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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.** ***The Journal of Immunology*, 2012, 188: 2338–2349.**

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.

Proteinases are crucial virulence factors produced by many periodontal pathogens. Apart from the generation of essential nutrients by host protein degradation, proteinases 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 proteinases C1s and C1r) to invading pathogens, either directly or via Igs. 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 proteinases 1, 2, and 3),

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Abbreviations used in this article: FB, factor B; GCF, gingival crevicular fluid; GMFI, geometric mean fluorescence intensity; GVB^{++}, barbiturate buffer with dextrose and gelatin; LB, Luria–Bertani; MAC, membrane attack complex; MBL, mannose-binding lectin; MMP, matrix metalloproteinase; NHS, normal human serum; ΔNHS, heat-inactivated NHS; pAb, polyclonal Ab; PVDF, polyvinylidene difluoride.

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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). 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). Karilysin is a metalloproteinase with a primary structure similar to matrix metalloproteinases (MMPs) and is able to cleave elastin, fibrinogen, and fibronectin (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 karilysin (Kly38, 38 kDa) and low molecular mass karilysin (Kly18, 18 kDa). All three forms of karilysin are active to varying degrees against substrates studied so far. Further characterization of karilysin 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 coopted 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 karilysin 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).

Karilysin as well as its inactive mutant proKly^{E136A} (the catalytic glutamic 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 Proteinase (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 karilysin were isolated and used in subsequent experiments: Kly48, high molecular mass karilysin (Kly38), low molecular mass karilysin (Kly18), and the inactive mutant proKly^{E136A}. 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-C1q, -C4c, and -C3d Abs (all from DakoCytomation), goat anti-MBL (R&D Systems), goat anti-C5 (Quidel), rabbit–anti-C5b9 neoepitope (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 Be70-14/2010 were grown on hemin *N*-acetylomuramic acid vitamin K agar plates at 37°C in an anaerobic chamber (Concept 400; Biotrace) with an atmosphere of 90% N_2, 5% CO_2, and 5% H_2. The purity and correct identity of the cultures was confirmed by Gram staining and 16S rDNA sequencing. For culture of *P. gingivalis* W83 and *P. intermedia* ATCC 25611, the same conditions were used, except that facultative anaerobic agar plates were used. *E. coli* laboratory strain DH5α (Invitrogen) was grown on standard Luria–Bertani (LB) agar plates or in LB broth. Bacterial strains used in this study are listed in Table I.

Bactericidal assay

Strain *E. coli* DH5α was cultured in LB broth until exponential growth phase. Cells were harvested, washed once in barbiturate buffer with dextrose and gelatin (GVB^{++}) (5 mM veronal buffer [pH 7.3], 140 mM NaCl, 0.1% gelatin, 1 mM $MgCl_2$, and 5 mM $CaCl_2$), and adjusted to an OD at 600 nm of 0.25. NHS was diluted in GVB^{++} to a concentration of 2% and incubated with various concentrations of karilysin variants for 20 min at room temperature. Thereafter, 10^6 bacteria cells were added and incubated with serum supplemented with karilysin for 20 min at 37°C in a total volume of 50 μl. After incubation, aliquots were removed, diluted serially, and spread 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 OD_{600} of 0.02–0.04, except from *P. intermedia* ATCC 25611, for which, OD_{600} of 0.2–0.4 was used. Thereafter, 5 μl 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 in 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-d-old agar plate cultures were harvested and washed once in GVB^{++} (classical/lectin pathway) or Mg-EGTA (2.5 mM veronal buffer [pH 7.3] containing 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM, $MgCl_2$, 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 Mg-EGTA and incubated anaerobically for 1 h at 37˚C in a total volume of 200 μl. ΔNHS (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% C1q-, C2-, and factor B (FB)-deficient serum, NHS and ΔNHS in GVB^{++}, and 10% FB-deficient serum, NHS, and ΔNHS in Mg-EGTA. Then, bacteria were washed once in FACS buffer (50 mM HEPES, 100 mM NaCl [pH 7.4], 1% BSA, and 30 mM NaN_3) and incubated with specific Abs against C3c, conjugated with FITC (DakoCytomation) for 45 min at room temperature. Finally, samples were washed twice, resuspended in fixing buffer (BD), diluted 1:10 in H_2O, and analyzed using flow cytometry (CyFlow space, Partec, Germany). Analogous experiments were performed to compare opsonization of different *T. forsythia* strains with human C3b and MAC deposition on bacteria. *T. forsythia* strains (ATCC 43037, ATCC 700198, and Be70-14/2010), prepared as above in GVB^{++} buffer, were incubated anaerobically with different serum concentrations for 45 min at 37˚C in a total volume of 200 μl. Then, bacteria were washed once in FACS buffer and incubated with specific Abs against C3c, conjugated with FITC for 45 min at room temperature or rabbit anti-C5b9 for 30 min at room temperature, followed by swine–anti-rabbit FITC-conjugated Abs for 30 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 10^8 cells/ml. Bacteria (50 μl) were mixed with 250 μl freshly collected human blood treated with 50 μg/ml Refludan (Pharmion), and incubated anaerobically for 15 min. After incubation, aliquots were removed, diluted serially, and plated on hemin *N*-acetylomuramic 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; Boehringverke; diluted 1:3000 in GVB^{++} buffer) at a concentration of 10^9 cells/ml for 20 min at 37˚C. After two washes with GVB^{++}, 5×10^8 cells/ml were incubated for 1 h at 37˚C with 1% NHS diluted in GVB^{++} buffer (total volume 150 μl). Before incubation with erythrocytes, NHS was preincubated with various concentrations of karilysin variants for 30 min at 37˚C. The samples were centrifuged, and the amount of lysed erythrocytes was determined by spectrophotometric measurement of the amount of released hemoglobin (405 nm; Varian 50 MPR Microplate Reader).

