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Sequence divergence in the *Treponema denticola* FhbB protein and its impact on factor H binding

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SUMMARY

Treponema denticola is an anaerobic spirochete whose abundance in the subgingival crevice correlates with the development and severity of periodontal disease. *T. denticola*'s ability to survive and thrive in the hostile environment of the periodontal pocket is due, at least in part, to its ability to bind factor H (FH), a negative regulator of the alternative complement pathway. The FH binding protein of *T. denticola* has been identified as FhbB and its atomic structure has been determined. The interaction of FH with *T. denticola* is unique in that FH bound to the cell surface is cleaved by the *T. denticola* protease, dentilisin. It has been postulated that FH cleavage by *T. denticola* leads to immune dysregulation in periodontal pockets. In this study, we conduct a comparative assessment of the sequence, properties, structure, and ligand binding kinetics of the FhbB proteins of strains 33521 and 35405. The biological outcome of the interaction of these strains with FH could differ significantly as 33521 lacks dentilisin activity. The data presented here offer insight into our understanding of the interactions of *T. denticola* with the host and its potential to influence disease progression.

Keywords

periodontitis; complement; factor H; immune evasion

INTRODUCTION

Periodontal disease is among the most common infections of adults. In the United States alone, approximately 116 million adults have some form of periodontal disease (Darveau, 2010, Beck, 1987). Periodontitis begins with bleeding and inflammation of the gums (gingivitis) and can progress to gingival degradation, alveolar bone loss and endentulism (tooth loss). As disease develops there is a significant transition in the overall composition of the oral microflora with *T. denticola* becoming a dominant species (Socransky et al., 1998, Ellen and Galimanas, 2005). *T. denticola* is a well characterized anaerobic spirochete

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that has been clearly linked to periodontal disease severity (Simonson et al., 1988, Loesche, 1988). Recent evidence suggests that expansion of the *T. denticola* population in the subgingival crevice leads to immune dysregulation and thereby creates conditions that exacerbate disease progression (McDowell et al., 2011, Miller et al., 2012).

The subgingiva are bathed in crevicular fluid, a serum and cell exudate rich in immune effectors including active complement (Boackle, 1991, Boackle et al., 1978, Schenkein and Genco, 1977, Schenkein, 1991). Consistent with its ability to thrive in periodontal pockets, *T. denticola* strain 35405 is resistant to human serum concentrations as high as 25% (McDowell et al., 2011). *T. denticola*'s ability to resist serum-mediated killing is due to the binding of factor H (FH) (McDowell et al., 2005), a negative regulator of the alternative complement pathway (Ruddy and Austen, 1969, Ruddy and Austen, 1971). FH is a 155 kDa glycoprotein that is composed of 20 imperfect repeat domains termed complement control protein (CCP) domains (Ferreira et al., 2010). In the healthy host, FH prevents complement activation by: 1) serving as a cofactor for the factor I-mediated cleavage of C3b (a critical opsonin), 2) blocking C3 convertase complex formation and 3) accelerating decay of preformed C3 convertase complex (Zipfel et al., 2002, Zipfel and Skerka, 2009). FH also contributes to regulation of complement activation by interacting with monomeric C-reactive protein (mCRP) (Jarva et al., 1999). The FH-mCRP complex enhances inactivation of C3b (Mihlan et al., 2009). In addition, FH-mCRP plays an important role in regulating inflammatory responses by inhibiting the production of pro-inflammatory cytokines (Mihlan et al., 2009). Several pathogens exploit the negative regulatory activity of FH. FH bound to the surface of pathogens functions to downregulate complement activation locally at the cell surface (reviewed in (Zipfel et al., 2007)).

FH binding proteins produced by different bacterial species represent a diverse group with little or no sequence or structural homology. The FH binding protein of *T. denticola* is an 11.4 kDa protein designated as FhbB (McDowell et al., 2005, McDowell et al., 2007). The atomic structure of FhbB has been solved at 1.7 Å resolution (Miller et al., 2012, Miller et al., 2011). The FhbB structure is unique consisting of an α fold with well defined negatively and positively charged faces. Mutagenesis studies demonstrate that FH interacts with the negatively charged face of FhbB (Miller et al., 2012). A unique aspect of the *T. denticola*-FH interaction is that bound FH is cleaved by dentilisin (McDowell et al., 2009). Dentilisin is a multi-subunit protease that is thought to mediate several aspects of *T. denticola* pathogenesis (Fenno and McBride, 1998, Fenno, 2012). It has been hypothesized that FhbB-dentilisin mediated cleavage of FH may deplete local FH concentrations resulting in immune dysregulation, thereby creating conditions that favor the progression of periodontal disease (Miller et al., 2012).

To date, studies that have sought to define the molecular basis of the interaction of FH with FhbB have been focused exclusively on the *T. denticola* type strain, 35405. Earlier sequence analyses of *fhbB* from strains 35405, GM1 and 33520 suggested that FhbB is highly conserved (McDowell et al., 2007). In this study we characterize a divergent FhbB ortholog derived from *T. denticola* 33521 (FhbB33521) and utilize this natural FhbB variant to further assess the molecular basis of the FH-FhbB interaction. The analyses presented here provide insight as to how different strains of *T. denticola* may influence the development and progression of periodontal disease.

