Capnocytophaga canimorsus:

Interaction with the innate immune system

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel

von

Salome Casutt-Meyer

aus Basel, Schweiz

Basel, September 2009

Namensnennung-Keine kommerzielle Nutzung-Keine Bearbeitung 2.5 Schweiz

---

Sie dürfen:

das Werk vervielfältigen, verbreiten und öffentlich zugänglich machen

Zu den folgenden Bedingungen:

Namensnennung. Sie müssen den Namen des Autors/Rechteinhabers in der von ihm festgelegten Weise nennen (wodurch aber nicht der Eindruck entstehen darf, Sie oder die Nutzung des Werkes durch Sie würden entlohnt).

Keine kommerzielle Nutzung. Dieses Werk darf nicht für kommerzielle Zwecke verwendet werden.

Keine Bearbeitung. Dieses Werk darf nicht bearbeitet oder in anderer Weise verändert werden.

- Im Falle einer Verbreitung müssen Sie anderen die Lizenzbedingungen, unter welche dieses Werk fällt, mitteilen. Am Einfachsten ist es, einen Link auf diese Seite einzubinden.

- Jede der vorgenannten Bedingungen kann aufgehoben werden, sofern Sie die Einwilligung des Rechteinhabers dazu erhalten.
- Diese Lizenz lässt die Urheberpersönlichkeitsrechte unberührt.

Die gesetzlichen Schranken des Urheberrechts bleiben hiervon unberührt.

Die Commons Deed ist eine Zusammenfassung des Lizenzvertrags in allgemeinverständlicher Sprache: http://creativecommons.org/licenses/by-nc-nd/2.5/ch/legalcode.de

Haftungsausschluss:
Die Commons Deed ist kein Lizenzvertrag. Sie ist lediglich ein Referenztext, der den zugrundeliegenden Lizenzvertrag übersichtlich und in allgemeinverständlicher Sprache wiedergibt. Die Deed selbst entfaltet keine juristische Wirkung und erscheint im eigentlichen Lizenzvertrag nicht. Creative Commons ist keine Rechtsanwaltsgesellschaft und leistet keine Rechtsberatung. Die Weitergabe und Verlinkung des Commons Deeds führt zu keinem Mandatsverhältnis.

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von:

Prof. Dr. Guy R. Cornelis

Prof. Dr. Christoph Dehio

Basel, den 13. Oktober 2009

Prof. Dr. Eberhard Parlow, Dekan.

© Salome Casutt-Meyer

In den Ziegelhöfen 181

CH-4054 Basel

Contents

Summary…………………………………………………………………………….. 1

1. Introduction………………………………………………………………………... 3

1.1 *Capnocytophaga canimorsus* 4

Capnocytophaga canimorsus caused infections 5

Studies on the pathogenesis of *Capnocytophaga canimorsus* 8

1.2 Phagocytosis 9

1.2.1 Phagocytosis by PMNs 10

Non-oxidative killing 10

Oxidative killing 11

Bacterial evasion strategies 12

1.3 Neutrophil extracellular traps 14

Bacterial evasion strategies 15

1.4 Complement 15

Bacterial evasion strategies 18

2. *C. canimorsus* is resistant to phagocytosis and killing by murine macrophages……………………………………………………………………. 21

2.1 Summary 22

2.2 Publication: *Capnocytophaga canimorsus* resists phagocytosis by macrophages and blocks the ability of macrophages to kill other bacteria 23

2.3 Additional results 34

Cc5 gtf mutant Y1C12 is sensitive to phagocytosis and killing by J774.1 macrophages 34

2.4 Materials and methods 35

2.5 Discussion 36

3. *C. canimorsus* blocks the oxidative burst in murine macrophages... 38

3.1 Summary 39

3.2 Introduction 40

3.3 Results 40

Cc5 needs to grow in the presence of cells to prevent *E. coli* killing 40

Cc5 blocks the oxidative bust 43

Block of the oxidative burst depends on the release of pitrilysin 47

3.4 Materials and methods 53

3.5 Discussion 59

4. Interaction of *C. canimorsus* with human complement and PMNs…. 64
4.1 Summary 65
4.2 Publication: Resistance of *Capnocytophaga canimorsus* to killing by human complement and polymorphonuclear leukocytes 66
4.3 Additional Results 77
Cc5 does not interfere with phagocytosis of *Y. enterocolitica* ΔYscN bacteria upon co-infection 77
Cc5 also resists phagocytosis and killing by dog PMNs 78
Cc5 is sensitive to NET-dependent killing 79
4.4 Materials and methods 83
4.5 Discussion 85
5. Heterogeneity of *C. canimorsus* strains………………………………… 88
5.1 Summary 89
5.2 Publication: Prevalence of *C. canimorsus* in dogs and occurrence of potential virulence factors 90
5.3 Additional Results 97
Serum resistance varies in *C. canimorsus* 97
C. canimorsus strains display outer membrane structure heterogeneity 97
5.4 Materials and methods 99
5.5 Discussion 100
6. Addendum: YadA confers NET sensitivity to *Y. enterocolitica*.......... 102
6.1 Summary 103
6.2 Manuscript in preparation: Oligomeric coiled-coil adhesin YadA is a double-edged sword 104
Appendix……………………………………………………………………….. 112
Abbreviations 113
Strains and plasmids 115
Oligonucleotides 119
Antibodies 120
References……………………………………………………………………….. 121
Acknowledgements………………………………………………………….. 138
Curriculum vitae..……………………………………………………………….. 141

Summary

We show that *Capnocytophaga canimorsus* strain 5 (*Cc5)* is even more resistant to phagocytosis and killing by murine macrophages (J774.1) and human polymorphonuclear neutrophils (PMNs) than *Yersinia enterocolitica*, which is known as a model bacterium for resistance against phagocytosis due to its type 3 secretion system (Grosdent *et al.*, 2002). We observed that *Cc5* even becomes completely resistant to phagocytosis at high multiplicity of infection (moi of 50). In addition, we demonstrate that the *Cc5* transposon mutant Y1C12, identified during a serum sensitivity screen, has an increased sensitivity to phagocytosis and killing by either murine macrophages or human PMNs even in the unopsonized state. This indicated that not an increased susceptibility for antibody binding or complement deposition led to an increased phagocytosis of the mutant, but that rather the outer surface was more readily recognized by the phagocytes.

Furthermore, we demonstrate that *Cc5* induces the formation of neutrophil extracellular traps upon infection of human PMNs *in vitro* and that *Cc5* is trapped and killed within neutrophil extracellular traps, indicating sensitivity of *Cc5* towards antimicrobial peptides present in PMN granules.

Analysis of serum resistance in *Cc5* revealed that serum resistance is probably linked to its lipopolysaccharide, which prevents deposition of the membrane attack complex on the bacterial surface.

Moreover, we have observed that upon growth in the presence of cells, *Cc5* releases or modifies factor(s) in the medium, which interfere with the killing ability of macrophages. Investigating the underlying mechanism, we could show that *Cc5* does not affect phagosome maturation, but blocks the oxidative burst. This capacity was shown to depend on the release of the zinc metallopeptidase pitrilysin by *Cc5*.

First analyses on the prevalence of the hypothetical virulence factors serum resistance and interference with the oxidative burst indicated that *C. canimorsus* strains might display strain variability. While 59% of the strains (50% of case strains, 61% of dog isolates) were able to block the killing ability of macrophages, 60% of the strains were highly serum resistant (100% of case strains, 54% of dog isolates). However, serum resistance could not be directly linked to a specific polysaccharide structure in *C. canimorsus*.

Salome Casutt-Meyer

Chapter 1

Introduction

1.1. *Capnocytophaga canimorsus*

The genus *Capnocytophaga* contains 7 species, all of them encountered in the oral cavity of humans or domestic animals, but *Capnocytophaga canimorsus* is the only one associated with severe human infections (Lion *et al.*, 1996). *Capnocytophaga* species are capnophilic, facultative anaerobic, strictly fermentative Gram-negative rods (Brenner *et al.*, 1989). They belong to the phylum of the *Bacteroidetes*, which is taxonomically far remote from Proteobacteria and the common human pathogens, and fall into two groups formerly known as Centers for Disease Control groups dysogenic fermenter (DF)-1 and DF-2. The DF-1 group species (*Capnocytophaga ochracea, Capnocytophaga sputigena, Capnocytophaga gingivalis, Capnocytophaga haemolytica, Capnocytophaga granulosa*) are members of the human oral flora and as opportunistic pathogens they can cause infections like endocarditis, bacteraemia, septicaemia, eye infections and peritonitis in both immunocompromised and immunocompetent patients (Buu-Hoi *et al.*, 1988; Campbell and Edwards, 1991; Esteban *et al.*, 1995; Font *et al.*, 1994; Parenti and Snydman, 1985; Rubsamen *et al.*, 1993). The normal habitat of DF-2 species (*C. canimorsus, Capnocytophaga cynodegmi*) is the oral cavity of dogs and cats.

The major characteristics of *C. canimorsus* include positive test results for oxidase, catalase, arginine dihydrolase, and *o*-nitrophenyl-β-D-galactopyranoside and negative reactions for urease, nitrates and indole. Fermentation of glucose, lactose, and maltose is often observed but not of raffinose and inulin (Janda *et al.*, 2006). However, especially differentiation between *C. cynodegmi* and *C. canimorsus* can be misleading if relying on fermentation assays (Mally *et al.*, 2009). Only one third of all *Capnocytophaga* isolates forwarded to the California's Microbial Disease Laboratory in the years 1972-2004 was submitted with the correct species identification. Many strains were received as either an un-identified Gram-negative rod or "identification unknown" (55%). 13% were submitted with incorrect identifications, such as *Streptobacillus* spp., *Legionella* spp., or *Haemophilus* spp., indicating the difficulty to properly diagnose *C. canimorsus* infections (Janda *et al.*, 2006). Nowadays, identification is mostly done by PCR amplification and 16S rDNA gene sequencing (Janda *et al.*, 2006). Nevertheless, it has to be noted that differentiation between *C. canimorsus* and

C. cynodegmi is still difficult as sequence similarity is very high (Mally *et al.*, 2009) and therefore may lead to misdiagnoses.

***C. canimorsus* caused infections**

C. canimorsus infections are associated with dog and cat bites (54% of cases), dog and cat scratches (8.5%), or close animal contact (27%), such as licking of human wounds (Lion *et al.*, 1996). Consequently, human-to-human transmissions have never been reported apart from one case where it could not be completely excluded (Risi and Spangler, 2006).

It has been estimated that every second person is bitten by an animal or by another human once per lifetime (Griego *et al.*, 1995; Yaqub *et al.*, 2004). The majority of these bite wounds is minor and does not need medical treatment but nevertheless, 1% of the total costs from emergency treatments in the US result from bite wound cases. Most of these infections are due to *Pasteurella*, *Streptococcus*, and *Staphylococcus* sp. and 80-90% of all bite wounds are inflicted by dogs, followed by cats. The annual mortality rate due to dog and cat bites in the US is 6.7 per 10^8 persons, albeit not all of these fatalities are caused by infections (Griego *et al.*, 1995; Yaqub *et al.*, 2004).

There are more than 160 described cases of human patients infected with *C. canimorsus* (Brenner *et al.*, 1989; Conrads *et al.*, 1997; Gaastra and Lipman, 2009; Macrea *et al.*, 2008; Tierney *et al.*, 2006) since its discovery in 1976 (Bobo and Newton, 1976), but there is only one reported case of a dog infected with *C. canimorsus* following a dog bite, even though it was isolated together with other organisms (Meyers *et al.*, 2007), and one case of a pet rabbit infection after dog bite (van Duijkeren *et al.*, 2006). No *C. canimorsus* infections of cats after a bite incidence have been reported, but there is a case report of a cat with chronic sinusitis and rhinitis due to a *C. canimorsus* infection (Frey *et al.*, 2003). As these wound infections are usually not reported, it is not possible to estimate the ability of *C. canimorsus* to cause infections in animals.

The prevalence of *C. canimorsus* in dogs ranges from 8% to 41% (Bailie *et al.*, 1978; Westwell *et al.*, 1989; Blanche *et al.*, 1998; Gaastra and Lipman, 2009) depending on the sampling method and there is a single report of the isolation of *C. canimorsus* from sheep and cattle (25-30%) (Westwell *et al.*, 1989).

Although there is a high occurrence of *C. canimorsus* in dogs, the number of documented clinical infections remains very low. Low virulence and susceptibility to antibiotics frequently used for post-dog bite prophylaxis may result in fast clearance after infection (Janda *et al.*, 2006). Systemic administration of antimicrobial agents is controversial for healthy persons, yet patients with a history of a dog bite or a wound licked by a dog are usually supplied with systemic prophylactic antibiotic treatment (Gaastra and Lipman, 2009; Macrea *et al.*, 2008). Nowadays, the first choice antibiotic for infection with *C. canimorsus* is penicillin G, although resistance of isolates has been mentioned (Gaastra and Lipman, 2009; Meybeck *et al.*, 2006). The spread of β-lactamase-producing strains limits the use of β-lactams as first-line treatments, underlying the necessity to test the *in vitro* susceptibility of clinical strains (Jolivet-Gougeon *et al.*, 2007).

In Denmark already between 1982 and 1995, the incidence of *C. canimorsus* infections was estimated to be 0.5 to 1 case per 1’000’000 inhabitants per year (Pers *et al.*, 1996). At present, there are several cases of human *C. canimorsus* infections in Switzerland each year (A. Trampuz, personal communication), but no statistics are available for this disease.

The incubation period of *C. canimorsus* infections is between 2-3 days, but in single cases incubation periods of up to 4 weeks were described (le Moal *et al.*, 2003; Lion *et al.*, 1996). The initial symptoms of clinical infections by *C. canimorsus* include fever (78% of the patients), malaise (26%), vomiting (31%), diarrhea (26%), myalgia (31%), abdominal pain (26%), dyspnoea (23%), chills (46%), confusion (23%) and headache (18%) (Pers *et al.*, 1996). Skin manifestations such as maculopapular rash and purpura are commonly associated to *C. canimorsus* infections (Hermann *et al.*, 1998; Lion *et al.*, 1996). Clinical infections generally appear as fulminant septicemia and peripheral gas gangrene (Pers *et al.*, 1996; Tierney *et al.*, 2006). Some patients also develop meningitis upon infection with *C. canimorsus*, which is accompanied by headache, but rarely by fever (le Moal *et al.*, 2003; Tierney *et al.*, 2006). Less commonly, *C. canimorsus* can also cause endocarditis and myocarditis with a mortality rate of 25% (Sandoe, 2004). Renal failure may also be associated to *C. canimorsus* infections, caused by disseminated intravascular coagulation or hypotension resulting from systemic infection (Mulder *et al.*, 2001). Disseminated intravascular coagulation can lead to peripheral gangrene and fulminant purpura often leading

to amputation (Hantson *et al.*, 1991). In one case, *C. canimorsus* could also be identified from cultures of pleural fluid from a patient that had developed pneumonia (Chambers and Westblom, 1992).

Fatality rate of systemic infections is as high as 30% (Lion *et al.*, 1996), while the mortality rate for meningitis is lower (5%) (de Boer *et al.*, 2007; le Moal *et al.*, 2003). In one case, a patient died from a secondary infection by *Aspergillus niger*, suggesting, that *C. canimorsus* might have induced some sort of immunosuppression (J.B. le Polain, personal communication). The overall clinical evolution of *C. canimorsus* infections suggests that the bacterium elicits little inflammation, at least in the early stages of infection, which would allow time for multiplication up to a stage at which it causes general sepsis and fatal shock.

While approximately 60% of the patients had a predisposing condition, 40% had no know risk factor. The most prevalent predisposing conditions were splenectomy (33% of systemic cases), alcohol abuse (24%), or other immunosuppression (5%) (Lion *et al.*, 1996). Haemolytic uremic syndrome, Waterhouse-Friedrichsen syndrome, Trauma, Hodgkin's disease, steroid therapy, chronic lung disease, and idiopathic thrombocytopenic purpura have also been described as identifiable factors for increased risk (Beebe and Koneman, 1995; Dire *et al.*, 1994; Lion *et al.*, 1996; Mirza *et al.*, 2000; Mulder *et al.*, 2001; Tierney *et al.*, 2006; Tobé *et al.*, 1999). An explanation as for why these conditions pose a risk for a *C. canimorsus* infection might be the elevated iron levels, which eventually support the growth of the poor iron scavenger (Weinberg, 2000). However, in a mouse model, addition of iron did not lead to an increased virulence (H. Shin, personal communication). Although the majority of cases are associated to immunocompromised patients, mortality is higher in patients without predisposing conditions (32% versus 28%). The reason for this phenomenon is not known (Beebe and Koneman, 1995; Lion *et al.*, 1996; Tierney *et al.*, 2006). Thus, infections with *C. canimorsus* cannot only be considered as opportunistic infections.

Studies on the pathogenesis of *C. canimorsus*

Few studies have so far investigated the molecular basis underlying severe infections caused by *C. canimorsus* (Fischer *et al.*, 1995; Mally *et al.*, 2008; Shin *et al.*, 2007). The bacterium was reported to multiply in mouse macrophages and

to be cytotoxic probably due to the production of a toxin (Fischer *et al.*, 1995). A later report could not detect cytotoxicity within 24 hours (h) of infection in 10 strains of *C. canimorsus* tested, including the same strain as in the original study (Shin *et al.*, 2007). However, there seems to be cytotoxicity at later stages of infection (48 h post infection) (S. Ittig, personal communication). Another study reported that a very low level of cytokine production was observed *in vitro* (Frieling *et al.*, 1997), which also could not be reproduced (Shin *et al.*, 2007).

Despite of its classification as a fastidious grower, *C. canimorsus* exhibits robust growth when in direct contact with mammalian cells including phagocytes (Mally *et al.*, 2009; Shin *et al.*, 2007). This property is dependent on a surface localized sialidase, which allows the bacterium to use host amino-sugars from glycan chains of host cell glycoproteins. This ability was shown to increase virulence in a mouse tissue cage infection model (Mally *et al.*, 2008).

Furthermore, it has been shown that neither live nor dead *C. canimorsus* did lead to the release of pro-inflammatory signals like interleukin (IL) 1-alpha, IL1-beta, IL-6, IL-8, macrophage inflammatory protein 1-beta, RANTES and tumor necrosis factor-alpha, from either naïve or activated murine macrophage cell line or human monocytes. This could be explained by the absence of Toll-like receptor (TLR) 4 activation, presumably due to a hypo-reactive lipopolysaccharide (LPS) (Shin *et al.*, 2007). Even more, one strain isolated from a fatal human case, *C. canimorsus* strain 5 (*Cc5)*, turned out to have a mechanism that actively blocks the pro-inflammatory signalling upon stimulation with endotoxic LPS (Shin *et al.*, 2007).

The clinical overview of *C. canimorsus* infections and the previous studies suggested that the bacterium might be able to avoid the immune system, at least in the early stages of infection. Nevertheless, it is unknown how *C. canimorsus* interacts with the innate immune system in order to avoid clearance by phagocytes, human complement or neutrophil extracellular traps (NETs), which might contribute to the establishment of infection.

In contrast to the high prevalence of *C. canimorsus* in dogs, there are only very little cases of human infections reported. Even though the patient's immune system probably is an important factor allowing or hindering infection, *C. canimorsus* strains may have some strain diversity, which might discriminate virulent and avirulent strains.

This thesis describes the findings on the interaction of *C. canimorsus* with the innate immune system, namely with phagocytes, NETs and complement, and shows first observations on strain variability in *C. canimorsus* with respect to hypothetical virulence functions.

1.2. Phagocytosis

Professional phagocytes such as polymorphonuclear neutrophils (PMNs), monocytes and macrophages use phagocytosis to internalize and destroy foreign objects, like pathogens. In addition to clearing of the infectious agent, phagocytosis (especially by macrophages and dendritic cells) initiates the process of antigen processing and presentation for the development of cellular immune responses.

The surface of phagocytes is adorned with many receptors that are able to recognize and decode their cognate ligands expressed on the surface of infectious agents and apoptotic cells leading to phagocytosis of the bound particle. The interaction of bacteria with a phagocyte can be direct, through the recognition of pathogen-associated molecular patterns (such as surface carbohydrates, peptidoglycans, or lipoproteins) by pathogen recognition receptors (PRRs), or indirect, through mediation by opsonins, such as immunoglobulin (Ig) G and components of the complement cascade like C3b, which attach to the pathogen surface where they are recognized by opsonin receptors, such as Fc receptor (FcR) gamma and complement receptor (CR) 3. Although the phagocytosis pathways initiated by the different receptors share common steps and lead to the destruction of the ingested pathogen, they may also differ depending on the particular receptor involved. While *e.g.* FcγR engagement is accompanied by a respiratory burst and initiation of a pro-inflammatory cascade, CR-mediated phagocytosis is not (reviewed by (Rabinovitch, 1995; Stuart and Ezekowitz, 2005)).

Emerging from pluripotent hematopoietic stem cells in the bone marrow, terminally differentiated PMNs are released into the peripheral blood where they circulate for 7-10 h. Upon infection, PMNs migrate into the affected tissues, where they have a life span of only a few hours. In contrast, monocytes that reach the extra-vascular compartment generally differentiate into macrophages

and – depending on their location in the body and on the functional demands placed on them – live for days, weeks, months or even years.

In addition to their role in phagocytosis, professional phagocytes secrete a variety of important immunomodulatory molecules, including cytotoxic radicals of oxygen and nitrogen (Nathan, 2003), enzymes that degrade the extracellular matrix (Borregaard and Cowland, 1997; Kang *et al.*, 2001; Owen and Campbell, 1999), and cytokines that can modify the behaviour of phagocytes and several other cell types (Bennouna *et al.*, 2003; Wittamer *et al.*, 2005).

While tissue resident macrophages only rely on the oxidative burst and the thereby produced reactive oxygen species (ROS) to kill the phagocytosed bacteria, PMNs additionally possess granules filled with antimicrobial peptides (AMPs) which constitute a non-oxidative killing mechanism for ingested microbes. Hence, we focus here on the events involved in microbial clearance by PMN-mediated killing.

1.2.1. Phagocytosis by PMNs

PMNs are the first immune cells recruited from the blood stream to the site of infection, thereby building the first line of defence against invading microorganisms such as bacteria, fungi, and protozoa (Kanthack and Hardy, 1895). In humans, roughly 100 billion PMNs enter and leave the circulating blood every day, outlining their importance in the immune system.

Upon contact, PMNs engulf microbes into a phagocytic vacuole, the phagosome. After phagocytosis, the phagosome matures in a series of fusion events with cytoplasmic granules to form a phagolysosome, wherein bacteria are killed by non-oxidative and oxidative killing mechanisms (reviewed by (Nathan, 2006; Rabinovitch, 1995; Stuart and Ezekowitz, 2005)).

Non-oxidative killing

Non-oxidative killing of invading pathogens is mediated by the fusion of neutrophil secretory granules with the phagosome and the subsequent release of antimicrobial granule contents into the phagolysosome. The granules to be discharged first are the specific granules (secondary granules) and the gelatinase granules (tertiary granules). Specific granules contain several AMPs including lysozyme (Cramer *et al.*, 1985) as well as the transmembrane units of the

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, gp91^{phox} and p22^{phox} (comprising flavocytochrome b_{558}) (Borregaard *et al.*, 1983; Jesaitis *et al.*, 1990). Gelatinase granules on the contrary contain *e.g.* the metalloproteases gelatinase and leukolysin (Kang *et al.*, 2001; Kjeldsen *et al.*, 1992; Lazarus *et al.*, 1968). These metalloproteases are not primarily thought to be important for killing of microbes, but to be essential for the degradation of extracellular matrix components of the vascular basement membranes during extravasation of PMNs (Borregaard and Cowland, 1997; Kang *et al.*, 2001; Owen and Campbell, 1999).

Next, the azurophilic granules (primary granules) are discharged. They contain α-defensins (Ganz *et al.*, 1985), which kill microorganisms by forming multimeric transmembrane pores (Wimley *et al.*, 1994), antibiotic proteases (*e.g.* cathepsin G, proteinase 3 and neutrophil elastase) (Campanelli *et al.*, 1990; Salvesen *et al.*, 1987; Sinha *et al.*, 1987) and small AMPs, including myeloperoxidase (MPO). MPO reacts with hydrogen peroxide (H_2O_2), formed by the NADPH oxidase, to form hypochlorous acid (HOCl), which is highly toxic for microorganisms (Klebanoff, 1999), building a link between oxidative and non-oxidative killing. Interestingly, azurophilic granules were not only shown to fuse with phagolysosomes, but also to be released at the site of contact with antibody (Ab)-opsonized bacteria even before closure of the phagosomal cup (Tapper *et al.*, 2002).

Oxidative killing

Oxidative killing of microbes depends on the generation of ROS by the NADPH oxidase. In resting cells, the NADPH oxidase is unassembled and inactive, having its protein components segregated into membranous and cytosolic parts of the cell. While the subunits p47^{phox}, p67^{phox}, p40^{phox} and the small GTPase Rac2 reside in the cytosol, flavocytochrome b_{558} (comprised of gp91^{phox} and p22^{phox}) is bound to the membranes of secondary granules. Upon activation and recruitment of secondary granules and secretory vesicles, flavocytochrome b_{558} locates to the phagolysosomal membrane or the cell surface, depending on the nature of the stimulus (DeCoursey and Ligeti, 2005). Assembly of the cytosolic components p67^{phox}, p40^{phox} and p47^{phox} with flavocytochrome b_{558} at the phagolysosomal membrane is triggered by hyperphosphorylation of p47^{phox} (El Benna *et al.*, 1994; Inanami *et al.*, 1998) and phosphorylation of p67^{phox} (Dusi and Rossi, 1993; El

Benna *et al.*, 1997) *via* several kinases including protein kinase C (PKC) (Cheng *et al.*, 2007; Ding *et al.*, 1993; Majumdar *et al.*, 1993; Nixon and McPhail, 1999; Reeves *et al.*, 1999) and Akt (Hoyal *et al.*, 2003). Upon interaction of $p67^{phox}$ with flavocytochrome b_{558}, $p67^{phox}$ binds to Rac2, which translocates independently to the assembling oxidase, where it is required for electron transfer in the active complex (Abo *et al.*, 1994; Clark *et al.*, 1990; Heyworth *et al.*, 1994; Iyer *et al.*, 1994; Park *et al.*, 1992; Quinn *et al.*, 1993).

After assembly, the NADPH oxidase transfers electrons from cytoplasmic NADPH to extracellular or intraphagosomal molecular oxygen (O_2), thereby generating superoxide (O_2^-). Within the phagosome, O_2^- is rapidly converted into H_2O_2 by superoxide dismutase (SOD). H_2O_2 further reacts to form other ROS such as singlet oxygen and hydroxyl radical ($OH^\bullet$). Furthermore, MPO can catalyze the H_2O_2-dependent oxidation of halides to form toxic chloramines and hypohalous acids, primarily HOCl (Babior, 1995). The importance of NADPH oxidase is highlighted by patients with chronic granulomatous disease, lacking a functional oxidase, which suffer from repeated life-threatening bacterial and fungal infections (Allen *et al.*, 1999; Curnutte, 1993; Dinauer, 1993).

Even though the phagocytic capability of macrophages is very efficient, their oxidative killing capacity is less marked, primarily because they lack MPO. However, it has been shown that concomitant with the uptake of apoptotic PMNs by macrophages, granule contents seemed to traffic to early endosomes and to co-localize with engulfed bacteria, increasing the killing efficiency of the macrophages (Tan *et al.*, 2006). Therefore, the transfer of AMPs from PMNs to macrophages could provide a cooperative defence strategy between innate immune cells against intracellular pathogens.

Bacterial evasion strategies

Pathogenic bacteria and fungi have evolved efficient strategies to avoid clearance by PMNs. The main strategies can be divided into six groups: (i) avoiding contact, (ii) preventing phagocytosis, (iii) inducing host cell death, (iv) escape into the cytoplasm, (v) interference with phagosome maturation, and (vi) increasing resistance to AMPs and oxidative stress (Figure 1).

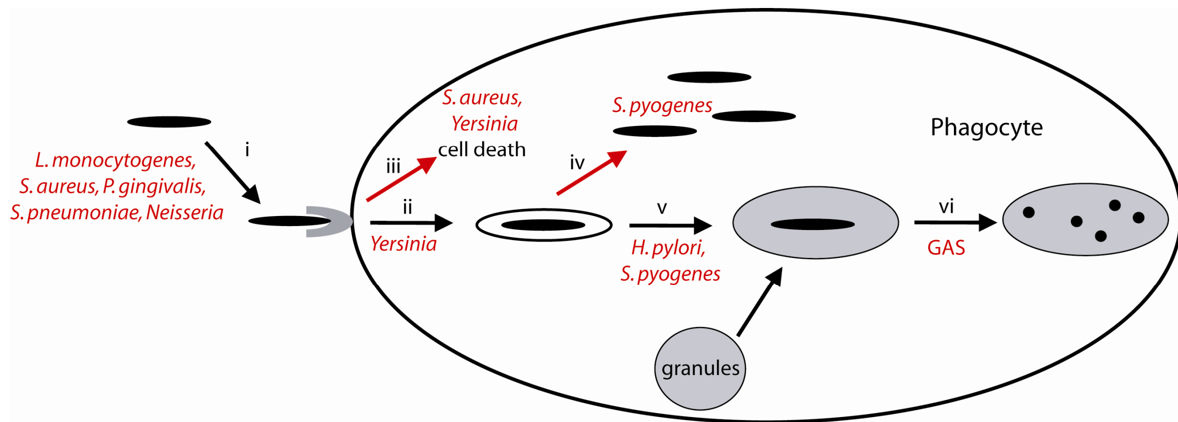


Figure 1. Bacterial strategies for evasion of phagocytic killing
Under normal circumstances, adherent bacteria are phagocytosed, the nascent phagosome matures via fusion with granules to form a phagolysosome, and the internalized bacteria are killed and degraded. Pathogens can perturb this system at several points, including avoiding contact or recognition by receptors (i), preventing phagocytosis (ii), induction of host cell death (iii), escape into the cytoplasm (iv), interference with phagosome maturation (v), or increasing resistance against AMPs and oxidative stress (vi). Adapted from (Allen, 2003).

There are two strategies known to avoid contact with PMNs: pathogens either remain in regions inaccessible to phagocytes, as does *e.g. Listeria monocytogenes*, which induces its own uptake into epithelial cells (Gaillard *et al.*, 1991), or they prevent the recruitment of PMNs to the site of infection by interfering with the proinflammatory response, *e.g.* by secreting chemotactic inhibitory protein (*Staphylococcus aureus*) (de Haas *et al.*, 2004).

Pathogenic bacteria are known to use three different approaches to prevent phagocytosis. First, they use physical barriers such as polysaccharide or polyglutamate capsules (*e.g*. *Porphyromonas gingivalis* (Sundqvist *et al.*, 1991), *Bacillus anthracis* (Keppie *et al.*, 1963)), thereby preventing recognition by the phagocyte's PRRs. Second, they block the phagocyte's actin cytoskeleton (*e.g. Yersinia* (Persson *et al.*, 2002)). Third, they interfere with opsonization. Bacteria can either interfere with complement deposition (*e.g*. *Streptococcus pneumoniae* (Horstmann *et al.*, 1988)), or they evade Ab-opsonization by degrading Abs (*Streptococcus* (Reinholdt *et al.*, 1990)), or by surface antigen variation (*e.g. Neisseria* (Hagblom *et al.*, 1985)), thereby preventing recognition by opsonin receptors.

Some pathogens also induce host cell death to avoid clearance by PMNs by secreting lysing toxins (*e.g. S. aureus* (Menestrina *et al.*, 2001)) or injecting

apoptosis inducing proteins (*e.g. Yersinia enterocolitica* (Ruckdeschel *et al.*, 1998)).

Interestingly, many bacteria survive inside PMNs after phagocytosis. The survival strategies range from inhibition or modification of phagosome maturation, as done *e.g.* by *Streptococcus pyogenes* (Staali *et al.*, 2006), degradation of AMPs (*e.g. P. gingivalis* (Carlisle *et al.*, 2009)) or relocation of NADPH oxidase (*e.g. Helicobacter pylori* (Allen *et al.*, 2005; Allen, 2007)), to the escape into the cytoplasm (*e.g. S. pyogenes* (Medina *et al.*, 2003)). Transcriptional analyses using whole genome microarrays have shown that group A *Streptococci* (GAS) *e.g.* up-regulate genes crucial for resistance to phagocyte-dependent killing, such as SOD, catalases and glutathione peroxidases, if attacked by PMNs (Voyich *et al.*, 2003).

1.3. NETs

PMNs have recently been shown to possess an alternative, phagocytosis-independent killing mechanism, called NETs. Upon activation (*e.g.* by phorbol myristate acetate (PMA), IL-8, LPS, bacteria, fungi, or activated platelets) PMNs release granule proteins and DNA that together form an extracellular fibrillar matrix that binds and kills Gram-positive and -negative bacteria (Brinkmann *et al.*, 2004; Clark *et al.*, 2007; Wartha *et al.*, 2007b).

The activation pathway leading to the formation of NETs can involve different receptors such as TLRs, as well as CRs and FcRs (Brinkmann *et al.*, 2004; Clark *et al.*, 2007). Stimulation of these receptors activates PKC, which initiates a signal transduction cascade that induces the assembly and activation of the NADPH oxidase complex. First, the nuclei of PMNs loose their shape and the eu- and heterochromatin homogenize and later, the nuclear envelope and the granule membranes disintegrate, allowing the mixing of NET components. Finally, the NETs are released as the cell membrane breaks (Fuchs *et al.*, 2007).

Structurally, NETs consist of smooth fibers with 15-17 nm diameter and globular domains of 25-28 nm and are composed of nuclear constituents such as chromatin and histones and granular peptides and enzymes, such as neutrophil elastase, cathepsin G and MPO, which are normally stored in typical neutrophil granules. In contrast, CD36, a granule membrane protein, the cytoplasmic markers annexin, actin and tubulin and various other cytoplasmic proteins were

excluded from NETs (Brinkmann *et al.*, 2004). It can be assumed that granule proteins and chromatin together form an extracellular structure that amplifies the effectiveness of its antimicrobial substances by ensuring a high local concentration.

Bacterial evasion strategies

Since NETs have only recently been discovered (Brinkmann *et al.*, 2004), there are only few studies addressing the interaction of NETs with pathogens. While *Candida albicans* has been shown to induce NET-formation and to be susceptible to NET-dependent killing (Urban *et al.*, 2006), both, Gram-positive (*e.g. S. aureus, S. pneumoniae*, and GAS) and -negative bacteria (*e.g. Salmonella enterica* serovar *Typhimurium* and *Shigella flexneri*) have been shown to be captured within NETs (Beiter *et al.*, 2006; Brinkmann *et al.*, 2004; Buchanan *et al.*, 2006). Whether viruses and parasites are killed by NETs is not yet known.

Whereas most bacteria studied so far became killed after being trapped within NETs, some bacteria were shown to be able to evade NET-dependent killing. The most studied evasion mechanism is the production of an extracellular DNase, *e.g.* by GAS, to degrade the DNA backbone (Beiter *et al.*, 2006; Buchanan *et al.*, 2006). Other possible mechanisms might involve polysaccharide capsules which prevent lysis by AMPs (*e.g.* in *Pneumococci*) (Wartha *et al.*, 2007a), or the secretion of proteases that degrade the NET-associated AMPs. Alternatively, one could also speculate that bacteria inhibiting the oxidative burst could prevent the formation of NETs.

1.4. Complement

The complement system is the major effector of the humoral branch of the immune system. While the membrane attack complex (MAC) mediates cell lysis, other complement components or split products participate in the inflammatory response, opsonization of antigen, viral neutralization, and clearance of immune complexes. We will focus here on the direct killing of bacteria by complement.

Complement-mediated opsonization and killing by MAC insertion depends on triggering of the classical, lectin and alternative pathways of complement activation. Complement activation by the classical pathway commonly begins with the binding of Abs to antigens, *e.g.* on a bacterial cell. The formation of antigen-

Ab complex induces conformational changes in the Fc portion of the IgM molecule that expose a binding site for C1. Binding and activation of C1 subunits then leads to the formation of the C3 convertase C4b2a by cleaving C4 and C2. The lectin pathway is activated by the binding of mannose-binding lectin (MBL) to mannose residues of glycoproteins or carbohydrates on the surface of microorganisms. Binding of MBL to a surface results in the recruitment of the MBL-associated proteases 1 and 2, which cause cleavage of C4 and C2 leading to the formation of the C3 convertase C4b2a. Finally, the alternative pathway contrasts with both the classical and the lectin pathway in that it is activated by direct interaction of C3 with the pathogen surface. C3, which contains an unstable thioester bond, undergoes slow spontaneous hydrolysis and the formed C3b then binds to foreign surface antigens or even to the host's own cells. Since sialic acids present on the membranes of most mammalian cells bind factor H (fH), C3b molecules bound on host cells are rapidly inactivated. Because many foreign antigenic surfaces have only low levels of sialic acid, C3b bound to these surfaces remains active for a longer time, which leads to the binding of factor B to C3b. Factor B is thereafter cleaved by factor D, leading to the formation of the C3 convertase C3bBb.

After assembly of a C3 convertase, which builds the major amplification step within the complement cascade, all three pathways converge in a single downstream process. First, C3 is cleaved into the small protein C3a and the large C3b. C3b can covalently associate with pathogen surfaces, leading to opsonization of the pathogens. Besides its function as opsonin, C3b also assembles with the C3 convertases to form a C5 convertase, which then cleaves C5 into C5a and C5b. While C5a is a potent pro-inflammatory molecule, C5b binds to the surface of the target cell and provides a binding site for the subsequent components of the MAC, C6, C7, C8, and C9 (reviewed by (Muller-Eberhard, 1988; Walport, 2001a, b)).

Upon binding of C6 and C7 to C5b, the resulting complex C5b67 undergoes structural changes exposing hydrophobic regions, which serve as biding sites for phospholipids, allowing the complex to insert into the phospholipid bilayer. Upon insertion of C8 to the C5b67 complex, a small pore is formed in the target cell membrane, but only upon binding and oligomerization of C9, the MAC forms a large channel through the membrane of the target cell, enabling ions and small molecules to diffuse freely across the membrane. Thereby, the affected cell

looses its osmotic stability and is killed by an influx of water and loss of electrolytes (Born and Bhakdi, 1986; Muller-Eberhard, 1986) (Figure 2).

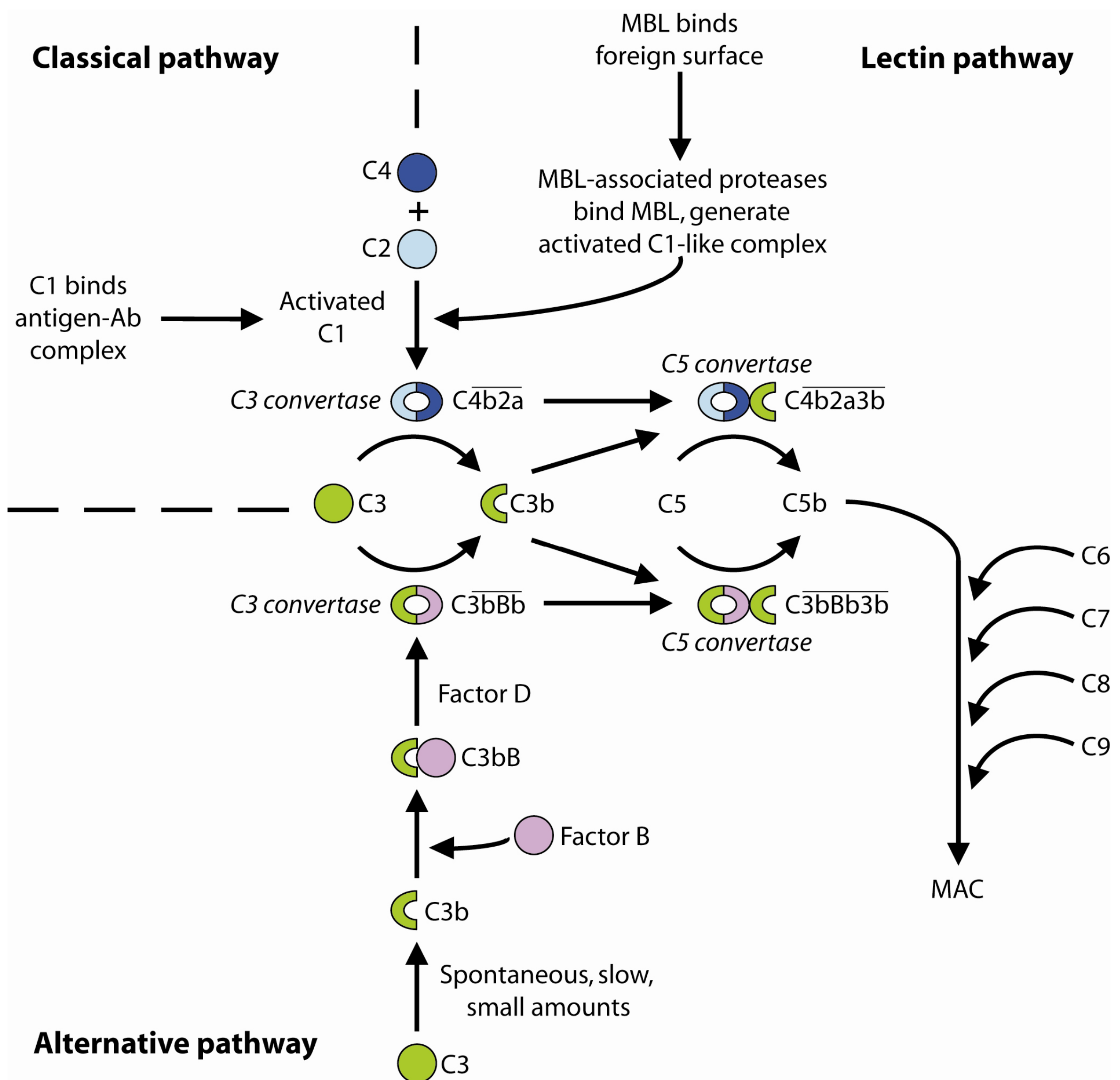


Figure 2. Overview of the complement activation pathways

The classical pathway is initiated when C1 binds to antigen-Ab complexes. The alternative pathway is initiated by binding of spontaneously generated C3b to activating surfaces. The lectin pathway is initiated by binding of the serum protein MBL to the surface of a pathogen. All three pathways generate C3 convertases and C5 convertases. C5b generated by the C5 convertases binds to the target cell membrane and is subsequently converted into a MAC by recruitment of C6, C7, C8 and C9. Adapted from (Goldsby *et al.*, 2003).

