

# Evidence that TP\_0144 of *Treponema pallidum* Is a Thiamine-Binding Protein

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Thiamine pyrophosphate (TPP), the biologically active form of thiamine (also known as vitamin B<sub>1</sub>), is an essential cofactor for several important enzymes involved in carbohydrate metabolism, and therefore, it is required for all living organisms. We recently found that a thiamine-binding protein (TDE\_0143) is essential for the survival of *Treponema denticola*, an important bacterial pathogen that is associated with human periodontitis. In this report, we provide experimental evidence showing that TP\_0144, a homolog of TDE\_0143 from the syphilis spirochete *Treponema pallidum*, is a thiamine-binding protein that has biochemical features and functions that are similar to those of TDE\_0143. First, structural modeling analysis reveal that both TDE\_0143 and TP\_0144 contain a conserved TPP-binding site and share similar structures to the thiamine-binding protein of *Escherichia coli*. Second, biochemical analysis shows that these two proteins bind to TPP with similar dissociation constant ( $K_d$ ) values (TDE\_0143,  $K_d$  of 36.50 nM; TP\_0144,  $K_d$  of 32.62 nM). Finally, heterologous expression of TP\_0144 in a  $\Delta$ TDE\_0143 strain, a previously constructed TDE\_0143 mutant of *T. denticola*, fully restores its growth and TPP uptake when exogenous thiamine is limited. Collectively, these results indicate that TP\_0144 is a thiamine-binding protein that is indispensable for *T. pallidum* to acquire exogenous thiamine, a key nutrient for bacterial survival. In addition, the studies shown in this report further underscore the feasibility of using *T. denticola* as a platform to study the biology and pathogenicity of *T. pallidum* and probably other uncultivable treponemal species as well.

Spirochetes comprise a monophyletic phylum that exhibits distinct long and coiled cell shapes and a characteristic corkscrew-like motility (1). The phylum comprises nine genera. One of these genera is *Treponema*, which contains several important human pathogens such as *Treponema pallidum*, the causative agent of syphilis (2, 3). The genus of *Treponema* consists of more than 60 different species, and many of these species cannot be cultivated *in vitro* (e.g., *T. pallidum* can be grown experimentally only in rabbit testes) (4–7). The lack of reliable *in vitro* cultivation systems has substantially hindered our understanding of the biology of *T. pallidum* and syphilis pathogenesis (3). Identifying virulence determinants of *T. pallidum* still depends on expressing potential virulence genes in heterologous systems such as *Escherichia coli* (8–11). However, the physiological differences between the spirochetes and *E. coli* have limited the use of *E. coli* systems for functional investigations.

Among the treponemal species, *Treponema denticola*, an oral spirochete associated with periodontitis, can be easily cultivated and is genetically manipulable (12–14). Due to the high physiological similarities between *T. denticola* and *T. pallidum*, the oral spirochete has been explored to study the physiology, biosynthetic pathways, and virulence determinants of *T. pallidum* (e.g., heterologous expression of *T. pallidum* genes in *T. denticola*) (15–17). ATCC 33520 and ATCC 35405 are two laboratory strains of *T. denticola*. The first one can be genetically manipulated more easily, and several vectors for heterologous gene expression have been developed (15, 17, 18). However, the genome of this strain has not been decoded, which has limited its application. The genome of ATCC 35405 has been sequenced (4). Comparative genomic studies revealed that more than two-thirds (728/1,041) of the predicted open reading frames (ORFs) in *T. pallidum* can be found in

the genome of ATCC 35405. On average, these homologues share 53% amino acid sequence identities and 71% similarities (4), which makes *T. denticola* a plausible model for *T. pallidum* research. Furthermore, we recently developed several new genetic tools for *T. denticola*, including a genetically modified strain that is more amenable to genetic manipulations (19), a modified gentamicin resistance marker (*aacC<sub>m</sub>*) for targeted mutagenesis (20), and a thiamine-riboswitch regulatory element (21). These tools make it possible to express *T. pallidum* homologues and then carry out functional studies in *T. denticola*.

Thiamine pyrophosphate (TPP) is essential for all living systems due to its cofactor roles for several important enzymes involved in carbohydrate and branched-chain amino acid metabolism (22–25). TPP biosynthesis is often linked to other central metabolic pathways (e.g., purine biosynthesis). Thus, studying TPP biosynthesis in a microorganism can help us to dissect its metabolic integrations (26). Bacteria either synthesize TPP via *de novo* biosynthesis pathways or import exogenous TPP from environments via specific transporters (22, 27). Both *T. denticola* and

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*T. pallidum* lack a *de novo* thiamine biosynthesis pathway (2, 4, 21). Thus, they must take up exogenous thiamine, a key nutrient for their survival. Bacteria typically import exogenous thiamine and its derivatives via primary ABC transporters, which consist of a thiamine-binding protein (Tbp), a transmembrane permease, and a cytosolic ATPase (28). The role of Tbp is to secure the ligand and deliver it to the permease, which uses energy from ATP hydrolysis to transport the ligand against a concentration gradient into the cytoplasm (25). TbpA, the thiamine-binding protein of *E. coli*, is essential for growth when its *de novo* thiamine biosynthesis pathway is disrupted (29). We recently identified a thiamine-binding protein (TDE\_0143) in *T. denticola*. Mutagenesis studies revealed that deletion of *TDE\_0143* dramatically impaired the ability of *T. denticola* to import exogenous TPP, and the mutant failed to grow in a medium with limited amounts of TPP (21). Similar to *T. denticola*, the genome of *T. pallidum* also encodes a thiamine-ABC transporter, which consists of TP\_0144 (thiamine-binding protein), TP\_0143 (transmembrane permease), and TP\_0142 (ATPase), suggesting that *T. pallidum* also needs to import exogenous thiamine for its metabolism (2, 21). The experiments in this report were specifically designed to address the following two questions. (i) Does TP\_0144 function as a thiamine-binding protein? (ii) By studying the function of TP\_0144, can we advance the genetics toolbox of *T. denticola* to allow us to establish a universal platform for expressing *T. pallidum* homologues and then conduct functional studies in *T. denticola*?