METHODS

Bacterial strains, DNA sequencing and generation of recombinant (r-) proteins

T. denticola strains 35405 and 33521 were grown in new oral spirochete (NOS) media under anaerobic conditions (McDowell et al., 2011). Cells were harvested by centrifugation and

genomic DNA was isolated as previously described (McDowell et al., 2007). PCR was performed using standard conditions with primers designed to amplify the full length *fhbB* gene including upstream and downstream flanking sequences. All primer sequences are provided in Table 1. The resulting amplicons were TA cloned and sequenced on a fee for service basis (Genewiz). To allow for production of r-FhbB, additional primers were designed to amplify the coding sequence (minus the sequence encoding the 23 amino acid leader peptide). Sequences that allow for ligase independent cloning (indicated by underlining in Table 1) with the pET32 Ek/LIC or pET46 Ek/LIC vectors (Novagen) were included in each primer. Proteins expressed using the pET32 Ek/LIC vector possess a 17 kDa N-terminal fusion containing 6x-His and S-tags. Proteins produced using pET46 Ek/LIC harbor a 3 kDa N-terminal fusion that contains a 6x-His tag. All cloning procedures were conducted as previously described (Miller et al., 2012). Recombinant FH proteins consisting of different CCP domains were produced with N-terminal 6x-His tags as previously described (Miller et al., 2012). Specifically, r-proteins spanning CCP1-4, CCP5-7, CCP6-8, CCP8-13, CCP14-18 and CCP19-20 were generated. R-FhbA, a FH binding protein derived from *Borrelia hermsii*, was generated in an earlier study (Hovis et al., 2008) and used as a control.

Generation of antisera

Anti-FhbB₃₅₄₀₅ and anti-FhbB₃₃₅₂₁ antisera were generated in C3H-HeJ mice (3 per antigen) as previously described (McDowell et al., 2011). In brief, 20 µg of r-protein in Alum (with two boosts) was administered to each mouse (n=3) (Earnhart et al., 2007). Anti-FhbB₃₅₄₀₅ and anti-FhbB₃₃₅₂₁ antisera were also generated in female Sprague-Dawley rats (2 per antigen). The rats were anesthetized using a cocktail containing ketamine/xylazine/acepromazine and then injected intraperitoneally with 40 µg of r-protein diluted in sterile phosphate buffered saline (PBS) and mixed 1:1 with complete Freund's adjuvant (total volume 0.5 mLs per rat) (Sigma-Aldrich). Three and 5 weeks after the primary immunization, animals were boosted with 40 µg of the same antigen diluted in PBS and mixed with incomplete Freund's adjuvant (Sigma-Aldrich). Two weeks following the final boost, animals were exsanguinated by cardiac puncture under anesthesia and serum harvested by centrifugation.

All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals (Eighth Edition) and in accordance with protocols peer-reviewed and approved by Virginia Commonwealth University and the University of Connecticut Health Center Institutional Animal Care and Use Committees.

Computer assisted structural modeling

A structural model of FhbB₃₃₅₂₁ was created using the known structure of FhbB₃₅₄₀₅ (pdb code 3R15) (Miller et al., 2012, Miller et al., 2011) and Modeler 9v1 (Sali, 1995, Sali et al., 1995). Briefly, ClustalW2 (Larkin et al., 2007) was used to align the FhbB₃₅₄₀₅ and FhbB₃₃₅₂₁ sequences (residues 24-101). Models were generated, compared to the FhbB₃₅₄₀₅ structure and evaluated by MolProbity to assess all-atom contacts and geometry (version 3.15) (Davis et al., 2007).

FH affinity ligand binding immunoblots, western blots and ELISA assays

FH affinity ligand binding immunoblot assays were performed as previously described (Miller et al., 2012). In brief, r-FhbB proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 15% Criterion gels (Bio-Rad) and transferred to Immobilon-P membranes by electroblotting. The membranes were overlaid with purified human FH (10 µg mL⁻¹; Complement Technologies) or the CCP constructs. Binding was detected using goat anti-FH antiserum (1:1000) (Complement Tech) with rabbit

anti-goat IgG serving as the secondary antiserum (1:20,000). Signal was detected by chemiluminescence using the SuperSignal ECL substrate (Thermo Scientific).

Western blot analyses were performed using standard, previously described methods (McDowell et al., 2011). Mouse anti-FhbB and rat anti-FhbB antisera were used at dilutions of 1:1000 and 1:5000, respectively. Secondary antibodies were used at a dilution of 1:40,000. Signal was detected by chemiluminescence using the SuperSignal ECL substrate (Thermo Scientific). ELISA assays were conducted as previously described (Miller et al., 2012). In brief, r-FhbB proteins, r-FhbA (positive control) and BSA (negative control) were immobilized in the wells of 96 well microtiter plates (in triplicate; 1 µg per well in 100 mM NaHCO₃, pH 9.6, overnight; 4°C). Non-specific binding was blocked with phosphate buffered saline with 0.2% Tween-20 (PBST) with 5% non-fat dry milk (PBSTM) (1 hour, room temperature). CCP constructs and full length FH (10 µg ml⁻¹ in PBST) were added to the wells (in triplicate) for 1 hr followed by 3 washes with PBST. Goat anti-human FH (1:800 in PBSTM; Complement Tech) was added to each well (1hr, room temperature). The plates were washed (3x) and then rabbit anti-goat IgG (1:20,000 dilution in PBSTM; Calbiochem) was added (1 hr). The plates were again washed (3x) and then antibody binding was detected using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 405 nm.

Indirect-immunofluorescence assays (IFAs)

IFAs were conducted as previously described (McDowell et al., 2005). In brief, duplicate slides were generated for each strain and air dried. One set of slides was treated with acetone to permeabilize the cells (i.e., fixed) while the second set was not treated (unfixed). Mouse anti-FhbB₃₅₄₀₅, mouse anti-FhbB₃₃₅₂₁ or goat anti-FlaA antiserum (1:500 dilution) was then added to the slides and antibody binding detected using Alexa 568-conjugated goat anti-mouse IgG antibody or Alexa 488-conjugated goat anti-rabbit IgG antibody (1:1000 in PBST, 3% BSA). Cells were also visualized by dark-field microscopy. Images were captured with a DP71 camera (Olympus).

