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

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SUMMARY

Treponema denticola, a periodontal pathogen, binds the complement regulatory protein, Factor H (FH). FhbB (FH binding protein B) 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 ~50kDa 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 has 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)fhbB), respectively. Both dentilisin and FhbB have been postulated to be key virulence factors that mediate complement evasion. Consistent with conditions in the sub-gingival crevice, an environment with a significant concentration of complement, Td35405wt was resistant to serum concentrations as high as 25%. Deletion of *fhbB* (Td35405)fhbB), 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 hours 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 while dentilisin is not. This report provides new insight into the novel complement evasion mechanisms of *T. denticola*.

Keywords

Periodontitis; FhbB; Factor H; *Treponema denticola*; Dentilisin

INTRODUCTION

Periodontitis is the most common infection of middle-aged adults affecting nearly 116 million individuals in the United States alone (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,

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host genetic predisposition and underlying disorders such as diabetes (Van Dyke and 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 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 (reviewed in (Ellen and Galimanas, 2005, Simonson et al., 1988)).

T. denticola binds the human complement regulatory protein, Factor H (FH) (McDowell et al., 2005, McDowell et al., 2009). The sole *T. denticola* FH binding protein is FhbB, an 11.4kDa, 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 (Kenedy et al., 2009, Alitalo et al., 2001, Hellwage et al., 2001, Kraiczy et al., 2001a, Meri et al., 2005, Grosskinsky et al., 2009, Hovis et al., 2008). FH serves as a cofactor for the factor I-mediated cleavage of C3b and inhibits the formation of, and accelerates the dissociation of, the C3 convertase complex (Ruddy and Austen, 1969, Ruddy and Austen, 1971, Zipfel and Skerka, 2009). Binding of pathogens to FH presented on the surface of host cells can also promote adherence and intracellular localization (Pandiripally et al., 2003, Hammerschmidt et al., 2007, Asakawa et al., 2003). *T. denticola* may also exploit FH in adherence and tissue invasion (McDowell et al., 2005, McDowell et al., 2007). Consistent with this, *T. denticola* can be found within a high percentage of gingival crevicular epithelial cells in periodontal disease patients (Colombo et al., 2007, Colombo et al., 2006). 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 and Zipfel, 2008).

The binding of FH to most bacteria occurs through a direct interaction with one or more surface proteins (Kraiczy and Würzner, 2005). However, there are unique aspects associated with FH binding to *T. denticola*. FH is bound to FhbB and then cleaved by the *T. denticola* protease, dentilisin to yield a 50kDa fragment that is retained on the cell surface (McDowell et al., 2009). Dentilisin is part of the larger chymotrypsin-like protease (CTLP) complex (Fenno et al., 1998a, Fenno et al., 1998b). CTLP is a multi-functional virulence factor that also contributes to cell to cell interactions, tissue destruction, activation of polymorphonuclear leukocytes and C3 cleavage (in vitro) (McDowell et al., 2009, Ellen et al., 2000, Yamazaki et al., 2006, Bamford et al., 2007, Miyamoto et al., 2006, Ishihara et al., 2004, Ishihara et al., 1998, Fenno et al., 1998a, Grenier et al., 1990, Ohta et al., 1986, Bian et al., 2005, Chi et al., 2003). 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, 1991, Schenkein and Genco, 1977, Boackle et al., 1978, Boackle, 1991).

Here we assess the relative contribution of dentilisin and FH binding in serum resistance through the analysis of *fhbB* (Td35405)fhbB) 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 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 PCR

T. denticola ATCC 35405 (Td35405wt) and isogenic mutants described below (and in Table 1) were grown in NOS broth (New Oral Spirochete broth; ATCC medium 1357) or NOS/GN semisolid medium (NOS with 0.5% gelatin, 0.5% Noble agar) as previously described (Chan et al., 1997, Haapasalo et al., 1991), with erythromycin (Em, 40 :g/ml) 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 10mL of NOS with 0.03 OD₆₀₀ units of cells collected at mid-log phase. Cell numbers were determined from OD₅₆₂ values (24 h intervals; 12 days). Strain Td35405wt was employed 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., 2009, McDowell et al., 2007, McDowell et al., 2005). *E. coli* JM109 and the pSTBlue-1 vector were employed (Novagen) for standard cloning purposes. Oligonucleotides used in this study are listed in table 2. PCR was performed using standard protocols.

