A Metalloproteinase Karilysin Present in the Majority of *Tannerella forsythia* Isolates Inhibits All Pathways of the Complement System

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A Metalloproteinase Karilysin Present in the Majority of *Tannerella forsythia* Isolates Inhibits All Pathways of the Complement System

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*Tannerella forsythia* is a poorly studied pathogen despite being one of the main causes of periodontitis, which is an inflammatory disease of the supporting structures of the teeth. We found that despite being recognized by all complement pathways, *T. forsythia* is resistant to killing by human complement, which is present at up to 70% of serum concentration in gingival crevicular fluid. Incubation of human serum with karilysin, a metalloproteinase of *T. forsythia*, resulted in a decrease in bactericidal activity of the serum. *T. forsythia* strains expressing karilysin at higher levels were more resistant than low-expressing strains. Furthermore, the low-expressing strain was significantly more opsonized with activated complement factor 3 and membrane attack complex from serum compared with the other strains. The high-expressing strain was more resistant to killing in human blood. The protective effect of karilysin against serum bactericidal activity was attributable to its ability to inhibit complement at several stages. The classical and lectin complement pathways were inhibited because of the efficient degradation of mannose-binding lectin, ficolin-2, ficolin-3, and C4 by karilysin, whereas inhibition of the terminal pathway was caused by degradation of C5. Interestingly, karilysin was able to release biologically active C5a peptide in human plasma and induce migration of neutrophils. Importantly, we detected the karilysin gene in >90% of gingival crevicular fluid samples containing *T. forsythia* obtained from patients with periodontitis. Taken together, the newly characterized karilysin appears to be an important virulence factor of *T. forsythia* and might have several important implications for immune evasion.

Periodontitis is a very common disease, and it is primarily the result of colonization of the subgingival surfaces of teeth by bacteria. The complex interaction between these bacteria, which harbor many virulence factors, and the host’s immune response results in localized chronic inflammation and subsequent destruction of the supporting structures of the tooth. Furthermore, growing evidence implies periodontitis as an important factor in development of cardiovascular diseases and rheumatoid arthritis (1, 2). *Tannerella forsythia* is an anaerobic Gram-negative bacterium, which is considered a major bacterial periodontal pathogen in humans. *T. forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola* form a “red complex” of bacterial species strongly associated with severe, chronic periodontitis (3). Many independent studies on different populations around the world have demonstrated a higher frequency of *T. forsythia* in subgingival plaque in patients with periodontitis, including aggressive periodontitis, compared with healthy volunteers (4). *T. forsythia* is very frequently present in subgingival plaques together with *P. gingivalis* (5). It appears that no single species is “etiologic” for periodontal diseases progression but that several bacterial species exist as complexes within the biofilm matrix in the oral cavity and that these complexes are required to initiate the disease.

Proteases are crucial virulence factors produced by many periodontal pathogens. Apart from the generation of essential nutrients by host protein degradation, proteases are also essential for protection of the bacteria from the host’s defenses, such as the complement system (6, 7). Complement is a major arm of the innate immune defense system, and one of its main functions is to recognize and destroy microorganisms (for a comprehensive review, see Ref. 8). The three pathways of human complement ensure that virtually any nonhost surface is recognized as hostile. The classical pathway is usually mediated by binding of the C1 complex (composed of recognition molecule C1q and two proteases C1r and C1s) to invading pathogens, either directly or via IgG. The lectin pathway is able to recognize, via collectins such as mannose-binding lectin (MBL)/ficolin complexes (composed of MBL or ficolins and MBL-associated proteases 1, 2, and 3),
polysaccharide molecules normally present only on microbial surfaces. Finally, complement can also be activated through the alternative pathway, which is not so much an activation pathway but a failure to appropriately regulate the constant low-level spontaneous activation of C3 as a result of the inherent instability of this protein. All three pathways lead to opsonization of the pathogen with an activated form of complement factor C3 (C3b), which enhances phagocytosis by phagocytes. Furthermore, anaphylatoxins C5a and C3a are released as byproducts to attract phagocytes to the site of infection. Finally, the end result of the complement cascade is formation of the membrane attack complex (MAC) and bacterial cell lysis. Host cells protect themselves from bystander damage following complement activation through the expression or recruitment of endogenous membrane-bound or soluble complement inhibitors.

The effect of complement on T. forsythia has not been studied, and it is unknown if the species is recognized by complement and whether it can resist a putative attack in a similar manner to P. gingivalis (6, 9) or Prevotella intermedia (10). However, it appears that every successful human pathogen able to establish persistent infection must develop a means to circumvent complement, and therefore, various strategies have been developed. Many bacteria are able to capture and use human complement inhibitors such as C4b-binding protein and factor H, thereby avoiding opsonization and lysis (11–13). Herpes viruses, in contrast, produce their own homologs of complement inhibitors (14). Many bacteria are able to capture and use human complement inhibitors as C4b-binding protein and factor H, thereby avoiding opsonization and lysis (11–13). Herpes viruses, in contrast, produce their own homologs of complement inhibitors (14). Furthermore, many bacteria use proteinases to incapacitate components of complement. For example, most strains of P. gingivalis are resistant to bacteriolytic activity of human serum (15, 16), and the gingipain proteinases are the major factor providing protection against complement (6, 9, 17–19). In a strong contrast to P. gingivalis and P. intermedia, very little is known about proteinases of T. forsythia. We have recently cloned and characterized a new proteinase karilysin from the T. forsythia ATCC 43037 strain (20). Karylsin is a metalloproteinase with a primary structure similar to matrix metalloproteinases (MMPs) and is able to cleave elastin, fibrinogen, and fibronecin (20). Full-length prokarilysin (proKly, 50 kDa) undergoes autocatalytic processing. After first cleavage of the N terminus at the Asn14–Tyr15 peptide bond, the fully active enzyme is generated (Kly48, 48 kDa). Sequentially, two cleavages at the C terminus release high molecular mass karylsin (Kly38, 38 kDa) and low molecular mass karylsin (Kly18, 18 kDa). All three forms of karylsin are active to varying degrees against substrates studied so far. Further characterization of karylsin was provided in our recent study solving the three-dimensional structure of Kly18. Interestingly, phylogenetic and sequence similarity analysis revealed much closer evolutionary relation of Kly18 to mammalian MMPs than to bacterial counterparts, and we proposed that this proteinase is a xenologue of mammalian MMPs co-opted by the bacterium through the very rare phenomenon of horizontal gene transfer between bacteria and humans (21).

