTCAAGAAGCTTTTCATATTC-3'), which contain engineered recognition sites (underlined) for SpeI and EcoRV, respectively. The amplicon was digested with SpeI and EcoRV and then cloned in similarly digested pCF693. In the resulting pCF728 plasmid (Fig. 1), transcription of the Bs2C gene and transcription of *cat* (encoding Cm^r) are both under transcriptional control of the *T. denticola* *tap1* promoter carried on pCF693.

DNA sequence analysis. DNA sequencing to confirm the deletion of the TdeIII recognition site in pCF693 and the Bs2C gene as cloned in shuttle plasmids was performed at the University of Michigan DNA Se-

quencing Core Facility and analyzed using DNASTAR sequence analysis software (DNASTAR Inc., Madison, WI).

Analysis of DNA digestion by *T. denticola* lysates. Sonicated cell lysates were prepared from 5-ml cultures of *T. denticola* as described previously (17). A 5- μ g volume of purified plasmid DNA was digested to completion with *Spe*I and then incubated with 20 μ l of crude cell lysate (boiled or not boiled) for 2 h. The entire digestion reaction mixture was separated on a 1% agarose gel, stained with ethidium bromide, and photographed under UV illumination.

Transformation of *T. denticola* with shuttle plasmids. Preparation and electroporation of competent *T. denticola* cells were conducted as previously described (1, 8), with the modifications described below. Competent cell preparations were made from *T. denticola* strains 33520, 35405, and 35405- Δ TDE0911. Four-day (late logarithmic phase; optical density at 600 nm, 0.4 to 0.5) *T. denticola* cultures were decanted into centrifuge tubes while in the oxygen-free chamber, and processing for subsequent washes and resuspension steps was conducted at 4°C. Sterile glycerol wash solution (10% [vol/vol] in H₂O) used for all wash steps was held under anoxic conditions for at least 12 h prior to use. Competent cells were stored in single-use aliquots at -80°C.

Methylated or unmethylated plasmid DNA (0.001 to 1 μ g in 5 μ l of 10 mM Tris-HCl [pH 8.5]) was electroporated into 50 μ l of *T. denticola* competent cells (approximately 10⁹ *T. denticola* cells) in a 1-mm-path-length cuvette using a GenePulser XCell (Bio-Rad, Hercules, CA) with a single pulse (1.8 kV, 200 Ω , 25 μ F), typically yielding a time constant of 5.1 ms. The *T. denticola* cells were then immediately suspended in 2 ml pre-reduced growth medium, placed in the oxygen-free chamber, and incubated overnight at 37°C. Transformed *T. denticola* cells were plated in prerduced NOS agar medium containing chloramphenicol (10 μ g ml⁻¹) for selection of transformants. For some experiments, transformed *T. denticola* cells were serially diluted prior to plating to facilitate colony counts. Cm^r colonies appearing after 7 to 14 days of anoxic incubation in the dark were picked for further analysis. The presence of pBFC or its derivatives in transformed *T. denticola* was determined in broth cultures grown from 10 colonies per transformation plate. Standard PCR using Platinum *Taq* polymerase (Life Technologies) and plasmid-specific oligonucleotide primers CX930 (5'-CGGCCAAGTTCATACGCTGATGA-3') and CX931 (5'-ATTGTTTCCAAAACACCTATACCTGA-3') were used to amplify a 2.5-kb region of the shuttle plasmid, including the 3' end of the Cm^r gene through the middle of the pTS1-derived REP gene. For further validation, plasmid DNA isolated from the same Cm^r *T. denticola* cultures was transformed back into *E. coli*. The restriction enzyme digestion patterns of plasmids subsequently isolated from Km^r *E. coli* transformants were compared with that of the original shuttle plasmid.

Measurement of transformation efficiency. The transformation efficiency of methylated or unmethylated shuttle plasmids in the three *T. denticola* host strains was measured by colony counts of serial dilutions of transformed cells in NOS semisolid agar medium containing appropriate selectable agents. Data are expressed as the mean levels of transformation efficiency (expressed in colonies per microgram of plasmid DNA) from at least three independent experiments.

