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Identification of the Gene Encoding the FhbB Protein of *Treponema denticola*, a Highly Unique Factor H-Like Protein 1 Binding Protein[▽]

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The gene encoding the *Treponema denticola* factor H-like protein 1 (FHL-1) binding protein, FhbB, was recovered and characterized. Sequence conservation, expression, and properties of FhbB were analyzed. The identification of FhbB represents an important step in understanding the contribution of FHL-1 binding in *T. denticola* pathogenesis and in development of periodontal disease.

Treponema denticola is an important contributor to the development of acute and chronic periodontal disease in humans (44, 48). Periodontal disease has been linked to systemic disease, including heart disease (21), low birth weight (34), and esophageal cancers (33). Periodontal disease affects nearly all individuals at some point in their lives. This disease results from the synergistic action of a polymicrobial population of endogenous bacteria in association with several host-determined factors. The association of spirochetes with periodontal disease has been firmly established (45). High numbers of *T. denticola* cells have been found in periodontal lesions and at the leading front of periodontitis-associated subgingival plaque (41).

T. denticola, a member of the "red microbial complex" (45), binds the complement regulatory protein, factor H-like protein 1 (FHL-1) (25). The molecular mass of the FHL-1 binding protein produced by *T. denticola* is ~12 kDa, and this protein has been tentatively designated FhbB (FHL-1 binding protein B). This protein is unique in that it binds FHL-1 but not the closely related factor H protein (FH) (25). FHL-1, which is derived from the FH mRNA via alternative splicing, consists of the N-terminal domain of FH (11, 50). Both FH and FHL-1, as well as other members of the FH protein family, have similar structural organizations in that they are comprised of a series 50- to 60-amino-acid repeat units called short consensus repeats (SCRs). FHL-1 is comprised of the first seven SCRs of FH, but in addition it has four unique C-terminal residues as a result of alternate splicing of the FH mRNA. In mammals, FH and FHL-1 contribute to regulation of the alternative complement pathway by serving as cofactors for factor I-mediated cleavage of C3b (39, 40). They also regulate complement by inhibiting the initial formation and accelerating the dissociation of the alternative pathway C3 convertase. While the importance of FH and FHL-1 binding by microbial pathogens as an immune evasion mechanism has been clearly demonstrated (for a review, see reference 20), some pathogens may also exploit the interaction as a way to facilitate adherence and intracellular localization (35). The different functional activities associated with these otherwise very similar proteins most likely result from the different ways that they fold and present individual SCR domains on their surfaces. Previously, we demonstrated that *T. denticola* cleaves C3b through a predominantly FHL-1-independent mechanism (25). This observation suggests that FHL-1 binding may contribute to other aspects of *T. denticola* pathogenesis, such as adherence to the extracellular matrix (ECM) or to anchorage-dependent cell types that present FHL-1. The interaction of *T. denticola* with cell- or ECM-anchored FHL-1 could promote biofilm formation, plaque development, and the progression of periodontal disease.

To allow future analysis of FhbB and the contribution of FHL-1 binding to *T. denticola* pathogenesis, the first goal of this study was to identify the gene that encodes FhbB. To do this, a proteomics-based approach was used. Since most spirochetal FH/FHL-1 binding proteins are lipoproteins (2, 15, 18), we focused on lipoprotein-encoding genes, of which there are more than 160 in strain 35405 (42, 43). In view of the fact that FH/FHL-1 binding proteins lack conserved primary sequence elements or an identifiable functional domain, we focused on the genes annotated as having unknown functions ($n = 63$). Of these 63 genes, 9 were predicted to encode proteins having molecular masses in a broad range (8 to 17 kDa) similar to the molecular mass of FhbB (~12 kDa). These nine open reading frames (ORFs) were then scanned for the presence of possible coiled-coil domains using the COILS program (22). Coiled coils have been demonstrated to be critical structural elements involved in FH/FHL-1 binding by several spirochetal proteins (16, 23, 27) and by the M protein of the group A streptococci (3). The predictive probability of coiled-coil formation was highest for tde0108 and tde1135 (Table 1). For one ORF (tde0851) no coiled-coil probability was predicted, and this ORF was not considered further. The remaining eight ORFs were the focus of additional screening analyses.