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Treponema denticola* ATCC 35405 (wild type) and its derivative mutant strains were grown in an oral bacterial growth medium (OBGM) supplemented with 10% heat-inactivated rabbit serum at 37°C in an AS-580 anaerobic chamber (Anaerobe Systems, Morgan Hill, CA) in the presence of 85% nitrogen, 5% carbon dioxide, and 10% hydrogen, as previously described (30). For the semi-solid growth medium, 0.7% low-melting-point SeaPlaque agarose (Lonza, Rockland, ME) was incorporated into the medium, which was then plated after bacterial suspensions were inoculated into the medium at 37°C (31). *Treponema pallidum* subsp. *pallidum* Nichols was extracted from rabbit testes after intratesticular infection and further purified by Percoll density gradient centrifugation as described before (5). The *Escherichia coli* TOP10 strain (Invitrogen, Carlsbad, CA) was used for routine plasmid construction and preparation. The *E. coli dam dcm* mutant strain (New England BioLabs, Ipswich, MA) was used to amplify and prepare unmethylated plasmids. The *E. coli* B21 Star(DE3) strain (Invitrogen) was used for recombinant protein expression and preparations. The *E. coli* strains were grown in lysogeny broth (LB) supplemented with appropriate concentrations of antibiotics.

**Preparations of TDE\_0143 and TP\_0144 recombinant proteins and antibodies.** To prepare the recombinant TDE\_0143 (rTDE\_0143) and TP\_0144 (rTP\_0144) proteins, the entire genes were PCR amplified using Platinum Pfx DNA polymerase (Invitrogen). The resulting amplicons were cloned into a pET100/D-TOPO expression vector (Invitrogen) with an N-terminal six-His (His<sub>6</sub>) fusion tag. To prepare mutated recombinant TDE\_0143 and TP\_0144 proteins, the codons for tyrosine 227 (TAC) in these two genes were mutated to alanines (GCC) (yielding rTP\_0143<sup>227Y/A</sup> and rTP\_0144<sup>227Y/A</sup>, respectively) using a QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The obtained plasmids were transformed into *E. coli* B21 Star(DE3) cells and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant proteins were purified using Ni-nitrilotriacetic acid (NTA)-agarose (Qiagen, Valencia, CA) under native conditions as previously described (21). The final purified proteins were dialyzed in 10 mM Tris buffer (pH 8.0) at 4°C overnight. Protein concentrations were

measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). To generate antibodies against TDE\_0143 and TP\_0144, mice were immunized with 1 mg of the recombinant proteins in three inoculations during a 1-month period (the antisera were produced by General Bioscience Corporation, Brisbane, CA). The obtained antisera were tested by immunoblotting as previously described (21).

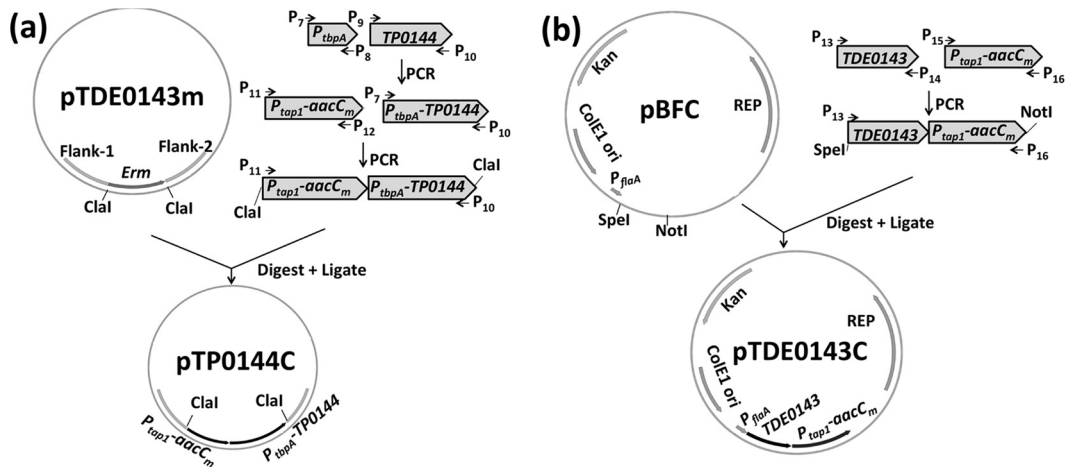
**TPP-binding assay.** Protein-TPP binding affinity was measured using an equilibrium dialysis method as described before (32). The assay was carried out using DispoBiodialyzer cassettes (The Nest Group, Southborough, MA), which have two chambers separated by a 5-kDa-cutoff membrane. Briefly, 60 μl of TPP (0 to 25 μM) was injected into one chamber, and then 60 μl (10 μM) of either the wild-type recombinant proteins or the mutated ones was injected into the other chamber. The loaded cassettes were maintained for 2 h with gentle rocking at room temperature to reach equilibrium. Then, 50 μl of samples from both sides of the cassettes was taken, pretreated, and subjected to reverse-phase high-performance liquid chromatography (HPLC) for measuring TPP concentrations as previously documented (21). The binding constants were calculated with GraphPad Prism, version 5, software (GraphPad Software, San Diego, CA).

**Complementation of a TDE\_0143 deletion mutant using TDE\_0143 and TP\_0144.** In our previous report, the *TDE\_0143* gene was deleted in frame (21), and the obtained mutant was designated ΔTDE\_0143. We first attempted to *cis* complement ΔTDE\_0143 using *TP\_0144*. The complementation vector pTP\_0144C was constructed by multiple-step PCR as illustrated in Fig. 1a. Briefly, the previously identified *TDE\_0143* promoter (*P<sub>tbpA</sub>*) (21), the *TP\_0144* gene, and the *P<sub>tap1</sub>-aacC<sub>m</sub>* cassette (20, 33) were PCR amplified with primers P<sub>7</sub>/P<sub>8</sub>, P<sub>9</sub>/P<sub>10</sub>, and P<sub>11</sub>/P<sub>12</sub>, respectively (Table 1 lists the primer sequences). *P<sub>tbpA</sub>* and *TP\_0144* were fused together by PCR with primers P<sub>7</sub>/P<sub>10</sub>. The resulting *P<sub>tbpA</sub>-TP\_0144* fragment was fused to *P<sub>tap1</sub>-aacC<sub>m</sub>* by PCR with primers P<sub>11</sub>/P<sub>10</sub>. The resulting fragments were cloned into a pGEM-T vector and then treated with ClaI to release *P<sub>tap1</sub>-aacC<sub>m</sub>-P<sub>tbpA</sub>-TP\_0144*. The released fragment was finally inserted into pTDE\_0143m, a previously constructed vector for inactivation of *TDE\_0143* (21), at an engineered ClaI cut site, yielding pTP\_0144C (Fig. 1a).