Triton X-114 extraction and phase partitioning

Cells were recovered by centrifugation (0.5 OD₆₀₀), extracted with 1% Triton X-114 and phase-partitioned (three times) as previously described (Roberts et al., 2000). The aqueous phase was concentrated 5-fold with Amicon Ultra 3,000 Da cut-off filters (Millipore). The detergent phase was concentrated by precipitation with 5 volumes of ice-cold acetone followed by centrifugation (15,000x g, 30 min, 4°C). The samples were solubilized with SDS-PAGE loading buffer, analyzed by SDS-PAGE, transferred to Immobilon-P membranes by electroblotting and screened with rat anti-FhbB₃₅₄₀₅, rat anti-FhbB₃₃₅₂₁ and rabbit anti-FlaA antisera using standard procedures. Antibody binding was detected using the appropriate secondary antibody and chemiluminescence.

Surface plasmon resonance (SPR)

FhbB-FH binding kinetic analyses were performed using a Biacore T100 and the data evaluated using BiaEvaluation V 1.1 (Biacore, Uppsala, Sweden) as previously detailed (Miller et al., 2012). In brief, r-FhbB proteins (ligand) were immobilized to an nitrilotriacetic acid (NTA) chip (GE Healthcare) via their N-terminal His-tag and increasing concentrations of purified FH (analyte; Complement Tech) were provided in the fluid phase. The kinetics data were fit to a Langmuir 1:1 binding model and averaged from at least two replicate experiments.

Site-directed mutagenesis

Site-directed mutagenesis was performed as previously described using a mutagenic PCR based approach with primers that allow for ligase independent cloning into the pET32 Ek/LIC and pET46 Ek/LIC vectors (Miller et al., 2012). All methods associated with the generation, production and purification of r-proteins were as previously described (Miller et al., 2012). FH binding to each r-protein was assessed using assays detailed above.

Measurement of dentilisin activity

The dentilisin activity of each strain was measured by monitoring hydrolysis of succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyne-p-nitroanilide (SAAPFNA) (Sigma-Aldrich) (Uitto et al., 1988, McDowell, 2011 #2471). In brief, aliquots of fresh, mid-log phase cultures were placed in buffer A (100 mM NaCl, 1 mM CaCl₂, 50 mM Tris pH 8.0) and transferred to the wells of a 96 well plate. SAAPFNA was added (1 mM in buffer A), the plates were incubated (30 min, 37°C) and SAAPFNA hydrolysis measured by monitoring absorbance at 405 nm. The dentilisin deficient strain, 35405-CCE (Bian et al., 2005), served as a control.

FH proteolysis assays

The ability of each strain to degrade purified FH was assessed as previously described (McDowell et al., 2011). In brief, 0.05 OD₆₀₀ of freshly harvested mid-log phase *T. denticola* cells were incubated in PBS with purified FH (80 µg ml⁻¹, 1 hr, 37°C). The samples were then solubilized, fractionated by SDS-PAGE, immunoblotted, and screened for FH proteolysis using anti-human FH antiserum (as described above).

Serum sensitivity assay

The sensitivity of strains 35405 and 33521 to killing by human serum was determined as previously described (McDowell et al., 2011). Cells from mid-log phase cultures were incubated with 25% complement-preserved normal human serum (NHS; Innovative Research) in NOS media (3 hr, 37°C, anaerobic conditions). As a control the strains were also incubated with 25% heat-inactivated NHS (HIS) (56°C, 30 min). In all cases the NOS media and human serum were equilibrated to anaerobic conditions prior to use. The percentage of morphologically intact cells (intact cells/total number of intact and morphologically disrupted cells) was determined by cell counting under dark field microscopy (3 fields; 400x magnification). Three biological replicates were performed and the results averaged.

RESULTS

Determination and analysis of the *T. denticola* strain 33521 *fhbB* sequence

Prior to this study, *fhbB* sequences from three different strains (35405, 33520, and GM-1) have been reported in the literature (McDowell et al., 2007). These sequences were found to be highly conserved with only a single nucleotide difference. Determination and analysis of the *fhbB*₃₃₅₂₁ sequence revealed 60.8% and 73.4% amino acid identity and similarity, respectively, with FhbB₃₅₄₀₅. An alignment of the sequences highlighting structural domains or motifs is presented in Fig. 1. Sequence differences are concentrated within the region analogous to the negatively charged 1 2 domain that forms the FH binding interface (Miller et al., 2012). The DNA sequences that flank the *fhbB* genes of strains 35405 and 33521, including the *fhbB* promoter, are highly conserved but harbor some polymorphisms. *fhbB*₃₃₅₂₁ possesses a 48 bp deletion downstream of the stop codon within the putative rho-independent terminator. It remains to be determined if this deletion influences the transcriptional expression of *fhbB* in strain 33521 (Data not shown).

FhbB₃₅₄₀₅ and FhbB₃₃₅₂₁ variants are antigenically distinct

To determine if FhbB₃₅₄₀₅ and FhbB₃₃₅₂₁ are antigenically distinct, antisera to both proteins were individually generated in rats and used to screen immunoblots of r-FhbB₃₅₄₀₅, r-FhbB₃₃₅₂₁ and r-FhbA. FhbA is a FH binding protein derived from the relapsing fever spirochete, *Borrelia hermsii* (Hovis et al., 2004), that served as a negative control (Fig. 2). The antisera proved to be variant specific with no cross-reactivity. Collectively, the data indicate that antibody responses are directed at variable amino acids within the putative FH interaction domain.

FhbB is an amphipathic surface exposed lipoprotein

Strains 35405 and 33521 were subjected to Triton X-114 extraction and phase partitioning. Immunoblot analysis of the resulting fractions using anti-FhbB₃₅₄₀₅, anti-FhbB₃₃₅₂₁ and anti-FlaA antiserum (control) revealed that FhbB partitions predominantly with the detergent phase indicating amphipathic properties consistent with a lipoprotein (Fig. 3). As expected, FlaA (a component of the inner membrane anchored-periplasmic flagella) partitioned with the insoluble phase.

