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**ORIGINAL ARTICLE**

# Inactivation of human coagulation factor X by a protease of the pathogen *Capnocytophaga canimorsus*

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## Essentials

- *Capnocytophaga canimorsus* causes severe dog bite related blood stream infections.
- We investigated if *C. canimorsus* contributes to bleeding abnormalities during infection.
- The *C. canimorsus* protease *Cc*DPP7 causes factor X dysfunction by N-terminal cleavage.
- *Cc*DPP7 inhibits coagulation *in vivo*, which could promote immune evasion and trigger hemorrhage.

**Summary.** *Background: Capnocytophaga canimorsus* is a Gram-negative bacterium that is present in the oral flora of dogs and causes fulminant sepsis in humans who have been bitten, licked, or scratched. In patients, bleeding abnormalities, such as petechiae, purpura fulminans, or disseminated intravascular coagulation (DIC), occur frequently. *Objective:* To investigate whether *C. canimorsus* could actively contribute to these bleeding abnormalities. *Methods:* Calibrated automated thrombogram and clotting time assays were performed to assess the anticoagulant activity of *C. canimorsus* 5 (*Cc*5), a strain isolated from a fatal human infection. Clotting factor activities were measured with factor-deficient plasma. Factor X cleavage was monitored with the radiolabeled zymogen and western blotting. Mutagenesis of *Cc*5 genes encoding putative serine proteases was performed to identify the protease that cleaves FX. Protein purification was performed with affinity chromatography. Edman degradation allowed the detection of N-terminal cleavage of FX. Tail bleeding times were measured in mice. *Results:* We found that *Cc*5 inhibited thrombin generation and increased the prothrombin time and the activated partial thromboplastin time of human plasma via FX cleavage. A mutant that was unable to synthesize a type 7 dipeptidyl peptidase (DPP7) of the S46 serine protease family failed to proteolyse FX. The purified protease (*Cc*DPP7) cleaved FX heavy and light chains from the N-terminus, and was active *in vivo* after intravenous injection. *Conclusions:* This is, to our knowledge, the first study demonstrating a detailed mechanism for FX inactivation by a bacterial protease, and it is the first functional study associating DPP7 proteases with a potentially pathogenic outcome.

**Keywords**: bacterial infection; coagulation; factor X; protease; sepsis.

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## Introduction

*Capnocytophaga canimorsus* is a Gram-negative bacterium belonging to the family *Flavobacteriaceae* in the phylum *Bacteroidetes* [1]. It is present in the oral flora of dogs [1,2], and causes rare but severe dog bite-related bloodstream infections in humans, with a mortality rate as high as 30% [3] (for reviews, see [3–5]). *C. canimorsus* sepsis can be accompanied by bleeding disorders, such as disseminated intravascular coagulation (DIC) (13–35% of all cases), which often results in gangrene (8–13%). Patients frequently show skin manifestations, including petechiae and purpura (11–46%) [3–6]. Furthermore, one-third of autopsies performed on deceased individuals reveal hemorrhage of adrenal glands [3].

The presence of pathogens or their products in the circulation provokes several procoagulant processes, such as tissue factor expression by monocytes and endothelial cells, fostering entrapment of bacteria inside fibrin clots [7]. The importance of coagulation in innate immunity is illustrated by *in vivo* data. For instance, infection of fibrin-deficient or coumadin-treated mice with *Yersinia enterocolitica* leads to an elevated pathogen burden and reduced survival [8]. Some bacteria interact with coagulation factors, thus modulating this first step of immune defense [9–11]. *Yersinia pestis*, for example, suppresses fibrin generation via the

plasminogen activator Pla, thereby reducing recruitment of leukocytes to the infection site [12].

In view of these facts and the frequent occurrence of bleeding abnormalities during *C. canimorsus* infection, we investigated the impact of *C. canimorsus* on hemostasis. We demonstrate that the blood culture isolate *C. canimorsus* 5 (*Cc*5) blocks coagulation via the *C. canimorsus* type 7 dipeptidyl peptidase (*Cc*DPP7), which inactivates coagulation factor X by proteolytic cleavage. We hypothesize that this could contribute to immune evasion of the bacteria and to the hemorrhage observed in patients.

## Materials and methods

### *Bacteria and growth conditions*

Bacterial strains are listed in Table S5. *Cc*5 was grown on blood agar plates (heart infusion agar [Difco, Franklin Lakes, NJ, USA], 5% defibrinated sheep blood [Oxoid, Basingstoke, UK], and gentamicin). *Escherichia coli* strains were routinely cultured in LB medium (Invitrogen, Waltham, MA, USA). The concentrations of antibiotics used were as follows: 10 μg $mL^{-1}$ erythromycin, 10 μg $mL^{-1}$ cefoxitin, 20 μg $mL^{-1}$ gentamicin, 50 μg $mL^{-1}$ kanamycin, 100 μg $mL^{-1}$ ampicillin, and 35 μg $mL^{-1}$ nalidixic acid.

### *Preparation of normal pooled plasma*

Human plasma was obtained from 16 healthy volunteers. Blood was drawn into vacutainer tubes (Venosafe; TERUMO, Tokyo, Japan) containing 0.109 mol $L^{-1}$ buffered sodium citrate, and centrifuged twice at 2500 × *g* for 15 min at room temperature. Normal pooled plasma (NPP) was snap-frozen and stored at − 80 °C. The study was approved by the ethics committee of Mont-Godinne, Belgium (Protocol number B039201316262), and performed according to the Declaration of Helsinki and the Belgian law on experiments in humans from 7 March 2004. Participants were informed about the aim of the study and risks of blood withdrawal, and gave written consent.

### *Calibrated automated thrombogram (CAT) assay*

All bacteria were grown on blood agar and incubated with NPP, and cell-free supernatants were added to MP- or PPP-Reagent (Thrombinoscope BV, Maastricht, the Netherlands). Control NPP was mixed with a thrombin calibrator (Thrombinoscope BV). Thrombin generation was monitored with a Fluoroskan Ascent (Thermo Scientific, Waltham, MA, USA) after FluCa (Thrombinoscope BV) injection. Calculations were performed with the Thrombinoscope software (Thrombinoscope BV).

### *Clotting time measurements*

Prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time and reptilase time were assessed with STA-Neoplastin R (Stago, Asnières-sur-Seine, France), HemosIL SynthASil (Instrumentation Laboratory, Bedford, MA, USA), STA-Thrombin (Stago BNL), or STA-Reptilase (Stago), repectively.

### *Coagulation factor activity assay*

Activities were determined with deficient plasma (Stago): STA-Immunodef FII (00740), STA-Deficient FV (00744), STA-Deficient FVII (00743), STA-Immunodef FIX (00734), STA-Deficient FX (00738), STA-Deficient FXI (00723), and STA-Immunodef FXII (00315). Standard curve measurements were performed according to the manufacturer's instructions. In brief, a series of NPP dilutions in Owren–Koller buffer (Stago) was mixed 1 : 1 with deficient plasma. A 1 : 10 dilution was assigned as 100% factor activity. PT was measured to determine FII, FV, FX and FVII activity, and APTT was measured to determine FIX, FXI and FXII activity. Clotting times were plotted against factor dilution. Plasma supernatants were diluted 1 : 10 and mixed 1 : 1 with factor-deficient plasma. Clotting was triggered immediately.

