molecular oral microbiology

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Identification of the primary mechanism of complement evasion by the periodontal pathogen, *Treponema denticola*

J.V. McDowell[1], J. Frederick[1], D.P. Miller[1], M.P. Goetting-Minesky[3], H. Goodman[1], J.C. Fenno[3] and R.T. Marconi[1,2]

1 Department of Microbiology and Immunology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA, USA
2 Center for the Study of Biological Complexity, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA, USA
3 Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, MI, USA

Correspondence: Richard T. Marconi, Department of Microbiology and Immunology, PO Box 980678, Virginia Commonwealth University, Richmond, VA 23298-0678, USA Tel.: +1 804 828 3779; fax: +1 804 828 9946; E-mail: rmarconi@vcu.edu

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Summary

Treponema denticola*, a periodontal pathogen, binds the complement regulatory protein Factor H (FH). Factor H binding protein B (FhbB) is the sole FH binding protein produced by *T. denticola*. The interaction of FhbB with FH is unique in that FH is bound to the cell and then cleaved by the *T. denticola* protease, dentilisin. A ~ 50-kDa product generated by dentilisin cleavage is retained at the cell surface. Until this study, a direct role for the FhbB–FH interaction in complement evasion and serum sensitivity had not been demonstrated. Here we assess the serum resistance of *T. denticola* strain 35405 (Td35405wt) and isogenic mutants deficient in dentilisin (Td35405-CCE) and FhbB production (Td35405-ΔfhbB), respectively. Both dentilisin and FhbB have been postulated to be key virulence factors that mediate complement evasion. Consistent with conditions in the subgingival crevice, an environment with a significant concentration of complement, Td35405wt was resistant to serum concentrations as high as 25%. Deletion of *fhbB* (Td35405ΔfhbB), which resulted in the complete loss of FH binding ability, but not inactivation of dentilisin activity (Td35405-CCE), rendered *T. denticola* highly sensitive to 25% human serum with 80% of the cells being disrupted after 4 h of incubation. Heat treatment of the serum to inactivate complement confirmed that killing was mediated by complement. These results indicate that the FH–FhbB interaction is required for serum resistance whereas dentilisin is not. This report provides new insight into the novel complement evasion mechanisms of *T. denticola*.

INTRODUCTION

Periodontitis is the most common infection of middle-aged adults, affecting nearly 116 million individuals in the USA alone (Beck *et al.*, 1987). The disease process is initiated by the formation and spread of a polymicrobial biofilm that ultimately progresses to plaque formation. Disease severity is determined by factors including composition of the oral flora, host genetic predisposition and underlying disorders such as diabetes (Van Dyke & Serhan, 2003). The human oral cavity contains in excess of 700 bacterial species (Paster *et al.*, 2006) with >400 associated with subgingival plaque (Paster *et al.*, 2001). Of the oral treponemes, *Treponema denticola* is the most abundant. In the healthy subgingival crevice, *T. denticola* represents <1% of the total bacterial population. However, in periodontal pockets this number can

exceed 40% (Dewhirst *et al.*, 2000). *T. denticola* is prevalent at the leading edge of plaque and there is a clear correlation between its abundance and disease severity (Simonson *et al.*, 1988; Ellen & Galimanas, 2005).

T. denticola binds the human complement regulatory protein, Factor H (FH; McDowell *et al.*, 2005, 2009). The sole *T. denticola* FH binding protein is FhbB, an 11.4-kDa, surface-exposed lipoprotein, that is universal among isolates (McDowell *et al.*, 2007). The binding of FH, a negative regulator of complement, to spirochetal surface proteins correlates with complement resistance (Alitalo *et al.*, 2001; Hellwage *et al.*, 2001; Kraiczy *et al.*, 2001a; Meri *et al.*, 2005; Hovis *et al.*, 2008; Grosskinsky *et al.*, 2009; Kenedy *et al.*, 2009). FH serves as a co-factor for the factor I-mediated cleavage of C3b and inhibits the formation of, and accelerates the dissociation of, the C3 convertase complex (Ruddy & Austen, 1969, 1971; Zipfel & Skerka, 2009). Binding of pathogens to FH presented on the surface of host cells can also promote adherence and intracellular localization (Asakawa *et al.*, 2003; Pandiripally *et al.*, 2003; Hammerschmidt *et al.*, 2007). *T. denticola* may also exploit FH in adherence and tissue invasion (McDowell *et al.*, 2005, 2007). Consistent with this, *T. denticola* can be found within a high percentage of gingival crevicular epithelial cells in patients with periodontal disease (Colombo *et al.*, 2006, 2007). The study of FhbB, the smallest known bacterially produced FH binding protein, could provide insight into the minimal molecular determinants required for FH binding, enhance our understanding of the molecular basis of bacterial serum resistance in general, and ultimately provide important information about human hereditary diseases that involve FH deficiencies, such as age-related macular degenerative disease (Jozsi & Zipfel, 2008).