Because many elements of the complement system are capable of attacking host cells as well as foreign cells and microorganisms, elaborate regulatory mechanisms have evolved to restrict complement activity to designated targets.

A general mechanism of regulation in all complement pathways is the inclusion of highly labile components like C3b that undergo spontaneous inactivation if they are not stabilized by reacting with other components. In addition, a series of regulator proteins can inactivate various complement components (*e.g.* C4 by C4b-binding protein (C4BP) and C3b by fH) or can avoid MAC-dependent lysis by preventing assembly of poly-C9 (*e.g.* protectin).

Bacterial evasion strategies

Gram-positive bacteria are naturally resistant to complement due to their capsules and the thick peptidoglycan layer, which sterically hinders the access of complement molecules to the bacterial surface. Even if the opsonin C3b is formed, it may become embedded deep in the capsular network thereby becoming inaccessible, which has been most clearly demonstrated with *S. aureus* (Wilkinson *et al.*, 1979). Some *Streptococci* were shown to shed lipoteichoic acids, which can bind to mammalian cells and not only sensitize them to autologous complement, but also redirect complement activation away from the bacterial surface (Beachey *et al.*, 1979; Hummell and Winkelstein, 1986).

Gram-negative bacteria have evolved different strategies to prevent killing by complement. The main strategies can be divided into three groups: (i) binding of regulators of the complement cascade, (ii) degradation of complement components, and (iii) inhibition of MAC-dependent lysis (Figure 3).

Many pathogens have been shown to bind complement regulatory proteins. Some bacteria decorate themselves with sialic acids to mimic host cells (*e.g. Meningococci* (Mandrell *et al.*, 1990)), as sialic acids were shown to interact with fH, a negative regulator of C3 convertases (Meri and Pangburn, 1990). A classical example of a specific factor responsible for complement resistance of a microbe is the M protein family of *S. pyogenes*. Besides binding fH (Horstmann *et al.*, 1988), many members of the M protein family can also bind C4BP, thereby disassembling the classical and the lectin pathway C3 convertase (Johnsson *et al.*, 1996; Thern *et al.*, 1995).

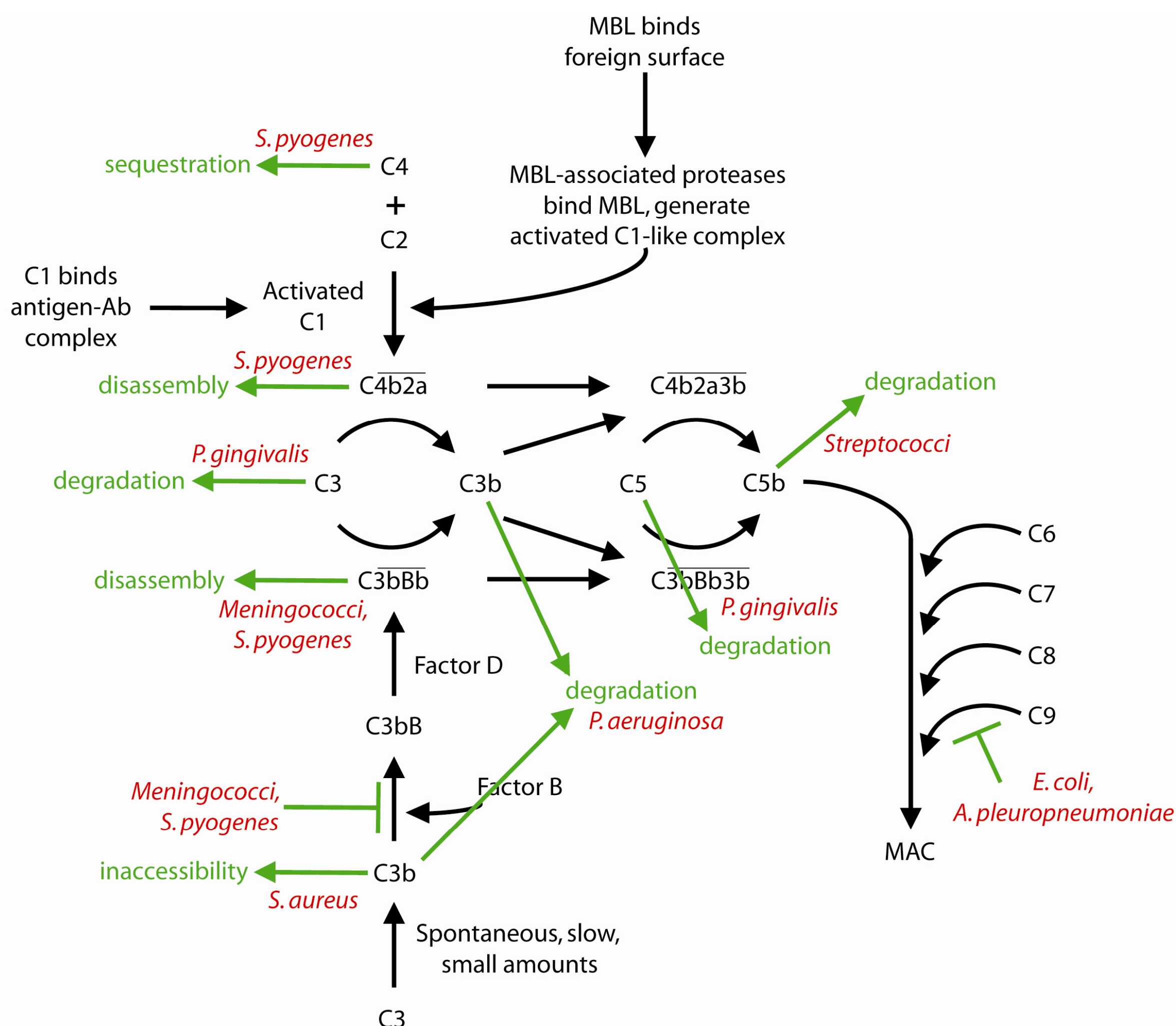


Figure 3. Bacterial evasion strategies

Bacteria interfere with complement activation and complement deposition at different stages. Adapted from (Goldsby *et al.*, 2003).

Many bacteria have proteases that can degrade complement proteins or inhibit their accumulation on bacterial surfaces. Some pathogenic *P. gingivalis* strains were shown to express an arginine-specific cysteine protease capable of cleaving C5 and C3, whereby C3b does not become bound onto the bacterial surface (Chen *et al.*, 1992; Schenkein, 1989). *P. aeruginosa* secretes active proteases in the form of alkaline protease and elastase that cleave C3b and thus inhibit C3b deposition and complement activation at the bacterial surface (Hong and Ghebrehiwet, 1992; Schmidtchen *et al.*, 2003). In addition, LPS variants of *P. aeruginosa* which are expressed at the pathogen surface, interfere with C3b deposition (Engels *et al.*, 1985; Schiller and Joiner, 1986). Alternatively, many strains of GAS and group B *Streptococci* produce a C5a-inactivating C5a-ase and

can therefore inhibit the inflammatory response and opsonophagocytosis (Takahashi *et al.*, 1995).

Some bacteria were also shown to prevent MAC-dependent lysis by either preventing insertion of the late complement components into the bacterial membrane, or by binding MAC regulators. Most bacteria interfere with the assembly of C9 to the MAC, thereby preventing pore formation and subsequent lysis. While capsular polysaccharides of *A. pleuropneumoniae e.g*. can limit the amount of bound C9 (Ward and Inzana, 1994), some pathogenic *Escherichia coli* strains can bind protectin (Rautemaa *et al.*, 1998), a negative host regulator of C9 assembly.

Chapter 2

C. canimorsus is resistant to phagocytosis and killing by murine macrophages

2.1. Summary

In this chapter we show that *Cc5* is even more resistant to phagocytosis and killing by murine macrophages (J774.1) than *Y. enterocolitica*, which is known as a model bacterium for resistance against phagocytosis due to its type 3 secretion system (T3SS) (Grosdent *et al.*, 2002). We observed that upon high multiplicity of infection (moi of 50), *Cc5* even completely resists phagocytosis and killing. Moreover, pre-opsonization with specific Abs only slightly increased phagocytosis and killing of *Cc5*.

In addition, we demonstrate that *Cc5* transposon (Tn) mutant Y1C12, identified during a serum sensitivity screen by Ch. Fiechter, has an increased sensitivity to phagocytosis and killing by J774.1 macrophages even in the unopsonized state. This indicated that not an increased susceptibility for Ab binding, or complement deposition led to an increased phagocytosis, but that the outer surface of the bacteria is more readily recognized by the macrophages.

Furthermore, we have observed that upon growth in the presence of cells, *Cc5* releases or modifies factor(s) in the medium, which interact with fresh macrophages and block their ability to kill bacteria after phagocytosis. This capacity was found in 6 out of 10 *C. canimorsus* strains tested.

2.2. Publication

Capnocytophaga canimorsus resists phagocytosis by macrophages and blocks the ability of macrophages to kill other bacteria

Immunobiology **213** (2008) 805-814

Salome Meyer, Hwain Shin and Guy R. Cornelis

Statement of my work. My contribution was the data of all figures except Fig. 6. Phagocytosis and killing of bacteria by murine macrophages and blocking of *E. coli* killing by *C. canimorsus*. Data of Fig. 6 was contributed by H. Shin.

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Immunobiology 213 (2008) 805–814

Immunobiology

www.elsevier.de/imbio

Capnocytophaga canimorsus resists phagocytosis by macrophages and blocks the ability of macrophages to kill other bacteria

Salome Meyer, Hwain Shin, Guy R. Cornelis*

Infection Biology, Biozentrum Basel, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Received 23 July 2008; accepted 23 July 2008

Abstract

Capnocytophaga canimorsus is a commensal bacterium from the canine oral flora, which can cause septicemia or meningitis in humans upon bite wound infections. *C. canimorsus* 5 (*Cc*5), a strain isolated from a patient with fatal septicemia, was used to investigate the interaction between *C. canimorsus* and J774.1 mouse macrophages. J774.1 cells infected at high multiplicity with *Cc*5 did not phagocytose nor kill *Cc*5 within 120 min of infection, unless the bacteria were opsonized with specific antibodies. Opsonization with complement, however, did not increase phagocytosis. Moreover, infection of J774.1 cells with live *Cc*5 led to the release of a soluble factor, which interfered with the ability of macrophages to kill other phagocytosed bacteria. These results provide an example of how *C. canimorsus* neutralizes the innate immune system.
© 2008 Elsevier GmbH. All rights reserved.

Keywords: *Capnocytophaga canimorsus*; Macrophages; Phagocytosis

Introduction

Capnocytophaga canimorsus is a Gram-negative commensal from the oral cavity of dogs and cats. The genus *Capnocytophaga* consists of nine species, all of which are living in the oral cavity of humans or domestic animals, but *C. canimorsus* is the only one associated with severe human infections. Since 1976, 160 cases of gangrene, septicemia or meningitis caused by *C. canimorsus* have been reported in humans that have been bitten, scratched or licked by a dog or cat (Blanche et al., 1998; Brenner et al., 1989; Tierney et al., 2006). Many of these cases are associated with splenectomy, alcohol abuse or immunosuppression history, but more than 40% of the patients have no known risk factor (Lion et al., 1996). Mortality rate is 30% for patients developing septicemia and 5% for patients with meningitis (le Moal et al., 2003). The overall clinical picture of infections with *C. canimorsus* implies that the bacterium elicits only weak inflammation, but very little is known about the pathogenesis of *C. canimorsus*. Recently, we showed that murine or human macrophages infected with 10 different *C. canimorsus* strains failed to release tumor necrosis factor (TNF) and interleukin (IL)-1α. Furthermore, macrophages infected with live or heat-killed (HK) *Cc*5 did not release IL-6, IL-8, interferon-γ, macrophage inflammatory protein-1β and nitric oxide (NO) (Shin et al., 2007). This absence of proinflammatory response was characterized by the inability of

*Abbreviations: Cc*5, *C. canimorsus* 5; HK, heat-killed; IL, interleukin; moi, multiplicity of infection; NO, nitric oxide; o/n, overnight; TLR, Toll-like receptor; TNF, tumor necrosis factor; Wt, wild type.

*Corresponding author. Tel.: +41 61 267 21 21, 41 61 267 21 10; fax: +41 61 267 21 18.

E-mail address: guy.cornelis@unibas.ch (G.R. Cornelis).

0171-2985/$ - see front matter © 2008 Elsevier GmbH. All rights reserved.
doi:10.1016/j.imbio.2008.07.019

Toll-like receptor (TLR) 4 to respond to *Cc*5. In addition, live *Cc*5 blocked the release of TNF and NO triggered by endotoxic lipopolysaccharide, while down regulating the expression of TLR4 and dephosphorylating p38 mitogen-activated protein kinase. These results highlight that *C. canimorsus* possesses active and passive mechanisms to prevent the onset of the inflammatory response (Shin et al., 2007).

In this article, we report the first investigation on phagocytosis of *C. canimorsus* by J774.1 mouse macrophage cells. We show that *C. canimorsus* is not only resistant against phagocytosis and killing but also blocks the killing of unrelated bacteria by J774.1 cells.

Materials and methods

Bacterial strains and media

C. canimorsus strains are listed in Table 1. *C. canimorsus* was routinely grown on heart infusion agar (Difco) supplemented with 5% sheep blood (Oxoid) for 2 days at 37 °C in the presence of 5% CO_2 (Shin et al., 2007). Wild-type (wt) *Y. enterocolitica* E40 (Cornelis et al., 1986) and knockout mutant *Y. enterocolitica* E40 ΔYscN were used as control bacteria. *Y. enterocolitica* were pre-grown overnight (o/n) at room temperature with continuous shaking (150 rpm) in brain–heart infusion. Before infection, type III secretion was induced as described (Letzelter et al., 2006). Streptomycin-resistant *Escherichia coli* TOP10 (Invitrogen) were pre-grown o/n at 37 °C with continuous shaking (150 rpm) in Luria–Bertani. Before infection, a fresh liquid culture was inoculated at OD_{600} of 0.2 and grown for 150 min at 37 °C with continuous shaking (150 rpm).

In vitro phagocytosis and killing assay

Mouse macrophage J774.1 cells were suspended in RPMI 1640 (Gibco) supplemented with 2% glutamine (Gibco) and 10% fetal bovine serum (Gibco), seeded in 24-well plates (1×10^5 cells/well) and incubated o/n at 37 °C and 5% CO_2. Bacteria were incubated either in the presence of J774.1 cells at different multiplicity of infection (moi) or without cells (reference sample). When specified, bacteria were opsonized for 30 min at 37 °C either with specific antibodies (rabbit sera specifically raised against HK *Cc*5 or anti *Y. enterocolitica* O:9, 1:500) or with heat-inactivated (60 min, 56 °C) guinea pig complement (Sigma, 10%) before infection. To synchronize the infection, 24-well plates were centrifuged immediately after infection for 5 min at 180*g* and 37 °C. At different time points after infection (0, 30, 60 and 120 min), the cells were scraped and resuspended. To differentiate between phagocytosis and killing, an aliquot was incubated with 0.1% saponin for 15 min at 37 °C in order to lyse the J774.1 cells. Aliquots from untreated and lysed samples were then plated at different dilutions to count the surviving bacteria. Samples from bacteria incubated without cells were plated in parallel as reference. Counts from lysed samples gave the total number of surviving bacteria, whereas counts from untreated cell samples gave the numbers of non-phagocytosed, extracellular bacteria. Since the number of individual colonies arising from intact phagocytes cannot exceed one per phagocyte, the number of phagocytosed but not killed bacteria was considered negligible in this assay. Numbers of phagocytosed and killed bacteria were calculated by subtracting the counts of extracellular or surviving bacteria, respectively, from the counts of the bacteria grown without cells (reference sample). Values are given as percentages of control values. Each value represents the mean of at least three independent experiments.

Phagocytosis by pre-infected macrophages

Two hours after seeding J774.1 (1×10^5 cells/well, as described previously), cells were pre-infected with *Cc*5 at a moi of 50. When specified, *Cc*5 was HK at 99 °C for 60 min before infection. Pre-infected J774.1 cells were incubated for indicated times at 37 °C and 5% CO_2. After pre-infection, cells were washed once and supplemented with fresh medium. Thereafter, J774.1 cells pre-infected with *Cc*5 were infected with streptomycin-resistant *E. coli* at a moi of 50. To determine the reference values for phagocytosis and killing of *E. coli* by macrophages, untreated macrophages were infected in parallel. At different time points after infection (0, 30, 60 and 120 min), the cells were scraped, resuspended and treated as described in the phagocytosis assay. The untreated and lysed aliquots were plated on streptomycin-containing plates. Samples from *E. coli* incubated without cells were plated in parallel as reference. The numbers of phagocytosed and killed *E. coli* were calculated as described here before.

Conditioning of the medium

Two hours after seeding J774.1 (1×10^5 cells/well), cells were infected for 24 h with *C. canimorsus* at a moi of 50. Supernatant was removed from cells and filtered through a nitrocellulose filter (0.2 μm pore size, Sarsted). Conditioned medium was then added to fresh J774.1 cells, which had been seeded 24 h prior (1×10^5 cells/well). J774.1 macrophages were incubated for 4 h at 37 °C and 5% CO_2 with the conditioned medium, washed once, supplemented with fresh RPMI medium and infected with streptomycin-resistant *E. coli* at a moi

Table 1. *C. canimorsus* strains used in this study and their anti-innate immunity properties

Strain	Collection number	Biological origin	Year of isolation	History and geographical origin	Reference	Anti-inflammatory activity	Anti-killing activity
*Cc*2	–	Human fatal septicemia after dog bite	1989	G. Wauters and M. Delmée, University Clinics St. Luc, Brussels, Belgium	Hantson et al. (1991)	No	No
*Cc*3	–	Human septicemia	1990	G. Wauters and M. Delmée, ←St. Jan Hospital, Brugge, Belgium	Vanhonsebrouck et al. (1991)	No	No
*Cc*5	–	Human fatal septicemia after dog bite	1995	G. Wauters and M. Delmée, ←Clinic of Libramont, Libramont, Belgium	Shin et al., (2007)	Yes	Yes
*Cc*7	–	Human septicemia	1998	G. Wauters and M. Delmée, ←KUL, Leuven, Belgium	Shin et al. (2007)	No	Yes
*Cc*9	BCCM/LMG 11510, CCUG 12569, CDC A3626	Human septicemia	1965	BCCM/LMG←CCUG←R. Weaver, CDC, Atlanta, Georgia←Virginia	Vandamme et al. (1996)	No	Yes
*Cc*10	BCCM/LMG 11541, CCUG 17234, ATCC 35978	Human septicemia after dog bite	–	BCCM/LMG←MCCM←ATCC←R. Weaver, CDC, Atlanta, Georgia←California Health Dept. (324-1-74)	Vandamme et al. (1996)	No	No
*Cc*11	BCCM/LMG 11551, MCCM 01373	Human septicemia	–	BCCM/LMG←MCCM←A. von Graevenitz, University Zürich, Zürich, Switzerland	Shin et al. (2007)	Yes	No
*Cc*12	ATCC 35979, CDC 7120	Human septicemia after dog bite	–	ATCC←R. Weaver, CDC, Atlanta, Georgia←California Health Dep.←San Antonio Community Hospital, San Antonio, Texas	Shin et al. (2007)	No	Yes
*Cc*13	–	Healthy dog's (Jackie) saliva	2005	Direct isolation, Basel, Switzerland	Shin et al. (2007)	No	Yes
*Cc*14	–	Healthy dog's (Pouchka) saliva	2005	Direct isolation, Basel, Switzerland	Shin et al. (2007)	No	Yes

of 50. The phagocytosis assay was then continued as described before.

Cytokine and NO assays

Experiments were performed as described previously (Shin et al., 2007).

Statistical analysis

For all experiments, means and standard deviation were calculated. Statistical significance was evaluated by using a two-tailed, unpaired Student's t test. Differences were determined to be significant when $p<0.05$.

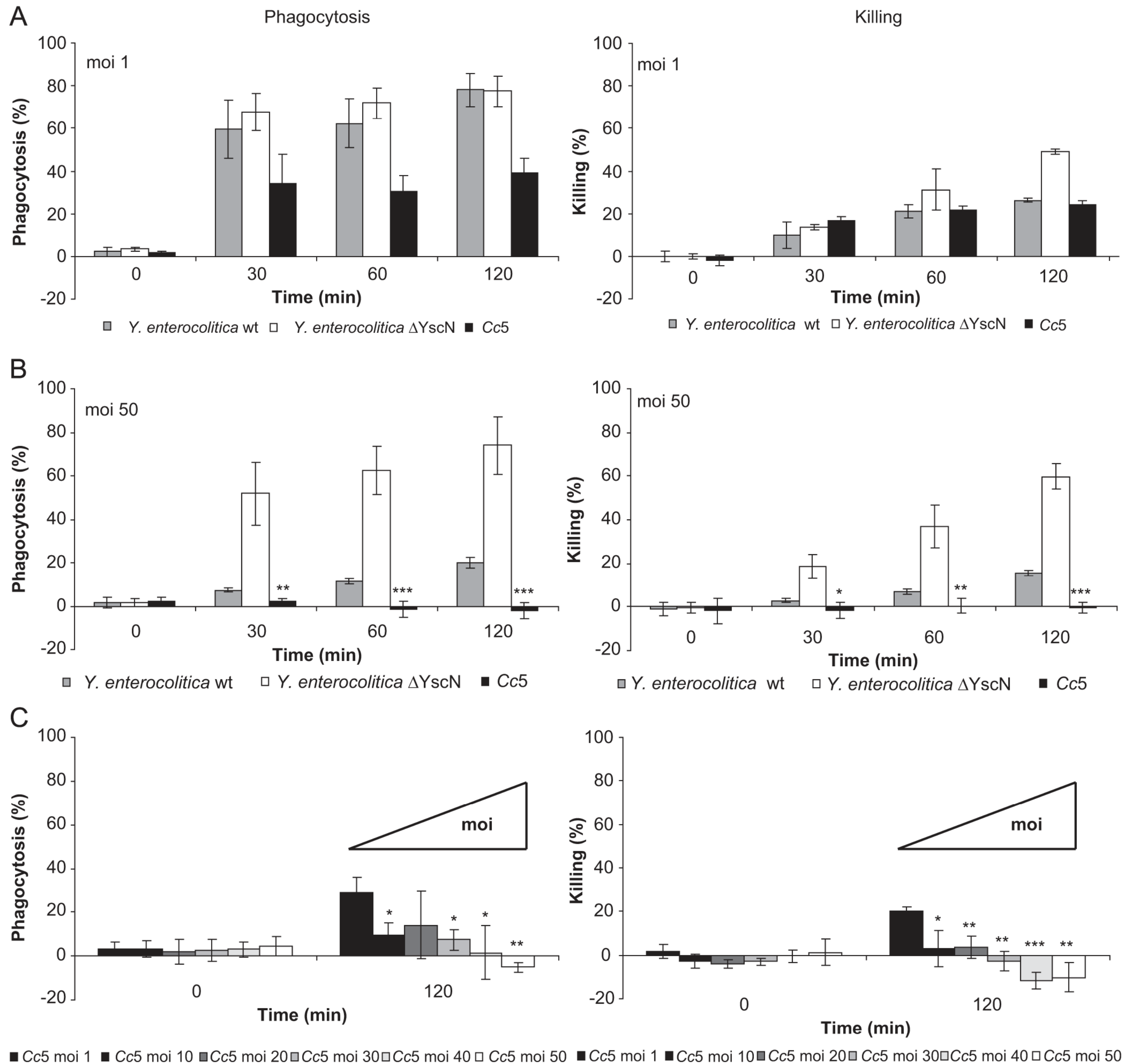


Fig. 1. Phagocytosis and killing of bacteria by J774.1 macrophage cells. Phagocytosis and killing were determined 0, 30, 60 and 120 min after infection of J774.1. (A) J774.1 cells were infected with either *Cc*5 (black), *Y. enterocolitica* wt (grey) or *Y. enterocolitica* ΔYscN (white) at moi of 1. (B) Infection with *Cc*5 (black), *Y. enterocolitica* wt (grey) or *Y. enterocolitica* ΔYscN (white) at moi of 50. Statistical significance is shown between phagocytosis and killing of *Cc*5 at moi of 1 (in panel A) and at moi of 50. (C) Infection with *Cc*5 at increasing moi (1–50). Statistical significance is shown for phagocytosis and killing of *Cc*5 at increasing moi compared to moi of 1 (all in panel C). Mean values from three or more experiments and standard deviations are shown including statistical significance with *$p>0.05$, **$p<0.01$ and ***$p<0.001$ using two-tailed, unpaired student's t test.

Results

*Cc*5 resists phagocytosis and killing by J774.1 mouse macrophages

We first investigated whether *Cc*5 is phagocytosed and killed by J774.1 mouse macrophages. As controls we included *Y. enterocolitica* E40 wt, which exhibits resistance to phagocytosis by injection of anti-phagocytotic effectors via its type III secretion system into the macrophage (Grosdent et al., 2002) and *Y. enterocolitica* E40 ΔYscN, which is sensitive to phagocytosis due to a loss of the ATPase of the type III Ysc machinery (Woestyn et al., 1994).

At a moi of 1, ~40% of the *Cc*5 bacteria were phagocytosed after 120 min of infection, while surprisingly ~80% of both wt and ΔYscN *Y. enterocolitica* were phagocytosed. About 20% of *Cc*5 were killed within 120 min of infection (Fig. 1A). While there was a strong and similar phagocytosis of the two

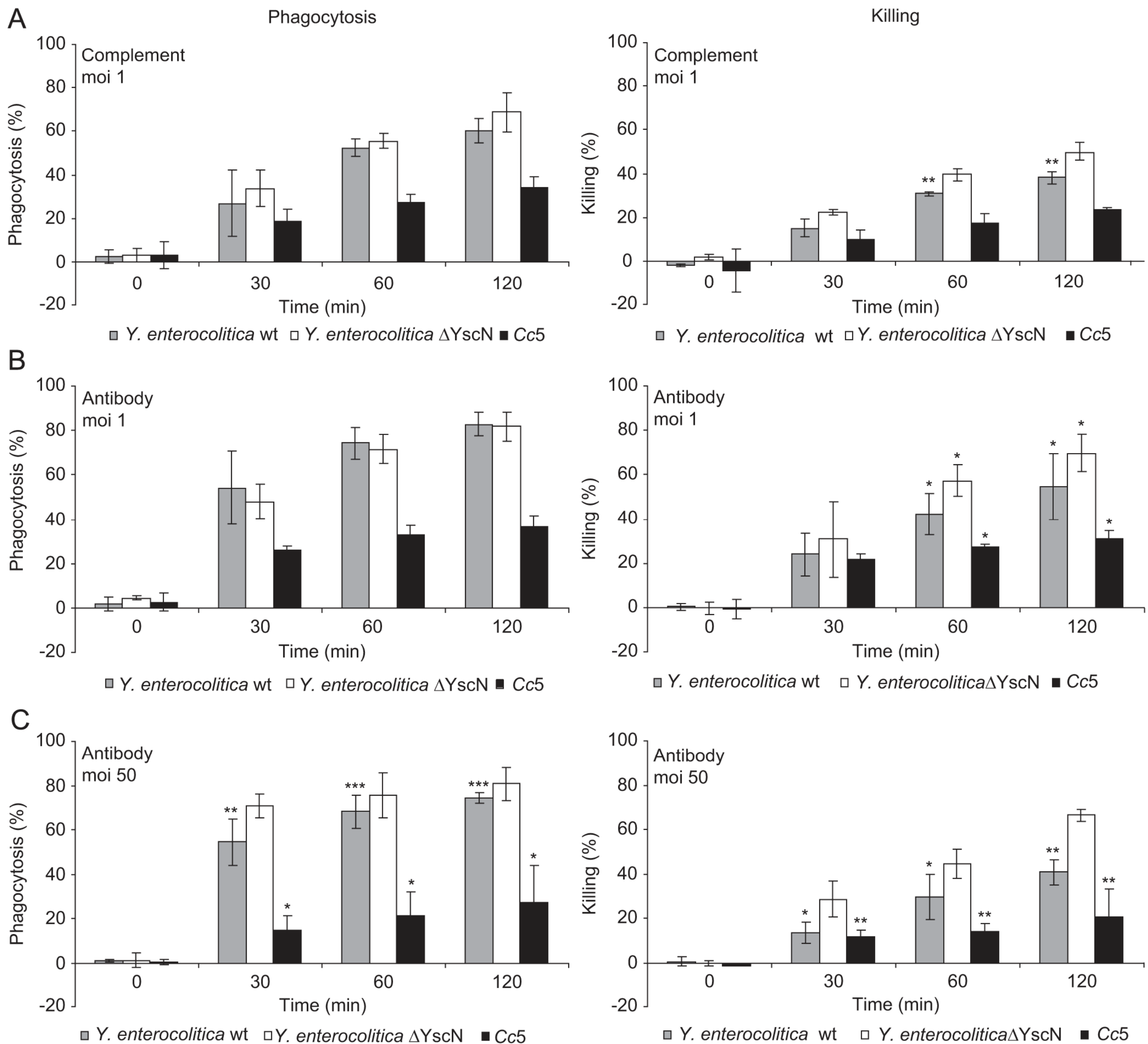


Fig. 2. Phagocytosis and killing of opsonized bacteria by J774.1 macrophage cells. Infection of J774.1 with pre-opsonized *Cc*5 (black), *Y. enterocolitica* wt (grey) or *Y. enterocolitica* ΔYscN (white) was done for 0, 30, 60 and 120 min. (A) Infection with complement opsonized bacteria (moi of 1). (B) Infection with antibody-opsonized bacteria (moi of 1). (C) Infection with antibody-opsonized bacteria (moi of 50). Statistical significance between bacterial phagocytosis and killing of un-opsonized bacteria and opsonized bacteria is indicated. Mean values from three or more experiments and standard deviations are shown including statistical significance with *$p<0.05$, **$p<0.01$, and ***$p<0.001$ using two-tailed, unpaired student's t test.

Y. enterocolitica strains, only ~20% of the *Y. enterocolitica* wt bacteria, but ~45% of the *Y. enterocolitica* ΔYscN bacteria, was killed within 120 min of infection.

Infection at a moi of 50 led to a drastic reduction of phagocytosis and killing of *Cc*5 by J774.1 macrophages compared to phagocytosis and killing at a moi of 1 ($p<0.05$, Fig. 1B). In contrast, still ~15% of *Y. enterocolitica* wt bacteria were killed by macrophages after 120 min. By increasing the moi (1, 10, 20, 30, 40 and 50), we observed a dose-dependent decrease in phagocytic uptake and killing of *Cc*5 by J774.1 ($p<0.05$, Fig. 1C). Hence, in high numbers *Cc*5 seems to prevent phagocytosis and killing by macrophages. This is in agreement with previous observations that *Cc5* survives and multiplies in the presence of J774.1 cells (Shin et al., 2007).

*Cc*5 is resistant against complement-dependent phagocytosis, but sensitive to antibody-mediated phagocytosis

Since *Cc*5 was able to resist phagocytosis and killing by macrophages, we investigated the effect of pre-opsonization of *Cc*5. Pre-opsonization of *Cc*5 with complement did not influence phagocytosis or killing by J774.1 cells (Fig. 2A). At a moi of 1, level of phagocytosis (~40%) and killing (~20%) of opsonized *Cc*5 within 120 min of infection were comparable to that of non-opsonized *Cc*5 (Fig. 1A). Accordingly, infection of J774.1 cells with complement-opsonized *Cc*5 at moi of 50 did not lead to any detectable phagocytosis or killing of the bacteria (data not shown). Complement opsonization only slightly increased the killing of *Y. enterocolitica* wt (~40%) compared to killing of

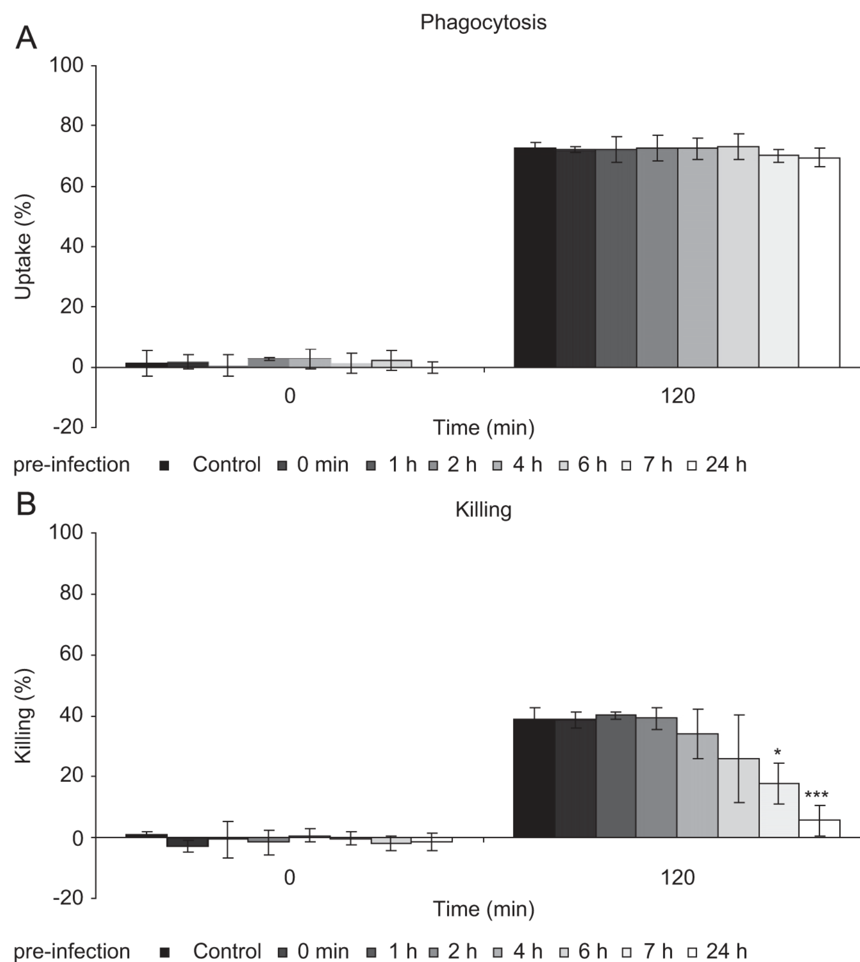


Fig. 3. Pre-infection of J774.1 macrophages with *Cc*5 influences the ability of the macrophages to kill other bacteria. J774.1 cells were pre-infected for different infection times (1, 2, 4, 6, 7 and 24 h) with *Cc*5 at a moi of 50, before infecting the macrophages with streptomycin-resistant *E. coli* (moi of 50). Phagocytosis and killing of untreated macrophages served as reference values. (A) Uptake of *E. coli* by J774.1. (B) Killing of *E. coli* by J774.1. Mean values from three or more experiments and standard deviations are shown including statistical significance between killing of *E. coli* by untreated macrophages and macrophages pre-infected for different infection times with *Cc*5 with *$p<0.05$ and ***$p<0.001$ using two-tailed, unpaired student's *t* test.

un-opsonized bacteria ($p<0.05$) as previously reported (Grosdent et al., 2002).

In contrast, pre-opsonization of *Cc*5 with rabbit sera specifically raised against HK *Cc*5 significantly increased killing of opsonized bacteria compared to killing of un-opsonized bacteria regardless of the moi ($p<0.05$, Fig. 2B and C). At a moi of 1, slightly more (i.e. ~30%) *Cc*5 were killed within 120 min of infection, whereas at moi of 50, ~30% of the antibody-opsonized *Cc*5 were phagocytosed and ~20% were killed by the macrophages after 120 min of infection. Phagocytosis and killing of both *Y. enterocolitica* strains were also increased upon antibody opsonization with rabbit anti *Y. enterocolitica* O:9. We conclude from these experiments that *Cc*5 is resistant to complement-dependent phagocytosis and killing, but sensitive to antibody-dependent phagocytosis and killing by macrophages.

Pre-incubation of macrophages with *Cc*5 impairs their capacity to kill phagocytosed bacteria

As we observed that *Cc*5 prevents its own uptake and killing at high moi (Fig. 1C), we investigated whether it could also influence the phagocytosis and killing of other bacteria. We first infected J774.1 mouse macrophages with *Cc*5 (moi of 50) for different time periods (1, 2, 4, 6, 7 and 24 h). Subsequently, *Cc*5-infected J774.1 cells were infected with streptomycin-resistant *E. coli* (moi of 50). Phagocytosis and killing of *E. coli* by untreated macrophages were used as reference values. While phagocytosis of *E. coli* by J774.1 cells was not influenced by pre-infection with *Cc*5 (Fig. 3A), killing of *E. coli* was reduced in a time-dependent manner (Fig. 3B). After 7 h of pre-infection with *Cc*5, killing of *E. coli* was reduced from ~35% to ~15%. After 24 h of pre-infection, <5% of the *E. coli* were killed.

Next, we determined whether only live *Cc*5 reduce the killing ability of macrophages. Thus, we pre-infected J774.1 cells for 24 h with HK *Cc*5 before infection with *E. coli*. Pre-infection with HK *Cc*5 did not influence phagocytosis and killing of *E. coli* (Fig. 4A).

We then investigated whether *Cc*5, which were pre-cultivated on J774.1 cells instead of blood agar plates, would be more efficient in blocking the killing of *E. coli*.