Construction of a *T. denticola fhbB* deletion clone (Td35405)fhbB)

DNA flanking *fhbB* (TDE0108), including most of TDE0107 (*malL*; alpha amylase-family protein) and TDE0109 (*pheS*; phenylalanyl-tRNA synthetase, alpha subunit), was PCR amplified 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 NdeI-XhoI fragment of pTDE0109 was ligated to similarly digested pTDE0107 to yield pTDE109-107. The 2.1 kb *ermF-ermB* (*erm^R*) cassette (Fletcher et al., 1995) derived from pSY118 (Kent et al., 2004) was released by NdeI digestion and ligated into the NdeI site located at the junction of TDE0107 and TDE0109 in pTDE109-107 to yield pCF537. The *erm^R* cassette in pCF537 is in opposite transcriptional orientation relative to TDE0108. For allelic replacement mutagenesis, pCF537 was digested with BamHI and XhoI, and electroporated into *T. denticola*. Recombinant strains were selected for *erm^R* (Fenno et al., 1998b, Li et al., 1996).

Measurement of dentilisin activity

PrTP (dentilisin) protease activity was measured by hydrolysis of succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (SAAPFNA) (Uitto et al., 1988). Four-day cultures were adjusted to an optical density of 0.25 (A₆₀₀) and diluted 1:8 in H₂O. Td35405-CCE served as a negative control for background activity (Bian et al., 2005). After incubation (37°C; 45 m), SAAPFNA hydrolysis was measured at A₄₅₀.

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 r-FhbB (25 :g in alum) at 0, 2 and 4 wks and sacrificed at wk 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 SDS-PAGE (15% Criterion Tris-HCl Gels; Biorad), 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). 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; Calbiochem), washed and bound FH detected using goat anti-human FH antiserum (dilution of 1:800; Calbiochem) with rabbit anti-goat IgG as the secondary (Pierce). 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). cDNA was generated using gene specific primers (Table 2) and Superscript III reverse transcriptase (Invitrogen). Real-time PCRs were carried out using Sybr green PCR master mix (Applied Biosciences) in an MJ Research Opticon 2 real-time thermocycler (95EC for 10 m followed by 40 cycles of 94EC for 15 s; 60EC for 30 s, and 72EC 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 is expressed as the total number of calculated transcripts divided by the total number of *flaA* transcripts.

Serum sensitivity assays

10^5 cells from a log phase cultures were incubated in the presence of 10, 25 or 40% complement certified normal human serum (in NOS; Innovative Research). Heat inactivation of complement (i.e., heat-inactivated serum; HIS) was achieved by incubation of serum at 56EC for 40min. The serum and NOS used in these analyses were equilibrated to anaerobic conditions by maintenance in anaerobic chamber prior to use and that all reactions and microscopic analyses were conducted under anaerobic conditions. Aliquots of cultures were assessed by darkfield microscopy after 1, 3, 5, and 7 h. The percentage of structurally intact cells (intact cells/total # of intact and morphologically disrupted cells) was determined (6 fields; 40 $\times$ magnification) (Nevin and Guest, 1967). Three biological replicates were performed.

FH proteolysis assays

T. denticola cells (mid-log phase) were recovered by centrifugation, washed, and cell density determined by at A_{600} . Aliquots (0.05 OD₆₀₀) were suspended in PBS (150 :l with 12.5 :g of FH) and incubated at 37 EC (1 h). Aliquots of the mixture were removed at different time points (ranging from 0 to 100 m), assessed by SDS-PAGE, immunoblotted, and screened with anti-human factor H 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 and Guest, 1967, ter Steeg, 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 hours at 40% serum concentration with intermediate serum survival (60 to 80%) occurring at 25% serum concentration (Figure 1). Less than 5% killing was observed at 10% serum concentrations after 4hrs and no

significant killing occurred with HIS. 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.