Primary gingival fibroblast cell cultures. Human primary gingival fibroblasts (HGF) were obtained by the direct cell outgrowth technique as previously described (19). HGF cells were maintained in minimal essential medium (α -MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% fungizone (Gibco/Life Technologies, Grand Island, NY, USA). For use in experiments, HGF from passage 3 or 4 were seeded at 3 $\times$ 10⁴ cells per well in 6-well plates containing sterile glass coverslips (22 mm²) and incubated at 37°C for 24 to 48 h to achieve 75% confluence. Acquisition and use of HGF for these studies were approved by the University of Michigan Health Sciences and Behavioral Sciences Institutional Review Board (HUM00100124).

Treatment of HGF with *T. denticola* expressing FbFP. *T. denticola* CF734 was grown in broth culture to an optical density of 0.3 at 600 nm and harvested by centrifugation at 4,000 $\times$ g for 10 min at 22°C. Following

centrifugation, *T. denticola* cells were washed with sterile phosphate-buffered saline (PBS) and centrifuged at 4,000 $\times$ g for 10 min at 22°C. Following aspiration of PBS solution, *T. denticola* was resuspended in serum-free α -MEM. Supplemented α -MEM was aspirated from the HGF cultures and replaced with serum-free α -MEM containing *T. denticola* CF734 (1 ml per well; multiplicity of infection [MOI] = 100). HGF cells and *T. denticola* cells were incubated (37°C, 5% CO₂) for 30 min, after which they were prepared for microscopic analysis.

Fluorescence microscopy. Microscopic images of *E. coli* colonies on LB agar plates were obtained at $\times$ 40 magnification using an Olympus DP-70 digital camera attached to a Leica MXFL III stereo fluorescence microscope. Bright-field images were obtained at an exposure of 1/20 s. Fluorescence images were obtained after a 2-s exposure (460-to-500-nm-wavelength excitation filter; 510-nm-wavelength emission filter).

For imaging of freshly grown, planktonic spirochetes, *T. denticola* cells were washed twice with PBS, placed on glass slides, allowed to dry, and then fixed with 3.5% glutaraldehyde-0.01% Triton X-100 for 3 min. The slides were then washed once each with PBS and water, stained according to the manufacturer's protocols with ProLong Gold Antifade mounting reagent with DAPI (4',6-diamidino-2-phenylindole) (Life Technologies, Carlsbad, CA), and imaged without further staining on a Nikon Eclipse Ti300 confocal instrument using a 40 $\times$ /1.30-numerical-objective (NA) oil objective lens, with laser excitation at wavelengths of 402 and 488 nm.

For imaging of HGF cells challenged with *T. denticola*, coverslips were recovered from the cocultured wells, washed with PBS, fixed with glutaraldehyde (3.5%, 10 min), and then washed again with PBS and placed on glass slides. The slides were washed once more with PBS and then stained according to the manufacturer's protocols with ProLong Gold Antifade mounting reagent with DAPI and Alexa Fluor 555 phalloidin (Life Technologies) to detect cell nuclei and cytoskeletal actin of HGF cells, respectively. Images were obtained on a Nikon Eclipse Ti300 confocal instrument using a Nikon Plan APO VC 60 $\times$ /1.40-NA oil differential interference contrast [DIC] Eclipse objective lens, with laser excitation at wavelengths of 402, 488, and 561 nm. Images were analyzed using Nikon EZ-C1 software.

RESULTS AND DISCUSSION

Prevalence and conservation of type II restriction enzymes in *Treponema*. Despite considerable effort by several investigators (references 4 and 16 and data not shown), there were no reports of shuttle plasmid transformation of *T. denticola* 35405 prior to the recent report by Bian and Li (3) that a 35405- Δ TDE0911 strain could be transformed with unmethylated shuttle plasmid pBFC. Those authors hypothesized that difficulties in transforming wild-type 35405 were due to the robust DNA restriction-modification (R-M) systems identified in its genome. These include two complete copies of a type I restriction system (*hsdRSM*) as well as genes annotated as encoding three type II restriction enzymes (TDE0228 [TdeII], TDE0911 [TdeIII], and TDE1268 [TdeI]). The specific DNA sequences recognized by these restriction enzymes have been predicted by REBASE (<http://rebase.neb.com/rebase/rebase.html>) (20), but only that encoded by TDE0911 has been reported in the literature (3). To further explore this issue, we performed BLAST searches of all available *Treponema* genomes, including 16 *T. denticola* strains in the Human Oral Microbiome Database (www.homd.org), which revealed the presence of TDE0911 homologs only in *T. denticola* strains 35405, 35404, and 33521, suggesting potential recent acquisition by horizontal gene transfer in these strains. The gene arrangement and DNA sequences of TDE0909 to TDE0911 are highly conserved in all three strains and are predicted to be transcribed as a single mRNA from a promoter sequence approximately 100 bp 5' to the TDE0909 start site. TDE0909 encodes a predicted DNA modifica-