Because of its unique growth requirements (22) and the fact that T. forsythia is difficult to culture, there have been few investigations into the virulence factors of this bacterial species. In the current study, we found that karylsin is an effective inhibitor of all complement pathways and one of the factors contributing to serum resistance of T. forsythia.

Materials and Methods
Ethics statement
The ethical board of Lund University (Malmö, Sweden) has approved collection of blood from healthy human volunteers. The ethical committee of Jena University (Jena, Germany) approved collection of periodontal plaques and gingival crevicular fluid (GCF). Written informed consent was obtained from patients and volunteers, and the investigation was performed according to the principles of the Declaration of Helsinki.

Sera
Normal human serum (NHS) was obtained from six healthy volunteers. Sera deficient from various complement components as well as matching NHS were obtained from Quidel. In all experiments, pooled sera were used, which were tested for the Ab titers against T. forsythia and Escherichia coli to ensure that they contain similar amounts of Abs against different bacteria, which is important in killing assays (data not shown).

Proteins
Purified complement proteins C4 and C5 were purchased from Complement Technology, whereas human MBL was purchased from the State Serum Institute (Copenhagen, Denmark). Recombinant human ficolins-2 and ficolin-3 were generated as previously described for ficolin-2 (23).

Karylsin as well as its inactive mutant proKlyE136A (the catalytic glutamatic acid was replaced by alanine) were expressed as GST-tagged recombinant proteins in E. coli and purified by affinity chromatography on glutathione-Sepharose 4 Fast Flow (Amersham Biosciences). The GST tag was removed from recombinant proteins bound to glutathione-Sepharose by cleavage with PreScission Proteaseinase (Amersham Biosciences). The second step of purification was size exclusion chromatography (Superdex 75 HiLoad 16/60; Pharmacia Biotech) performed to remove the GST-cleaving enzyme. The following forms of karylsin were isolated and used in subsequent experiments: Kly48, high molecular mass karylsin (Kly38), low molecular mass karylsin (Kly18), and the inactive mutant proKlyE136A. Arginine-specific (HRgpA and RgpB) and lysine-specific (Kgp) gingipains were purified from the P. gingivalis HG66 strain culture fluid as described previously (6). Before using in any assay, gingipains were preactivated for 15 min by incubation in a buffer specific for the particular assay supplemented with 2 mM DTT.

Abs
The following Abs against human Ags were used throughout this study: polyclonal (pAb) rabbit anti-clq, -C4c, and -C3d Abs (all from DakoCytomation), goat anti-MBL (R&D Systems), goat anti-C5 (Quidel), rabbit-anti-C5b9 neopeptioid (CompTech), and mouse anti–ficolin-2 (24) or anti–ficolin-3 (25) mAbs. FITC-labeled Abs anti-C3c, as well as secondary pAb conjugated with HRP against rabbit, goat, or mouse, and secondary pAb conjugated with FITC, were from DakoCytomation.

Bacterial strains and their culture
T. forsythia strains ATCC 43037, ATCC 700198, and BC70-14/2010 were grown on hemin-N2 agar plates or in LB broth. Bacterial strains used in this study are listed in Table I.

Bactericidal assay
Strain E. coli DH5a was cultured in LB broth until exponential growth phase. Cells were harvested, washed once in barbiturate buffer with dextrose and gelatin (GVBB) (5 mM veronal buffer [pH 7.3], 140 mM NaCl, 0.1% gelatin, 1 mM MgCl2, and 5 mM CaCl2), and adjusted to an OD of 0.500 nm of 0.25. NHS was diluted in GVB+ to a concentration of 2% and incubated with various concentrations of karylsin variants for 20 min at room temperature. Thereafter, 106 bacteria cells were added and incubated with serum supplemented with karylsin for 20 min at 37˚C in a total volume of 50 µL. After incubation, aliquots were removed, diluted serially, and plated onto LB agar plates. Heat-inactivated NHS (ΔNHS) (56˚C, 30 min) was used as a negative control. Plates were incubated for 12 h in 37˚C, after which, colonies were counted, and the numbers of surviving bacteria (colony-forming units per milliliter) were calculated.

All bacterial strains (Table I) were harvested from agar plates and washed once in GVB+ and adjusted to OD600 of 0.02–0.04, except from P. intermedia ATCC 25611, for which, OD600 of 0.2–0.4 was used. Thereafter, bacteria was mixed anaerobically with 30 and 75% NHS diluted in GVB+ or as control 30% and 75% ΔNHS in a total volume of 110 µL. The samples were incubated anaerobically at 37˚C for 1.5 h, shaking vigorously. After incubation, aliquots were removed, serially diluted, and spread
on appropriate agar plates. Plates were incubated in an anaerobic chamber for 5–7 d (P. gingivalis and P. intermedia) or 10–14 d (T. forsythia) or aerobic conditions for 24 h (E. coli strains), after which, colonies were counted, and bacterial survival was calculated.