FhbB surface localization was also assessed by IFA. Labeling of non-permeabilized cells with anti-FhbB antibody was observed for strains 35405 and 33521 but not 35405 *fhbB* (Fig. 4). Cell integrity was verified by screening slides with anti-FlaA antiserum. FlaA is a major component of the inner membrane anchored-periplasmic flagella. Only acetone permeabilized cells were labeled with the anti-FlaA antibody. Collectively these analyses demonstrate that FhbB is a surface exposed outer membrane protein with properties consistent with a lipoprotein.

FhbB₃₅₄₀₅ and FhbB₃₃₅₂₁ are similar in structure and surface charge distribution

The FhbB₃₃₅₂₁ sequence was threaded onto the previously determined high resolution (1.7 Å) atomic structure of FhbB₃₅₄₀₅ (Miller et al., 2012, Miller et al., 2011). Five models each with an average root mean-squared deviation (RMSD) of 0.17 Å were obtained. The models were then evaluated using MolProbity (Chen et al., 2010). The model depicted in Fig. 5 for FhbB₃₃₅₂₁ had the fewest all-atom clashes with 98% of residues in favored Ramachandran phi-psi angles. These analyses indicate a strong fit for the deduced structural model for FhbB₃₃₅₂₁ and suggest that in spite of significant sequence diversity, the overall structures of FhbB₃₃₅₂₁ and FhbB₃₅₄₀₅ are highly similar. The surface charge distribution for each protein was also compared. A stronger and more widely dispersed region of negative charge is predicted at the FH binding interface of FhbB₃₃₅₂₁.

Identification of the FhbB interaction site on FH

As noted above, sequence differences between FhbB₃₃₅₂₁ and FhbB₃₅₄₀₅ are concentrated within the FH binding interface. These differences could result in different molecular contact points with FH. FhbB₃₅₄₀₅ interacts with CCP7 of FH (McDowell et al., 2012, Miller et al., 2012). To determine if FhbB₃₃₅₂₁ interacts with the same FH domains as FhbB₃₅₄₀₅, ELISA based binding analyses were conducted using full length FH and r-constructs consisting of CCP1-4, CCP5-7, CCP6-8, CCP8-13, CCP14-18, and CCP19-20 (Fig. 6). FhbB₃₃₅₂₁ bound CCP5-7, CCP6-8 and full length FH but not to other CCPs, localizing the binding site within CCPs 6 and 7. BSA served as a negative control and as expected, it did not bind to FH or to any of the CCP constructs. The binding results obtained through ELISA assays was subsequently confirmed using affinity ligand binding immunoblot assays (data not shown). The integrity of the CCP fragments used in these binding assays was confirmed by SDS-PAGE and the ability of anti-FH antibody to recognize all of the CCP constructs was demonstrated by immunoblot analyses (data not

shown). In addition, the biological competence of the r-CCP1-4 fragment to serve as a co-factor in the factor I-mediated cleavage of C3b was verified using a C3b cleavage assay (data not shown).

FH binding kinetics

To compare the binding kinetics of FH with r-FhbB₃₃₅₂₁ and FhbB₃₅₄₀₅, surface plasmon resonance analyses were performed. The FhbB proteins were coupled to NTA-chips and purified FH was provided in the fluid phase. The K_D for the r-FhbB₃₃₅₂₁-FH interaction was determined to be $9.01 \pm 0.09 \times 10^{-7} \text{M}$ with a k_a of $3.41 \pm 0.72 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ and a k_d of $0.0307 \pm 0.0062 \text{s}^{-1}$ (Fig. 7). In contrast the K_D for the r-FhbB₃₅₄₀₅-FH interaction was determined to be $1.51 \pm 0.02 \times 10^{-6} \text{M}$ with a k_a of $7.56 \pm 0.26 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ and k_d of $0.11 \pm 0.01 \text{s}^{-1}$ (Miller et al., 2012). Hence, the FhbB₃₃₅₂₁-FH interaction is characterized by slower on- and off-rates and a stronger binding affinity (relative to the FhbB₃₅₄₀₅-FH interaction).

Site-directed mutagenesis of FhbB: identification of residues involved in the FhbB-FH interaction

Earlier site-directed mutagenesis analyses of FhbB₃₅₄₀₅ and FH identified residues in both proteins that are important molecular determinants in the binding interaction (McDowell et al., 2005, McDowell et al., 2009, Miller et al., 2012). To identify residues that play a role in the FH-FhbB₃₃₅₂₁ interaction, site-directed mutagenesis analyses were conducted. The following individual substitutions were introduced into FhbB₃₃₅₂₁: E43K, E43D, E43G, D45K, D45E, D45G, D45A, D48A, D58A, and D61A. The spatial location of each residue in the FhbB structure is indicated in Fig. 5. R-proteins harboring these individual substitutions were generated, purified and tested for FH binding using affinity ligand binding immunoblot assays (Fig. 8). Residue E43 was targeted because in FhbB₃₅₄₀₅ residues E43 and K88 form a salt-bridge between 1 and 3. These residues are conserved suggesting that the E43-K88 salt bridge is an important structural component in FH binding (McDowell et al., 2007, Miller et al., 2012). However, E43D, E43K, and E43G substitutions had little or no effect on FH binding demonstrating that the E43-K88 salt bridge is not required for FH binding. Residue D48 was targeted because it is a negatively charged residue located within the predicted FH-binding domain of FhbB. Substitution of D48 with A had no effect on FH binding. In an earlier study, other nearby negatively charged residues (E45, D58 and E62) were demonstrated to influence the FhbB₃₅₄₀₅-FH interaction (Miller et al., 2012). In FhbB₃₃₅₂₁, the identity of the residues at positions 45 (D) and 58 (E) differs from strain 35405 (E and D, respectively) but negative charge is retained. Substitution of these residues with Ala abolished FH binding, confirming the previously reported critical requirement to maintain negative charge at these positions (McDowell et al., 2007, Miller et al., 2012). The importance of D/E45 and D/E58 in binding is consistent with their spatial placement in FhbB. These residues sit on the surface accessible faces of 1 and 2. In FhbB₃₅₄₀₅, E62 was identified as a critical residue for FH binding (Miller et al., 2012). When substituted with Ala, FH binding was completely lost. Interestingly, while FhbB₃₃₅₂₁ has a Leu at position 62, position 61 is an Asp residue. We speculated that negative charge at residue 61 compensates for the absence of negative charge at residue 62. However, a D61A substitution mutant retained FH binding, indicating subtle differences in the residues required for FH binding between these FhbB orthologs. The data presented here provide additional insight into the molecular basis of the FhbB-FH interaction and support the conclusion that the 1 2 face of FhbB serves as the interaction platform for FH.