r-Protein was generated for each of the eight ORFs listed in Table 1 using the pET32 Ek-LIC cloning vector and methods that have been described previously (8). The primers used in

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TABLE 1. *T. denticola* ORFs analyzed in this study

ORF	Predicted protein molecular mass (kDa)	Highest coiled-coil formation probability[a]
tde0108	11.4	0.702
tde0429	8.5	0.235
tde0940	16.0	0.039
tde1135	13.5	0.295[b]
tde1190	16.6	0.142[c]
tde1191	16.3	0.101
tde1361	12.7	0.200[d]
tde2448	16.7	0.040[e]

[a] Unless indicated otherwise, probabilities were determined using a 14-amino-acid window without weighting of the a and d positions of the coiled-coil heptad repeat sequence (positions a to g).
[b] For a 28-amino-acid window, the probability was 0.852.
[c] For a 21-amino-acid window, the probability was 0.269.
[d] Probability with a and d positions weighted.
[e] For a 21-amino-acid window (a and d positions weighted), the probability was 0.085.

TABLE 2. Oligonucleotide primers

Primer	Oligonucleotide sequence[a]
TDE0108FLIC	GACGACGACAAGATTACTTTC AAAATGAATACTGCAC
TDE0108RLIC	GAGGAGAAGCCCGGTTTACTT TATCTTTTTGGGTAT
TDE1135FLIC	GACGACGACAAGATCTGCACA AGAAGCGGAATA
TDE1135RLIC	GAGGAGAAGCCCGGTTCAATC TTCTTTTGCTTTTCC
TDE2448FLIC	GACGACGACAAGATTAAGAGC CGCCGAATCGCCGAAC
TDE2448RLIC	GAGGAGAAGCCCGGTTTATTT CTTTTCGATTTTGCG
TDE0429FLIC	GACGACGACAAGATTAAAACA ACCGATACAAGTAAAA
TDE0429RLIC	GAGGAGAAGCCCGGTTTAGAT AGGCTTCAATATAAGC
TDE1191FLIC	GACGACGACAAGATTTCTAAG ACAGCGATAAAGGC
TDE1191RLIC	GAGGAGAAGCCCGGTTTAGTA CTCTCCACTATTGAGC
TDE1190FLIC	GACGACGACAAGATTAAAACA AATGAGAAAAAAAATGCTC
TDE1191RLIC	GAGGAGAAGCCCGGTCTAATA TTCCGTATGCTTAAAATC
TDE1361FLIC	GACGACGACAAGATTAAACAA TTTATTGCCGATATTG
TDE1361RLIC	GAGGAGAAGCCCGGTTTAAGC TCGTAGTCGGTACCATTG
TDE0940FLIC	GACGACGACAAGATCAAGACA AAGCAAATTCAGCC
TDE0940RLIC	GAGGAGAAGCCCGGTCTATAA TTCGATATTAAAAACATTC
FhbB RT F	ACGCGCTTGAGAATGAATTA
FhbB RT R	AATCTAATGCAAGGGCTTCAG
FlaA RT F	GCTCAGGTTGATGATCAGG
FlaA RT R	GCAATTGATTTGATAACGCCG
TDE0109R1	GCTCATCAGCTTGCAAAGGC
TDE0109R2	CGATATTCATGACGTTTACTAC
FhbB Up	CTCTTGACAGTACGTATAGTG
FhbB78R	GGGTTTTTTATCCACAATTTG

[a] Underlining indicates the tail sequences added to allow annealing into the pET32 Ek/LIC vector.