**Complement activation on T. forsythia**

*T. forsythia* ATCC43037 from 10- to 14-old agar plate cultures were harvested and washed once in GVB++ (classic/lectin pathway) or MgEGTA (2.5 mM veronal buffer [pH 7.3] containing 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM MgCl₂, and 10 mM EGTA; alternative pathway) and adjusted to an OD 0.5-0.7 at 600 nm. Thereafter, 140 µl bacteria was mixed with 1–30% NHS diluted in GVB++ or 2–30% NHS diluted in MgEGTA and incubated anaerobically for 1 h at 37°C in a total volume of 200 µl. ANHS (30%) was used as a control. Similar experiments were performed using commercial NHS and deficient sera (Quidel). *T. forsythia* ATCC43037, treated as above, was incubated with 10% Clq-C2, and factor B (FB)-deficient serum, NHS, and ANHS in GVB++ and 10% FB-deficient serum, NHS, and ANHS in MgEGTA. Then, bacteria were washed once in FACS buffer (50 mM HEPES, 100 mM NaCl [pH 7.4], 1% BSA, and 30 mM NaNO₃) and incubated with specific Abs against C3c, conjugated with FITC (DakoCytomation) for 45 min at room temperature. The geometric mean fluorescence intensity (GMFI) was calculated for all the samples using FlowJo software (Tree Star).

**Whole-blood killing assay**

*T. forsythia* strains (ATCC 43037, ATCC 700198, and Be70-14/2010) were washed in PBS buffer and adjusted to the approximate concentration of 10⁶ cells/ml. Bacteria (50 µl) were mixed with 250 µl freshly collected human blood treated with 50 µg/ml Reffudan (Pharmion), and incubated anaerobically for 15 min. After incubation, aliquots were removed, diluted serially, and plated on hemin N-acetylglucosamine acid vitamin K medium. The survival was calculated from the numbers of colonies.

**Hemolytic assays**

To assess activity of the classical pathway, sheep erythrocytes were washed three times with GVB++ buffer. The cells were incubated with a complement-fixing Ab (amboceptor; Boehringer) diluted 1:1000 in GVB++ buffer and at a concentration of 10⁶ cells/ml. Bacteria (50 µl) were mixed with 250 µl freshly collected human blood treated with 50 µg/ml Reffudan (Pharmion), and incubated anaerobically for 15 min. After incubation, aliquots were removed, diluted serially, and plated on hemin N-acetylglucosamine acid vitamin K medium. The survival was calculated from the numbers of colonies.

**Degradation assay**

C4 and C5 (0.2 µg/ml each) were incubated with Kly48 at concentrations ranging from 0.25 to 4 µM Kly48 and 4 µM proKly48 (MBL) or 0.06 to 1 µM Kly48 and 1 µM proKly48 (ficolins). After overnight incubation at 37°C in 50 mM HEPES [pH 7.4], 150 mM NaCl, and 4% SDS. After separation, the gels were stained with silver salts to visualize separated proteins.

**Chemotaxis assay**

For C5a chemotaxis assays, plasma was used, because serum may contain C5a and C5adesArg, which are produced during blood coagulation (27). Blood was collected with 50 µg/ml Reffudan (Pharmion), a recombinant hirudin anticoagulant that does not affect complement activation (28), and spun at 2000 rpm for 10 min, and the plasma was stored in aliquots at −80°C. To isolate neutrophils, human blood from healthy volunteers was diluted using heparinized blood collection tubes (BD Vacutainer) and left for 15 min at room temperature. Subsequently, blood was layered on equal volume of Histopaque-1119 (Sigma-Aldrich) and centrifuged for 20 min at 800 × g at room temperature. Polymorphonuclear cell-rich interphase was washed once in 0.5% human albumin (Sigma-Aldrich) in PBS (HyClone), placed onto 65–85% Percoll gradient (GE Healthcare), and centrifuged for 20 min at 800 × g at room temperature. The interphase between the 70–75% Percoll layers was collected and washed once in 0.5% albumin solution, and the cells were adjusted to the concentration of 1 × 10⁵ cells/ml in the PBS solution of 4% heat-inactivated (30 min, 56°C) hirudin-treated human plasma. Purity of neutrophils (>70%) was determined by flow cytometry using staining with anti–CD16 mAb labeled with allophycocyanin (ImmunoTools).

**Chemotactic activity**

Cleaning activity was measured in a disposable 96-well cell migration system with 3-µm polycarbonate membranes (ChemoTx; NeuroProbe). Serial dilutions of karylsin (Kly48) and its inactive mutant (proKly48) were incubated with 4% heat-inactivated human plasma (the same as for neutrophil suspension) for 30 min at 37°C and thereafter applied to the wells of the ChemoTx microplate. Recombinant human C5a (Sigma-Aldrich) at 12.5 nM diluted in 4% heat-inactivated human plasma served as a positive control, whereas 2 µM Kly48 and proKly48 was used on the negative controls. A volume of 50 µl 1 × 10⁷ neutrophils/ml in 4% heat-inactivated human plasma was applied on each well of the filter top. The microplate was incubated for 60 min 37°C in humidified air with 5% CO₂ and then, the membrane was removed. Samples were transferred to the new flat-bottom 96-well plate (Sterilin) and mixed with 30 µl cell lysis buffer (0.5% hexadecyl trimethyl ammonium bromide, 2% Tween 20, 0.5% Triton X-100, and 120 mM NaCl). Similarly, 30 µl cell lysis buffer was added to all the wells of the empty ChemoTx microplate. Both plates were incubated for 30 min at room temperature, and subsequently, the solutions from corresponding wells were pooled together. Activity of
neutrophil-associated myeloperoxidase was detected in the lysates using 1,2-phenylenedianiline dihydrochloride tablets (DakoCytomation), and the absorbance was measured at 490 nm.