The binding of FH to most bacteria occurs through a direct interaction with one or more surface proteins (Kraiczy & Würzner, 2005). However, there are unique aspects associated with FH binding to *T. denticola*. Factor H is bound to FhbB and then cleaved by the *T. denticola* protease, dentilisin to yield a 50-kDa fragment that is retained on the cell surface (McDowell *et al.*, 2009). Dentilisin is part of the larger chymotrypsin-like protease complex (Fenno *et al.*, 1998a, b). Chymotrypsin-like protease is a multifunctional virulence factor that also contributes to cell-to-cell interactions, tissue destruction, activation of polymorphonuclear leucocytes and C3 cleavage (*in vitro*) (Ohta *et al.*, 1986; Grenier *et al.*, 1990; Fenno *et al.*, 1998a; Ishihara *et al.*, 1998, 2004; Ellen *et al.*, 2000; Chi *et al.*, 2003; Bian *et al.*, 2005; Miyamoto *et al.*, 2006; Yamazaki *et al.*, 2006; Bamford *et al.*, 2007; McDowell *et al.*, 2009). Dentilisin-mediated C3 cleavage activity may modulate complement responses to *T. denticola* (Yamazaki *et al.*, 2006). Hence, two possible mechanisms of complement modulation have been described; dentilisin-mediated cleavage of C3 and cell-bound FH-mediated regulation of the alternative cascade. Circumvention of complement would facilitate survival in gingival crevicular fluid, blood and saliva where complement is present and active (Schenkein & Genco, 1977; Boackle *et al.*, 1978; Boackle, 1991; Schenkein, 1991).

Here we assess the relative contribution of dentilisin and FH binding in serum resistance through the analysis of *fhbB* (Td35405ΔfhbB) and dentilisin (Td35405-CCE) mutants (Bian *et al.*, 2005). The results demonstrate that serum resistance is dependent on the binding of FH to FhbB and not dependent on dentilisin activity. The results presented here further our understanding of the interaction between complement, FhbB and dentilisin and provide insight into the unique complement evasion mechanisms of this important, dominant periodontal pathogen.

METHODS

Bacterial strains, bacterial growth cloning and polymerase chain reaction

Treponema denticola ATCC 35405 (Td35405wt) and the isogenic mutants described below (and in Table 1) were grown in New Oral Spirochete (NOS) broth (ATCC medium 1357) or NOS/GN semi-solid medium (NOS with 0.5% gelatine, 0.5% Noble agar) as previously described (Haapasalo *et al.*, 1991; Chan *et al.*, 1997), with erythromycin (40 μg ml^{-1}) added as appropriate. Cultures were examined by phase-contrast or dark-field microscopy for purity, motility and typical strain morphology. Growth curves were determined (in triplicate) by inoculating 10 ml NOS with 0.03 optical density at 600 nm (OD_{600}) units of cells collected at mid-log phase. Cell numbers were determined from OD_{562} values (24-h intervals; 12 days). Strain Td35405wt was employed

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant features	Source or reference
Td35405wt	Type strain; parent for mutagenesis	ATCC
Td35405-CCE	Dentilisin deficient mutant	Bian *et al.* (2005)
Td35405ΔfhbB	fhbB mutant	This study
Plasmid		
pSY118	*ermF-ermAM* cassette	Kent *et al.* (2004)
pCF537	*ermF-ermAM* replacing 5′ and central portion of *fhbB* between *malL* and *pheS*	This study

ATCC, American type culture collection, Rockville, MD. Plasmid construction, with intermediate steps, is described in Methods section.

for these analyses because its complete genome sequence is known (Seshadri *et al.*, 2004) and its complement interactions have been previously characterized (McDowell *et al.*, 2005, 2007, 2009). *Escherichia coli* JM109 and the pSTBlue-1 vector were employed (Novagen, Gibbstown, NJ) for standard cloning purposes. Oligonucleotides used in this study are listed in Table 2. Polymerase chain reaction (PCR) was performed using standard protocols.