FIG 2 Degradation of pBFC by *T. denticola* requires TdeIII activity and the recognition site. Plasmids pBFC and pCF693 were prepared in *E. coli* strains with (m+) or without (m-) the ability to methylate adenine and cytosine residues. Each agarose gel lane contains 5 μ g plasmid DNA that was linearized with *Spe*I and then incubated for 2 h with cell lysates of each of *T. denticola* strains 35405, 33520, and 35405- Δ TDE0911 (Δ 0911). In the indicated lanes, lysates were boiled prior to incubation with plasmid DNA. *Spe*I-linearized plasmid controls (0.6 μ g) are in the first lane of each gel. Molecular mass standards quantified in kilobases (Kb) are shown for each gel.

tion methyltransferase (TdeIII-M), while TDE0910 encodes a hypothetical protein. No TDE0911 homologue was found in the ATCC 33520 genome sequence, consistent both with the absence of signal in a Southern blot of 33520 DNA probed with TDE0911 and with the inability of ATCC 33520 cell extracts to cleave linear DNA fragments carrying the putative TdeIII recognition site (3) (Fig. 2). Interestingly, in addition to strains 35405, 35404, and 33521, TDE0911 homologs are present in only one other spirochete (*Brachyspira pilosicoli* (57% identity) and several oral *Capnocytophaga* strains (>65% identity) and in various environmental isolates (>50% identity), including *Chlorobium* and *Thermodesulfovibrio* spp.

TDE0228 (TdeII) is highly conserved in the same three *T. denticola* strains that contain TDE0911 but is absent in other *Treponema* spp. The closest homolog (32% identity) is found in *Dolosigranulum pigrum*, a *Gemella*-like organism isolated from diverse human infections (21).

TDE1268 (TdeI) is highly conserved in 9 of 16 sequenced *T. denticola* strains, including 33521, 35404, and 35405, but was not detected in *T. denticola* 33520. TDE1268 homologs are present at >50% identity in numerous strains of *Streptococcus anginosus*, *Moraxella catarrhalis*, and oral *Capnocytophaga* and at somewhat lower identity in numerous pathogenic and oral commensal streptococci.

Type II restriction activity in *T. denticola*. Of the three type II restriction enzymes encoded in the ATCC 35405 genome, only the enzyme activity of TdeIII encoded by TDE0911 has been verified in *T. denticola*, as shown by the inability of a *T. denticola* Δ TDE0911 mutant to cleave the sequence GGCCC (3) (Fig. 2).

This is one of four potential TdeIII recognition sites (GGNCC) predicted in the REBASE database. Based on comparative activity analyses of the cloned gene products, the recognition sites of TdeI ($G^{m}ATC$) and TdeII (CTCTTC) are also predicted in REBASE, but neither has been directly verified in *T. denticola* (20; R. Roberts, personal communication). In addition to a single GGCCC TdeIII site, pBFC also contains two GGTC sites, but no GGACC or GGGCC sites. There are 5 instances of predicted TdeII recognition sites in pBFC. While the pattern of adenine methylation in *T. denticola* is not known, pBFC contains 19 potential recognition sites for TdeI.

We assayed susceptibility of *Spe*I-linearized pBFC and pCF693 to cleavage and degradation in freshly prepared sonicated lysates of *T. denticola* strains 35405, 35405- Δ TDE0911, and 33520. If TdeIII were to recognize both GGCCC and GGTC sites, the expected restriction patterns would be as follows: for pBFC, 1.25 kb, 1.33 kb, 1.44 kb, and 2.5 kb; and for pCF693, 1.33 kb, 1.44 kb, and 3.7 kb. As shown in Fig. 2, the 35405 lysate cleaved both pBFC and pCF693, independently of plasmid DNA methylation status, yielding bands consistent with at least partial cleavage at each of the predicted GGCCC and GGTC sites. Consistent with the absence of the TdeIII (GGCCC) site in pCF693, the 2.5-kb band was absent in the pCF693 digest. In contrast, 35405- Δ TDE0911 exhibited very weak digestion activity against methylated plasmid DNA, yielding only faint digestion products whose size differed from those generated by 35405. The sizes of these fragments (approximately 1.4 to 2.1 kb) are consistent with predicted TdeII restriction activity. 35405- Δ TDE0911 did not cleave unmethylated DNA. These re-

sults suggest that TDE0911 encodes the predominant type II restriction activity in *T. denticola* 35405.