### *Experiments with radiolabeled FX*

Radiolabeling of 50 μg of FX (Haematologic Technologies, Essex Junction, VT, USA) with $Na^{125}I$ (1 mCi) (Perkin Elmer, Waltham, MA, USA) was performed with a precoated iodination tube (cat. no. 28601; Pierce, Waltham, MA, USA). Iodine incorporation constituted 3.7% of total iodine. Bacteria were incubated with [$^{125}$I]FX in NPP for 1 h at 37 °C. 4-(2-aminoethyl)Benzenesulfonyl fluoride) (AEBSF) treatment was performed with 2 × $10^9$ bacteria $mL^{-1}$ at room temperature. Cell-free supernatants were

**Fig. 1.** *Capnocytophaga canimorsus* 5 (*Cc*5) inhibits thrombin generation and leads to increased clotting times. (A, B) Thrombin generation profiles of normal pooled plasma (NPP) after incubation with different concentrations of *Cc*5 for 15 min (A) and 1 h (B). Phosphate-buffered saline (PBS) only (ctrl) or bacterial suspensions in PBS were diluted 1 : 10 in NPP to make the final dilutions of $10^6$–$10^9$ *Cc*5 bacteria $mL^{-1}$ and incubated at 37 °C. (C) Thrombin generation profiles of NPP after incubation for 15 min at 37 °C with PBS (ctrl) or *Escherichia coli* MG1655, *Yersinia enterocolitica* E40, live *Cc*5, or paraformaldehyde-fixed *Cc*5, all at $10^9$ $mL^{-1}$. Thrombin generation was monitored with a Fluoroskan Ascent after addition of MP-Reagent (4 μM phospholipids) or PPP-Reagent (5 pM tissue factor, 4 μM phospholipids), and FluCa (fluorogenic thrombin substrate + $CaCl_2$). Representative results from three independent experiments performed in triplicate are shown. (D, E) *Cc*5 bacteria ($10^9$ $mL^{-1}$) or PBS (ctrl) were incubated at 37 °C with NPP for 1 h. For prothrombin time (PT) measurements, coagulation was induced with STA-Neoplastin R (D); for activated partial thromboplastin time (APTT) measurements, HemosIL SynthASil was used (E). Average clotting times of at least three independent experiments performed in duplicate are shown. Error bars represent the standard deviation. ***$P \leq 0.001$ as compared with control, determined with Student's *t*-test. [Color figure can be viewed at wileyonlinelibrary.com]

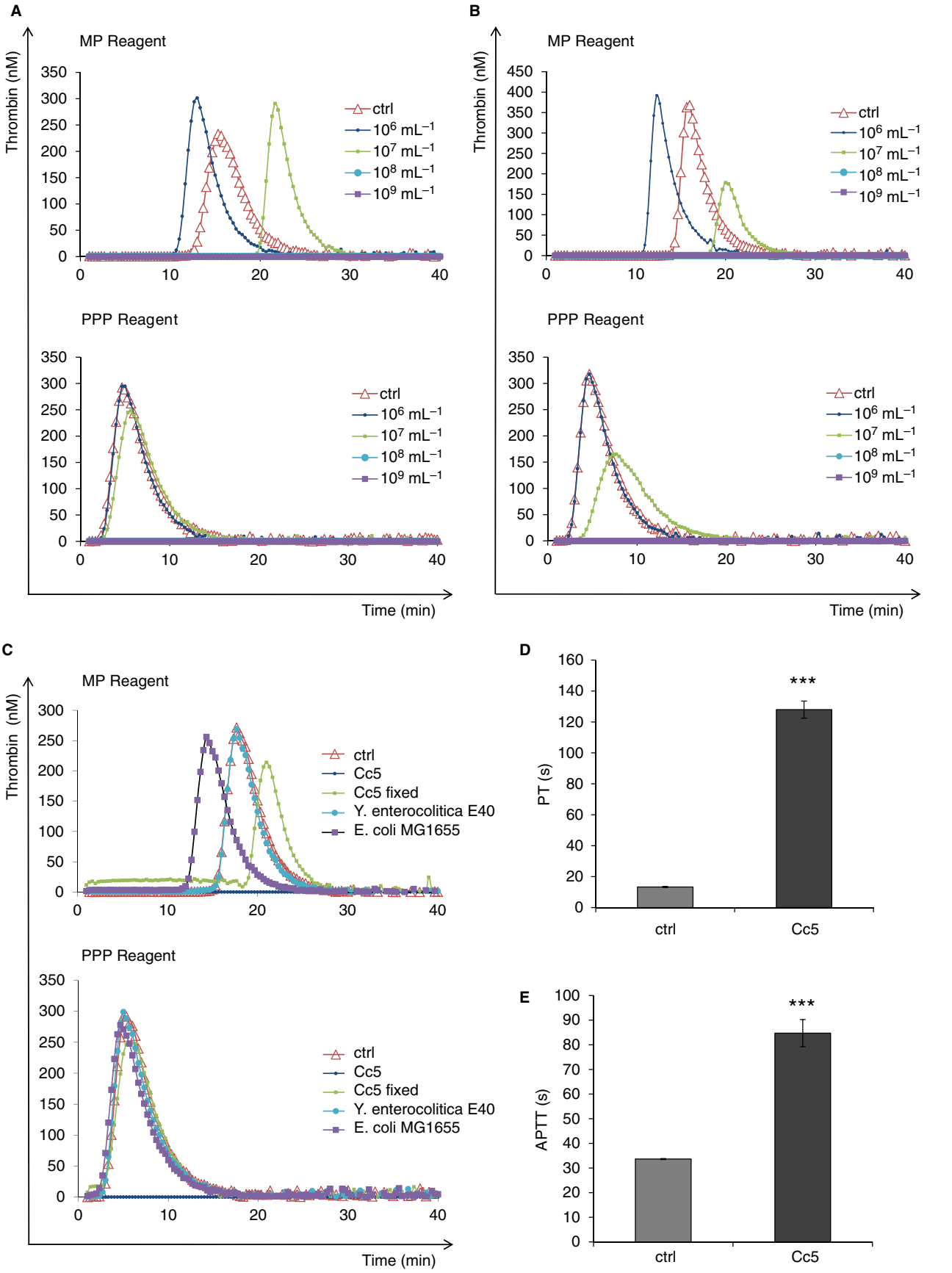

**Table 1** (A) Activity of selected coagulation factors (A) and thrombin and reptilase times (B) of normal pooled plasma (NPP) incubated for 1 h at 37 °C with $10^9$ *Capnocytophaga canimorsus* 5 (*Cc*5) bacteria $mL^{-1}$ or phosphate-buffered saline (PBS) as control.

| | NPP + PBS | NPP + *Cc*5 |
|---|---|---|
| (A) Factor activities (%)* ± SD | | |
| FII | 92 ± 7 | 44 ± 4 ($P = 0.0006$)† |
| FV | 95 ± 17 | 96 ± 14 |
| FVII | 100 ± 4 | 45 ± 1 ($P < 0.0001$) |
| FIX | 105 ± 0 | 1 ± 0 ($P < 0.0001$) |
| FX | 81 ± 6 | 1 ± 0 ($P < 0.0001$) |
| FXI | 64 ± 5 | 71 ± 6 |
| FXII | 98 ± 13 | 122 ± 17 |
| (B) Clotting times (s) ± SD | | |
| Thrombin time | 17.3 ± 0.1 | 20.1 ± 1.8 |
| Reptilase time | 17.5 ± 0.5 | 18.3 ± 1.2 |

SD, standard deviation. *Factor activities were determined with factor-deficient plasma. Sample plasma was diluted 1 : 10 in Owren–Koller buffer, and mixed 1 : 1 with factor-deficient plasma, and the prothrombin time (FII, FV, FVII, and FX) or activated partial thromboplastin time (FIX, FXI, and FXII) was measured. The activity was calculated by means of a standard curve obtained by mixing different dilutions of a calibrator NPP, starting from 1 : 10, with equal volumes of the appropriate factor-deficient plasma. The 1 : 10 dilution of standard NPP was arbitrarily assigned to a factor activity of 100%. †For statistical analysis, one-way ANOVA followed by a Bonferroni test was carried out. *P*-values represent statistical significance as compared with the control (NPP + PBS).

run on a 12% Nupage Novex Bis-Tris gel (Life Technologies) with MOPS buffer (Life Technologies, Waltham, MA, USA). The dried gel was exposed to a Phosphor Screen (multisensitive; Perkin Elmer), which was scanned with a Cyclone Phosphor Imager (Perkin Elmer).