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 *erm^R* cassette (Figure 2; panel A). Several clones harboring the desired mutations were obtained and verified through PCR. The resulting clone selected for further analysis was designated as Td35405)fhbB. 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 (Slivinski-Gebhardt et al., 2004; Chi et al., 2002). 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 35405 but absent in strain 33520 (Marconi, R.T., 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 (Figure 3; panel A) and Td35405-CCE (data not shown) but not in Td35405)fhbB. Consistent with FhbB being the sole FH binding protein of *T. denticola*, no FH binding to Td35405)fhbB was observed while high level binding was observed with all other strains (Figure 3; panel A). 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 effect on dentilisin activity and as expected, no activity was observed in the Td35405-CCE strain (Figure 3; panel B). To rule out polar effects on genes flanking *fhbB* which encode an alpha amylase family protein (TDE0107; MalL) and a phenylalanyl tRNA transferase (TDE0109; PheS), real time RT-PCR was performed (the organization of genes flanking TDE0108 is depicted in Figure 2). *FlaA* transcript levels of each strain served as the normalization control (Figure 3; panel C). As expected, no *fhbB* mRNA was detected in Td35405)fhbB while *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 Td35405)fhbB and Td35405-CCE. Lastly, to determine if inactivation of *fhbB* influences growth rate, growth curves were generated (Figure 3; panel D). The cells were grown under standard conditions in NOS media (supplemented with 2% rabbit serum). While the growth rates were similar, Td35405)fhbB did grow slightly slower than Td35405wt. The minor differences observed could result from physiological changes associated with the loss of FhbB production or the expression of the *erm^R* 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 Td35405)fhbB were incubated with 25% NHS. The Td35405wt and Td35405-CCE strains showed greater than 80% and 95% cell survival, respectively, after 7 hr (Figure 4). In stark contrast, by 5 hr only 15% of the Td35405)fhbB cells were found to be motile and

structurally intact. The role of complement in killing was confirmed as detailed above using HIS. 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 so as to allow for cleavage by dentilisin, Td35405wt, Td35405fhbB and Td35405-CCE were incubated with human serum. Aliquots were removed at 10 min intervals out to 100 minutes, separated by SDS-PAGE, immunoblotted and screened with anti-FH antiserum. Td35405wt, which is FhbB+ dentilisin+, rapidly cleaved FH. In contrast the kinetics of cleavage were dramatically slower with Td35405fhbB. Nearly complete cleavage of the input FH did not occur until the 100 minute timepoint. Minimal, if any, cleavage of FH was observed with Td35405-CCE (Figure 5). This observation indicates that dentilisin mediated degradation of FH occurs at the cell surface of *T. denticola* in a FhbB dependent manner and that FH cleavage is due to dentilisin and not attributable to a secreted factor.

DISCUSSION

FH binding contributes to the serum resistance of numerous microbial pathogens including spirochetal species associated with Lyme disease and relapsing fever (Alitalo et al., 2001, Rogers et al., 2009, Hovis et al., 2004, Hovis et al., 2006, Kraiczy et al., 2001b). *T. denticola*, binds FH via its FhbB protein (McDowell et al., 2007) and then utilizes dentilisin, a *T. denticola* protease (Ishihara et al., 1996, Ellen et al., 2000, Chi et al., 2003, Ishihara et al., 1998), 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*, compare the relative contribution of FhbB dependent-FH binding and the anti-complement activity of dentilisin in serum resistance (Yamazaki et al., 2006) and 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 1960's, 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 and Guest, 1967). *B. vincentii* and *T. microdentium* N39 were completely killed while *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 later study from the 1980's demonstrated that an oral treponeme biofilm (of undetermined species composition) was killed by 100% serum but not by heat-inactivated serum (ter Steeg, 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. *B. burgdorferi* for example, which produces as many as 5 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 *fhhB* deletion mutant (Td35405)fhhB) was generated. The successful deletion of *fhhB* was confirmed using multiple approaches. Analyses of the serum sensitivity of Td35405wt, Td35405-CCE and Td35405)fhhB strains revealed that deletion of *fhhB* renders cells highly susceptible to complement mediated killing. Percent intact values for Td35405wt, Td35405-CCE and Td35405)fhhB after 7 hrs 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 from 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* utilizes cell bound FH domains to facilitate binding to host cells and thus aid in the expansion of the subgingival oral biofilm (McDowell et al., 2009).