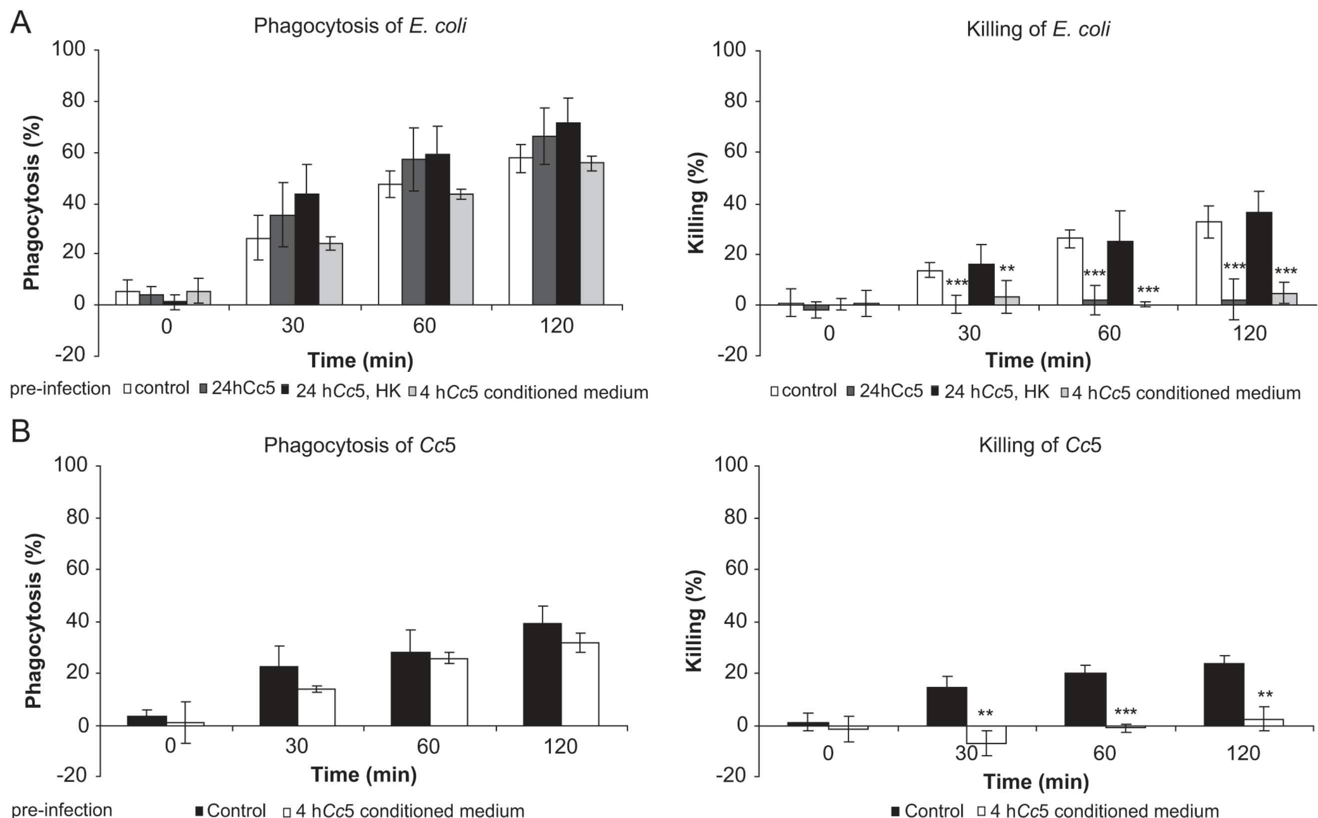


Fig. 4. *Cc*5 releases a soluble factor blocking killing of *E. coli* by J774.1 macrophages. (A) Macrophages were infected either directly with *E. coli* (white), or were pre-infected for 24 h with live *Cc*5 (dark grey), HK *Cc*5 (black), or were pre-treated for 4 h with *Cc*5 conditioned medium (light grey) before infection with *E. coli*. (B) Macrophages were infected either directly with *Cc*5 (moi of 50, black) or pre-treated for 4 h with *Cc*5 conditioned medium before infection with *Cc*5 (moi of 50, white). Mean values from three or more experiments and standard deviations are shown including statistical significance between killing of bacteria by untreated macrophages and pre-infected macrophages, with **$p<0.01$ and ***$p<0.001$ using two-tailed, unpaired student's t test.

We infected J774.1 cells for 24 h with *Cc*5 at a moi of 50. After 24 h, we isolated the *Cc*5 bacteria by centrifugation. These conditioned bacteria were then used to pre-infect fresh J774.1 for 4 h before infection with *E. coli* and they were found to abolish killing of *E. coli* bacteria within 120 min of infection (data not shown). This was in contrast to *Cc*5 grown on plates, which needed more than 7 h of pre-infection to block the killing of *E. coli* (Fig. 3B). Taken together, this suggests that *Cc*5 needs to be primed by macrophages to interfere with the killing of *E. coli* by macrophages.

We next investigated whether a released soluble factor was involved in this phenomenon. Therefore, we filtered the supernatant of the J774.1 culture that had been infected for 24 h with *Cc*5 and we used this conditioned medium to pre-incubate fresh J774.1 cells. After pre-treatment with this conditioned medium, the J774.1 cells were unable to kill *E. coli* (Fig. 4A). A shorter incubation of the J774.1 with conditioned medium did not prevent *E. coli* killing (data not shown).

We then examined whether *Cc*5 needs to interact specifically with macrophages in order to produce this soluble factor. Therefore, we pre-treated J774.1 cells with medium conditioned by the growth of *Cc*5 in the presence of Rat-1 fibroblast cells, and we observed again the absence of *E. coli* killing (data not shown). Thus, *Cc*5 does not necessarily need to be in contact with J774.1 cells to produce this effect.

We then investigated whether *Cc*5 themselves could be protected by this mechanism. Thus, we pre-treated J774.1 cells for 4 h with conditioned medium, before infecting cells at a moi of 1 with *Cc*5 resuspended from plate. The killing rate went down from 20% by untreated macrophages to <5% by macrophages pre-treated with conditioned medium (Fig. 4B).

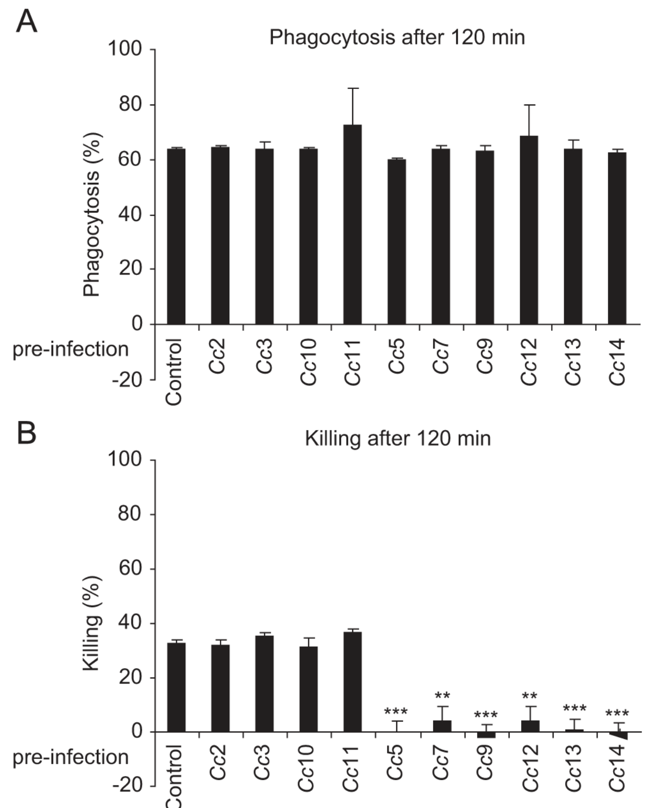


Fig. 5. Six out of 10 *C. canimorsus* strains can block the killing of bacteria by macrophages. J774.1 macrophage cells were pre-treated for 4 h with conditioned medium from different *C. canimorsus* strains before infection with *E. coli* (moi of 50). Phagocytosis and killing of untreated macrophages served as reference values. (A) Uptake of *E. coli* by macrophages. (B) Killing of *E. coli* by macrophages. Mean values from three or more experiments and standard deviations are shown including statistical significance between killing of bacteria by untreated macrophages and macrophages pre-infected 4 h with conditioned medium from different *C. canimorsus* strains with $^{**}p<0.01$ and $^{***}p<0.001$ using two-tailed, unpaired student's t test.

The ability to block the killing capacity of macrophages is not found in every *C. canimorsus* strain

We then tested whether this ability of *Cc*5 to block the killing of *E. coli* by macrophages is also found in other *C. canimorsus* strains. We used a collection of 10 *C. canimorsus* strains (Table 1), which were isolated either from human bite wound infections or from the oral cavity of healthy dogs (Shin et al., 2007). None of the strains was cytotoxic upon infection of J774.1 cells for 24 h (data not shown). Only 6 out of the 10 *C. canimorsus* strains tested (*Cc*5, *Cc*7, *Cc*9, *Cc*12, *Cc*13 and *Cc*14) were able to block the killing of *E. coli* by J774.1 cells, while *Cc*2, *Cc*3, *Cc*10 and *Cc*11 did not display this property (Fig. 5).

Inhibition of proinflammatory response induced by HK *Y. enterocolitica* by *C. canimorsus*

Recently, we demonstrated that *Cc*5 actively blocks the release of TNF and NO induced with HK *Y. enterocolitica* (Shin et al., 2007). Since the "anti-killing" activity of *Cc*5 was not found in all isolates tested, we wondered whether the "anti-inflammatory" mechanism would also be restricted to the same strains. We therefore tested the same *C. canimorsus* strains for their capacity to block the proinflammatory response induced by HK *Y. enterocolitica*. J774.1 cells were infected with live *C. canimorsus* (moi 20) together with HK *Y. enterocolitica* (moi 20), and supernatants from 24 h after infection were tested for TNF and NO release. Only *Cc*5

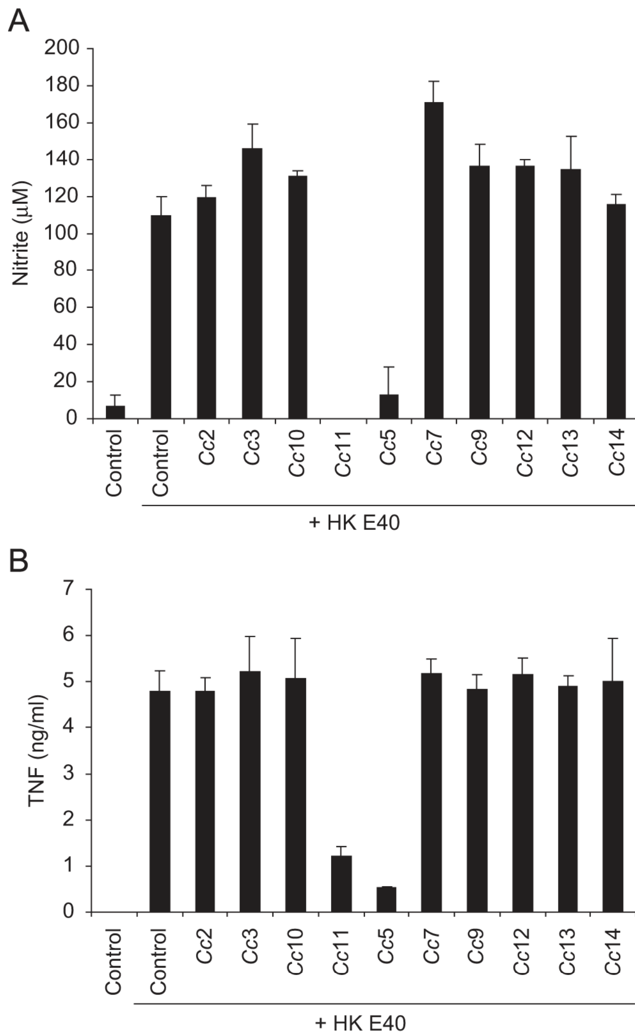


Fig. 6. *Cc*5 and *Cc*11 can inhibit the proinflammatory response induced by HK *Y. enterocolitica*. J774.1 macrophage cells were infected with the indicated *C. canimorsus* strains (moi of 20) together with HK *Y. enterocolitica* wt (E40, moi of 20) for 24 h. The proinflammatory response induced by HK *Y. enterocolitica* in the absence of *C. canimorsus* served as reference value. Culture supernatants were analyzed for the release of NO (A) and TNF (B). Results represent the mean of at least three independent experiments. Error bars represent the standard deviation.

and *Cc*11 blocked the release of TNF and NO (Fig. 6). Thus, these two properties are not coincident and they probably involve two different mechanisms.

Discussion

The clinical overview of *C. canimorsus* infection suggests that *C. canimorsus* escapes the innate immune system, at least in the early stages of infection. The present study on the interaction of *C. canimorsus* with murine macrophages leads to three major observations. First, at moi of 1, both *Cc*5 and *Y. enterocolitica* were susceptible to phagocytosis. This is somewhat surprising for *Y. enterocolitica* as it is an example of resistant bacteria. Thus, we observed that the type III secretion system is not completely protective at very low moi. This observation, to our knowledge, was not reported before. At a moi of 50, the situation was very different. Both *Cc*5 and *Y. enterocolitica* wt were significantly more resistant than *Y. enterocolitica* mutant deprived of its type III secretion system. Thus, at high moi, the type III secretion system indeed protects wt *Y. enterocolitica* from phagocytosis and killing as shown before (Grosdent et al., 2002). *Cc*5 was always less phagocytosed and killed than wt *Y. enterocolitica*. This could result from a lack of adhesion. However, the fact that the amount of phagocytosis and killing went down significantly when the moi was increased suggested that there might be another mechanism affecting phagocytosis. To our knowledge, there is no type III secretion system present in *C. canimorsus* (unpublished data), implying that *Cc*5 would resist phagocytosis by other mechanism(s). So far, we have no evidence on the mechanism(s) that could be involved.

Second, opsonization with complement did not have a detectable effect on the phagocytosis of *Cc5*. This raises the question whether *Cc*5 might prevent the deposition of C3. Further investigations on the interaction between *Cc*5 and complement are in progress. Opsonization with antibodies, however, increased phagocytosis of *Cc*5.

Third, our findings suggest that four out of eight clinical isolates of *C. canimorsus* and two out of two isolates from healthy dogs are able to block the macrophage's ability to kill phagocytosed bacteria. Surprisingly, infection of macrophages with these *C. canimorsus* isolates did not influence the phagocytosis of other bacteria, but it prevented the killing of the ingested bacteria. The ability of some strains of *C. canimorsus* to block the killing ability of macrophages suggests that these strains might be more pathogenic than the others. In this respect, it would be interesting to investigate more strains isolated from dog mouths and from human infections to compare the capacity to block the killing of phagocytosed bacteria. This ‘‘anti-killing’’ property depends on a soluble factor present in the culture medium of infected macrophages. At this stage, we can only speculate that this factor is of bacterial origin, rather than released by macrophages. The fact that this factor is also present in the supernatant of Rat-1 cells infected by *Cc*5 (unpublished data) suggests that this factor is of bacterial origin and not produced by macrophages, but this needs to be further investigated. Results available so far do not allow making hypotheses concerning the biochemical nature of this factor. It could be a protein, a carbohydrate or even a secondary metabolite. Experiments are in progress to clarify this

point. It is also impossible at this stage to speculate on how this factor prevents killing. It could interfere with the activation of the macrophage or it could interfere with signalling leading to one or more specific killing mechanisms. An interesting question is the relation between this factor and the mechanism that prevents the onset of the proinflammatory response that we described earlier (Shin et al., 2007). Interestingly, not all strains interfering with the macrophage's killing ability can also block the release of TNF and NO and vice-versa, which indicates that *C. canimorsus* is endowed with at least two different mechanisms blocking the innate immunity at different stages.

It is noteworthy that *C. canimorsus* is a commensal but has the same capacity as a pathogen to down-regulate the innate immune system. This is however not unprecedented since *Bacteroides thetaiotaomicron* has also been shown to down-regulate the innate immunity (Kelly et al., 2004). In its natural environment, the oral cavity of dogs and cats, *C. canimorsus* is challenged by the anti-bacterial action of saliva as well as by the presence of tissue resident macrophages. *C. canimorsus* seems to have perfectly adapted to its environment by down regulating the innate immune system in order to guarantee its survival. Therefore, the study of commensals like *C. canimorsus* may help to get a better understanding of commensalism and its relation to pathogenesis.

Acknowledgements

We thank R. Landmann and M. Mally for stimulating discussions and critical reading of the manuscript. This work was funded by the Swiss National Science Foundation Grant no. 310000-113333. The authors have declared that no competing interests exist.

Author contributions: SM, HS and GC conceived and designed the experiments. SM and HS performed the experiments. SM, HS and GC wrote the paper.

References

Blanche, P., Bloch, E., Sicard, D., 1998. *Capnocytophaga canimorsus* in the oral flora of dogs and cats. J. Infect. 36, 134.

Brenner, D.J., Hollis, D.G., Fanning, G.R., Weaver, R.E., 1989. *Capnocytophaga canimorsus* sp. nov. (formerly CDC group DF-2), a cause of septicemia following dog bite, and *C. cynodegmi* sp. nov., a cause of localized wound infection following dog bite. J. Clin. Microbiol. 27, 231–235.

Cornelis, G.R., Sory, M.-P., Laroche, Y., Derclaye, I., 1986. Genetic analysis of the plasmid region controlling virulence in *Yersinia enterocolitica* 0:9 by Mini-Mu insertion and lac gene fusions. Microb. Pathogenesis 1, 349–359.

Grosdent, N., Maridonneau-Parini, I., Sory, M.-P., Cornelis, G.R., 2002. Role of Yops and adhesins in resistance of *Yersinia enterocolitica* to phagocytosis. Infect. Immun. 70, 4165–4176.

Hantson, P., Gautier, P.E., Vekemans, M.-C., Fievez, P., Evrard, P., Wauters, G., Mahieu, P., 1991. Fatal *Capnocytophaga canimorsus* septicemia in a previously healthy woman. Ann. Emerg. Med. 20, 93–94.

Kelly, D., Campbell, J.I., King, T.P., Grant, G., Jansson, E.A., Coutts, A.G., Pettersson, S., Conway, S., 2004. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. Nat. Immunol. 5, 104–112.

Le Moal, G., Landron, C., Grollier, G., Robert, R., Burucoa, C., 2003. Meningitis due to *Capnocytophaga canimorsus* after receipt of a dog bite: case report and review of the literature. Clin. Infect. Dis. 36, e42–e46.

Letzelter, M., Sorg, I., Mota, L.J., Meyer, S., Stalder, J., Feldman, M., Kuhn, M., Callebaut, I., Cornelis, G.R., 2006. The discovery of SycO highlights a new function for type III secretion effector chaperones. EMBO 25, 3223–3233.

Lion, C., Escande, F., Burdin, J.C., 1996. *Capnocytophaga canimorsus* infections in human: review of the literature and cases report. Eur. J. Epidemiol. 12, 521–533.

Shin, H., Mally, M., Kuhn, M., Paroz, C., Cornelis, G.R., 2007. Escape from immune surveillance by *Capnocytophaga canimorsus*. J. Infect. Dis. 195, 375–386.

Tierney, D.M., Strauss, L.P., Sanchez, J.L., 2006. *Capnocytophaga canimorsus* mycotic abdominal aortic aneurysm: why the mailman is afraid of dogs. J. Clin. Microbiol. 44, 649–651.

Vandamme, P., Vancanneyt, M., van Belkum, A., Segers, P., Quint, W.G.V., Kersters, K., Paster, B.J., Dewhirst, F.E., 1996. Polyphasic analysis of strains of the genus *Capnocytophaga* and centers for disease control group DF-3. Int. J. Syst. Bacteriol. 46, 782–791.

Vanhonsebrouck, A.Y., Gordts, B., Wauters, G., van Landhuyt, H.W., 1991. Fatal septicemia with *Capnocytophaga canimorsus* in a compromised host. A case report with review of the literature. Acta Clin. Belg. 46, 364–370.

Woestyn, S., Allaoui, A., Wattiau, P., Cornelis, G.R., 1994. YscN, the putative energizer of the Yersinia Yop secretion machinery. J. Bacteriol. 176, 1561–1569.

2.3. Additional results

Cc5 glycosyltransferase (*gtf*) mutant Y1C12 is sensitive to phagocytosis and killing by J774.1 macrophages

During the serum sensitivity screen on our *Cc5* Tn mutant library, Ch. Fiechter has identified *Cc5* Tn mutant Y1C12 to be serum sensitive. To enlarge our knowledge on this mutant, we decided to determine phagocytosis resistance of Y1C12 in comparison to *Cc5*. We therefore tested phagocytosis and killing of Y1C12 by J774.1 mouse macrophages at low and high moi (moi 1 and moi 50, respectively).

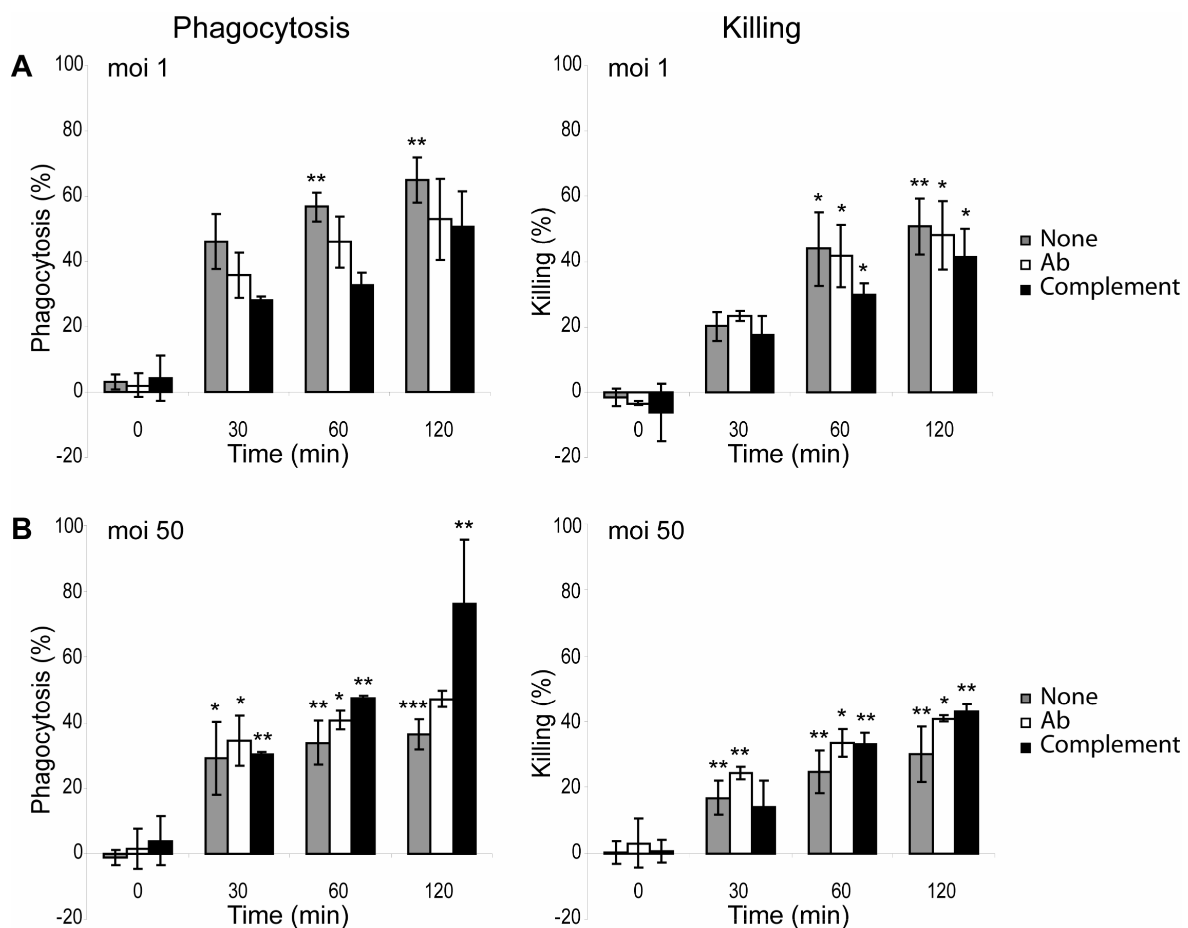


Figure 1. Phagocytosis and killing of Y1C12 bacteria by J774.1 macrophages
Infection of macrophages with unopsonized (grey), Ab pre-opsonized (white), or complement pre-opsonized (black) Y1C12 was done for 0, 30, 60 and 120 minutes (min). (**A**) Infection at an moi of 1. (**B**) Infection at an moi of 50. Mean values and standard deviations from at least three independent experiments are shown including statistical significance in comparison to *Cc5* (Meyer *et al.*, 2008) with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ using two-tailed, unpaired Student's *t* test.

At an moi of 1 *Cc5* bacteria were only phagocytosed and killed by ~40% and ~25%, respectively (Meyer *et al.*, 2008), while ~65% Y1C12 bacteria were phagocytosed within 2 h and ~50% were killed (Figure 1.A). At an moi of 50, *Cc5* was completely resistant to phagocytosis and killing by murine macrophages (Meyer *et al.*, 2008) whereas ~35% Y1C12 bacteria were phagocytosed and ~30% were killed (Figure 1.B). Comparably to Ab pre-opsonization of *Cc5* (Meyer *et al.*, 2008), Abs increased killing of Y1C12 by about 10% at high moi. In contrast, pre-opsonization with heat-inactivated (HI) guinea pig complement did neither at low nor at high moi significantly increase killing of Y1C12 bacteria compared to killing of unopsonized Y1C12 bacteria. Though, it needs to be noted that HI guinea pig complement only leads to opsonization with pre-formed C3b, which strongly decreases complement opsonization.

Overall, these observations show that – compared to *Cc5* – Y1C12 mutant bacteria have an increased sensitivity against phagocytosis and killing by mouse macrophages at low and high moi.

2.4. Materials and methods

Bacterial strains and media

Growth of bacteria and preparation of cRPMI (Gibco) was done as described (Meyer *et al.*, 2008).

Selective agents

To select for strains or transposons, antibiotics were added at the following concentration: 10 μg/ml Em, 20 μg/ml Gm.

In vitro phagocytosis and killing assay

The assay was performed as described (Meyer *et al.*, 2008).

Statistical analysis

For all experiments, means and standard deviations were calculated. Statistical significance was evaluated using two-tailed, unpaired Student's *t* test. Differences were considered to be significant when $p < 0.05$ with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2.5. Discussion

We could demonstrate that *Cc5* is even more resistant to phagocytosis and killing by murine macrophages than *Y. enterocolitica*, which is considered a model organism for phagocytosis resistance due to its T3SS (Grosdent *et al.*, 2002). However a *Cc5* Tn mutant discovered in a screening for serum sensitivity, Y1C12, was observed to have an increased sensitivity towards phagocytosis and killing by murine macrophages at low and high moi (moi 1 and 50, respectively) compared to *Cc5*. Interestingly, we could observed that *Cc5* mutant Y1C12 is more susceptible to phagocytosis even in the unopsonized state. This indicated that not an increased susceptibility for Ab binding or complement deposition led to an increased phagocytosis, but that the outer surface of the bacteria is more readily recognized by the macrophage's PRRs, leading to an increased phagocytosis and therefore to an increased killing of the mutant bacteria. As the mutant has been found in a screen on serum sensitivity (performed by Ch. Fiechter), this further indicated that the mutant might be affected in its outer membrane structure.

More analysis on the gene hit by the Tn in mutant Y1C12 needs to be done to understand its increased susceptibility to complement and phagocytosis by murine macrophages. In addition, LPS of *Cc5* and Y1C12 should be compared in order to verify whether the mutant indeed displays an altered outer membrane structure thus explaining increased phagocytosis and serum sensitivity.

Human PMNs are among the first line of defence bacteria encounter upon infection and they are the most efficient professional phagocytes of the human immune system. Therefore, resistance of *Cc5* and Y1C12 against phagocytosis and killing by human PMNs should be investigated.

Interestingly, we observed that four out of eight clinical isolates and two out of two isolates from healthy dogs were able to block the ability of murine macrophages to kill phagocytosed bacteria. It would thus be interesting to identify the mechanisms and the bacterial factor(s) by which *C. canimorsus* interferes with the killing ability of macrophages. Additionally, determination of the prevalence of this capacity within *C. canimorsus* strains would allow achieving further information on the hypothetical contribution of this property to virulence.

To summarize, we provide evidence that *Cc5* is highly resistant to phagocytosis and killing by murine macrophages and additionally interferes with

the macrophage's killing ability upon prolonged exposure. This might be beneficial for bacterial survival in its natural environment, the oral cavity of dogs, which is naturally enriched by tissue resident macrophages.

Chapter 3

C. canimorsus blocks the oxidative burst in murine macrophages

3.1. Summary

As we have shown previously that *Cc5* blocks the killing ability of macrophages (Meyer *et al.*, 2008), we investigate here that *Cc5* needs to grow in the presence of cells in order to generate conditioned (cond.) medium or cond. bacteria, which are then able to block the killing ability of fresh murine macrophages upon 4 h of pre-treatment.

Investigating the mechanism by which *Cc5* interferes with the killing ability of macrophages, we could demonstrate that *Cc5* does neither affect lysosome associated protein 1 (LAMP-1) recruitment to phagosomes nor acidification of phagolysosomes, indicating that phagosome maturation is not affected by *Cc5*. In contrast, *Cc5* prevents the oxidative burst normally induced upon phagocytosis of *E. coli*. Whether *Cc5* prevents the assembly of the NADPH oxidase, or whether *Cc5* redirects the oxidase away from the phagolysosomes could not be determined yet.

In another approach, we wanted to identify factor(s) involved in blocking the NADPH oxidase activity. We demonstrate here that protein(s) and divalent cations are involved in interfering with the killing ability of macrophages. S. Ittig has prepared medium from uninfected and *Cc5* infected HeLa cells and SDS-PAGE analysis revealed a band appearing after infection with *Cc5*. This band was analyzed by mass spectrometry (MS) by S. Ittig and P. Jenö and one of the proteins identified corresponded to open reading frame (ORF) 958 (*pitrilysin*), having a predicted metallopeptidase activity and a Zinc-binding motif. S. Ittig thereafter constructed a *Cc5* pitrilysin mutant (Δ*pit*) and a complementation plasmid (cΔ*pit*). Δ*pit* bacteria could not prevent the induction of an oxidative burst upon phagocytosis of *E. coli*, indicating that pitrilysin is indeed involved in the mechanism by which *Cc5* interferes with the NADPH oxidase. Further investigations on the biochemical properties of pitrilysin are ongoing as well as attempts to identify the mechanism by which pitrilysin interacts with macrophages.

3.2. Introduction

As we have shown previously that *Cc5* blocks the killing ability in macrophages (Meyer *et al.*, 2008), we wanted to determine the underlying mechanism and the involved factor(s).

3.3. Results

Cc5* needs to grow in the presence of cells to prevent killing of *E. coli

As we observed that – upon growth in the presence of cells – *Cc5* released secreted factor(s), which prevented killing but not phagocytosis of *E. coli* by J774.1 macrophages (Meyer *et al.*, 2008), we wanted to verify the conditions required to observe this block of killing.

Therefore, we first determined how long cond. bacteria (bacteria grown for 24 h in the presence of J774.1) needed to be in contact with macrophages in order to interfere with their killing ability. Thus, fresh macrophages were pre-infected with cond. bacteria for different pre-infection times and killing of *E. coli* (moi 50) by macrophages was determined thereafter. While 2 h pre-infection with cond. *Cc5* did not affect killing of *E. coli*, 4 h pre-infection with cond. bacteria prevented killing of *E. coli* within 2 h of infection (Figure 1). As *Cc5* resuspended from plate could only interfere with the killing ability of macrophages after ~7 h of pre-infection (Meyer *et al.*, 2008), this indicated that upon growth in the presence of cells, *Cc5* become more competent to block the killing ability of macrophages.

In order to exclude that the above findings were due to a mass effect as *Cc5* grows in the presence of macrophages upon 24 h of infection (Shin *et al.*, 2007), we determined the OD_{600} of cond. bacteria before pre-infection of fresh macrophages. Starting from an OD_{600} of 0.0125, the bacteria had grown up to an OD_{600} of 1.73 ± 0.64 within 24 h. Thus, fresh *Cc5* from plate were prepared at the appropriate concentration and fresh macrophages were pre-infected for 4 h before infection with *E. coli* at an moi of 50. 4 h pre-infection with high amounts of fresh *Cc5* did not block the killing of *E. coli* within 2 h of infection (Figure 1). This was in contrast to 4 h pre-infection with cond. *Cc5*, indicating that growth in the presence of cells and not the moi is important to make *Cc5* capable of blocking the killing ability of macrophages (Figure 1).

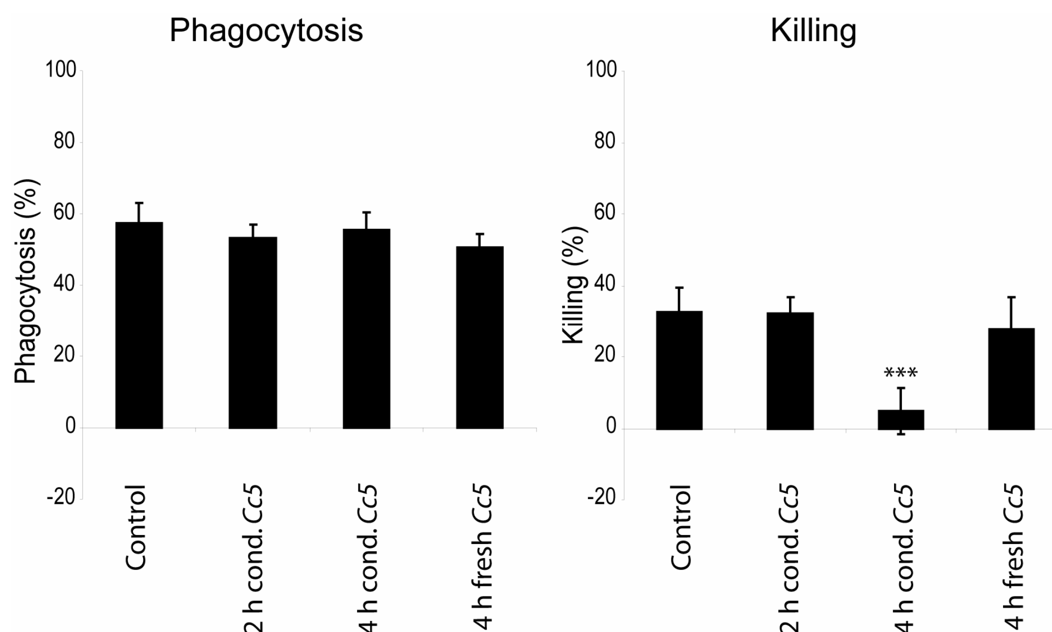


Figure 1. *Cc5* needs to grow in the presence of cells to prevent killing of *E. coli*
J774.1 macrophages were either left untreated (control) or pre-infected for either 2 or 4 h with either conditioned or fresh *Cc5*. After pre-infection, macrophages were infected for 2 h with *E. coli* at an moi of 50 and phagocytosis and killing of *E. coli* were determined by survival plating. Mean values and standard deviations from at least three independent experiments are shown including statistical significance comparing to phagocytosis and killing, respectively, by untreated macrophages with *** $p < 0.001$ using two-tailed, unpaired Student's *t* test.

As we observed that – upon growth in the presence of murine macrophages – *Cc5* released or modified factor(s) in the medium, which were able to block the killing ability of macrophages in the absence of whole bacteria (Meyer *et al.*, 2008), we decided to continue working with cond. medium, *i.e*. medium gained after infection of macrophages for 24 h.

Therefore, we wanted to determine, how long cond. medium needs to be applied to fresh cells in order to block the killing ability of macrophages. Thus, J774.1 macrophages were pre-treated for either 2 or 4 h with *Cc5* cond. medium before infection with *E. coli* (moi 50). As already observed for cond. *Cc5* (Figure 1), *Cc5* cond. medium had to be added for 4 h to fresh macrophages in order to block their killing ability (Figure 2).

As the bacteria need to be grown for 24 h in the presence of cells to gain cond. medium, we wanted to exclude that the age of the medium itself influenced the killing ability of the macrophages. Therefore, fresh macrophages were pre-treated for 4 h with old cRPMI (medium which was previously for 24 h in contact with cells)

before infection with *E. coli* (moi 50). 4 h pre-treatment with old cRPMI had no influence on the killing ability of the macrophages (Figure 2), indicating that indeed *Cc5* released or modified factor(s) in the medium upon growth in the presence of cells, which interfere with the macrophages.

As the effect on the killing ability of macrophages was only observed after at least 4 h of pre-treatment with either cond. bacteria (Figure 1) or cond. medium (Figure 2), we tested whether the concentration of the cond. medium is also relevant. Thus, macrophages were pre-treated for 4 h with *Cc5* cond. medium diluted 1:2 with fresh cRPMI before infection with *E. coli* (moi 50). Interestingly, diluted *Cc5* cond. medium could not prevent killing of *E. coli*, suggesting that concentration of the factor(s) is critical (Figure 2).

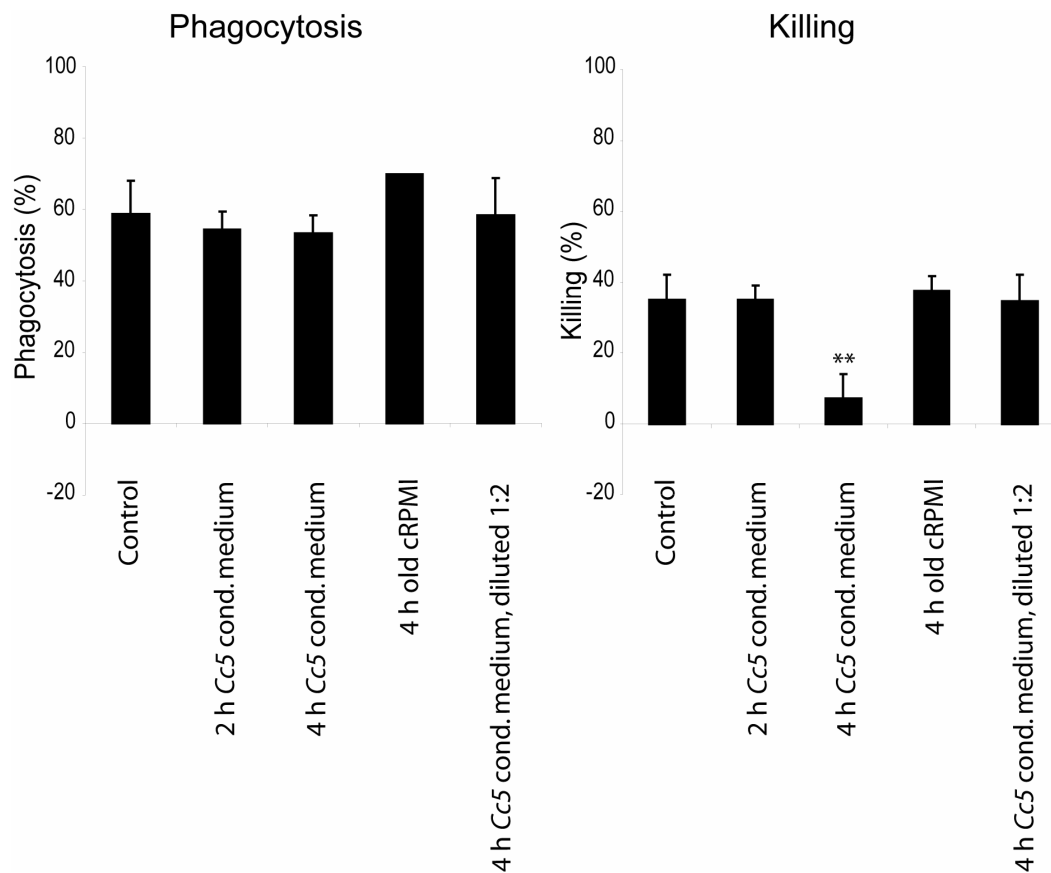


Figure 2. Concentration of cond. medium is essential

J774.1 macrophages were either left untreated (control) or pre-treated as indicated before infection with *E. coli* at an moi of 50. 2 h after infection, phagocytosis and killing of *E. coli* were determined by survival plating. Mean values from three or more independent experiments and standard deviations are shown including statistical significance comparing to phagocytosis and killing, respectively, by untreated macrophages with ** $p < 0.01$ using two-tailed, unpaired Student's *t* test.

As we observed that *Cc5* interfered with the killing ability of murine macrophages, we wanted to explore, whether *Cc5* could also block the killing ability of human PMNs. Thus, freshly isolated PMNs were either left untreated or pre-treated for 4 h with *Cc5* cond. medium before infection with *E. coli* (moi 50). As PMNs are very short lived after isolation from human blood, PMNs died before the end of the experiment and it therefore could not be determined yet, whether *Cc5* has an effect on the killing ability of human PMNs.

_Cc5_ blocks the oxidative burst

Upon phagocytosis by macrophages, bacteria are engulfed within phagosomes, which mature to acidic phagolysosomes *via* a series of vesicle fusion events (for a review on phagocytosis see (Stuart and Ezekowitz, 2005)). As we have shown that *Cc5* blocks the killing ability of macrophages without affecting phagocytosis (Meyer *et al.*, 2008), we wanted to determine whether *Cc5* interferes with the maturation of the phagosomes.

In a first step we thus used LAMP-1 as a marker for lysosomes and localization of *E. coli* (expressing enhanced green fluorescent protein (eGFP)) within phagolysosomes was determined. Therefore, either fresh (control) or *Cc5* cond. medium pre-treated macrophages were infected with *E. coli* (moi 10). After 2 h of infection, cells were fixed and phagosome maturation was determined by immunofluorescence.

We observed that ~70% of the *E. coli* localized within LAMP-1 positive lysosomes after 2 h of infection. This percentage did not differ whether the macrophages were fresh (control) or pre-treated with *Cc5* cond. medium (Figure 3).

