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A Metalloproteinase Mirolysin of *Tannerella forsythia* Inhibits All Pathways of the Complement System

Monika Jusko,* Jan Potempa, †‡ Danuta Mizgalska, † Ewa Bielecka, *‡ Miroslaw Ksiazek, † Kristian Riesbeck, ․ Peter Garred, ․ Sigrun Eick, ‡ and Anna M. Blom*

Recent reports focusing on virulence factors of periodontal pathogens implicated proteinases as major determinants of remarkable pathogenicity of these species, with special emphasis on their capacity to modulate complement activity. In particular, bacteria-mediated cleavage of C5 and subsequent release of C5a seems to be an important phenomenon in the manipulation of the local inflammatory response in periodontitis. In this study, we present mirolysin, a novel metalloproteinase secreted by *Tannerella forsythia*, a well-recognized pathogen strongly associated with periodontitis. Mirolysin exhibited a strong effect on all complement pathways. It inhibited the classical and lectin complement pathways due to efficient degradation of mannose-binding lectin, ficolin-2, ficolin-3, and C4, whereas inhibition of the alternative pathway was caused by degradation of C5. This specificity toward complement largely resembled the activity previously characterized metalloproteinase of *T. forsythia*, karilysin. Interestingly, mirolysin released the biologically active C5a peptide in human plasma and induced migration of neutrophils. Importantly, we demonstrated that combination of mirolysin with karilysin, as well as a cysteine proteinase of another periodontal pathogen, *Prevotella intermedia*, resulted in a strong synergistic effect on complement. Furthermore, mutant strains of *T. forsythia*, devoid of either mirolysin or karilysin, showed diminished survival in human serum, providing further evidence for the synergistic inactivation of complement by these metalloproteinases. Taken together, our findings on interactions of mirolysin with complement significantly add to the understanding of immune evasion strategies of *T. forsythia* and expand the knowledge on molecular mechanisms driving pathogenic events in the infected periodontium.

**P**eriodontal disease is one of the most common infectious inflammatory diseases. With >30% prevalence in the adult population and ~5% being severe cases (leading to a complete loss of a dentition), the disease presents substantial economic burden (1). The influence of periodontitis on systemic health and its likely contribution to cardiovascular diseases, diabetes, as well as rheumatoid arthritis (2–4) underscores a crucial role of inflammation in the disease mechanism and emphasize the need of innovative and effective therapies. Recent advances in understanding the molecular basis of interaction between periodontal pathogens and host innate immunity system revealed a double-edged sword role of complement in chronic inflammatory events driving periodontal tissue destruction (5).

Complement is produced in the body both locally and systemically, and it provides the first arm of defense against pathogens. This involves pattern-recognition molecules of the classical pathway, that is, the C1 complex (composed of recognition molecule C1q and two proteinases C1s and C1r), and the lectin pathway, that is, mannose-binding lectin (MBL)/ficolin complexes (composed of MBL or ficolins and three MBL-associated proteinases MASP-1, MASP-2, and MASP-3). These molecules ensure efficient recognition of the invading pathogen with subsequent activation of the complement cascade. Additionally, complement can be activated on microbial or non-self surfaces, which lack complement inhibitors, through the alternative pathway, based on the constant low level of spontaneous hydrolysis of C3 to C3(H2O). The latter is further stabilized in the presence of factors B and D into alternative pathway C3 convertase, providing substantial amplification loop, accounting for ≥80% of the total complement activation (6). All three pathways lead to opsonization of the pathogen with C3b (an activated form of complement factor C3), which facilitates phagocytosis. Furthermore, anaphylatoxins released during activation of the cascade, C3a and C5a, stimulate inflammatory cells and attract phagocytes to the site of infection by involvement of specific G-coupled receptors C3a receptor (C3aR) and C5a receptor (C5aR), respectively. Finally, the end result of the complement cascade is formation of the membrane attack complex (MAC) and bacterial cell lysis. Expression of membrane-bound as well as recruitment of soluble complement...
inhibitors provides the protection for the host cells when this powerful system is activated. Unfortunately, this sophisticated system is often corrupted by microbial pathogen–derived proteinases (7), which in the case of periodontitis may contribute to tissue damage by chronic inflammation driven by subgingival plaque bacteria.