To assess activity of the alternative pathway, rabbit erythrocytes were washed three times with GVB^{++} buffer and then used at a concentration of 5×10^8 for incubation for 1 h at 37˚C with 8% C1q-depleted human serum (Quidel) diluted in GVB^{++} buffer (total volume 150 μl). The serum used was pretreated with karilysin variants for 15 min at 37˚C. The samples were centrifuged, and the amount of lysed erythrocytes was determined spectrophotometrically (405 nm) using a Varian 50 MPR Microplate Reader and Cary 50 Bio UV-Visible Spectrophotometer (Varian).

Complement activation assays

Microtiter plates (Maxisorp; Nunc) were incubated overnight at 4˚C with 50 μl of a solution containing 2 μg/ml human aggregated IgG (Immuno), 100 μg/ml mannan (M-7504; Sigma-Aldrich), 20 μg/ml zymosan (Z-4250; Sigma-Aldrich) in 75 mM sodium carbonate (pH 9.6), or 10 μg/ml acetylated BSA (Sigma-Aldrich; acetylated as described in Ref. 26) in PBS. Between each step of the procedure, the plates were washed four times with 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5). The wells were blocked with 1% BSA (Sigma-Aldrich) in PBS for 2 h at room temperature. NHS (classical and lectin pathway) was diluted in GVB^{++} buffer and used at a concentration of 2% for measurement of deposition of C1q, 3% for C3b and C4b in the classical pathway, 2% for C3b, C4b, ficolin-2, and ficolin-3 in the lectin pathway, and 4% for MBL. In the case of the alternative pathway, C1q-depleted serum was used at a concentration of 2% for C3b and 4% for C5. These concentrations were chosen on the basis of initial titrations. The serum used was mixed with various concentrations of karilysin variants, preincubated for 25 min (NHS) or 15 min (C1q-depleted serum) at 37˚C, and incubated in the wells of microtiter plates for 45 min at 37˚C for C1q, MBL, ficolin-2, and ficolin-3, 20 min at 37˚C for C3b and C4b (classical and lectin pathway), and 30 min for C3b and C5 (alternative pathway). Complement activation was assessed by detecting deposited complement factors using specific Abs against C1q, C4b, C3d, C5, MBL, ficolin-2, and ficolin-3 and diluted in the blocking buffer. Bound Abs were detected with HRP-labeled anti-rabbit, anti-goat, or anti-mouse secondary pAbs. Bound HRP-labeled pAbs were detected with 1,2-phenylenediamine dihydrochloride tablets (DakoCytomation), and the absorbance was measured at 490 nm.

Degradation assay

C4 and C5 (0.2 μM each) were incubated with Kly48 at concentrations ranging from 0.06 to 1.92 μM, 1.92 μM proKly^{E136A}, or alone. Incubations were carried out for 3 h (C4) or 5 h (C5) in GVB^{++} buffer at 37˚C. The proteins were separated by SDS-PAGE electrophoresis using the standard Laemmli procedure and 12% gels. Prior to electrophoresis, the samples were boiled for 5 min at 95˚C in a reducing sample loading buffer containing 25 mM DTT and 4% SDS. After separation, the gels were stained with silver salts to visualize separated proteins.