To exclude that extracellular *E. coli* interfered with the evaluation of *E. coli* localized in lysosomes, macrophages were additionally pre-treated with Cytochalasin (Cyt.) D, which blocks the actin cytoskeleton, thereby preventing phagocytosis. Upon pre-treatment with Cyt. D, less than 15% of the *E. coli* were still found to be localized within LAMP-1 positive lysosomes (Figure 3). These might represent bacteria phagocytosed by macrophages, which were not blocked by Cyt. D, or extracellular *E. coli* which were incorrectly counted to be localized in LAMP-1 positive lysosomes due to their localization on the cell. However, treatment of macrophages with Cyt. D strongly and equally reduced the lysosomal

localization of *E. coli* in control as well as in *Cc5* cond. medium pre-treated macrophages. This indicated that the ~70% lysosomal localization determined previously represented predominantly phagocytosed *E. coli* localized within LAMP-1 positive lysosomes. These results suggested that *Cc5* did not affect phagolysosomal maturation until the recruitment of LAMP-1.

As acidification is the latest step in phagolysosome maturation, we wanted to determine whether LAMP-1 stained only acidic lysosomes. Thus, macrophages were pre-treated with Bafilomycin, which prevents acidification of lysosomes. Upon pre-treatment with Bafilomycin, LAMP-1 positive lysosomal localization of *E. coli* was reduced from ~70% to ~50% and ~40% in control macrophages and *Cc5* cond. medium pre-treated macrophages, respectively (Figure 3). As still between 40-50% of the *E. coli* were localized within LAMP-1 positive lysosomes even if acidification was prevented by the addition of Bafilomycin, this indicated that LAMP-1 was recruited before acidification of the lysosomes. Therefore LAMP-1 is not a suitable marker to determine acidification of phagolysosomes.

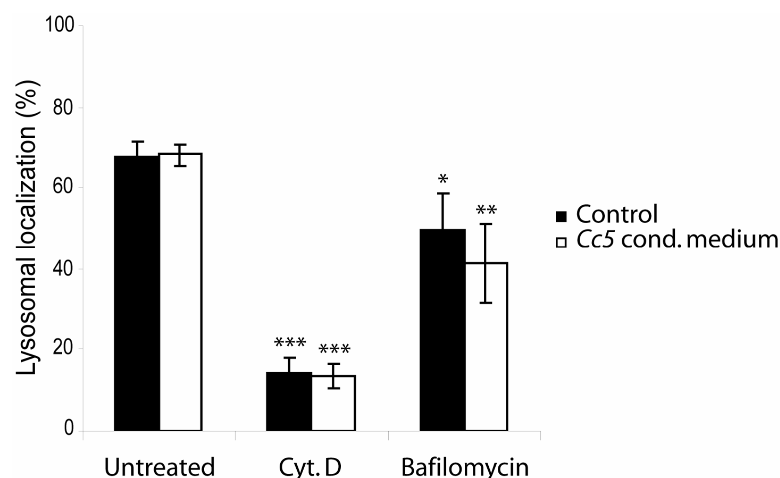


Figure 3. *Cc5* does not affect LAMP-1 recruitment

Lysosomal localization of *E. coli* 2 h after infection of macrophages at an moi of 10. Lysosomal localization of *E. coli* after phagocytosis by 4 h *Cc5* cond. medium pre-treated macrophages is shown in white, lysosomal localization after phagocytosis by fresh macrophages (control) is shown in black. Lysosomal localization was determined by calculating the percentage of *E. coli* found in LAMP-1-positive lysosomes (immunofluorescence). If indicated, macrophages were additionally pre-treated with either Cyt. D to block phagocytosis, or with Bafilomycin to prevent acidification of the lysosomes. Mean values and standard deviations from at least three independent experiments are shown including statistical significance comparing to lysosomal localization in untreated macrophages with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ using two-tailed, unpaired Student's *t* test.

In order to test, whether acidification of the lysosomes still occurred after pre-treatment with *Cc5* cond. medium, we investigated the localization of *E. coli* within acidic lysosomes by lysotracker staining. Lysotracker is a red fluorescent, cell-permeant dye, accumulating in acidic environments and organelles. After infecting macrophages for 2 h with *E. coli* at an moi of 10, ~60% of the *E. coli* localized within lysotracker-stained, acidic lysosomes irrespective of whether the macrophages were fresh (control) or pre-treated for 4 h with *Cc5* cond. medium (Figure 4).

To exclude that extracellular *E. coli* interfered with the evaluation of *E. coli* localized in acidic lysosomes, macrophages were pre-treated with Cyt. D. After pre-treatment with Cyt. D, less than 11% of the bacteria were still found to be localized within acidic lysosomes (Figure 4). These might represent *E. coli* phagocytosed by macrophages, which were not blocked by Cyt. D, or extracellular *E. coli* which were incorrectly counted to be localized within acidic lysosomes due to their extracellular attachment to the cell. However, the strong and equal reduction in lysosomal localization of *E. coli* after treatment with Cyt. D in control macrophages as well as in *Cc5* cond. medium pre-treated macrophages indicated that the lysosomal localization in untreated macrophages represented phagocytosed *E. coli* localized within lysotracker-stained lysosomes.

To determine the specificity of lysotracker for acidic lysosomes, macrophages were pre-treated with Bafilomycin. Upon pre-treatment with Bafilomycin, acidic lysosomal localization of *E. coli* was reduced from ~60% to less than 8% in control macrophages and *Cc5* cond. medium pre-treated macrophages (Figure 4), indicating that lysotracker indeed specifically accumulated in acidic lysosomes and therefore suggesting that *Cc5* did not affect acidification of lysosomes and thus did not affect phagolysosomal maturation.

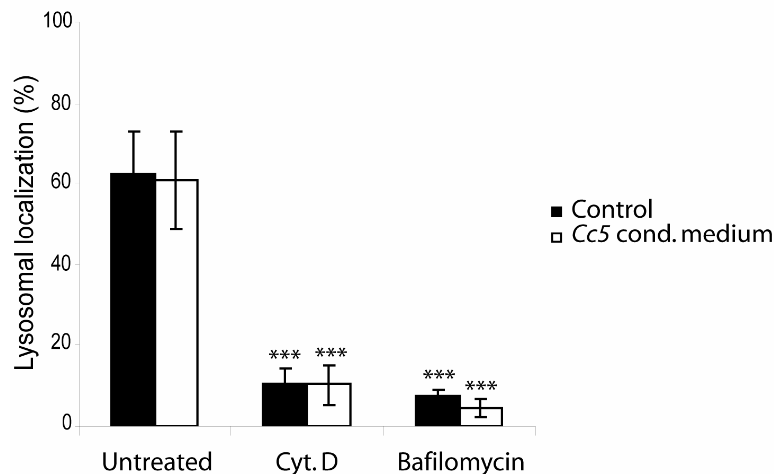


Figure 4. *Cc5* does not affect acidification of lysosomes
Lysosomal localization of *E. coli* 2 h after infection of macrophages at an moi of 10. Lysosomal localization of *E. coli* after phagocytosis by 4 h *Cc5* cond. medium pre-treated macrophages is shown in white, lysosomal localization after phagocytosis by fresh macrophages (control) is shown in black. Lysosomal localization was determined by calculating the percentage of *E. coli* recruitment to lysotracker-positive lysosomes (immunofluorescence). If indicated, macrophages were additionally pre-treated with either Cyt. D to block phagocytosis, or with Bafilomycin to prevent acidification of the lysosomes. Mean values and standard deviations from at least three independent experiments are shown including statistical significance comparing to lysosomal localization in untreated macrophages with *** $p < 0.001$ using two-tailed, unpaired Student's *t* test.

As we observed that pre-treatment of murine macrophages with *Cc5* cond. medium blocked the killing ability of macrophages (Meyer *et al.*, 2008), but not the maturation of phagolysosomes (Figures 3 and 4), we wanted to examine whether *Cc5* has an influence on the oxidative burst induced upon activation of the NADPH oxidase in the acidified phagolysosome. We therefore infected either fresh (control) or 4 h *Cc5* cond. medium pre-treated J774.1 macrophages for 30 min with *E. coli* at an moi of 50. After addition of the cell permeant dye dihydrorhodamine 1.2.3 (DHR), the strength of the oxidative burst was determined by measuring the mean fluorescence intensity of the macrophages, as DHR becomes fluorescent and membrane-impermeant upon oxidation.

While infection of fresh macrophages induced an oxidative burst and therefore induced oxidation of DHR, macrophages pre-treated for 4 h with *Cc5* cond. medium were not able to induce an oxidative burst upon infection with *E. coli* (Figure 5). These results suggested that *Cc5* specifically prevented the oxidative burst normally induced upon phagocytosis.

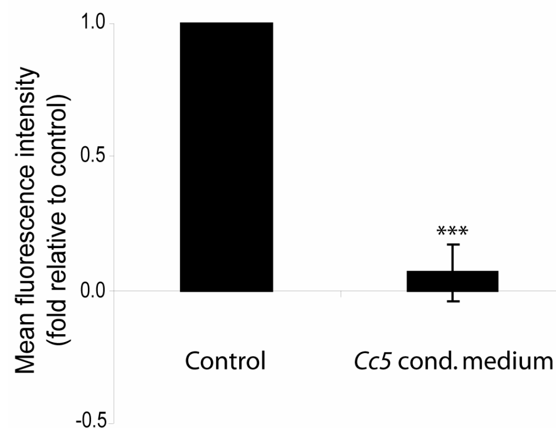


Figure 5. *Cc5* blocks the oxidative burst

The strength of the oxidative burst induced after 30 min infection with *E. coli* at an moi of 50 was determined either in untreated macrophages (control) or in 4 h *Cc5* cond. medium pre-treated macrophages. The oxidative burst was measured by the addition of DHR and the mean fluorescence intensity of the macrophages was determined by FACS analysis. Background fluorescence of uninfected macrophages was subtracted. The values represent means and standard deviations of at least three independent experiments. Statistical significance is shown comparing *Cc5* cond. medium pre-treated macrophages to control macrophages with *** $p < 0.01$ using two-tailed, unpaired Student's *t* test.

In a next step, we wanted to examine how *Cc5* interfered with the NADPH oxidase. Therefore, we intended to analyze the expression and localization of four NADPH oxidase subunits ($p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and $gp91^{phox}$). Unfortunately, determination of NADPH oxidase subunit expression and localization in fresh and 4 h *Cc5* cond. medium pre-treated macrophages before and after infection with *E. coli* at an moi of 50 was not successful, by reasons of experimental limits due to the insufficient quality of commercially available Abs.

Block of the oxidative burst depends on the release of pitrilysin

After we identified that *Cc5* blocked the oxidative burst (Figure 5), we wanted to identify the factor(s) involved in this mechanism.

In a first step, we intended to identify whether protein(s) are essential for interfering with the killing ability of macrophages. Thus, *Cc5* cond. medium was either HI or treated with proteinase K before addition to fresh macrophages for 4 h. Analysis of subsequent *E. coli* killing revealed that neither HI, nor proteinase K-treated *Cc5* cond. medium could prevent the killing of *E. coli* by macrophages

within 2 h of infection. This indicated that protein(s) might be important for blocking the killing ability of macrophages (Figure 6).

Next, we were interested whether the binding or translocation of divalent cations might be required for interference with the killing ability of macrophages. Hence, we supplemented *Cc5* cond. medium with 1 mM EDTA before adding it for 4 h to fresh macrophages. Interestingly, EDTA supplemented *Cc5* cond. medium could not block the killing ability of macrophages (Figure 6), indicating that indeed divalent cations are important for the mechanism underlying the blocking of the macrophage's killing ability.

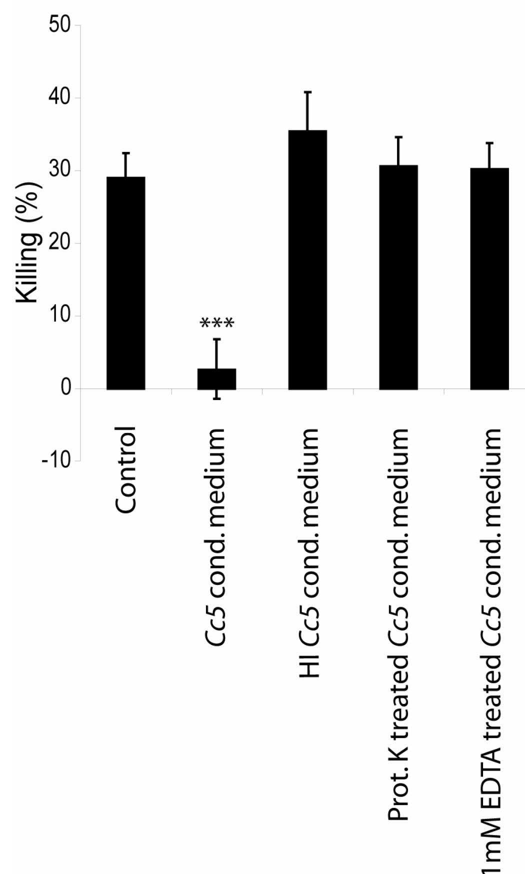


Figure 6. Chelating divalent cations prevents blocking of the killing ability of macrophages
J774.1 macrophages were either left untreated (control) or pre-treated for 4 h as indicated before infection with *E. coli* at an moi of 50. Killing of *E. coli* after 2 h of infection was determined by survival plating. Mean values and standard deviations from at least three independent experiments are shown including statistical significance comparing to killing by untreated macrophages with *** $p < 0.001$ using two-tailed, unpaired Student's *t* test.

As the previous results indicated that protein(s) might be necessary, we wanted to determine the size of the involved protein(s) by applying size exclusion centrifugation steps on *Cc5* cond. medium. As the flowthrough of a 10 kilo Dalton (kDa) cut off filter still blocked the killing ability of macrophages (Figure 7), we applied a second centrifugation step with a 3 kDa cut off filter. Therefore, *Cc5* cond. medium was passed through a 10 kDa filter and the flowthrough was further passed through a 3 kDa filter. The retention sample of the second filtration step still blocked the killing of *E. coli* (Figure 7). These findings indicated that factor(s) involved in blocking the killing ability of macrophages can pass a 10 kDa filter, but are retained by a 3 kDa filter.

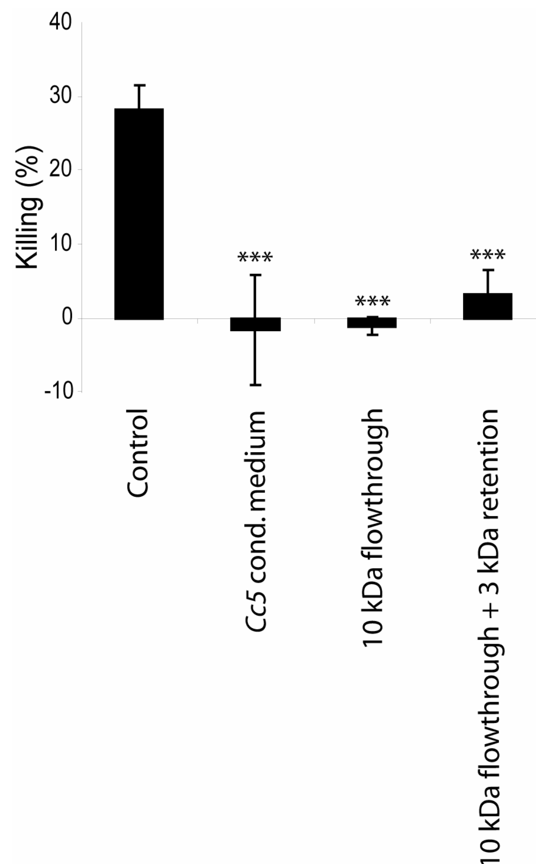


Figure 7. Size exclusion centrifugation of *Cc5* cond. medium

J774.1 macrophages were either left untreated (control) or pre-treated for 4 h as indicated before infection with *E. coli* at an moi of 50. Killing of *E. coli* was determined 2 h after infection by survival plating. Mean values and standard deviations from at least three independent experiments are shown including statistical significance comparing to killing by untreated macrophages with *** $p < 0.001$ using two-tailed, unpaired Student's *t* test.

SDS-PAGE analysis of cond. medium filtered through a 10 kDa filter by S. Ittig revealed that filtration did not prevent proteins between 10-50 kDa from passing the membrane, but only reduced the amount of higher molecular weight proteins (data not shown). Thus, we concluded that at least one factor involved has a molecular size between 3 and 50 kDa.

S. Ittig prepared medium from *Cc5* infected and from untreated HeLa cells and compared them by SDS-PAGE followed by silver staining. He identified a band running at ~54 kDa, which was absent in medium from untreated cells, but appeared in medium of cells infected with *Cc5* (Figure 8). Upon MS analysis (P. Jenö), S. Ittig identified peptides belonging to proteins of 3 ORFs (Table 1) based on the *Cc5* genome provided by P. Manfredi (unpublished data).

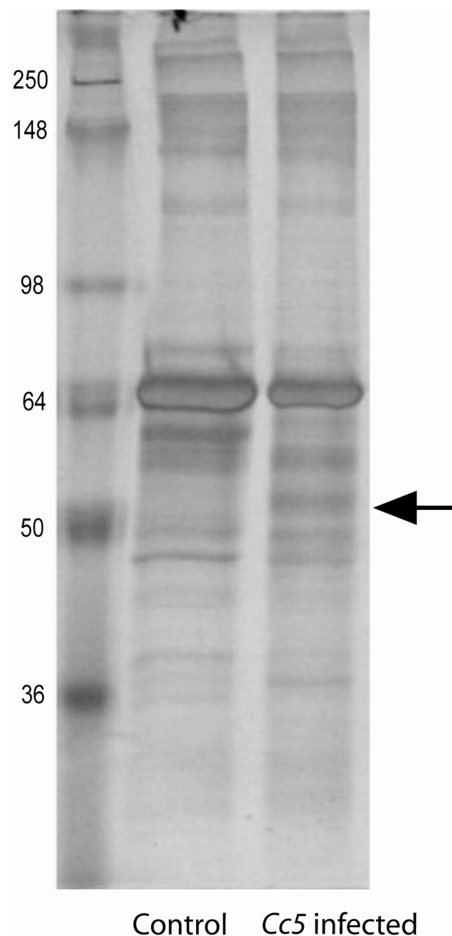


Figure 8. Comparison of uninfected and *Cc5* infected medium

Silver staining of an SDS-PA gel. In the first lane, sDMEM from uninfected HeLa cells was loaded (control). In the second lane, sDMEM from 4 h *Cc5* infected HeLa cells was loaded. The arrow indicates a band appearing after infection with *Cc5*, which was cut for MS analysis.

Predicted ORF	Predicted size	Predicted function	Signal peptide
1890	48 kDa	C-terminal: OmpA/MotB like; N-terminal: thrombospondin type III repeat	Yes
958	50 kDa	Metallopeptidase, Zn-binding motif (HXXEH), insulinase/pitrilysin	Yes
1975	50 kDa	Fumarat hydratase, aspartate-ammonia lyase	No

Table 1. ORFs corresponding to MS data

Based on the previous findings that chelating divalent cations in *Cc5* cond. medium prevented the block of the killing ability in macrophages (Figure 6), we decided to knockout ORF #958 (Δ*pit*) and – as control – ORF #1890 (Δ*1890*). *Cc5* knockouts were generated by S. Ittig. The two knockouts were subsequently tested for their ability to prevent killing of *E. coli* by murine macrophages. Therefore, cond. medium of either mutant was added for 4 h to fresh macrophages and macrophages were then infected for 2 h with *E. coli* at an moi of 50.

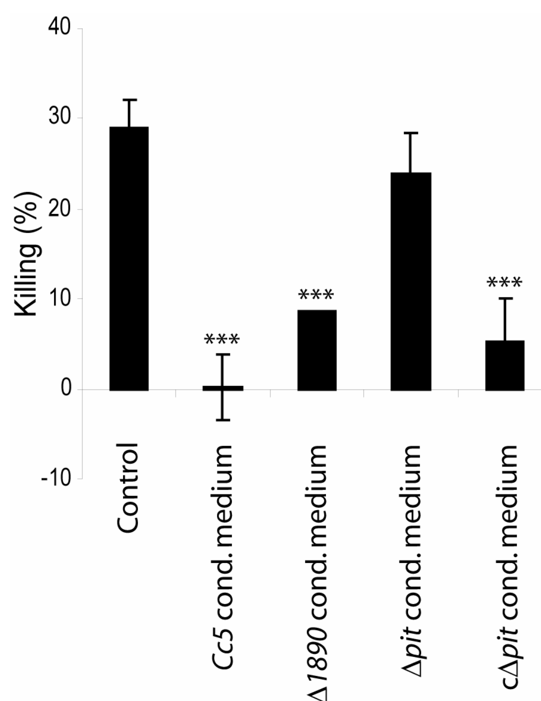


Figure 9. Δ*pit* cond. medium cannot block the killing ability of macrophages

J774.1 macrophages were either left untreated (control) or pre-treated for 4 h as indicated before infection with *E. coli* at an moi of 50 for 2 h. Killing of *E. coli* was determined by survival plating. Mean values and standard deviations from at least three independent experiments are shown including statistical significance comparing to killing by untreated macrophages with *** $p < 0.001$ using two-tailed, unpaired Student's *t* test.

Cond. medium from Δ*1890* bacteria blocked the killing of *E. coli*, whereas cond. medium of Δ*pit* bacteria did not (Figure 9). As complementation of the Δ*pit* mutation by expressing *pitrilysin* from pSI04 *in trans* in Δ*pit* (cΔ*pit*) again prevented the killing of *E. coli*, this indicated that the predicted metallopeptidase pitrilysin might be involved in blocking the killing ability of macrophages (Figure 9).

As the pitrilysin protein seemed to be involved in blocking the killing ability of macrophages, we next determined whether pitrilysin is also important for blocking the oxidative burst. Therefore either fresh (control) or Δpit cond. medium pre-treated J774.1 macrophages were infected for 30 min with *E. coli* at an moi of 50. After addition of DHR, the strength of the oxidative burst was determined by measuring the mean fluorescence intensity of the macrophages, as DHR becomes fluorescent upon oxidation.

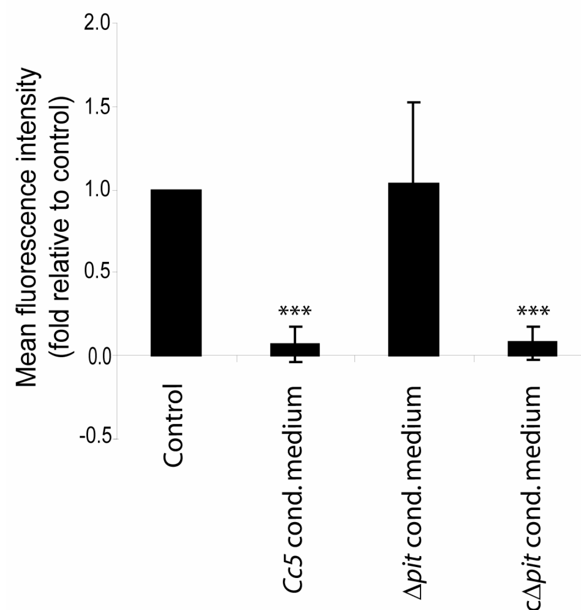


Figure 10. Δ*pit* cond. medium cannot block the oxidative burst

The strength of the oxidative burst induced after 30 min infection with *E. coli* at an moi of 50 was determined either in untreated macrophages (control) or in macrophages pre-treated for 4 h as indicated. The oxidative burst was measured by the addition of DHR and the mean fluorescence intensity of the macrophages was determined by FACS analysis. Background fluorescence of uninfected macrophages was subtracted. The values represent means and standard deviations of at least three independent experiments. Statistical significance is shown in comparison to control macrophages with *** $p < 0.01$ using two-tailed, unpaired Student's *t* test.

While macrophages pre-treated with *Cc5* cond. medium were not able to induce an oxidative burst upon infection with *E. coli*, infection of untreated macrophages induced an oxidative burst and thus induced oxidation of DHR. Pre-treatment of macrophages with Δ*pit* cond. medium did not prevent the oxidative burst induced upon infection with *E. coli*. In contrast, cond. medium from a Δ*pit* mutant expressing *pitrilysin in trans* (cΔ*pit*) again blocked the oxidative burst (Figure 10). These results suggested that *Cc5* specifically prevented the oxidative burst induced upon phagocytosis dependent on the release of pitrilysin into the medium.

3.4. Materials and methods

Bacterial strains and media

Growth of bacteria and preparation of cRPMI (Gibco) was done as described (Meyer *et al.*, 2008). HeLa type Kyoto cells were grown in DMEM (Gibco) supplemented with 2% glutamine (Gibco) and 10% fetal calf serum (FCS) (cDMEM). For infection with bacteria, medium was replaced with DMEM (Gibco) supplemented with 2% glutamine (Gibco) and Hepes (10 mM, Gibco) (sDMEM).

Selective agents

To select for strains, plasmids or transposons, antibiotics were added at the following concentration: 10 μg/ml Cf, 10 μg/ml Em, 20 μg/ml Gm and 10 μg/ml Sm.

Phagocytosis and killing by pre-infected macrophages

The assay was performed as described (Meyer *et al.*, 2008).

Conditioning of bacteria

2 h after seeding J774.1 macrophages (10^5 cells/well in a 24-well plate, Falcon), cells were infected for 24 h with *C. canimorsus* at an moi of 50. The supernatant was thoroughly mixed to detach *C. canimorsus* bacteria and removed from cells. The supernatant was centrifuged (2 min, 13000 rounds per minute (rpm), room temperature (RT)) and the pellet was resuspended in 100 µl PBS. Cond. bacteria were then added to fresh J774.1 cells, which had been seeded 24 h prior (10^5 cells/well in a 24-well plate, Falcon). J774.1 macrophages were incubated for indicated times at 37 °C and 5% CO_2 with the cond. bacteria,

washed once and supplemented with fresh cRPMI medium before infection with Sm-resistant *E. coli* at an moi of 50. The phagocytosis assay was then continued as described (Meyer *et al.*, 2008).

Conditioning of the medium

Cond. medium was generated as described (Meyer *et al.*, 2008).

Manipulation of cond. medium

Cond. medium was generated as described (Meyer *et al.*, 2008) and manipulated as indicated. Heat-inactivation was done for 1 h at 56 °C. Proteinase K (0.1 mg/ml, Roche) was added for 2 h at 37°C. Divalent cations were chelated by incubation of cond. medium for 30 min at 37°C with 1 mM EDTA. Filtration *via* a 10 kDa cut off filter (Amicon Ultra) was done by centrifugation (19 min, 4000 g, RT). The cond. medium filtered through a 10 kDa filter (10 kDa flowthrough) was then loaded on a 3 kDa filter (Amicon Ultra) if indicated and centrifuged again (30 min, 4000 g, RT). The retained medium from the 3 kDa filter was re-diluted to the loading volume with fresh cRPMI and then used for pre-treatment of fresh macrophages.

Isolation of human PMNs

Human PMNs were isolated from healthy volunteers using the dextran-Percoll protocol, adapted with modifications described previously (Jepsen and Skottun, 1982). Contaminating erythrocytes were removed by hypotonic lysis with “aqua ad iniectabilia” (Bichsel).

Phagocytosis and killing by pre-treated PMNs

Freshly isolated human PMNs were resuspended at 10^7 ml^{-1} in D-PBS (Gibco) supplemented with 10% Ab-depleted HI NHS (Scipac Ltd.) and pre-treated for 4 h with *Cc5* cond. medium. After pre-treatment, PMNs were infected with *E. coli* at an moi of 50. As control, untreated PMNs were infected. Samples were analyzed 2 h after infection. To distinguish between phagocytosis and killing, an aliquot was incubated with “aqua ad iniectabilia” (Bichsel) for 1 min in order to lyse the PMNs. Aliquots from untreated and lysed samples were then plated at different dilutions to count the surviving bacteria. Samples from bacteria incubated without cells were plated in parallel as a reference. Counts from lysed samples gave the total

number of surviving bacteria, whereas counts from untreated cell samples gave the numbers of non-phagocytosed, non-adhering, extracellular bacteria. Numbers of phagocytosed and killed bacteria were calculated by subtracting the counts of extracellular or surviving bacteria, respectively, from the counts of the bacteria grown without cells (reference sample).

Localization of *E. coli* in LAMP-1 positive lysosomes

All samples were prepared in triplicates. J774.1 macrophages were seeded in 96 well black-clear TC-treated plates (approx. 2x 10^4 cells/well in 200 μl cRPMI, Costar) and incubated overnight (o/n) at 37 °C and 5% CO_2 in a humidified incubator. 24 h after seeding, the medium was replaced with 200 μl *Cc5* cond. medium if indicated and incubated another 4 h. If indicated, cells were pre-treated with either Cyt. D (10 μg/ml, Sigma) for 20 min or with Bafilomycin (20 nM, Sigma) for 30 min before infection with eGFP expressing *E. coli* at an moi of 10. To synchronize the infection, plates were centrifuged (5 min, 1000 rpm, 37 °C). Plates were incubated for 2 h at 37 °C and 5% CO_2 in a humidified incubator. To stop the infection, cells were washed 2x with pre-warmed PBS and fixed by the addition of 200 μl pre-warmed PFA (3%) for 13 min at 37 °C. After fixation, cells were washed 2x with PBS and permeabilized with 0.1% Saponin supplemented with 2% bovine serum albumin (Saponin/BSA) for 30 min at RT. After permeabilization, cells were incubated 40 min at RT with α-1D4B (α-LAMP-1 Ab, rat monoclonal, 1:200, gift from J. Pieters) diluted in Saponin/BSA. After washing 4x with Saponin/BSA, cells were incubated for 30 min at RT with Cy3-conjugated Affini Pure goat anti-rat IgG (1:100, Jackson Immuno Research, gift from Ch. Dehio) diluted in Saponin/BSA. After washing 3x with PBS, DNA was stained by the addition of Hoechst (1:10000, Sigma) for 30 min at RT. Cells were washed again 3x with PBS and stored at 4 °C in the dark until use. The plates were analyzed using an automated microscope ImageXpressMICRO (Molecular Devices Corporation). Pictures were taken at 12 sites/well using filters for DAPI (10 ms), Texas Red (1500 ms) and GFP (400 ms). Mean inner LAMP-1 fluorescence was recorded at the same position as eGFP signals. Mean outer LAMP-1 fluorescence was recorded within a 5 μm circle around eGFP signals. Data was analyzed using MetaXpress 2.0.1.18. *E. coli* were determined to be

localized within LAMP-1 positive lysosomes if the average of the mean outer fluorescence divided by the mean inner fluorescence was < 0.5.

Localization of *E. coli* in acidified lysosomes (lysotracker staining)

All samples were prepared in triplicates. J774.1 macrophages were seeded in 96 well black-clear TC-treated plates (approx. 2x 10^4 cells/well in 200 µl cRPMI, Costar) and incubated o/n at 37 °C and 5% CO_2 in a humidified incubator. 24 h after seeding, the medium was replaced with 200 µl *Cc5* cond. medium if indicated and incubated another 4 h. Thereafter, the medium was replaced by 200 µl cRPMI supplemented with 60 nM lysotracker red. After 30 min incubation, cells were pre-treated if indicated with either Cyt. D (10 µg/ml, Sigma) for 20 min or with Bafilomycin (20 nM, Sigma) for 30 min before infection with eGFP expressing *E. coli* at an moi of 10. To synchronize the infection, plates were centrifuged (5 min, 1000 rpm, 37 °C). Plates were incubated for 2 h at 37 °C and 5% CO_2 in a humidified incubator. To stop the infection, cells were washed 2x with pre-warmed PBS and fixed by the addition of 200 µl pre-warmed PFA (3%) for 13 min at 37 °C. After fixation, cells were washed 1x with PBS and DNA was stained by the addition of Hoechst (1:10000, Sigma) for 30 min at RT. Cells were washed again 3x with PBS and stored at 4 °C in the dark until use. The plates were analyzed as described for LAMP-1 staining.

Western blot analysis of NADPH oxidase subunits

J774.1 murine macrophages were seeded into 6 well plates (6x 10^5 cells/well in 2 ml cRPMI, Falcon) and incubated for 24 h at 37 °C and 5% CO_2 in a humidified incubator. If needed, cells were pre-treated for 4 h with *Cc5* cond. medium and subsequently infected with *E. coli* at an moi of 50. To synchronize the infection, plates were centrifuged (5 min, 1000 rpm, 37 °C) and then incubated for 30 min at 37 °C and 5% CO_2 in a humidified incubator. To stop the infection, the cells were washed with PBS and the well plate was cooled on ice. After washing the cells with ice-cold PBS, the cells were scraped and transferred into an Eppendorf tube. After centrifugation (10 min, max. speed, 4 °C), the cells were lysed by the addition of 100 µl RIPA buffer supplemented with 1% Triton X-100 and complete protease inhibitors Mini mix (Roche). To allow complete lysis, the cells were incubated for 30 min on ice and mixed repeatedly. To trash the cell debris, the

samples were centrifuged (5 min, max. speed, 4 °C) and the supernatant was transferred into a fresh Eppendorf tube and mixed with 25 μl 5x SDS-PAGE buffer. Samples were boiled 5 min at 99 °C and stored at -20 °C until use. To detect expression of NADPH oxidase subunits, samples were loaded onto 12% SDS-PAGE gels and separated (15 min at 125 V, followed by 75 min at 195 V). Proteins were transferred onto a nitrocellulose membrane (Hybond^{TM}, Amershan Biosciences) by semi-dry Western blotting (90 min, 25 V) and the membrane was blocked o/n at 4 °C with PBS/Tween (0.2% Tween) supplemented with 5% skimmed milk. Next, the blot was washed 4x with PBS/Tween followed by 1 h incubation at RT with the primary Ab diluted in PBS/Tween. After washing 6x with PBS/Tween, the membrane was incubated another h at RT with the corresponding secondary Ab diluted in PBS/Tween. The membranes were again washed 6x with PBS/Tween and developed for 1 min by the addition of ECL (GE Healthcare). Primary Abs used were α-p22^{phox} (rabbit), α-p47^{phox} (goat), α-p67^{phox} (goat) and α-gp91^{phox} (goat) (used at various dilutions ranging from 1:200 to 1:10000, Santa Cruz) and secondary Abs used were swine anti-rabbit Ig/HRP and rabbit anti-goat Ig/HRP (1:5000, DAKO).

Localization of NADPH oxidase subunits by immunofluorescence

J774.1 macrophages were seeded in 8 well chamber slides (10^4 cells/well in 200 μl cRPMI, Falcon) and incubated 24 h at 37 °C and 5% CO_2 in a humidified incubator. If needed, cells were pre-treated for 4 h with *Cc5* cond. medium and subsequently infected with *E. coli* at an moi of 50. To synchronize the infection, plates were centrifuged (5 min, 1000 rpm, 37 °C) and then incubated for 2 h at 37 °C and 5% CO_2 in a humidified incubator. To stop the infection, cells were fixed by the addition of 3% PFA. After fixation, cells were washed 2x with PBS and permeabilized with 0.1% Saponin supplemented with 2% BSA (Saponin/BSA) for 40 min at RT. After permeabilization, cells were incubated 60 min at RT with the primary Ab diluted in Saponin/BSA. After washing 4x with Saponin/BSA, the cells were incubated for 30 min at RT with the corresponding secondary Ab diluted in Saponin/BSA. After washing 3x with PBS, Hoechst was added (1:10000, Sigma) and the cells were incubated for another 20 min at RT. After washing 3x with PBS and 1x with ddH_2O, the chambers were removed and the slide was mounted using VectaShield Hard Cover Mounting Medium (Vector). Slides were analyzed on an

Olympus IX81F-3 microscope mounted with a high speed Yokogawa spinning head and pictures were analyzed using AndorIQ software. Primary Abs used were α-p22^{phox} (rabbit), α-p47^{phox} (goat), α-p67^{phox} (goat) and α-gp91^{phox} (goat) (used at various dilutions ranging from 1:100 to 1:500, Santa Cruz) and secondary Abs used were rabbit anti-goat Ig/FITC (1:200, SBA) and goat anti-rabbit Ig/FITC (1:200, Santa Cruz) respectively.

Measurement of NADPH oxidase activity

NADPH oxidase activity was determined by measuring oxidation of DHR (Molecular Probes), a cell permeant dye which becomes fluorescent and cell impermeant upon oxidation. Therefore, murine macrophage J774.1 cells were seeded in a 24-well plate (10^5 cells/well in 1 ml cRPMI, Falcon) and incubated 24 h at 37 °C and 5% CO_2 in a humidified incubator. If needed, cells were pre-treated for 4 h with cond. medium and subsequently infected with *E. coli* at an moi of 50. To synchronize the infection, plates were centrifuged (5 min, 1000 rpm, 37 °C) and then incubated for 30 min at 37 °C and 5% CO_2 in a humidified incubator. After 30 min of infection, DHR (2 μM) was added for 15 min to the cells. Thereafter, cells were washed 3x with PBS containing 2% FCS. Cells were scraped and FACS analysis was performed determining the mean fluorescence intensity of macrophages. The oxidative burst was determined after subtraction of the background values (uninfected macrophages).

Preparation of culture medium samples for SDS-PAGE

HeLa type Kyoto cells were seeded in a 6-well plate (2x 10^5 cells/well in 2 ml cDMEM, Falcon) and incubated 24 h at 37°C and 5% CO_2 in a humidified incubator. After incubation, cells were washed once with sDMEM and 2 ml sDMEM were added to the cells. HeLa cells were either left uninfected, or infected for 4 h with *Cc5* at an moi of 50. To synchronize the infection, plates were centrifuged (5 min, 1000 rpm, 37 °C). After infection, the supernatant was collected and centrifuged (10 min, 14000 rpm, 4 °C). The supernatant was transferred into a fresh Eppendorf tube and proteins were precipitated o/n at 4 °C by the addition of 10% TCA. After centrifugation (60 min, 14000 rpm, 4 °C), the pellet was resuspended in 300 μl ice-cold acetone and incubated 2 min at RT. After another step of centrifugation (60 min, 14000 rpm, 4 °C), the acetone was

removed and the pellet was air dried. For SDS-PAGE analysis, the pellet was resuspended in 60 μl Tris/HCl (pH 8.0) and 15 μl 5x SDS-PAGE loading dye. Samples were boiled (10 min, 99 °C) and after cooling to RT, 2 μl 0.5 M iodoacetamide in Tris/HCl (pH 8.0) (gift from P. Jenö) were added. 35 μl of each sample were loaded onto 15% SDS-PA gels for analysis.

Silver periodic acid staining

SDS-PA gels were fixed for 10 min in 40% EtOH, 10% acetic acid. After rinsing with water for 10 min, the gels were sensibilized with 0.05% glutaraldehyde, 0.01% formaldehyde and 40% EtOH for 5 min. After rinsing 20 min with 40% EtOH, gels were rinsed for 20 min with water and were sensibilized for 1 min with 0.02% sodium-thiosulfate. After rinsing 2x with water, gels were coloured with 0.1% silver nitrate for 20 min in the dark. After rinsing with water, gels were developed by the addition of 2.5% sodium-carbonate, 0.04% formaldehyde and the colour reaction was stopped by replacing the solution with 5% acetic acid.

Directed gene replacement by allelic exchange

Directed gene replacement by allelic exchange was performed as described (Mally and Cornelis, 2008). The *ermF* resistance cassette was amplified from pEP4351 as template and the upstream and downstream flanking regions of genes *1890* and *pitrilysin* were amplified from 100 ng *Cc5* genomic DNA as template. Genomic DNA from *Cc5* was isolated with the GenElute bacterial genomic DNA kit (Sigma) following the manufacturers instructions. For generating upstream and downstream flanking regions and the *ermF* resistance cassette containing homologous extensions for Δ*1890*, primer pairs 5094+9095, 5096+5097 and 5098+5099 were used. The final PCR product was generated using primers 5094+5099 and was digested with *Pst*I and *Spe*I for cloning into the appropriate sites of *C. canimorsus* suicide vector pMM25 giving pSI03. For generating upstream and downstream flanking regions and the *ermF* resistance cassette containing homologous extensions for Δ*pit*, primer pairs 5100+9101, 5102+5103 and 5104+5105 were used. The final PCR product was generated using primers 5100+5105 and was digested with *Pst*I and *Spe*I for cloning into the appropriate sites of *C. canimorsus* suicide vector pMM25 giving pSI02. The

resulting plasmids were transferred by conjugative transfer into *Cc5* (Mally and Cornelis, 2008).

Electroporation

Electroporation of *C. canimorsus* was done as described (Mally and Cornelis, 2008).

Statistical analysis

For all experiments, means and standard deviations were calculated. Statistical significance was evaluated using two-tailed, unpaired Student's *t* test. Differences were considered to be significant when $p < 0.05$ with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.5. Discussion

We could show that upon growth in the presence of cells, *Cc5* releases soluble factor(s) into the supernatant, which interfere with the oxidative burst in murine macrophages, thereby preventing the killing of phagocytosed bacteria. As only 10 strains have been tested so far for their ability to interfere with the killing ability of macrophages (Meyer *et al*., 2008), the prevalence of this ability should be analyzed on a collection of *C. canimorsus* strains derived not only from human cases, but also isolated from healthy dogs in order to determine the prevalence of this capacity in *C. canimorsus* and the potential relevance for virulence.