**Determination of karilysin expression in laboratory strains of T. forsythia**

Total RNA was purified from 14-d-old agar cultures of T. forsythia (ATCC 43037, ATCC 700198, and Be70-14/2010) using RNase Protect Bacteria Mini Kit (Qiagen). cDNA was synthesized from 1.2 μg total RNA using the Omniscript kit (BioSource; Invitrogen) in a single round of reverse transcription. Random hexamers (Applied Biosystems) were used at a final concentration of 2.5 μM. Subsequently, 2 μl of the total reverse transcription mixture was used for two parallel PCRs for each strain—one with specific primers amplifying a fragment of karilysin catalytic domain (sense primer 5'-GTC TGC GAT CAA GCA ACC T-3' and antisense primer 5'-TCC ATA TTC TCC TTG AGG TGT C-3') and the other one with primers amplifying control gene (waaA) and 3-deoxy-t-manno-oct-2-ulosonic acid (Kdo) transferase (sense primer, 5'-CTC GCT CGG TGA GTT TGA A-3', and antisense primer, 5'-ATG GCG AAA AGA ACG TCA AC-3'). The PCR was performed for 45 cycles, with one cycle consisting of denaturation at 94°C for 30 s, annealing at 56°C for 25 s, and polymerization at 72°C for 30 s. The amplified PCR products were then analyzed by electrophoresis in 1% agarose gel.

**Determination of karilysin expression in vivo**

Patients with diagnosed chronic periodontitis attending the Clinic of Periodontology at the University Hospital of Jena were recruited for this study. For detection of T. forsythia in clinical samples, GCF samples were obtained from patients with severe periodontitis (aggressive periodontitis [n = 17] and chronic periodontitis [n = 37]) and 72 healthy controls (Table II). Two paper points were inserted in each pocket for 20 s, and DNA was subsequently extracted using the Genomic Mini System (A&A Biotechnology), according to the manufacturer’s recommendations. PCR for detection of T. forsythia was carried out as described previously (29). To determine whether the karilysin gene was transcribed in vivo, part of GCF was kept frozen at −20°C until mRNA was extracted for RT-PCR analysis. Total RNA from ∼50 μg GCF was purified using an RNeasy kit (Qiagen), and cDNA was synthesized from 1 μg total RNA using the Omniscript kit, according to the manufacturer’s instructions. Oligonucleotide primers (sense primer, 5'-GTC TGC GAT CAA GCA ACC T-3', and antisense primer, 5'-TCC ATA TTC TCC TTG AGG TGT C-3') were used at a final concentration of 5 pmol. The PCR with Taq polymerase was performed for 30 cycles, consisting of denaturation at 94°C for 30 s, annealing at 56°C for 25 s, and polymerization at 72°C for 30 s. The amplified PCR products were then analyzed by electrophoresis on 2% agarose gel.

**Statistical analysis**

One-way ANOVA (InStat) was used to calculate the p values to estimate whether the observed differences between experimental results were statistically significant.

**Results**

T. forsythia activates all pathways of complement

T. forsythia was inoculated with NHS, a source of human complement diluted in two different buffers: GVB**+** that allows activation of the classical and the lectin pathways (low NHS concentrations) or all complement pathways (high NHS concentrations) or Mg-EGTA buffer that allows only alternative pathway activation. Then, bacteria were washed, and deposited C3b was detected with specific Abs using flow cytometry. C3b was deposited on T. forsythia cells in both GVB**+** (Fig. 1A) and Mg-EGTA buffers (Fig. 1B), indicating activation of the classical/lectin and alternative pathways, respectively. No significant opsonization of the bacteria with C3b from ΔNHS was observed, as expected. To confirm these results and to determine the role of particular complement pathways in the activation of complement by T. forsythia, human sera deficient in C1q, C2, or FB were used to determine deposition of C3b. We found that deposition of C3b in GVB**+** was diminished from C1q-deficient and C2-deficient sera in comparison with NHS (Fig. 1C), indicating that classical/lectin pathway, and particularly C1q, is important in the activation of complement by T. forsythia. Furthermore, depletion of FB from serum prevented deposition of C3b compared with NHS in both Mg-EGTA and GVB**+** buffer (Fig. 1C), indicating that T. forsythia activates the alternative complement pathway as well and that this pathway accounts for the important amplification loop for the overall complement activation.

T. forsythia is resistant to killing by complement

Because T. forsythia is efficiently recognized by complement while being a successful pathogen, it must have developed effective complement evasion strategies. To verify the serum resis-
tance of *T. forsythia* strains and to compare this resistance with that of previously analyzed periodontal pathogens, *T. forsythia* ATCC 43037, ATCC 700198, Be70-14/2010, as well as *P. intermedia* ATCC 25611 and *P. gingivalis* W83 strains were incubated with NHS and control ΔNHS. Surviving bacteria were determined by colony counting. A serum-sensitive *E. coli* DH5a strain was used as a control. *T. forsythia* ATCC 43037 was not killed even by 75% NHS, similar to serum-resistant *P. gingivalis* W83 (Fig. 2A). *T. forsythia* Be70-14/2010 was more sensitive to NHS but also showed a significant degree of survival even in 75% NHS, comparable to *P. intermedia* ATCC 25611 (Fig. 2A). *T. forsythia* ATCC 700198 was most sensitive to NHS of the *T. forsythia* strains tested, but even this strain showed some survival in 75% serum, indicating high resistance of the bacterium to human complement. *E. coli* DH5a strain was entirely lysed at 30% NHS. The *T. forsythia* with highest serum resistance, ATCC 43037 and Be70-14/2010, were found to strongly express the metalloproteinase karilysin, as determined at mRNA level by RT-PCR (Fig. 2B), whereas the expression of karilysin in the more serum-sensitive *T. forsythia* strain ATCC 700198 was undetectable.