33521 lacks dentilisin activity and FH cleavage

There are conflicting reports regarding the dentilisin phenotype of strain 33521 (Wyss et al., 2004, Heuner et al., 2001, Goetting-Minesky et al., 2012). Wyss *et al.* and Goetting-Minesky

et al. determined strain 33521 to be negative for dentilisin activity while Heuner *et al.* reported it to be dentilisin positive. To independently assess dentilisin activity, SAAPFNA assays were conducted (Fig 9). Strain 35405 (dentilisin positive) and 35405-CCE (a dentilisin-deficient strain) (Bian *et al.*, 2005) served as positive and negative controls, respectively. An absorbance change of 0.71 ± 0.02 was measured for 35405-CCE, representing a baseline for this assay. Strain 35405, which has strong dentilisin activity, resulted in an absorbance change of 3.51 ± 0.07 . Strain 33521 had an absorbance change below baseline, 0.56 ± 0.02 , indicating that 33521 does not have dentilisin activity. Dentilisin has been demonstrated to cleave FH bound to the *T. denticola* surface (McDowell *et al.*, 2009). Hence we also used FH cleavage as a readout of potential dentilisin activity (Fig. 9). While the positive control strain (35405) completely cleaved the input FH, FH cleavage was not observed with 33521 or 35405-CCE. Collectively, these results demonstrate that strain 33521 is incapable of FH cleavage.

Strain 33521 is resistant to human serum

Incubation of 35405, 33521, and 35405 *fhhB* with 25% NHS resulting in 12%, 8% and 49% killing respectively (Fig. 10). No significant killing was observed with HIS. The serum sensitivity levels observed here for strains 35405 and 35405 *fhhB* are consistent with an earlier report that demonstrated the importance of FhbB in evasion of complement dependent-serum mediated killing (McDowell *et al.*, 2011). The slightly better percent survival rate of the 33521 strain, relative to a dentilisin positive strain (35405) suggests that dentilisin may slightly decrease complement resistance presumably as a consequence of FH cleavage.

DISCUSSION

Complement evasion mediated by the binding of FH is an important virulence mechanism for a diverse range of pathogens (Zipfel *et al.*, 2008) including the periopathogen, *T. denticola* (McDowell *et al.*, 2012). While more than 70 species of *Treponemes* have been identified in the subgingival crevice (Paster *et al.*, 2006), *T. denticola* is the only species shown to bind FH (McDowell *et al.*, 2005). In addition, it is the only bacterial species that has been demonstrated to cleave FH bound to its surface (McDowell *et al.*, 2009). The cleavage of FH may lead to its local depletion resulting in a cascade of events that favors the growth of the periopathogen consortium and the progression of periodontal disease (Miller *et al.*, 2012, McDowell *et al.*, 2012). Understanding how FH interacts with different *T. denticola* strains will enhance our understanding of the role of FH binding and the epidemiology of periodontal disease.

In this study we have assessed the properties of a unique variant of FhbB carried by *T. denticola* strain 33521. To date, analyses of the FhbB-FH interaction have focused exclusively on strain 35405 (McDowell *et al.*, 2009, McDowell *et al.*, 2007, McDowell *et al.*, 2011, McDowell *et al.*, 2005, McDowell *et al.*, 2012, Miller *et al.*, 2012, Miller *et al.*, 2011). The *fhhB* gene was PCR amplified from strain 33521 using primers designed based on the strain 35405 genome sequence that target flanking regions of *fhhB*. Sequence analyses revealed that *fhhB*₃₃₅₂₁ differs significantly from previously reported *fhhB* sequences (McDowell *et al.*, 2007) sharing only 60.8% amino acid identity and 73.4% similarity with FhbB₃₅₄₀₅. Sequence differences were concentrated within the FH binding interface domain. Within this domain FhbB₃₃₅₂₁ displays 53% and 64% amino acid identity and similarity, respectively, with FhbB₃₅₄₀₅. In contrast, the identity and similarity values of the opposite face of the protein are 74% and 90%, respectively. It is noteworthy that although the FH binding interface is the most variable domain of the protein, FhbB₃₃₅₂₁ retains FH binding ability. Although considerable sequence differences exists between these

FhbB orthologs, molecular modeling analyses predict that FhbB₃₃₅₂₁ and FhbB₃₅₄₀₅ are of similar structure.

It has been postulated that the interaction between bacterially produced FH binding proteins and FH is dependent on the presentation of the proper electrostatic environment and not on a strictly defined primary sequence (Miller et al., 2012). The divergence within the FH binding interface of these FhbB orthologs supports this suggestion. The electrostatic surface charge maps of FhbB₃₃₅₂₁ and FhbB₃₅₄₀₅ are similar with well defined positively (1 3 2) and negatively charged (1 2) faces. The stronger and more widely dispersed negative-surface charge region of the 1 2 face of FhbB₃₃₅₂₁, compared with FhbB₃₅₄₀₅, suggests potentially greater affinity for FH. To assess this possibility, surface plasmon resonance analyses were conducted and binding kinetics measured. FhbB₃₃₅₂₁ bound FH with tighter affinity than FhbB₃₅₄₀₅ with K_D values of 0.90 and 1.51 μ M, respectively. As discussed above, the tighter binding to FhbB₃₃₅₂₁ is most likely due to the more concentrated negative charge at the FH binding interface (refer to Fig. 5). The potential impact of this altered charge distribution is discussed in detail below.