We also attempted to complement the mutant with *TDE\_0143* using pBFC, a shuttle vector of *T. denticola* (17), as illustrated in Fig. 1b. The *TDE\_0143* gene and the *tap1* promoter-driven gentamicin resistance marker (*P<sub>tap1</sub>-aacC<sub>m</sub>*) were PCR amplified with primers P<sub>13</sub>/P<sub>14</sub> and P<sub>15</sub>/P<sub>16</sub>, respectively. The resulting fragments were fused together by PCR using primers P<sub>13</sub>/P<sub>16</sub>. The fused *TDE\_0143-P<sub>tap1</sub>-aacC<sub>m</sub>* fragment was cloned into a pGEM-T Easy vector and then released using SpeI and NotI. The released DNA fragment was finally cloned into pBFC downstream of the *flaA* promoter (*P<sub>fla</sub>*) (17, 33), yielding the pTDE\_0143C vector (Fig. 1b). The obtained plasmids were amplified and purified from the *E. coli dam dcm* mutant strain (19). For complementation of the ΔTDE\_0143 mutant, the mutant was separately transformed with 10-μg amounts of unmethylated pTP\_0144C and pTDE\_0143C and then plated on OBGM semisolid agar plates containing 40 μg ml<sup>-1</sup> erythromycin and/or 20 μg ml<sup>-1</sup> gentamicin for screening of positive colonies as previously documented (32, 34).

**Southern blotting.** Total genomic DNA of *T. denticola* was isolated using an Illustra Bacteria Genomic Prep kit (GE Healthcare, Little Chalfont, United Kingdom). Southern blot analysis was carried out as previously described (19). Briefly, the purified genomic DNA was first digested with HindIII, separated on a 1.0% agarose gel, and transferred to Hybond-N<sup>+</sup> membranes (GE Healthcare). To prepare DNA probes, *aacC<sub>m</sub>*, *TP\_0144*, and *TDE\_0143* were amplified by PCR, and the obtained amplicons were labeled with digoxigenin (DIG). The DNA labeling, hybridization, and detection were carried out using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnosis GmbH, Mannheim, Germany), according to the manufacturer's instructions.

**SDS-PAGE and Western blotting.** Equal amounts of *T. denticola* and *T. pallidum* whole-cell lysates (ranging from 10 to 20 μg) were separated



**FIG 1** Schematic illustration of vector construction. Construction of vectors pTP\_0144C (a) and pTDE\_0143C (b) is shown. pTP\_0144C was constructed to *cis* complement  $\Delta$ TDE\_0143 using *TP\_0144*, and pTDE\_0143C was constructed to *trans* complement the mutant using *TDE\_0143*. The vector pTDE\_0143m was previously constructed to delete *TDE\_0143* in frame (21). pBFC is a shuttle vector of *T. denticola* (17). Arrows indicate the relative positions of PCR primers for constructing these vectors. The sequences of these primers are listed in Table 1. *P<sub>tap1</sub>*, *tap1* promoter; *P<sub>tbpA</sub>*, *tbpA* promoter; *aacC<sub>m</sub>*, modified gentamicin resistance marker; REP, replication origin of *T. denticola*.

on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). The blots were probed using antibodies against TDE\_0143 (anti-TDE\_0143), TP\_0144 (anti-TP\_0144), or FlaA (anti-FlaA) and then developed using horseradish peroxidase-conjugated secondary antibody with an enhanced chemiluminescence (ECL) luminol assay as previously described (21). Signals were quantified using a Molecular Imager ChemiDoc XRS system with Image Lab software (Bio-Rad Laboratories).

**Measuring *T. denticola* growth rates.** The previously developed TPP assay medium (TA medium) (21) was used to measure growth of *T. denticola*. Briefly, 5  $\mu$ l of late-logarithmic-phase *T. denticola* cultures ( $\sim 5 \times 10^8$  cells ml<sup>-1</sup>) was inoculated into 5 ml of TA medium supplemented with different amounts of thiamine or TPP and grown at 37°C in an anaerobic chamber. The cultures were enumerated every 24 h using a

Petroff-Hausser counting chamber (Hauser Scientific, Horsham, PA) for up to 7 days. Counts were repeated in triplicate with at least three independent experiments. The results were expressed as mean cell numbers  $\pm$  standard deviations (SD).

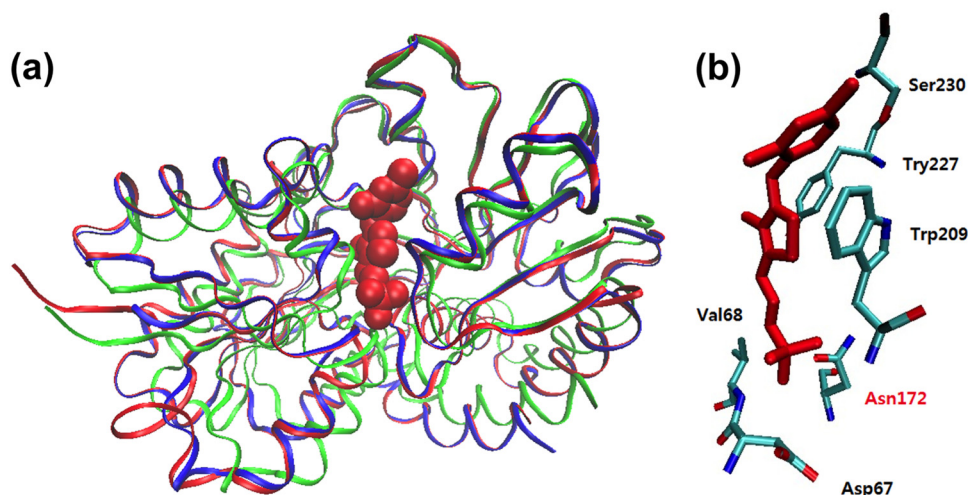
**Detection of intracellular thiamine and its derivatives by HPLC.** Concentrations of thiamine and its derivatives (i.e., thiamine monophosphate [TMP] and TPP) in the *T. denticola* cells were measured by HPLC as previously described (21, 35). Briefly, *T. denticola* strains were cultivated in the TA medium with different amounts of TPP to stationary phase. Bacterial cells from 50 ml of the cultures were harvested by centrifugation at 6,000  $\times$  g for 10 min. The collected cell pellets were washed twice using phosphate-buffered saline (PBS, pH 7.4) and then resuspended in PBS. The final cell densities were adjusted to 10<sup>10</sup> cells ml<sup>-1</sup>. The obtained samples were lysed with sonication and fractionated by centrifugation at