It has been long recognized that chronic periodontitis is an infectious disease; however, no single bacterial species has been identified as “etiologic” for progression of the disease. Several bacterial clusters within a complex periodontal biofilm have been described, with three major consensus periodontal pathogens composing the so-called “red complex”: T. forsythia, Porphyromonas gingivalis, and Treponema denticola (8). Recent advancement in periodontal studies finally refuted the perception of periodontitis as a classical infection, triggered by a single or limited number of microorganisms. Rather, periodontal disease is the result of dysbiosis in the oral microbiota, leading to the formation of pathogenic biofilm of altered composition and increased bacterial counts, which, in turn, causes complement-dependent inflammation of periodontium and consequently bone loss (9, 10). In contrast to the previous hypothesis implicating a crucial contribution of red complex species in the pathogenesis of periodontal disease, the new model of polymicrobial synergy and dysbiosis proposes a concept of low-abundant keystone pathogens capable of breaking down periodontal homeostasis and changing normal benign oral biofilm into a dysbiotic one (9). This novel concept was introduced based on the findings involving the most intensively studied prototype periodontal pathogen P. gingivalis (11). To disrupt host homeostasis and induce dysbiosis, this bacterium engages two receptors: complement receptor C5aR, activated by P. gingivalis–induced C5a, and TLR2. The resulting C5aR–TLR2 cross-talk impairs the killing functions of macrophages and neutrophils, alters the composition of oral biofilm, and leads to upregulation of inflammatory and bone resorptive cytokines (11–14). These studies changed the understanding of periodontal disease and left open the question as to identification of other keystone pathogens. It appears plausible that one important prerequisite of such a master pathogen in periodontitis is the expression of a proteinase of C5 convertase activity, because P. gingivalis deficient in its C5a-releasing proteinases, gingipains, did not induce dysbiosis in a mouse periodontitis model (11). As we demonstrated before, P. gingivalis has not one but three proteinases that can generate biologically active C5a (15). T. forsythia, which is very important but so far a much less studied component of the classical red complex, appears to be equipped with proteinases of C5 convertase activity as well. We previously showed that its metallloproteinase karilysin, among other complement-targeting activities, can release C5a and induce migration of neutrophils (16). A recent study on T. forsythia revealed the existence of an entire array of genes encoding putative secretory proteinases with similarity to karilysin, all possessing a nearly identical C-terminal domain that ends with a -Lys-Leu-Ile-Lys-Lys motif. These proteins, referred to as KLKK proteinases, may function as “etiological” C-terminal domain that ends with a -Lys-Leu-Ile-Lys-Lys motif.

**Sera and proteins**

Normal human serum (NHS) was obtained from eight healthy volunteers. Heat-inactivated NHS was made by incubating NHS for 30 min at 56°C. Sera deficient in various complement components as well as matching NHS were obtained from Quidel. Purified complement proteins C3, C4, and C5 were purchased from Complement Technology. Mirolysin, cloned from the T. forsythia ATCC 43037 genome, as well as its inactive mutant MirE341A (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 (GE Healthcare). The GST tag was removed from recombinant proteins bound to GSH-Sepharose by cleavage with PreScission Protease (GE Healthcare). Tag-free mirolysin and inactive mutant MirE341A were subsequently purified by size exclusion chromatography using a Superdex 75 HiLoad 16/60 (Pharmacia Biotech) column. The metalloproteinase karilysin forms Kly48, high-molecular-mass karilysin (Kly38), and low-molecular-mass karilysin (Kly18) were purified as described (16). Interpain A (InfA) was expressed and purified as in Potempa et al. (18).

**Abs**

The following Abs against human Ags were used throughout this study: polyclonal (pAbs) rabbit anti-C1q, C4c, and C3d Abs (all from Dako), goat anti-MLB (R&D Systems), goat anti-C5 (Quidel), mAb mouse anti-ficolin-2 (19) or anti-ficolin-3 (20), and mouse anti-C9 neoantigen Abs (HyCult Biotech). Secondary pAbs conjugated with HRP against rabbit, goat, or mouse were from Dako.

**Bacterial strains and their culture**

T. forsythia strain ATCC 43037 was grown on hemin N-acetylmuramic acid vitamin K (HNK) agar plates at 37°C in an anaerobic chamber (Concept 400, Biotrace) with an atmosphere of 90% N2, 5% CO2, and 5% H2. The purity and correct identity of the cultures were confirmed by Gram staining and 16S rDNA sequencing. T. forsythia mutant strains lacking mirolysin (Δmir) or karilysin (Δkly) were grown similarly, with supplementation of 10 μg/ml chloramphenicol or 5 μg/ml erythromycin in the agar plates, respectively.

**Construction of proteinase deletion mutants**

Deletional inactivation of mir (BFO_2661; formerly known as T0341) gene encoding mirolysin metallloproteinase in T. forsythia. The plasmid for deletion of the mir gene (BFO_2662) was based on two synthesized DNA fragments (GeneArt Strings, Life Technologies) and the pUC19 plasmid backbone. Each fragment contained a portion of 341 adjacent genomic sequence and part of the chloramphenicol acetyltransferase (cat) gene truncated in the vicinity of an internal EcoRI restriction site. The first DNA fragment contained a 5′ SacI restriction site, followed by a 778-bp DNA fragment upstream of the BFO_2661 start codon followed by a 221-bp DNA sequence encoding cat. The second DNA fragment consisted of 449-bp of the cat gene, followed by 551-bp of a BFO_2661′ 3′ truncated region, terminated with a KpnI restriction site. These DNA fragments were ligated after EcoRI digestion and cloned into the SacI and KpnI sites of pUC19. The correct orientation of the DNA fragments in the plasmid was confirmed by sequencing.