Consistent with the extensive diversity between FhbB₃₅₄₀₅ and FhbB₃₃₅₂₁ these orthologs were found to be antigenically distinct from each other. Initial immunoblot analyses of r-FhbB proteins using antisera generated in mice against r-FhbB₃₅₄₀₅ revealed only weak detection of r-FhbB₃₃₅₂₁. To investigate this further, antisera to r-FhbB₃₅₄₀₅ and FhbB₃₃₅₂₁ (as individual proteins) was generated in rats and tested in immunoblot and IFA assays for their ability to detect r-FhbB₃₅₄₀₅ and FhbB₃₃₅₂₁. Antibody binding occurred strictly with the homologous protein. While the negatively charged 1 2 face of FhbB is the most divergent region of the protein, the positively charged face of the protein (1 3 2) is well conserved and presumably surface exposed in the r-protein. Hence the absence of antibody in hyperimmune serum from both mice and rats that targets this face of the protein is somewhat surprising. FhbB has recently been proposed as a candidate for the development of a periodontal disease vaccine (McDowell et al., 2012). It will be essential to further investigate and consider the antigenic diversity of FhbB in vaccine development efforts.

The presence of a spirochetal lipobox (Setubal et al., 2006) in the FhbB sequence and the binding of FH to the surface of *T. denticola* (McDowell et al., 2005) imply that FhbB is a surface-exposed lipoprotein. However, this has not been directly demonstrated. Surface exposure and membrane localization were assessed using IFA and Triton-X114 extraction and phase partitioning approaches. IFA analyses using anti-FhbB antibody revealed uniform labeling and hence uniform distribution of FhbB on the cell surface. Consistent with the immunoblot analyses discussed above, efficient surface labeling using the anti-FhbB₃₅₄₀₅ and anti-FhbB₃₃₅₂₁ antiserum proved to be strain specific. The surface presentation demonstrated by IFA is consistent with Triton-X114 extraction and phase partitioning analyses which revealed that FhbB is amphipathic and concentrated in the detergent phase, properties consistent with it being an outer membrane lipoprotein.

The molecular basis of the interaction between FH and bacterial FH binding proteins has been intensively studied (Zipfel et al., 2007, Ferreira et al., 2010, Lambris et al., 2008). Focusing on those produced by spirochetes, the majority interact with either CCP6-8 or CCP19-20 domains of FH (reviewed in (McDowell et al., 2012). The interaction site for FhbB₃₅₄₀₅ was previously localized to CCP6-8 (McDowell et al., 2005). CCP7 is an important functional domain of FH that interacts with host derived glycosaminoglycans and the inflammatory marker, C-reactive protein (Giannakis et al., 2003, Hebecker et al., 2010). Through a combination of modeling and mutational analyses, the FhbB₃₅₄₀₅ interaction site on FH has been mapped to CCP7. Collectively, the data presented here indicate that FhbB₃₃₅₂₁ interacts with CCPs 6 and 7 of FH. Mutational analyses were also conducted to

identify residues of FhbB₃₃₅₂₁ that are involved in the FH binding interaction. Non-conservative substitutions at residues D45 and E58 abolished FH binding. Subtle differences in residues involved in FH binding exist between FhbB₃₅₄₀₅ and FhbB₃₃₅₂₁ as substitution of FhbB₃₅₄₀₅ E62 resulted in a loss of FH binding, while substitution of FhbB₃₃₅₂₁ D61 did not. It is noteworthy that in FhbB₃₅₄₀₅, the negative charge is more concentrated on a single helix, whereas in FhbB₃₃₅₂₁, the negative charge spans the termini of the two helices. With D61 at the edge of this negative charge pool, it is not surprising that its substitution had a minimal effect on FH binding. In FhbB₃₅₄₀₅, E62 is centrally located within the negatively charged FH binding interface presented by 1–2. Its substitution would interrupt the continuity of negative charge required for binding. The data presented support the hypothesis that structure and charge, as opposed to a strict primary amino acid sequence are the dominant determinants in FH binding.

Dentilisin is an important virulence determinant that mediates several processes critical in *T. denticola* pathogenesis (Bamford et al., 2007, Bian et al., 2005, Fenno et al., 1998, Miao et al., 2011). As described here and in earlier reports, FhbB binds FH to the cell surface where it is then cleaved by dentilisin (McDowell et al., 2009). Using a *T. denticola* strain 35405 dentilisin deficient mutant (designated as CCE), we previously demonstrated that dentilisin is the sole protease responsible for FH cleavage (McDowell et al., 2011). The ability, or lack thereof, of *T. denticola* strains to cleave FH could significantly influence the host pathogen interaction and possibly the development, progression and severity of periodontal disease. There have been conflicting published reports regarding dentilisin activity of strain 33521 (Wyss et al., 2004, Heuner et al., 2001). Using the SAAPFNA cleavage assay, here we confirm a study by Wyss *et al.* that reported strain 33521 lacks dentilisin activity (Wyss et al., 2004). Consistent with this, when purified human FH was incubated with strain 33521, cleavage was not observed. Hence, *in vivo*, various strains of *T. denticola* may not participate in FH cleavage.