Construction of a *T. denticola fhbB* deletion clone (Td35405ΔfhbB)

The DNA flanking *fhbB* (TDE0108), including most of TDE0107 (*malL*; α-amylase-family protein) and TDE0109 (*pheS*; phenylalanyl-transfer RNA synthetase, α-subunit), was amplified by PCR from genomic DNA using primer pairs CX653–CX654 and CX655–CX656, respectively. The amplicons were individually cloned in pSTBlue-1 to yield pTDE0107 and pTDE0109. The 1.8-kb *Nde*I–*Xho*I fragment of pTDE0109 was ligated to similarly digested pTDE0107 to yield pTDE109-107. The 2.1-kb *ermF-ermB* (*ermR*) cassette (Fletcher *et al.*, 1995) derived from pSY118 (Kent *et al.*, 2004) was released by *Nde*I digestion and ligated into the *Nde*I site located at the junction of TDE0107 and TDE0109 in pTDE109-107 to yield pCF537. The *ermR* cassette in pCF537 is in opposite transcriptional orientation relative to TDE0108. For allelic replacement mutagene-

Table 2 Oligonucleotide primers used in this study

Primer (description)	Sequence (5′–3′)[1]
CX653 (TDE0107 3′ end-R w/*Nde*I)	AACATATGACTACACTATACGTACTG
CX654 (TDE0107 region-F)	CAAAGGCGGGAAATACATAG
CX655 (TDE0109 3′ end-F w/*Nde*I)	GCATATGGTACTAAAGCAGCTG
CX656 (TDE0109 5′ end-R)	TTAGCACACAACCGACAC
CX669 (TDE0108 F for mutant screen)	CCCGTCATTGTTTGCATC
CX670 (TDE0108 R for mutant screen)	CATGGCTCTTGGCTTAAAC
CX234 (ermF/AM 5′ end-R)	ACTCTGAATGGATAAAGTTTGC
CX235 (ermF/AM 3′ end-F)	AGGAAATAATTCTATGAGTCGC
0107F (qRT-PCR primer)	ACTCTCCGCCGTCTTAAATTCGGT
0107R (qRT-PCR primer)	ACGGTATGGGAGTTCTTGCATGGT
RT 0109F (qRT-PCR primer)	CAATTCCATGCAGCCCGCGATATT
RT 0109R (qRT-PCR primer)	ATAATTCCAGCCTCGGCTTCCTGT
RT 0108F (qRT-PCR primer)	ACGCGCTTGAGAATGAATTA
RT 0108R (qRT-PCR primer)	AATCTAATGCAAGGGCTTCAG
RT FlaAF (qRT-PCR primer)	GCTCAGGTTGATGATCAGG
RT FlaAR (qRT-PCR primer)	GCAATTGATTTGATAACGCCG
1191F-LIC (TDE1191 5′ end)	GACGACGACAAGATTTCTAAGACAGCGATAAAGGC
1191R-LIC (TDE1191 3′ end)	GAGGAGAAGCCCGGTTTAGTACTCTCCACTATTGAGC
107-108Int (5′ upstream *fhbB*; primer 3[2])	CTCTTGACAGTACGTATAGTG
0109R (TDE0109 3′ end; primer 4)	CGATATTCATGACGTTTACTAC
FhbB23F-LIC (*fhbB* 5′ end; primer 1)	GACGACGACAAGATTACTTTCAAAATGAATACTGCAC
FhbB78R-LIC (*fhbB* internal; primer 2)	GAGGAGAAGCCCGGTTTAGGGTTTTTTATCCACAATTTG
FhbB102R-LIC (*fhbB* 3′ end)	GAGGAGAAGCCCGGTTTACTTTATCTTTTTGGGTAT

[1]Engineered restriction enzyme site underlined. [2]Primers 1–4 refer to numbering in Fig. 2A.

sis, pCF537 was digested with *Bam*HI and *Xho*I, and electroporated into *T. denticola.* Recombinant strains were selected for ermR (Li *et al.*, 1996; Fenno *et al.*, 1998b).

Measurement of dentilisin activity

The PrtP (dentilisin) protease activity was measured by hydrolysis of succinyl-L-alanyl-L-alanyl-Lprolyl-L-phenylalanine-*p*-nitroanilide (SAAPFNA) (Uitto *et al.*, 1988). Four-day cultures were adjusted to an optical density of 0.25 [absorbance at 600 nm (A_{600})] and diluted 1 : 8 in H_2O. Td35405-CCE served as a negative control for background activity (Bian *et al.*, 2005). After incubation (at 37°C for 45 min), SAAPFNA hydrolysis was measured at A_{450}.

Generation of anti-FhbB antisera

Recombinant FhbB was generated using methods that have been previously described (McDowell *et al.*, 2007). For Anti-FhbB antiserum generation, mice were inoculated with recombinant (r-) FhbB (25 μg in alum) at 0, 2 and 4 weeks and sacrificed at week 6. Mice were terminally bled and the serum was harvested (Metts *et al.*, 2003).