### *Generation of knock-out mutants*

Primers are listed in Table S6. Site-directed mutagenesis was performed as described by Mally *et al*. [13] with primers 1.1 and 1.2 to amplify the upstream flanking region (fragment 1) and primers 2.1 and 2.2 to amplify the downstream flanking region (fragment 2) of the gene to be knocked out. Primers 3.1 and 3.2 were used to amplify the *ermF* resistance cassette (fragment 3). In contrast to Mally *et al*., we first fused fragment 1 with fragment 3 in a separate PCR reaction before fusing this to fragment 2.

### *Construction of complementation and expression plasmids*

Plasmids and primers are listed in Tables S7 and S6, respectively. The *dpp7* gene was amplified with primers 7982 and 7983 and inserted into pMM47 [13], generating the complementation plasmid pKH28. *Dpp7* was amplified with primers 7982 and 7989, introducing a C-terminal Strep-His tag, and inserted into pPM5 [14] to generate plasmid pKH32. Plasmids were purified from One Shot TOP10 Electrocomp *E. coli* (Thermo Scientific), and transformed into *Cc*5 Δ*dpp7* bacteria. Positive clones were isolated on cefoxitin blood agar plates. Primers 5451 and 4730 or 7809 and 4730 were used for sequencing of inserts in pMM47 or pPM5, respectively.

### *Construction of the catalytic CcDPP7 mutant*

We introduced a point mutation in the *dpp7* gene, replacing the active site serine of *Cc*DPP7 with an alanine (S649A), and added a C-terminal Strep-His tag for purification. *Dpp7* was amplified in two fragments by the use of primers 7982 and 7999 or 7998 and 7989, which were then fused with primers 7982 and 7989 (Table S6). The product was inserted into pPM5, generating pKH33. We then proceeded as described above.

### *Protein purification*

*Cc*5 Δ*dpp7* (pKH32) and *Cc*5 Δ*dpp7* (pKH33) bacteria were disrupted with a French press. Cleared lysates were purified with a HisTALON resin (Clontech Laboratories, Mountain View, CA, USA) and a Streptactin resin (Iba), according to the manufacturer's instructions. Purity was assessed by silver staining as described previously [15] (Fig. S1).

### *Immunoblotting*

The following primary antibodies were used: rabbit antiserum raised against purified *Cc*DPP7-Strep-His (1 : 5000; this work), mouse mAb HFX-LC and mAb HFX-HC (1 : 1000 and 1 : 1500, respectively; Enzyme Research Laboratories, South Bend, IN, USA), and rabbit anti-F10A cleaved Ala41 (1 : 1000; AssaybioTech, Sunnyvale, CA, USA). The following secondary antibodies were used: swine anti-rabbit horseradish peroxidase (HRP) (1 : 5000; Dako, Santa Clara, CA, USA) and goat anti-mouse HRP (1 : 5000; Dako). Lumiglo Reserve Chemoluminiscent Substrate (KPL, Gaithersburg, MD, USA) was used for development, according to the manufacturer's instructions.

### *Quantification of CcDPP7 release*

One hundred and sixty microliters of supernatants of NPP or heat-inactivated plasma (HIP) treated with $10^9$ wild-type (WT) *Cc*5, with Δ*dpp*7 or with phosphate-buffered saline (PBS) were added to 40 μL of L-methionyl-L-leucyl-4-methylcoumaryl-7-amide (Peptanova, Sandhausen, Germany; final concentration of 250 ng $mL^{-1}$) and incubated for 8 min at 37 °C. Fluorescence was read on a Fluoroskan Ascent (excitation/emission: 355 nm/460 nm). A dilution series of a *Cc*DPP7 standard was added for quantification purposes.

### *N-terminal sequencing*

Twenty-five micrograms of FX in PBS was incubated with or without *Cc*DPP7 for 30 min at 37 °C. Concentrated samples

(Amicon Ultra-0.5 10K MWCO filters; Millipore, Billerica, MA, USA) were applied to a Nupage Novex Bis Tris gel (Life Technologies). Proteins were transferred to a PVDF-membrane (Amersham, UK), and visualized with Coomassie R250 brilliant blue. Sequencing was carried out at the laboratory for Protein Biochemistry and Biomolecular Engineering at the University of Ghent, Belgium.

### *Effect of CcDPP7 on clotting factors*

Purified clotting factors (Haematologic Technologies) were incubated with *Cc*DPP7 or $CcDPP7_{S649A}$ in PBS. FIX and FX were diluted 1 : 5 in factor-deficient plasma to 5 μg $mL^{-1}$ and 10 μg $mL^{-1}$, respectively. FVII (0.5 μg $mL^{-1}$) and FII (10 μg $mL^{-1}$) were mixed 1 : 1 with factor-deficient plasma.

### *Evaluation of tail bleeding time*

Balb/c mice were obtained from the University of Namur and Harlan Laboratories (the Netherlands), and housed at the animal facility of the University of Namur. Experiments were performed in accordance with institutional guidelines and animal bioethics consent (approval no. UN LE 13/205). Mice were injected with 200 μL of 1 mg $kg^{-1}$ *Cc*DPP7 or with 200 μL of the vehicle PBS. Warfarin was administered for 3 days with the drinking water. After anesthesia with ketamine/Domitor (82.5 mg $kg^{-1}$/0.55 mg $kg^{-1}$), a 5-mm-long piece of the tail tip was removed, and the tail was submerged in warm PBS (37 °C). The total bleeding time represents all bleeding periods that occurred within 10 min.

## Results

### *Cc5 inhibits thrombin generation*

To determine whether *C. canimorsus* interferes with coagulation, we performed a CAT assay, in which thrombin generation was monitored with a fluorogenic thrombin substrate. We incubated NPP with different amounts of *Cc*5 bacteria, and induced thrombin generation via the intrinsic pathway (MP-Reagent) or the extrinsic pathway (PPP-Reagent) at different time points. Both pathways were affected at concentrations from $10^7$ bacteria $mL^{-1}$, and completely blocked at $10^8$ bacteria $mL^{-1}$. The inhibitory effect already occurred after 15 min (Fig. 1A; Table S1), and increased slightly when the incubation time was prolonged to 1 h (Fig. 1B; Table S2). A slight, but insignificant, acceleration of thrombin generation occurred with MP Reagent, which might have resulted from the activation of the intrinsic pathway on the

