Analysis of the complement sensitivity of oral treponemes and the potential influence of FH binding, FH cleavage and dentilisin activity on the pathogenesis of periodontal disease

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SUMMARY

Treponema denticola, a periopathogen, evades complement-mediated killing by binding the negative complement regulatory protein factor H (FH) to its surface via the FhbB protein. Paradoxically, bound FH is cleaved by T. denticola’s dentilisin protease, a process hypothesized to trigger localized dysregulation of complement activation in periodontal pockets. The ability of other oral treponemes to evade complement-mediated killing and bind and cleave FH has not been assessed. In this report, we demonstrate that representative isolates of Treponema socranskii, Treponema medium, Treponema pectinovorum and Treponema maltophilum are also serum resistant, whereas Treponema vincentii and Treponema amylovorum are serum sensitive. Although T. denticola’s ability to evade complement-mediated killing is strictly dependent on FH binding, other serum-resistant treponemal species lack FhbB and do not bind FH, indicating an FH-independent mechanism of complement evasion. To assess the influence of FhbB sequence variation on FH binding and cleavage by T. denticola, fhbB sequences were determined for 30 isolates. Three distinct phyletic types were identified. All T. denticola strains bound FH and were serum resistant, but differences in binding kinetics, dentilisin activity and FH cleavage ability were observed. Based on these analyses, we hypothesize that the composition of the T. denticola population is a determining factor that influences the progression and severity of periodontal disease.

INTRODUCTION

Periodontitis is a chronic inflammatory disease initiated by a complex microbial biofilm that results in destruction of the periodontium, culminating in alveolar bone resorption and edentulism (Darveau, 2010). Distinct bacterial consortia have been identified that correlate with either healthy or diseased tissue. Elevated numbers of bacteria in the ‘red-complex’ (Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola) correlate strongly with periodontal disease progression and severity (Socransky et al., 1998). Oral treponemes, including T. denticola, are anaerobic spirochetes that are found at the...
interface between the subgingival plaque and the periodontal epithelium (Ellen & Galimanas, 2005). *Treponema denticola* is a minor component of the bacterial population in the healthy subgingiva but may exceed 40% of the total bacterial population in diseased periodontal pockets (Ellen & Galimanas, 2005).

The subgingival crevice is bathed in crevicular fluid that is rich in serum proteins, antimicrobial peptides and active complement (Schenkein & Genco, 1977b; Boackle, 1991). Complement is a key part of the innate immune system (Ricklin et al., 2010). Host cells are protected from activated complement by membrane-bound and fluid-phase complement regulators including factor H (FH). FH is a 155-kDa glycoprotein that consists of 20 complement control protein (CCP) domains and binds to host cell glycosaminoglycans via CCPs 6–7 and 19–20 (Ferreira et al., 2010). Although FH is a key regulator of the alternative complement pathway, its ability to influence several immunological pathways and processes that are relevant to periodontal disease is now clear (Miilan et al., 2009; Zipfel & Skerka, 2009; Ferreira et al., 2010; Barthel et al., 2012).

*Treponema denticola* strain 35405 exploits the negative complement regulatory activity of FH by binding to an 11.4-kDa surface exposed lipoprotein designated as FhbB (McDowell et al., 2005, 2007). The binding interaction occurs between the negatively charged face of FhbB and the CCP 7 domain of FH (McDowell et al., 2005, 2007; Miller et al., 2011, 2012). Using a *T. denticola* 35405 *fhbB* deletion mutant (35405Δ*fhbB*), it was demonstrated that serum resistance is strictly dependent on this interaction (McDowell et al., 2011). Paradoxically, FH bound to the *T. denticola* surface is cleaved by the protease dentilisin (McDowell et al., 2009). It has been hypothesized that as the *T. denticola* population expands with disease progression, cleavage of FH by dentilisin leads to local dysregulation of complement activation initiating a cascade of destructive downstream events that result in tissue destruction and bone resorption (Miller et al., 2012). This in turn creates a favorable environment for the survival of other periodontal pathogens.

In this study, we assessed the complement sensitivity of six oral treponeme species in addition to a comprehensive panel of *T. denticola* strains. All *T. denticola* strains were determined to be serum resistant and to bind FH. However, other species that were serum resistant did not produce FhbB or bind FH, indicating that they employ alternative methods for complement evasion. Since FhbB and FH binding has, to date, been assessed in only four *T. denticola* strains (35405, 33520, 33521 and GM1), in this study we determined *fhbB* sequences and assessed FH binding, dentilisin activity and FH cleavage for a large panel of *T. denticola* isolates (McDowell et al., 2005, 2007). The influence of *fhbB* sequence variation on FH binding and serum resistance was also assessed and the kinetics of the FhbB–FH interaction was determined for representative recombinant FhbB proteins using surface plasmon resonance. This study provides a comprehensive analysis of the serum resistance of oral treponemes. The results demonstrate that some oral treponemes employ an FH-independent mechanism to evade complement-mediated destruction. In addition, the data indicate significant phenotypic variation among *T. denticola* isolates. This observation is of epidemiological and pathogenic relevance because it suggests that the composition of the *T. denticola* population may influence the progression and severity of disease.