Immunoblot analyses and FH affinity ligand-binding immunoblot (ALBI) assays

r-proteins or cell lysates were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); 15% Criterion Tris–HCl Gels; Bio-Rad, Hercules, CA), transferred to membranes and screened with anti-Msp (Fenno *et al.*, 1998a), anti-PrtP, anti-FlaA (flagellar filament protein) (Fenno *et al.*, 1998a; Bian *et al.*, 2005), or anti-FhbB antiserum using standard methods. Secondary antibody was detected by chemiluminescence using the SuperSignal west pico substrate (Thermo Scientific, Waltham, MA). FH binding was assessed using the FH ALBI assay (McDowell *et al.*, 2003). In brief, membrane immobilized proteins were incubated with purified human FH (10 ng ml^{-1}; Calbiochem, San Diego, CA), washed and bound FH detected using goat anti-human FH antiserum (dilution of 1 : 800; Calbiochem) with rabbit anti-goat immunoglobulin G as the secondary (Pierce, Rockford, IL). As above, detection was by chemiluminescence.

Qualitative and quantitative reverse transcription-PCR (RT-PCR)

RNA was extracted from *T. denticola* strains using the RNeasy RNA extraction kit (Qiagen, Valencia, CA). Complementary DNA was generated using gene-specific primers (Table 2) and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCRs were carried out using Sybr green PCR master mix (Applied Biosciences, Carlsbad, CA) in an MJ Research Opticon 2 real-time thermocycler (95°C for 10 min followed by 40 cycles of 94°C for 15 s; 60°C for 30 s, and 72°C for 30 s). Cycle thresholds were normalized to the *flaA* standard curve based on amplification of genomic DNA, as described previously (Frederick *et al.*, 2008). The data were further normalized against *flaA* (a constitutively expressed gene) transcript levels (McDowell *et al.*, 2007). Data are expressed as the total number of calculated transcripts divided by the total number of *flaA* transcripts.

Serum sensitivity assays

Cells (10^5) from log-phase cultures were incubated in the presence of 10, 25 or 40% complement-certified normal human serum (in NOS; Innovative Research, Novi, MI). Heat inactivation of complement (i.e. heat-inactivated serum) was achieved by incubation of serum at 56°C for 40 min. The serum and NOS used in these analyses were equilibrated to anaerobic conditions by maintenance in anaerobic chamber before use and all reactions and microscopic analyses were conducted under anaerobic conditions. Aliquots of cultures were assessed by dark-field microscopy after 1, 3, 5 and 7 h. The percentage of structurally intact cells (intact cells/total number of intact and morphologically disrupted cells) was determined (six fields; 40 × magnification) (Nevin & Guest, 1967). Three biological replicates were performed.

Factor H proteolysis assays

T. denticola cells (mid-log phase) were recovered by centrifugation, washed, and cell density determined at A_{600}. Aliquots (0.05 OD_{600}) were suspended in phosphate-buffered saline (150 μl with 12.5 μg FH) and incubated at 37°C (1 h). Aliquots of the mixture were removed at different time-points (ranging from 0 to 100 min), assessed by SDS-PAGE, immunoblotted

and screened with anti-human FH antisera using standard procedures.

RESULTS

Analysis of *T. denticola* serum sensitivity

The serum sensitivity of *T. denticola* has not previously been assessed in detail and the strains that were employed in earlier analyses (as discussed below) are, to our knowledge, no longer available (Nevin & Guest, 1967; ter Steeg *et al.*, 1988). To assess the serum sensitivity of *T. denticola* and determine if serum sensitivity is influenced by dentilisin, Td35405wt and Td35405-CCE were incubated with increasing concentrations of human serum. In all experiments, complement-certified human serum was employed. High sensitivity of both strains (<25% survival) was observed at 4 h at 40% serum concentration with intermediate serum survival (60–80%) occurring at 25% serum concentration (Fig. 1). Less than 5% killing was observed at 10% serum concentrations after 4 h and no significant killing occurred with heat-inactivated serum. The results presented here indicate that *T. denticola* is sensitive to physiologically relevant concentrations of complement and that dentilisin is not required for serum resistance.

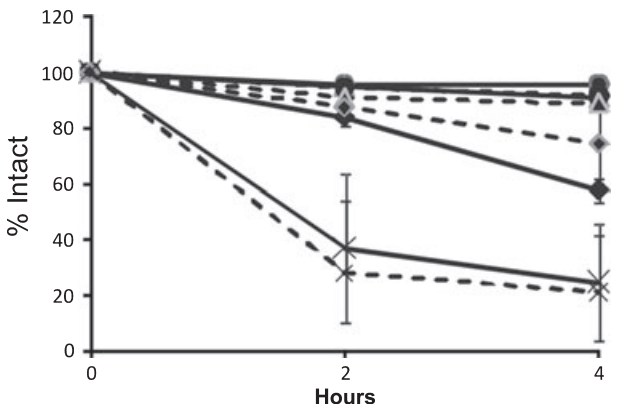

Figure 1 Analysis of the sensitivity of *T. denticola* to human serum. Td35405wt (solid lines) and Td35405-CCE (dashed lines) were incubated in 10% (filled triangle), 25% (filled diamond), and 40% (cross) 'complement certified' normal human serum or in 25% (filled circle) of the same serum lot after heat-inactivation (i.e. heat inactivated serum). At the time-points indicated, % intact was determined through dark-field microscopic analysis of the cultures. The data are presented as the average of three separate experiments with error bars indicating the standard deviations. All methods are detailed in the text.