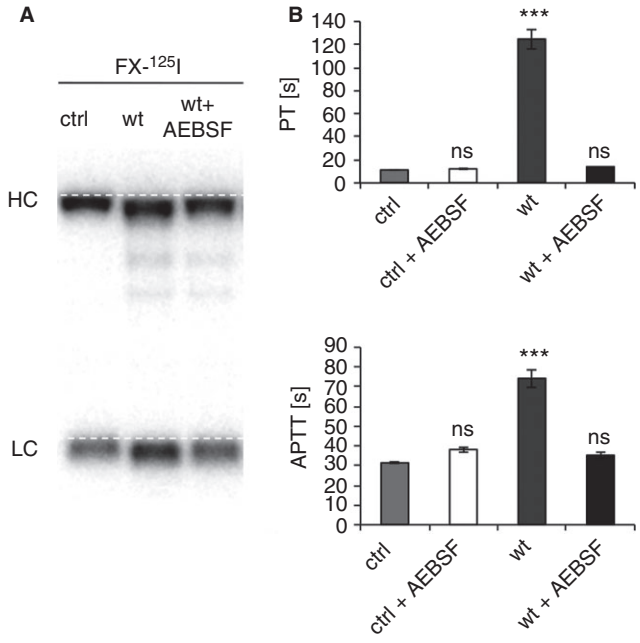


**Fig. 2.** A serine protease mediates factor X cleavage and causes increased clotting times. (A) Autoradiography of [$^{125}$I]FX. Iodinated FX (3 μg $mL^{-1}$) was added to normal pooled plasma (NPP), and incubated either with phosphate-buffered saline (PBS) (ctrl) or with wild-type (WT) bacteria in PBS preincubated for 30 min in the presence or absence of 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). The dashed line indicates the size shift. A representative image of three independent experiments is shown. (B) Prothrombin time (PT) and activated partial thromboplastin time (APTT) measurements of NPP treated with *Capnocytophaga canimorsus* 5 WT bacteria in PBS preincubated for 30 min in the presence or absence of 1 mM AEBSF. As controls (ctrl), either PBS or 1 mM AEBSF in PBS were added to NPP. Mean values (± standard deviation) of three independent experiments, each performed in triplicate, are shown (***$P \leq 0.001$ as compared with control). A concentration of $10^9$ bacteria $mL^{-1}$ was used for all conditions, and the incubation was always performed for 1 h at 37 °C. One-way-ANOVA with a Bonferroni *post hoc* test was used for statistical analysis. HC, heavy chain; LC, light chain; NS, not significant.

surface of *Cc*5, as shown for other bacteria [16]. Two other Gram-negative control strains, *E. coli* MG1655 and *Y. enterocolitica* E40, did not inhibit thrombin generation, and paraformaldehyde fixed *Cc*5 only slightly reduced the amount of generated thrombin (Fig. 1C; Table S3), suggesting that the inhibitory effect was specific to *Cc*5 and mediated by an active mechanism.

### Cc5 prolongs the PT and APTT

We next measured the PT and the APTT of *Cc*5-treated NPP; these allow the detection of deficiencies in the extrinsic and the common pathway, or in the intrinsic and the common pathway, respectively [17,18]. As this method is less sensitive than the CAT assay, $10^9$ bacteria $mL^{-1}$ were used and incubated for 1 h. NPP treated with *Cc*5 showed a 10-fold increased PT (Fig. 1D) and a two-fold to three-fold increased APTT (Fig. 1E), thus confirming that *Cc*5 exerts an inhibitory effect on the coagulation cascade and that all pathways are impaired. When we tested the activity of different coagulation factors, we found that four vitamin K-dependent (VKD) clotting factors were affected. FX and FIX activities were strongly decreased to 1% of normal, and FII and FVII activities by > 50%, hinting at either dysfunction or depletion of these factors by the bacteria (Table 1A). There was no effect on fibrin(ogen), as confirmed by thrombin time and reptilase time assays, both of which allow the detection of fibrin(ogen) deficiencies (Table 1B).

### FX is cleaved by a bacterial serine protease

We investigated the fate of FX, one of the most affected VKD factors. When we incubated *Cc*5 bacteria with NPP spiked with radiolabeled FX, we observed protein modifications, with a slight band shift of the FX heavy chain (HC) and light chain (LC). These no longer occurred upon prior treatment of the bacteria with the irreversible serine protease inhibitor AEBSF (Fig. 2A), suggesting cleavage by a bacterial serine protease. In addition, there was *Cc*5-mediated generation of two faint bands below the HC, which were still visible after AEBSF treatment of the bacteria, indicating that another bacterial factor, potentially another protease or a deglycosylase, also affected FX. As preincubation of *Cc*5 with AEBSF completely abolished the increased clotting times (Fig. 2B),

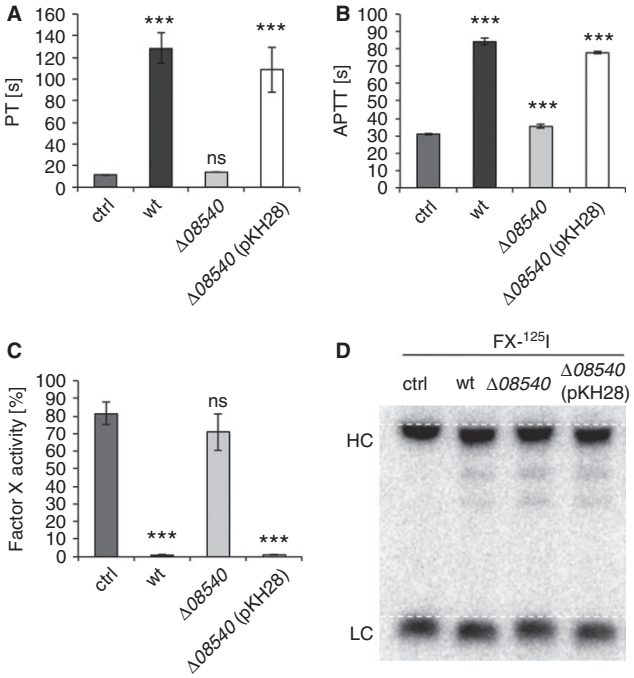


**Fig. 3.** Deletion of the gene *Ccan_08540* restores normal prothrombin time (PT) and activated partial thromboplastin time (APTT) values, factor X function, and FX protein integrity. Complementation of Δ*Ccan_08540* with the *Ccan_08540* expression plasmid pKH28 leads again to increased clotting times and impaired FX activity. (A, B) PT and APTT values of normal pooled plasma (NPP) incubated with phosphate-buffered saline (PBS) (ctrl) or $10^9$ bacteria $mL^{-1}$ of wild-type (WT) *Capnocytophaga canimorsus* 5, Δ*Ccan_08540* (Δ*08540*), or the complemented strain Δ*Ccan_08540* (pKH28) (Δ*08540* [pKH28]). Mean values (± standard deviation [SD]) of at least four independent experiments, each performed in duplicate, are shown. (C) FX activity of NPP incubated in the absence or presence of $10^9$ bacteria $mL^{-1}$ was measured with factor-deficient plasma. Mean values (± SD) of three independent experiments, each performed in duplicate, are shown. Statistical analysis was performed by comparison with the control (***$P \leq 0.001$). (D) Representative autoradiography of [$^{125}$I]FX in NPP incubated with PBS (ctrl) or $10^9$ bacteria $mL^{-1}$. The dashed line indicates the size shift. All experiments were performed for 1 h at 37 °C. One-way-ANOVA with a Bonferroni *post hoc* test was used for statistical analysis. HC, heavy chain; LC, light chain.

serine protease-mediated FX LC and HC degradation seems to be the main cause of the increase in the APTT and PT, and will be the focus of this article.