**TABLE 1** Oligonucleotide primers used in this study

Primer	Sequence <sup>a</sup>	Orientation <sup>b</sup>	Description and/or purpose
P <sub>1</sub>	CACCGCTCACCGTATGCGTGTT	F	TP_0144 recombinant protein
P <sub>2</sub>	TCATGGGGTTGATGCGTG	R	TP_0144 recombinant protein
P <sub>3</sub>	GCCGCACTGGTACTTGTAGTCCACCACAAGTGC	F	Site mutagenesis of TP_0144
P <sub>4</sub>	GCACTTGTGGTGGCACTAAGTACCAGTGCGGC	R	Site mutagenesis of TP_0144
P <sub>5</sub>	GCTCCCATAGCCATTTCTGCCACAAGCAGCC	F	Site mutagenesis of TDE_0143
P <sub>6</sub>	GGCTGCTTGTGGCAGAAATGGCTATGGGAGC	R	Site mutagenesis of TDE_0143
P <sub>7</sub>	CGCCACCTAAGGACAACCTGTTATTATGCAC	F	Complementation, <i>P<sub>tbpA</sub></i>
P <sub>8</sub>	CATACGGTGAGCCATATGCTCCCTCCGCCG	R	Complementation, <i>P<sub>tbpA</sub></i>
P <sub>9</sub>	GGGAGCATATGGCTCACCGTATGCGTGTGTCTTC	F	Complementation, TP_0144
P <sub>10</sub>	ACTAGTTCATGGGGTTGATGCGTGTAATGC	R	Complementation, TP_0144
P <sub>11</sub>	ACTAGTCATCGTTCATATTATACTTCTCC	F	Complementation, <i>P<sub>tap1</sub>-aacC<sub>m</sub></i>
P <sub>12</sub>	CAAGTTGTCTTAGGTGGCGGTACTTGGGTC	R	Complementation, <i>P<sub>tap1</sub>-aacC<sub>m</sub></i>
P <sub>13</sub>	ACTAGTATGAAAAATCATTACGCTC	F	Complementation, TDE_0143
P <sub>14</sub>	GAACGATGTTATTTTGTAAAACATTGATTACGGC	R	Complementation, TDE_0143
P <sub>15</sub>	CAATGTTTTACAAAAATAACATCGTTCATATTATATTCTCC	F	Complementation, <i>P<sub>tap1</sub>-aacC<sub>m</sub></i>
P <sub>16</sub>	GCGGCCGCTTAGGTGGCGGTACTTGGGTC	R	Complementation, <i>P<sub>tap1</sub>-aacC<sub>m</sub></i>
P <sub>17</sub>	CGGTGTGCCGCTCTTTTTCAG	F	TDE_0143 DNA probe
P <sub>18</sub>	GCAAAATAGCCGTAGTCAAAGGG	R	TDE_0143 DNA probe
P <sub>19</sub>	CTCACCGTATGCGTGTGTGC	F	TP_0144 DNA probe
P <sub>20</sub>	CGGATGGCACGATGAATAGC	R	TP_0144 DNA probe

<sup>a</sup> The engineered restriction enzyme sites are underlined.

<sup>b</sup> F, forward; R, reverse.



**FIG 2** Structural modeling of TP\_0144. (a) Comparison of overall structures of TDE\_0143 (blue), TP\_0144 (red), and the TbpA protein of *E. coli* (green). The TbpA protein of *E. coli* (PDB ID 2QRY) (36) was used as the template in structural modeling. The TPP ligand is shown in a Van der Waals representation. (b) A putative thiamine-binding site is displayed with predicted binding residues and the bound TPP (red).

10,000  $\times$  g for 10 min. The supernatants were collected and pretreated for HPLC analysis as previously reported (21). Quantification of thiamine and its derivatives was conducted on an Agilent 1260 Infinity series HPLC system (Agilent) equipped with a Zobex Eclipse Plus C<sub>18</sub> Rapid Resolution HT analytical column (3.0 by 50 mm; 1.8- $\mu$ m particle size) under the same detection conditions as previously documented (35). The calibration linear range of thiamine, TMP, and TPP was 10 to 500 nmol on the column. Three independent experiments were conducted, and the intracellular thiamine and its derivative concentrations were expressed in molecules per cell.

**Construction of a homology model of TP\_0144 and TDE\_0143.** Chain A from the crystal structure of *E. coli* TbpA (Protein Data Bank identification code [PDB ID] 2QRY) (36) together with TPP was used as the template for constructing both homology models in Modeller, version 9v7 (37). Sequence alignment among TP\_0144, TDE\_0143, and the template was conducted using Clustal X. The automodel module with the conjugate gradient optimization method was adopted to produce the final model with the best objective function value among the five resultant candidates. Thiamine-binding residues were selected at the relative positions comparable to those in 2QRY. All the diagrams were produced in VMD, version 1.8.7.