The restriction digestion patterns generated by *T. denticola* 33520 were notably distinct from those of 35405 and 35405- Δ TDE0911. As shown in Fig. 2, 33520 did not cleave methylated plasmid DNA but yielded the same pattern of 5 bands from unmethylated pBFC and pCF693. These results are consistent with those reported by Bian and Li (3). Taken together with the absence of identifiable homologues of the three 35405 type II enzymes in 33520, this suggests that the *T. denticola* 33520 genome encodes at least one highly active restriction enzyme with a defined recognition site of greater than 4 bp and specificity for unmethylated DNA. Further studies are required to characterize this enzyme activity in strain 33520, as well as the activities of TdeI and TdeII in strain 35405.

Modified electroporation protocol enhances *T. denticola* transformation efficiency. The existing protocol for genetic transformation of *T. denticola*, first developed by the Kuramitsu group (8, 22), closely follows a basic protocol for electrotransformation of *E. coli* and has remained essentially unchanged for over 15 years (8, 22). Due to the wide range of microbial physiology, species- and strain-specific electroporation protocols have been developed to optimize electrotransformation in numerous organisms (23–27). While we have not yet addressed electrical parameters of electroporation (voltage, resistance, capacitance), we identified several areas that could potentially affect *T. denticola* transformation efficiency, including both pre- and postelectroporation steps.

In a recent discussion of strategies for culturing anaerobes, Bacic and Smith (28) stated that “Anaerobic culturing is as much a philosophy as it is a technique and the researcher needs to scrutinize all aspects of an experiment to ensure that reasonable consideration has been given to maintaining anaerobiosis at all stages of growth.” During preparation of electroporation-competent cells, exposure of *T. denticola* to oxygen and cellular damage during centrifugation and wash steps were the most likely sources of reduced viability. For this reason, all water washes were replaced with sterile, prerduced 10% glycerol that had been held under anoxic conditions for at least 12 h. In addition, all processing steps in preparing electrocompetent *T. denticola* cells were conducted so as to minimize exposure to oxygen.

Exposure to oxygen during and after electroporation may also contribute to reduced *T. denticola* viability. Competent cells were thawed on ice, mixed with DNA samples, and subjected to electroporation without delay. This is a departure from the published protocol, which calls for incubation of competent cells with DNA for 30 min. We deemed this unwise due to both the oxygen exposure issue and the likelihood of DNA degradation due to the activity of endonucleases, either secreted or released by cell lysis. Exposure to oxygen via growth medium that has not been made anoxic also likely contributes to reduced postelectroporation *T. denticola* viability. This is especially true for the soft agar medium used for plating, which solidifies at the temperature maintained in oxygen-free chambers. Conversely, mixing *T. denticola* with molten agar at temperatures much above 37°C subjects the cells to heat shock, which also reduces viability (data not shown). For these reasons, we equilibrated NOS agar medium in a calibrated 40°C heat block in the oxygen-free chamber for 18 h before plating electroporated cells. It has been suggested that heat treatment of competent cells (50°C, 30 min) to temporarily inactivate restric-

tion-modification systems might increase *T. denticola* transformation efficiency (8). While this strategy was reported to be effective in *E. coli* and *Staphylococcus* (29, 30) and has been used in *T. denticola* (4), its effects have never been empirically tested in *T. denticola*. Due to the sensitivity of *T. denticola* to heat shock, this approach was deemed unattractive and was not pursued.

Finally, instability of some selective agents during long-term incubation can contribute to “false-positive” transformation results. In particular, chloramphenicol analysis has documented light sensitivity and limited activity after the long-term incubation required for *T. denticola* colony formation (31). For this reason, fresh chloramphenicol was added to NOS agar medium just before use and transformation plates were incubated in the dark. *T. denticola* plasmid transformation was confirmed both by shuttle plasmid-specific PCR and by transformation of *E. coli* with plasmid DNA isolated from *T. denticola* transformants followed by confirmation of the expected restriction digest pattern of plasmids recovered from *E. coli* (data not shown).