**METHODS**

**Bacterial strains and generation of recombinant proteins**

*Treponema medium* (ATCC 700293), *Treponema socorskii* (ATCC 35535), *Treponema vincentii* (ATCC 35580) and all strains of *T. denticola* were grown in new oral spirochete media under anaerobic conditions as previously described (McDowell et al., 2005). *Treponema amylovorum* (ATCC 700288), *Treponema maltophilum* (ATCC 51939) and *Trepnema pectinovorum* (ATCC 33768) were grown in OMIZ-P4 under anaerobic conditions. Recombinant (r-) FhbB proteins were generated, as previously described, using primers designed to amplify the mature protein (removing the 23 amino acid signal peptide) with sequences that allow for ligase-independent cloning with the pET46 Ek/LIC vector (Table 1) (Miller et al., 2012). Recombinant FH proteins consisting of different CCP domains and r-FhbA, an FH-binding protein from *Borrelia hermsii*, were generated in earlier studies (Hovis et al., 2008; Miller et al., 2013).
DNA sequencing, phylogenetic and structural analyses

The fhbB gene was amplified using polymerase chain reaction and sequenced from 30 T. denticola strains as previously described (McDowell et al., 2007). The primers used for the polymerase chain reaction are listed in Table 1. Additional sequences, generated as part of the Human Microbiome U54 initiative (broadinstitute.org) derived from T. denticola isolates AL-2, F0402, H-22, H-1, MYR, US-Trep1, SP23, SP32, SP33, SP37 and SP34 were also analysed. FhbB sequences were aligned using CLUSTALOMEGA (Sievers et al., 2011) and phylogenetic analyses conducted using MEGAS (Tamura et al., 2011). Phylogenetic trees were constructed using the neighbor-joining method and the standard errors of distances were computed by bootstrap analysis using 500 replications. The sequences determined as part of this study were assigned GenBank accession numbers HM991217–HM991226. The structural relationships among FhbB variants were assessed using computer-assisted structural modeling. A structural model of different FhbB types was created using the known structure of FhbB35405 (pdb code 3R15) (Miller et al., 2011, 2012). Briefly, CLUSTALW2 (Larkin et al., 2007) was used to align the FhbB sequences (residues 24–101). Structural models were generated and compared with the FhbB35405 structure using MODELER9v1 (Sali, 1995; Sali et al., 1995). MOLEPROBITY (version 3.15) (Davis et al., 2004) was used to assess all-atom contacts and geometry. CONSURF was used to map the location of conserved and divergent residues using the FhbB35405 as the reference structure (Ashkenazy et al., 2010).

Analyses of the FhbB–FH interaction using affinity ligand-binding immunoblot assays, sucrose octasulfate competitive binding analyses, enzyme-linked immunosorbent assays, and surface plasmon resonance

FH affinity ligand-binding immunoblot (ALBI) assays (a membrane overlay approach) were performed as previously described (McDowell et al., 2005). In brief, cell lysates of each strain or recombinant protein were solubilized and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Equal loading of recombinant proteins and protein integrity were assessed by staining with Coomassie blue. Proteins were transferred to polyvinylidene fluoride, overlaid with purified human FH (10 μg ml⁻¹ in phosphate-buffered saline with 0.2% Tween-20 (PBST); Complement Tech, Tyler, TX) and bound FH was detected using goat anti-FH antiserum (1 : 800 dilution, Complement Tech). Horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin-G (1 : 5000 dilution, Abcam) was used as the secondary antibody. Bound antibody was detected using horseradish peroxidase-conjugated donkey anti-rabbit IgG (1 : 5000 dilution, Abcam) and 4-chloro-1-naphthol (Sigma). The FH protein concentration was determined using the Lowry method (Lowry et al., 1951). The concentration of FH used for the ALBI assays was approximated based on the 1:1 binding stoichiometry of FH and FhbB35405. The complexity of the FH–FhbB interaction was assessed by bidirectional ligand-binding immunoblot assays (McDowell et al., 2005). Table 1 Oligonucleotide primers (5′–3′) used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDE0107Up</td>
<td>TTTATTCCACCATCCATACATC</td>
</tr>
<tr>
<td>TDE0109Rev</td>
<td>CGATATTATGAGCCTTTACTAC</td>
</tr>
<tr>
<td>FhbB1 LIC (+)</td>
<td>GACGACGACAAAGATTACTTCCAAATGAAATACGAC</td>
</tr>
<tr>
<td>FhbB1 LIC (−)</td>
<td>GAGGAGAAGCGGCTTTTATTTCCTTTTTGGTAT</td>
</tr>
<tr>
<td>FhbB2 LIC (+)</td>
<td>GACGACGACAAAGATTACTTCCAAATGAAATACGAC</td>
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<td>GAGGAGAAGCGGCTTTTATTTCCTTTTTGGTAT</td>
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<td>FhbB3 LIC (+)</td>
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</table>

1The segment of each primer that was included to allow for annealing with the pET46 Ek/LIC vector is indicated by underlining.
lin G antibody served as the secondary antibody (1:20,000 dilution, Calbiochem, San Diego, CA). *Treponema denticola* strains 35405 and 35405\(\text{fhbB}\) served as positive and negative controls for the FH-binding assays, respectively.