Inactivation of *fhbB* and characterization of the resulting mutant

Allelic exchange mutagenesis was performed to delete *fhbB* (along with 50 bp of its upstream sequence) and replace it with an *ermR* cassette (Fig. 2A). Several clones harboring the desired mutations were obtained and verified through PCR. The resulting clone selected for further analysis was designated as Td35405ΔfhbB. Note that repeated attempts to restore FhbB by complementation *in trans* were not successful. As reported in earlier studies, complementation of deleted Td35405 genes remains a technically challenging task (Kuramitsu *et al.*, 2005) and to date complementation has been accomplished only in the *T. denticola* 33520 strain (Chi *et al.*, 2002; Slivienski-Gebhardt *et al.*, 2004). The 35405 strain, and not 33520, was used in this study because it is the best characterized strain of *T. denticola* and its genome sequence has been determined (Seshadri *et al.*, 2004). Comparative genome microarray analyses suggest that efforts to complement 35405 are most likely complicated by a restriction modification system that is present in strain 35405 but absent in strain 33520 (R. T. Marconi, unpublished data).

To assess the properties of the strains, immunoblot, FH binding and dentilisin activity assays were conducted. Using anti-FhbB antiserum, FhbB was readily detected in Td35405wt (Fig. 3A) and Td35405-CCE (data not shown) but not in Td35405ΔfhbB. Consistent with FhbB being the sole FH binding protein of *T. denticola*, no FH binding to Td35405ΔfhbB was observed whereas high-level binding was observed with all other strains (Fig. 3A). As controls for the production of other virulence factors, identical immunoblots were screened for Msp (major sheath protein), FlaA and the dentilisin subunit, PrtP. To measure the dentilisin activity of each strain SAAPFNA assays were performed. Deletion of *fhbB* had no affect on dentilisin activity and as expected, no activity was observed in the Td35405-CCE strain (Fig. 3B). To rule out polar effects on genes flanking *fhbB*, which encode an α-amylase family protein (TDE0107; MalL) and a phenylalanyl transfer RNA transferase (TDE0109; PheS), real-time RT-PCR was performed (the organization of genes flanking TDE0108 is depicted in Fig. 2). *FlaA* transcript levels of each strain served as the

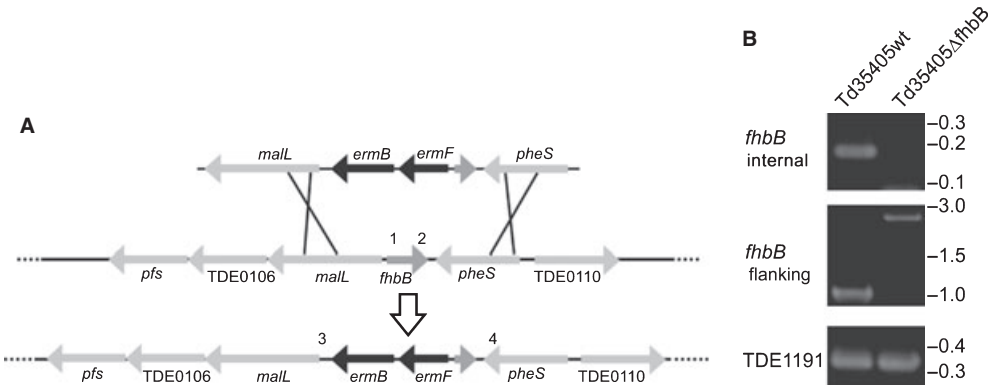

Figure 2 Generation of a *T. denticola* 35405 FhbB deletion mutant (Td35405ΔfhbB). An *fhbB T. denticola* deletion mutant was generated as detailed in the text. The steps that resulted in the double cross-over event are depicted in schematic form in (A). The DNA fragment used to replace *fhbB* consisted of an *ermR* cassette flanked by genes that reside upstream and downstream of *fhbB.* The locations of the primers used for the polymerase chain reaction (PCR) screening are indicated. In (B) the results of PCR analyses designed to confirm deletion of *fhbB* and insertion of the *ermR* cassette are presented. Primers that amplify within *fhbB* (primers 1–2), across the insertion site (primers 3–4) or a control gene (TDE1191) were employed (as indicated in the figure). The migration position of DNA size standards (in kb) are indicated to the right.