As shown in Table 1, using the modified protocol, we routinely obtained 10^3 to 10^4 colonies (CFU) using 1 μ g input plasmid DNA. This is at least 100-fold-higher efficiency than previous reports, most of which reported using 5 to 10 μ g plasmid DNA and recovering <100 CFU (and in most cases <10 CFU) in any *T. denticola* host strain (2, 4, 5). While a linear relationship between the amount of input DNA and the efficiency of transformation by electroporation is reported for *E. coli* (23), we and others have found an inverse relationship between input DNA amount and transformation efficiency in other microbes (24, 32). Using the modified protocol described here, we have obtained >5,000 CFU using as little as 50 ng plasmid DNA. As shown in Table 2, we were able to obtain the highest efficiency (CFU per microgram) in transforming *T. denticola* 35405 with 1 ng methylated pCF693. Our results suggest that the typical high dose of input DNA used by most *Treponema* researchers (10 μ g per transformation) is far from optimal and may be counterproductive for successful transformation.

Shuttle plasmid modification to enhance transformation efficiency in *T. denticola* 35405. Having demonstrated high-efficiency transformation of *T. denticola* 35405, we then compared the transformation efficiency of pBFC with that of pCF693, which differs from pBFC only in the absence of a documented recognition site for TdeIII restriction endonuclease. As shown in Table 1, we did not observe markedly higher transformation efficiency of 35405 with pCF693 compared with pBFC, nor were there major differences in the abilities of these plasmids to transform either 35405- Δ TDE0911 or 33520, both of which lack genes encoding TdeIII. It should be noted that the calculated values presented in Table 1 do not reflect experiments in which transformation efficiency could only be estimated as $>10^4$ CFU per μ g. In light of the results shown in Fig. 2, it is likely that the two alternate TdeIII recognition sites present in both pBFC and pCF693 (GGTCC, Fig. 1) are susceptible to TdeIII activity and that absence of one TdeIII site in pCF693 does not make a large difference in transformation. It would be of interest to determine whether removal of the two remaining TdeIII sites from pCF693 would enhance transformation efficiency.

Plasmid DNA methylation has no effect on *T. denticola* transformation efficiency. Because plasmid DNA methylation status was reported to affect *T. denticola* transformation (3), we performed transformations using plasmid DNA prepared in *E.*

TABLE 1 *T. denticola* transformation efficiency using different strains and plasmids

<i>T. denticola</i> strain	Plasmid	Methylation status ^a	CFU ^b	No. of expts with CFU TNTC ^c
35405	pBFC	+	$1.6 \times 10^3 (\pm 400)$	2
		—	$1.7 \times 10^3 (\pm 649)$	0
	pCF693	+	$1.8 \times 10^3 (\pm 283)$	4
		—	$2.4 \times 10^3 (\pm 1.1 \times 10^3)$	1
35405-ΔTDE0911	pBFC	+	$[>4 \times 10^4]$	3
		—	$4.9 \times 10^2 (\pm 466)$	3
	pCF693	+	$[>4 \times 10^4]$	3
		—	$9.2 \times 10^2 (\pm 875)$	3
33520	pBFC	+	$8.5 \times 10^2 (\pm 981)$	2
		—	$5.9 \times 10^2 (\pm 0)$	4
	pCF693	+	80 (± 0)	2
		—	$[>4 \times 10^4]$	4

^a Plasmid DNA was prepared in *E. coli* JM109 (*dam*⁺ *dcm*⁺; methylation +) or JM110 (lacking *dam* and *dcm*; methylation —).

^b CFU ($\pm$ standard deviation) recovered following transformation with 1 μ g plasmid DNA. Three or more independent experiments were conducted for each condition. CFU values in brackets [$>4 \times 10^4$] represent conditions for which all plates yielded CFU too numerous to count at the highest dilution tested, with no standard deviation calculated.

^c Number of experiments with CFU too numerous to count (TNTC) at the highest dilution ($>4 \times 10^4$ CFU per μ g). These experiments are not included in calculations of CFU per microgram and standard deviations.

coli strains with and without the ability to methylate adenine and cytosine residues. As shown in Table 1, no clear pattern emerged of an effect of plasmid DNA methylation status in any of the *T. denticola* strains tested. However, as noted above, CFU calculations do not include data from experiments in which $>10^4$ CFU were recovered. Our results are in contrast to those of a previous study which reported that 35405 transformation required unmethylated plasmid DNA whereas 33520 could accept only methylated plasmid DNA (3). As discussed above, the conditions under which *T. denticola* transformations are conducted greatly affect efficiency. Under transformation conditions that yield very low efficiency, small differences are magnified and can make the difference between successful and unsuccessful transformation.