Binding of full-length FH and CCP constructs to r-FhbB proteins was assessed by enzyme-linked immunosorbent assay (ELISA) as previously described (Miller *et al.*, 2013). Recombinant FhbB proteins were immobilized overnight at 4°C in triplicate wells of ELISA plates (1 μg per well in 100 mM NaHCO\(_3\), pH 9.6). *Borrelia hernsii* FH-binding protein, FhbA, and bovine serum albumin were also immobilized and served as positive and negative controls, respectively. Non-specific binding was blocked for 1 h with 5% non-fat dry milk in PBST. Purified full-length human FH and r-CCP constructs (10 μg ml\(^{-1}\) in PBST) were added to the wells for 1 h followed by three washes with PBST. Goat anti-human FH (1:800 in PBST + 5% milk; Complement Tech) was added to each well for 1 h, followed by three washes and application of rabbit anti-goat immunoglobulin G conjugated to horseradish peroxidase (1:20,000 dilution, Calbiochem, San Diego, CA). Antibody binding was detected using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; 405 nm). The data were normalized to r-FhbB\(_{35405}\) and averaged across three plates.

To determine whether sucrose octasulfate (SOS) can inhibit FH binding, plates were coated and non-specific binding was blocked as described above. FH (5 μg ml\(^{-1}\)) was incubated with increasing concentrations of SOS (0–50 μm) in PBST for 1 h with constant gentle agitation. The immobilized FhbB was overlaid with the FH-SOS solution for 1 h. The wells were washed three times with PBST and antibody binding was detected using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; 405 nm). The data were normalized to r-FhbB\(_{35405}\) and averaged across three plates.

The kinetics of the FhbB–FH interaction was assessed by surface plasmon resonance using a BiaCore T200 and the data were evaluated using BIAEVALUATION V1.1 (Biacore, Uppsala, Sweden) (Miller *et al.*, 2012, 2013). Briefly, r-FhbB proteins (ligand) were immobilized on nitrotriacetic acid chips (GE Healthcare, Chalfont St Giles, UK) via their N-terminal His-tag and increasing concentrations of purified human FH (analyte; Complement Tech) were added in the fluid phase. The kinetics data were fitted to a Langmuir 1:1 binding model and averaged from three replicate experiments.

### Measurement of dentilisin activity and FH cleavage

The dentilisin activity of each strain was determined by measuring its efficiency in hydrolysis of succinyl-L-alanine-L-alanine-L-prolyl-L-phenylalanine-p-nitroanilide (SAAPFNA; Sigma-Aldrich, St Louis, MO) (Uitto *et al.*, 1988; Miller *et al.*, 2013). Aliquots of mid- to late-log phase cultures were pelleted by centrifugation (5000 g, 15 min, 4°C), suspended in buffer A (100 mM NaCl, 1 mM CaCl\(_2\), 50 mM Tris–HCl, pH 8.0) and transferred to 96-well plates. SAAPFNA (1 μm in buffer A) was added to each well to a final concentration of 500 μM and the absorbance was measured at 405 nm every 5 min for 60 min. The data presented are the average of the measurement of three wells per biological replicate and are representative of three replicate experiments. The data are presented as the change in absorbance between the 0-min and 60-min time-points. The dentilisin-mutant strain 35405-CCE (Bian *et al.*, 2005) served as a negative control. Statistical significance was assessed using Student's *t*-test.

*Treponema denticola* strains were assessed for their ability to degrade FH. In brief, 0.1 OD\(_{600}\) of freshly harvested, mid-log phase cells were incubated with 40 μg ml\(^{-1}\) human FH (Complement Tech) in PBS (50-μl reaction volume). A sample was immediately removed at 0 min and the remainder was incubated for 120 min at 37°C. The samples were fractionated by SDS–PAGE, transferred to polyvinylidene fluoride and screened with anti-human FH antiserum (dilution of 1:1000; Complement Tech). Antibody binding was detected using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) at 405 nm.