The gene *kly* encoding karilysin is commonly present in *T. forsythia*

To determine whether the *kly* gene coding for karilysin was expressed in *T. forsythia* strains in vivo, we first performed PCR on GCF samples of patients with periodontitis and healthy controls to determine the presence of *T. forsythia*. We found that the majority of patients with both chronic and aggressive periodontitis carried *T. forsythia* (Table II). The *kly* gene, as determined by PCR, was present in >90% of the patients positive for *T. forsythia*. Furthermore, it was detected in the samples from all five healthy controls in which we also found *T. forsythia*. To ascertain that the *kly* gene was also transcribed in vivo, we analyzed GCF samples isolated from patients with periodontitis by RT-PCR and found that all analyzed samples in which the *kly* gene was detected also contained karilysin mRNA (Fig. 2C).

**FIGURE 2.** *T. forsythia* is resistant to lysis by human serum. (A) *T. forsythia* strains: ATCC 43037, ATCC 700198, Be70-14/2010, *P. gingivalis* W83, *P. intermedia* ATCC 25611, and *E. coli* DH5a were incubated with 30 and 75% NHS and ΔNHS. The surviving bacteria were enumerated after anaerobic culture on appropriate agar plates. The survival of bacteria was calculated as a percentage of growth compared with ΔNHS. Means of three independent experiments are presented with bars indicating SD. (B) RT-PCR determination of karilysin expression. Karilysin-specific and control *WaaA*-specific mRNA was amplified from total RNA of three *T. forsythia* strains, and products were separated in 2% agarose gels. Lanes 1–3, Karilysin-specific amplification (291 bp). Lanes 5–7, Control gene *WaaA*-specific amplification (95 bp). (C) Karilysin expression in GCF from patients with chronic periodontitis. Karilysin-specific mRNA was amplified by RT-PCR and separated on agarose gel. Lanes 1–5, GCF samples from five representative patients carrying *T. forsythia* and karilysin gene. The size of the product was 291 bp. (D and E) *T. forsythia* strains were incubated for 45 min with several concentrations of NHS or high concentrations of ΔNHS diluted in GVB++. Deposited C3b (D) and MAC (E) were detected with specific Abs using flow cytometry. Deposition of C3b and MAC is shown as GMFI, and means of three independent experiments are presented with bars indicating SD. (F) *T. forsythia* strains were incubated for 15 min with human blood. The survival of bacteria was calculated as a percentage of growth compared with the inoculum. In (A) and (D)–(F), statistical significance of observed differences between *T. forsythia* strains was estimated using one-way ANOVA and a Tukey posttest; *p < 0.05, **p < 0.01, ***p < 0.001 (only significant differences indicated). M, polynucleotide size marker; NC, negative control in which reverse transcriptase was omitted; PC, positive control (i.e., RNA isolated from cultured *T. forsythia*).
manner and rescued *E. coli* that are otherwise very sensitive to killing by NHS (Fig. 3A). All three active karilysin forms showed a significant effect on the survival of the bacteria in the presence of NHS, whereas the inactive mutant proKlyE136A had no effect.

**Karilysin interferes with classical and alternative complement pathways**

To understand how karilysin destroys the bactericidal activity of NHS (i.e., complement), the enzyme was incubated at various concentrations with human serum, and hemolytic assays were used to assess activity of the classical and alternative pathways of complement in the pretreated sera. Karilysin was found to be an efficient inhibitor of the classical pathway, whereas the inactive mutant proKlyE136A did not show any inhibition (Fig. 3B). Kly48 and Kly38 were equally effective and inhibited the classical pathway by 95% when present at nanomolar concentrations (150 nM), whereas Kly18 was 2-fold less efficient. To assess the effect of karilysin on the alternative pathway, a modified hemolytic assay was used, because karilysin requires calcium ions for activity, whereas the standard buffer used for the alternative pathway assay contains EGTA, which chelates calcium. Therefore, GVB⁺⁺ buffer and C1q-depleted NHS were used for incubation with rabbit erythrocytes. Under such conditions, Kly48 and Kly38 inhibited the alternative pathway (Fig. 3C), whereas Kly18 had no appreciable activity. The inactive mutant proKlyE136A did not affect the alternative pathway.

**Karilysin interferes with the classical pathway at the level of C4**

Each complement pathway is a cascade of events activated in a consecutive manner. To assess which complement factor(s) were affected by karilysin, a microtitre plate-based assay was used. In this assay, complement activation was initiated by various lipids, depending on the activation pathway analyzed, and the deposition of successive complement factors was then detected with specific Abs. In the case of the classical pathway, complement activation was initiated by aggregated human IgS. We found that deposition of C1q was not affected, but the subsequent deposition of C4b and C3b from 2% NHS was decreased by all three forms of karilysin (Fig. 4). The inactive proKlyE136A mutant had no effect on activation and deposition of any complement factor in the classical pathway.

**Karilysin interferes with the lectin pathway at the level of MBL, ficolin-2, and ficolin-3**

For assessment of the lectin pathway, we used plates coated with mannan (ligand for MBL) or acetylated BSA (ligand for ficolins). In this case, Kly48 and Kly38 inhibited deposition of the lectin pathway recognition molecules: ficolin-2 (Fig. 5A), ficolin-3 (Fig. 5B), MBL (Fig. 5C), and the ensuing factors such as C4b (Fig. 5D) and C3b (Fig. 5E), whereas the proKlyE136A mutant had no effect (Fig. 5). Kly18 was less efficient than the other two forms but still had significant effects at the highest concentrations used.