Deletional inactivation of kly (BFO_2683; formerly known as T0367) gene encoding karilysin metallloproteinase in T. forsythia. To obtain a plasmid for kly (BFO_2683) deletion, an erythromycin cassette was flanked by two DNA fragments amplified from T. forsythia genomic DNA. The 972-bp fragment was amplified with primers 5′-TGTGAATTCGGACGAGCGAAGGATGAACTCCTCTC-3′ and 5′-GATCCCCGTGTAGTCGTCGTATACCCGGGACAACGAATTATCTCCTTAA-3′. The 1235-bp long downstream fragment was amplified with primers 5′-GATGTGCGACTTGAAGATGTGACGCGCTTCG-3′ (containing a SalI site) and 5′-GCTCGCCAATAGAATACAAACAAAGCTTGA-3′ (containing a HindIII site). An erythromycin resistance cassette (ermF-ermA) was amplified from plasmid pDestB (21) with the following primers: 5′-CTCGTCGACGACTTTTATTGCCAAGTTCTAATGC-3′ (with a SalI restriction site) and 5′-CTCGTTCGACTTTTATTGCGAAGTTCTAATGC-3′ (with a SalI restriction site). All DNA fragments were digested by indicated restriction enzymes and cloned into pUC19 digested with EcoRI and HindIII. The correct placement of DNA fragments was confirmed by sequencing.

Electroporation of T. forsythia. Electrocompetent T. forsythia cells were obtained by a modified procedure as described in Sakakibara et al. (22). Briefly, cells from 5-d-old plates were collected to a 50-ml conical tube and washed twice with 20 ml electroporation buffer (1 mM MgCl2, 10% glycerol) and resuspended in 1 ml of the same buffer. One microgram plasmid DNA was electroporated to 100 μl bacterial slurry in a Micro...
Pulser electroporation apparatus (Bio-Rad Laboratories) (2.5 kV, 4 ms) followed by incubation in growth medium for 12 h. Thereafter, bacteria were collected and plated on selective media containing 10 μg/ml chloramphenicol or 5 μg/ml erythromycin. Then, 8-d resistant clones were subcultured and tested for correct cassette placement by sequencing.

**Confirmation of correct gene deletion in T. forsythia** Genomic DNA from the parental strain (ATCC 43037) and its mutants lacking mirolysin (Δmir) and karilysin (Δkly) were isolated using a genomic DNA purification kit (Thermo Scientific). PCR amplification of genes BFO_2661 (mir), BFO_2683 (kly), and a control gene BFO_2665 [encoding another KLKK proteinase mirolase, formerly known as TF0347 (17)] was performed with primers listed in Table I. The PCR with Taq polymerase (Thermo Scientific) was performed for 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 56°C for 20 s, and polymerization at 72°C for 30 s. The amplified PCR products were then analyzed by electrophoresis on 2% agarose gel.

**Determination of mirolysin 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; chronic periodontitis, n = 37) and healthy controls (n = 6). 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 (23). To determine whether the mirolysin gene (mir) 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′-GGGAACGAAGGACA-3′ and anti-sense primer 5′-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 thereafter analyzed by electrophoresis on 2% agarose gel.

**MAC deposition on T. forsythia**

T. forsythia was harvested, washed once in PBS, adjusted to OD₆₀₀ of 0.6, and incubated for 30 min with 5 μM CFSE (Sigma-Aldrich). After pre-staining with CFSE, bacteria were washed once in GVB⁺⁺ buffer (5 mM veronal buffer [pH 7.3], 140 mM NaCl, 0.1% gelatin, 1 mM MgCl₂, and 5 mM CaCl₂) and adjusted to an OD₆₀₀ of 0.6. Thereafter, 120 μl bacteria was mixed with 10–40% NHS diluted in GVB⁺⁺ and incubated anaerobically for 30 min at 37°C in a total volume of 200 μl. For ΔNHS, 40% was used as a control. Thereafter bacteria were washed once in flow cytometry buffer (50 mM HEPES, 100 mM NaCl [pH 7.4], 1% BSA, and 30 mM Na₂S) and incubated with specific mAb against C9 neoantigen, followed by secondary pAbs conjugated with Alexa Fluor 647 (Life Technologies). Finally, samples were washed twice, resuspended in fixing buffer (BD Biosciences) diluted 1:10 in H₂O, and analyzed using flow cytometry (CyFlow space, Partec, Munster, Germany). The geometric mean fluorescence intensity for 10,000 CFSE⁺ cells was calculated for all the samples using FlowJo software (Tree Star).