### Serum sensitivity analysis

Resistance to complement-mediated killing was determined as described in previous studies (McDowell *et al.*, 2011; Miller *et al.*, 2013) with minor modifications. Cells from mid-log phase cultures were incubated with 40% complement-preserved human serum (NHS; Innovative Research, Novi, MI) in the appropriate growth media (either new oral spirochete medium or OMIZ-P4; 37°C; anaerobic conditions; 0–3 h). To determine if potential serum-mediated killing was
complement dependent, strains were also incubated with 40% serum that had been incubated at 56°C for 30 min to inactivate complement (heat-inactivated serum; HIS). The percentage of intact cells (e.g. % survival) was determined by cell counts using dark field microscopy (average of 10 fields of view under 1000 × magnification). The values presented represent the number of morphologically intact cells divided by the total number of morphologically intact and disrupted cells (intact cell/intact and disrupted cells × 100). Cells with multiple outer sheath blebs were determined to be morphologically disrupted.

RESULTS

Complement resistance of oral treponemes

A BLAST search of determined oral treponeme genome sequences (Human Microbiome U54 initiative, Broad Institute, Cambridge, MA; broadinstitute.org), revealed that sequences with significant primary sequence homology to fhbB are found only in T. denticola. To determine if other oral treponeme species may bind FH via a protein that is unrelated to FhbB, FH ALBI assays were performed (Fig. 1A). Of the seven species tested, only T. denticola 35405 bound human FH and binding occurred to an 11-kDa protein consistent with the molecular mass of FhbB35405. As expected, T. denticola 35405 δ fhbB did not bind FH.

To determine if the inability of some treponemal species to bind FH correlates with serum sensitivity, representative strains were incubated with 40% NHS or HIS and percent survival was determined (Fig. 1B). Less than 5% of T. amylovorum and T. vincentii cells survived in the presence of NHS, whereas >95% of the cells survived in HIS. Based on these data, it can be concluded that serum-mediated killing of these species is complement-dependent. Treponema medium, T. maltophilum, T. pectinovorum, and T. socranskii were resistant to 40% NHS with >90% survival. These results indicate that five of the seven oral treponemes tested in this study are highly resistant to complement-mediated killing. While FH binding is essential to the survival of T. denticola, it does not contribute to the serum resistance of several other oral treponeme species.

Identification and phylogenetic analysis of fhbB variants

The fhbB sequences of T. denticola isolates 35405, GM-1 and 33520 were previously determined and found to be nearly identical (McDowell et al., 2007). Recently, a divergent FhbB protein produced by isolate 33521 was identified and demonstrated to bind FH (Miller et al., 2013). To conduct comprehensive analyses of fhbB phylogenetics, sequences derived from 43 isolates of T. denticola were aligned. Four were previously determined, nine were from determined genomes sequences and 30 were determined as part of this study. Phylogenetic analyses delineated three FhbB phyletic clusters that are well

![Figure 1](image-url)
supported by bootstrap analysis (Fig. 2). Twenty-one of the sequences (48.8%) were type 1 FhbB (designated as FhbB1), 14 (32.6%) were type 2 (FhbB2) and eight (18.6%) were type 3 (FhbB3). Throughout the manuscript, the isolate of origin for each FhbB sequence, where appropriate to specify, is indicated by subscript (i.e. FhbB135405). Sequences within the FhbB1 and FhbB2 clusters are highly conserved at the intra-type level while FhbB3 sequences displayed greater divergence. An alignment of representative sequences is presented in Fig. 2. The structure of FhbB135405 was previously determined at 1.7 Å.

**Figure 2** Identification of three major *Treponema denticola* FhbB phyletic clusters. The phylogenetic relationships among FhbB sequences (*n* = 43) were assessed using the neighbor-joining method (top panel). The results of bootstrap analyses (500 bootstrap replicates) are indicated at each node. The *Borrelia hermsii* FhbA screen FH binding protein was used to root the tree. The middle panel presents an FhbB amino acid alignment with FhbB135405 serving as the reference strain. Identical residues are shown as dots. Secondary structure elements as inferred from the known atomic structure of FhbB135405 (Miller et al., 2011, 2012) are indicated above the alignment. Alpha helices 1–2 map within the negatively charged face of FhbB that has been demonstrated to serve as the FH interaction domain. The bottom panel presents a matrix of amino acid identity and similarity values (upper and lower quadrants, respectively).

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resolution (Miller et al., 2011, 2012). An amino acid identity/similarity matrix is also presented in Fig. 2. All sequences are predicted to encode lipoproteins with a conserved 23 amino acid leader peptide that, after processing, yields a ~11.4-kDa mature protein that is tethered to the cell surface via its lipid moiety.

To assess potential structural conservation among FhbB variants, representative FhbB1, FhbB2 and FhbB3 sequences were threaded onto the FhbB135405 atomic structure. The models obtained were analysed by MOLPROBITY to identify those with the fewest all-atom clashes and the highest percentage of residues with favored Ramachandran phi-psi angles. All FhbB variants analysed displayed a high confidence-strong structural fit with the FhbB135405 structure. Only minor structural variations were observed (Fig. 3A). Analyses of surface charge distribution maps (overlaid on the structures in Fig. 3) revealed that consistent with FhbB135405, all FhbB variants have well defined positively and negatively charged surfaces.