To determine if FH must bind to FhbB in order to be cleaved by dentilisin, each strain was incubated with purified FH and then FH cleavage was assessed over time. FH incubated with the Td35405wt strain was almost completely cleaved 30 minutes. In contrast the same level of FH degradation in the presence of the Td35405)fhhB strain required 3 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 in order 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 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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Figure 1. Analysis of the sensitivity of *T. denticola* to human serum

Td35405wt (solid lines) and Td35405-CCE (dashed lines) were incubated in 10% (▼), 25% (⊗), and 40% (▲) “complement certified” normal human serum (NHS) or in 25% (⊠) of the same serum lot after heat-inactivation (i.e., heat inactivated serum; HIS). At the time points indicated, percent 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 deviation. All methods are detailed in the text.

Figure 2. Generation of a *T. denticola* 35405 FhbB deletion mutant (Td35405)fhbB)

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 Panel A. The DNA fragment used to replace *fhbB* consisted of an *erm^R* cassette flanked by genes that reside up and downstream of *fhbB*. The location of primers used for PCR screening are indicated. In Panel B the results of PCR analyses designed to confirm deletion of *fhbB* and insertion of the *erm^R* 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.

Figure 3. Characterization of Td35405)fhbB and Td35405-CCE mutants

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

Figure 4. Deletion of *fhhB* but not dentilisin results in increased serum sensitivity

Equal numbers of Td35405wt, Td35405ΔfhhB, 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.

Figure 5. Proteolytic digestion of factor H by *T. denticola* is dependent on dentilisin and is facilitated by the production of FhbB

Cells of each strain were incubated with purified human FH and aliquots were collected at 10 minute intervals as indicated above each lane. The aliquots were fractionated by SDS-PAGE, transferred to membranes and FH degradation 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.

Table 1

Strains and plasmids used in this study

Strain or plasmid	Relevant features	Source or reference ^a
Td35405wt	Type strain; parent for mutagenesis	ATCC
Td35405-CCE	Dentilisin deficient mutant	(Bian et al., 2005)
Td35405)fhbB	)fhbB mutant	This study
Plasmid		
pSY118	<i>ermF-ermAM</i> cassette	(Kent et al., 2004)
pCF537	<i>ermF-ermAM</i> replacing 5' and central portion of <i>fhbB</i> between <i>malL</i> and <i>PheS</i>	This study

^a ATCC, American Type Culture Collection, Rockville, MD.

^b Plasmid construction, with intermediate steps, described in Methods.

Table 2

Oligonucleotide primers used in this study

Primer (Description)	Sequence (5'-3') ^a
CX653 (TDE0107 3'end-R w/NdeI)	AACATATGACTACACTATACGTACTG
CX654 (TDE0107 region-F)	CAAAGGCGGGAAATACATAG
CX655 (TDE0109 3'end-F w/NdeI)	<u>G</u> CATATGGTACTAAAGCAGCTG
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)	ACTCTGAATGGATAAAAGTTTGC
CX235 (ermF/AM 3'end-F)	AGGAAATAATTCTATGAGTCGC
0107F (qRT-PCR primer)	ACTCTCCGCCGTCTTAAATTCGGT
0107R (qRT-PCR primer)	ACGGTATGGGAGTTCTTGCATGGT
RT 0109F (qRT-PCR primer)	CAATTCCATGCAGCCCGGATATT
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)	GACGACGACAAGATTCTAAGACAGCGATAAAGGC
1191R-LIC (TDE1191 3'end)	GAGGAGAAGCCCGTTTAGTACTCTCCACTATTGAGC
107–108Int (5'Upstream <i>fhbB</i> ; primer 3 ^b)	CTCTTGACAGTACGTATAGTG
0109R (TDE0109 3'end; primer 4)	CGATATTCATGACGTTTACTAC
FhbB23F-LIC (<i>fhbB</i> 5'end; primer 1)	GACGACGACAAGATTACTTTCAAATGAATACTGCAC
FhbB78R-LIC (<i>fhbB</i> internal; primer 2)	GAGGAGAAGCCCGTTTAGGGTTTTTATCCACAATTTG
FhbB102R-LIC (<i>fhbB</i> 3' end)	GAGGAGAAGCCCGTTTACTTTATCTTTTGGGTAT

^aEngineered restriction enzyme site underlined.^bPrimers 1–4 refer to numbering in Figure 2A.