Most cell types use their NADPH oxidase to produce ROS for signalling purposes. However, NADPH oxidase of phagocytes plays a crucial role in host defence by producing ROS that are intended to kill invading microbes. As we observed that *Cc5* blocks the oxidative burst in macrophages, we thus tried to investigate how *Cc5* interacts with the NADPH oxidase of murine macrophages. In a resting phagocyte, the NADPH oxidase complex is disassembled, with its components segregated into different parts of the cell. To activate the NADPH oxidase, the following events must occur: (i) Agonists (*e.g.* bacteria) bind their plasma membrane receptor. (ii) Protein kinases – including PKC – phosphorylate activating components, the most critical and extensively phosphorylated being $p47^{phox}$, which unfolds from an autoinhibitory conformation. (iii) Phospholipase A_2 is activated and generates arachidonic acid, which activates NADPH oxidase. (iv) The four cytosolic components comprising the heterotrimer of $p67^{phox}$, $p47^{phox}$

and p40^{phox}, as well as Rac2, translocate to the phagosome or plasma membrane where flavocytochrome b_{558} is located and NADPH binds to the assembled complex. (v) Proton channels are converted to readily open "active" state, perhaps by arachidonic acid or by phosphorylation of PKC, or tyrosine kinase (DeCoursey and Ligeti, 2005; Lee *et al.*, 2003).

Some bacteria like *Francisella tularensis* have been shown to prevent the accumulation of both membraneous and soluble NADPH oxidase components on the phagosomal membrane (McCaffrey and Allen, 2006), and others like *H. pylori* redirect the majority of NADPH oxidase complexes to the plasma membrane, whereby ROS are released into the extracellular milieu (Allen *et al.*, 2005). Interestingly, *N. gonorrhoeae* have been shown to suppress the oxidative burst in PMNs, but the mechanism remains unknown (Criss and Seifert, 2008).

Once the NADPH oxidase is assembled, control over the rate of enzyme activity could be exerted at a number of points: (i) NADPH biosynthesis, (ii), NADPH binding to the reactive site on gp91^{phox}, (iii) $NADP^+$ release, (iv) electron transfer from NADPH to O_2, (v) O_2 binding to its pocket, (vi) O_2^- release and (vii) dismutation of O_2^- to H_2O_2, which might prevent reversed electron flow. It should be noted that NADPH must be regenerated continuously during the respiratory burst, which is believed to occur mainly via the hexose monophosphate shunt in PMNs, but for sure from intracellular glycogen breakdown (DeCoursey and Ligeti, 2005; Lee *et al.*, 2003).

In order to examine how *Cc5* interferes with the oxidative burst in murine macrophages, it is noteworthy that cond. medium needs to be in contact with fresh macrophages for at least 4 h to block the oxidative burst. This could eventually suggest that *Cc5* reduces the expression of single NADPH oxidase subunits, the level of free NADPH in the cells, or that *Cc5* interferes with the signalling cascade. *Cc5* has been shown to feed on aminosugars present on cell surfaces (Mally *et al.*, 2008) and there are indications that *Cc5* deglycosylates TLR4 (S. Ittig, personal communication). These observations might be further indications, that *Cc5* possibly degrades receptors involved in the signalling cascade normally leading to the activation of the NADPH oxidase subunits, thereby preventing the assembly of the NADPH oxidase.

On the other hand, it is also possible that *Cc5* interferes with signalling without preventing the assembly of the NADPH oxidase, but rather redirecting the localization of the NADPH oxidase away from the phagolysosomes towards the outer membrane. Since *Cc5* itself is highly resistant against phagocytosis by murine macrophages, it remains questionable whether release of ROS into the extracellular milieu would indeed be beneficial for the survival of *C. canimorsus*.

By analyzing *Cc5* cond. medium, we could demonstrate that one of the factors involved in preventing the oxidative burst is pitrilysin, a putative metallopeptidase containing an inverted zinc-binding motif.

Zinc-containing metalloproteases are widely distributed from prokaryotes to eukaryotes and bacterial metalloproteases may fall into three families: thermolysin, serralysin and neurotoxin families of which prototype enzymes are produced by *Bacillus thermoproteolytics*, *Serratia marcescens*, or *Clostridium tetani* respectively (Miyoshi and Shinoda, 2000). The majority of these zinc binding enzymes catalyze hydrolysis or closely related transfer reactions. In a protein environment, tetrahedral or 5-coordinated Zn^{2+} is ideally suited to activate coordinated water or hydroxide as a nucleophile for attacking the carbonyl carbon of a peptide bond, the carbon CO_2 or the phosphorus of a phosphate ester (Coleman, 1998).

Pitrilysin from *C. canimorsus* belongs to one of the at least four families of zinc proteases that do not contain the classical HEXXH motif. Besides the so-called pitrilysin family, these include the carboxypeptidase A family, the zinc D-Ala-D-Ala carboxypeptidases, and the aminopeptidases (Coleman, 1998).

The periplasmic zinc oligopeptidase pitrilysin from *E. coli* (previously also called protease III and protease Pi, among others) is the founding member of this family (Roth, 2004; Swamy and Goldberg, 1982). These proteases contain an inverted zinc-metalloprotease core motif (HXXEH) that is typically located within 200 residues of the N-terminal, as well as a pair of glutamate residues that are located 70 and 77 aa distal to the core motif. The conserved residues of this extended metalloprotease motif ($HXXEHX_{69}EX_6E$) are either catalytic or coordinate the enzyme's catalytic zinc ion and are essential for activity (Becker and Roth, 1992).

Pitrilysin from *E. coli* is the only protease of this family whose structure has been determined (PDB ID 1Q2L). This revealed that the enzyme has two nearly

identically sized concave domains that are tethered by a linker region of 20-30 residues (Maskos *et al.*, 2005). *In vitro* studies have demonstrated that pitrilysin from *E. coli* is a non-essential enzyme which cleaves insulin, glucagons, Aβ peptide, β-galactosidase fragments and certain short synthetic peptides (Alper *et al.*, 2006; Cornista *et al.*, 2004; Roth, 2004). However, these substrates have been used as surrogate substrates for pitrilysin since its physiological targets are unknown.

We could show so far that upon growth of *Cc5* in the presence of cells, pitrilysin is released into the supernatant and cond. medium from a ∆pit mutant can no longer block the oxidative burst induced upon infection with *E. coli*, indicating that pitrilysin is involved in blocking the NADPH oxidase. Biochemical characterization of pitrilysin from *Cc5* is needed to understand its properties *in vitro* and purification of active pitrilysin could help identifying its interaction partners thereby giving hints on the mechanism how *Cc5* blocks the oxidative burst.

If pitrilysin can be purified, it might also be possible to analyze the effect of pitrilysin on human PMNs in order to understand whether *Cc5* can also interfere with the oxidative burst in human PMNs. However, it should be noted that in contrast to tissue resident macrophages, PMNs also contain granules filled with AMPs, which contribute to a non-oxidative killing of phagocytosed bacteria. But as *C. canimorsus* encounters rather tissue resident macrophages than PMNs in its natural habitat, it might be of more importance for *C. canimorsus* to interfere with the oxidative burst.

Chapter 4

Interaction of *C. canimorsus* with human complement and PMNs

4.1. Summary

We show here that *Cc5* is resistant to phagocytosis and killing by human and dog PMNs and to killing by human and dog complement. We furthermore demonstrate that complement resistance seems to be due to prevention of MAC deposition even though *Cc5* becomes opsonized by C3b.

Additionally, Ch. Fiechter isolated Y1C12, a *Cc5* Tn mutant that is hypersensitive to killing by complement *via* the Ab-dependent classical pathway. The mutation inactivated a putative *gtf* gene, suggesting that the Y1C12 mutant is affected at the level of a capsular polysaccharide or LPS structure. Indeed, M. Mally and U. Zaehringer could show that *Cc5* appeared to have several polysaccharidic structures, one being altered in Y1C12.

We here provide evidence that increased serum sensitivity of Y1C12 bacteria might be due to an increased deposition of MAC. Besides, we also show that Y1C12 bacteria are more sensitive to phagocytosis and killing by human PMNs than *Cc5* wild-type (wt) bacteria, thereby strengthening the observations that the mutant is affected in the outer membrane structure of the bacteria.

Even if *Cc5* was found to be completely resistant to phagocytosis by human PMNs at an moi of 50, *Cc5* could not prevent the phagocytosis and killing of *Y. enterocolitica* ΔYscN bacteria upon co-infection of human PMNs. This indicated that resistance observed at high moi is not due to a general interference with the phagocytosis ability of the cells.

We demonstrate in addition that *Cc5* induces the formation of NETs upon infection of human PMNs *in vitro* and that *Cc5* is trapped and killed within NETs, indicating sensitivity of *Cc5* towards AMPs present in PMN granules, which become embedded in NETs.

4.2. Publication

Resistance of *Capnocytophaga canimorsus* to killing by human complement and polymorphonuclear leukocytes

Infection and Immunity **77** (2009) 2262-2271

Hwain Shin*, Manuela Mally*, Salome Meyer*, Chantal Fiechter*, Cécile Paroz, Ulrich Zaehringer and Guy R. Cornelis

* Contributed equally to this work

Statement of my work. My contribution was the data of figures: Fig. 1a (*E. coli* and *S. enterica* data), Fig. 1b, Fig. 1d, Fig. 2, Fig. 3a, Fig. 3b (*S. enterica* data), Fig. 3c, Fig. 3d, Fig. 4c and Fig. 5; Phagocytosis and killing of bacteria by human PMNs, analysis of Ab, fH, C4BP, C3b and MAC deposition on bacteria, determination of Abs present in pooled NHS and analysis of serum resistance of *Cc11*, *Cc12*, *E. coli* and *S. enterica*.
Data for Fig. 1d, 3c, 3d, 4c, as well as data concerning fH and C4BP binding was generated in collaboration with C. Paroz.
Data of Fig. 1a (*Cc5* data), Fig. 1c, Fig. 3b (*Cc5*, Y1C12, cY1C12 data), Fig. 4a, Fig. 4b and Table 1 were contributed by H. Shin and Ch. Fiechter. Data of Fig. 6 and Fig. 7 was provided by M. Mally.

INFECTION AND IMMUNITY, June 2009, p. 2262–2271 Vol. 77, No. 6
0019-9567/09/$08.00+0 doi:10.1128/IAI.01324-08
Copyright © 2009, American Society for Microbiology. All Rights Reserved.

Resistance of *Capnocytophaga canimorsus* to Killing by Human Complement and Polymorphonuclear Leukocytes[▽]

Hwain Shin,[1]† Manuela Mally,[1]† Salome Meyer,[1]† Chantal Fiechter,[1]† Cécile Paroz,[1] Ulrich Zaehringer,[2] and Guy R. Cornelis[1]*

Infection Biology, Biozentrum, University of Basel, Klingelbergstrasse 50/70, CH-4056 Basel, Switzerland,[1] and Leibnitz Zentrum für Medizin und Biowissenschaften, Forschungszentrum Borstel, Parkallee 1-40, D-23845 Borstel, Germany[2]

Received 30 October 2008/Returned for modification 15 December 2008/Accepted 12 March 2009

***Capnocytophaga canimorsus* is a bacterium of the canine oral flora known since 1976 to cause rare but severe septicemia and peripheral gangrene in patients that have been in contact with a dog. It was recently shown that these bacteria do not elicit an inflammatory response (H. Shin, M. Mally, M. Kuhn, C. Paroz, and G. R. Cornelis, J. Infect. Dis. 195:375–386, 2007). Here, we analyze their sensitivity to the innate immune system. Bacteria from the archetype strain Cc5 were highly resistant to killing by complement. There was little membrane attack complex (MAC) deposition in spite of C3b deposition. Cc5 bacteria were as resistant to phagocytosis by human polymorphonuclear leukocytes (PMNs) as *Yersinia enterocolitica* MRS40, endowed with an antiphagocytic type III secretion system. We isolated Y1C12, a transposon mutant that is hypersensitive to killing by complement via the antibody-dependent classical pathway. The mutation inactivated a putative glycosyltransferase gene, suggesting that the Y1C12 mutant was affected at the level of a capsular polysaccharide or lipopolysaccharide (LPS) structure. Cc5 appeared to have several polysaccharidic structures, one being altered in Y1C12. The structure missing in Y1C12 could be purified by classical LPS purification procedures and labeled by tritiated palmitate, indicating that it is more likely to be an LPS structure than a capsule. Y1C12 bacteria were also more sensitive to phagocytosis by PMNs than wild-type bacteria. In conclusion, a polysaccharide structure, likely an LPS, protects *C. canimorsus* from deposition of the complement MAC and from efficient phagocytosis by PMNs.**

Since its discovery in 1976, *Capnocytophaga canimorsus* has been regularly isolated from severe human infections transmitted by dogs or cats (3). The genus *Capnocytophaga* belongs to the family *Flavobacteriaceae* and the phylum *Bacteroidetes*. This phylum is taxonomically remote from the *Proteobacteria*, which includes most common human pathogens but includes the family *Bacteroidaceae*, which contains some major commensals of the mammalian intestinal system such as *Bacteroides thetaiotaomicron* and *Bacteroides fragilis* (6). The phylum *Bacteroidetes* includes *Porphyromonas gingivalis*, a common agent of periodontal disease in humans (33), as well as three animal pathogens: *Flavobacterium psychrophilum*, the causative agent of cold-water disease in salmonid fish (7); *Ornithobacterium rhinotracheale*, causing respiratory disease in poultry (29); and *Riemerella anatipestifer*, the agent of "duckling disease" in waterfowl and turkeys (30, 35). The genus *Capnocytophaga* includes a variety of commensal bacteria that can be found in the oral flora of humans and animals. Seven *Capnocytophaga* species are found in humans, while *C. canimorsus* and *Capnocytophaga cynodegmi* are found in dogs and cats (4, 9).

Although *C. canimorsus* has been reported to be present in up to 25.5% of dogs (1, 2, 41), there is only one report in the literature of a dog suffering from a *C. canimorsus* infection, and it was not lethal (24). In contrast, more than 160 cases of severe human infections have now been reported (15, 36), and most cases are not reported any more. The number of cases of human infections is estimated to be around 1 per 1 million inhabitants per year (26). A pet rabbit infection after a dog bite has also been mentioned in the literature (39). Generally, infections result in fulminant septicemia, peripheral gangrene, or meningitis, with a mortality rate as high as 30%. Splenectomy, alcohol abuse, and immunosuppression have been associated with a number of cases, but more than 40% of the patients had no obvious risk factor (19, 36). Severe infections generally do not occur after dramatic bite injuries, which are preventively treated with antibiotics, but rather after apparently benign bites, scratches, or even licks.

In spite of the fact that these infections are now very well known to clinicians throughout the world, very few studies have addressed the molecular mechanisms of *C. canimorsus* pathogenesis. Recently, we showed that *C. canimorsus* cells exhibit robust growth when they are in direct contact with mammalian cells including phagocytes. This property depends on a surface-exposed sialidase allowing *C. canimorsus* to utilize internal amino sugars of glycan chains from host cell glycoproteins. Although sialidase most likely evolved to sustain commensalism, by releasing carbohydrates from mucosal surfaces, it also contributes to bacterial persistence in a murine infection model. Even more, there is evidence that in the mouse, *C. canimorsus* cells feed on polymorphonuclear leukocytes (PMNs) by deglycosylating host glycans (21). This observation implies

* Corresponding author. Mailing address: Biozentrum, Klingelbergstrasse 50/70, CH-4056, Basel, Switzerland. Phone: 41 (0) 61 267 21 21. Fax: 41 (0) 61 267 21 18. E-mail: guy.cornelis@unibas.ch.

† H.S., M.M., S.M., and C.F. contributed equally to this work.

▽ Published ahead of print on 23 March 2009.

that *C. canimorsus* cells escape the primary lines of defense. In agreement with this idea, we also showed that *C. canimorsus* cells are not detected by the usual sentinels of the innate immune system. Macrophages infected with live or heat-killed (HK) bacteria from *C. canimorsus* strain Cc5, a strain isolated from a patient with fatal septicemia (31), do not release tumor necrosis factor, interleukin-1α (IL-1α), IL-6, IL-8, gamma interferon, macrophage inflammatory protein 1b, and nitric oxide. This absence of a proinflammatory response is characterized by the inability of Toll-like receptor 4 to respond to Cc5 (31). Moreover, live, but not HK, Cc5 cells block the release of tumor necrosis factor and nitric oxide induced by HK *Yersinia enterocolitica* cells. In addition, live Cc5 cells downregulate the expression of Toll-like receptor 4 and dephosphorylate p38 mitogen-activated protein kinase (31). *C. canimorsus* cells also resist phagocytosis by a murine macrophage cell line, and some strains, like Cc5, even block the killing of unrelated preys like *Escherichia coli* by macrophages (23).

Following this investigation of the interaction between *C. canimorsus* and the innate immune system, we address here the question of the susceptibility to killing by human complement and PMNs. We show that *C. canimorsus* cells are resistant to complement killing as well as to PMN-mediated phagocytosis and killing. Finally, by isolating and characterizing a highly sensitive mutant, we show that these properties depend on a polysaccharidic structure, likely a lipopolysaccharide (LPS).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The following bacterial strains were used: *C. canimorsus* strains Cc5, Cc11, and Cc12 (ATCC type strain), all isolated from human infections as described previously (31); *E. coli* Top10 (Invitrogen); *Yersinia enterocolitica* MRS40 (34); *Salmonella enterica* serovar Typhimurium strain SL1344 *aroA* (14); and *S. enterica* SL1344 *aroA rfaG* (a gift of D. Bumann). *C. canimorsus* bacteria were routinely grown on heart infusion agar (Difco) supplemented with 5% sheep blood (Oxoid) for 2 days at 37°C in the presence of 5% CO_2. For the genetic screen, Cc5 mutant bacteria were grown in 100 μl heart infusion broth (Difco) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen) in 96-well plates for 48 h without shaking in a 37°C incubator with 5% CO_2. *Y. enterocolitica* cells were grown and type III secretion was induced as described elsewhere previously (5, 18). *S. enterica* cultures were started (optical density at 600 nm [OD_{600}] of 0.2) from a culture grown overnight and were grown for 2 h at 37°C. Selective agents were added at the following concentrations: ampicillin at 100 μg/ml (*E. coli* pCF1 and *E. coli* pCF3), cefoxitin at 10 μg/ml (*C. canimorsus* cY1C12), erythromycin at 10 μg/ml (*C. canimorsus* Y1C12), gentamicin at 20 μg/ml (all *C. canimorsus* strains), kanamycin at 20 μg/ml (*S. enterica rfaG* strain), nalidixic acid at 3.5 μg/ml (*Y. enterocolitica* MRS40), and streptomycin at 10 μg/ml (*E. coli* and wild-type [wt] *S. enterica*).

Antisera. Anti-C3c-fluorescein isothiocyanate (FITC) was obtained from Dako, and anti-human factor H (fH) was obtained from Calbiochem. Anti-C4b-binding protein (C4BP) was a kind gift from Anna Blom (Lund University, Lund, Sweden). Anti-human immunoglobulin G (IgG) (Fc specific)-FITC was obtained from Sigma. Anti-dog IgA-FITC was obtained from Bethyl Laboratories. Mouse anti-human C5B-9 antibody (Ab) was obtained from Dako, and anti-mouse IgG-FITC Ab was obtained from Southern Biotech. Anti-goat IgG-FITC was obtained from Santa Cruz, and anti-rabbit IgG-FITC was obtained from Southern Biotech. Polyclonal serum against Cc5 was generated from rabbits by immunization with HK Cc5 (Laboratoire d'Hormonologie, Marloie, Belgium). Polyclonal serum against *Y. enterocolitica* LPS O:9 was generated from rabbit by immunization with lysate from total *Y. enterocolitica* E40 pYV^- bacteria after 30 min of boiling. The Y1C12-absorbed serum was prepared by incubating an excess amount of Y1C12 mutant bacteria (harvested from blood plates and washed in phosphate-buffered saline [PBS]) with anti-Cc5 serum at 4°C for 4 h. Bacteria were removed by centrifugation and filtration through a 0.2-μm filter.

Detection of human Abs at the bacterial surface. Bacteria from blood agar plates were washed with PBS and adjusted to an OD_{600} of 0.1 in PBS. Five hundred microliters of bacteria (2.5×10^7 CFU) was pelleted by centrifugation (6,000 × *g* for 2 min) and resuspended in 500 μl PBS supplemented with 10% heat-inactivated (HI) normal human serum (NHS). After 30 min of incubation at 37°C, bacteria were washed twice with PBS. Anti-human IgG (Fc specific)-FITC Ab was added to the pellet at a 1:500 dilution and incubated for 15 min at room temperature (RT) in the dark. Bacteria were washed twice with PBS, and the final pellet was resuspended in 500 μl PBS prior to fluorescence-activated cell sorter (FACS) analysis.

Sensitivity to killing by serum. Bacteria were harvested by gently scraping colonies off the agar surface, washed, and resuspended in PBS to an OD_{600} of 0.4. NHS from healthy volunteers was pooled, aliquoted, and stored at −80°C. Animal sera were purchased from Innovative Research. Serum was HI at 55°C for 30 min. A total of 1×10^7 bacteria were incubated in 10% NHS in PBS at 37°C with 5% CO_2. Serial dilutions were plated onto blood plates, and viable colonies were counted after 24 h (*S. enterica* serovar Typhimurium and *E. coli*) or 48 h (*C. canimorsus*) of incubation. When indicated, NHS was incubated with 10 mM $MgCl_2$ and 10 mM EGTA for 10 min at 37°C before usage. Abs were depleted from NHS by incubation with GammaBind Sepharose (GE Healthcare) for 3 h at 4°C.

Detection of C3b, MAC, fH, and C4BP binding by flow cytometry analysis. Bacteria from blood agar plates were washed with PBS and adjusted to an OD_{600} of 1 in PBS. For the detection of C3b, 90 μl of bacterial suspension (4.5×10^7 CFU) was incubated with 10 μl C7-depleted human serum (Sigma) for 30 min at 37°C. After incubation, bacteria were pelleted by centrifugation (6,000 × *g* for 1 min) and washed once with PBS. Anti-C3c-FITC was added to the pellet at a 1:200 dilution and incubated for 30 min at RT in the dark. For detection of MAC, 90 μl of bacterial suspension (4.5×10^7 CFU) was incubated with 1 μl NHS and 9 μl PBS for 15 min at 37°C. After incubation, bacteria were pelleted by centrifugation (6,000 × *g* for 1 min) and washed once with PBS. Anti-C5B-9 Ab was added to the pellet at a 1:200 dilution and incubated at RT for 30 min. After washing with PBS, anti-mouse IgG-FITC-conjugated Ab was added to the pellet at a 1:200 dilution and incubated for 15 min at RT in the dark. For the detection of fH and C4BP, 90 μl of bacteria (4.5×10^7 CFU) was incubated with 10 μl HI NHS for 60 min at 37°C. After incubation, bacteria were pelleted by centrifugation (6,000 × *g* for 1 min) and washed once with PBS. Anti-fH Ab or anti-C4BP Ab was added to the pellet at a 1:200 dilution and incubated for 30 min at RT. After washing with PBS, anti-goat IgG-FITC-conjugated Ab or anti-rabbit IgG-FITC-conjugated Ab was added to the pellet at a 1:200 dilution and incubated for 15 min at RT in the dark. For all detections, bacteria were washed twice in PBS, and the final pellet was resuspended in 1 ml of PBS prior to FACS analysis.

Detection of fH and C4BP deposition by immunoblotting. Bacteria from blood agar plates were washed once with PBS–0.05% Tween and adjusted to an OD_{600} of 14. One hundred microliters of bacterial suspension (2.8×10^8 CFU) was incubated with 100 μl HI NHS at 37°C for 1 h. Bacteria were washed extensively with PBS–0.05% Tween. Bound proteins were eluted using 100 μl 1 M glycine (pH 2.2) for 20 min at RT. Eluted proteins were neutralized and analyzed by immunoblotting.

Immunofluorescence. Falcon culture slides (Becton Dickinson) were coated with poly-D-lysine. Bacteria from blood agar plates were adjusted to an OD_{600} of 1 in PBS. Slides were washed four times with PBS. One hundred microliters of bacterial suspension, corresponding to 5×10^7 CFU, was added to the slides and incubated for 1 h at 37°C. Slides were washed four times with PBS before being fixed with 3% paraformaldehyde for 15 min at RT. Bacteria were labeled with anti-Cc5 or Y1C12-absorbed anti-Cc5 antiserum at the appropriate dilution for 30 min at 4°C. Slides were washed four times in PBS. FITC-conjugated secondary Ab was added, and slides were incubated at 37°C for 20 min. Slides were washed four times with PBS, mounted with antifade reagent (Vector Laboratories), and analyzed by use of a Leica Dmire2 microscope. Pictures were taken with a digital camera (Hamamatsu Photonics) and OpenLab software (version 3.1.2).

Isolation of human PMNs. Human PMNs were isolated from healthy volunteers using the dextran-Percoll protocol, adapted with modifications described previously (16). Contaminating erythrocytes were removed by hypotonic lysis with "aqua ad iniectabilia" (Bichsel).

In vitro phagocytosis and killing assay. Freshly isolated human PMNs were resuspended at 1×10^7 ml^{-1} in D-PBS (Gibco) supplemented with 10% Ab-depleted HI NHS (Scipac Ltd.) and infected with bacteria at the indicated multiplicity of infection (MOI). Bacteria were incubated at 37°C either in the presence of PMNs or without cells as the reference sample. When specified, bacteria were opsonized for 30 min at 37°C either with 10% HI NHS (as a source of Abs and spontaneously formed C3b) or with 10% C7-depleted human serum (as a source of C3b; Sigma) before infection. Samples were analyzed 120 min after infection. To differentiate between phagocytosis and killing, an aliquot was incubated with "aqua ad iniectabilia" (Bichsel) for 1 min in order to lyse the

PMNs. Aliquots from untreated and lysed samples were then plated at different dilutions to count the surviving bacteria. Samples from bacteria incubated without cells were plated in parallel as a reference. Counts from lysed samples gave the total number of surviving bacteria, whereas counts from untreated cell samples gave the numbers of nonphagocytosed, nonadhering, extracellular bacteria. Numbers of phagocytosed and killed bacteria were calculated by subtracting the counts of extracellular or surviving bacteria, respectively, from the counts of the bacteria grown without cells (reference sample). Values are given as percentages of control values.

Tn mutant screen for serum bactericidal assay. Random Tn*4351* mutants were generated as described previously (20).Transposon (Tn) mutant bacteria were grown in 96-well plates (see above) and subsequently diluted in a new plate to an OD_{590} of 0.025. Fifty microliters of bacterial suspension (6.25×10^5 CFU) was incubated with either 10% NHS or HI NHS as a control in a 100-μl total volume. After 3 h of incubation, a microliter-range aliquot was spotted onto blood agar using a metal stamper and incubated for 48 h. The insertion site of the Tn was mapped by arbitrary PCR as described previously (21). The Tn integration site for mutant Y1C12 was confirmed by using primers 5′-GGACATTGT CTCTCTTTCC-3′ and 5′-CGAGCGTCCAGAGGCAATG-3′, complementary to the glycosyltransferase (*gtf*) sequence.

Construction of complementation plasmid. Full-length *gtf* was amplified with primers 5′-GGAATTCTCTATGCCCAATG TGTCGG-3′ and 5′-ATGGATCC CAAAACCCGAAACTCCTG-3′ and inserted into the EcoRI/BamHI sites (underlined) of vector pUC19, resulting in pCF1. Full-length *gtf* was amplified from pCF1 using primers 5′-AATTCCATGGGAAAAGTACTTATAGTAACAC-3′ and 5′-GCTCTAGAAATTTTTTTAAATTAAGTATTCG-3′ inserted into the corresponding sites of *E. coli*-*C. canimorsus* shuttle vector pMM47 (20) using NcoI and XbaI (underlined), leading to the insertion of a glycine at position 2 and a C-terminal six-histidine tag (pCF3). All constructs were sequenced with an ABI sequencer.

Outer membrane proteins were isolated by a sarcosyl extraction method described previously (21).

Preparation of proteinase K-resistant samples. Bacteria were washed with PBS and adjusted to an OD_{600} of 1.5 in PBS. Five hundred microliters of bacterial suspension (3.75×10^8 CFU) was pelleted (20,000 × *g* for 1 min), and bacteria were resuspended in 100 μl loading buffer (1% sodium dodecyl sulfate [SDS], 10% glycerol, 50 mM dithiothreitol, 0.02% bromophenol blue, 45 mM Tris (pH 6.8). Samples were boiled at 99°C for 10 min and then incubated at 37°C overnight after proteinase K (Roche) had been added to a final concentration of 50 μg/ml. After incubation, samples were boiled again for 10 min at 99°C, and proteinase K was added to a final concentration of 100 μg/ml. Samples were then incubated at 55°C for 3 h, boiled again for 5 min at 99°C, and loaded onto a 15% SDS-polyacrylamide gel electrophoresis (PAGE) gel or 16% Tricine-SDS-PAGE gel. Samples were analyzed by immunoblotting or silver-periodic acid staining (37), respectively.

LPS isolation. wt and Y1C12 mutant bacteria were grown on 500 blood agar plates, harvested by scraping, and treated with 0.2% phenol. Bacterial pellets were washed with 1 liter each of ethanol, acetone, and diethyl ether with stirring (RT for 60 min). After centrifugation, cells were dried in air, giving 11.2 g (wt) and 30.8 g (Y1C12) of biomass. The isolation of LPS was achieved after phenol-water extraction (40), whereby the LPS was identified in the phenol phase, which was then extracted by the phenol-chloroform-petroleum ether method (11), giving 70 mg (wt) and 200 mg (Y1C12) LPS, respectively.

In vivo radiolabeling with [^{3}H]palmitate and fluorography. Bacteria were inoculated into a culture of HeLa epithelial cells (ATCC CCL-2) in complete Dulbecco's modified Eagle's medium at 37°C with 5% CO_2 at an MOI of 20. Sixteen hours postinfection, [9,10-^{3}H]palmitic acid (48 Ci/mmol; Perkin-Elmer Life Sciences) was added to a final concentration of 50 μCi/ml, and incubation was continued for 8 h, by which time the bacterial culture had reached approximately 10^8 bacteria/ml, as described elsewhere previously (21). Supernatants of 2 by 1 ml were collected without detaching epithelial cells from the wells. Bacteria were then collected by centrifugation, and pellets were combined from 2 ml and stored at −20°C until processing. Total cell extracts were obtained by solubilizing a pellet for 3 min at 85°C in 60 μl of loading buffer (1% SDS, 10% glycerol, 50 mM dithiothreitol, 0.02% bromophenol blue, 45 mM Tris [pH 6.8]). For LPS analysis, total cell extracts were digested with 50 μg/ml of proteinase K for 2 h at 60°C and heat treated for 5 min at 85°C. Samples of total cell extracts or proteinase K digests (20 μl/lane) were separated by Tricine-SDS-PAGE using 16% polyacrylamide (28). Gels were fixed in 25:65:10 isopropanol:water:acetic acid overnight and subsequently soaked for 30 min in Amplify (Amersham). Gels were vacuum dried and exposed to SuperRX autoradiography film (Fuji) for 18 h at −70°C.

Statistical analyses. For all experiments, means and standard deviations were calculated. For phagocytosis assays and FACS analyses, statistical significance was evaluated using a two-tailed, unpaired Student's *t* test. Differences were considered significant when the *P* value was <0.05 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Nucleotide sequence accession number. The sequence of the Y1C12 locus was deposited in the GenBank database under accession number FJ214098.

RESULTS

***C. canimorsus* cells are resistant to killing by complement.** We first tested the sensitivity of *C. canimorsus* to complement-mediated killing. Cc5 cells were incubated for various periods of time with 10% NHS and plated, and the survival rate was determined by viable counting. For comparison, *E. coli* Top 10 cells were used as serum-sensitive bacteria, and *S. enterica* serovar Typhimurium SL1344 cells were used as serum-resistant bacteria. Within 30 min, 10% NHS reduced the number of live *E. coli* cells by 5 $\log_{10}$ units, but it reduced the number of live Cc5 cells by only 0.1 $\log_{10}$ units and the number of *S. enterica* serovar Typhimurium cells by 0.5 $\log_{10}$ units (Fig. 1A). After 3 h of incubation, Cc5 counts were even higher than in the inoculum, while *E. coli* counts went down by almost 6 $\log_{10}$ units and *S. enterica* serovar Typhimurium counts were reduced by 0.7 $\log_{10}$ units (Fig. 1A). *E. coli* cells survived incubation with HI NHS (data not shown), demonstrating that killing was indeed complement mediated. Two other *C. canimorsus* strains isolated from human infections, Cc11 and Cc12 (ATCC type strain), were then tested and found to be as resistant as Cc5 (Fig. 1B), indicating that complement resistance is a common property of *C. canimorsus* strains.

Sera obtained from different species of mammals are known to possess different levels of bactericidal activity against gram-negative bacteria (13, 22). Since *C. canimorsus* is a dog commensal and since mice were shown to be quite resistant to experimental infection by *C. canimorsus* (21), we tested the sensitivity of Cc5 to normal dog serum and normal mouse serum. As shown in Fig. 1C, it appeared that Cc5 also resists killing by complement from dog and mouse.

We then tested whether this serum resistance was due to the recruitment of negative regulators that are known to interfere with C3b deposition. We first performed a serum absorption experiment in which whole bacteria were incubated with HI NHS, followed by elution of bound serum proteins at a low pH and analysis of eluates by immunoblotting. By this method, some fH, but no C4BP, was found to be recruited at the surface of Cc5, while none was recruited at the surface of *E. coli* (data not shown). We then tried to confirm this observation by direct flow cytometry analysis with an FITC-conjugated secondary Ab directed against anti-fH or anti-C4BP Abs. However, neither fH nor C4BP could be detected at the bacterial surface by this approach (data not shown). This suggests that the fH deposition observed by the first approach might not be sufficient to prevent C3b deposition.

We next analyzed whether there was C3b deposition. Bacteria were incubated for 30 min with C7-depleted NHS, and deposited C3b was detected by flow cytometry with an FITC-conjugated anti-C3c Ab. There was indeed a significant deposition of C3b on Cc5 bacteria as well as on *E. coli* bacteria (Fig. 1D). Hence, the resistance of *C. canimorsus* to complement killing was not the result of a lack of C3b deposition but was

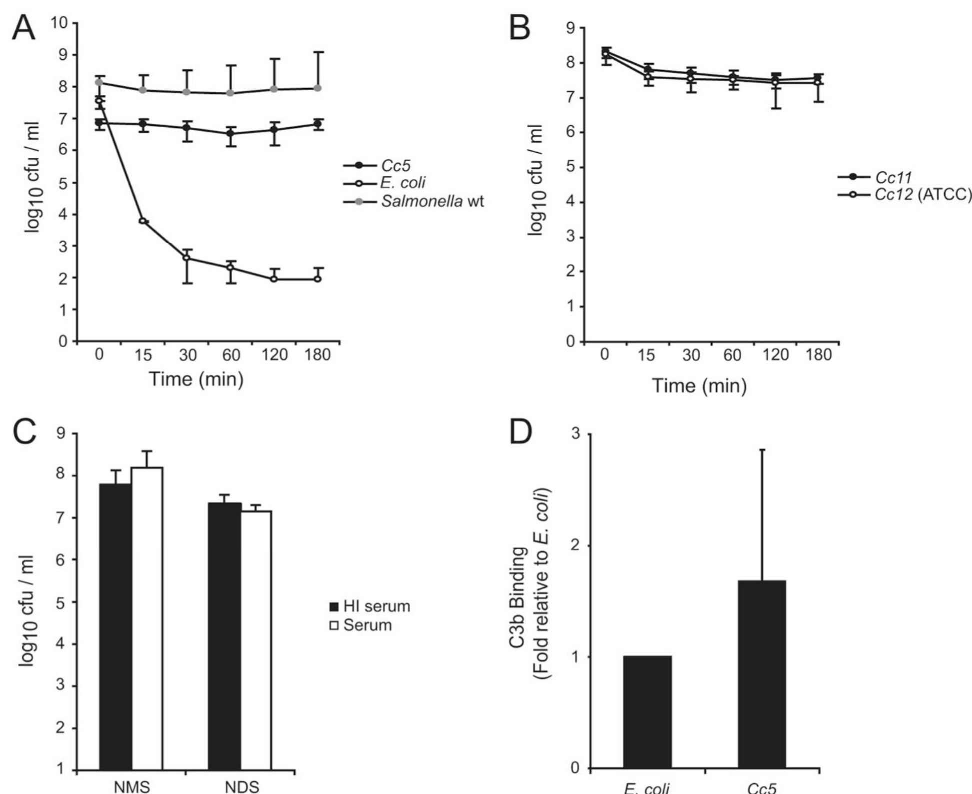


FIG. 1. *C. canimorsus* cells are resistant to complement-mediated killing. (A) Total CFU present after incubation of Cc5 (black circles), *E. coli* (white circles), and wt *S. enterica* serovar Typhimurium (gray circles) in 10% NHS for 0, 15, 30, 60, 120, and 180 min at 37°C. (B) Survival of Cc11 (black circles) and Cc12 (white circles) in 10% NHS for 0, 15, 30, 60, 120, and 180 min at 37°C. (C) Total CFU present after incubation of Cc5 in 10% HI (black) or active (white) normal mouse serum (NMS) or normal dog serum (NDS) for 180 min at 37°C. (D) Binding of C3b by Cc5 or *E. coli* after 30 min incubation with HI NHS. FACS analysis was performed using anti-C3c polyclonal antiserum to quantify the level of C3b on the bacterial surface. The values are the means of three independent experiments ± standard deviations (bars).

likely the result of a lack of MAC insertion. Indeed, very little, if any, C5B-9 was detected at the surface of Cc5 bacteria by flow cytometry (mean fluorescence intensity increased by about 1.3-fold compared to background fluorescence).

Cc5 bacteria resist phagocytosis and killing by PMNs. We next examined the interaction between *C. canimorsus* and human PMNs. We used wt *Y. enterocolitica* as a control for phagocytosis-resistant bacteria (12, 43). In the absence of opsonization and at an MOI of 1, the levels of phagocytosis of Cc5 and wt *Y. enterocolitica* reached about 30% and 40%, respectively. About 20% of Cc5 and 30% of *Y. enterocolitica* bacteria were killed by PMNs (Fig. 2). At an MOI of 50, Cc5 was completely resistant to phagocytosis and killing by PMNs (both <5%), whereas 28% of wt *Y. enterocolitica* cells were phagocytosed, and 17% were killed (data not shown).

Preopsonization with C7-depleted NHS increased the level of phagocytosis and killing of Cc5 and wt *Y. enterocolitica* by about twofold at an MOI of 1. To test the opsonizing effect independent of C3b, we compared the effect of HI-NHS (Abs and spontaneously preformed C3b) to the effect of C7-depleted NHS (activated C3b plus Abs). As shown in Fig. 2, HI NHS increased phagocytosis and killing of Cc5 by 5%, suggesting that there could be anti-*C. canimorsus* Abs in the pool of NHS. We monitored the presence of such Abs by FACS analysis using anti-human IgG-FITC and found that, indeed, the opsonization of Cc5 by HI NHS led to a sixfold increase in the mean fluorescence intensity (data not shown). In comparison, preopsonization of *Y. enterocolitica* with C7-depleted human serum led to ~60% phagocytosis and 55% killing of the bacteria at an MOI of 1. At an MOI of 50, Cc5 preopsonization with C7-depleted NHS led to 20% phagocytosis, while *Y. enterocolitica* was phagocytosed at 60% (data not shown).

In conclusion, Cc5 bacteria were as resistant as or even more resistant to phagocytosis and killing by human PMNs than *Y. enterocolitica* endowed with the anti-phagocytic type III secretion system.

Isolation of a Cc5 mutant that is sensitive to killing by complement and PMNs. By screening a library of Tn*4351* mutants of Cc5, we isolated a clone (designated Y1C12) whose survival rate in NHS was severely decreased compared to that of the wt. As shown in Fig. 3A, 10% NHS reduced the viable counts of Y1C12 by more than 2 $\log_{10}$ units already within 30 min and by almost 5 $\log_{10}$ units after 3 h of incubation. Y1C12 mutant bacteria survived incubation with HI NHS, meaning that the observed serum sensitivity involved complement-dependent killing (Fig. 3B). The Y1C12 mutant was even more sensitive to killing by complement than the *rfaG* mutant of *S. enterica* serovar Typhimurium deficient in O-chain formation of LPS (17). Deposition of C3b onto Y1C12 mutant bacteria was not significantly increased compared to that of the wt (Fig.