Using all known FhbB amino acid sequences, a sequence conservation map was generated using FhbB135405 as the reference structure (Fig. 3B). The greatest degree of sequence diversity among variants maps within residues 20 through 66 (middle of α helix 1 to the C-terminus of α helix 2). It is this region of FhbB1 that has been demonstrated to serve as the FH interaction domain (McDowell et al., 2009; Miller et al., 2011, 2013).

Mapping of the FH interaction site for FhbB1, FhbB2 and FhbB3 proteins

Previous studies demonstrated that the FH-binding site for FhbB135405 is within CCP 6–8 and overlaps with a glycosaminoglycan (GAG) -binding site (Giannakis et al., 2003; Miller et al., 2012). In this report, ELISAs were used to investigate the binding of full-length purified human FH and r-FH CCP constructs to each FhbB type (Fig. 4A). All FhbB proteins bound full-length FH

Figure 3 Structural models, charge distribution maps and sequence conservation of FhbB variants. Comparative modeling of the three-dimensional structures of each FhbB type (A) was performed using MODELLER (University of California San Francisco, San Francisco, CA) based upon the known structure of FhbB135405 (PDB 3R15). The ribbon diagrams of FhbB (gray) were overlaid with color-coded, transparent electrostatic surface maps. Residues of negative and positive charge are shown in red and blue, respectively. Each structure was rotated through 180° to show both the front and back view of each model. (B) A conservation map using the FhbB135405. A conservation score was calculated for each residue using the Consurf algorithm. Conserved, variable and highly variable residues shown in gray, light green and dark green, respectively. All methods are described in the text.
and r-FH constructs consisting of CCP 6–8, CCP 6–7, and CCP 6–7 but not to CCP 19–20. The FH- and plasminogen-binding protein from *Borrelia hermsii*, FhbA (Hovis et al., 2004; Rossmann et al., 2007), which exclusively interacts with CCP 19–20 of FH (Hovis et al., 2006), served as a binding specificity control. Bovine serum albumin served as a negative control. FhbB2 displayed the lowest level of binding to FH and the CCP 6–7 domain. To further verify that the FhbB variants bind within the GAG-binding site of CCP 6–7, competitive binding analyses were conducted using SOS, a synthetic GAG-analog (Fig. 4B) (Prosser et al., 2007). SOS inhibited binding of FH binding to each FhbB type in a dose-dependent manner. Surface plasmon resonance approaches were employed to compare the binding kinetics of the three major FhbB types with FH (Fig. 4C). The $K_D$ value for the FhbB2–FH interaction was similar to that reported for Fhb1 and FhbB3 FH interactions (Miller et al., 2012, 2013). In comparing the kinetics of the interaction of each FhbB type with FH, the $K_D$ value for the FhbB–FH interaction is largely a reflection of a slower off rate. This could compensate for lower affinity binding and allow for more efficient cleavage of FH by *T. denticola* strains producing FhbB2 proteins.

**Serum resistance does not correlate with FhbB type or proteolytic activity**

To determine if dentilisin activity, and thus FH cleavage, correlates with levels of serum resistance, the dentilisin activity of 16 *T. denticola* strains was...
determined using the SAAPFNA cleavage assay (Fig. 5A). Dentilisin activity varied widely among strains with some having no discernable activity under the conditions tested. Hence, based on this observation we speculated that strains will differ significantly in their ability to cleave FH. To test this, the

![Image](image.png)

**Figure 5** Quantification of the dentilisin activity of *Treponema denticola* strains producing FhbB1, FhbB2, and FhbB3 proteins. (A) Results of SAAPFNA assays. Mid-log phase cultures were incubated with SAAPFNA and absorbance was read at 0 and 60 min. The absorbance value obtained at 0 min (background) was subtracted from the value obtained after 1 h and the average of three assays was plotted. (B) Results of factor H (FH) cleavage assays are presented. Each strain (as indicated above the panel) was incubated with FH and aliquots of the supernatant were collected at time 0 and 120 min were fractionated by SDS–PAGE, the proteins were transferred to membranes and the blots were screened with anti-human FH antiserum as detailed above. The absence of a band at 150 kDa is indicative of FH cleavage. * P < 0.01.