**Bactericidal assay**

T. forsythia strain ATCC 43057 and its mutants Δkly and Δmir were harvested from agar plates, washed once in GVB⁺⁺, and adjusted to an OD₆₀₀ of 0.01. Thereafter, 10 μl (~3 × 10⁸ bacteria) were mixed anaerobically with 10 and 30% NHS or, as control, 30% ΔNHS diluted in GVB⁺⁺, in a total volume of 100 μl. The samples were incubated anaerobically at 37°C for 40 min with shaking. After incubation, aliquots were removed, serially diluted, and spread on appropriate agar plates. Plates were incubated in an anaerobic chamber for 7–10 d and the survival at different conditions was calculated as percentage of input CFU for each strain. Resupplementation experiments of knock out strains with recombinant proteins were performed with only small modifications. Briefly, 90 μl NHS (7.5%) diluted in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM MgCl₂, and 5 mM CaCl₂ was pre-incubated for 30 min at 37°C with recombinant Mir or Kly38, after which 10 μl relevant knockout bacteria were added. The samples were further incubated for 30 min and processed as above.

**Hemolytic assays**

To assess activity of the classical pathway, sheep erythrocytes were washed three times with GVB⁺⁺ buffer and then used at a concentration of 5 × 10⁶ cells/ml for 1 h incubation at 37°C with 3% C1q-depleted human serum (Quidel) diluted in GVB⁺⁺ buffer (total volume 150 μl). The serum was used pretreated with mirolysin variants for 25 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).

**Complement activation assays**

Microroller plates (MaxiSorp, Nunc) were incubated overnight at 4°C with 50 μl of a solution containing 2 μg/ml human aggregated IgG (Immun, 100 μg/ml mannan (Sigma-Aldrich, M-7504)), and 20 μg/ml zymosan (Sigma-Aldrich, Z-4250) in 75 mM sodium carbonate (pH 9.6) or 10 μg/ml acetylated BSA (Sigma-Aldrich, acetylated as described in Ref. 24) 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 (Saveen Werner) in PBS for 2 h at room temperature. NHS (classical and lectin pathways) was diluted in GVB⁺⁺ buffer and used at a concentration of 2% for measurement of deposition of C1q, 1% 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 4% for both C3b and C5b. These concentrations were chosen on the basis of initial titrations. The serum used was mixed with various concentrations of mirolysin variants, pre-incubated for 25 min at 37°C, and incubated in the wells of microroller plates for 45 min at 37°C for C1q, MBL, ficolin-2, and ficolin-3, 30 min at 37°C for C3b and C4b (classical and lectin pathways), and 35 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 35 min for C3b and C5 (alternative pathway). The samples were incubated with HRP-labeled anti-rabbit, anti-goat, or anti-mouse secondary pAbs. Bound HRP-labeled pAbs were detected with 1,2-phenylenediamine dihydrochloride tablets (Dako), with absorbance measured at 490 nm.

**Degradation assay**

C3, C4, and C5 (0.1 μM each) diluted in 50 mM HEPES, 75 mM NaCl, and 5 mM CaCl₂ (pH 7.4) were incubated with Mir at concentrations ranging from 0.06 to 2 μM Mir or 2 μM Mir alone for 1 h at 37°C.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BFO_2661 (mir)</strong></td>
<td>5′-GTTAATTCTGCAACCCATGCGA-3′</td>
<td>5′-GCGAATTTCCGAGTACATT-3′</td>
<td></td>
</tr>
<tr>
<td><strong>BFO_2683 (kly)</strong></td>
<td>5′-GCCAATTTCTGCAAGATTTC-3′</td>
<td>5′-CGTTAATTCTGCAAGATTTC-3′</td>
<td></td>
</tr>
<tr>
<td><strong>BFO_2665 (KLKK proteinase, mirolase)</strong></td>
<td>5′-AGGGCTGTCATTGTCGTTCGG-3′</td>
<td>5′-AGGGCTGTCATTGTCGTTCGG-3′</td>
<td></td>
</tr>
</tbody>
</table>
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.

Chemotaxis assay

For C5a chemotaxis assays, plasma was used, as serum may contain C5a and C5a des-Arg produced during blood coagulation (25). Human neutrophils were isolated as described (26), stained with 1 μM CFSE, washed, and suspended in 4% solution of heparin-inactivated human plasma (30 min, 56°C) to the final concentration of 5 × 10⁵ cells/ml. Chemotactic activity was measured in a disposable 96-well cell migration system with 3-μm polycarbonate membranes (ChemoTx, Neuro Probe). Serial dilutions of Mir and its inactive mutant MirE341A were incubated with 4% heparin-inactivated human plasma (the same as for neutrophil suspensions) for 25 min at 37°C and thereafter applied to the wells of a ChemoTx microplate. Plasma-purified human C5a (Complement Technology) diluted in 4% heparin-inactivated human plasma served as a positive control whereas 2 μM Mir and MirE341A in PBS and PBS alone were used as negative controls. A volume of 50 μl neutrophil suspension was applied on each well of the filter top. The microplate was incubated for 60 min at 37°C in humidified air with 5% CO₂ and then the membrane was removed. Samples were transferred to the new flat-bottom black 96-well plates (Nunc) and the emptied wells were washed twice with 30 μl PBS, with subsequent pooling of washes with the respective samples (final volume 90 μl). Fluorescence at 535 nm was collected using Wallac 1420 Victor2 microplate reader (PerkinElmer).