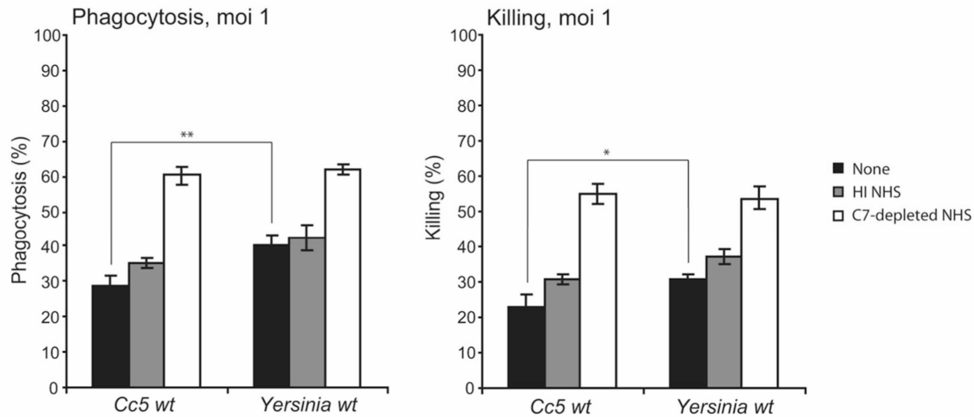


FIG. 2. Cc5 cells are resistant to PMN-mediated killing. Levels of phagocytosis and killing were determined 120 min after infection of human PMNs. PMNs were infected with either Cc5 or wt *Y. enterocolitica* at an MOI of 1. Infection with nonopsonized (black), HI NHS-opsonized (gray), or C7-depleted NHS-opsonized bacteria (white) are shown. Results are the means of at least three independent experiments ± standard deviations (bars). The statistical significance of the difference between Cc5 and wt *Y. enterocolitica* (unopsonized) is given, with * indicating a *P* value of <0.05 and ** indicating a *P* value of <0.01, using a two-tailed unpaired Student's *t* test.

3C), reinforcing our above-described observation that resistance of the wt to complement does not result from a lack of C3b deposition. However, as expected, there was significantly more MAC formed at the surface of Y1C12 bacteria. Already after 15 min of incubation with NHS, there was twofold more MAC inserted onto the mutant bacteria than onto wt Cc5. Since the already-lysed bacteria are not detected by FACS analysis, this difference is probably even underestimated. In

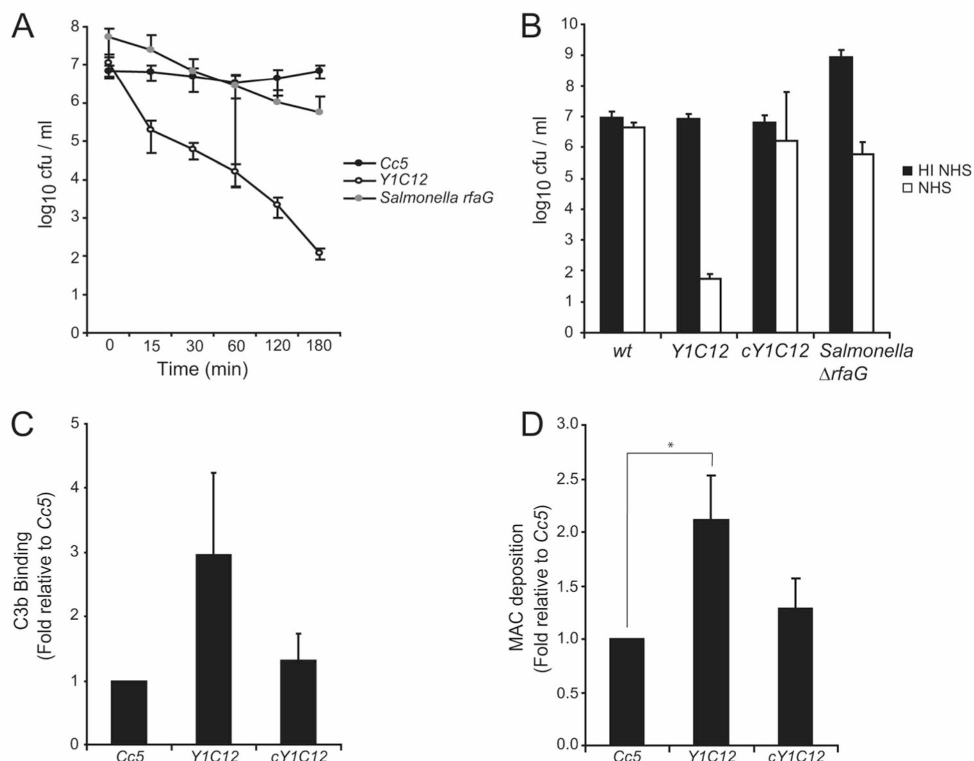


FIG. 3. Characterization of the serum-sensitive Tn mutant of Cc5. (A) Total CFU present after incubation of Cc5 (black circles), Y1C12 (white circles), or an *S. enterica* serovar Typhimurium *rfaG* mutant (gray circles) in 10% NHS for 0, 15, 30, 60, 120, and 180 min at 37°C. (B) Total CFU present after incubation of Cc5, Y1C12, cY1C12, or an *S. enterica* serovar Typhimurium *rfaG* mutant in 10% HI NHS (black) or 10% NHS (white) for 180 min at 37°C. (C) Binding of C3b by Cc5, Y1C12, or cY1C12 after 30 min incubation with C7-depleted NHS. FACS analysis was performed using anti-C3c polyclonal antiserum to quantify the level of C3b on the bacterial surface. (D) MAC deposition on Cc5, Y1C12, or cY1C12 after 15 min of incubation with NHS (longer incubation leads to significant lysis). FACS analysis was performed using mouse anti-C5B-9 primary and anti-mouse-FITC secondary Abs to quantify the level of MAC insertion into the bacterial surface. The statistical significance of the difference in MAC insertion between Y1C12 and wt *C. canimorsus* is given, with * indicating a *P* value of <0.05 using a two-tailed unpaired Student's *t* test. Results are the means of at least three independent experiments ± standard deviations (bars). cY1C12, Y1C12 mutant bacteria complemented with the *gtf* gene in *trans*.

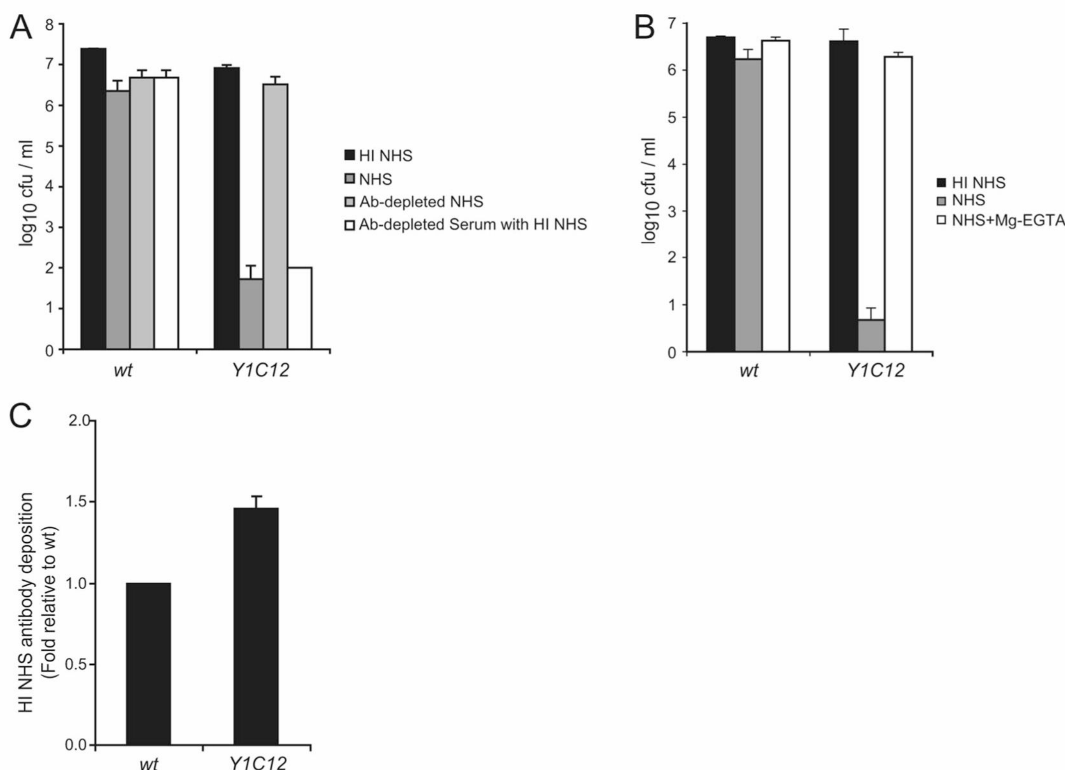


FIG. 4. Killing of Y1C12 occurs via an IgG-dependent classical pathway. (A) Total CFU present after incubation of wt or Y1C12 *C. canimorsus* in 10% HI NHS (black), NHS (dark gray), Ab-depleted NHS (light gray), or Ab-depleted NHS supplemented with HI NHS as a source of Abs (white) for 180 min at 37°C. (B) Total CFU present after incubation of wt or Y1C12 *C. canimorsus* in 10% HI NHS (black), NHS (gray), or Mg-EGTA-treated NHS (white) for 180 min at 37°C. (C) Binding of human serum Abs to wt, Y1C12, or cY1C12 *C. canimorsus* after 30 min incubation with HI NHS. The level of Ab deposition on bacteria was quantified by FACS analysis using anti-human IgG-FITC Ab. Results are the means of at least three independent experiments ± standard deviations (bars).

conclusion, mutant Y1C12 bacteria are killed by complement because they allow more MAC deposition onto their surface (Fig. 3D).

Killing of the serum-sensitive Y1C12 mutant involves the Ab-dependent classical pathway. Since the NHS pool contained Abs recognizing Cc5, and as these Abs favored phagocytosis, we investigated the role of these Abs in the killing of Y1C12 mutant bacteria by complement in NHS. There was no significant difference in the survival rates of wt bacteria in 10% NHS, HI NHS, Ab-depleted NHS, and Ab-depleted NHS supplemented with HI NHS (as a source of Abs) (Fig. 4A). In contrast, Y1C12 mutant bacteria survived in HI NHS and Ab-depleted NHS but were readily killed by both NHS and Ab-depleted NHS supplemented with HI NHS (Fig. 4A). These results thus indicate that the killing of Y1C12 bacteria by complement is dependent on the presence of specific Abs recognizing *C. canimorsus*.

To further dissect which complement pathway is responsible for killing Y1C12 mutant bacteria, NHS was treated with Mg-EGTA, which blocks the classical and the lectin pathways (8). After 180 min of incubation with 10% Mg-EGTA-treated NHS, the number of viable bacteria was reduced only by less than 0.5 $\log_{10}$ units, in comparison to 6 $\log_{10}$ units in untreated NHS (Fig. 4B). Thus, it appears that the killing of Y1C12 bacteria occurs primarily by the Ab-dependent classical pathway. However, as already seen for C3b deposition, the deposition of human serum Abs onto Y1C12 mutant bacteria was increased only by 1.5-fold (Fig. 4C), in good agreement with the hypothesis that the increased sensitivity is due essentially to differences in the sensitivity to MAC deposition.

Serum-sensitive Y1C12 mutant bacteria have an increased sensitivity to phagocytosis by PMNs. At an MOI of 1, the levels of PMN-mediated phagocytosis and killing of nonopsonized Y1C12 reached ~50% and ~40%, respectively (Fig. 5), compared to ~30% and ~20% for wt Cc5. Preopsonization of the Y1C12 mutant bacteria with C7-depleted NHS increased the level of phagocytosis and killing by PMNs by 40%. When Y1C12 mutant bacteria were preopsonized with HI NHS, phagocytosis increased by only ~10%, indicating that Abs present in NHS were responsible for only a slight increase in levels of phagocytosis and killing of Y1C12. In conclusion, Y1C12 mutant bacteria were approximately twice as sensitive to phagocytosis and killing by PMNs as the wt.

The Y1C12 mutant is affected in a glycosyltransferase. The transposon integration site in Y1C12 was found to be located after the first 26 bp of a gene encoding a putative glycosyltransferase (*gtf*). A locus of 11 kb was sequenced, and BLAST search analysis revealed that eight of nine surrounding genes are predicted to be involved in sugar biosynthesis and that one was predicted to be a prokaryote-type ATPase (Fig. 6 and Table 1).

First, we wanted to confirm that the serum sensitivity of the

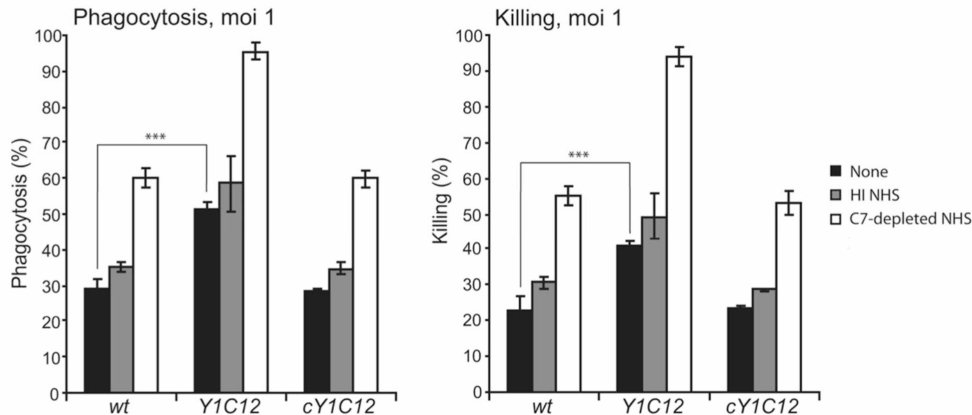


FIG. 5. Tn mutant Y1C12 cells exhibit increased sensitivity to phagocytosis and killing by PMNs. Phagocytosis and killing were determined 120 min after infection of human PMNs. PMNs were infected with wt, Y1C12, or cY1C12 *C. canimorsus* at an MOI of 1. Infections with nonopsonized (black), HI NHS-opsonized (gray), or C7-depleted NHS-opsonized bacteria (white) are shown. Results are the means of at least three independent experiments ± standard deviations (bars), including statistical significance between phagocytosis and killing of the wt and Y1C12 (unopsonized), with *** indicating a *P* value of <0.001 using a two-tailed, unpaired Student's *t* test.

Y1C12 mutant is due to a disruption in the hypothetical *gtf* gene and not due to polar effects of the transposon. The *gtf* gene was therefore cloned into a *C. canimorsus* expression shuttle vector (20), and the resulting plasmid, pCF3, was introduced into Y1C12 in *trans*. The complemented Y1C12 (designated cY1C12) bacteria exhibited a phenotype similar to that of wt bacteria with regard to C3b deposition, resistance to complement, and resistance to PMN-mediated killing (Fig. 3B and C, 4, and 5). Hence, the serum sensitivity of Y1C12 resulted from the disruption of the *gtf* gene.

The Y1C12 mutant is affected in the biosynthesis of a lipopolysaccharidic structure. Glycosyltransferases are enzymes that are involved in the formation of polysaccharide structures such as capsules or O antigens of LPS. To gain better insights into possible alterations of the bacterial surface on Y1C12 bacteria, we first absorbed the Cc5 antiserum with a large excess of intact Y1C12 in order to generate an antiserum recognizing the structure affected by the *gtf* mutation specifically. The efficacy of the serum absorption was controlled by immunofluorescence: while crude anti-Cc5 antiserum recognized wt, Y1C12, and cY1C12 bacteria, the Y1C12-absorbed anti-Cc5 antiserum recognized only wt and cY1C12 bacteria (data not shown). Next, we purified polysaccharide structures by digesting whole bacteria with proteinase K followed by immunoblotting using crude and absorbed anti-Cc5 antiserum (Fig. 7). Immunoblot analysis with anti-Cc5 antiserum of the proteinase K-treated samples from the wt revealed four major bands, labeled A, B, C, and D in Fig. 7A. Bands A, B, and D were present in the proteinase K-treated samples from Y1C12 bacteria, but band C was not, which was replaced by a band migrating faster (C*) (Fig. 7A), suggesting that band C corresponds to the structure altered in Y1C12. In good agreement with this, only band C was visualized in samples from wt and cY1C12 bacteria when the Y1C12-absorbed anti-Cc5 antiserum was used (Fig. 7B). We conclude from this result that a surface structure (band C) is altered in Y1C12 bacteria.

To determine the nature of this structure, we specifically extracted and purified the LPS from wt and Y1C12 bacteria and analyzed it by SDS-PAGE followed by either immunoblotting (Fig. 7A) or periodic acid-silver staining (Fig. 7C). In the periodic acid-silver-stained gel, the LPS preparation from wt bacteria appeared as three bands, with two migrating like bands C and D in the proteinase K-treated samples. In contrast, the LPS preparation from Y1C12 bacteria lacked the C band but exhibited a band migrating like band C*. When analyzed by immunoblotting with complete crude anti-Cc5 serum, the LPS from the wt appeared to contain bands B, C, and D, while the LPS from Y1C12 showed only band D clearly (Fig. 7A). When analyzed by immunoblotting using the Y1C12-absorbed serum, band C appeared in the LPS preparation of wt bacteria but not in the LPS preparation of Y1C12 (Fig. 7B). These results suggested that the polysaccharide structure (band C) altered by the *gtf* mutation is likely to be an LPS, although it does not exhibit the typical ladder pattern by

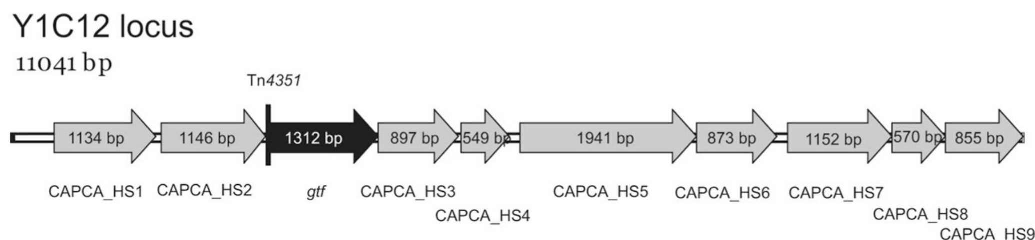


FIG. 6. Y1C12 is affected in a gene encoding a putative glycosyltransferase. Shown is the genetic locus of Y1C12 including its upstream and downstream genes (GenBank accession number FJ214098).

TABLE 1. Sequence analysis of the Y1C12 genomic locus

Locus tag or gene product	Best-hit BLAST accession no., description (species)[a]	Score	E value	InterProScan accession no. (description)[b]
CAPCA_HS1	YP_001296180.1, FnlB protein involved in UDP-l-FucpNAc biosynthesis (a nucleotide sugar precursor for antigen-O biosynthesis) (*Flavobacterium psychrophilum* JIP02/86)	554	4e−156	IPR000888 (dTDP-4-dehydrorhamnose 3,5-epimerase related), IPR016040 [NAD(P) binding]
CAPCA_HS2	YP_001296179.1, FnlC protein involved in UDP-l-FucpNAc biosynthesis (a nucleotide sugar precursor for antigen-O biosynthesis), UDP-2-acetamino-2,6-dideoxy-l-talose 2-epimerase (*Flavobacterium psychrophilum* JIP02/86)	707	0.0	IPR003331 (UDP-*N*-acetylglucosamine 2-epimerase)
Gtf	ZP_03015063.1, hypothetical protein BACINT_02652 (*Bacteroides intestinalis* DSM 17393)	441	7e−122	IPR001296 (glycosyltransferase, group 1)
CAPCA_HS3	YP_001192692.1, NAD-dependent epimerase/dehydratase (*Flavobacterium johnsoniae* UW101)	315	4e−84	IPR001509 (NAD-dependent epimerase/dehydratase), IPR016040 [NAD(P) binding]
CAPCA_HS4	ZP_01043213.1, sugar transferase (*Idiomarina baltica* OS145)	265	1e−69	IPR003362 (bacterial sugar transferase)
CAPCA_HS5	YP_862017.1, capsular polysaccharide biosynthesis protein (*Gramella forsetii* KT0803)	623	2e−176	IPR003869 (polysaccharide biosynthesis protein CapD), IPR016040 [NAD(P) binding]
CAPCA_HS6	YP_001302048.1, putative glucose-1-phosphate thymidyltransferase (*Parabacteroides distasonis* ATCC 8503)	508	1e−142	IPR005907 (glucose-1-phosphate thymidylyltransferase, long form), IPR005835 (nucleotidyl transferase)
CAPCA_HS7	ZP_02034053.1, hypothetical protein PARMER_04094 (*Parabacteroides merdae* ATCC 43184)	384	6e−105	IPR011579 (prokaryotic ATPase)
CAPCA_HS8	NP_810251.1, dTDP-4-dehydrorhamnose-3,5-epimerase (*Bacteroides thetaiotaomicron* VPI-5482)	281	2e−74	IPR000888 (dTDP-4-dehydrorhamnose-3,5-epimerase related), IPR014710 (RmlC-like jelly roll fold)
CAPCA_HS9	YP_001299685.1, dTDP-4-dehydrorhamnose reductase (*Bacteroides vulgatus* ATCC 8482)	335	2e−90	IPR005913 (dTDP-4-dehydrorhamnose reductase), IPR016040 [NAD(P) binding]

[a] Nonredundant database as of August 2008.
[b] InterPro accession number and description as of August 2008.

electrophoresis. In contrast, structure B, which does migrate as a ladder, is not present in the LPS preparation and, hence, probably does not represent an LPS structure.

To check whether this structure has a lipid anchor, we incorporated [9,10-^{3}H]palmitic acid into wt or Y1C12 bacteria. To do this, we cultured bacteria in the presence of HeLa epithelial cells because this is the best way to obtain robust growth in a synthetic medium (21). Labeled bacteria were treated with proteinase K and analyzed by Tricine-SDS-PAGE followed by autoradiography (Fig. 7D). A band migrating like band C appeared in wt Cc5 bacteria (Fig. 7A) but not in Y1C12 mutant bacteria, where a band migrating more slowly appeared (C*). These data suggest that the structure altered in Y1C12 has a lipid anchor, reinforcing the idea that it represents a form of LPS. Structure B did not appear to be palmitoylated and, hence, probably does not correspond to LPS in spite of its migration profile. It could represent a capsule. Structure D was also lipidated, suggesting that Cc5 could have two different LPS structures (structures C and D) besides other polysaccharidic structures (structures A and B).

DISCUSSION

In an effort to understand the pathogenesis of human *C. canimorsus* infections, we investigated here the sensitivity of *C. canimorsus* to killing by complement and phagocytosis by human PMNs. The archetypal *C. canimorsus* strain Cc5 and two other strains isolated from human infections turned out to be highly resistant to killing by the human, dog, and mouse complement systems. C3b deposition was not prevented, but nevertheless, there was very little MAC assembly. This suggested that some surface structure prevents MAC assembly. This hypothesis was reinforced by the fact that a mutant that was hypersensitive to complement killing turned out to undergo significantly more MAC deposition than the wt. The mutation turned out to affect a glycosyltransferase, hinting that a polysaccharide structure is responsible for complement resistance. In good agreement with this prediction, the mutant turned out to be affected in the glycosidic moiety of a lipidated structure. However, this analysis revealed the presence of more than one polysaccharide structure. Since there was no obvious change in the patterns of outer membrane proteins themselves (data not shown), we thus conclude that a polysaccharide structure,

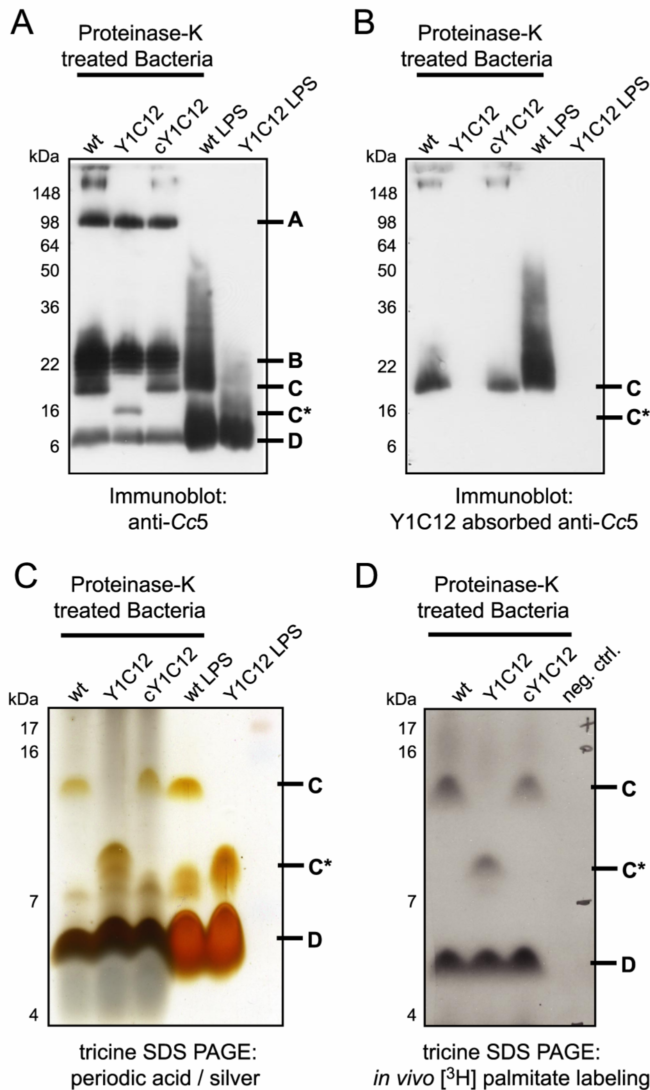


FIG. 7. Y1C12 has an altered lipidated polysaccharide structure. (A) Immunoblotting analysis of proteinase K-treated Cc5, Y1C12, and cY1C12 bacteria and of LPS isolated from Cc5 and Y1C12 using anti-Cc5. (B) Immunoblotting analysis as described above (A) using Y1C12-absorbed anti-Cc5 antiserum. (C) Silver-periodic acid staining of Tricine gel electrophoresis of the same samples as above (A and B). (D) Proteinase K-resistant structures of wt Cc5, Y1C12, and cY1C12 bacteria that had been labeled in vivo using [9,10-^{3}H]palmitic acid.

likely LPS, prevents the formation or insertion of the MAC and makes *C. canimorsus* resistant to complement killing. Although the critical structure is lipidated, one cannot formally exclude that it could be a capsule rather than the LPS. Indeed, *Neisseria meningitidis* polysialic acid capsules have been shown to have a lipid anchor (38). More work is thus required to characterize the surface structure of *C. canimorsus* that is responsible for complement resistance. Polysaccharide surface structures are well known for protecting gram-negative bacteria, including the closely related *P. gingivalis* (32), from complement killing, either by steric hindrance of the MAC or by binding fH, eventually through decoration with sialic acid residues (10, 27). According to our data, the first mechanism is the most likely here, but the presence of sialic acid on a surface structure cannot be definitely excluded so far. We detected some fH binding on wt Cc5, but the hypersensitive mutant could still bind fH, reinforcing the idea that fH binding is not key to complement resistance. The hypersensitive mutant was found to be killed by the classical or lectin pathway rather than by the alternative pathway, and the pools of NHS used as a complement source were found to contain Abs recognizing Cc5. This observation is not surprising since pools of human serum have been reported to contain Abs recognizing *Capnocytophaga* sp. belonging to normal human oral flora (42), and we confirmed that the pools of HI NHS used in this study contained Abs recognizing *Capnocytophaga ochracea*, *Capnocytophaga gingivalis*, and *C. cynodegmi* (data not shown).

C. canimorsus also turned out to be quite resistant to phagocytosis and killing by human PMNs. The complement-sensitive *gtf* mutant bacteria were more sensitive to phagocytosis and killing by human PMNs than wt Cc5 bacteria, indicating that the same polysaccharide structure protects from complement killing and from phagocytosis. The resistance to phagocytosis was more pronounced at a high MOI, and this suggests that a factor other than the polysaccharide might contribute to this resistance. In conclusion, by virtue of a particular polysaccharidic structure, *C. canimorsus* can resist the assaults from the innate immune system. This resistance, combined with the low proinflammatory and even immunosuppressive properties described previously (31) as well as a capacity to feed in vivo from host glycans (21), explains easily that *C. canimorsus* may cause extremely severe infections. However, in view of these virulence properties and the high prevalence of *C. canimorsus* in dogs, one must question why human infections are so rare. One possibility would be that the *C. canimorsus* strains colonizing most dogs would not be endowed with all these virulence functions or would have only some of them. Dog strains are presently being collected and analyzed to address this hypothesis. Besides this, a thorough immunological investigation of patients who survived a *C. canimorsus* infection would be of the utmost interest but is difficult to organize given the low prevalence of infections. These two approaches may shed some light on this problem, but one should keep in mind that the same haunting question applies to many pathogens causing severe diseases, like *Neisseria meningitidis*, to cite only one (25).

ACKNOWLEDGMENTS

We thank Regine Landmann and Naja Jann for their help and advice concerning the isolation and handling of PMNs and Anna Blom for the gift of the anti-C4BP serum.

This work was supported by the Swiss National Science Foundation (grant 32-65393.01). We declare that no competing interests exist.

REFERENCES

1. **Bailie, W. E., E. C. Stowe, and A. M. Schmitt.** 1978. Aerobic bacterial flora of oral and nasal fluids of canines with reference to bacteria associated with bites. J. Clin. Microbiol. **7:**223–231.
2. **Blanche, P., E. Bloch, and D. Sicard.** 1998. *Capnocytophaga canimorsus* in the oral flora of dogs and cats. J. Infect. **36:**134.
3. **Bobo, R. A., and E. J. Newton.** 1976. A previously undescribed gram-negative bacillus causing septicemia and meningitis. Am. J. Clin. Pathol. **65:**564–569.
4. **Brenner, D. J., D. G. Hollis, G. R. Fanning, and R. E. Weaver.** 1989. *Capnocytophaga canimorsus* sp. nov. (formerly CDC group DF-2), a cause of septicemia following dog bite, and *C. cynodegmi* sp. nov., a cause of localized wound infection following dog bite. J. Clin. Microbiol. **27:**231–235.
5. **Cornelis, G., J. C. Vanootegem, and C. Sluiters.** 1987. Transcription of the yop regulon from *Y. enterocolitica* requires trans acting pYV and chromosomal genes. Microb. Pathog. **2:**367–379.

6. **Coyne, M. J., and L. E. Comstock.** 2008. Niche-specific features of the intestinal *Bacteroidales*. J. Bacteriol. **190:**736–742.
7. **Duchaud, E., M. Boussaha, V. Loux, J. F. Bernardet, C. Michel, B. Kerouault, S. Mondot, P. Nicolas, R. Bossy, C. Caron, P. Bessieres, J. F. Gibrat, S. Claverol, F. Dumetz, M. Le Henaff, and A. Benmansour.** 2007. Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. Nat. Biotechnol. **25:**763–769.
8. **Fine, D. P., S. R. Marney, Jr., D. G. Colley, J. S. Sergent, and R. M. Des Prez.** 1972. C3 shunt activation in human serum chelated with EGTA. J. Immunol. **109:**807–809.
9. **Frandsen, E. V., K. Poulsen, E. Kononen, and M. Kilian.** 2008. Diversity of *Capnocytophaga* species in children and description of *Capnocytophaga leadbetteri* sp. nov. and *Capnocytophaga* genospecies AHN8471. Int. J. Syst. Evol. Microbiol. **58:**324–336.
10. **Frank, M. M., K. Joiner, and C. Hammer.** 1987. The function of antibody and complement in the lysis of bacteria. Rev. Infect. Dis. **9**(Suppl. 5)**:**S537–S545.
11. **Galanos, C., O. Luderitz, and O. Westphal.** 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. **9:**245–249.
12. **Grosdent, N., I. Maridonneau-Parini, M. P. Sory, and G. R. Cornelis.** 2002. Role of Yops and adhesins in resistance of *Yersinia enterocolitica* to phagocytosis. Infect. Immun. **70:**4165–4176.
13. **Hanski, C., M. Naumann, A. Grutzkau, G. Pluschke, B. Friedrich, H. Hahn, and E. O. Riecken.** 1991. Humoral and cellular defense against intestinal murine infection with *Yersinia enterocolitica*. Infect. Immun. **59:**1106–1111.
14. **Hoiseth, S. K., and B. A. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238–239.
15. **Janda, J. M., M. H. Graves, D. Lindquist, and W. S. Probert.** 2006. Diagnosing *Capnocytophaga canimorsus* infections. Emerg. Infect. Dis. **12:**340–342.
16. **Jepsen, L. V., and T. Skottun.** 1982. A rapid one-step method for the isolation of human granulocytes from whole blood. Scand. J. Clin. Lab. Investig. **42:**235–238.
17. **Kadam, S. K., A. Rehemtulla, and K. E. Sanderson.** 1985. Cloning of *rfaG*, *B*, *I*, and *J* genes for glycosyltransferase enzymes for synthesis of the lipopolysaccharide core of *Salmonella typhimurium*. J. Bacteriol. **161:**277–284.
18. **Letzelter, M., I. Sorg, L. J. Mota, S. Meyer, J. Stalder, M. Feldman, M. Kuhn, I. Callebaut, and G. R. Cornelis.** 2006. The discovery of SycO highlights a new function for type III secretion effector chaperones. EMBO J. **25:**3223–3233.
19. **Lion, C., F. Escande, and J. C. Burdin.** 1996. *Capnocytophaga canimorsus* infections in human: review of the literature and cases report. Eur. J. Epidemiol. **12:**521–533.
20. **Mally, M., and G. R. Cornelis.** 2008. Genetic tools for studying *Capnocytophaga canimorsus*. Appl. Environ. Microbiol. **74:**6369–6377.
21. **Mally, M., H. Shin, C. Paroz, R. Landmann, and G. R. Cornelis.** 2008. *Capnocytophaga canimorsus*: a human pathogen feeding at the surface of epithelial cells and phagocytes. PLoS Pathog. **4:**e1000164. doi:10.1371/journal.ppat.1000164.
22. **Marcus, S., D. W. Esplin, and D. M. Donaldson.** 1954. Lack of bactericidal effect of mouse serum on a number of common microorganisms. Science **119:**877.
23. **Meyer, S., H. Shin, and G. R. Cornelis.** 2008. *Capnocytophaga canimorsus* resists phagocytosis by macrophages and pages the ability of macrophages to kill other bacteria. Immunobiology **213:**805–814.
24. **Meyers, B., J. P. Schoeman, A. Goddard, and J. Picard.** 2008. The bacteriology and antimicrobial susceptibility of infected and non-infected dog bite wounds: fifty cases. Vet. Microbiol. **127:**360–368.
25. **Parkhill, J., M. Achtman, K. D. James, S. D. Bentley, C. Churcher, S. R. Klee, G. Morelli, D. Basham, D. Brown, T. Chillingworth, R. M. Davies, P. Davis, K. Devlin, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, S. Leather, S. Moule, K. Mungall, M. A. Quail, M. A. Rajandream, K. M. Rutherford, M. Simmonds, J. Skelton, S. Whitehead, B. G. Spratt, and B. G. Barrell.** 2000. Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. Nature **404:**502–506.
26. **Pers, C., B. Gahrn-Hansen, and W. Frederiksen.** 1996. *Capnocytophaga canimorsus* septicemia in Denmark, 1982–1995: review of 39 cases. Clin. Infect. Dis. **23:**71–75.
27. **Rautemaa, R., and S. Meri.** 1999. Complement-resistance mechanisms of bacteria. Microbes Infect. **1:**785–794.
28. **Schagger, H.** 2006. Tricine-SDS-PAGE. Nat. Protoc. **1:**16–22.
29. **Schuijffel, D. F., P. C. van Empel, A. M. Pennings, J. P. van Putten, and P. J. Nuijten.** 2005. Successful selection of cross-protective vaccine candidates for *Ornithobacterium rhinotracheale* infection. Infect. Immun. **73:**6812–6821.
30. **Segers, P., W. Mannheim, M. Vancanneyt, K. De Brandt, K. H. Hinz, K. Kersters, and P. Vandamme.** 1993. *Riemerella anatipestifer* gen. nov., comb. nov., the causative agent of septicemia anserum exsudativa, and its phylogenetic affiliation within the *Flavobacterium-Cytophaga* rRNA homology group. Int. J. Syst. Bacteriol. **43:**768–776.
31. **Shin, H., M. Mally, M. Kuhn, C. Paroz, and G. R. Cornelis.** 2007. Escape from immune surveillance by *Capnocytophaga canimorsus*. J. Infect. Dis. **195:**375–386.
32. **Slaney, J. M., A. Gallagher, J. Aduse-Opoku, K. Pell, and M. A. Curtis.** 2006. Mechanisms of resistance of *Porphyromonas gingivalis* to killing by serum complement. Infect. Immun. **74:**5352–5361.
33. **Socransky, S. S., and A. D. Haffajee.** 2005. Periodontal microbial ecology. Periodontol. 2000 **38:**135–187.
34. **Sory, M. P., and G. R. Cornelis.** 1994. Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. Mol. Microbiol. **14:**583–594.
35. **Subramaniam, S., B. Huang, H. Loh, J. Kwang, H. M. Tan, K. L. Chua, and J. Frey.** 2000. Characterization of a predominant immunogenic outer membrane protein of *Riemerella anatipestifer*. Clin. Diagn. Lab. Immunol. **7:**168–174.
36. **Tierney, D. M., L. P. Strauss, and J. L. Sanchez.** 2006. *Capnocytophaga canimorsus* mycotic abdominal aortic aneurysm: why the mailman is afraid of dogs. J. Clin. Microbiol. **44:**649–651.
37. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
38. **Tzeng, Y. L., A. K. Datta, C. A. Strole, M. A. Lobritz, R. W. Carlson, and D. S. Stephens.** 2005. Translocation and surface expression of lipidated serogroup B capsular polysaccharide in *Neisseria meningitidis*. Infect. Immun. **73:**1491–1505.
39. **van Duijkeren, E., C. van Mourik, M. Broekhuizen, M. Leuven, W. Gaastra, and D. Houwers.** 2006. First documented *Capnocytophaga canimorsus* infection in a species other than humans. Vet. Microbiol. **118:**148–150.
40. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5:**83–91.
41. **Westwell, A. J., K. Kerr, M. B. Spencer, and D. N. Hutchinson.** 1989. DF-2 infection. BMJ **298:**116–117.
42. **Wilson, M. E., R. Burstein, J. T. Jonak-Urbanczyk, and R. J. Genco.** 1985. Sensitivity of *Capnocytophaga* species to bactericidal properties of human serum. Infect. Immun. **50:**123–129.
43. **Woestyn, S., A. Allaoui, P. Wattiau, and G. R. Cornelis.** 1994. YscN, the putative energizer of the *Yersinia* Yop secretion machinery. J. Bacteriol. **176:**1561–1569.

Editor: J. B. Bliska

4.3. Additional results

Cc5 does not interfere with phagocytosis of *Y. enterocolitica* ΔYscN bacteria upon co-infection

As we have observed that *Cc5* completely resists phagocytosis at high moi (moi of 50), but is phagocytosed up to ~40% at low moi (moi of 1) (Meyer *et al.*, 2008; Shin *et al.*, 2009), we intended to determine, whether *Cc5* also interferes with phagocytosis of other bacteria upon co-infection of human PMNs. We thus co-infected freshly isolated human PMNs with *Y. enterocolitica* ΔYscN, a phagocytosis-sensitive strain due to inactivation of its T3SS (Meyer *et al.*, 2008; Woestyn *et al.*, 1994), at an moi of 1 and either *Cc5* (moi of 50) or – as a control – *E. coli* (moi of 50). As rates of phagocytosis and killing of bacteria can depend on the moi, we additionally infected PMNs with *Y. enterocolitica* ΔYscN bacteria at an moi of 50.

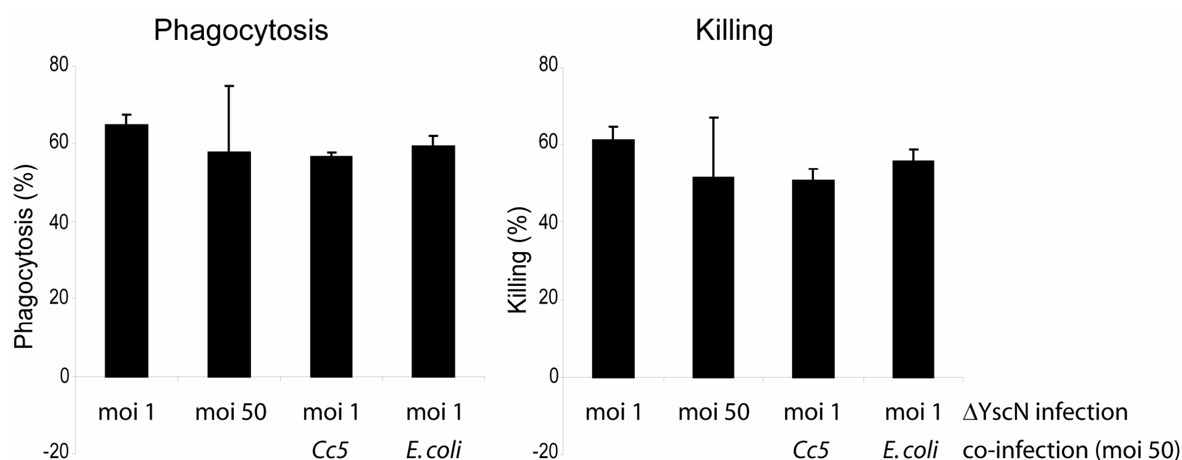


Figure 1. *Cc5* does not interfere with phagocytosis of ΔYscN bacteria upon co-infection
Freshly isolated human PMNs were infected with *Y. enterocolitica* ΔYscN bacteria at either an moi of 1 or an moi of 50. In addition, PMNs were co-infected with either *Cc5* or *E. coli* at an moi of 50 if indicated. Phagocytosis and killing of *Y. enterocolitica* ΔYscN was determined by survival plating 2 h after infection. Means and standard deviations of three independent experiments are shown.