**Table 2** Data summary

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<th>Isolate</th>
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<th>Dentilisin activity</th>
<th>Factor H cleavage</th>
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<th>% Survival (HIS)</th>
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<td>35405</td>
<td>1</td>
<td>1.59 ± 0.01</td>
<td>+</td>
<td>78.3 ± 3.6</td>
<td>97.6 ± 1.5</td>
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<td>MS25</td>
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<td>92.6 ± 3.3</td>
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<tr>
<td>SP48</td>
<td>2</td>
<td>0.54 ± 0.01</td>
<td>+</td>
<td>94.9 ± 1.2</td>
<td>96.5 ± 2.5</td>
</tr>
<tr>
<td>SP55</td>
<td>2</td>
<td>0.01 ± 0.02</td>
<td>–</td>
<td>83.4 ± 20.4</td>
<td>97.2 ± 0.8</td>
</tr>
<tr>
<td>SP54</td>
<td>2</td>
<td>0.01 ± 0.01</td>
<td>–</td>
<td>82.0 ± 13.2</td>
<td>98.1 ± 0.8</td>
</tr>
<tr>
<td>SP50</td>
<td>2</td>
<td>0.08 ± 0.01</td>
<td>–</td>
<td>86.9 ± 9.0</td>
<td>95.0 ± 4.9</td>
</tr>
<tr>
<td>SP49</td>
<td>2</td>
<td>0.26 ± 0.02</td>
<td>+</td>
<td>90.0 ± 14.6</td>
<td>95.4 ± 4.8</td>
</tr>
<tr>
<td>SP72</td>
<td>2</td>
<td>0.01 ± 0.01</td>
<td>–</td>
<td>68.7 ± 10.2</td>
<td>95.1 ± 1.4</td>
</tr>
<tr>
<td>35404</td>
<td>3</td>
<td>0.09 ± 0.01</td>
<td>+</td>
<td>88.4 ± 5.7</td>
<td>98.2 ± 0.3</td>
</tr>
<tr>
<td>SP64</td>
<td>3</td>
<td>0.69 ± 0.01</td>
<td>+</td>
<td>82.6 ± 3.3</td>
<td>99.0 ± 0.2</td>
</tr>
<tr>
<td>33521</td>
<td>3</td>
<td>0.01 ± 0.01</td>
<td>–</td>
<td>98.4 ± 1.7</td>
<td>97.4 ± 0.2</td>
</tr>
<tr>
<td>CF171</td>
<td>3</td>
<td>1.02 ± 0.01</td>
<td>+</td>
<td>93.8 ± 3.9</td>
<td>97.3 ± 1.7</td>
</tr>
<tr>
<td>CF170</td>
<td>3</td>
<td>1.44 ± 0.03</td>
<td>+</td>
<td>94.5 ± 3.5</td>
<td>95.7 ± 1.5</td>
</tr>
</tbody>
</table>
FH cleavage ability of each strain was analysed. The majority of strains that produced FhbB2 or FhbB3 proteins either lacked or were attenuated in their ability to cleave FH (Fig. 5B). This suggests that there may be significant differences in the potential of strains expressing different FhbB types to cause local dysregulation of complement activation.

To determine if production of a given FhbB type protein influences the ability to evade complement-mediated killing, each strain was exposed to 40% complement-preserved NHS. In this assay, differences in FH cleavage activity would not be expected to influence serum resistance as the concentration of FH is high in serum. As a negative control for this assay, cells were also exposed to HIS, which possesses no complement activity. The data are presented in Table 2. All T. denticola isolates were highly resistant to serum-mediated killing regardless of the FhbB type they produced or dentilisin activity level. The mean percent survival was 89.0% for FhbB1-producing strains, 84.3% for FhbB2-producing strains, and 91.5% for FhbB3-producing strains. These results indicate a trend that mirrors the ability to evade complement mediated killing regardless of the FhbB type they produced or surface using the protease, dentilisin (McDowell et al., 2009). Based on this, it was hypothesized that FH cleavage will result in its local depletion triggering dysregulation of complement activation and the downstream destructive processes that culminate in periodontal disease. To better understand the role of FH binding in survival of treponemes in the subgingival environment, FH binding and serum sensitivity of seven oral Treponema species were tested. In addition, we assessed variation in specific phenotypic properties of T. denticola that are relevant to immune evasion and the potential to cause immune dysregulation. FH binding by representative strains of seven oral treponeme species (T. denticola strain 35405, T. socianskii, T. medium, T. pectinovorum, T. maltophilum, T. vincentii and T. amylovorum) was tested using ALBI assays. Among this panel of isolates only T. denticola 35405 bound FH. FH-binding analyses of 15 additional T. denticola isolates revealed that this phenotype is shared by 13 of 15 isolates (87%). While FH bound only weakly to isolates CF170 and CF171 using the ALBI assay approach, r-FhbB3 proteins derived from these strains bound FH at a level similar to other r-FhbB proteins and FhbB expression was clearly detected using immunoblot analyses (data not shown). Consistent with the inability of other cultivable treponemal species to bind FH, a homolog of FhbB was not detected in available genome sequences. It can be concluded that among the major cultivable oral treponemes, the FH-binding phenotype is unique to T. denticola.