Our results show that *T. denticola* strains with widely differing capabilities for restricting foreign DNA based on sequence specificity and methylation pattern can be consistently transformed with shuttle plasmids at reasonably high efficiency. Molecular mechanisms of transformation by allelic replacement mutagenesis differ substantially from those of plasmid transformation, but the same considerations and strategies can be applied. While we have not explored the effects of DNA concentration on allelic replacement, we have had some success using the strategies described here to construct mutations in *T. denticola* genes that had resisted prior efforts (data not shown).

DNA restriction systems specifically recognize “foreign” DNA by a combination of sequence specificity and nucleotide methylation patterns. Once a plasmid replicates within *T. denticola*, it has the appropriate (as-yet-undefined) methylation pattern and is

thus effectively *T. denticola* DNA, regardless of presence of specific DNA sequences. This can be illustrated by a cursory examination of numerous *T. denticola* gene sequences that contain the experimentally confirmed 5'-GGCCC-3' recognition site for TdeIII (3, 6, 17). As long as host DNA polymerase recognizes a plasmid replicon and any essential genes carried on the plasmid are expressed, one can approach the problem of plasmid transformation as a “race” between host cell restriction systems and DNA polymerase. For this reason, our strategy combines limiting targets of *T. denticola* restriction activity with optimizing the methodology for efficiency of transformation for this spirochete.

Expression of a flavin mononucleotide-based fluorescent protein in *T. denticola*. The ability to express fluorescent markers has become a key technique in *in vivo* imaging of cells and tissue (33). Since its development as a molecular tool 20 years ago (34), the green fluorescent protein (GFP) and its various derivatives have become some of the most widely used reporters for *in vitro* and *in vivo* gene expression. A major limitation of this system is the strict requirement for molecular oxygen as a cofactor for the active chromophore of GFP and related proteins. Because *T. denticola* is an obligate anaerobe, expression of active oxygen-dependent chromophores is problematic. Early studies showed that detection of GFP or X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) chromophore activity in *T. denticola* required several days of incubation in air (35, 36), which severely limited the utility of these technologies. Development of a widely applicable technology for fluorescent probe expression in the absence of oxygen (37) has greatly expanded the range of chromogenic detection systems beyond the canonical versions of GFP and their various oxygen-dependent derivatives (37). This provides opportunities for major advances in molecular microbiology of obligate anaerobes (38, 39) as well as in analysis of hypoxic responses in a wide range of biological systems (40–42). While highly promising, this technology has not yet become widely used in anaerobic microbes.

To demonstrate flavin mononucleotide-based fluorescent protein expression in *T. denticola*, we utilized a derivative of the Bs2

TABLE 2 Effect of plasmid DNA amount on transformation efficiency^a

Plasmid DNA amt	CFU at 1:200 dilution	CFU per μ g
1.0 μ g	>200	$>4 \times 10^4$
100 ng	>200	$>4 \times 10^4$
10 ng	55	1.1×10^6
1 ng	19	3.8×10^6

^a *T. denticola* ATCC 35405 was transformed with (methylated) pCF693 as described in Materials and Methods.

FIG 3 Expression of FbFP in *E. coli*. *E. coli* JM109 bacteria carrying plasmids of interest were grown on LB agar with appropriate antibiotics. The agar plates were then photographed under normal illumination to visualize colonies and under UV light (460- to 500-nm-wavelength excitation filter; 510-nm-wavelength emission filter) to visualize FbFP expression. Bar, 2 mm. pGlow, JM109/pGlow-CK^{XXN}-Bs2 carrying FbFP gene downstream of a *Clostridium* promoter; pCF693, JM109/pCF693 (*E. coli*-*T. denticola* shuttle plasmid); pCF728, JM109/pCF728 (pCF693 with FbFP gene downstream of *T. denticola* *tap1* promoter).