Purified human MBL and recombinant ficolin-2 and ficolin-3 (10 μg/ml each) were incubated with karilysin at concentrations ranging from 0.25 to 4 μM Kly48 and 4 μM proKly^{E136A} (MBL) or 0.06 to 1 μM Kly48 and 1 μM proKly^{E136A} (ficolins). After overnight incubation at 37˚C in 50 mM HEPES (pH 7.4), 150 mM NaCl, and 5 mM $CaCl_2$, samples were mixed with reducing sample loading buffer and boiled for 5 min at 95˚C. Proteins were separated on 15% Tricine-SDS polyacrylamide gel using the von Jagow procedure (MBL) or 10% gel using the Laemmli procedure (ficolins) and subsequently transferred on a polyvinylidene difluoride (PVDF) membrane (Pall). MBL and ficolins were detected using specific Ab, followed by HRP-conjugated secondary pAbs. The signal was developed using a diaminobenzidine solution (Sigma-Aldrich).

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 Refludan (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 drawn 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 1×10^7 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 was measured in a disposable 96-well cell migration system with 3-μm polycarbonate membranes (ChemoTx; Neuro-Probe). Serial dilutions of karilysin (Kly48) and its inactive mutant (proKly^{E136A}) were incubated with 4% heat-inactivated human plasma (the same as for neutrophils 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 proKly^{E136A} in PBS and PBS alone were used as negative controls. A volume of 50 μl 1×10^7 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% CO_2, 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 [Sigma-Aldrich] in PBS). Similarly, 30 μl cell lysis buffer was added to all the wells of the emptied 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-phenylenediamine 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 RNeasy 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-D-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 1.5% 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 μl 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 incubated with NHS, a source of human complement diluted in two different buffers: GBV^{++} 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.

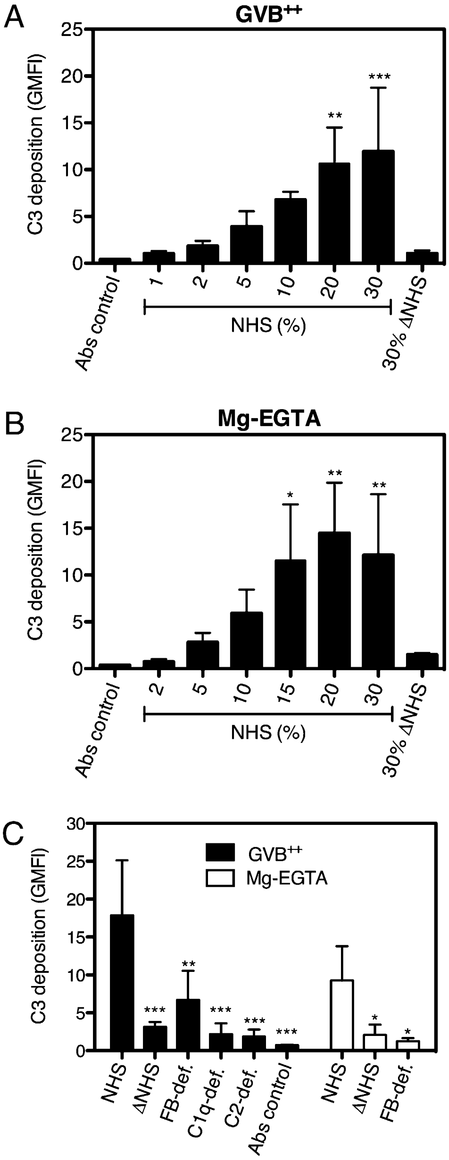

FIGURE 1. *T. forsythia* activates all complement pathways. *T. forsythia* was incubated for 1 h with several concentrations of NHS or 30% ΔNHS diluted in GVB^{++} (**A**) or Mg-EGTA (**B**). Similarly, *T. forsythia* was incubated with NHS, C1q-, C2-, FB-deficient serum and ΔNHS diluted in GVB^{++} or with NHS, ΔNHS, and FB-deficient serum diluted in Mg-EGTA (**C**). Deposited C3b was detected on bacterial surface with specific Abs conjugated with FITC using flow cytometry. Deposition of C3b is shown as GMFI. Means of three independent experiments are presented with bars indicating SD. Statistical significance of observed differences was estimated using one-way ANOVA and a Dunnett posttest (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). In (A) and (B), the statistical significance of differences is shown compared with Abs control (bacteria incubated with Abs only); in (C), compared with NHS in the respective buffers.