**Karilysin interferes with the terminal pathway at the level of C5**

The alternative pathway was activated by immobilized zymosan in GVB⁺⁺ buffer and C1q-deficient serum. All forms of karilysin were able to inhibit deposition of C5 (Fig. 6B) but not C3b (Fig. 6A), whereas the inactive proKlyE136A mutant had no effect. These results indicated that unlike the classical and the lectin pathways, karilysin does not inhibit the early stages of alternative pathway activation but does have a significant effect on the common terminal pathway of complement.

**Karilysin cleaves complement recognition molecules of the lectin pathway: MBL, ficolin-2, and ficolin-3**

To confirm that decreased deposition of MBL and ficolins on their ligands was due to degradation by karilysin, purified human MBL
and recombinant ficolin-2 and ficolin-3 were incubated with karilysin at various molar ratios. The proteins were then separated by SDS-PAGE and transferred to a PVDF membrane upon which collectins were visualized using specific Abs. Upon reduction, the majority of the oligomers of native MBL dissociates and migrates as 32-kDa monomers. Karilysin cleaved MBL in a dose-dependent manner releasing several products ranging from 12 to 30 kDa. The cleavage was already observed at the lowest concentration of karilysin used (0.25 \text{mM}; karilysin/MBL ratio 0.8:1) (Fig. 7A). Similarly to MBL, reduced ficolins dissociate to monomers of 34 kDa. Dose-dependent cleavage of both ficolin-2 and ficolin-3 was observed after incubation with karilysin. The cleavage of ficolin-3 by karilysin first released truncated protein with the molecular mass decreased by only a few kilodaltons, but at a higher concentration of 1 \text{mM} (karilysin/ficolin ratio 3.3:1), caused total degradation to small fragments not detectable by Abs (Fig. 7C). In the case of ficolin-2, weak traces of cleavage product truncated by a few kilodaltons also could be observed, indicating a similar pattern, although the cleavage efficiency was lower than in case of ficolin-3 (Fig. 7B). The Abs used for detection of collectins did not show any cross-reactivity with purified karilysin (data not shown).

**FIGURE 3.** Karilysin destroys bactericidal and hemolytic activity of human serum. (A) *E. coli* DH5α were incubated with 2% NHS pretreated with increasing concentrations of karilysin variants as well as the inactive mutant proKlyE136A, and the surviving bacteria were enumerated after overnight culture on LB agar plates. The survival was expressed as a logarithm of colony-forming units. As a control, ΔNHS was used. Statistical significance of observed differences (compared with 2% NHS) was estimated using one-way ANOVA and a Tukey posttest: *p < 0.05, **p < 0.01 (only significant differences indicated). (B) Classical pathway. NHS (1%) was supplemented with various concentrations of karilysin variants and preincubated for 30 min at 37°C, after which, sheep erythrocytes sensitized with Abs and diluted in GVB++ were added. (C) Alternative pathway. C1q-depleted human serum (8%) was preincubated with increasing concentrations of karilysin variants for 15 min at 37°C. Serum was then added to sheep erythrocytes diluted in GVB++. For both (B) and (C), after 1 h of incubation, the degree of lysis was estimated by measurement of released hemoglobin (absorbance at 405 nm). Lysis obtained in the absence of karilysin was set as 100%. In (A)–(C), an average of three independent experiments is presented with bars indicating SD.

**FIGURE 4.** Karilysin inhibits the classical pathway of complement. Karilysin variants were incubated for 25 min with 2% (C1q) or 3% (C3b, C4b) NHS diluted in GVB++ and added to microtiter plates coated with IgGs. After 20 min (C3b and C4b) and 45 min (C1q) of incubation, the plates were washed, and deposited C1q (A), C4b (B), and C3b (C) were detected with specific pAbs. Absorbance obtained in the absence of karilysin was set as 100%. An average of three independent experiments is presented with bars indicating SD.

Karilysin cleaves preferentially α-chains of C4 and C5 and generates biologically active C5a

To assess the cleavage pattern by karilysin, purified C4 and structurally related C5 were incubated with Kly48 at various molar ratios. The proteins were then separated by SDS-PAGE and visualized using silver staining (Fig. 8A, 8B). C4 is composed of covalently linked α-, β-, and γ-chains, whereas C5 contains α- and β-chains. For both proteins, karilysin first released truncated protein with the molecular mass decreased by only a few kilodaltons, but at a higher concentration of 1 \text{μM} (karilysin/ficolin ratio 3.3:1), caused total degradation to small fragments not detectable by Abs (Fig. 7C). In the case of ficolin-2, weak traces of cleavage product truncated by a few kilodaltons also could be observed, indicating a similar pattern, although the cleavage efficiency was lower than in case of ficolin-3 (Fig. 7B). The Abs used for detection of collectins did not show any cross-reactivity with purified karilysin (data not shown).
Because karilysin acted preferentially on the α-chain of C5 and apparently was able to produce a band of molecular mass corresponding to C5b, it was interesting to assess whether incubation of purified protein with heat-inactivated human plasma would indeed result in the generation of chemotactic peptide C5a. To verify this hypothesis, heat-inactivated human plasma was incubated with several concentrations of Kly48 and proKlyE136A and then applied to lower wells of a ChemoTx plate. Freshly purified human neutrophils (5 × 10⁵/well) diluted in 4% heat-inactivated human plasma were allowed to migrate through the filter toward the samples containing karilysin-treated plasma. C5a served as a positive control. Karilysin (Kly48) stimulated neutrophil migration with chemotactic activity peaking at 0.5 μM Kly48, for which the achieved migration was comparable to that toward 12.5 nM C5a (Fig. 8C). At the highest concentrations of Kly48, the migration dropped compared with the peak of activity, which is typical for effect exerted by excess of C5a. The inactive mutant proKlyE136A did not generate chemotactic activity in plasma. Neither Kly48 nor proKlyE136A (2 μM) showed any chemotactic activity when used alone (data not shown), similar to PBS.