### *Identification of the bacterial serine protease affecting FX activity*

According to the MEROPS protease database, the *Cc*5 genome [19] encodes 27 potential serine proteases (Table S4), which we individually deleted by gene-directed mutagenesis. All mutants behaved like WT bacteria (data not shown), except for one, Δ*Ccan_08540*, which no longer led to an increased PT (Fig. 3A). There was still a small increase in the APTT (Fig. 3B), which might have resulted from the hypothetical second factor modifying the FX HC or from an interaction of *Cc*5 with molecules in the APTT reagent. The normalization of the PT and APTT in Δ*Ccan_08540* bacteria-treated NPP coincided with intact FX function (Fig. 3C) and the disappearance of the HC and LC band shift of FX (Fig. 3D). A complemented strain of Δ*Ccan_08540*, Δ*Ccan_08540* (pKH28), behaved similarly to WT *Cc*5, meaning that the mutation of *Ccan_08540* was indeed responsible for the phenotype (Fig. 3A–C). These findings indicate that *Ccan_08540* encodes a serine protease that is responsible for FX cleavage and inhibition of coagulation.

### Ccan_08540 *encodes a DPP7 homolog*

In MEROPS, the protein encoded by *Ccan_08540* is annotated as a dipeptidyl peptidase 11 (DPP11)-like protease, a member of the S46 family of serine proteases. When we aligned the amino acid sequence of the Ccan_08540 protease with that of the DPP11 homolog of *Porphyromonas gingivalis* (*Pg*DPP11) [20] (Fig. S2), we found that Ccan_08540 has a glycine instead of an arginine at P667. As this arginine is a hallmark of DPP11 proteases [21], its substitution by a glycine, as in *Pg*DPP7,

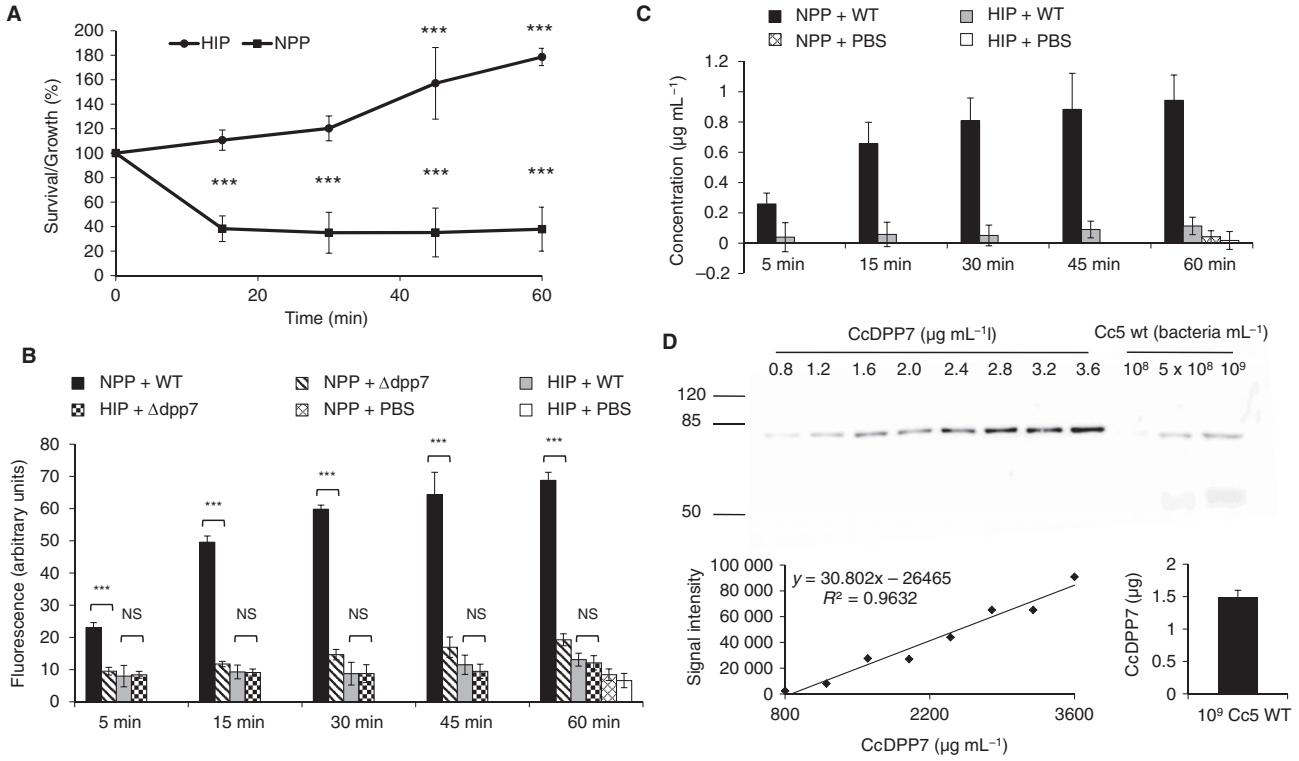


**Fig. 4.** *Capnocytophaga canimorsus* type 7 dipeptidyl peptidase (*Cc*DPP7) release into normal pooled plasma (NPP) is mediated by lysis of *C. canimorsus* 5 (*Cc*5). (A) *Cc*5 is killed in NPP. Wild-type (WT) *Cc*5 bacteria ($10^9$ $mL^{-1}$) were incubated for the indicated time points with NPP or heat-inactivated plasma (HIP), and colony-forming units were quantified by plating. The percentage of surviving/growing bacteria relative to the inoculum is shown. ***$P \leq 0.001$. (B) *Cc*DPP7 release into plasma is triggered by bacterial lysis. WT *Cc*5 or Δ*dpp7* bacteria ($10^9$ $mL^{-1}$) were incubated with NPP or HIP for the indicated time points. Phosphate-buffered saline (PBS) only was used as control. *Cc*DPP7 release was monitored with a fluorogenic L-methionyl-L-leucyl-4-methylcoumaryl-7-amide substrate. The average fluorescence (± standard deviation [SD]) of three independent experiments is shown. NS, not significant; ***$P \leq 0.001$ versus NPP/HIP incubated with Δ*dpp7* bacteria. (C) The average *Cc*DPP7 concentration (± SD) in NPP and HIP was calculated on the basis of the fluorescence generated in each of the three experiments shown in panel B, with purified *Cc*DPP7 as a standard. (D) Quantification of *Cc*DPP7 expression by *Cc*5. An anti-*Cc*DPP7 western blot is shown. Ten microliters of purified *Cc*DPP7 or WT *Cc*5 bacteria at the indicated concentrations in sample buffer were loaded onto an SDS-PAGE gel. A representative blot and standard curve of three independent experiments, as well as the average *Cc*DPP7 amount (± SD) expressed by $10^9$ WT *Cc*5 bacteria, are shown. The band intensities were quantified with AMERSHAM IMAGER 600 software, and the average amount of *Cc*DPP7 expressed by $10^9$ WT *Cc*5 bacteria was calculated from three independent experiments.

another *P. gingivalis* dipeptidyl peptidase, indicates that Ccan_08540 is, rather, a member of the DPP7 subgroup of S46 proteases [21]. We therefore called it *Cc*DPP7. The *Ccan_08540* gene, which we named *dpp7*, could be identified in all 26 *C. canimorsus* strains that we have sequenced so far, with a gene identity of > 99% (Fig. S3A). *Cc*5 also possesses a DPP11 homolog, which is encoded by *Ccan_11580*, but it is not implicated in the anticoagulant effect (data not shown).

*Cc*DPP7 homologs are present in the genomes of other *Capnocytophaga* species, such as *Capnocytophaga cynodegmi* and *Capnocytophaga canis*, that cause wound infections, but no sepsis (Fig. S3A) [1,22]. Twelve further *C. canimorsus* and three *C. canis* strains that we tested increased the PT and APTT in a similar manner. Three tested *C. cynodegmi* strains were less efficient in affecting the PT, but prolonged the APTT like *C. canimorsus* (Fig. S3B).