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 resistance 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* DH5α 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* DH5α 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).

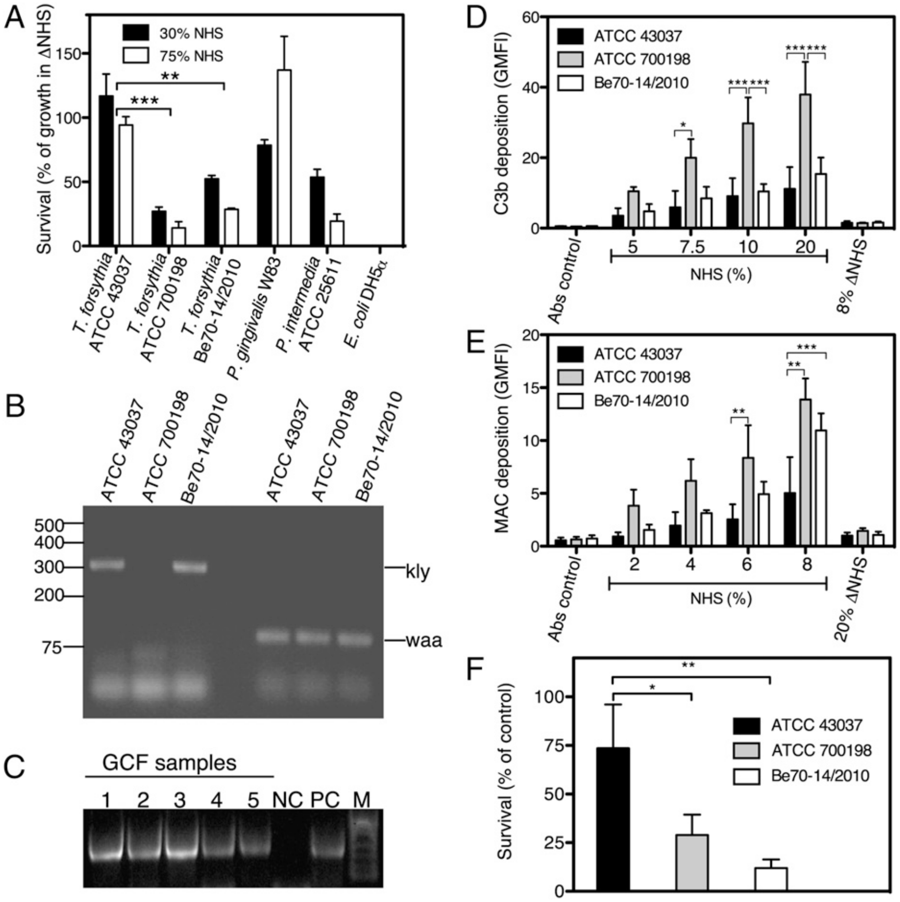

FIGURE 2. *T. forsythia* is resistant to lysis by human serum. (**A**) *T. forsythia* strains: ATCC 43037, ATCC 700198, Be70-14/2010, *P. gingivalis* W83, *P. intermedia* ATCC 25611, and *E. coli* DH5a were incubated with 30 and 75% NHS and ΔNHS. The surviving bacteria were enumerated after anaerobic 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*).

Table I. Description of bacterial strains used in this study

Bacterial Strain	Characteristics
E. coli DH5a	Common laboratory strain
T. forsythia ATCC 43037	Human periodontal pocket isolate
T. forsythia ATCC 700198	Subgingival plaque isolate from juvenile periodontitis
T. forsythia Be70-14/2010	Severe peri-implantitis isolate
P. gingivalis W83	Periodontal abscess isolate
P. intermedia ATCC 25611	Periodontal empyema isolate

The strains expressing karilysin are more resistant to opsonization with C3b and deposition of MAC, which might contribute to their survival in whole blood

Because distinct expression of karilysin was found in the available laboratory strains of *T. forsythia* and there was some correlation between higher serum resistance and karilysin expression (Fig. 2A, 2B), we further investigated the interactions of all three strains with complement. *T. forsythia* ATCC 43037, ATCC 700198, and Be70-14/2010 were incubated with different NHS concentrations, and subsequently, opsonization with C3b as well as deposition of MAC on the bacterial surface were analyzed using specific Abs and flow cytometry. The strain ATCC 700198, which had undetectable levels of Kly RNA transcripts, was found to be opsonized with C3b in significantly larger amounts compared with the other two strains. The strain Be70-14/2010 showed only slightly higher C3b opsonization than the highly serum-resistant strain ATCC 43037, which had lowest levels of deposited C3b (Fig. 2D). Accordingly, ATCC 700198 contained the highest level of MAC on the surface. The most highly resistant strain, ATCC 43037, had significantly less deposited MAC, whereas the strain of intermediate survival, Be70-14/2010, was resistant to MAC deposition at lower serum concentrations but at higher concentrations reached the same MAC deposition as ATCC 700198 (Fig. 2E).