It stands to reason that *T. denticola* strains deficient in dentilisin activity would be more efficient in evading complement mediated killing. Using 33521 as an example, this strain binds FH with higher affinity and since it does not cleave FH, bound FH would be retained at the cell surface longer. Consistent with this hypothesis strain 33521, which does not cleave FH was slightly more serum resistant than 35405. The dentilisin deficient phenotype could be beneficial for *T. denticola* in a healthy sub-gingival crevice prior to the onset of active periodontal disease. In such an environment the ability to evade complement mediated killing would facilitate low level persistence. As periodontal disease begins to develop and the relative numbers of *T. denticola* increase, immune evasion may become less important due to the physical protection provided by the highly developed oral biofilm. The possible proliferation of *T. denticola* strains that cleave FH in periodontal pockets could lead to local FH depletion. In the absence of sufficient FH, immune dysregulation would occur. Specifically FI-mediated cleavage of C3b and decay of the C3bBb convertase complex would be inhibited, resulting in accumulation of C3b on host cells. Deposition of C3b on host cells would disrupt normal self-recognition mechanisms and lead to destruction of the periodontium. Local FH depletion could also impact regulation of the classical complement cascade. FH has been demonstrated to bind to monomeric C-reactive protein (mCRP). CRP is highly elevated in periodontal disease patients (Gomes-Filho et al., 2011). The FH-mCRP complex functions to negatively regulate the C5 convertase complex on apoptotic cell surfaces to allow for clearance of damaged cells in a controlled mechanism, limiting immune activation (Mihlan et al., 2009). In the absence of such regulation there would a significant increase in membrane attack complex formation and further cellular damage. There would also be an increase in the potent anaphylatoxin C5a resulting in the production of proinflammatory cytokines and inhibited clearance of damaged cells. Hence if FH were limiting, tissue repair would be inefficient while inflammation and tissue destruction would

ensue. Interestingly, one of the binding sites on FH for mCRP is within CCP7 (Perkins et al., 2010a, Perkins et al., 2010b, Giannakis et al., 2003, Okemefuna et al., 2010) which is the binding site for the *T. denticola* FhbB protein (this study; (McDowell et al., 2005). It remains to be determined if FhbB competes with CRP for binding to FH. The affinity of the CRP-FH interaction has been determined to be 4.2 μ M (Okemefuna et al., 2010), weaker than the FhbB-FH interaction (Miller et al., 2012) indicating that FhbB could competitively inhibit CRP binding to FH.

The periopathogen community may benefit from FH depletion through several different mechanisms. First, enlargement of periodontal pockets as a result of immune mediated destruction would expand the anaerobic environment and promote growth of periopathogens, which are mostly strict anaerobes. In addition, the destruction of tissue would release nutrients that promote growth.

There is much to be learned about the interplay between periopathogens and the host immune system. The local degradation of immune regulatory molecules by some members of the periopathogen community may prove to be a key driving force in disease progression. It has recently been suggested that *Porphyromonas gingivalis* also subverts the host immune response in a manner that favors the growth of periopathogens (Hajishengallis et al., 2011, Hajishengallis et al., 2012, Hajishengallis, 2011). Intervention strategies that disrupt these pathogen induced-host damaging processes may offer novel non-invasive approaches to controlling periodontal disease.

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	<u>Leader Peptide</u>	<u>$\alpha 1$</u>	<u>$\alpha 2$</u>	
35405	MKNKKIFTVLFLLAVSALLFTSCTFKMNTAQKAHYEKFINALENELKTRHIPAGAVIDM			
33521	.....VL.....S..A.....A..SG...DV.DNPMT.QN.KEG			
	<u>$\alpha 2$</u>	<u>$\beta 1$</u>	<u>$\alpha 3$</u>	<u>$\beta 2$</u>
35405	LAEINTEALALDYQIVDKKPGTSIAQGTAAAALRRRFIPKKIK			
33521	.DLA.VG.A..NFK.....A..E..K.....E.....V....-			

Fig. 1.

FhbB sequence alignment. The DNA sequences of *fhbB*₃₅₄₀₅ and *fhbB*₃₃₅₂₁ were determined as described in the text and translated. The amino acid sequences were aligned using ClustalW. The leader peptide and structural domains previously determined through X-ray crystallography for the FhbB₃₅₄₀₅ protein are indicated above the sequences. The FH binding interface of FhbB₃₅₄₀₅ extends from alpha helix 1 (1) through alpha helix 2 (2). Identical residues are indicated by periods and gaps by (-).

Fig. 2.

FhbB variants are antigenically distinct. Recombinant proteins (indicated above each lane) were produced as 6X-His-tagged fusions, purified, subjected to SDS-PAGE and transferred to membranes. The membranes were screened with antiserum as indicated below each panel. Molecular weights are indicated to the left. All methods are detailed in the text.

Fig. 3.

Triton X-114 extraction and phase partitioning of *T. denticola* demonstrate that FhbB is amphipathic. *T. denticola* strains 35405 and 33521 were cultivated, harvested, washed, extracted with Triton X114 and phase partitioned as detailed in the text. The resulting fractions (as indicated above each lane) were assessed by SDS-PAGE, transferred to PVDF membranes and screened with anti-FhbB antisera or anti-FlaA antisera. Molecular weight standards are indicated to the left.

Fig. 4.

FhbB is presented on the *T. denticola* cell surface. Immunofluorescence assays were performed as detailed in the text. Intact (unfixed) or acetone permeabilized (fixed) *T. denticola* 35405, 33521 and 35405 FhbB cells (as indicated to the right of each row of images) were screened with anti-FhbB (α-FhbB) and anti-FlaA (α-FlaA) antiserum. Antibody binding was detected using fluorescently-conjugated antibodies. Cells within each field of view were also visualized by dark-field microscopy.

Fig. 5. Structural modeling of FhbB. The determined and predicted structures for FhbB₃₅₄₀₅ (top panel, PDB 3R15) and FhbB₃₃₅₂₁ (bottom panel) are shown as ribbon diagrams with surface electrostatic charges (red-negative charge; blue-positive charge). The left panel presents a head on view of the FH binding interface. The images in the right panels are rotated 180° around the y-axis. The location of residues that were targeted for site-directed mutagenesis or that are discussed in the text are indicated.

Fig. 6.