all PCRs, including those used for cloning, are shown in Table 2. Protein expression in isopropyl-β-D-thiogalactopyranoside (IPTG)-induced *Escherichia coli* was demonstrated by screening an immunoblot with S-protein (Fig. 1). All immunoblot assays were conducted exactly as described previously (24). All expressed r-proteins were the predicted size and exhibited little or no degradation. The ability of the proteins to bind FHL-1 was assessed using the affinity ligand binding immunoblot (ALBI) assay (32). The r-protein derived from ORF tde0108 displayed strong FHL-1 binding, while no binding was detected to other r-proteins (Fig. 1). As a control, an identical blot was screened using the ALBI assay except that no FH/FHL-1 was added; as expected, no signal was observed. From these analyses we concluded that tde0108 encodes the FhbB protein previously described by McDowell et al. (25).

Analysis of the *fhbB* gene sequence revealed that it is 309 bp long and encodes a putative lipoprotein with a predicted molecular mass of 11.4 kDa and a pI of 10.6. The gene has a strong ribosomal binding site (AAGGA) and is followed 43 bp downstream by a rho-independent transcriptional terminator with the sequence 5′-CCATCGGAAGATTCCGTCCTCCGA TGG-3′. At the protein level, FhbB lacks potential transmembrane-spanning helices and is predicted to be presented on the surface of the cell, anchored by a lipid moiety (lipidation signal peptide motif, MKNKKIFTVLFLLAVSALLFTSC) (42). FHL-1 binding to the surface of *T. denticola* cultivated in vitro has been demonstrated previously (25).

FhbB differs from other FH/FHL-1 binding proteins in terms of its binding specificity (it binds only FHL-1) and predicted structure. It has a single coiled coil, whereas other FH/FHL-1 binding proteins of spirochetes contain two or more coiled coils (16, 23, 27). Multiple coiled coils within a protein could mediate intramolecular interactions that define or present the FH/FHL-1 binding pocket. The occurrence of only a single coiled coil in FhbB raises the possibility that this domain is involved in an intermolecular interaction that is necessary for FH/FHL-1 binding. The interaction could be a direct interaction with FHL-1 or could facilitate FhbB dimer formation which allows for presentation of the FHL-1 binding pocket. It is important to note that coiled-coil interactions are very stable and are resistant to heat and sodium dodecyl sulfate (17, 46). This could explain why the FhbB protein retains FHL-1 binding activity even after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting.

To determine if *fhbB* is present in other *T. denticola* strains, PCR analyses were conducted. The *fhbB* gene was successfully amplified from *T. denticola* strains 35405, 33520, and GM-1 (Fig. 2A). Sequence analyses of the amplicons revealed that the gene is highly conserved, suggesting that it has an important functional role in *T. denticola* biology. The *fhbB* genes from the 35405 and 33520 strains had identical sequences, while GM-1 *fhbB* differed at one nucleotide position (G-to-A transition), which results in a His-to-Arg change at position 95. To determine if *fhbB* has the same orientation relative to its flanking sequence in other strains, PCR analyses were performed. The binding site for each primer tested is shown in Fig. 2B. Amplicons that were the same size were obtained from all three strains tested, indicating that the gene orientation is conserved. *fhbB* is located between tde0107, which encodes an alpha-amylase family protein, and tde0109, which encodes the