Taking into account the increased opsonization of *T. forsythia* ATCC 700198 (poor karilysin producer) with complement fragments, compared with the other two strains, we tested how this would translate to the survival of bacteria in whole human blood. For this purpose, bacteria were incubated for 15 min at 37°C in fresh human blood, and the survival was assessed by colony counting from serial dilutions (Fig. 2F). The strain ATCC 43037 (kly positive) was found to survive best in this experimental setup, whereas the other two strains ATCC 700198 (kly negative) and Be70-14/2010 (kly positive) were killed more efficiently, indicating most probably contribution of other factors than karilysin to the final survival of the three strains.

Karilysin destroys the bactericidal activity of human serum

To quantitatively assess the effect of purified karilysin variants on the bactericidal activity of human serum, an *E. coli* DH5α model system was used whereby cells were incubated with NHS pretreated with various concentrations of the three forms of karilysin or its inactive mutant (proKly^{E136A}). Surviving bacterial cells were determined by colony counting. Karilysin was found to destroy the bactericidal activity of human serum in a dose-dependent 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 proKly^{E136A} had no effect.

Table II. Prevalence of *T. forsythia* and the presence of karilysin in subgingival plaque samples

Group (*n*)	Prevalence of *T. forsythia* (%)	*Karilysin* Gene Presence in *T. forsythia*-Positive Samples (%)
Healthy controls (72)	5 (7)	5/5 (100)
Aggressive periodontitis (17)	15 (88)	14/15 (93)
Chronic periodontitis (37)	28 (76)	27/28 (96)

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 proKly^{E136A} 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 proKly^{E136A} 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 microtiter plate-based assay was used. In this assay, complement activation was initiated by various ligands, 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 proKly^{E136A} 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 proKly^{E136A} 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 proKly^{E136A} 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

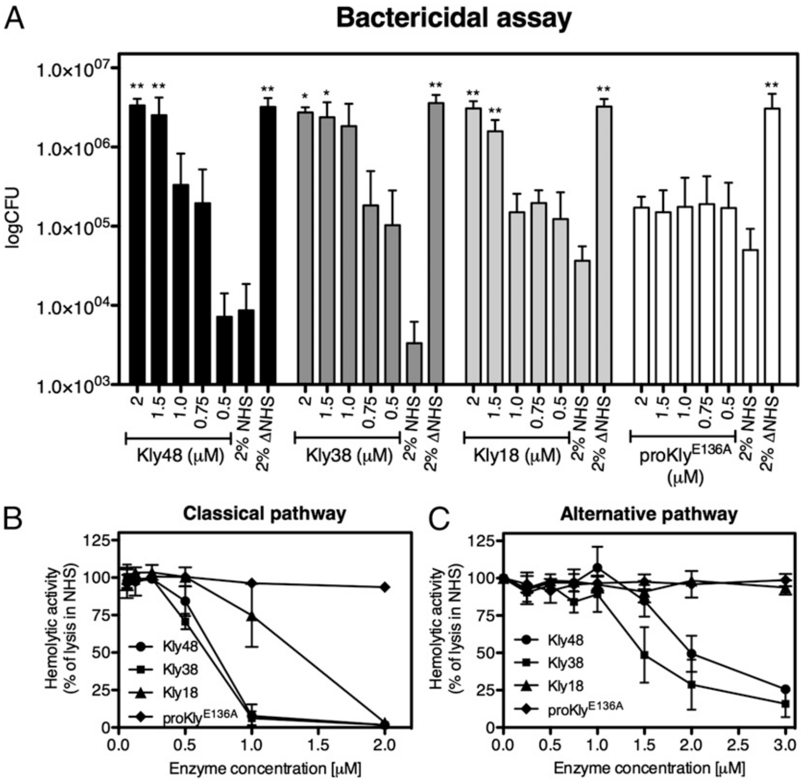

FIGURE 3. Karilysin destroys bactericidal and hemolytic activity of human serum. (**A**) *E. coli* DH5α were incubated with 2% NHS pretreated with increasing concentrations of karilysin variants as well as the inactive mutant proKly^{E136A}, and the surviving bacteria were enumerated after overnight 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.