Statistical analysis

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

Results

The gene mir encoding mirolysin is commonly present in T. forsythia

Given the pivotal role of periodontal bacteria proteases to the pathogenic mechanism of periodontitis via modulating complement activity, we sought to determine the expression of novel proteases by a series of T. forsythia strains. Previously, we demonstrated a key role of a metalloprotease karilysin of T. forsythia in the defense against complement (16). The sequenced genome of T. forsythia ATCC 43037 (available at: http://www.ncbi.nlm.nih.gov/gene/) contains several putative proteases, which share a unique multidomain structure with karilysin and contain characteristic C-terminal domains ending with 5a-aa residues (Lys-Leu-Ile-Lys-Lys), for which they were collectively named KLIJK proteases. One of the KLIJK proteases, a metalloprotease of the M43 family of proteolytic enzymes, is encoded by the gene BFO_2661. This novel metalloprotease named mirolysin was cloned from the T. forsythia ATCC 43037 genome. To evaluate its relevance in vivo, we isolated GCF samples of patients with periodontitis and on healthy controls. To further prove the role of both mirolysin and karilysin in complement evasion of T. forsythia, we generated single knockout strains of these proteases and confirmed deletional inactivation of respective genes by PCR (Fig. 1B). Next, we tested the ability of these strains to resist complement attack. Strikingly, both knockout strains ∆kly and ∆mir showed increased deposition of the terminal complement product, MAC (Fig. 1C), compared with their parental strain. Increased complement attack of bacterial strains devoid of single metallopeptinases translated into their largely abolised survival in human serum (Fig. 1D). Whereas the parental strain proliferated equally well in NHS and heat-inactivated NHS, both mutants showed diminished growth in 10% NHS and were completely eradicated in 30% NHS (Fig. 1D). These results clearly indicate that both karilysin as well as mirolysin play a major role in complement evasion by T. forsythia. Furthermore, to restore the phenotype of knockout strains, we supplemented these with recombinant proteases and incubated them with 7.5% NHS. For ∆mir, already as low as 0.6 μM recombinant enzyme was sufficient to provide protection against complement, and at higher concentrations of the enzyme, the knockout strain reached proliferation ability of the parental bacteria (Fig. 1E). An analogical experiment performed for ∆kly yielded similar results (Fig. 1F).

Mirolysin interferes with classical and alternative complement pathways

To find out how the novel metalloprotease mirolysin can contribute to complement-modulating ability of T. forsythia, we tested its activity in well-established hemolytic assays. The enzyme was incubated at various concentrations with human serum, and the hemolytic activity of the classical and alternative pathways of complement was analyzed in the pretreated sera. Mirolysin exerted dramatic inhibition of classical pathway–mediated hemolysis, in contrast to its inactive mutant MirE341A, which had no effect (Fig. 2A). Next, we used a modified hemolytic assay to test the activity in the alternative pathway, as mirolysin 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, Mir strongly inhibited the alternative pathway (Fig. 2B) whereas the inactive mutant MirE341A had no effect.

Mirolysin inhibits classical pathway at the level of C4

The triggering of complement takes place via distinct cascade pathways (classical, lectin, or alternative), which proceed in a consecutive manner and converge at the level of C3 activation. To assess which stages of complement pathways were affected by

### Table II. Prevalence of T. forsythia and the presence of mirolysin in subgingival plaque samples

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Prevalence of T. forsythia</th>
<th>Mirolysin Gene Presence in T. forsythia-Positive Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls (6)</td>
<td>1/6 (17%)</td>
<td>0/1 (0%)</td>
</tr>
<tr>
<td>Aggressive periodontitis (17)</td>
<td>15/17 (88%)</td>
<td>13/15 (87%)</td>
</tr>
<tr>
<td>Chronic periodontitis (37)</td>
<td>28/37 (76%)</td>
<td>24/28 (86%)</td>
</tr>
</tbody>
</table>

mRNA as well (Fig. 1A, Table II). The pattern of expression of mir in T. forsythia strains appeared to correlate with kly mRNA expression, which we analyzed in our previous study (16).