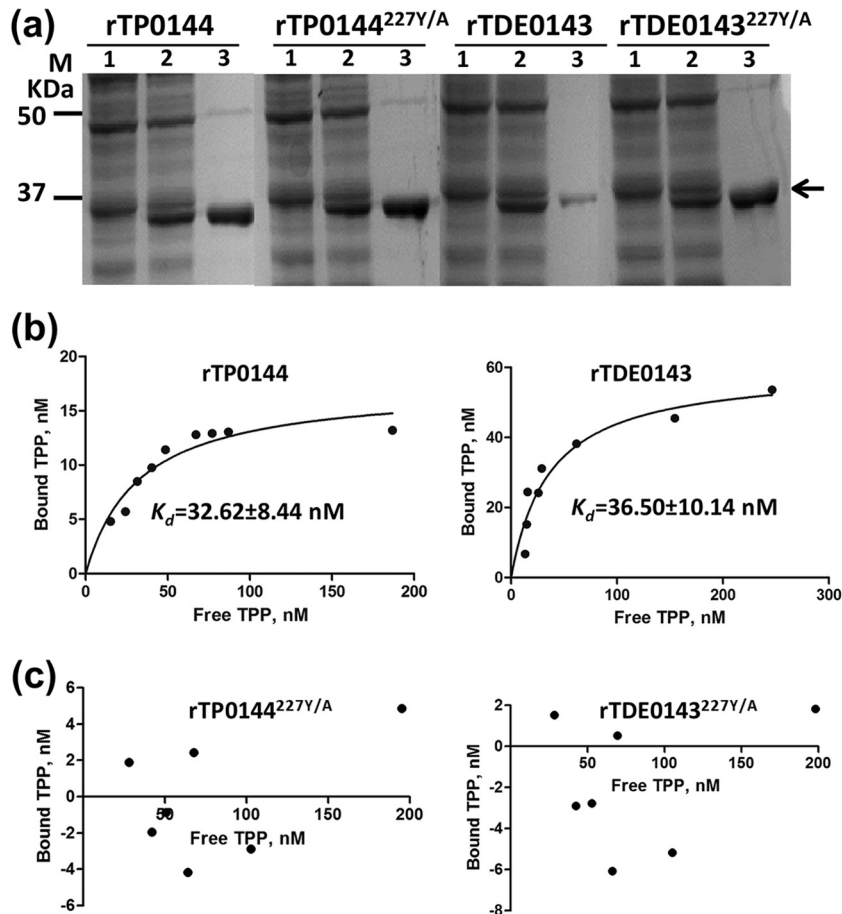
## RESULTS AND DISCUSSION

**TP\_0144 is a thiamine-binding protein.** TP\_0144 is annotated as a thiamine-binding periplasmic protein in the genome of *T. pallidum* (Nichols) (2). It consists of 335 amino acids (aa) with a predicted molecular mass of 36.8 kDa. The amino acid sequence of TP\_0144 was used as a query to search the Pfam database (38) and the Conserved Domain Database (CDD) (39). The results showed that TP\_0144 is a member of PRK11205, a family of thiamine transporter substrate-binding subunits. Within this family, TP\_0144 shares a high amino acid sequence similarity to TDE\_0143 (38% identity and 59% similarity). Structure homology modeling further revealed that TP\_0144 could possibly bear a structure similar to that of TDE\_0143 and TbpA of *E. coli* (Fig. 2a) and that TPP could conformably sit in the active site with well-conserved binding residues (Fig. 2b) (36), suggesting that TP\_0144 is a thiamine-binding protein.

To further ascertain that TDE\_0143 and TP\_0144 are thiamine-binding proteins, the binding affinities of purified

rTDE\_0143 and rTP\_0144 to TPP were measured. As shown in Fig. 3, these two recombinant proteins bound to TPP with similar affinities; the dissociation constant ( $K_d$ ) value of rTP\_0144 is  $32.62 \pm 8.44$  nM, and the  $K_d$  value of rTDE\_0143 is  $36.50 \pm 10.14$  nM. Previous mutational and structural studies indicate that tyrosine 215 (Y215) in the TPP-binding site of TbpA is essential for TPP binding activity (36). To confirm that the observed binding activity is specific, the corresponding tyrosine 227 (Y227) residues in the predicted TPP-binding sites of rTDE\_0143 and rTP\_0144 (Fig. 2b) were replaced with alanines, yielding two mutated recombinant proteins (rTDE\_0143<sup>227Y/A</sup> and rTP\_0144<sup>227Y/A</sup>). We found that the mutation completely abolished their ability to bind TPP (Fig. 3c). Collectively, these results indicate that TDE\_0143 and TP\_0144 are thiamine-binding proteins with similar binding affinities to TPP.

**Heterologous expression of TP\_0144 in *T. denticola*.** In our previous studies, we constructed a TDE\_0143 mutant (designated  $\Delta$ TDE\_0143) in which the entire gene was replaced in frame by an erythromycin resistance marker (*erm*). This mutant is defective in TPP transport and is unable to grow when exogenous TPP in the growth medium is limited (21). *T. pallidum* cannot be continuously cultivated *in vitro* and is not genetically manipulable (5). To study the biological role of TP\_0144 *in vivo*, we decided to complement the  $\Delta$ TDE\_0143 mutant using TP\_0144. If it plays a role similar to that of TDE\_0143, we anticipated that heterologous expression of TP\_0144 in the mutant would restore its wild-type phenotype. The strategy illustrated in Fig. 1a was developed to *cis* complement  $\Delta$ TDE\_0143 with TP\_0144. The suicide plasmid pTP\_0144C was specifically designed to replace the *erm* cassette in the mutant chromosome with *P<sub>tapI</sub>-aacC<sub>m</sub>-P<sub>tbpA</sub>-TP\_0144*. To complement the  $\Delta$ TDE\_0143 strain, purified unmethylated pTP\_0144C was transformed into the mutant by electroporation. Seven days after the transformation, more than 20 gentamicin-resistant colonies appeared on the plates. Six colonies were randomly selected and subjected to PCR using a pair of primers specific to the TP\_0144 gene. All six colonies were positive (data not shown), indicating that TP\_0144 was integrated into the chromosomes of these colonies. One colony (TP\_0144<sup>com</sup>, where com



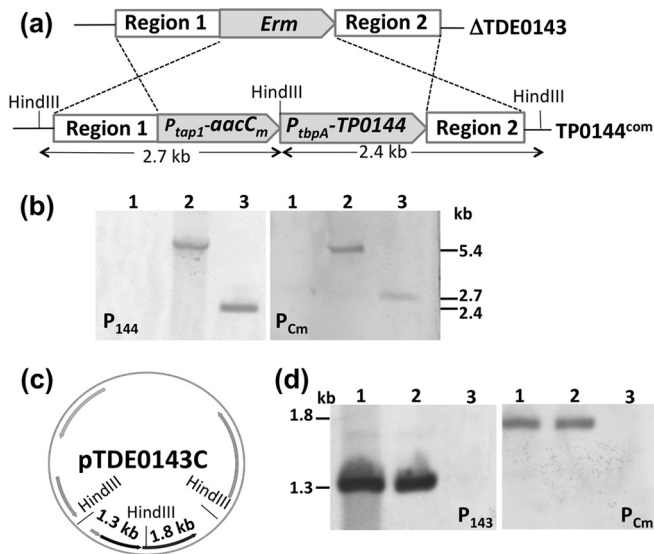
**FIG 3** Measuring the TPP-binding affinity of TP\_0144 and TDE\_0143. (a) SDS-PAGE analysis of purified recombinant TP\_0144 (rTP\_0144) and TDE\_0143 (rTDE\_0143) proteins and their mutated versions (rTP\_0144<sup>227Y/A</sup> and rTDE\_0143<sup>227Y/A</sup>). For each protein, lanes 1 and 2 represent uninduced and induced *E. coli* lysates, respectively; lane 3 for each protein represents the purified recombinant protein. (b and c) Plots of equilibrium binding of the recombinant proteins and TPP. Equilibrium dialysis was performed as previously described (21). The binding constants were determined with GraphPad Prism, version 5 (one site, specific binding).