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 μM; karilysin/MBL ratio 0.8:1) (Fig. 7A).

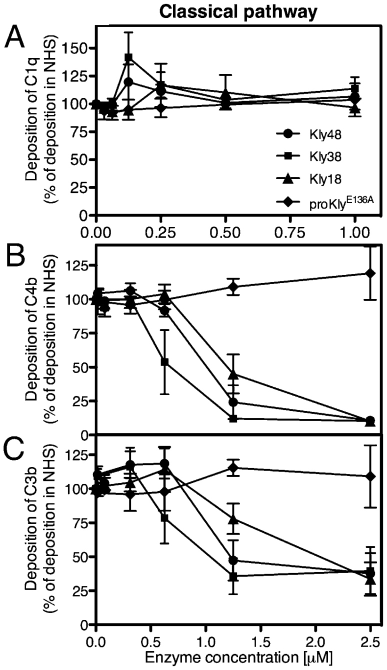

FIGURE 4. Karilysin inhibits the classical pathway of complement. Karilysin variants were incubated for 25 min with 2% (C1q) or 3% (C3b, C4b) NHS diluted in GVB^{++} and added to microtiter plates coated with IgGs. After 20 min (C3b and C4b) and 45 min (C1q) of incubation, the plates were washed, and 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.

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 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).

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 proteolytically cleaves the α-chain, whereas the β-chain (and γ-chain of C4) is relatively resistant, which is similar to what has previously been observed for gingipains (6). C4 was degraded at lower concentrations of karilysin compared with C5. The proKly^{E136A} mutant did not cause any degradation of C4 or C5 (Fig. 8A, 8B). Furthermore, no degradation of a human serum albumin under the same conditions was observed (data not shown).

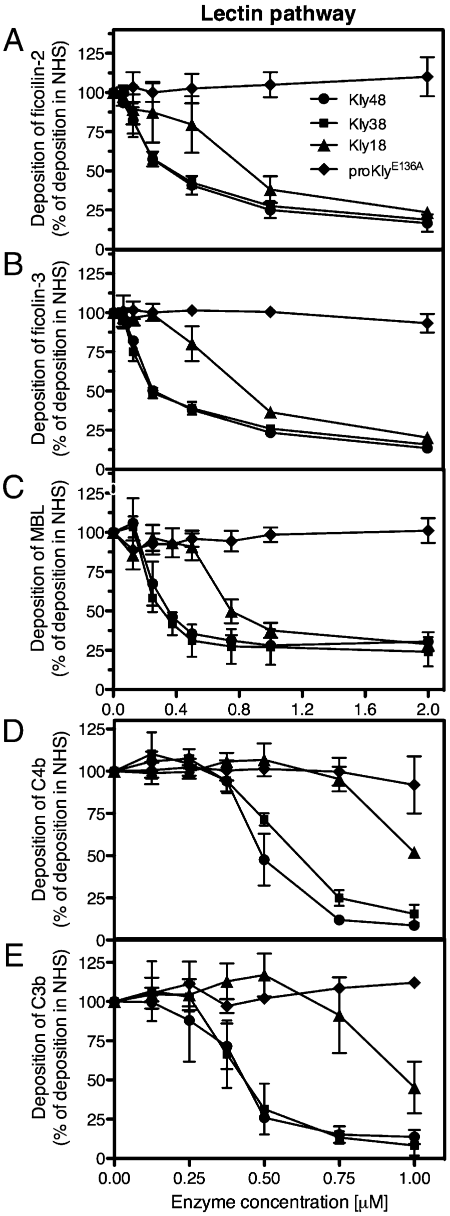

FIGURE 5. Karilysin inhibits the lectin pathway of complement. Karilysin variants were incubated for 25 min with 4% (MBL) or 2% (C3b, C4b, ficolin-2, and ficolin-3) NHS diluted in GVB^{++} and added to microtiter plates coated with mannan (MBL, C3b, and C4b) or acetylated BSA (ficolins). After 20 min (C3b and C4b) or 45 min (MBL, ficolin-2, and ficolin-3) of incubation, the plates were washed, and deposited ficolin-2 (**A**), ficolin-3 (**B**), MBL (**C**), C4b (**D**), and C3b (**E**) were detected with specific Abs. Absorbance obtained in the absence of karilysin was set as 100%. An average of three independent experiments is presented with bars indicating SD.