Mirolysin contributes to serum resistance of T. forsythia strains and appears to exert a synergistic effect in combination with karilysin
Mirolysin, 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 various ligands, depending on the presence of mirolysin (up to 320%; Fig. 3A). However, subsequent deposition of C4b (Fig. 3B) and C3b (Fig. 3C) was inhibited by mirolysin, indicating its effect on C4 level. The active MirE136A mutant was devoid of any activity on the classical pathway (Fig. 3A–C). Additionally, when human serum was preincubated with mirolysin in the absence of any immobilized C1 activator and added to empty microtiter plate blocked with BSA, we detected increasing C1q deposition on this inert surface with increasing Mir concentration, whereas there was no C1q deposition from NHS alone. This effect was not observed for the inactive mutant MirE341A (Fig. 3D).

**Mirolysin 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). Deposition of the lectin pathway recognition molecules ficolin-2, and ficolin-3 by mirolysin was inhibited, followed by inhibition of the ensuing factors such as C4b (Fig. 4D) and C3b (Fig. 4E). The MirE341A mutant had no effect (Fig. 4).

**Mirolysin targets the terminal pathway at the level of C5**

The alternative pathway was activated by immobilized zymosan in GVB++ buffer and C1q-deficient serum. Whereas C3b deposition was not affected by Mir (Fig. 5A), the deposition of C5b was...
inhibited (Fig. 5B), indicating that the early stages of alternative pathway activation are not targeted by this metalloproteinase, but the common terminal pathway is inhibited. The inactive mutant MirE341A had no effect on C3b or C5b deposition (Fig. 5).

*Mirolysin cleaves preferentially α-chains of C3, C4, and C5 and generates biologically active C5a*

As observed in our previous studies on periodontal proteinases, these enzymes preferentially target α-chains of major complement factors C3, C4, and C5 (15, 16, 18). To verify whether this was the case for mirolysin, we incubated purified complement factors C3, C4, and C5 with different concentrations of Mir. The proteins were then separated by SDS-PAGE and visualized using silver staining (Fig. 6A–C). C4 is composed of covalently linked α-, β-, and γ-chains whereas C3 and C5 contain α- and β-chains. For all these proteins, mirolysin first proteolytically cleaved the α-chain whereas the β-chains and γ-chain of C4 were relatively resistant.
The degradation of C3 was very limited, with little truncation of the α-chain that did not appear to be processed further (Fig. 6A). In case of α-chains of C4 and C5, the cleavage by Mir was very efficient, as even at the lowest enzyme concentration there was a clear effect of the proteinase (Fig. 6B, 6C). The inactive mutant did not cause any degradation of C3, C4 or C5 (Fig. 6A–C). Of note, mirolysin is susceptible to autocatalytic processing during incubation, hence the presence of two bands on SDS-PAGE. The cleavage of C5 by mirolysin indicated that the proteinase produced a pattern potentially corresponding to C5b (truncated α-chain and unprocessed β-chain) (Fig. 6C). At higher concentrations of the enzyme, C5b was degraded further, because the truncated α-chain was degraded completely, whereas β-chains became slightly reduced in intensity. C5b generation under physiological conditions is always accompanied by a release of small chemotactic peptide C5a from C5α-chain. Therefore we assessed whether incubation of purified protein with heat-inactivated human plasma would indeed result in generation of functional chemotactic peptide C5a. Such a phenomenon has previously been shown with karilysin or gingipains (15, 16). To this end, heat-inactivated human plasma was incubated with several concentrations of Mir and MirE341A and then applied to lower wells of a ChemoTx plate. Freshly purified human neutrophils were allowed to migrate through the filter toward the samples containing mirolysin-treated plasma. C5a served as a positive control. Mirolysin stimulated neutrophil migration in a dose-dependent manner, with chemotactic activity peaking at 0.75 μM Mir, for which the achieved migration was comparable to that toward 12.5 nM C5a (Fig. 6D). The inactive mutant MirE341A did not generate chemotactic activity in plasma. At the highest concentration Mir had no effect on neutrophil migration, most probably owing to generation of an excessive amount of C5a that desensitizes C5aR, causing a decrease of chemotactic activity to the level comparable to that toward 125 nM C5a. In parallel to PBS, neither Mir nor MirE341A (2 μM) showed any chemotactic activity when used alone (not shown).

FIGURE 5. Mirolysin inhibits the alternative pathway of complement. Mirolysin was incubated for 25 min with 4% 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 mirolysin was set as 100%. An average of three independent experiments is presented with bars indicating SD. Statistical significance of observed differences was estimated using two-way ANOVA and a Bonferroni posttest. ***p < 0.001.

FIGURE 6. Mirolysin degrades preferentially α-chains of C3, C4, and C5 and generates biologically active C5a. C3 (A), C4 (B), and C5 (C) (0.2 μM each) were incubated with increasing concentrations of mirolysin or 2 μM MirE341A. Incubations were carried out for 1 h and the proteins were then separated by SDS-PAGE electrophoresis. The gels were stained with silver salts. (D) Increasing concentrations of mirolysin or MirE341A were incubated with 4% heat-inactivated human plasma and then placed in the wells of a ChemoTx microplate. Fluorescence at 485 nm corresponding to the number of CFSE-stained neutrophils that migrated through the membrane was measured after 1 h incubation. PBS was used as negative control, and human C5a (12 and 125 nM) was used as a positive control. Fluorescence obtained for the highest migration in the assay (achieved for 12 nM C5a) was set as 100%. An average of four independent experiments is presented with bars indicating SD. Statistical significance of observed differences was estimated using one-way ANOVA and a Dunnett posttest. **p < 0.01 and calculated compared with untreated plasma (0 μM proteinase).