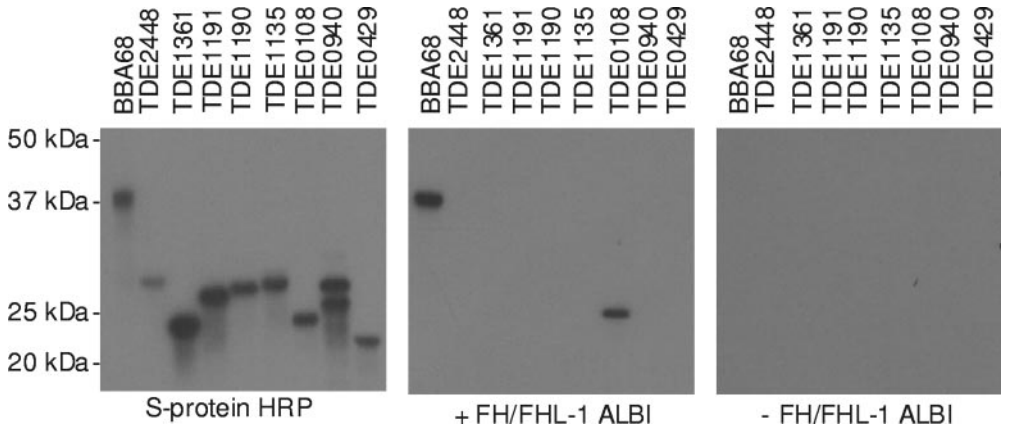

FIG. 1. Identification of the *T. denticola* strain 35405 ORF that encodes the FHL-1 binding protein, FhbB. S-tagged fusion proteins were generated for potential FHL-1 binding proteins. Expression was verified using horseradish peroxidase (HRP)-conjugated S protein (left panel), and FHL-1 binding was assessed using the ALBI assay (center panel) (32). As a negative control one blot was screened with primary and secondary antibodies without FH/FHL-1 added (right panel). The positions of molecular mass markers are indicated on the left, and the ORF designations are indicated above the lanes. r-BBA68 protein, a *Borrelia burgdorferi* FH binding protein, served as the positive control.

alpha subunit of phenylalanyl tRNA synthetase. The localization of *fhbB* between genes that encode housekeeping functions suggests that *fhbB* is a gene that has a bacterial origin and is not a gene that was recently acquired by, or subject to, lateral transfer. This is in contrast to the FH binding OspE proteins of the Lyme disease spirochetes, which are carried by prophage (9, 49).

To verify that *fhbB* is transcribed by strains 35405, 33520, and GM-1 during anaerobic cultivation in NOS medium (ATCC medium 1357), spirochetes were cultivated at 37°C in an anaerobe jar for ~8 days, RNA was extracted, and real-time reverse transcription (RT)-PCR was performed. All methods used in these analyses have been described previously (49). Standard curves generated using cloned PCR amplicons allowed calculation of transcript numbers. *fhbB* was determined to be highly expressed, and the transcript levels ranged from 0.1% to 0.5% of the transcript levels of *flaA* (Fig. 3A). There was no significant difference in the level of *fhbB* expression between strains 35405, GM-1, and 33520. In a previous study it was demonstrated that the composition of the growth medium

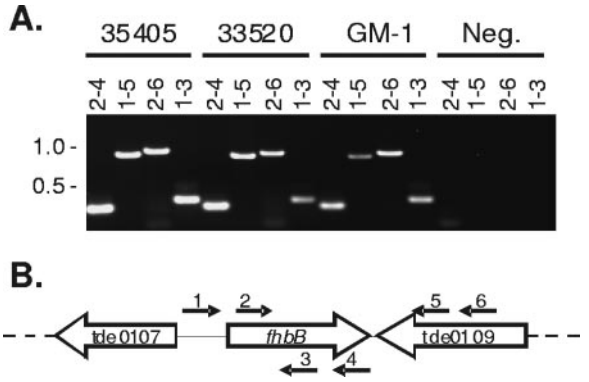

FIG. 2. PCR analyses of FhbB and its flanking regions in diverse *T. denticola* strains. Regions internal to or flanking *fhbB* were PCR amplified from *T. denticola* strains 35405, 33520, and GM-1. Control reactions were performed with no DNA template added (Neg.) (A). The primers used are indicated above the lanes. The primer numbers correspond to the following primers: primer 1, FhbB Up; primer 2, TDE0108FLIC; primer 3, FhbB78R; primer 4, TDE0108RLIC; primer 5, TDE0109R1; and primer 6, TDE0109R2. All primer sequences are shown in Table 2. The target sites for the primers are indicated in panel B.