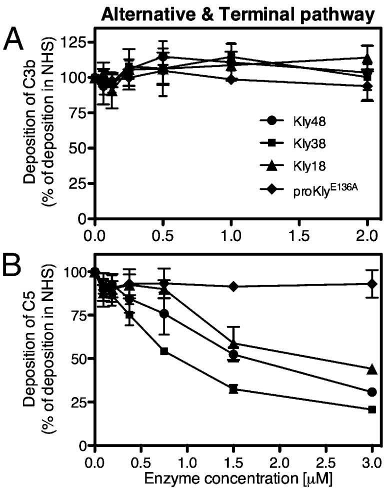

FIGURE 6. Karilysin inhibits the terminal pathway of complement. Karilysin variants were incubated for 15 min with 2% (C3b) and 4% (C5) C1q-depleted serum diluted in GVB^{++} and added to microtiter plates coated with zymosan. After 30 min of incubation, the plates were washed, and deposited C3b (**A**) or C5b (**B**) was 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.

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 proKly^{E136A} and then applied to lower wells of a ChemoTx plate. Freshly purified human neutrophils (5×10^5/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 proKly^{E136A} did not generate chemotactic activity in plasma. Neither Kly48 nor proKly^{E136A} (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

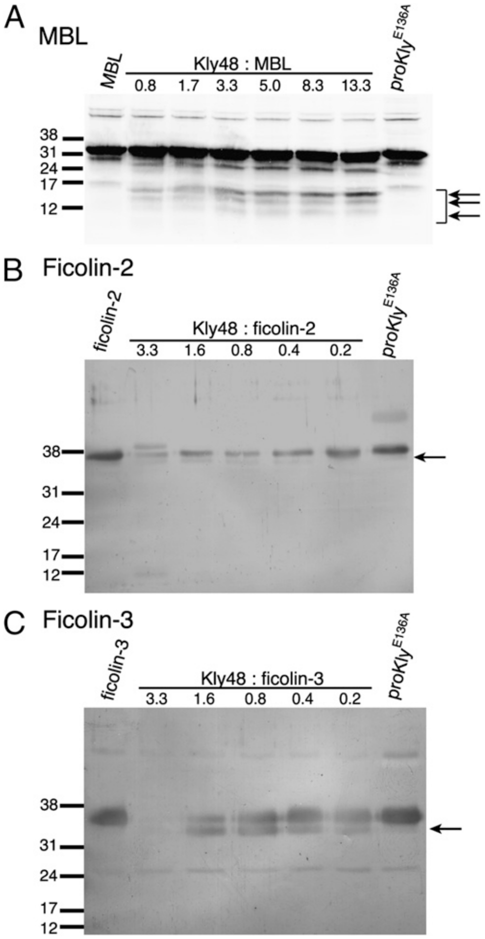

FIGURE 7. MBL, ficolin-2, and ficolin-3 are cleaved by karilysin. Purified human MBL (**A**) and recombinant human ficolin-2 (**B**) and ficolin-3 (**C**) (10 μg/ml each) were incubated overnight at 37°C with increasing concentrations of Kly48 or a single high concentration of proKly^{E136A}. The molar ratios of collectins to karilysin are indicated in the figure. The samples were then reduced, and proteins were separated by electrophoresis on 15% Tris-Tricine gel (MBL) or 10% Laemmli gel (ficolins) and transferred to PVDF membrane. Monomers of MBL (A), ficolin-2 (B), and ficolin-3 (C) were detected with specific Abs. Arrows indicate positions of degradation products.