indicates the complemented strain) was selected for further confirmation by Southern blotting. The chromosomal DNA of the TP\_0144<sup>com</sup> strain was extracted, digested with HindIII, and then hybridized with two DNA probes: one is a TP\_0144-specific probe ( $P_{144}$ ), and the other is an *aacC<sub>m</sub>*-specific probe ( $P_{Cm}$ ). If the *erm* cassette in TP\_0144<sup>com</sup> is replaced by *P<sub>tap1</sub>-aacC<sub>m</sub>-P<sub>tbpA</sub>-TP\_0144* as expected, a 2.4 kb-HindIII-digested DNA fragment should be detected by  $P_{144}$ , and a 2.7-kb DNA fragment should be detected by  $P_{Cm}$  (Fig. 4a). As shown in Fig. 4b, these two DNA fragments were detected in the TP\_0144<sup>com</sup> strain (lane 3) but not in its parental mutant strain (lane 1), indicating that the *P<sub>tap1</sub>-aacC<sub>m</sub>-P<sub>tbpA</sub>-TP\_0144* fragment had integrated to the original locus of TDE\_0143 in the chromosome of *T. denticola* as designed.

As a control, we also complemented the  $\Delta$ TDE\_0143 mutant with TDE\_0143 using the vector pTDE\_0143C shown in Fig. 1b. After the transformation, only two gentamicin-resistant colonies appeared on the plates. Restriction digestion analysis of the plasmids isolated from these two colonies revealed that they both harbored pTDE\_0143C. The plasmids isolated from one of these colonies (TDE\_0143<sup>com</sup>) were further digested with HindIII and then hybridized with a TDE\_0143-specific probe ( $P_{143}$ ) and  $P_{Cm}$ . As shown in Fig. 4d, both of these probes hybridized to the plasmids

(a 1.3-kb DNA fragment was detected by  $P_{143}$ , and a 1.8-kb fragment was detected by  $P_{Cm}$ ) isolated from the TDE\_0143<sup>com</sup> (lane 2) strain and to the pTDE\_0143C vectors isolated from *E. coli* (lane 1, positive control) but not to the shuttle vector pBFC (lane 3, negative control). These results indicate that pTDE\_0143C was successfully transformed into  $\Delta$ TDE\_0143.

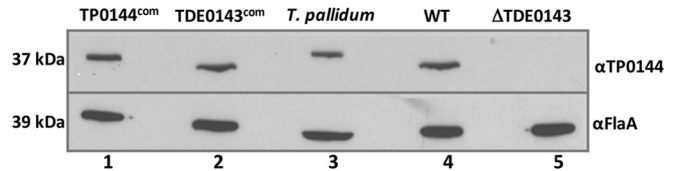
**TP\_0144 is expressed in the TP\_0144<sup>com</sup> strain.** Western blot analysis was carried out to determine if TP\_0144 is expressed in the TP\_0144<sup>com</sup> strain. We first raised an antiserum against TP\_0144 (anti-TP\_0144) in mice and then carried out the Western blot analysis. As shown in Fig. 5, a positive band near 37 kDa was detected in the whole-cell lysates of the TP\_0144<sup>com</sup> strain (lane 1) and *T. pallidum* (lane 3), indicating that TP\_0144 was successfully expressed in the complemented strain. Western blotting also detected a positive band (approximate 36 kDa) in the whole-cell lysates of the TDE\_0143<sup>com</sup> (lane 2) and *T. denticola* wild-type (lane 4) strains but not in the lysate of the  $\Delta$ TDE\_0143 mutant (lane 5), indicating that TDE\_0143 was cross-reactive with anti-TP\_0144 and that the expression of TDE\_0143 was restored in the TDE\_0143<sup>com</sup> strain. We repeated this experiment using an antibody against TDE\_0143 (anti-TDE\_0143), and a similar pattern was observed (data not shown), suggesting that



**FIG 4** Southern blot analysis of TP\_0144<sup>com</sup> and TDE\_0143<sup>com</sup>. (a) The diagram shows how the *erm* cassette in the ΔTDE\_0143 strain is replaced by *P<sub>tap1</sub>-aacC<sub>m</sub>-P<sub>tbpA</sub>-TP\_0144* via allelic exchange recombination, generating the TP\_0144<sup>com</sup> strain. Predicted HindIII cut sites and the sizes of DNA fragments generated by HindIII near the locus of *TDE\_0143* are labeled. (b) Southern blot analysis. Purified chromosomal DNA and plasmids were treated with HindIII and then hybridized against two DNA probes: one is specific to TP\_0144 (*P<sub>144</sub>*), and the other is specific to *aacC<sub>m</sub>* (*P<sub>Cm</sub>*). Lane 1, ΔTDE\_0143 chromosomal DNA (negative control); lane 2, pTP\_0144C (positive control); lane 3, TP\_0144<sup>com</sup> chromosomal DNA. The numbers on the right represent the sizes of detected DNA fragments. (c) Physical map of pTDE\_0143C. Predicted HindIII cut sites and the sizes of HindIII-digested DNA fragments are shown on the map. Additional details are depicted in Fig. 1b. (d) Southern blot analysis. The isolated plasmid DNAs were digested with HindIII and then hybridized against *P<sub>Cm</sub>* and a *TDE\_0143*-specific probe (*P<sub>143</sub>*). Lanes 1, pTDE\_0143C isolated from *E. coli* (positive control); lanes 2, the plasmids isolated from TDE\_0143<sup>com</sup>; lanes 3, pBFC (negative control). The numbers on the left represent the sizes of detected DNA fragments.

there is an antigenic cross-reaction between TP\_0144 and TDE\_0143. This result is consistent with the high sequence similarity between these two proteins.