normalization control (Fig. 3C). As expected, no *fhbB* mRNA was detected in Td35405ΔfhbB whereas *fhbB* expression in Td35405wt and Td35405-CCE occurred at levels equivalent to that previously reported for this gene (McDowell *et al.*, 2007). Relative to Td35405wt, no significant differences in mRNA levels for *malL* and *pheS* were observed in both Td35405ΔfhbB and Td35405-CCE. Lastly, to determine if inactivation of *fhbB* influences growth rate, growth curves were generated (Fig. 3D). The cells were grown under standard conditions in NOS media (supplemented with 2% rabbit serum). Although the growth rates were similar, Td35405ΔfhbB did grow slightly more slowly than Td35405wt. The minor differences observed could result from physiological changes associated with the loss of FhbB production or the expression of the *ermR* resistance cassette. Alternatively, it is possible that the presence of FhbB in the *T. denticola* outer membrane enhances growth in the presence of serum. It is important to note that the rabbit serum added to standard *T. denticola* cultivation media is not typically heat treated to inactivate complement.

Comparative analysis of serum sensitivity

To determine if deletion of *fhbB* alters serum susceptibility the Td35405wt, Td35405-CCE and Td35405ΔfhbB were incubated with 25% normal human serum. The Td35405wt and Td35405-CCE strains showed >80% and >95% cell survival, respectively, after 7 h (Fig. 4). In stark contrast, by 5 h, only 15% of the Td35405ΔfhbB cells were found to be motile and structurally intact. The role of complement in killing was confirmed as detailed above using heat-inactivated serum. These results provide the first direct evidence for a correlation between FhbB production, FH binding and serum resistance. It can be concluded that dentilisin is not a significant contributor in serum resistance and that FH binding mediated by FhbB is the dominant mechanism.

FhbB is required for dentilisin cleavage of FH

To determine if FH must be bound to the cell surface in an FhbB-dependent manner to allow for cleavage by dentilisin, Td35405wt, Td35405ΔfhbB and Td35405-CCE were incubated with human serum. Aliquots were removed at 10-min intervals up to 100 min, separated by SDS-PAGE, immunoblotted and screened with anti-FH antiserum. Td35405wt, which is $FhbB^+$ and $dentilisin^+$, rapidly cleaved FH. In contrast the kinetics of cleavage was dramatically slower with Td35405ΔfhbB. Almost complete cleavage of the input FH did not occur until the 100-minute time-point. Minimal, if any, cleavage of FH was observed with Td35405-CCE (Fig. 5). This observation indicates that dentilisin-mediated degradation of