T. forsythia mirolysin acts synergistically with karilysin, as well as with InpA, a cysteine protease of Prevotella intermedia

Karilysin and mirolysin are expressed together at similar levels by T. forsythia in GCF (Fig. 1A). Therefore, we can assume that they...
would be found together at the sites of infection. Additionally, owing to coaggregation and strict correlation of major periodontal bacteria, it is also likely that proteinases of other periodontal bacteria are secreted at similar sites as mirolysin. Hence, we evaluated how they act on complement when incubated in combinations. To this end, Mir and the three karilysin forms (Kly48, Kly38, and Kly18), as well as cysteine proteinases of *P. gingivalis* (gingipains: HRgpA and RgpB are arginine-specific gingipains whereas Kgp is lysine-specific) and *P. intermedia* (InpA), were preincubated with 2% NHS at concentrations chosen to affect the activity of the lectin pathway by only 10–30%. The deposition of C3b was assessed. There was no synergy of mirolysin with any of the gingipains, for which the combined effect was at most additive (data not shown). In contrast, we found that all forms of karilysin acted synergistically with mirolysin, because the deposition of C3b in the presence of combinations of mirolysin and Kly48/Kly38/Kly18 was lower than predicted if the effects of the proteinases were added separately (Fig. 7). The same was observed for the combination of mirolysin with InpA.

**Discussion**

Numerous studies addressing interactions of oral bacteria with immune defenses have increased our understanding of the pathogenesis of periodontal disease. It has become evident that the attempts to identify a single bacterial species etiologic for this disease will likely never be successful owing to complexity of both bacterial as well as host factors shaping the final homeostatic state in the gingival crevice. At health, homeostasis in periodontal tissues is maintained by a controlled low-grade inflammatory state, confining the bacterial plaque to the gingival margin and keeping limited flow of GCF, substantial to supply the periodontium with essential nutrients (27). Along with the nutrients, various plasma components are extruded to the gingival crevice, including complement proteins, which increase in their concentrations once bacterial biofilm manages to proliferate (e.g., during poor oral hygiene) and spreads down to the crevice. At the inflammatory conditions complement is found at 70–80% of its serum concentration, with elevation of certain components reflecting their local production (28, 29). Although complement is a key host defense against pathogens, its activation must be tightly controlled because when deregulated, it may excessively amplify inflammation and contribute to immunopathology. Periodontal bacteria disrupt the fine balance between complement activation and inhibition in the periodontium, and they cause a shift toward exacerbated chronic inflammation. In turn, this results in a dysbiosis of the whole oral plaque in both composition and numbers, presumably due to the overgrowth of those species that are better adapted to inflammatory conditions (9, 30).

Bacterial proteinases targeting complement appear to be hallmark factors of successful periodontal pathogens. In this regard, it has been shown that in contrast to a gingipains-deficient strain, wild-type *P. gingivalis* can impair host defenses, avoid immune clearance, and induce development of pathogenic oral plaque, which then causes destruction of affected periodontal tissues (11, 12) However, the aforementioned results were obtained in mouse models in the absence of other key pathogens strictly associated with periodontitis normally simultaneously found in humans. Given the polymicrobial nature of this disease, identification of virulence mechanisms contributing to disruption of immune homeostasis shared by different key periodontal pathogens could give rationale for developing effective therapeutics. All three microorganisms of the classical red complex strongly associated with periodontitis exhibit similar correlations with the prevalence and severity of the disease (8, 31). In this study, we provide evidence that *T. forsythia* expresses virulence factors that allow it to modulate complement, and in particular exploit the C5α/C5αR axis, the importance of which has been recently highlighted in the pathogenesis of periodontitis (13, 32). Its metalloprotease mirolysin appears to act synergistically with previously described karilysin (16) and exert biphasic effect on complement. The prevalence of mirolysin in *T. forsythia* strains is very high (Table II) and similar to that of karilysin. Mirolysin, similar to karilysin, inhibits deposition of members of collagen-containing lectin family of proteins, MBL and ficolins on mannan-coated surfaces (Fig. 4). The lectin pathway of complement allows recognition of pathogens in the absence of specific Abs via direct binding of MBL and ficolins to their surface carbohydrates. *T. forsythia* exposes a whole array of carbohydrate moieties in glycans attached to its cell surface layer (S-layer) (33). Hence, mirolysin together with karilysin can help the bacterium to avoid this pathway, both at the stage of recognition, but also downstream by targeting C4 (Fig. 3). In the case of the classical pathway, C1q deposition from serum was enhanced in the presence of mirolysin, both on aggregated IgG (Fig. 3A), but also on a nonactivating surface coated with BSA (Fig. 3D). However, downstream from this step, we observed inhibition of the classical pathway at the level of C4. Induction of C1q deposition on inert surfaces or bacterial surfaces was demonstrated before for other periodontal bacterial proteinases, gingipains of *P. gingivalis*, and InpA of *P. intermedia* (15, 18). However, whereas this effect could be recreated in vitro using purified C1 for gingipains (15), InpA required serum to be present for this to occur (18). In the case of mirolysin, C1q deposition on BSA-coated plates occurred only in the presence of NHS, but not for C1 alone (data not shown), similar to InpA. Thus, it appears that mirolysin may require a third protein/serum factor to induce C1 deposition. Furthermore, mirolysin does not cleave C1q (data not shown), further indicating that its effect is not direct. These data collectively show that periodontal pathogens have a versatile influence on C1/C1q. Whereas benefits of limiting C1q deposition appear obvious, one potential advantage of increased C1q deposition would be when this effect could selectively be targeted on the