gene carried on pGlow-CK^{XXN}-Bs2. The synthetic gene construct on this plasmid, designated Bs2C, is derived from the photoactive N-terminal light oxygen voltage domain of *B. subtilis* YtvA (37). Bs2C has been codon optimized for efficient expression in clostridia, which would presumably facilitate its expression in other low-GC-content microbes such as *T. denticola*. The Bs2C gene was cloned between the *Spe*I and *Eco*RV sites in pCF693 to yield pCF728 (Fig. 1), such that transcription of genes encoding both Bs2C and chloramphenicol resistance (Cm^r) in pCF728 is under transcriptional control of the *tap1* promoter (Ptap1) from *T. denticola* ATCC 33520 carried on the vector (16). The Cm^r gene on the shuttle plasmid (isolated from *Staphylococcus aureus*) does not confer chloramphenicol resistance in *E. coli*. We hypothesized that this might be due to lack of function of Ptap1 in *E. coli*. In order to test this, we compared expression of Bs2C-mediated FbFP fluorescence in *E. coli* JM109 carrying pGlow-CK^{XXN}-Bs2, pCF693, or pCF728. As shown in Fig. 3, strong FbFP fluorescence was detected in *E. coli* carrying pGlow-CK^{XXN}-Bs2 but not in *E. coli* carrying either pCF693 or pCF728. These data suggest that Ptap1 on pBFC and pCF693 does not function as a transcriptional promoter in *E. coli*. This is a significant and previously unnoted feature of this shuttle plasmid, in that it should permit cloning of genes whose expression is toxic in *E. coli* (43) for expression in *T. denticola* from a shuttle plasmid. In concert with improved shuttle plasmid transformation efficiency, this feature will facilitate mutant complementation studies (heretofore at best problematic in *T. denticola*) because it should now be possible to clone full-length “toxic” genes in the shuttle plasmid for subsequent expression in cognate *T. denticola* mutants.

To demonstrate flavin mononucleotide-based fluorescent protein expression in *T. denticola*, shuttle plasmid pCF728 carrying the gene encoding Bs2C under the transcriptional control of Ptap1 was electroporated into *T. denticola* 35405. Cm^r colonies were analyzed for plasmid presence and expression of fluorescence activity. A Cm^r *T. denticola* 35405 strain carrying pCF728 (designated strain CF734) was chosen for further analysis. As shown in Fig. 4, *T. denticola* CF734 emits a strong green fluorescence signal when subjected to excitation at 488 nm, while *T. denticola* 35405 exhibits a very low level of autofluorescence. As shown in Fig. 5, *T. denticola* CF734 cells were readily visualized in experiments in which HGF cells were challenged with *T. denticola*. The green

FIG 4 Visualization of *T. denticola* CF734 expressing FbFP. *T. denticola* 35405 and CF734 were stained with DAPI and visualized in confocal microscopy. Images shown were obtained at $\times 400$ magnification under laser excitation to visualize FbFP (488-nm wavelength) and DAPI (402-nm wavelength) and to produce a merged image.

fluorescence activity of *T. denticola* CF734 permits its use in studying *T. denticola* interactions with host cells without the time and expense required to perform complex multistep immunological assays. We are currently employing *T. denticola* CF734 in studies of oral cell responses to *T. denticola* as well as in studies of multi-species oral biofilm formation and interspecies interactions (data not shown).

We have demonstrated here two significant additions to the *T. denticola* genetic toolkit. We showed that the plasmid transformation efficiency in *T. denticola* can be greatly increased, primarily by gentle treatment of cells and careful attention to maintenance of anoxic conditions at all stages of the transformation process. Doing so resulted in the first efficient and repeatable transformation of the wild-type *T. denticola* 35405 strain with a shuttle plasmid. Using this shuttle plasmid, we constructed a *T. denticola* strain expressing fluorescence activity under normal (anoxic) growth conditions. This simple methodological advance expands the repertoire of genetic tools available in *T. denticola* to include routine complementation of chromosomal mutations and expression of heterologous proteins under anoxic conditions. These methodol-

FIG 5 Merged confocal image showing a human gingival fibroblast following a brief (30-min) challenge with *T. denticola* CF734. The fibroblast cell nucleus and cytoskeletal actin were stained with DAPI and Alexa Fluor 555 phalloidin, respectively. The image shown represents merged green, blue, and red channels. Bar, 5 μ m.

ogies will be utilized in ongoing and planned studies of *T. denticola* gene regulation and cell-cell interactions. Finally, our results suggest that expanded characterization of *T. denticola* restriction systems may yield further advances in transformation efficiency.

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