Karilysin acts synergistically with gingipains

Because karilysin and gingipains are most often present simultaneously at the sites of infection colonized with *T. forsythia* and *P. gingivalis*, we assessed how they acted on complement when present together. To this end, Kly48 and the three gingipains (HRgpA and RgpB are arginine-specific gingipains, whereas Kgp is lysine specific) were preincubated with 2% NHS at concentrations chosen to affect the activity of the lectin pathway by only 10–30%. The deposition of C4b was assessed, and we found that the arginine-specific but not the lysine-specific proteinases acted synergistically with karilysin, because the deposition of C4b in the presence of combinations of karilysin and the HRgpA and RgpB gingipains was lower than predicted if the effects of the proteinases were added separately (Fig. 9). For example, karilysin alone decreased the deposition of C4b by 10% at the concentration used, whereas RgpB yielded a 30% decrease. When used together at the same concentrations, karilysin and RgpB decreased C4b deposition by 90% instead of 40%, which would be expected if these proteinases had only additive effects.

**Discussion**

The factors governing *T. forsythia* infection are poorly studied when compared with other periodontal pathogens such as *P. gingivalis*. However, they are important to study because current treatment for severe periodontal disease is only partially effective and entails intensive use of antibiotics, which contributes to spreading of antibiotic resistance. It is becoming apparent that all successful human bacterial pathogens must develop strategies to circumvent complement attack (12). Microorganisms in gingival...
sulcus are immersed in GCF, which is a serum-derived tissue exudate. Because complement components are present in GCF at up to 70% of serum concentration (30) and, in vivo, there is a high level of complement activation in gingival fluid of patients with periodontitis (31, 32), successful evasion of complement is paramount for the survival of *T. forsythia* in the periodontal pockets. One such strategy of defense against complement developed by *T. forsythia* appears to depend on the production of karilysin, which we now show is not only able to degrade complement factors C4 and C5 but also MBL and ficolins-2 and ficolin-3, which are crucial molecules enabling recognition of the majority of pathogens without a need of previous exposure and presence of specific Abs. It appears that karilysin has a rather broad specificity because it also degrades casein, gelatin, fibrinogen, fibronectin, and elastin at pH ranging from neutral to slightly alkaline (20). Importantly, we found that MBL, ficolins, C4, and C5 but not all other complement components are targeted by karilysin in the presence of whole serum (in deposition assays), suggesting a high degree of specificity.

The proteolytic activities of oral bacteria are thought to play important roles in the etiology of periodontitis and dental abscesses. These proteinases may contribute to tissue destruction, increase availability of nutrients, and impair host defense by degrading IgGs and components of complement. *T. forsythia* ex-
presses peptidase degrading benzoyl-DL-arginine naphthylamide, the activity of which appears to be related to sites of periodontal tissue destruction (33). Apart from serine proteinase activity, there is also evidence for expression of cysteine proteinase PrtH by T. forsythia (34). PrtH has recently been identified to be identical to a protein named forsythia detaching factor (35), and it appears to be related to caspases (36). Besides the above, T. forsythia can probably express and secrete many other peptidases because it harbors genes encoding putative secretory enzymes that have not been functionally studied yet. So far, karilysin’s role in the immune system evasion by T. forsythia was shown in a study determining that karilysin is able to cleave the antimicrobial peptide LL-37, significantly reducing its bactericidal activity (37).

Karilysin undergoes autocatalytic processing at both the N and C termini. Fully active high molecular mass karilysin (Kly 38) is further processed into low molecular mass karilysin (Kly18), containing the catalytic domain, because of truncation at the C terminus (20). In most of our assays, Kly18 was less active than lesser processed forms. Interestingly, Kly38 was the most active form of karilysin in most assays in this study. Considering that Kly38 differs from Kly18 by a 277-aa C-terminal domain, one may postulate a certain role for the C terminus of karilysin in inactivation of complement proteins. Kly18 is similar to mammalian MMPs and is proposed to have become incorporated to bacterial genome from an external mammalian or mammalian blood-fed insect source, whereas its unique flanking regions including the C-terminal domain are postulated to have evolved in a bacterial environment (21). However, a word of caution is required in the interpretation of these results because the various forms of Kly used in this study undergo a certain degree of further autoprocessing during the incubation required for various assays (as can be seen in incubation assays followed by silver staining [Fig. 8]). We can be only certain of the initial form of Kly used at the start of the assay. Because clear differences between karilysin forms were detected, they could not have been all entirely processed to Kly18. On the basis of evidence from periodontal pathogens studies, we can speculate that the concentrations of enzymes used in our assays are in range of karilysin concentrations found in vivo. First, gingipains from P. gingivalis are detected at the level of 0.4 μM in GCF from the inflamed sites of chronic periodontitis patients (38). The bacterial counts of P. gingivalis and T. forsythia found in chronic periodontitis patients before treatment are very similar (39), so we can assume that the karilysin concentrations in vivo are similar to these of gingipains. In the current study, we show convincingly that both the karilysin gene and its mRNA transcript are present in GCF at sites infected with T. forsythia. In addition, we also show that karilysin acts synergistically with gingipains, and previously, we have shown the synergy between gingipains and interpain A from P. intermedia. Therefore, we hypothesize that because different periodontal bacteria are present together at infection sites, relatively low expression of different synergistic proteinases could be sufficient to exert a significant effect on complement.