Some pathogens evade complement-mediated killing using non-FH-dependent mechanisms including the binding of complement regulatory proteins C4BP and vitronectin (Potempa et al., 2008; Barbosa et al., 2009; Grosskinsky et al., 2010; Singh et al., 2010; Schwab et al., 2013). To determine if serum-resistant species that do not bind FH employ non-FH-dependent complement evasion mechanisms, the serum sensitivity of representative isolates of the seven species listed above was assessed. Cells were exposed to 40% NHS or 40% HIS for 3 h and cell survival was measured. A serum concentration of 40% is consistent with the estimated concentration of serum in gingival crevicular fluid (Schenkein & Genco, 1977a,b). All isolates of T. denticola displayed significant serum

DISCUSSION

The impact of periodontal disease is not confined to the oral cavity as it correlates with increased risk for several systemic diseases (Lopez, 2008; Taylor & Borgnakke, 2008; Kavoussi et al., 2009; Inaba & Amano, 2010). Hence, a detailed understanding of phenotypic variation, mechanisms of persistence and serum resistance of oral treponemes is an essential step forward in devising strategies to combat these important pathogens and minimize their impact on overall human health. For oral treponemes to survive and thrive in periodontal pockets they must possess the ability to evade complement-mediated destruction as gingival crevicular fluid possesses levels of complement proteins similar to that of serum. This study is the first comprehensive assessment of the serum sensitivity of oral treponemes associated with periodontal disease and of T. denticola phenotypic differences relevant to FH binding, cleavage and dentilisin activity.

It was previously demonstrated that T. denticola strain 35405 evades serum-mediated killing by binding FH via the FhbB protein (McDowell et al., 2007, 2011). Paradoxically, T. denticola strain 35405 has been demonstrated to cleave FH that is bound to its surface using the protease, dentilisin (McDowell et al., 2009). Based on this, it was hypothesized that FH cleavage will result in its local depletion triggering dysregulation of complement activation and the downstream destructive processes that culminate in periodontal disease. To better understand the role of FH binding in survival of treponemes in the subgingival environment, FH binding and serum sensitivity of seven oral Treponema species were tested. In addition, we assessed variation in specific phenotypic properties of T. denticola that are relevant to immune evasion and the potential to cause immune dysregulation. FH binding by representative strains of seven oral treponeme species (T. denticola strain 35405, T. socianskii, T. medium, T. pectinovorum, T. maltophilum, T. vincentii and T. amylovorum) was tested using ALBI assays. Among this panel of isolates only T. denticola 35405 bound FH. FH-binding analyses of 15 additional T. denticola isolates revealed that this phenotype is shared by 13 of 15 isolates (87%). While FH bound only weakly to isolates CF170 and CF171 using the ALBI assay approach, r-FhbB3 proteins derived from these strains bound FH at a level similar to other r-FhbB proteins and FhbB expression was clearly detected using immunoblot analyses (data not shown). Consistent with the inability of other cultivable treponemal species to bind FH, a homolog of FhbB was not detected in available genome sequences. It can be concluded that among the major cultivable oral treponemes, the FH-binding phenotype is unique to T. denticola.
Oral treponeme resistance to human serum

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resistance and per cent survival in NHS ranging from 68 to 98%. Although T. socranskii, T. medium, T. maltophilia and T. pectinovorum do not produce FhbB or bind FH, more than 90% of the cells survived after 3 h of exposure to NHS. In contrast, the per cent survival of T. vincentii was 40% after 1 h, dropping to less than 5% after 3 h. Treponema amylovorum proved to be the most susceptible species with complete killing occurring after 1 h exposure to NHS. The role of complement in the killing process was confirmed using HIS. No killing was observed with any isolate upon exposure to HIS. The data indicate that T. socranskii, T. medium, T. maltophilia and T. pectinovorum employ FhbB- and FH-independent mechanisms to evade complement-mediated killing. To determine if serum-resistant species that do not bind FH, bind C4BP and or vitronectin, ALBI assays were performed using 20% human serum as the source for C4BP and vitronectin. Specific binding was not observed (data not shown). Future analyses will focus on identifying the molecular basis of serum resistance for T. socranskii, T. medium, T. maltophilia and T. pectinovorum.

Prior to this study, the impact of fhbB sequence variation on FH binding had been assessed in only two isolates of T. denticola (35405 and 35321) (Miller et al., 2013). In this study, phylogenetic relationships among fhbB sequences obtained from 43 different T. denticola isolates were assessed. Three distinct phyletic clusters were delineated. The proteins encoded by each cluster were designated as FhbB1, FhbB2 and FhbB3. Sequences within the FhbB1 and FhbB2 clusters were found to be highly conserved with a significantly greater degree of variation among FhbB3 sequences. In spite of extensive sequence differences, molecular modelling revealed that all FhbB types possess the negatively charged surface region previously shown to form the FH-binding domain of FhbB135405 (Miller et al., 2012). However, the highest degree of primary sequence divergence mapped within the FH-binding interface. Recombinant FhbB proteins representing each FhbB type were generated and tested to determine if sequence variation influences binding to FH or to specific CCP domains. ELISA analyses with the r-FhbB proteins immobilized in the wells revealed similar binding of each protein to full-length FH. All FhbB variants bound to the region of FH consisting of CCP domains 6 and 7, but not to an FH fragment consisting of CCP19–20. Hence, the FH interaction domain of diverse FhbB proteins appears to be similar to that of FhbB135405 (Miller et al., 2012).