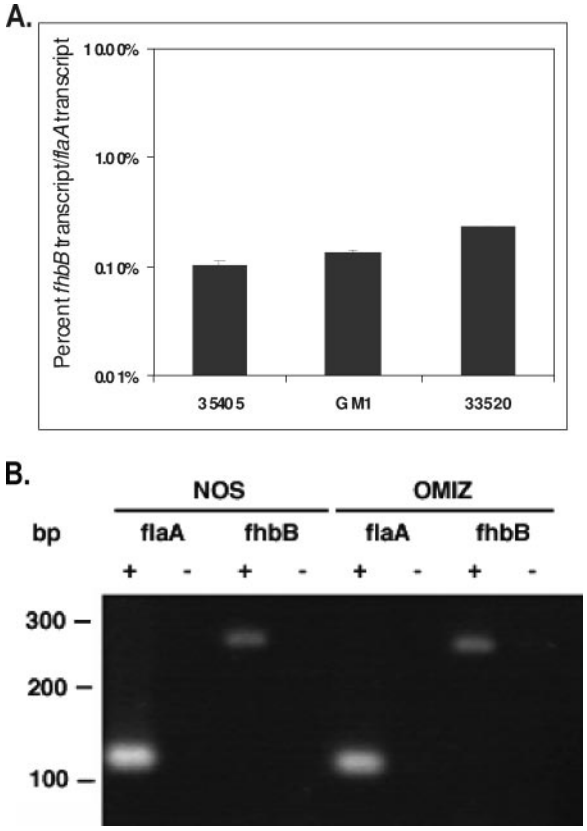

FIG. 3. Analysis of *fhbB* expression using RT-PCR. Real-time RT-PCR analyses were performed as described in the text. (A) Data for strains 35405, GM-1, and 33520. (B) RT-PCR performed to assess *fhbB* expression by strain 35405 grown in either NOS or OMIZ medium. The amplicons were analyzed by agarose gel electrophoresis in 2% MetaPhor agarose gels.

can influence protein expression profiles of *T. denticola* (38). To assess *fhbB* expression in the two most commonly used *T. denticola* growth media, RNA was extracted from strain 35405 grown in NOS or OMIZ medium (5), and RT-PCR was performed. Detection of the constitutively produced *flaA* transcript served as a positive control, and reactions in which RT was omitted served as a negative control. Expression of *fhbB* was observed in spirochetes cultivated in both media (Fig. 3B). The constitutive expression of *fhbB* suggests that FhbB has an important role in *T. denticola* biology.

Identification of the gene encoding FhbB is an important step that will facilitate future analyses of the role of FHL-1 binding in *T. denticola* pathogenesis. The importance of FH and/or FHL-1 binding as a microbial virulence mechanism is becoming increasingly apparent. Numerous viruses, parasites, and bacteria, including several spirochetes, exploit FH and/or FHL-1 binding as a means of facilitating C3b cleavage and hence immune evasion (1, 6, 7, 10, 12–14, 16, 19, 25, 26, 28–32, 35–37). However, we previously demonstrated that while *T. denticola* cleaves C3b, this activity is not dependent on FHL-1 binding (25). C3b cleavage by *T. denticola* appears to be due to dentilisin, a chymotrypsin-like protease which is one of several identified proteases produced by *T. denticola* (47). FHL-1 binding may instead be more important in adherence and tissue invasion, as has been demonstrated for some streptococci (35) and *Actinobacillus* (4). We previously demonstrated that *T. denticola* binds to SCR7 of FHL-1. Our hypothesis is that *T. denticola* binds primarily to cell- or ECM-anchored FHL-1, an interaction mediated by the RGD motif contained in SCR4, via FhbB. The outcome of this interaction may facilitate biofilm and plaque formation and thus development and progression of periodontal disease. Future analyses will seek to test this hypothesis.

The GenBank accession numbers for sequences determined for this study are EF032155 and EF032156.

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