Upon 2 h of infection at an moi of 1, ~65 % *Y. enterocolitica* ΔYscN bacteria were phagocytosed and ~60% were killed by human PMNs. At an moi of 50, rates of phagocytosis and killing were with ~60% and ~50%, respectively, slightly lower but not significantly different. Phagocytosis and killing of ΔYscN bacteria was

neither significantly affected if PMNs were co-infected with *Cc5* at an moi of 50, nor if PMNs were co-infected with *E. coli* at an moi of 50 (Figure 1). This indicated that *Cc5* can prevent its own phagocytosis at high moi (Meyer *et al.*, 2008; Shin *et al.*, 2009), but not phagocytosis of other bacteria.

Cc5 resists phagocytosis and killing by dog PMNs

We have shown previously, that *Cc5* is even more resistant to phagocytosis and killing by human PMNs than *Y. enterocolitica*, and that phagocytosis resistance is increased at increasing moi (Shin *et al.*, 2009). We were therefore interested, whether *Cc5* is also resistant to phagocytosis and killing by dog PMNs. Thus, we infected freshly isolated dog PMNs at low and high moi (1 and 50, respectively) and determined phagocytosis and killing of *Cc5* within 2 h of infection by survival plating.

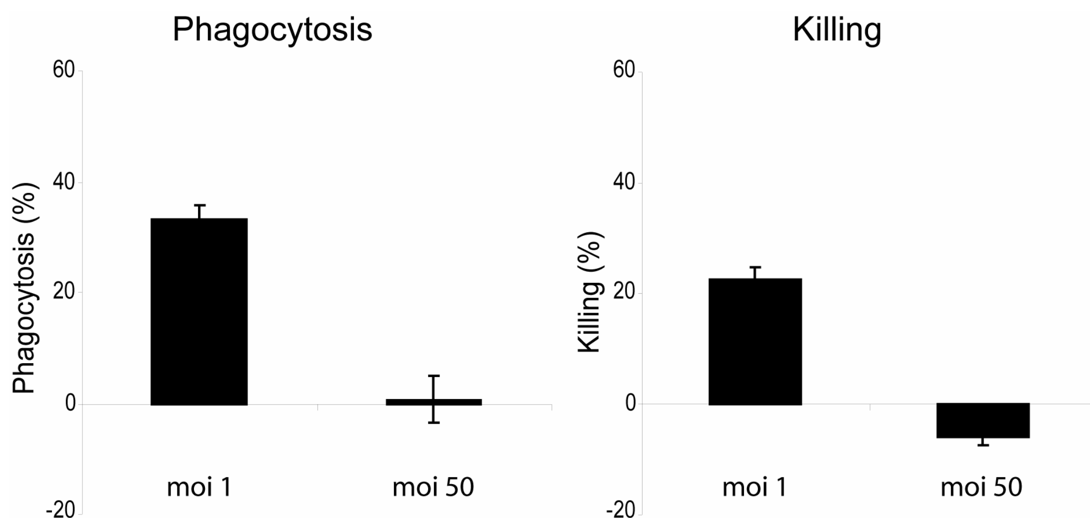


Figure 2. *Cc5* is resistant to phagocytosis and killing by dog PMNs at high moi
Freshly isolated dog PMNs were infected with *Cc5* at the indicated moi. 2 h after infection, phagocytosis and killing were determined by survival plating. Means and standard deviations of four independent experiments are shown.

Comparably to phagocytosis and killing by human PMNs (Shin *et al.*, 2009), ~33% *Cc5* bacteria were phagocytosed and ~22% were killed within 2 h of infection at low moi, whereas *Cc5* bacteria were completely resistant to phagocytosis and killing by dog PMNs at high moi (Figure 2). These findings suggested that *Cc5* also resist phagocytosis by PMNs in their natural habitat, the oral cavity of dogs, which may help the bacteria to reside inside the dog's mouth.

***Cc5* is sensitive to NET-dependent killing**

As it has been shown recently that PMNs form NETs upon infection, which trap and kill extracellular bacteria (Brinkmann *et al.*, 2004), we were interested whether *Cc5* also resists killing by NETs.

In order to test if we can artificially induce NET formation, we triggered NET formation by the addition of PMA and observed DNA release by staining with Sytox, a cell impermeant green fluorescent DNA binding dye. Analysis of DNA release with a fluorescent plate reader showed that PMNs indeed released DNA upon PMA stimulation (Figure 3.A).

In a next step, we wanted to see, whether the released DNA formed NETs. We thus analyzed untreated and PMA treated PMNs either by immunofluorescence, staining DNA with Hoechst, or by scanning electron microscopy (SEM) and we could thereby observe formation of NETs upon PMA stimulation (Figure 3.B-E).

As we could artificially induce NET formation, we wanted to examine whether *Cc5* and the *Cc5* Tn mutant Y1C12 induced NET formation upon infection. Thus, we infected human PMNs at an moi of 1 with either *Cc5* or Y1C12 bacteria. As negative control, we used untreated PMNs and as positive control, PMNs were treated with PMA. Infection of PMNs for 3 h with either *Cc5* or Y1C12 bacteria strongly induced DNA release as observed by Sytox staining of extracellular DNA (Figure 4.A). In a next step, we wanted to know, whether the release of DNA upon infection with *Cc5* or Y1C12 is only triggered by viable bacteria, or whether HI bacteria also induce DNA release. Analysis of DNA release after infection of PMNs for 3 h at an moi of 1 with HI bacteria revealed that both, HI *Cc5* as well as HI Y1C12 bacteria induced the release of DNA (Figure 4.A), suggesting that *C. canimorsus* passively induced NET formation upon infection of human PMNs.

We thus analyzed by SEM, whether the observed DNA release upon infection with *C. canimorsus* corresponded to NET formation. Hence, we infected human PMNs for 2 h with either *Cc5* or Y1C12 bacteria at an moi of 1. We observed that *Cc5* and Y1C12 both induced the formation of NETs (Figure 4.B-C). As bacteria were found to be covered within NETs, this indicated that the bacteria might be bound by NETs and therefore might be in contact with the AMPs bound to the NET DNA backbone.

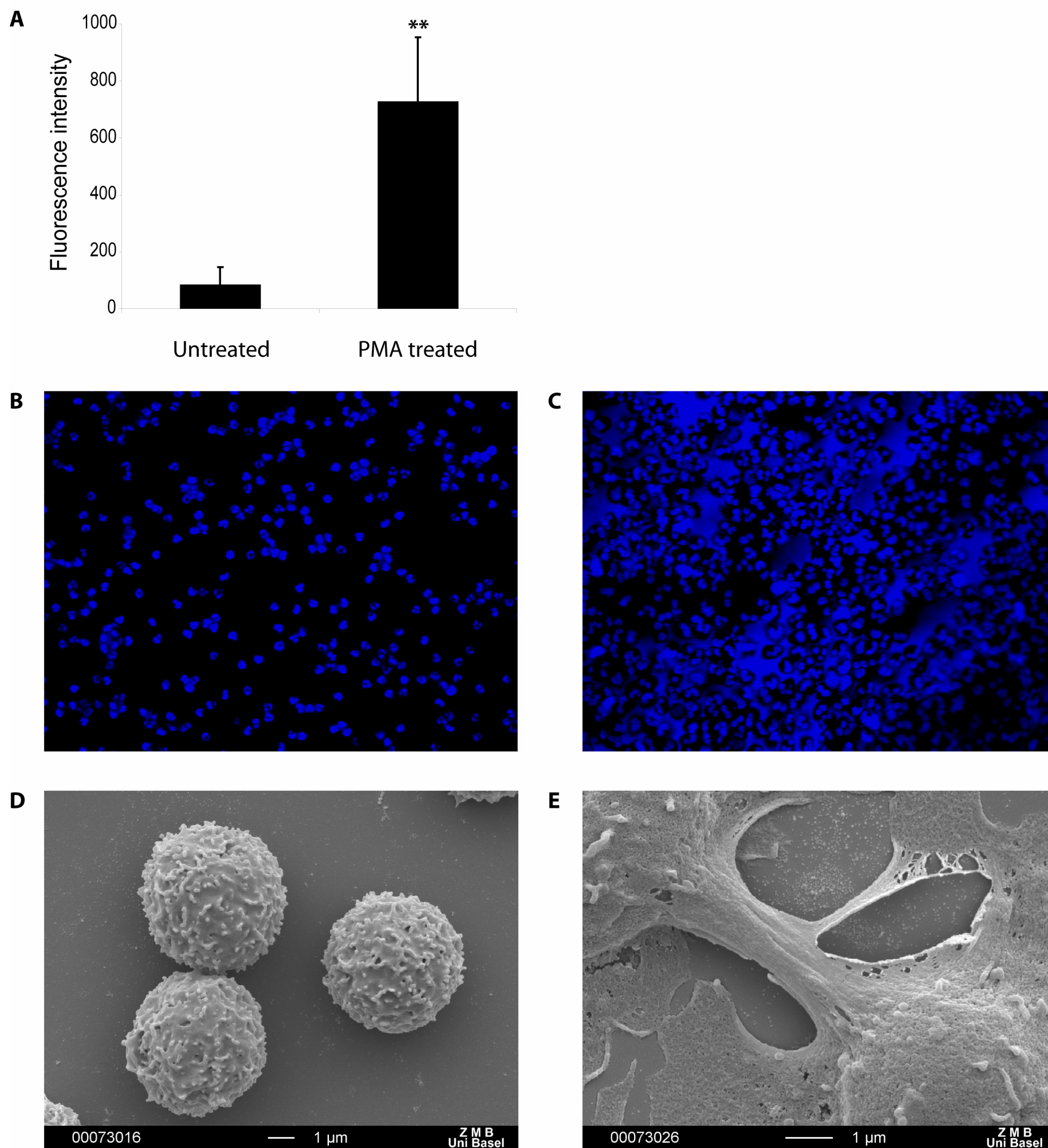


Figure 3. PMNs form NETs upon stimulation with PMA

(**A**) DNA release was quantified by Sytox staining of untreated PMNs and PMA treated PMNs. Mean values from three independent experiments and standard deviation are shown including statistical significance in comparison to untreated PMNs with ** $p < 0.01$ using unpaired, two-tailed Student's *t* test. (**B**) Untreated PMNs were stained with Hoechst and analyzed on a Leica Dmire2 microscope. (**C**) PMA-treated PMNs were stained and analyzed as in (B). (**D**) Untreated PMNs analyzed by SEM. (**E**) PMA treated PMNs analyzed by SEM.

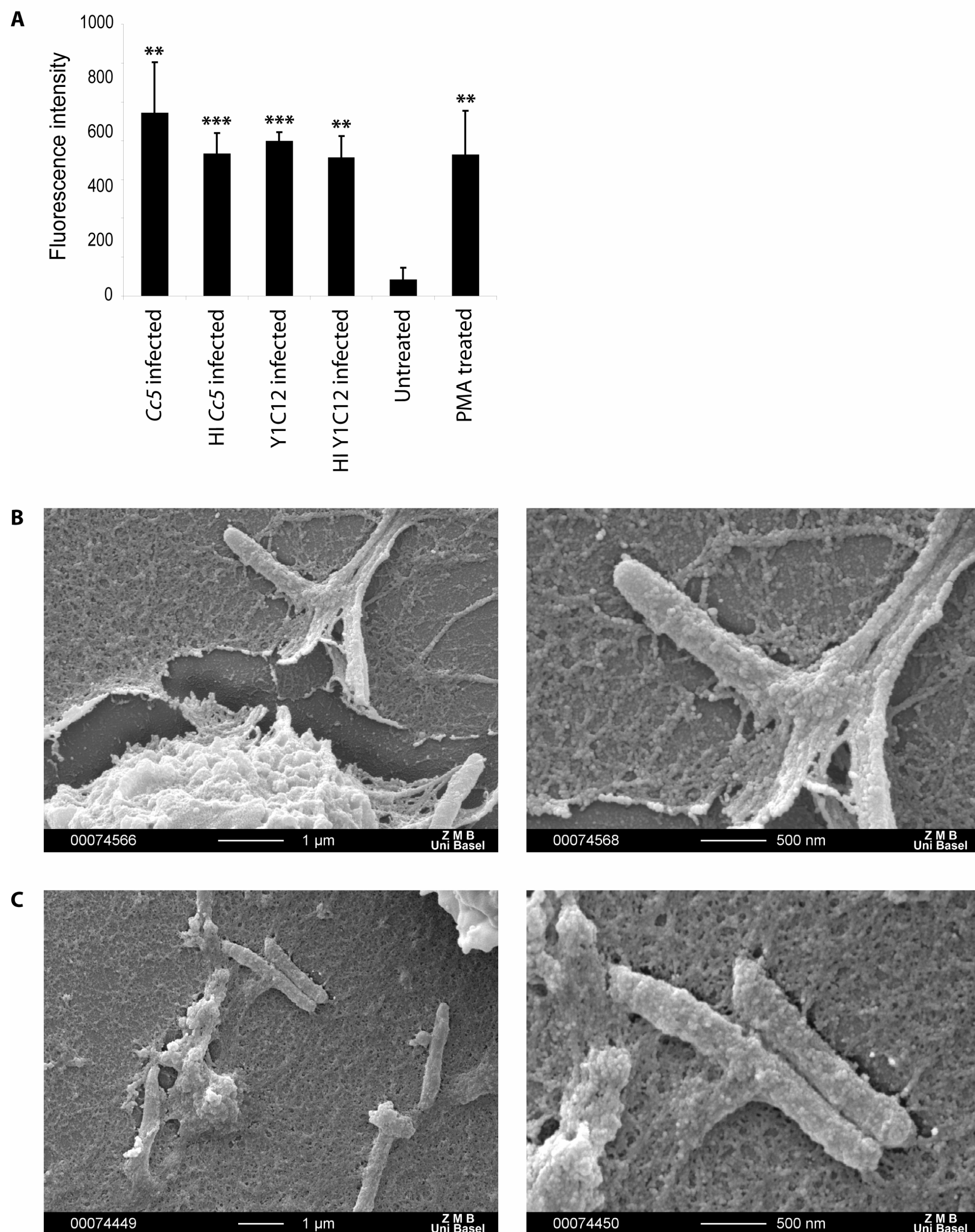


Figure 4. ***Cc5*** **and Y1C12 induce NET formation** ***in vitro***

(**A**) PMNs were infected for 3 h with *Cc5* or Y1C12 bacteria at an moi of 1. DNA release was quantified by Sytox staining. Untreated PMNs were used as negative control and NET formation was induced by PMA as positive control. Mean values from three independent experiments and standard deviations are shown including statistical significance in comparison to untreated PMNs with ** $p < 0.01$ and *** $p < 0.001$ using unpaired, two-tailed Student's *t* test. (**B**) Human PMNs were infected for 2 h with *Cc5* at an moi of 1 and analyzed by SEM. Right panel shows a magnified detail of left panel. (**C**) Human PMNs were infected for 2 h with Y1C12 at an moi of 1 and analyzed by SEM. Right panel shows a magnified detail of left panel.

In order to analyze whether *Cc5* and Y1C12 bacteria were sensitive to NET-dependent killing, we infected PMA stimulated and Cyt. D treated (block of the actin cytoskeleton, thereby preventing phagocytosis) human PMNs for 2 h with bacteria at an moi of 1 and determined extracellular killing by survival plating. In order to prevent NET degradation by human serum, we reduced the serum concentration from 10% to 2% compared to normal phagocytosis assays. We observed that ~30% *Cc5* and ~40% Y1C12 bacteria were killed within 2 h. To exclude that extracellular killing of the bacteria was NET-independent, we additionally pre-treated PMNs with DNase to degrade NETs, which completely prevented killing of bacteria. This suggested that bacteria were killed by NETs upon infection of PMA and Cyt. D treated PMNs (Figure 5).

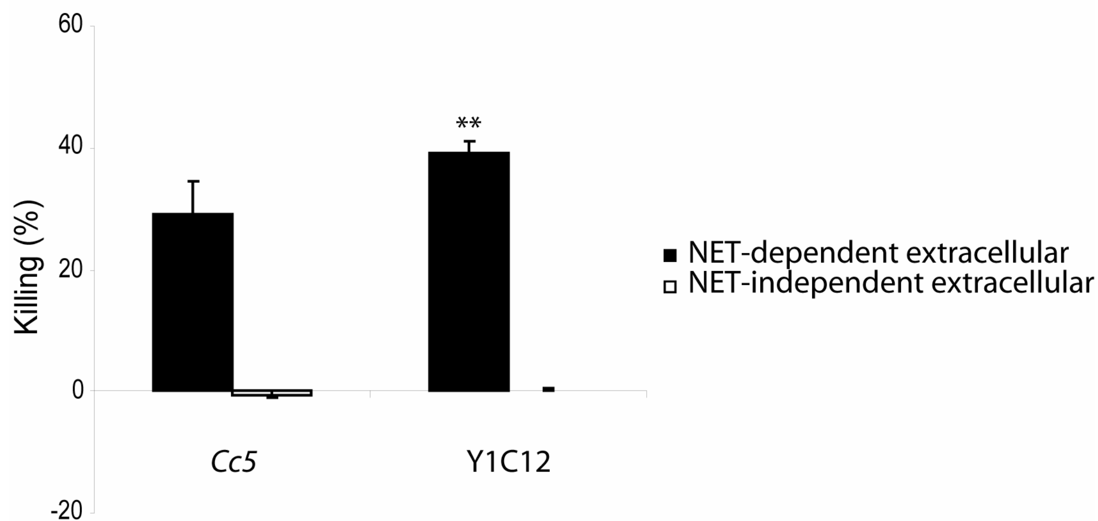


Figure 5. *Cc5* and Y1C12 are sensitive to NET-dependent killing

Extracellular killing of bacteria was determined 2 h after infection at an moi of 1 by survival plating. NET formation was stimulated by pre-treatment of PMNs with PMA and phagocytosis was prevented by the addition of Cyt. D (black). As control, NETs were degraded by the addition of DNase before infection (white). Mean values from three or more independent experiments and standard deviation are shown. Statistical significance compared to *Cc5* is shown with ** $p < 0.01$ using two-tailed, unpaired Student's *t* test.

4.4. Materials and methods

Bacterial strains and growth conditions

Bacteria were grown as described (Meyer *et al.*, 2008).

Selective agents

To select for strains and transposons, antibiotics were added at the following concentration: 10 μg/ml Em, 20 μg/ml Gm, 3.5 μg/ml Na and 10 μg/ml Sm.

Isolation of PMNs

Human or dog PMNs were isolated from healthy donors using the dextran-Percoll protocol, adapted with modifications described previously (Jepsen and Skottun, 1982). Contaminating erythrocytes were removed by hypotonic lysis with "aqua ad iniectabilia" (Bichsel).

Co-infection of human PMNs

Freshly isolated human PMNs were resuspended at 10^7 ml^{-1} in D-PBS (Gibco) supplemented with 10% Ab-depleted HI NHS (Scipac Ltd.) and infected with bacteria as indicated. *In vitro* phagocytosis and killing assay was continued as described (Shin *et al.*, 2009).

In vitro phagocytosis and killing assay with dog PMNs

Freshly isolated dog PMNs were resuspended at 10^7 ml^{-1} in D-PBS (Gibco) supplemented with 10% Ab-depleted HI NHS (Scipac Ltd.) and *in vitro* phagocytosis and killing assay was continued as described (Shin *et al.*, 2009).

Quantification of DNA released by activated PMNs

The assay was adapted from (Gupta *et al.*, 2005). Briefly, freshly isolated human PMNs were resuspended in D-PBS (Gibco) supplemented with 2% Ab-depledet HI NHS (Scipac Ltd.). The cells were seeded into 96-well microtiter plates (2 x 10^5 cells/well, Falcon) and either stimulated with PMA (25 nM, Sigma) for 30 min, or infected with bacteria at an moi of 1 for 3 h at 37 °C and 5% CO_2 in a humidified incubator. After incubation, Sytox green (10 µM, Molecular Probes), a cell impermeant DNA binding dye, was added to the cells to detect extracellular DNA. Untreated PMNs were used as control. Fluorescence was

determined at 485 nm and detected with a Wallac Victor^2 1420 Multilabel counter (Perkin Elmer).

Immunofluorescence analysis of DNA release

Falcon culture slides (Becton Dickinson) were coated with poly-D-lysine. Freshly isolated human PMNs were prepared in RPMI 1640 (Invitrogen) supplemented with 2% Ab-depleted HI NHS (Scipac Ltd.) and 2 mM L-glutamine (hRPMI) at a concentration of 10^6 ml^{-1}. Slides were washed 3x with D-PBS (Gibco). 500 μl of the PMN suspension were added to the slides and incubated for 1 h at 37 °C and 5% CO_2 in a humidified incubator. If necessary, PMNs were thereafter incubated for 30 min with 25 nM PMA (Sigma) to induce NET formation. Slides were fixed with 4% PFA for 1 h at 37 °C. Slides were then washed 2x with D-PBS and blocked o/n at 4 °C with PBS containing 3% BSA. DNA was labelled by incubation with Hoechst (1:10000, Sigma) for 30 min at RT in the dark. Slides were washed 3x with PBS and once with ddH_2O before mounting with VectaShield Hard Cover Mounting Medium (Vector). Slides were analyzed by use of a Leica Dmire2 microscope at 40x magnification. Pictures were taken with a digital camera (Hamatsu Photonics) and OpenLab software (version 3.1.2).

SEM analysis of NET formation

SEM analysis of NET formation was adapted from (Gupta *et al.*, 2005). In brief, freshly isolated human PMNs were resuspended in D-PBS (Gibco) supplemented with 2% Ab-depledet HI NHS (Scipac Ltd.). The cells were seeded on 12 mm 0.001% poly-D-lysine coated coverslips in 24-well microtiter plates (10^6 cells/well, Falcon). The medium was replaced with hRPMI and bacteria were added at an moi of 1. After 2 h of infection at 37 °C and 5% CO_2, samples were fixed with 2.5% glutaraldehyde and dehydrated with graded ethanol series (30%, 50%, 70%, and 100%). After dehydration and critical-point drying, the specimens were coated with 2 nm platinum-film and analyzed on a Philips XL-30 ESEM scanning electron microscope at the ZMB, Biozentrum, University of Basel.

***In vitro* analysis of NET-dependent killing**

The protocol was adapted from (Urban *et al.*, 2006). Freshly isolated human PMNs were resuspended at 10^6 ml^{-1} in D-PBS (Gibco) supplemented with 2% Ab-depleted HI NHS (Scipac Ltd.). To induce NET formation, PMNs were

incubated for 30 min with 25 nM PMA (Sigma) at 37 °C and 5% CO_2. Cyt. D (10 µg/ml, Sigma) was added for 20 min before infection to block phagocytosis. Bacteria were added at an moi of 1 to PMNs. To determine killing, samples were taken at 2 h after infection, diluted in ddH_2O and plated. As control, NETs were degraded by incubation with 50 U/ml of RNase-free and protease-free DNase-1 (Worthington) prior to the addition of bacteria. To determine reference values, an equal number of bacteria was incubated without PMNs. The survival rate was calculated by reference to the sample without PMNs.

Statistical analysis

For all experiments, means and standard deviations were calculated. Statistical significance was evaluated using two-tailed, unpaired Student's *t* test. Differences were considered to be significant when $p < 0.05$ with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

4.5. Discussion

We could demonstrate that *Cc5* is even more resistant to phagocytosis and killing by human PMNs than *Y. enterocolitica*, which is considered a model organism for phagocytosis resistance due to its T3SS (Grosdent *et al.*, 2002). This resistance of *Cc5* was decreased upon disruption of a putative *gtf* gene by a Tn in *Cc5* mutant Y1C12, which led to an altered outer membrane structure. These findings go in line with the observation that *Cc5* is highly resistant to phagocytosis and killing by murine macrophages (Meyer *et al.*, 2008), whereas Y1C12 is not. In addition, we observed that *Cc5* also resists phagocytosis and killing by dog PMNs, which suggests, that *Cc5* also resists phagocytosis in its natural habitat, the oral cavity of dogs.

We also show that *Cc5* is highly resistant to killing by human complement, as the bacteria prevent the deposition of MAC by virtue of their LPS. This was supported by the observation that Y1C12 mutant bacteria were serum sensitive due to an increased MAC deposition. All these findings suggest that *Cc5* has a highly protective outer membrane structure shielding the bacteria not only from MAC deposition, but also preventing phagocytosis by professional phagocytes. Thereby the bacteria maybe not only promote their survival within their natural habitat, but also within patients.

Complement resistance of *C. canimorsus* was analyzed so far for strains *Cc5*, *Cc11* and *Cc12* and all of them displayed a high serum resistance. This was in contrast to an old study on the serum sensitivity of oral *Capnocytophaga* spp. isolates, which indicated that these bacteria might be sensitive to the classical complement pathway (Wilson *et al.*, 1985). However, in a following study, oral and blood derived *Capnocytophaga* strains were tested for their serum sensitivity and an increased serum-resistance was observed in blood derived strains. This observation correlated with different electrophoretic mobilities of the examined LPS, indicating that serum resistance might be a virulence trait (Wilson *et al.*, 1987). As the three *C. canimorsus* strains tested here were all derived from human cases, further analysis should be done on human case strains in comparison to strains isolated from healthy dogs. Comparison of the outer membrane structure composition and the prevalence of serum resistance in case strains and in dog isolates could eventually reveal more information about this potential virulence trait in *C. canimorsus*.

As we observed that *Cc5* displays an increasing resistance to phagocytosis by either murine macrophages (Meyer *et al.*, 2008) or human PMNs with increasing moi, we suggested that *Cc5* might interfere with the phagocytosis ability of the phagocytes. We therefore co-infected human PMNs with *Cc5* (moi of 50) and *Y. enterocolitica* ∆YscN bacteria (moi of 1), but this did not affect phagocytosis and killing of ∆YscN bacteria. This suggested that *Cc5* actively prevents its own phagocytosis, but does not generally interfere with phagocytosis. This result is in good agreement with the previous observation that upon prolonged infection of murine macrophages with *Cc5* (moi of 50), killing, but not phagocytosis of *E. coli* bacteria, is prevented (Meyer *et al.*, 2008).

All together, our findings implicate that *Cc5* possesses at least three mechanisms by which the bacteria interfere with phagocytosis by professional phagocytes. First, *Cc5* seems to have an unreactive outer membrane structure, which is not readily recognized by PRRs on phagocytes, as unopsonized *Cc5* are highly resistant to phagocytosis and killing by professional phagocytes. Second, we observed that *Cc5* bacteria display an increased phagocytosis resistance at increasing moi, but do not prevent phagocytosis of other bacteria. This implements that *Cc5* bacteria actively interfere with their own uptake in a dose-dependent manner, but do not generally interfere with the ability of phagocytes to

engulf bacteria. And third, we demonstrated that *Cc5* can interfere with the killing ability of murine macrophages (Meyer *et al.*, 2008) by blocking the oxidative burst normally induced upon phagocytosis. This capacity does not affect phagocytosis of other bacteria and was found to be dependent on the release of the putative metallopeptidase pitrilysin.

Interestingly, not only Y1C12 mutant bacteria, but also *Cc5* was found to induce the formation of NETs upon infection of human PMNs at an moi of 1 *in vitro*. Both strains were observed to be sensitive to NET-dependent killing, even if Y1C12 mutant bacteria displayed an increased sensitivity compared to *Cc5* bacteria. This might be due to the altered outer membrane structure of Y1C12 bacteria, rendering the bacteria more accessible and therefore more sensitive to the antimicrobial activity of AMPs bound to NETs.

As we have shown that *Cc5* bacteria are sensitive to trapping and killing by NETs, these findings indicated that the bacteria display some sensitivity against AMPs. These results are in good agreement with another study where *C. sputigena* ATCC 33123, *C. gingivalis* ATCC 33124 and *C. ochracea* ATCC 27872 were shown to be sensitive to the bactericidal activity of synthetic LL-37, a cathelicidin, indicating sensitivity of *Capnocytophaga* spp. to AMPs (Tanaka *et al.*, 2000).

However, it has been shown recently that formation of NETs requires the production of ROS *in vivo* (Fuchs *et al.*, 2007). As we have discovered that *Cc5* can block the NADPH oxidase in murine macrophages upon prolonged infection *in vitro*, it might be interesting to examine whether *Cc5* can also block the oxidative burst in human PMNs and whether the bacteria thereby also prevent the formation of NETs. This could help *C. canimorsus* establishing a systemic infection in humans and might thus contribute to virulence. As examination of this mechanism still requires a 4 h pre-treatment of the macrophages with *Cc5* cond. medium, these questions could so far not be addressed as human PMNs quickly die after isolation. A study on the prevalence of the capability of blocking the killing ability of macrophages could give further hints on whether this mechanism might contribute to virulence in humans and should therefore be conducted.

Chapter 5

Heterogeneity of *C. canimorsus* strains

5.1. Summary

In this chapter we provide evidence that *C. canimorsus* strains have strain variability with respect to hypothetical virulence functions. A collection of 69 strains (8 human case isolates and 61 dog isolates) were analyzed in collaboration with L. Soussoula for their ability to prevent killing of *E. coli* by murine macrophages. 59% of the strains (50% of case strains, 61% of dog isolates) displayed this phenotype. As the 2 *C. cynodegmi* control strains also had this property, this indicated that this capability might rather be a species trait than a capacity uniquely linked to virulence.

M. Mally determined sialidase activity and found this to be present in all strains outlining the importance of sialidase for the survival of the bacteria in their natural habitat. Additionally, M. Mally provided evidence that thorough sequence analysis is needed in order to correctly distinguish *C. canimorsus* from *C. cynodegmi* strains as they share high sequence similarity and she revealed the existence of a new clade of *C. canimorsus*. Furthermore, M. Mally demonstrated that analysis of carbohydrate fermentation as a tool for species identification is unreliable. H. Shin demonstrated that the ability to suppress NO release in macrophages is a rare property.

Screening of the *C. canimorsus* library for serum resistance in collaboration with C. Paroz revealed that 60% of the strains were highly serum resistant (100% of case strains, 54% of dog isolates), indicating that serum resistance is widespread among *C. canimorsus* strains, but necessary for establishing an infection in humans.

As we have shown that serum resistance is linked to a polysaccharidic structure in *Cc5*, we were interested whether there is a specific structure in *C. canimorsus*, which might be responsible for serum resistance. Analysis of the polysaccharidic structures of all strains in collaboration with C. Paroz showed a high strain heterogeneity which did not obviously correlate with serum resistance.

5.2. Publication

Prevalence of *Capnocytophaga canimorsus* in dogs and occurrence of potential virulence factors

Microbes and Infection **11** (2009) 509-514

Manuela Mally*, Cécile Paroz*, Hwain Shin*, Salome Meyer*, Lavinia Soussoula, Ueli Schmiediger, Caroline Saillen-Paroz and Guy R. Cornelis

* Contributed equally to this work

Statement of my work. My contribution was the data on the ability of the *C. canimorsus* strains to block the killing of *E. coli* by J774.1 macrophages (summarized in Table 1).
Data on suppression of the killing ability was generated in collaboration with L. Soussoula.
Data on 16srDNA sequence, carbohydrate fermentation and sialidase activity was contributed by M. Mally in collaboration with C. Paroz. Data on suppression of NO release was contributed by H. Shin in collaboration with C. Paroz. Strains were collected by U. Schmiediger and C. Saillen-Paroz.

ELSEVIER

INSTITUT PASTEUR

Microbes and Infection 11 (2009) 509−514

www.elsevier.com/locate/micinf

Original article

Prevalence of *Capnocytophaga canimorsus* in dogs and occurrence of potential virulence factors

Manuela Mally [a,1], Cécile Paroz [a,1], Hwain Shin [a,1], Salome Meyer [a,1], Lavinia V. Soussoula [a], Ueli Schmiediger [b], Caroline Saillen-Paroz [c], Guy R. Cornelis [a,*]

[a] *Infection Biology, Biozentrum, University of Basel, Klingelbergstrasse 50/70, CH-4056 Basel, Switzerland*
[b] *Zwinglistrasse 10, 4137 Birsfelden, BL, Switzerland*
[c] *Rue du Molage 34, 1860 Aigle, VD, Switzerland*

Received 30 January 2009; accepted 20 February 2009
Available online 11 March 2009

Abstract

Capnocytophaga canimorsus is a Gram-negative commensal of dog's mouth causing severe human infections. A strain isolated from a human fatal infection was recently shown to have a sialidase, to inhibit the bactericidal activity of macrophages and to block the release of nitric oxide by LPS-stimulated macrophages. The present study aimed at determining the prevalence of *C. canimorsus* in dogs and the occurrence of these hypothetical virulence factors. *C. canimorsus* could be retrieved from the saliva of 61 dogs out of 106 sampled. Like in clinical isolates, all dog strains had a sialidase and 60% blocked the killing of phagocytosed *Escherichia coli* by macrophages. In contrast, only 6.5% of dog strains blocked the release of nitric oxide by LPS-challenged macrophages, suggesting that this property might contribute to virulence. The comparative analysis of 69 16S rDNA sequences revealed the existence of *C. canimorsus* strains that could be misdiagnosed.
© 2009 Elsevier Masson SAS. All rights reserved.

Keywords: Pathogenesis; Inflammation; Macrophage

1. Introduction

Capnocytophaga canimorsus is a Gram-negative bacterium from the family of *Flavobacteriaceae* [1] responsible for severe human infections transmitted by dogs or cats [2−4]. Animal infections have not been reported except for a pet rabbit that had been bitten by a dog [5]. Infections in humans generally consist of septicemia, with peripheral gangrene or meningitis, with a high mortality [2]. Infections do not necessarily occur after severe injuries, which are generally followed by a preventive antibiotic treatment, but rather after small bites, scratches, or even licks [6]. A number of patients infected by *C. canimorsus* have an immunosuppression history [6,7], but a significant number of patients have no obvious risk factor [2,7].

It is not clear yet why *C. canimorsus* can be so aggressive for humans but a number of clues could be collected recently. Quite remarkably, *C. canimorsus* scavenge aminosugars from glycoproteins present at the surface of animal cells and this property depends on the presence of a bacterial surface-exposed sialidase, which was found to contribute to persistence of *C. canimorsus* in the experimentally infected mouse [8]. Due to their LPS, *C. canimorsus* are resistant to killing by human complement as well as to phagocytosis by human PMNs [25] and by mouse macrophages [9]. They are not cytotoxic for macrophages but nevertheless, they do not trigger an inflammatory response as they do not react with Toll-like receptor 4 [10]. In addition to this capacity to passively escape immune detection, some strains, like *C. canimorsus* 5 (*Cc5*), also incapacitate the pro-inflammatory

* Corresponding author. Tel.: +41 (0)61 267 21 21 (secret), +41 (0)61 267 21 10 (direct); fax: +41 (0)61 267 21 18.
E-mail address: guy.cornelis@unibas.ch (G.R. Cornelis).
[1] Contributed equally to this work.

1286-4579/$ - see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2009.02.005

response of macrophages by preventing p38 MAPK phosphorylation and the release of nitric oxide (NO), TNF and other cytokines upon LPS stimulation [10]. Moreover, when challenged by *Escherichia coli*, these macrophages can no longer kill phagocytosed *E. coli* [9]. Two strains out of eight from human origin (*Cc5* and *Cc11*) displayed the anti-inflammatory mechanism [9,10] while four strains (*Cc5, Cc7, Cc9, Cc12*), displayed the anti-killing mechanism [9]. This small survey thus showed that the *C. canimorsus* strains are not identical in terms of potential virulence factors.

In the present study, we carried out a survey of the prevalence of *C. canimorsus* in dogs in Switzerland and we analyzed the dog strains for the three different virulence properties.

2. Materials and methods

2.1. Isolation of C. canimorsus *from dog's saliva and bacterial growth conditions*

The saliva from dogs was collected using a swab, which was rubbed against the gingival and cheek mucosa. Swabs were immediately dipped into a Port-a-cul™ (BD Biosciences) transport medium. Within 24 h, swabs were used to inoculate Heart Infusion Agar plates (Difco) supplemented with 5% sheep blood (Oxoid) and 20 µg/ml gentamicin, which is not active against the genus *Capnocytophaga* [7,11–13]. Plates were incubated for 2 days (d) at 37 °C with 5% CO_2. Four single colonies resembling the ATCC type strain of *C. canimorsus*, with regard to colony shape, colour and smell, were further analyzed.

2.2. 16S rDNA sequencing and analysis

A single colony was resuspended in 100 µl ddH_2O and boiled for 15 min at 98 °C. One µl was used as template for amplification of 1.07 kb of the 16S rDNA. Primers 27F (5′-agagtttgatcctggctcag-3′) and 1100R (5′-gggttgcgctcgttg-3′), binding in the conserved region [14,15], were used at 0.4 µM concentration with 200 µM dNTP and 1 U Taq polymerase (NEB). PCR was carried out for 5 initial cycles (94 °C for 30 s, 60 °C for 2 min, 72 °C for 3 min) in which the annealing temperature was reduced by 1.5 °C/cycle, followed by 30 cycles (94 °C for 30 s, 52 °C for 90 s, 72 °C for 3 min) and final elongation for 10 min at 72 °C. The 1.1 kb PCR product was extracted from a 1.2% agarose gel by NucleoSpin® (Machery Nagel). For sequencing with BigDye Terminator Ready Reaction (PE Biosystems), 20–50 ng of the cleaned PCR product was analyzed with primers 27F, 685R (5′-tctacgcatttcaccgctac-3′) [14,15] and 1100R. Primary data was aligned using ContigExpress and AlignX (Vector NTI software package; Invitrogen). The consensus sequences were then used for sequence analysis using the Ribosomal Database Project II-Release 10 tools "Seqmatch" and "Tree Builder" (http://rdp.cme.msu.edu/) [16].

2.3. Carbohydrate fermentation

Bacteria were harvested from plates, washed in PBS and adjusted to 3×10^9 cfu/ml. Phenol red broth was supplemented with 1% of raffinose or sucrose (Sigma). Fermentation media with or without carbohydrates were inoculated with 1.5×10^8 cfu and 3×10^8 cfu of bacterial suspension giving 200 µl final volume in microtiter plates. Acid production was monitored by the phenol red pH indicator, after 48 h and 7 d incubation at 37 °C with 5% CO_2.

2.4. Sialidase activity detection

Sialidase activity detection was done as described [8].

2.5. Suppression of the NO release ability of macrophages

J774.A1 murine macrophages (ATCC TIB-67) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1 mM sodium pyruvate (=cRPMI). Cells were seeded at a density of 10^5/ml in 24 well plates 15 h before infection. *E. coli* Top10 (Invitrogen) were washed and resuspended in PBS, heat killed (HK) for 2 h at 99 °C and adjusted to 4×10^7 cfu/ml in cRPMI. *C. canimorsus* grown on plates were harvested, washed in PBS and adjusted to 4×10^7 cfu/ml in cRPMI. Live *C. canimorsus* bacteria and HK *E. coli* bacteria (100 µl each) were added to 10^5 J774.1 cells, corresponding to a multiplicity of infection (moi) of 40 for each. 24 h post infection, cell free supernatants were obtained by centrifugation at $20,000 \times g$ for 10 min. NO release was analyzed by detection of stable sodium nitrite using a colorimetric assay with Griess reagent (Sigma).

2.6. Suppression of the killing ability of macrophages by C. canimorsus

Suppression of the killing ability of macrophages was determined as described in Ref. [9] for conditioned medium.

3. Results

3.1. Prevalence of C. canimorsus *in dogs*

We sampled the saliva from 105 dogs from Switzerland (areas of Basel, Lausanne and Valais), irrespective of race, age and sex of the animal. We also swabbed the dog of a patient who suffered from a *C. canimorsus* infection (Zurich). Colonies with a morphology evoking *C. canimorsus* were analyzed by comparing 1.07 kb of the 16S rDNA (from nucleotide 27 to 1100, *E. coli* 16S rDNA numbering) to all 16S RNA genes in the database [16]. Up to four colonies per dog were analyzed but only one *C. canimorsus* strain was kept per dog. Three strains with a different morphology, evoking *Capnocytophaga cynodegmi* were also analyzed for comparison. For most of the 61 strains analyzed, the archetypal *C. canimorsus* ATCC 35979 strain (*Cc12,* accession number L14637) gave the

Table 1
Strains and phenotypes.