CCP6–7 have been demonstrated to be a contact point for several bacterially produced FH-binding proteins (reviewed in Zipfel et al., 2002, 2007; Zipfel & Skerka, 2009). This region of FH is also a key interaction site for host-produced GAG (Giannakis et al., 2001, 2003; Prosser et al., 2007). SOS, a GAG analog, binds to multiple sites on FH including CCP7. To further refine the interaction site for FhbB variants on FH, SOS competitive binding analyses were performed. SOS inhibited binding of all variants to FH or FH-derived fragments, providing further evidence that all FhbB variants use similar contact points with CCP7. Binding kinetics of the FH–FhbB interaction were also assessed using surface plasmon resonance. While the $K_D$ values for the FH interaction with each FhbB type were similar, the on-off rates ($k_\alpha$ and $k_\beta$) differed significantly.

Treponema denticola strain 35405 has been demonstrated to cleave FH in an FhbB–dentilisin-dependent manner (McDowell et al., 2009). To date, comparative analyses of dentilisin activity have been limited to a few strains (Goetting-Minesky et al., 2013) and a potential correlation between dentilisin activity and FH cleavage in natural isolates has not been assessed. In this study, the dentilisin activity of 16 T. denticola isolates was measured by monitoring cleavage of the chromogenic substrate, SAAPFNA. Activity varied widely among isolates with some displaying no or minimal activity. This observation is consistent with an earlier study that demonstrated significant differences of dentilisin activity among strains (Goetting-Minesky et al., 2013). Four of the isolates investigated in this study (35405, 33520, 35404 and 33521) were also analysed by Goetting-Minesky et al. (2013). The results obtained in these two studies are in excellent agreement; however, differences in the methods of data analysis resulted in slight differences in conclusions. In this study, we subtracted the absorbance obtained at time zero from the absorbance measured at 2 h whereas Goetting-Minesky et al. did not. Using this approach we conclude that isolates 35404 and 33521 are devoid of activity as opposed to the conclusion that they possess low-level activity. Dentilisin is considered to be a major virulence factor for T. denticola (Ishihara et al., 1996, 1998; Ellen et al., 2000; Chi et al., 2003; Miyamoto et al., 2006;
Yamazaki et al., 2006; Bamford et al., 2007; McDowell et al., 2009). Variation in its activity among strains could prove to be of considerable epidemiological value and could influence disease progression. It remains to be determined if strains that display higher levels of dentilisin activity are more virulent. The relative proportion of strains with high vs. low activity within a specific anatomical site could potentially be a contributing factor in disease severity. In analyses of strain 35405, efficient FH cleavage was demonstrated to be directly dependent on FhbB and dentilisin (McDowell et al., 2009). The ability of a broad panel of T. denticola strains to directly cleave FH, and the dependence of this activity on dentilisin, were assessed. Dentilisin activity was found in all FhbB1 strains tested, whereas FhbB2 and FhbB3 strains did not all yield measurable dentilisin activity. Strains that produce FhbB1 proteins were found to have significantly greater FH cleavage ability than strains that produce FhbB2 and FhbB3 proteins. However, there were some exceptions as some strains with low dentilisin activity were able to cleave (SP55 and SP54) or partially cleave (35404) FH. It can be concluded that while dentilisin is a key contributor to FH cleavage, in some strains it is possible that other proteases may also play a role.

In conclusion, this report demonstrates that the serum-resistance phenotype first described for T. denticola is shared by several oral treponemes. However, the molecular mechanisms responsible for serum resistance differ among species. Treponema denticola uses an FH-dependent mechanism while other serum-resistant species employ a yet to be defined, FH-independent mechanism. All T. denticola isolates tested, in spite of significant FhbB sequence variation within their FH interaction domain, bound FH, but not all strains cleaved FH. The differences in FH–FhbB binding kinetics observed for the three FhbB phyletic types and the wide range in dentilisin activity noted among T. denticola strains was striking and suggests that in vivo the T. denticola population is heterogeneous with regard to these key virulence-related activities. It is our hypothesis that strains that produce FhbB1 proteins and have higher dentilisin activity will display greater virulence. Hence, the relative proportions of strains present in the subgingival crevice that differ in these activities could significantly influence disease progression and severity. Studies are currently underway to directly address these questions. A human FH transgenic mouse model that has been extensively used to study the role of FH binding in the pathogenesis of Neisseria meningitidis (Beernink et al., 2011; Vu et al., 2012) is now being employed by our laboratory to determine if fhbB genotype, FH binding, FH cleavage and dentilisin activity influence alveolar bone loss during periodontal disease.

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