**Heterologous expression of TP\_0144 in ΔTDE\_0143 restores its growth and TPP uptake.** Our previous studies demonstrated that inactivation of *TDE\_0143* impaired TPP uptake, and the mutant failed to grow when exogenous TPP was limited in the growth medium (21). These results indicate that *T. denticola* primarily depends on its thiamine-ABC transporter to acquire TPP for its survival and growth. To determine if TP\_0144 plays a role similar to that of TDE\_0143 *in vivo*, we cultivated the TP\_0144<sup>com</sup> and TDE\_0143<sup>com</sup> strains in TA medium containing defined amounts of TPP (100 nM, 500 nM, and 10 μM) or thiamine (200 nM, 500 nM, and 10 μM). Similar to our previous report, the ΔTDE\_0143 strain could grow only when a high concentration of TPP or thiamine (i.e., 10 μM) was supplemented in the medium (Fig. 6c and f). Under the low concentrations of TPP (100 nM and 500 nM) or thiamine (200 nM and 500 nM), the mutant completely stopped growing (Fig. 6a, b, d, and e). In contrast, the TP\_0144<sup>com</sup> and TDE\_0143<sup>com</sup> strains partially restored growth at a concentration of 100 nM TPP or 200 nM thiamine (Fig. 6a and d); they restored growth almost to the same rate as that of the wild type at a concentration of 500 nM TPP or thiamine (Fig. 6b and e). These results indicate that heterologous expression of TP\_0144 in



**FIG 5** Detection of TP\_0144 and TDE\_0143 by Western blotting. The blots were probed with an antibody against *T. pallidum* TP\_0144 (αTP\_0144) or an antibody against *T. denticola* flagellar filament sheath protein FlaA (αFlaA). FlaA was used as a loading control. WT, wild type.

the mutant could compensate for the absence of TDE\_0143, demonstrating that TP\_0144 plays a role similar to that of TDE\_0143 *in vivo*.

The *T. denticola* wild-type and complemented strains but not the ΔTDE\_0143 mutant could grow in TA medium supplemented with a limited amount of thiamine (Fig. 6d and e), suggesting that TDE\_0143 and TP\_0144 are also involved in thiamine uptake. As mentioned earlier, TPP is the active form of thiamine. Besides the *de novo* biosynthesis pathways, some bacterial species can take up exogenous thiamine and then convert it to TPP through different salvage pathways (27). In *E. coli*, thiamine is converted to TPP by a two-step reaction that is catalyzed by ThiK (thiamine kinase) and ThiL (thiamine phosphate kinase) (40). In *Bacillus subtilis*, YloS (also known as ThiN), a thiamine pyrophosphokinase, catalyzes the direct conversion of thiamine to TPP (41). Interestingly, genome mining revealed that while *T. denticola* contains a homolog of ThiK (TDE\_1260), *T. pallidum* encodes a homolog of YloS (TP\_0518), suggesting that they might convert thiamine to TPP by different mechanisms.

To further investigate whether the heterologous expression of TP\_0144 restores TPP uptake, the intracellular level of thiamine, TMP, and TPP in the TP\_0144<sup>com</sup> strain was measured by HPLC and compared to that of the wild-type and TDE\_0143<sup>com</sup> strains. Parallel to the measurement of growth curves (Fig. 6), the bacterial cells were harvested and subjected to HPLC analysis. As shown in Table 2, with the supplement of 10 μM TPP, the intracellular level of TPP in the TP\_0144<sup>com</sup> strain ( $18.89 \times 10^3 \pm 0.18 \times 10^3$  molecules/cell) was almost identical to that of the wild-type ( $19.91 \times 10^3 \pm 0.51 \times 10^3$  molecules/cell) and TDE\_0143<sup>com</sup> ( $20.44 \times 10^3 \pm 0.66 \times 10^3$  molecules/cell) strains. With supplements of 100 nM and 500 nM, the intracellular level of TPP in TP\_0144<sup>com</sup> ( $3.72 \times 10^3$  and  $10.57 \times 10^3$  molecules/cell, respectively) was slightly lower than that in the wild type ( $4.79 \times 10^3$  and  $14.08 \times 10^3$  molecules/cell, respectively), but the difference was not statistically significant. Of note, with the supplement of 100 nM, the intracellular level of TPP in the TDE\_0143<sup>com</sup> strain ( $8.11 \times 10^3 \pm 0.45 \times 10^3$  molecules/cell) was higher than that of the wild type ( $4.79 \times 10^3 \pm 0.56 \times 10^3$  molecules/cell). This is probably due to the *trans* complementation, which might elevate the expression level of TDE\_0143. This, in turn, could increase TPP uptake. The ΔTDE\_0143 strain was able to grow when 10 μM TPP was supplemented (Fig. 6c). However, its intracellular TPP level ( $14.23 \times 10^3 \pm 0.16 \times 10^3$  molecules/cell) was lower than the levels of the wild type and the complemented strains (Table 2), suggesting that the lack of TDE\_0143 compromised the function of the TPP-ABC transporter in TPP uptake. Taken together, these results indicate that heterologous expression of TP\_0144 in the ΔTDE\_0143 strain restores its growth and TPP uptake when exogenous TPP is