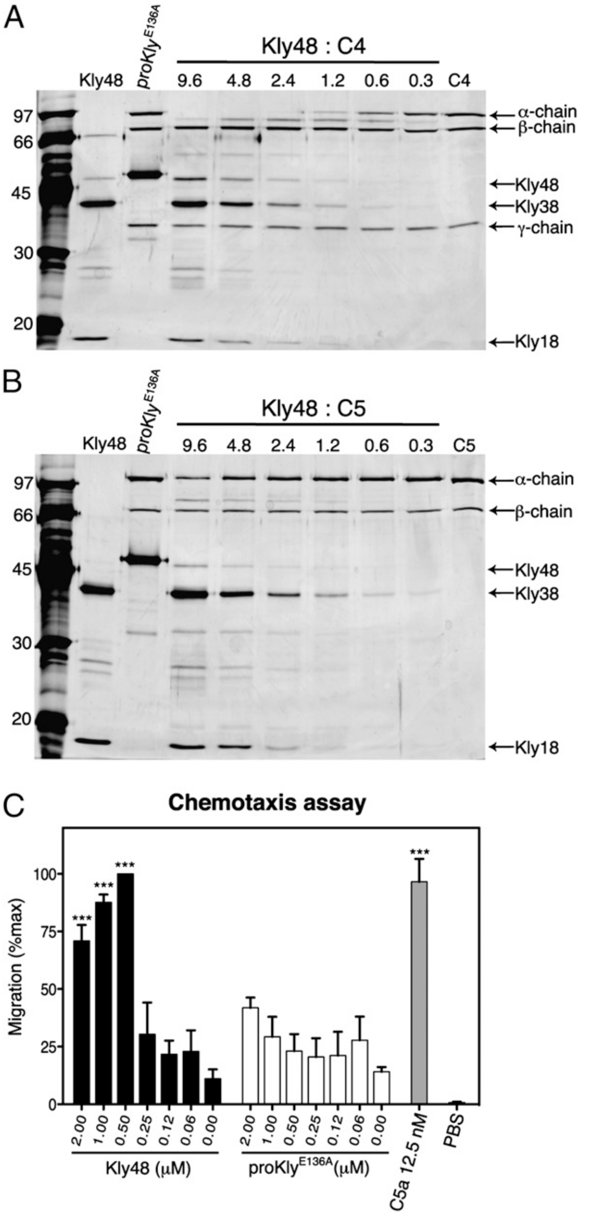

FIGURE 8. Karilysin degrades preferentially α-chains of C4 and C5 and generates biologically active C5a. C4 (**A**) and C5 (**B**) (0.2 μM each) were incubated with increasing concentrations of Kly48 or 1.92 μM proKly^{E136A}. The molar ratios of complement proteins to karilysin are indicated in the figure. Incubations were carried out for 3 h (C4) or 5 h (C5) in GVB^{++} buffer at 37°C, and the proteins were then separated by SDS-PAGE electrophoresis. The gels were stained with silver salts. Arrows indicate positions of intact components of C3/C4 as well as the various forms of karilysin. (**C**) Increasing concentrations of Kly48 and proKly^{E136A} were incubated with 4% heat-inactivated human plasma and then placed in the wells of ChemoTx microplate. Neutrophil migration was measured after 1 h as an activity of neutrophil-associated myeloperoxidase. PBS was used as a negative control, and human C5a (12.5 nM) was used as a positive control. Absorbance obtained for the highest migration in the assay (achieved for 0.5 μm Kly48) was set as 100%. An average of three independent experiments is presented with bars indicating SD. Statistical significance of observed differences was estimated using one-way ANOVA and a Dunnett posttest (***$p < 0.001$) and calculated compared with untreated plasma (0 μM proteinase).

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 ficolin-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 Igs and components of complement. *T. forsythia* ex-

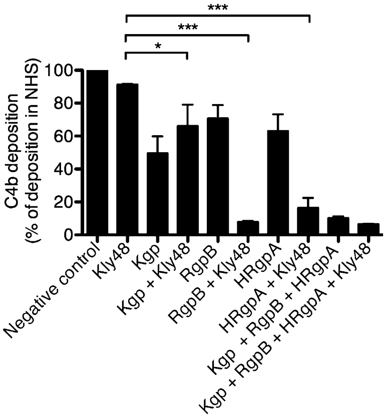

FIGURE 9. Karilysin and gingipains act synergistically. Mannan was immobilized on microtiter plates and allowed to activate 2% NHS containing 0.4 μM Kly48 and three gingipains, Kgp (44 nM), RgpB (66 nM), and HRgpA (33 nM), alone or mixed together. After 25 min of incubation, the plates were washed, and deposited C4b was detected with specific Abs. An average of three independent experiments is presented with bars indicating SD. Statistical significance of observed differences was estimated using one-way ANOVA and a Tukey posttest; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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 ≤1.5 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 C5a. 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 mannan, indicating efficacious abolition of 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 opsonized 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 the expression of other putative complement inhibitors by the analyzed strains, and certainly, there might be other factors varying in expression between the strains such as those playing a role in complement inhibition downstream from C3 convertases. These potential differences were also confirmed in the whole-blood killing assay in which the strain ATCC 43037 survived the best again, whereas the killing of the other two strains was significantly more efficient and comparable, although they differed in karilysin expression. Therefore, we have to stress that there must be other 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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Disclosures

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