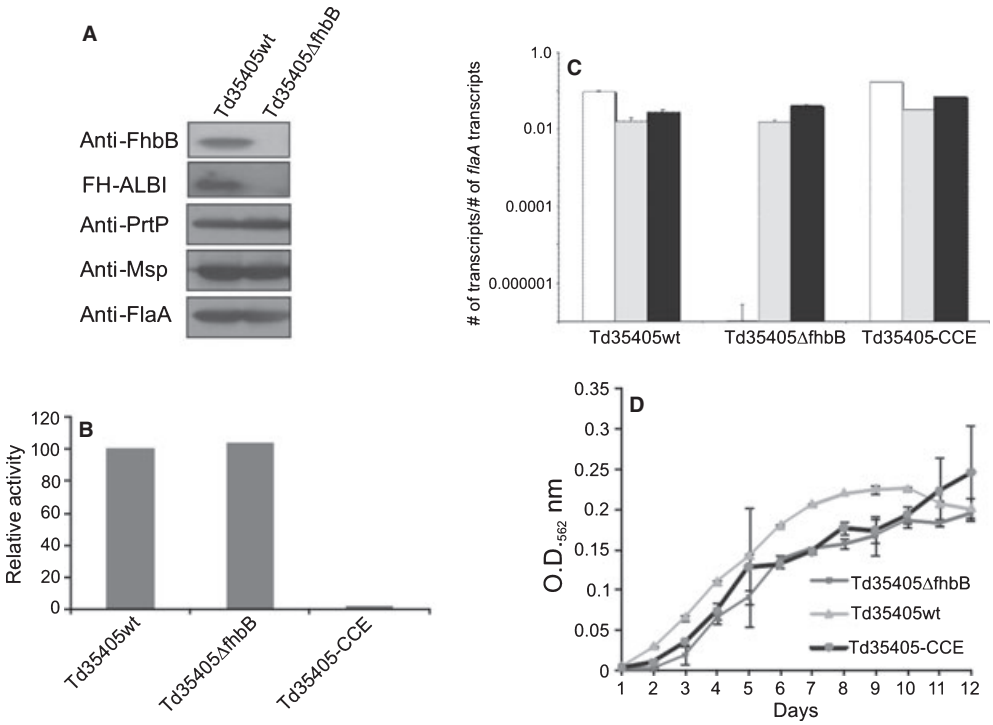

Figure 3 Characterization of Td35405ΔfhbB and Td35405-CCE mutants. (A) presents the results of immunoblot analyses. Cell lysates from Td35405ΔfhbB and Td35405wt were screened with protein-specific antisera as indicated in the figure. FH binding was assessed using the FH ALBI approach. In (B) dentilisin activity was measured by quantifying SAAPFNA hydrolysis. Data shown are mean values of triplicate samples from a representative experiment. (C) Quantitative real-time RT-PCR analyses in which the transcriptional levels of *fhbB* (white bars), *malL* (grey), *pheS* (black) and *flaA* were determined for Td35405wt, Td35405ΔfhbB and Td35405-CCE. The data were normalized against the *flaA* values. In (D), growth curves for each strain are presented. All methods are detailed in the text.

FH occurs at the cell surface of *T. denticola* in an FhbB-dependent manner and that FH cleavage is the result of dentilisin and not attributable to a secreted factor.

DISCUSSION

Factor H binding contributes to the serum resistance of numerous microbial pathogens including spirochete species associated with Lyme disease and relapsing fever (Alitalo *et al.*, 2001; Kraiczy *et al.*, 2001b; Hovis *et al.*, 2004, 2006; Rogers *et al.*, 2009). *T. denticola*, binds FH via its FhbB protein (McDowell *et al.*, 2007) and then uses dentilisin, a *T. denticola* protease (Ishihara *et al.*, 1996, 1998; Ellen *et al.*, 2000; Chi *et al.*, 2003), to cleave FH and generate a 50-kDa FH fragment. This fragment is retained at the cell surface (McDowell *et al.*, 2005). The goals of this study were to assess the complement sensitivity of *T. denticola*, to compare the relative contribution of FhbB-dependent FH binding and the anti-complement activity of dentilisin in serum resistance (Yamazaki *et al.*, 2006), and to determine if FH cleavage by dentilisin is required for serum resistance.

The first step in these analyses was to measure the sensitivity of Td35405wt and Td35405-CCE (dentilisin-deficient mutant) to human serum. To date, the sensitivity of oral treponemes to human serum has not been rigorously assessed. In a study from the 1960s, a *Borrelia vincentii* strain (later reclassified as *T. vincentii*) and two *T. microdentium* strains (MRB and N39) (subsequently reclassified as *T. denticola*) were tested for sensitivity to 40% guinea-pig serum (Nevin & Guest, 1967). *Borrelia vincentii* and *T. microdentium* N39 were killed whereas *T. microdentium* MRB was resistant. Sensitivity to human serum was not tested and the molecular basis for the observed differing serum sensitivities of these strains was not determined. We previously demonstrated that *T. vincentii* lacks *fhbB* (McDowell *et al.*, 2005), which may explain the serum sensitivity of the *B. vincentii* isolate used in the above-mentioned study. A

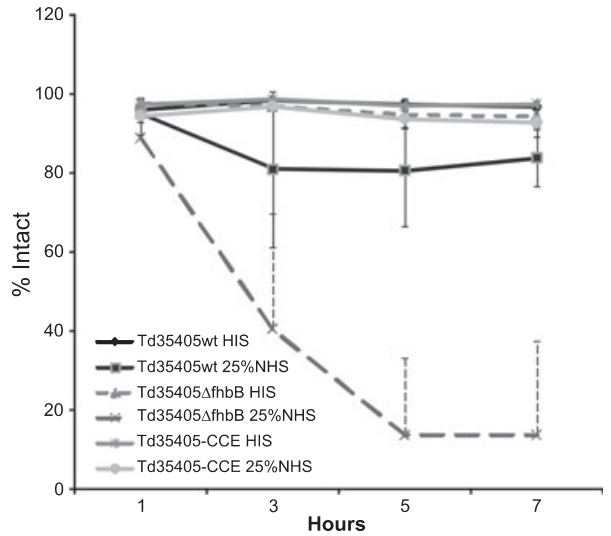

Figure 4 Deletion of *fhbB* but not dentilisin results in increased serum sensitivity. Equal numbers of Td35405wt, Td35405ΔfhbB and Td35405-CCE cells were incubated in 25% normal human serum (NHS) or in 25% heat-inactivated human serum (HIS) for the time interval indicated. The cultures were assessed by dark-field microscopy and the percentages of intact spirochetes were determined at each time-point.