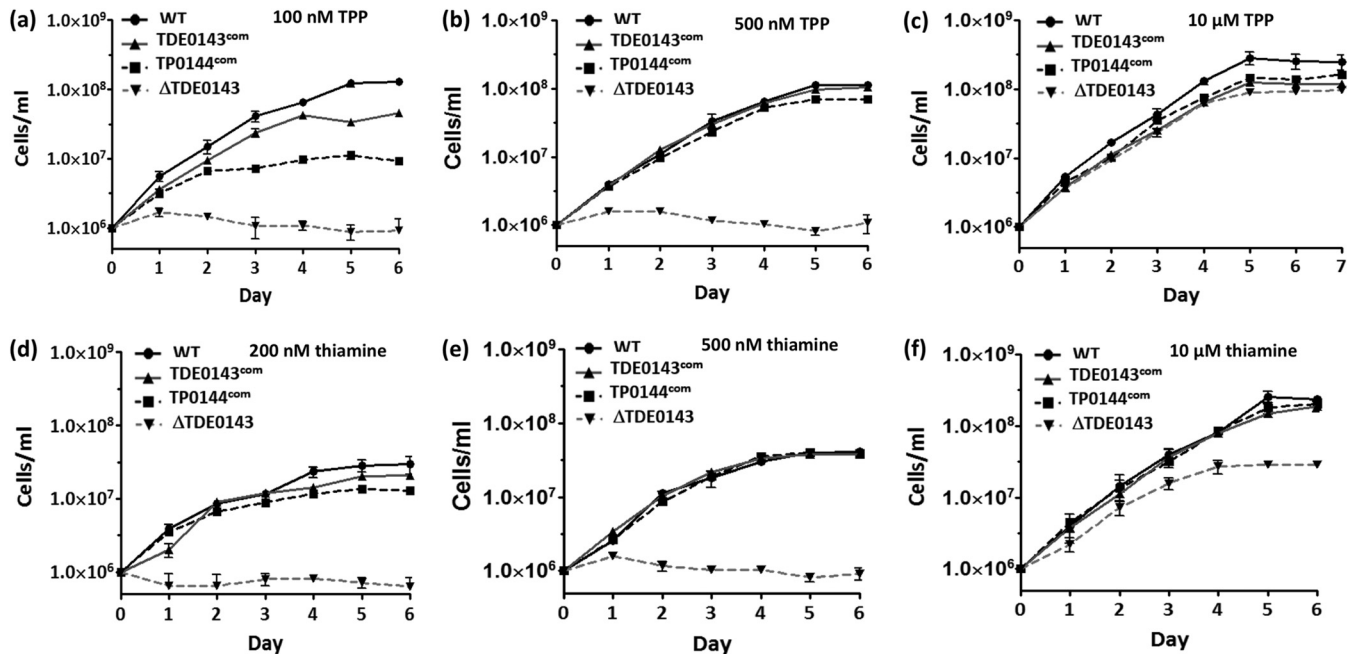


FIG 6 Measuring *T. denticola* growth in TA medium with a defined amount of TPP and thiamine. Four *T. denticola* strains were cultivated in TA medium supplemented with three different amounts of TPP (a, b, and c) or thiamine (d, e, and f). Cell counting was repeated in triplicate from at least three independent experiments, and the results shown here are representatives of one experiment. The results are expressed as mean cell numbers  $\pm$  standard deviations. WT, wild type.

limited, which is indicative of the important role of TP\_0144 in TPP acquisition of *T. pallidum*.

**Conclusion.** Although tremendous efforts have been undertaken during the last several decades, *T. pallidum* still cannot be continuously cultivated *in vitro* (5, 42, 43). This is primarily due to

a lack of understanding of its nutrient requirements and metabolism. Thiamine is a key nutrient that is required by all living organisms (25). However, little is known about its metabolism in *T. pallidum*. To our knowledge, this report is the first attempt to investigate thiamine metabolism of *T. pallidum*. Based on the genomic information of *T. pallidum* (2) and the evidence provided here, we conclude (i) that it lacks a *de novo* thiamine biosynthesis pathway, which means that *T. pallidum* must acquire this key nutrient from the host, and (ii) that it most likely depends on its thiamine-ABC transporter (TP\_0144-TP\_0143-TP\_0142) to import exogenous thiamine. The studies from this report also tell us that thiamine, as an essential nutrient, should be supplemented in the growth medium in future attempts to develop *in vitro* cultivation methods for *T. pallidum*.

It has long been of high interest to use cultivable *Treponema* species as a vehicle to express heterologous genes of *T. pallidum* and then study their biological and/or pathogenic functions (1, 15, 44). To date, *T. denticola* ATCC 33520 and *Treponema phagedenis* biotype Kazan strains have been used (15, 44). However, the genome information of these two strains remains unknown, which makes it more difficult to conduct genetic operations and interpret the functions of heterologous genes. Moreover, in the process of infection, a given virulence factor often acts in concert with other virulence determinants (45). *T. phagedenis* is nonpathogenic, which limits its usefulness to study the virulence factors of *T. pallidum*. In contrast to ATCC 33520 and *T. phagedenis*, the genome of *T. denticola* ATCC 35405 has been sequenced (4). This strain is pathogenic, and its pathogenicity can be tested in animal models (46–48). Most importantly, more than two-thirds of *T. pallidum* genes can be found in the genome of ATCC 35405 (4), including the majority of known *T. pallidum* virulence factors (10, 16, 44, 49–53). These advantages make this strain an ideal model

TABLE 2 Detection of intracellular TPP and its analogs in *T. denticola* using HPLC

Treatment and strain <sup>b</sup>	Intracellular concn (10 <sup>3</sup> molecules/cell) <sup>a</sup>		
	TPP	TMP	Thiamine
100 nM TPP			
WT	4.79 $\pm$ 0.56	0.69 $\pm$ 0.09	2.76 $\pm$ 0.41
TP0_144 <sup>com</sup>	3.72 $\pm$ 0.82	0.14 $\pm$ 0.04	0.56 $\pm$ 0.12
TDE_0143 <sup>com</sup>	8.11 $\pm$ 0.45	0.32 $\pm$ 0.01	0.56 $\pm$ 0.04
$\Delta$ TDE_0143	ND	ND	ND
500 nM TPP			
WT	14.08 $\pm$ 0.13	0.50 $\pm$ 0.02	4.10 $\pm$ 0.23
TP0_144 <sup>com</sup>	10.57 $\pm$ 0.26	0.55 $\pm$ 0.58	1.30 $\pm$ 0.03
TDE_0143 <sup>com</sup>	15.17 $\pm$ 0.14	1.22 $\pm$ 0.03	0.86 $\pm$ 0.02
$\Delta$ TDE_0143	ND	ND	ND
10 $\mu$ M TPP			
WT	19.11 $\pm$ 0.51	1.35 $\pm$ 0.13	1.05 $\pm$ 0.11
TP0_144 <sup>com</sup>	18.89 $\pm$ 0.18	4.32 $\pm$ 0.14	1.30 $\pm$ 0.04
TDE_0143 <sup>com</sup>	20.44 $\pm$ 0.66	5.39 $\pm$ 0.50	1.42 $\pm$ 0.17
$\Delta$ TDE_0143	14.23 $\pm$ 0.16	4.73 $\pm$ 0.17	1.31 $\pm$ 0.01

<sup>a</sup> Parallel to the growth curve measurements shown in Fig. 6, *T. denticola* cells were harvested at the stationary growth phase, lysed by ultrasonication, pretreated, and subjected to HPLC analysis as previously described (21). Three independent assays were conducted. ND, not detected due to the mutant incapable of growth.

<sup>b</sup> Strains were grown in TA medium supplemented with the indicated concentration of TPP.

for *T. pallidum* research. However, this strain is notoriously challenging for genetic manipulation (20, 54). For example, it contains multiple DNA modification systems and does not accept the shuttle vectors developed for ATCC 33520 (19). Fortunately, the genetic tools that we recently developed allow us to genetically manipulate this strain (19, 20, 30, 54). In this report, by investigating the function of TP\_0144, we demonstrate that ATCC 35405 can be a useful platform to study the biology and pathogenicity of *T. pallidum* and probably other uncultivable treponemal species as well.

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