Divergent FhbB variants interact with CCP6-7 of FH. R-FhbB₃₅₄₀₅, FhbB₃₃₅₂₁, FhbA and BSA were immobilized in the wells of ELISA plates and screened with r-proteins spanning different CCP domains of FH (indicated in the insert). FhbA, which has been demonstrated to bind to CCP19-20, served as a positive control. BSA, which does not bind FH served as a negative control. Binding of the CCP constructs was measured using an ELISA format as detailed in the text.

Fig. 7. Analysis of the kinetics of the interaction of FH with FhbB₃₅₄₀₅ and FhbB₃₃₅₂₁. Binding kinetics were assessed using surface plasmon resonance. R-FhbB proteins were immobilized on a NTA chip and various concentrations of FH were added in the fluid phase. The binding curve obtained using FH at a concentration of 2.5 μ M is presented.

Fig. 8.

Identification of FhbB residues required for FH binding. Site-directed amino acid substitutions were introduced into FhbB using a mutagenic PCR approach. Recombinant proteins were generated, purified, fractionated by SDS-PAGE and transferred to membranes. Note that the substitution mutants were generated using two different expression vectors and hence the results are presented in two panels. The proteins tested in panel A were generated in pET32 Ek/LIC while those in panel B were generated using pET46 Ek/LIC vector. To verify protein loading, the top blot in panel A was screened with S-Protein as previously described (McDowell et al., 2002). In panel B the protein loading was assessed by staining of a duplicate gel with coomassie brilliant blue G250 (CBB). The membranes were incubated with purified FH and binding detected with FH-specific antisera. As a loading controls, identical blots were screened with HRP-conjugated S-protein to detect the N-terminal expression tag.

Fig. 9.

Strain 33521 lacks dentilisin activity and fails to degrade bound FH. (A) Dentilisin activity of strain 33521 was analyzed by measuring SAAPFNA cleavage. Strain 35405 and 35405-CCE (a dentilisin deficient strain) served as positive and negative controls, respectively. All methods were as detailed in the text. (B) The ability of each strain to cleave FH was assessed through immunoblot analysis of FH that had been incubated with whole cells (as indicated). FH breakdown was monitored using anti-human FH antiserum.

Fig. 10.

Analysis of the sensitivity of *T. denticola* strains to human serum. Strains 35405 (black), 33521 (dashed lines), and 35405 *f*hbB (white) were incubated in 25% complement-preserved NHS or in HIS for 3 hrs. The percentage of intact cells was determined through dark-field microscopy. The data presented are an average of three individual experiments.

Table 1

Oligonucleotide primers (5'-3') used in this study

TDE0107Up	TTTATTCCACCATTCCATACATC
TDE0109Rev	CGATATTCATGACGTTTACTAC
FhbB3352LIC (+)	<u>GACGACGACAAGATT</u> CGTTTAAAAATGAATACTGCAC
FhbB3352LIC (-)	<u>GAGGAGAAGCCCGTT</u> ACTTTTCTTGGGTACAAAG
CCP1LIC(+)	<u>GACGACGACAAGGA</u> AGATTGCAATGAACCTCCTCC
CCP4LIC(-)	<u>GAGGAGAAGCCCGTT</u> CATGATTTTCTTCACATGAAGGCAACG
CCP5LIC(+)	<u>GACGACGACAAGAA</u> ATCATGTGATAATCCTTATATTC
CCP7LIC(-)	<u>GAGGAGAAGCCCGTT</u> CAGACACGGATGCATCTGGGAGTAG
CCP8LIC(+)	<u>GACGACGACAAGAAAA</u> AAACATGTTCAAATCAAGTATAG
CCP13LIC(-)	<u>GAGGAGAAGCCCGTT</u> CATGCCATTGAGCAGTTCCTTC
CCP14LIC(+)	<u>GACGACGACAAGAA</u> ACAATTATGCCACCTCCACCTC
CCP18LIC(-)	<u>GAGGAGAAGCCCGTT</u> CAAGAATCTTTCATTGAGGTGGTTC
CCP19LIC(+)	<u>GACGACGACAAGAT</u> CAAAGATTCTACAGGAAAATGTG
CCP20LIC(-)	<u>GAGGAGAAGCCCGTT</u> CTATCTTTTGCACAAGTTGGATAC
521E43K(+)	GGTCTTAAAAATGATGTAAAAGATAA
521E43K(-)	TTATCTTTTACATCATTTTAAAGACC
521E43D(+)	GGTCTTGACAATGATGTAAAAGATAA
521E43D(-)	TTATCTTTTACATCATTTGCAAGACC
521E43G(+)	GGTCTTGGAATGATGTAAAAGATAA
521E43G(-)	TTATCTTTTACATCATTTCCAAGACC
521D45K(+)	GGTCTTGAAAATAAAGTAAAAGATAA
521D45K(-)	TTATCTTTTACTTTATTTTCAAGACC
521D45E(+)	GGTCTTGAAAATGACGTAAAAGATAA
521D45E(-)	TTATCTTTTACGTCATTTTCAAGACC
521D45G(+)	GGTCTTGAAAATGGTGAAAAGATAA
521D45G(-)	TTATCTTTTACACCATTTTCAAGACC
521D45A(+)	GGTCTTGAAAATGCTGTAAAAGATAATCCAATGACTGC
521D45A(-)	GCAGTCATTGGATTATCTTTTACAGCATTTTCAAGACC
521D48A(+)	GGTCTTGAAAATGATGTAAAAGCTAATCCAATGACTGC
521D48A(-)	GCAGTCATTGGATTAGCTTTTACATCATTTTCAAGACC
521E58A(+)	CAAAATGTGAAAGCAGGGCTTGATCTTGC
521E58A(-)	GCAAGATCAAGCCCTGCTTTCACATTTTG
521D61A(+)	GAAGGGCTTGCTCTTGCTAATGTCTG
521D61A(-)	CGACATTAGCAAGAGCAAGCCCTTC

The portions of each primer that were included to allow for annealing with the pET LIC vectors are indicated by underlining.