### Cc*DPP7 is released into plasma via bacterial lysis*

Being devoid of a signal peptide, *Cc*DPP7 is likely to be cytoplasmic. As the *Cc*5 genome contains no evidence of machinery allowing active secretion, except for the gliding motility system [19], and as *C. canimorsus* is killed by complement at high plasma concentrations [23], we speculated that complement-mediated lysis could induce *Cc*DPP7 release. When we incubated $10^9$ bacteria $mL^{-1}$ in NPP, we observed that 60% of the bacteria had died within 15 min (Fig. 4A). No more killing occurred up to 1 h. When complement was inactivated by heat treatment of plasma, there was no killing and even slight growth. Using a fluorogenic dipeptide substrate cleaved by *Cc*DPP7, we observed a strong time-dependent increase of fluorescence in the supernatants of NPP, but not in HIP, incubated with WT *Cc*5 bacteria (Fig. 4B), suggesting the liberation of *Cc*DPP7 by bacterial lysis. In the same conditions, Δ*dpp7* bacteria resulted in low fluorescence, confirming that substrate cleavage was mainly caused by *Cc*DPP7. Using serial dilutions of purified *Cc*DPP7 (Fig. S1), we calculated that $10^9$ WT *Cc*5 $mL^{-1}$ of NPP release an average of 942 ng of *Cc*DPP7 (Fig. 4C). This might be a slight overestimation, given the low activity of other *Cc*5 proteases on the substrate. However, if we assume 60% lysis (Fig. 4A), the value is in the same range as that obtained by a quantitative western blot assay, which showed that $10^9$ bacteria contain,

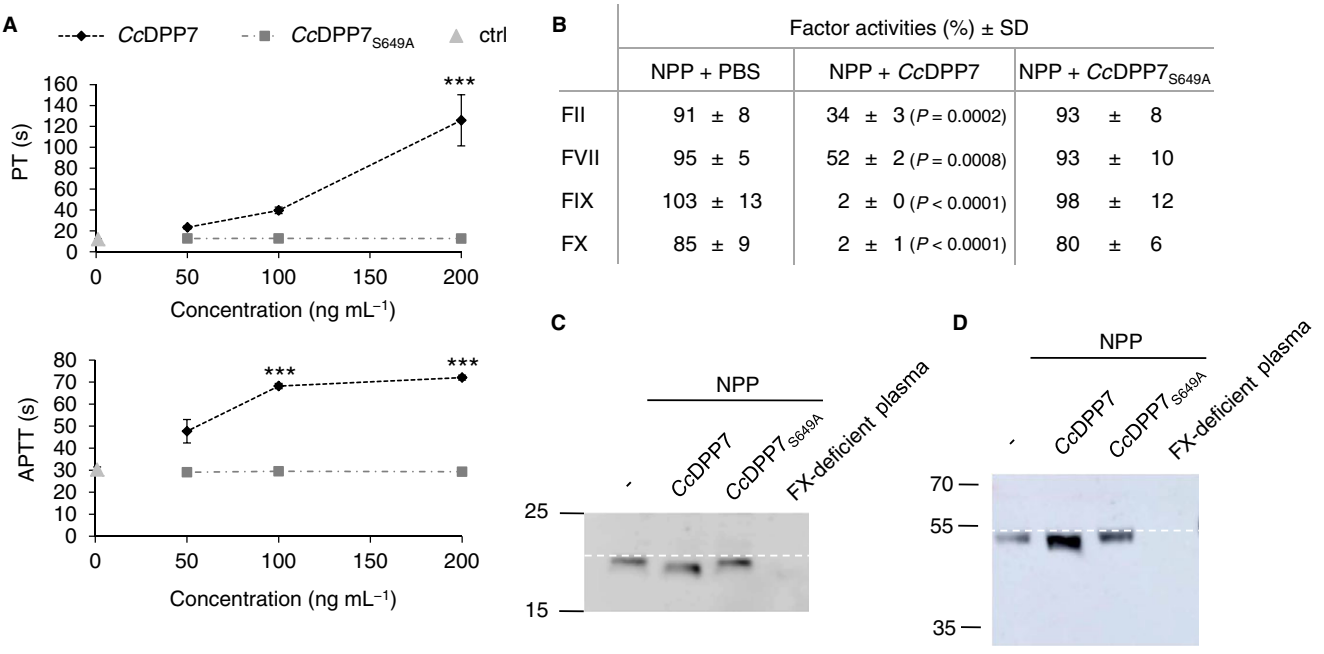


**Fig. 5.** Addition of purified Capnocytophaga canimorsus type 7 dipeptidyl peptidase (CcDPP7) to normal pooled plasma (NPP) leads to prolonged clotting times, decreased activity of vitamin K dependent clotting factors and cleavage of factor X heavy chain (HC) and light chain (LC). (A) *Cc*DPP7, *Cc*DPP7$_{S649A}$ or phosphate-buffered saline (PBS) was diluted 1 : 10 in NPP to the indicated concentrations, and incubated at 37 °C for 1 h; the PT and APTT were then measured. One-way ANOVA with a Bonferroni *post hoc* test was performed for statistical analysis by comparison with the untreated control; ***$P \leq 0.001$. (B) The activities of FII, FVII, FIX and FX in NPP treated for 1 h at 37 °C with PBS or 200 ng $mL^{-1}$ *Cc*DPP7 or *Cc*DPP7$_{S649A}$ were measured with factor-deficient plasma. The results of three independent experiments (± standard deviation [SD]), each performed in duplicate, are shown. For statistical analysis, one-way ANOVA with a Bonferroni *post hoc* test was performed by comparison with NPP + PBS. (C, D) *Cc*DPP7 causes FX light chain (LC) and heavy chain (HC) cleavage in NPP. Cropped images of anti-FX LC (C) or HC (D) western blots of NPP incubated for 1 h at 37 °C in the presence or absence of 200 ng $mL^{-1}$ *Cc*DPP7 or *Cc*DPP7$_{S649A}$ are shown. Ten microliters of plasma samples diluted 1 : 20 were loaded. Ten microliters of FX-deficient plasma diluted 1 : 20 served as a negative control. Dashed lines indicate the band shift. A representative result of three independent experiments is shown. Ctrl, control. [Color figure can be viewed at wileyonlinelibrary.com]

on average, 1.49 μg of *Cc*DPP7 (Fig. 4D). To conclude, *Cc*DPP7 is rapidly released into plasma via bacterial lysis.

### Cc*DPP7 cleaves the N-termini of FX LC and HC*

We next characterized the cleavage of FX by purified *Cc*DPP7. We first confirmed the anticoagulant action of the purified protease. Indeed, NPP incubated with *Cc*DPP7 showed dose-dependent increases in PT and APTT (Fig. 5A), reduced VKD factor activity (Fig. 5B), and an altered molecular weight of FX (Fig. 5C,D). A catalytic mutant of *Cc*DPP7, *Cc*DPP7$_{S649A}$, in which the active serine had been replaced by an alanine, was inactive. To test the efficiency of *Cc*DPP7 in cleaving FX, we performed a PT assay with FX-deficient plasma, which we supplemented with FX that was pretreated with *Cc*DPP7 at different concentrations. We observed a significant increase in the PT starting from an enzyme/substrate ratio of 1 : 50 (by weight) as compared with the control (Fig. 6A), which corresponds to the ratio needed to significantly increase the PT in NPP (Fig. 5A), assuming a plasma FX concentration of 10 μg mL$^{-1}$. The PT maximum was reached at ratios of 1 : 10 to 1 : 4. To exclude any interference of *Cc*DPP7 with FX-deficient plasma at the moment of induction, we added a control, incubating FX and *Cc*DPP7 separately and only mixing them upon addition to FX-deficient plasma when clotting was induced. The PT was normal in this condition, meaning that the effect was only triggered by the interaction of *Cc*DPP7 with FX.