Interestingly, karilysin preferentially cleaved α-chains of C4 and C5, similarly to what was previously observed for gingipains and interpain A. At low concentrations, gingipains from P. gingivalis cause activation of complement factors C3, C4, and C5 by preferentially cleaving the α-chains of these proteins to cause the release of anaphylatoxins C3a and C5a as well as the activated forms C3b, C4b, and C5b (6). Similarly, interpain A from P. intermedia also releases C3a and C4a as confirmed by N-terminal sequencing of generated C3 and C4 fragments (10). At higher concentrations, gingipains and interpain A digested these three complement components to smaller, inactive fragments. In degradation assays for C4 and C5 conducted with karilysin, the degradation pattern observed on silver-stained gels was similar to that previously obtained for gingipains (C5) or interpain A (C4), which indicates an analogous mode of action. Furthermore, we have confirmed that karilysin is able to release substantial chemotactic activity in heat-inactivated human plasma, which is consistent with the release of biologically active C5α. This suggests that periodontal pathogens share common mechanisms for controlling complement, mediated by bacterial peptidases. At low concentrations of enzymes, complement factors are cleaved at positions required for subsequent activation of the system, which may be linked to early infection stages when complement activation may actually be beneficial for the pathogen, because it provides access to nutrients in inflammatory exudates. At later stages of infection with higher numbers of bacteria and higher production of enzymes, complement is inactivated by multiple cleavages of vital factors.

A recent study showed that C5a generated by P. gingivalis protects these bacteria from phagocytosis by macrophages that become overwhelmed by released C5a (40). Thus, P. gingivalis uses cross-talk between TLR2 and the C5a receptor for immune subversion. It might be interesting to verify whether T. forsythia is able to exploit the same mechanism. Importantly, the dominant cellular population in the gingival sulcus is neutrophils, which tend to form a specific structure called a “leukocyte wall” along the margins of the periodontal plaque (41). Despite their high concentrations, neutrophils seem to be inefficient in controlling the bacterial infection in periodontitis, and the reason for this remains unclear. One possible explanation might be that the local generation of high C5a concentrations could paralyze the crevicular neutrophils, which express high levels of the C5a receptor. There are numerous reports showing that neutrophils become immunologically incompetent in the presence of high concentrations of C5a (42–44). In contrast, stimulation of inflammatory cells with C5a might contribute to amplified periodontal tissue damage. In this regard, C5a stimulation causes enhanced reactive oxygen species production by neutrophils (42), which might be detrimental to host tissues, whereas periodontal bacterium P. gingivalis was shown to be resistant to reactive oxygen species-mediated killing (45, 46). Moreover, C5a was shown to induce the
release of MMP-9 from human eosinophils and neutrophils (47), and elevated MMP-9 in oral fluids is one of the biomarkers of periodontal disease (48). Therefore, C5a production by bacteria brings numerous possibilities of host immune cell modulation.

MBL and ficolins are members of the collagen-containing lectin family of proteins (collectins), and we hypothesize that their cleavages by karilysin may take place within collagen-like domains. Further analysis is required to determine the exact cleavage positions; however, there are some observations supporting this hypothesis. First, karilysin was previously shown to cleave collagen substrates (20). Second, it is a member of the family of MMPs, and a specific cleavage of the MBL collagen-like domain was shown for bacterial and human metalloproteinases in some conditions, such as denaturation caused by point mutations found in partial MBL deficiency (49). Third, we found that the degradation of MBL by karilysin was much more efficient when MBL was denatured by heat (data not shown). Because denaturation results in perturbation of the triple helical fold of the collagen-like domains of MBL, this may indicate that karilysin cleavage sites are indeed located within collagen-like domains, therefore allowing for more efficient proteolysis of denatured MBL compared with the native molecule. It is important to emphasize that the experiment with denatured MBL does not demonstrate that native MBL degradation by karilysin in vivo is not efficient. MBL cleavage in vivo is affected by many factors, which may render MBL susceptible to cleavage, such as ligand binding and complex formation with MBL-associated peptidases, and the results from the complement deposition assay performed in human serum showed efficient inhibition of MBL deposition on mammalian cells by the lectin pathway by karilysin. In addition, we found that karilysin cleaves ficolin-2 and ficolin-3, which share a collagen-like domain with MBL. Interestingly, it was previously shown that recombinant and serum-purified ficolin-3 were highly resistant to collagenase digestion, in contrast to other ficolins and MBL (50). In case of karilysin, both ficolin-3 and ficolin-2 were degraded, and ficolin-3 degradation was even more efficient that ficolin-2.

A comparison of the degree of complement deposition on the bacteria (Fig. 2D, 2E) supports the role of karilysin in the serum resistance of T. forsythia. The strain ATCC 700198 lacking karilysin was significantly more osonized with C3b compared with the other two strains with good karilysin expression. This clearly shows that karilysin expression gives an advantage to the bacteria, because inhibition of C3b opsonization should result in a decrease of phagocytosis. The same strain was also significantly more coated with terminal MAC. The differences in MAC deposition (reflecting the events downstream from C3b opsonization) between the strains were not so clear-cut though, because the clinical isolate Be70-14/2010 expressing karilysin did not manage to prevent MAC deposition as equally well as the other karilysin expressing strain ATCC 43037 and was closer in phenotype to the ATCC 700198. However, karilysin should not be the only anticomplement virulence factor expressed by T. forsythia. There are no data available yet regarding their virulence factors expressed by T. forsythia that also contribute to its virulence, perhaps acting synergistically with karilysin.

Taken together, the fact that karilysin was able to cleave several crucial complement components and that it appears to be expressed by the majority of strains of T. forsythia in vivo imply that this enzyme may be crucial for survival of this periodontal pathogen.

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