**FIGURE 7.** Mirolysin acts synergistically with karilysin of *T. forsythia* as well as InpA of *P. intermedia*. Mannan was immobilized on microtiter plates and allowed to activate 2% NHS containing 0.3 μM Mir, three karilysin forms Kly48 (0.4 μM), Kly38 (0.4 μM), Kly18 (0.8 μM), and InpA (0.3 μM), alone or mixed together. After 25 min of incubation, the plates were washed and deposited C3b 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 < 0.001.
Staphylococcus aureus disease. In this regard, we observed that aureolysin of and thus contribute to the dysbiosis of the plaque observed in the potentially lead to the eradication of some complement-sensitive species surfaces of certain commensal oral bacteria. This could very vital for the survival of this pathogen. Furthermore, since knocking out just one proteinase in T. forsythia (Mir or Kly) resulted in drastically diminished complement resistance of the plaque site can lead to the local generation of high C5a levels. C5a is a very active molecule, and in principle it can contribute to periodontal tissue damage via several mechanisms, mainly increased vasodilation and vascular permeability together with the chemotactic recruitment of inflammatory cells, including neutrophils. Neutrophil accumulation in periodontal pockets and infiltrating the junctional epithelium is one of the hallmark features of periodontitis, and these cells appear to be involved in the immunopathogenesis of the disease (34, 35). Similarly to deregulated complement activation, deviations in neutrophil activity (both impaired or exacerbated activity and/or recruitment) result in the disrupted homeostasis in the periodontium and manifest in humans by various forms of periodontitis (35, 36). Local production of C5a by bacterial proteinases could certainly affect both the migration and the activity of neutrophils in the gingival crevice. The impact of C5a on neutrophils is not straightforward, as it strictly depends on the exact levels of the anaphylatoxins. Whereas low levels of C5a induce neutrophil chemotaxis and increase their antibacterial activity, the high levels evoke their “immune paralysis” demonstrated by inhibited migration and diminished performance (37, 38). Hence, taking into account synergistic action of several periodontal bacterial proteinases, there is a high probability for local generation of high C5a levels that could corrupt neutrophils populating the periodontium. Additionally, the bacteria can further shut down immune defenses when bridging two signaling pathways, that is, TLRs and C5aR. Induction of such a cross-talk between TLR2 and C5aR in human and mouse neutrophils as well as mouse macrophages by P. gingivalis resulted in abolished intracellular killing of bacteria and had a profound impact on the oral bacterial community in mice (11, 13, 14). It remains to be elucidated whether such a subversive cross-talk can occur in the presence of other periodontal bacteria. However, identification of C5a convertase-like enzymes in other long-recognized pathogens of human periodontitis, as T. forsythia, underscores the importance of this virulence mechanism. As C5a appears to act in favor for key periodontal pathogens and the bystander species, a new therapeutic concept emerged aiming to counteract C5a signaling by implementing C5aR antagonists in periodontitis (32). Future studies will soon bring more evidence proving efficiency of such a treatment. Of note, the existence of two proteinases inactivating complement in a similar way stresses the importance of complement evasion by T. forsythia. It also highlights that the described mechanisms are very vital for the survival of this pathogen. Furthermore, since knocking out just one proteinase in T. forsythia (Mir or Kly) resulted in drastically diminished complement resistance of the pathogen, we can hypothesize that these enzymes do act synergistically and both are vital for the bacterium. Furthermore, putting presented data into the context of other similar studies (15, 16, 18), proteinases of periodontal bacteria with their fine-tuned effects on complement emerge to be key virulence factors of these pathogens.

Disclosures

The authors have no financial conflicts of interest.

References