We additionally monitored FX degradation by SDS-PAGE followed by silver staining, and found that a *Cc*DPP7/FX ratio of 1 : 1000 was already sufficient to

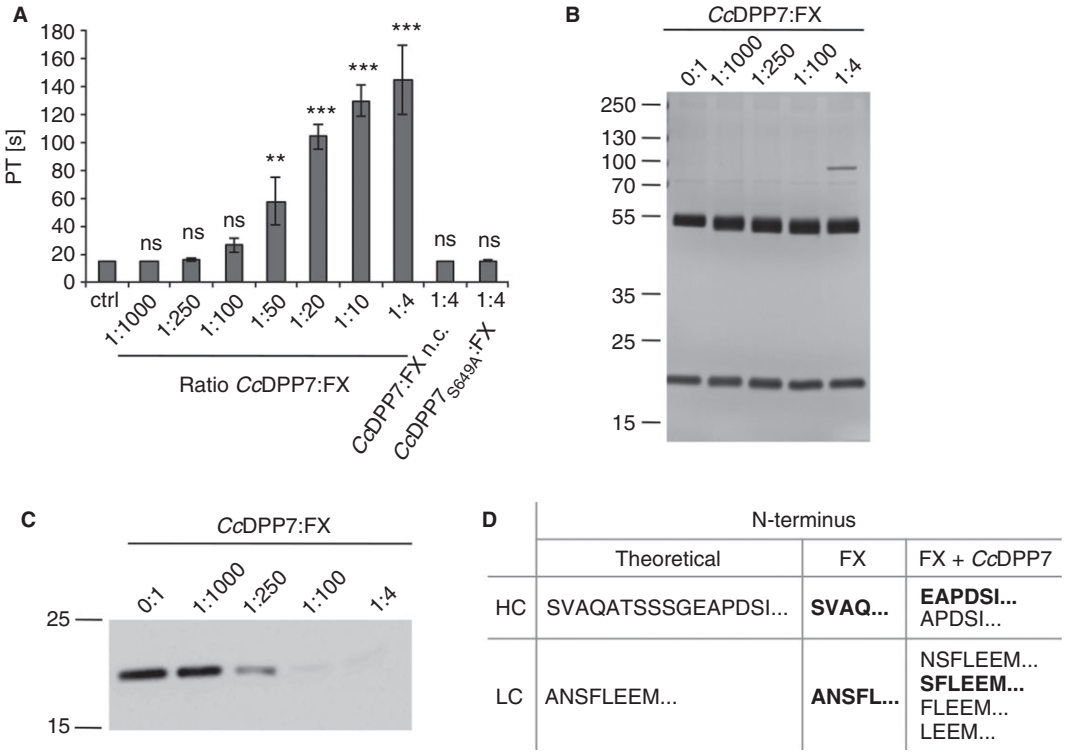


**Fig. 6.** Factor X is degraded by N-terminal cleavage. (A) *Capnocytophaga canimorsus* type 7 dipeptidyl peptidase (*Cc*DPP7) affects FX function in a dose-dependent manner. FX (50 μg mL$^{-1}$) was incubated for 30 min at 37 °C in the presence or absence of *Cc*DPP7 or *Cc*DPP7$_{S649A}$ at the indicated enzyme/substrate ratios (by weight), and subsequently added to FX-deficient plasma at a concentration of 10 μg mL$^{-1}$. To exclude any interaction of *Cc*DPP7 with FX-deficient plasma at the moment of induction, a control was included for which FX and *Cc*DPP7 were first incubated separately and only mixed upon addition to FX-deficient plasma when the inducer was added (n.c., no coincubation). Mean values and standard deviations of three independent experiments, each performed in triplicate, are shown. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ versus control. One-way-ANOVA with a Bonferroni *post hoc* test was used for statistical analysis. (B) Degradation of pure human FX by *Cc*DPP7 happens in a dose-dependent manner, but reaches a limit at high doses. FX (50 μg mL$^{-1}$) in phosphate-buffered saline (PBS) was incubated with *Cc*DPP7 at the indicated ratios (by weight) for 30 min at 37 °C. Ten microliters of each sample in sample buffer was loaded. Representative silver staining of three independent experiments is shown. (C, D) FX is degraded by *Cc*DPP7 via N-terminal cleavage. (C) Western blot for amino acids 1–20 of the FX light chain (LC). Purified human FX (50 μg mL$^{-1}$) in PBS was incubated with the indicated concentrations of *Cc*DPP7 or *Cc*DPP7$_{S649A}$ for 30 min at 37 °C. Ten microliters of each sample in sample buffer was loaded. Representative results of three independent experiments are shown. (D) Purified human FX (50 μg mL$^{-1}$) in PBS was incubated in the presence or absence of 12.5 μg mL$^{-1}$ *Cc*DPP7. Automated N-terminal Edman degradation was performed on the LC and heavy chain (HC). Amino acid sequences from two independent experiments are shown. Sequences in bold were the most abundant in both experiments. ctrl, control; NS, not significant; PT, prothrombin time.

initiate a clearly visible band shift of the FX HC (Fig. 6B). The LC shift was more obvious from 1 : 250, indicating that *Cc*DPP7 could have a higher specificity for the HC. The discrepancy between this test and the PT assay may result from the high sensitivity of the silver staining, which detected partial degradation, and the low sensitivity of the neoplastin reagent, which contains high amounts of TF.

The DPP7 homolog of *P. gingivalis* is an aminopeptidase [24]. To explore whether *Cc*DPP7 degrades the FX N-terminus, we incubated FX with *Cc*DPP7 at different ratios, and analyzed the FX LC by western blotting with an antibody recognizing the first 20 amino acids. There was reduced antibody binding from a 1 : 250 ratio, indicating N-terminal cleavage (Fig. 6C). $CcDPP7_{S649A}$ did not induce N-terminal cleavage of the LC (Fig. S4). N-terminal sequencing was performed to confirm this result and to investigate the fate of the HC. Because, at a ratio of 1 : 4, no more binding of the LC antibody occurred and the FX HC and LC were shifted to a maximum, suggesting that all FX molecules had been cleaved (Fig. 6B), we decided to use this condition for sequencing. We found that, mainly, 10 N-terminal amino acids of the HC and two amino acids of the LC were missing (Fig. 6D), confirming the aminopeptidase nature of *Cc*DPP7.

Apart from FX, *Cc*DPP7 also acted on FIX (Figs 5B and S5C) and FVII, but the latter action was weaker and less specific (Figs 5B and S5B). It is likely that the N-termini of FIX and FVII undergo limited cleavage, but, so far, we have no evidence for this. *Cc*DPP7 reduced FII activity in NPP (Fig. 5B), but did not reduce the activity of purified FII (Fig. S5A). This raises the question of whether the impact of *Cc*DPP7 on FII observed in NPP was overestimated, owing to an effect on multiple coagulation factors, or whether our assay with purified FII was not sensitive enough.

### *Cc*DPP7 is active in vivo

In order to confirm the anticoagulant activity of *Cc*DPP7 *in vivo*, we administered the enzyme to Balb/c mice by intravenous injection. One hour after *Cc*DPP7 administration, the median tail bleeding time was two-fold higher than in the control (Fig. 7). Mice of the positive control group, which had received warfarin, showed a three-fold increase in the median tail bleeding time as compared with the mock-treated control group.