later study from the 1980s demonstrated that an oral treponeme biofilm (of undetermined species composition) was killed by 100% serum but not by heat-inactivated serum (ter Steeg *et al.*, 1988) indicating that complement is the likely basis for the observed serum-mediated killing. Unfortunately, the specific isolates employed in the analyses described above are not, to our knowledge, available for study. Relative to that of other spirochetes, the serum resistance level of *T. denticola* can be described as moderate. *Borrelia burgdorferi* for example, which produces as many as five FH binding proteins, is highly resistant and no killing occurs in the presence of 50% human serum (Hellwage *et al.*, 2001; Kraiczy *et al.*, 2001c; Alitalo *et al.*, 2002; McDowell *et al.*, 2003). The moderate serum resistance level of *T. denticola* is consistent with, and sufficient for, survival in the levels of complement that are present in the subgingival crevice.

To test the hypothesis that FH binding by FhbB is the key determinant of *T. denticola* complement resistance, an *fhbB* deletion mutant (Td35405ΔfhbB) was generated. The successful deletion of *fhbB* was confirmed using multiple approaches. Analyses of the serum sensitivity of Td35405wt, Td35405-CCE and Td35405ΔfhbB strains revealed that deletion of *fhbB* renders cells highly susceptible to complement-mediated killing. Percentage intact values for Td35405wt, Td35405-CCE and Td35405ΔfhbB after 7 h in 25% serum were 85, 90 and 15%, respectively. These results provide the first direct evidence for the important role that the FH–FhbB interaction plays in complement evasion. In addition, the results demonstrate that dentilisin activity is not required for serum resistance. This suggests that even though dentilisin has inherent C3 cleavage activity, the net effect of this activity is minor and does not influence serum sensitivity as has been previously postulated (Yamazaki *et al.*, 2006). Furthermore, it can also be concluded that FH cleavage by dentilisin is not required for the serum resistance phenotype. Future studies will seek to determine what potential biological role the surface-associated 50 kDa FH fragment plays in *T. denticola* pathogenesis. We previously postulated that *T. denticola* uses cell-bound FH domains to facilitate binding to host cells and so aid in the expansion of the subgingival oral biofilm (McDowell *et al.*, 2009).

To determine if FH must bind to FhbB to be cleaved by dentilisin, each strain was incubated with purified FH and then FH cleavage was assessed over time. Factor H incubated with the Td35405wt strain was almost completely cleaved after 30 min. In contrast, the same level of FH degradation in the presence of the Td35405ΔfhbB strain required three

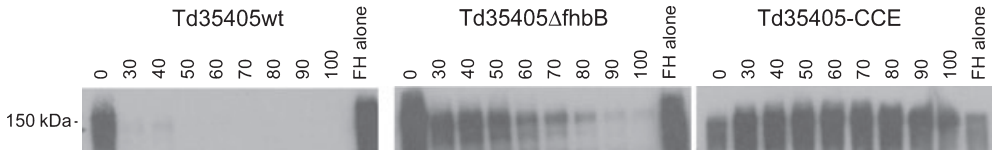

Figure 5 Proteolytic digestion of FH by *T. denticola* is dependent on dentilisin and is facilitated by the production of FH binding protein B (FhbB). Cells of each strain were incubated with purified human FH and aliquots were collected at 10-min intervals as indicated above each lane. The aliquots were fractionated by SDS-PAGE, transferred to membranes and FH degradation was assessed using anti-FH antisera as detailed in the text. As a control, FH was incubated without cells (FH alone) to assess possible non-specific degradation.

times longer to occur and no cleavage was observed with the Td35405-CCE dentilisin mutant. These results demonstrate several things. First, it is clear that dentilisin is the sole *T. denticola* protease involved in FH cleavage. The efficiency of FH cleavage is clearly dependent on the presence of FhbB. It is our hypothesis that FhbB must bind FH to orient FH at the cell surface in a manner that allows for FH cleavage by dentilisin.

In summary, this study provides new insight into the mechanistic basis of serum resistance of *T. denticola.* Using dentilisin and FhbB-deficient strains we demonstrate that serum resistance is strictly dependent on the production of FhbB and not on the production of dentilisin. This study is the first to provide direct evidence of a central role of FH binding in the serum resistance of an oral spirochete. As bleeding increases in patients with periodontal disease and complement levels rise in the periodontal pocket, there would be a clear selective advantage for those species that are serum resistant. The inherent serum resistance of *T. denticola* could be an important factor that allows for its significant overgrowth relative to that of other species in the periodontal pocket.

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