## Discussion

Although coagulation disorders classically accompany generalized infections, the bleeding and coagulation abnormalities are particularly severe in the case of *C. canimorsus* infection, often leading to therapeutic amputations [25–29]. When we investigated the possible interaction between *C. canimorsus* and hemostasis, we found that *Cc*5 inhibited coagulation of human plasma via a bacterial serine protease of the DPP7 type (*Cc*DPP7), which caused cleavage of the N-termini of the FX LC and HC. Removal of all but three amino acids of the FX activation peptide hampers the activation by the intrinsic FXase [30]. The HC cleaved by *Cc*DPP7 lacks only 10 amino acids, but this could be sufficient to decrease the rate of FX activation. *Cc*DPP7 predominantly cleaves two amino acids from the N-terminus of the LC, which comprises a γ-carboxyglutamic acid (Gla) domain. Even the absence of the very first alanine (A1) can affect FX activity by preventing the formation of hydrogen bonds with Gla16, Gla20, Thr21, and Gla21, which normally mediate conformational stability of the Gla domain [31]. In addition, A1 contributes to the generation of a Ω-loop, which stretches until Gly11 [31] and is necessary for the interaction of the

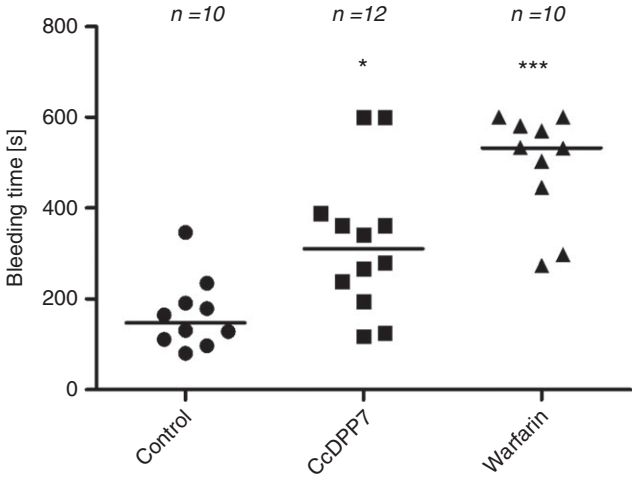


**Fig. 7.** *Capnocytophaga canimorsus* type 7 dipeptidyl peptidase (*Cc*DPP7) prolongs the bleeding time *in vivo*. *Cc*DPP7 (1 mg $kg^{-1}$) or phosphate-buffered saline as control was injected into the tail vein of male Balb/c mice. Bleeding times were measured 1 h after injection. The positive control group received 5 mg $L^{-1}$ warfarin in the drinking water for 3 days. $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$ versus vehicle-treated control. One-way-ANOVA with a Bonferroni *post hoc* test was used for statistical analysis.

Gla domain with membrane surfaces [32]. This suggests that, although *Cc*DPP7 cleaves only a few residues from the FX HC and LC N-termini, it could be sufficient for FX inactivation.

It would be of great interest to identify the mechanism of interaction of *Cc*DPP7 with FX, which should help to clarify its preference for certain factors, and to investigate why *Cc*DPP7 stops cleaving after a limited number of amino acids from the HC and LC. Even with very high enzyme concentrations, no further cleavage occurs (Fig. 6B). A possible cause could be post-translational modifications causing steric hindrance, such as glycans, or a complex secondary structure making further amino acids less accessible.

Owing to its intracellular location, *Cc*DPP7 might essentially be a housekeeping protease that could nevertheless play a role in cleaving clotting factors in plasma, because it is strongly released by the complement-mediated lysis of *Cc*5 bacteria. As CcDPP7 is also expressed by strains not causing invasive infections, it is clear that the possession of *Cc*DPP7 alone does not suffice to make *C. canimorsus* a pathogenic bacterium. Other factors, such as the weakly endotoxic lipopolysaccharide [33], the resistance to phagocytosis [34], the capacity to capture iron [14], and the N-glycoprotein deglycosylation [35], cooperate to determine pathogenicity. Unfortunately, the investigation of virulence factors has been hampered by the lack of animal models permitting the investigation of pathogenesis *in vivo*. Rabbits [36], mice and hamsters (unpublished data) clear bacteria completely. *In vitro*, in human plasma, *Cc*DPP7 was effective in the nanogram range, and $10^7$ bacteria $mL^{-1}$ sufficed to affect thrombin generation. In the mouse, ~ 17 μg of pure *Cc*DPP7 $mL^{-1}$ blood was required to increase the tail bleeding time. We have no obvious explanation for this discrepancy, but protein stability, the presence of inhibitors or other unknown targets in murine blood could play a role.

Some clinical reports mention the presence of extracellular bacteria in peripheral blood smears [37,38], indicating a high bacterial load. However, in most cases, *C. canimorsus* was only detected by culture, suggesting lower bacterial numbers. This raises the question of whether *Cc*DPP7 could have systemic activity.

A generalized anticoagulant effect would probably only occur at advanced stages of sepsis, when bacteremia is high and many bacteria have lysed, releasing their intracellular contents. *Cc*DPP7 levels are probably not sufficient to prevent DIC; there could be, at most, aggravation of DIC-induced hemorrhage.

*C. canimorsus* adheres to various cell types, including endothelial cells (unpublished data), and vegetations have been found within the heart [39]. Local inhibition of coagulation by agglomerates of adhering bacteria in tissues, organs or blood vessels releasing *Cc*DPP7 seems to be more probable. Brenner *et al.* mentioned a *C. canimorsus* isolate derived from a petechial lesion [1], pointing to the presence of *C. canimorsus* in the skin, which is interesting with respect to the skin hemorrhage observed in patients. It is tempting to speculate that *C. canimorsus* could directly induce skin manifestations, like *Neisseria meningitidis* [40,41], or that it could reduce the inflammatory response and clearance, like *Y. pestis* [12], by locally blocking hemostasis.

To conclude, we demonstrate a novel mechanism for the inactivation of FX by a DPP7-type protease. This effect on FX could contribute to local inhibition of coagulation, or perhaps to aggravation of DIC-induced bleeding. It could equally be a mechanism by which *C. canimorsus* evades detection and killing by the immune system.

## Addendum

K. Hack performed the experiments. K. Hack, J. M. Douxfils, and G. R. Cornelis wrote the paper. K. Hack, J. M. Douxfils, G. R. Cornelis, E. Hess, F. Renzi, F. Lauber, and J. Douxfils contributed to the design of experiments and to the interpretation of data.

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## Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Purification of *Cc*DPP7 and *Cc*DPP7$_{S649}$.
**Fig. S2.** Sequence alignment of *Pg*DPP7 and *Pg*DPP11 with Ccan_08540 and Ccan_11580.
**Fig. S3.** DPP7 is expressed in all analyzed *C. canimorsus*, "C. canis" and *C. cynodegmi* strains.
**Fig. S4.** N-terminal cleavage of FX LC.
**Fig. S5.** *Cc*DPP7 affects FIX and FVII, but not FII activity.
**Table S1.** Effect of *Cc*5 on lag time, ETP and peak thrombin concentration after 15 min in NPP.
**Table S2.** Effect of *Cc*5 on lag time, ETP and peak thrombin concentration after 1 h in NPP.

**Table S3.** Effect on thrombin generation after incubation of $10^9$ bacteria $mL^{-1}$ or PBS as control for 15 min in NPP.
**Table S4.** The 27 serine protease-encoding genes of *Cc*5.
**Table S5.** Bacterial strains used in this study.
**Table S6.** Oligonucleotides used in this study.
**Table S7.** Plasmids used in this study.

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