Strain	ATCC 35979, *Capnocytophaga canimorsus*[a]		ATCC 49044, *Capnocytophaga cynodegmi*		Fermentation of[b]			Inhibition of	
	Similarity score	S_ab score	Similarity score	S_ab score	Raffinose	Sucrose	Sialidase[c]	NO release[d]	*Escherichia coli* killing[e]
C. canimorsus clinical isolates									
Cc2	**0.966**	**0.808**	0.957	0.743	−	−	+	−	−
Cc3	**0.994**	**0.908**	0.979	0.842	−	−	+	−	−
Cc5	**0.987**	**0.934**	0.976	0.855	−	−	+	+	+
Cc7	**0.994**	**0.931**	0.979	0.827	−	−	+	−	+
Cc9	**0.985**	**0.904**	0.975	0.829	−	−	+	−	+
Cc10	**0.991**	**0.883**	0.981	0.808	−	−	+	−	−
Cc11	**0.987**	**0.879**	0.979	0.826	−	−	+	+	−
Cc12	**0.985**	**0.911**	0.981	0.834	−	−	+	−	+
C. canimorsus isolated from dogs									
*CcD*57	**0.998**	**0.879**	0.983	0.806	−	−	+	−	+
*CcD*34	**0.996**	**0.947**	0.983	0.886	−	+	+	−	+
*CcD*68	**0.996**	**0.896**	0.981	0.814	−	−	+	−	+
*CcD*20	**0.995**	**0.947**	0.984	0.839	−	−	+	−	−
*CcD*37	**0.994**	**0.947**	0.980	0.870	−	−	+	+	+
*CcD*39	**0.994**	**0.916**	0.978	0.822	−	v	+	−	−
*CcD*69	**0.994**	**0.888**	0.981	0.818	−	−	+	−	+
*CcD*106	**0.993**	**0.938**	0.982	0.843	−	−	+	−	+
*CcD*16	**0.992**	**0.908**	0.982	0.839	−	−	+	−	−
*CcD*25	**0.992**	**0.906**	0.977	0.818	−	−	+	−	+
*CcD*33	**0.992**	**0.917**	0.980	0.824	−	−	+	+	+
*CcD*10	**0.991**	**0.917**	0.976	0.850	−	−	+	−	+
*CcD*53	**0.990**	**0.920**	0.981	0.834	−	−	+	−	+
*CcD*58	**0.990**	**0.869**	0.982	0.830	−	−	+	−	+
*CcD*80	**0.990**	**0.874**	0.985	0.845	−	−	+	+	−
*CcD*96	**0.990**	**0.928**	0.981	0.843	−	−	+	−	+
*CcD*101	**0.990**	**0.908**	0.977	0.817	−	−	+	−	−
*CcD*35	**0.989**	**0.927**	0.978	0.831	−	−	+	−	+
*CcD*44	**0.988**	**0.897**	0.979	0.845	−	−	+	−	+
*CcD*40	**0.987**	**0.913**	0.974	0.783	−	−	+	−	+
*CcD*73	**0.987**	**0.893**	0.979	0.810	−	−	+	−	+
*CcD*13	**0.986**	**0.929**	0.976	0.819	−	−	+	−	+
*CcD*18	**0.986**	**0.874**	0.977	0.796	−	−	+	+	+
*CcD*105	**0.986**	**0.911**	0.977	0.817	−	−	+	−	+
*CcD*3	**0.985**	**0.913**	0.978	0.836	−	−	+	−	+
*CcD*77	**0.983**	**0.827**	0.979	0.801	−	−	+	−	−
*CcD*71	0.982	**0.885**	**0.984**	0.878	−	−	+	−	+
*CcD*52	**0.981**	**0.823**	0.974	0.762	−	−	Reduced	−	−
*CcD*63	**0.981**	**0.919**	0.969	0.824	−	−	+	−	+
*CcD*5	**0.980**	**0.916**	0.971	0.827	−	−	+	−	+
*CcD*43	**0.979**	**0.847**	0.972	0.821	−	−	+	−	+
*CcD*47	**0.978**	**0.857**	0.969	0.788	−	−	+	−	−
*CcD*84	**0.978**	**0.794**	0.970	0.762	−	−	+	−	−
*CcD*89	**0.977**	**0.912**	0.970	0.825	−	−	+	−	+
*CcD*38	**0.977**	0.802	0.975	**0.814**	−	+	+	−	+
*CcD*88	**0.977**	0.802	0.975	**0.817**	−	−	+	−	+
*CcD*104	**0.976**	**0.926**	0.968	0.836	−	−	+	−	+
*CcD*95	**0.976**	0.790	0.973	**0.806**	v	+	+	−	−
*CcD*51	**0.975**	**0.876**	0.966	0.798	−	−	+	−	+
*CcD*66	**0.975**	**0.810**	0.972	0.806	−	+	+	−	−
*CcD*50	**0.975**	0.774	0.970	**0.788**	−	−	+	−	+
*CcD*82	**0.974**	0.744	0.971	**0.763**	−	−	+	−	−
*CcD*94	**0.974**	0.767	0.972	**0.785**	v	v	+	−	−
*CcD*6	**0.973**	**0.904**	0.957	0.788	−	−	+	−	−
*CcD*93	**0.972**	0.752	0.970	**0.770**	−	−	+	−	+
*CcD*103	**0.972**	0.789	0.969	**0.793**	−	−	+	−	−
*CcD*36	0.971	0.787	**0.972**	**0.820**	−	−	+	−	−
*CcD*76	**0.970**	**0.819**	0.952	0.730	+	+	+	−	+
*CcD*7	**0.970**	0.775	0.965	**0.781**	−	−	+	−	−
*CcD*11	**0.970**	0.799	0.969	**0.810**	−	+	+	−	−

(*continued on next page*)

Table 1 (*continued*)

Strain	ATCC 35979, *Capnocytophaga canimorsus*[a]		ATCC 49044, *Capnocytophaga cynodegmi*		Fermentation of[b]			Inhibition of	
	Similarity score	S_ab score	Similarity score	S_ab score	Raffinose	Sucrose	Sialidase[c]	NO release[d]	*E*scherichia *coli* killing[e]
*CcD*64	0.970	0.783	**0.972**	**0.797**	−	−	+	−	+
*CcD*1	0.969	0.800	**0.970**	**0.805**	−	+	+	−	−
*CcD*75	**0.969**	0.733	**0.969**	**0.771**	−	−	+	−	−
*CcD*46	0.968	0.779	**0.970**	**0.786**	−	−	+	−	−
*CcD*97	**0.968**	0.727	0.964	**0.739**	v	+	+	−	−
*CcD*81	**0.967**	**0.861**	0.958	0.798	−	−	+	−	+
*CcD*102	0.967	0.842	**0.969**	**0.861**	−	−	+	−	−
*CcD*79	**0.966**	0.783	0.965	**0.801**	−	−	+	−	+
*CcD*4	0.964	**0.779**	**0.965**	0.764	−	+	+	−	+
*CcD*85	**0.951**	**0.733**	**0.951**	0.728	+	+	+	−	−
*CcD*54	**0.948**	0.591	0.943	**0.617**	+	+	+	−	+
Typical *C. cynodegmi* isolated from dogs									
*Ccy*19	0.983	0.806	**0.992**	**0.852**	−	+	+	−	+
*Ccy*46	0.977	0.849	**0.991**	**0.897**	+	−	+	−	+
*Ccy*74	0.986	0.878	**0.997**	**0.968**	+	+	Reduced	−	+

[a] Scores are determined by the RDP tool SeqMatch, whereas the similarity score reports the percent sequence identity over all pairwise comparable positions and the S_ab score represents the number of unique 7-base oligomers shared between the query sequence and the given type strains sequence divided by the lowest number of unique oligos in either of the two sequences. Highest similarity scores and S_ab scores are depicted boldface. *CcD* strains are listed from the highest similarity score calculated compared to *C. canimorsus* ATCC 35979 in descending order.

[b] v, varied.

[c] Analyzed by 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid cleavage.

[d] NO release is tested by co-infection of J774.1 with specified isolate and HK *E. coli*.

[e] Monitored by viable counting after pre-infection of J774.1 with specified isolate subsequently challenged by *E. coli*.

highest S_ab scores and *C. cynodegmi* ATCC 49044 type strain (accession number X97245) gave the second highest scores (Table 1). For the three hypothetical *C. cynodegmi* strains, the *C. cynodegmi* type strain gave the highest score. For a number of strains however, both scores were very close (Table 1), making the identification uncertain. We then built a phylogeny tree of the 16S rDNA sequences [16] of the sequences from all strains isolated from dogs and from human infections as well as from the type strains from all *Capnocytophaga* sp. [10]. The phylogeny tree gave an unambiguous answer: 61 strains from dogs appeared in one single large clade including the *C. canimorsus* ATCC 49044 type strain and the eight clinical isolates. Interestingly, 22 strains, which could not be typed simply by sequence homologies clustered as a separate group within the *C. canimorsus* clade (Fig. 1).

We also monitored the fermentation of sucrose and raffinose, which is reported to be negative for *C. canimorsus* and 89% positive for *C. cynodegmi* [4]. However, as shown in Table 1, these tests did not give results consistent with the results of the 16S rDNA analysis. Given the clarity of the 16S rDNA phylogeny tree, we decided to maintain our identification based from 16S rDNA analysis. Hence, a *C. canimorsus* strain could be isolated from 61 dogs out of 106 sampled (57.5%). There was no correlation between the state of carrier and race, age, sex or environment (data not shown).

3.2. Presence of sialidase activity

The 61 *C. canimorsus* strains isolated from dogs were tested for the different virulence properties reported earlier and compared to 8 clinical isolates of *C. canimorsus* [9,10]. All 61 *C. canimorsus* strains had sialidase activity, indicating that sialidase is a stable trait of the species. The four *C. cynodegmi* strains including the type strain tested also had sialidase activity.

3.3. Inhibition of the bactericidal activity of macrophages

We next tested the capacity of *C. canimorsus* to interfere with the innate immune response. At first, we checked whether the *C. canimorsus* and *C. cynodegmi* strains included in this study were cytotoxic for cultured J774.1 macrophages. As we observed earlier with strains isolated from human patients [10] and in contrast with a previous report [17], we did not observe any cytotoxicity from *C. canimorsus*. We then monitored the capacity of *C. canimorsus* to block the killing of *E. coli* after phagocytosis [9]. 37 out of the 61 (61%) strains inhibited the killing of phagocytosed *E. coli* (Table 1, Fig. 1). This property was found in 50% of the clinical isolates as well as in the 3 typical *C. cynodegmi* strains. This phenotype is thus widely distributed in *C. canimorsus* and not limited to *C. canimorsus*.

3.4. Capacity to block the NO release by macrophages

We finally monitored the capacity of *C. canimorsus* to block the release of NO by LPS-stimulated macrophages [10]. Only 4 out of the 61 dog strains (6.5%) were active. In contrast, 2 out of 8 clinical isolates were positive for this property. Considering an occurrence of 6.5%, the probability of finding 2 positive strains in a sample of 8 strains is 0.09. None of the three *C. cynodegmi* had this property.

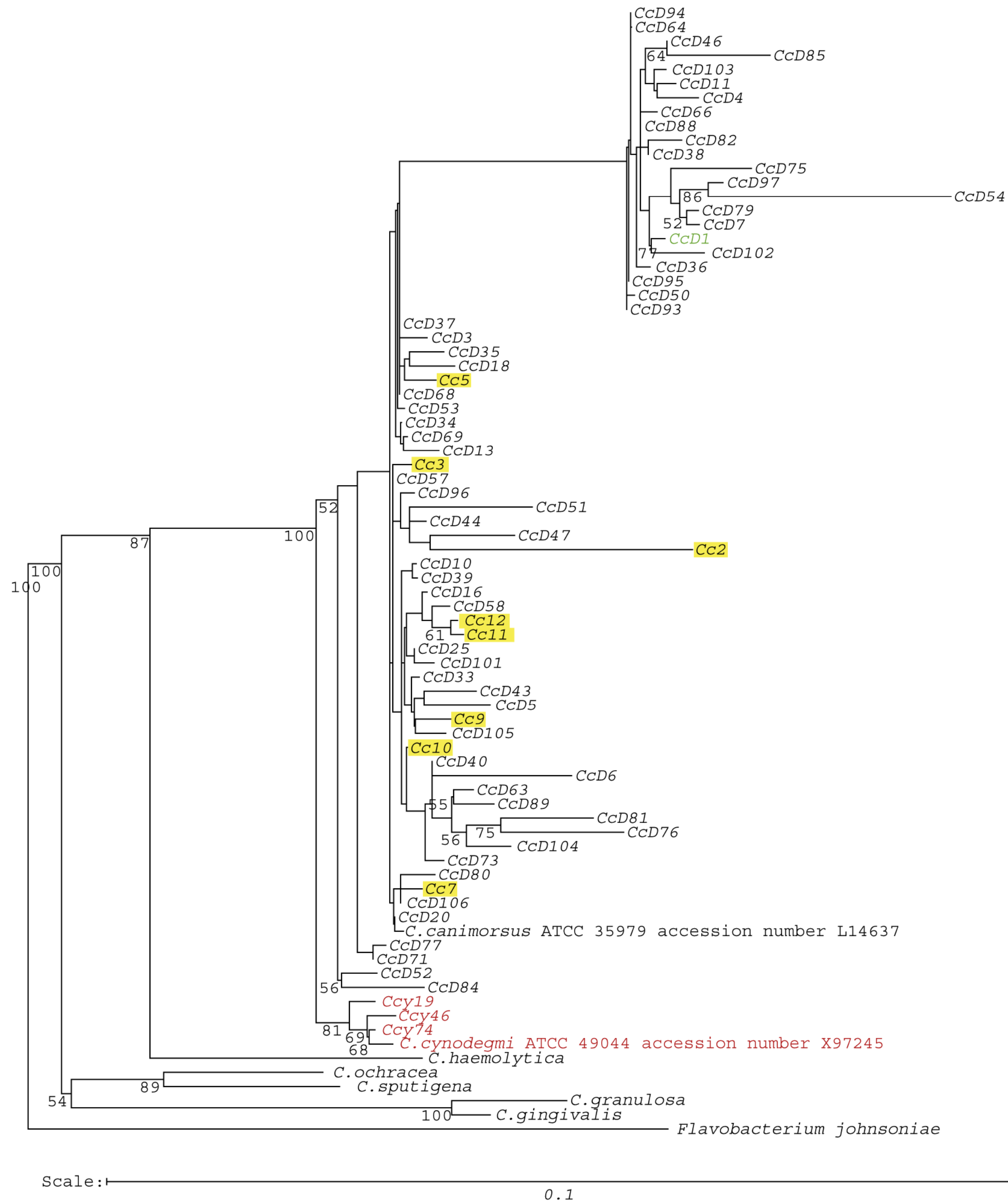


Fig. 1. 16S rDNA tree using RDP Tree Builder. The majority consensus tree is displayed showing the bootstrap value at the nodes if higher than 50%. The human isolates are highlighted in yellow. Strain *CcD1*, highlighted in green, is the strain that was isolated from a dog, which caused a human infection. *Flavobacterium johnsoniae* (accession number M59051) was selected as an outgroup.

4. Discussion

Previous reports showed that *C. canimorsus* are part of the commensal flora of up to 25.5% of the dogs [18–20]. In contrast, the number of human infection is very low (0.5–1 per million per year [21]), suggesting that other factors are required to generate disease. Immunosuppression of the patient is a clear predisposing factor but a significant number of patients have no known immunosuppression [2,6]. The strikingly low infection rate could also result from some heterogeneity in the population of *C. canimorsus*. The population could be composed of "common" strains that could behave as opportunistic pathogens and "more aggressive" strains that could infect even non-immuno-compromised patients. To address this question, we investigated here whether the "immuno-suppressive" properties we discovered in a strain isolated from a fatal case [9,10] would be commonly found among the dog's isolates.

We could retrieve by culture *C. canimorsus* from *ca* 60% of the dogs. Since we isolated *C. canimorsus* by culture, we infer that these 60% are probably an underestimate of the prevalence in dogs. This figure is higher than the ones published before [18–20], but this difference results probably from the screening technique and the typing difficulty, which is

apparent from Table 1 and Fig. 1 and is not unprecedented in the genus *Capnocytophaga* [22–24]. On the basis of their 16S rDNA sequence, some *C. canimorsus* strains may indeed be confused with *C. cynodegmi* if no phylogeny tree is considered. In addition, the metabolism of sugars does not clarify the situation, as already mentioned for *Capnocytophaga ochracea* and *Capnocytophaga sputigena* for instance [23,24].

Our first observation with dog strains is that they all produce an active sialidase, like the clinical isolates. We infer that sialidase is essential for the metabolism of *C. canimorsus* in the dog's mouth environment. This idea is reinforced by the observation that a sialidase was also found in the other dog commensal, *C. cynodegmi*. The capacity to prevent macrophages from killing ingested *E. coli* seemed to be very widespread among the dog's strains as well as in the clinical isolates and the proportion was not very different in both groups.

Finally, the capacity to block the onset of the macrophage pro-inflammatory response was found in only 6.5% of the dog strains while it was found in 2 out of 8 strains isolated from human infections, suggesting that this property might be more common in strains from human infections than in strains from dogs. Although the small number of clinical isolates that could be analyzed does not allow drawing any firm conclusion, it gives a hint that this rare property could be a factor favoring an infection, at least in immunocompetent patients. The molecular analysis of this phenomenon, presently in progress, is leading, among others, to a straightforward diagnostic test allowing the detection of these potentially more dangerous strains. It will allow testing more clinical isolates and hence to determine whether these strains are indeed more common among clinical isolates than among dog strains. In that case, one could envision a detection of these strains in dogs and eventually the treatment of carrier dogs in order to protect their owner.

Acknowledgements

We would like to thank A. Junker and M. Kuhn for organizing sampling from 2 dogs and E. van Nimwegen for calculating probabilities. This work was supported by the Swiss National Science Foundation (grant 32-65393.01).

References

[1] J.F. Bernardet, Y. Nakagawa, B. Holmes, Proposed minimal standards for describing new taxa of the family Flavobacteriaceae and emended description of the family, International Journal of Systematic and Evolutionary Microbiology 52 (2002) 1049–1070.

[2] J.M. Janda, M.H. Graves, D. Lindquist, W.S. Probert, Diagnosing *Capnocytophaga canimorsus* infections, Emerging Infectious Diseases 12 (2006) 340–342.

[3] R.A. Bobo, E.J. Newton, A previously undescribed gram-negative bacillus causing septicemia and meningitis, American Journal of Clinical Pathology 65 (1976) 564–569.

[4] D.J. Brenner, D.G. Hollis, G.R. Fanning, R.E. Weaver, *Capnocytophaga canimorsus* sp. nov. (formerly CDC group DF-2), a cause of septicemia following dog bite, and *C. cynodegmi* sp. nov., a cause of localized wound infection following dog bite, Journal of Clinical Microbiology 27 (1989) 231–235.

[5] E. van Duijkeren, C. van Mourik, M. Broekhuizen, M. Leuven, W. Gaastra, D. Houwers, First documented *Capnocytophaga canimorsus* infection in a species other than humans, Veterinary Microbiology 118 (2006) 148–150.

[6] D.M. Tierney, L.P. Strauss, J.L. Sanchez, *Capnocytophaga canimorsus* mycotic abdominal aortic aneurysm: why the mailman is afraid of dogs, Journal of Clinical Microbiology 44 (2006) 649–651.

[7] C. Lion, F. Escande, J.C. Burdin, *Capnocytophaga canimorsus* infections in human: review of the literature and cases report, European Journal of Epidemiology 12 (1996) 521–533.

[8] M. Mally, H. Shin, C. Paroz, R. Landmann, G.R. Cornelis, *Capnocytophaga canimorsus*: a human pathogen feeding at the surface of epithelial cells and phagocytes, PLoS Pathogens 4 (2008) e1000164 [doi: 1000110.1001371/journal.ppat.1000164].

[9] S. Meyer, H. Shin, G.R. Cornelis, *Capnocytophaga canimorsus* resists phagocytosis by macrophages and blocks the ability of macrophages to kill other bacteria, Immunobiology 213 (2008) 805–814.

[10] H. Shin, M. Mally, M. Kuhn, C. Paroz, G.R. Cornelis, Escape from immune surveillance by *Capnocytophaga canimorsus*, The Journal of Infectious Diseases 195 (2007) 375–386.

[11] T. Butler, R.E. Weaver, T.K. Ramani, C.T. Uyeda, R.A. Bobo, J.S. Ryu, R.B. Kohler, Unidentified gram-negative rod infection. A new disease of man, Annals of Internal Medicine 86 (1977) 1–5.

[12] M. Mally, G.R. Cornelis, Genetic tools for studying *Capnocytophaga canimorsus*, Applied and Environmental Microbiology 74 (2008) 6369–6377.

[13] B. Kristensen, H.C. Schonheyder, N.A. Peterslund, S. Rosthoj, N. Clausen, W. Frederiksen, *Capnocytophaga* (*Capnocytophaga ochracea* group) bacteremia in hematological patients with profound granulocytopenia, Scandinavian Journal of Infectious Diseases 27 (1995) 153–155.

[14] D.J. Lane, 16S/23S rRNA Sequencing, in: E.G. Stackebrandt, M. Goodfellow (Eds.), Nucleic Acid Techniques in Bacterial Systematics, John Wiley & Sons, New York, 1991, pp. 115–174.

[15] J.L. Johnson, Similarity Analysis of rRNAs, in: P. Gerhard, R.G.E. Murray, G.A. Wood, N.R. Krieg (Eds.), Methods for General and Molecular Bacteriology, American Society for Microbiology, Washington, DC, 1994, pp. 683–700.

[16] J.R. Cole, Q. Wang, E. Cardenas, J. Fish, B. Chai, R.J. Farris, A.S. Kulam-Syed-Mohideen, D.M. McGarrell, T. Marsh, G.M. Garrity, J.M. Tiedje, The Ribosomal Database Project: improved alignments and new tools for rRNA analysis, Nucleic Acids Research (2008).

[17] L.J. Fischer, R.S. Weyant, E.H. White, F.D. Quinn, Intracellular multiplication and toxic destruction of cultured macrophages by *Capnocytophaga canimorsus*, Infection and Immunity 63 (1995) 3484–3490.

[18] W.E. Bailie, E.C. Stowe, A.M. Schmitt, Aerobic bacterial flora of oral and nasal fluids of canines with reference to bacteria associated with bites, Journal of Clinical Microbiology 7 (1978) 223–231.

[19] P. Blanche, E. Bloch, D. Sicard, *Capnocytophaga canimorsus* in the oral flora of dogs and cats, Journal of Infection 36 (1998) 134.

[20] A.J. Westwell, K. Kerr, M.B. Spencer, D.N. Hutchinson, DF-2 infection, BMJ (Clinical research ed.) 298 (1989) 116–117.

[21] C. Pers, B. Gahrn-Hansen, W. Frederiksen, *Capnocytophaga canimorsus* septicemia in Denmark, 1982–1995: review of 39 cases, Clinical Infectious Diseases 23 (1996) 71–75.

[22] M. Ciantar, H.N. Newman, M. Wilson, D.A. Spratt, Molecular identification of *Capnocytophaga* spp. via 16S rRNA PCR-restriction fragment length polymorphism analysis, Journal of Clinical Microbiology 43 (2005) 1894–1901.

[23] J.E. Kristiansen, A. Bremmelgaard, H.E. Busk, O. Heltberg, W. Frederiksen, T. Justesen, Rapid identification of *Capnocytophaga* isolated from septicemic patients, European Journal of Clinical Microbiology 3 (1984) 236–240.

[24] H. Speck, R.M. Kroppenstedt, W. Mannheim, Genomic relationships and species differentiation in the genus *Capnocytophaga*, Zentralblatt fur Bakteriologie, Mikrobiologie, und Hygiene 266 (1987) 390–402.

[25] H. Shin, M. Mally, S. Meyer, C. Fiechter, C. Paroz, U. Zaehringer, G.R. Cornelis. Resistance of Capnocytophaga canimorsus to killing by 1 human complement 2 and polymorphonuclear leukocytes. Infection and Immunity, in press.

5.3. Additional results

Serum resistance varies in *C. canimorsus* strains

As we have shown previously that the three human case isolates *Cc5*, *Cc11* and *Cc12* are highly resistant to killing by human complement (Shin *et al.*, 2009), we wanted to examine whether this is a species property, or whether complement resistance might be a virulence determinant.

Hence, C. Paroz incubated *C. canimorsus* case isolates (*Cc*) and *C. canimorsus* dog strains (*CcD*) with either HI NHS or NHS and compared colony forming units (cfu) after 3 h of infection. We observed that 100% of the human case strains (8/8) and 54% of the dog isolates (33/61) were highly resistant to killing by human complement (reduced by less than 1 $\log_{10}$) (Table 1). These findings indicated that serum resistance might indeed be an important virulence determinant.

C. canimorsus strains display outer membrane structure heterogeneity

As we have shown evidence that serum resistance of *Cc5* is linked to the polysaccharidic structure of the bacteria (Shin *et al.*, 2009), we were interested whether there is a specific polysaccharidic structure in *C. canimorsus*, which is linked to serum resistance. C. Paroz therefore analyzed proteinase K-treated samples of all *C. canimorsus* strains by tricine SDS-PAGE and either periodic acid/silver staining (Figure 1) or immunoblotting with α-*Cc5* Ab (data not shown).

It has been previously shown that *Cc5* comprises 4 polysaccharidic structures purified by proteinase K digest (Shin *et al.*, 2009). As bands A and B were not present in an LPS purification, this suggested that those two polysaccharidic structures probably do not represent LPS, but maybe a capsule. Bands C and D were identified in an LPS purification indicating that these two bands might represent two LPS structures of *Cc5* (Shin *et al.*, 2009). Even though serum sensitivity of *Cc5 gtf* mutant Y1C12 could be linked to band C (Shin *et al.*, 2009), *C. canimorsus* strains displayed a high polysaccharide structure heterogeneity (Figure 1) which did not obviously correspond to serum resistance (Table 1).

Strain	Serum sensitivity [a]	Strain	Serum sensitivity [a]
CcD88	-0.267 ± 0.549	*CcD35*	0.863 ± 0.289
CcD103	0.073 ± 0.136	*Cc10* [b]	0.877 ± 0.120
CcD79	0.077 ± 0.028	*Cc11* [b]	0.880 ± 0.162
CcD85	0.088 ± 0.217	*CcD93*	0.885 ± 0.230
CcD50	0.096 ± 0.024	*CcD77*	0.952 ± 0.068
CcD46	0.130 ± 0.091	*Cc12* [b]	0.971 ± 0.063
CcD71	0.136 ± 0.087	*CcD33*	1.101 ± 0.206
CcD82	0.147 ± 0.048	*CcD80*	1.185 ± 0.156
CcD102	0.159 ± 0.159	*CcD5*	1.202 ± 0.451
CcD1	0.167 ± 0.114	*CcD18*	1.245 ± 0.163
CcD75	0.208 ± 0.135	*CcD51*	1.297 ± 0.267
CcD11	0.212 ± 0.162	*CcD54*	1.361 ± 0.149
CcD36	0.216 ± 0.194	*CcD57*	1.406 ± 0.240
CcD64	0.235 ± 0.295	*CcD84*	1.449 ± 0.261
CcD4	0.246 ± 0.019	*CcD39*	1.466 ± 0.211
Cc9 [b]	0.296 ± 0.076	*CcD40*	1.694 ± 0.215
CcD95	0.301 ± 0.124	*CcD81*	1.823 ± 0.089
CcD94	0.303 ± 0.142	*CcD104*	1.924 ± 0.038
CcD97	0.339 ± 0.064	*CcD106*	1.926 ± 0.087
CcD76	0.372 ± 0.117	*CcD10*	1.969 ± 0.188
CcD63	0.391 ± 0.109	*CcD101*	1.970 ± 0.166
CcD34	0.393 ± 0.181	*CcD3*	2.063 ± 0.096
CcD38	0.416 ± 0.162	*CcD105*	2.136 ± 0.219
CcD89	0.437 ± 0.132	*CcD13*	2.205 ± 0.520
Cc5 [b]	0.439 ± 0.140	*CcD58*	2.209 ± 0.227
CcD7	0.502 ± 0.139	*CcD37*	2.243 ± 0.073
CcD66	0.512 ± 0.253	*CcD25*	2.243 ± 0.278
CcD43	0.530 ± 0.101	*CcD69*	2.272 ± 0.467
CcD53	0.569 ± 0.258	*CcD73*	2.321 ± 1.175
Cc2 [b]	0.589 ± 0.156	*CcD52*	2.366 ± 0.324
Cc3 [b]	0.636 ± 0.059	*CcD6*	2.514 ± 0.166
CcD16	0.662 ± 0.126	*CcD44*	2.736 ± 0.321
CcD96	0.808 ± 0.221	*CcD20*	2.801 ± 0.176
CcD68	0.833 ± 0.247	*CcD47*	3.042 ± 0.309
Cc7 [b]	0.853 ± 0.124		

Table 1. Serum sensitivity of *C. canimorsus* strains

[a] Log_{10} reduction and standard deviations analyzed after 3 h of incubation with NHS.

[b] Human case strains are highlighted in yellow.

Strains reduced by less than 1 log_{10} were considered to be serum resistant. Means and standard deviations of at least three independent experiments are shown.

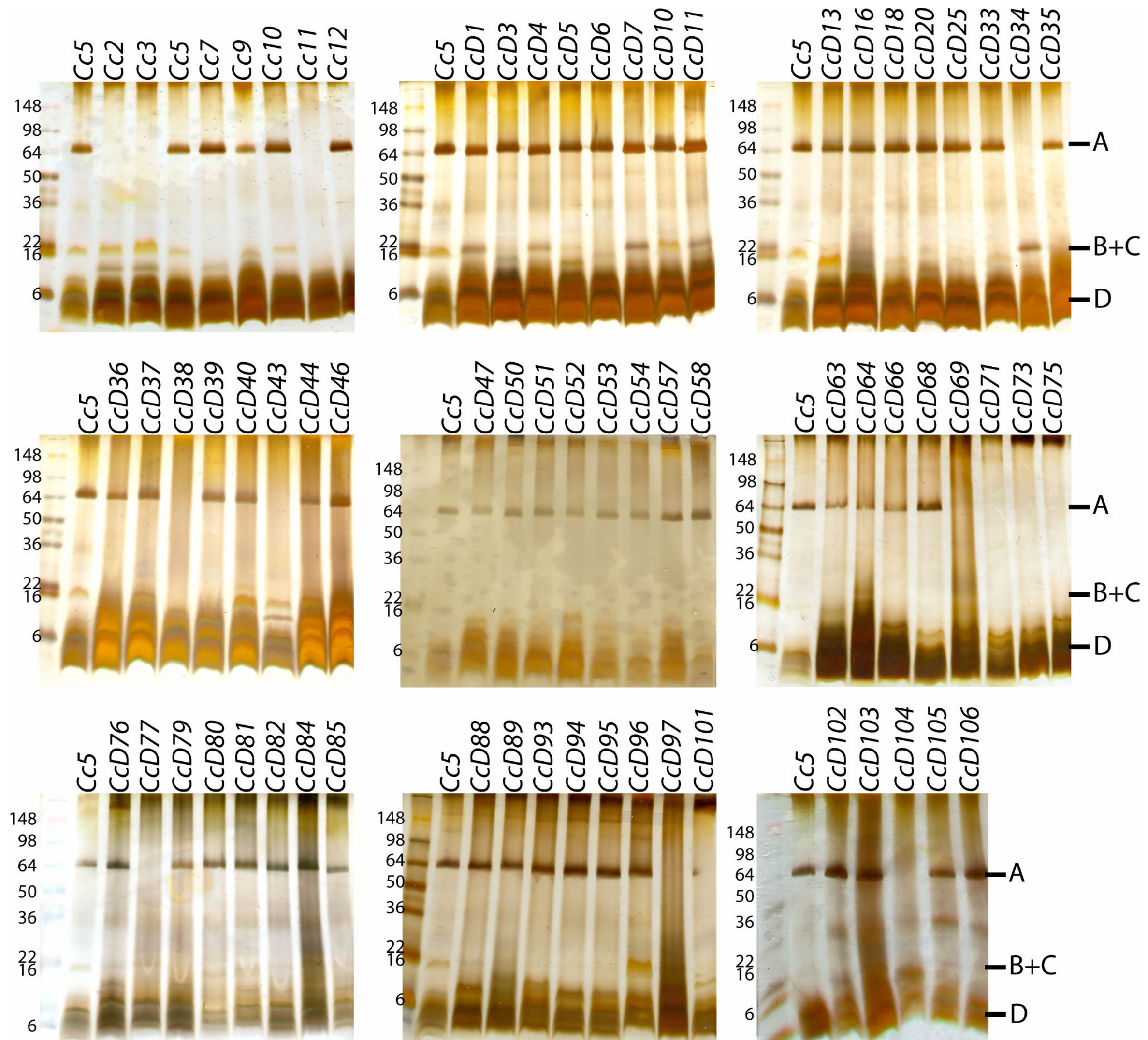


Figure 1. ***C. canimorsus*** **strain heterogeneity in outer membrane structure**

Silver periodic acid staining analysis of Tricine gel electrophoresis of proteinase K-treated *C. canimorsus* strains.

5.4. Materials and methods

Bacterial strains and media

Bacteria were grown as described (Shin *et al.*, 2007).

Selective agents

To select for *C. canimorsus* strains, Gm was added to a concentration of 20 μg/ml.

Serum sensitivity

Serum sensitivity was determined as published (Shin *et al.*, 2009). Means and standard deviations of at least three independent experiments are shown.

Preparation of proteinase K-resistant samples

Samples were prepared as described (Shin *et al.*, 2009) and loaded onto a 16% Tricine-SDS-PA gel. Analysis was done by immunoblotting with α-*Cc5* Ab (Shin *et al.*, 2009) or silver-periodic acid staining (Tsai and Frasch, 1982).

5.5. Discussion

As we observed that *Cc5* resists killing by human complement (Shin *et al.*, 2009), we were interested whether serum resistance is a common trait in *C. canimorsus* or whether this capacity is linked to virulence. Previous studies on the serum sensitivity of oral *Capnocytophaga* spp. isolates indicated that these bacteria might be sensitive to the classical complement pathway (Wilson *et al.*, 1985). Additionally, it has been shown that blood derived strains displayed a higher serum resistance compared to oral isolates, coming along with different electrophoretic mobility of LPS (Wilson *et al.*, 1987). This suggested that indeed serum resistance might be a virulence trait in *Capnocytophaga* spp.. We observed that 100% of the human case strains, but only 54% of the dog isolates were highly resistant to killing by human complement. These findings indicated that even if serum resistance is widespread among *C. canimorsus* strains, it is necessary for establishing an infection in humans. As our findings are in good agreement with a previous study on blood-derived *Capnocytophaga* spp. strains (Wilson *et al.*, 1987), this suggested that serum resistance is not necessary for survival of *Capnocytophaga* spp. in their natural habitat, but is an important virulence trait.

Gram-negative bacteria, including closely related *P. gingivalis* (Slaney *et al.*, 2006), are well known to prevent killing by human complement by virtue of their polysaccharidic structures, either by steric hindrance of MAC deposition or by binding fH, eventually through decoration with sialic acid residues (Frank *et al.*, 1987; Rautemaa and Meri, 1999). As our data suggested that *Cc5* rather sterically prevents MAC deposition (Shin *et al.*, 2009), we analyzed the polysaccharidic outer structures of *C. canimorsus* human case strains and isolates from healthy dogs by silver periodic acid staining and immunoblotting of proteinase K-treated

samples. Interestingly, *C. canimorsus* strains displayed a high polysaccharidic outer structure heterogeneity, which did not obviously correlate with serum resistance. More detailed analysis of operons involved in LPS or capsule formation and hypothetical sialic acid decoration is therefore needed to clarify the origin of serum resistance in *C. canimorsus*.

Chapter 6

Addendum:

YadA confers NET sensitivity to *Y. enterocolitica*

6.1. Summary

Studying the interaction of *Cc5* with NETs, we also observed that *Y. enterocolitica* wt bacteria were readily trapped and killed within NETs, whereas *Y. enterocolitica* ∆YadA bacteria were neither trapped nor killed by NETs, even if both bacteria induced formation of NETs *in vitro*. Binding of *Y. enterocolitica* to NETs was found to be dependent on the collagen binding sites of *Yersinia* adhesin A (YadA). In addition, we could provide evidence for the presence of collagen within or closely associated to NET structures. These findings indicated that binding to NETs is essential for rendering the bacteria accessible to AMPs present on the DNA backbone of the NETs. As it has been discovered previously that YadA only exists as a pseudogene in *Yersinia pestis*, which increased the virulence of these bacteria (Rosqvist *et al.*, 1988), we determined NET-sensitivity of *Y. pestis*. Interestingly, *Y. pestis* EV76 bacteria were resistant to killing by NETs but became sensitive upon the expression of *yadA in trans*. These observations provide an example that virulence factors may always have a cost for the pathogen, thereby displaying the close evolutionary relation between pathogens and their virulence factors on side and the human immune system on the other side.

6.2. Manuscript in preparation

Oligomeric coiled-coil adhesin YadA is a double-edged sword

Manuscript in preparation

Salome Casutt-Meyer and Guy R. Cornelis

Statement of my work. My contribution included all data; *In vitro* induction of NET formation upon infection of human PMNs and sensitivity of bacteria to NET-dependent killing. In addition, I analyzed the presence of collagen in NETs and the serum resistance of the different strains.

Abstract

YadA (*Yersinia* adhesin A) is an important virulence factor for the food-borne pathogens *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. Surprisingly, *yadA* is a pseudogene in *Yersinia pestis*. Even more intriguing, the introduction of a functional *yadA* gene correlates with a decrease of the virulence of *Y. pestis*. Here we show that YadA makes *Yersinia* sensitive to killing by the neutrophil extracellular traps (NETs) produced by leukocytes. By providing an explanation as for why evolution selected for the inactivation of *yadA* in *Y. pestis*, this observation clarifies an old enigma. It also illustrates the complexity of host-pathogen interactions.

Adhesins are adherence factors essential to the virulence of many pathogenic bacteria (1). YadA (*Yersinia* adhesin A) from the food-borne pathogens *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* is the archetype of a family of coiled-coil oligomeric protein adhesins also encountered in *Neisseria* (NadA), *Moraxella* (UspAs), *Haemophilus* (Hia) and *Bartonella* (BadA) (2). It is encoded on the same virulence plasmid as the type-III secretion system (T3SS) and its expression is co-regulated with that of the T3SS, suggesting an important role in pathogenesis (3). Indeed, YadA promotes docking to animal cells and hence facilitates T3S. It also confers adherence to extracellular matrix components, the capacity to invade non-phagocytic cells, resistance to phagocytosis by polymorphonuclear leukocytes (PMNs) and resistance to complement (4). It thus came as a surprise that *yadA* is a pseudogene in the flea-borne *Yersinia pestis*, the causative agent of plague (5, 6). Even more intriguing, the introduction of a functional *yadA* gene (originally called *yopA*) correlates with a decrease of the virulence of *Y. pestis* in a mouse model (5). These results imply that YadA imposes a cost to *Yersinia* in the host, which led to its disappearance by natural selection. The cost of this was however, not known so far.

Here we show that YadA makes *Yersinia* sensitive to killing by neutrophil extracellular traps (NETs) (7). Upon contact with human PMNs, *Y. enterocolitica* E40 bacteria induced the formation of NETs (Fig. 1A and S1A) and became trapped in fibers (Fig. 1A). In agreement with this, ~40% of bacteria were killed (Fig. 1C). *yadA* knockout (ΔYadA) *Y. enterocolitica* E40 bacteria also induced the

formation of NETs (Fig. 1B and S1A) but in contrast to wt bacteria, they were neither trapped (Fig. 1B) nor killed (Fig. 1C). This experiment revealed, for the first time, that YadA has some negative effect for the pathogen. To test whether this negative effect could help explaining why *yadA* is a pseudogene in *Y. pestis* EV76, we set out to test if the original YadA from *Y. pestis* had this property. We expressed YadA from a *Y. pseudotuberculosis* strain (94% similar to frame-shift corrected YadA from *Y. pestis* EV76) (YadA$_{\Psi tb}$) in ΔYadA *Y. enterocolitica* and found that indeed, ΔYadA *Y. enterocolitica* E40 bacteria expressing YadA$_{\Psi tb}$ regained the sensitivity to NETs (Fig. 1C). Consistently, wt *Y. pestis* EV76 were resistant to attachment (Fig. S1B) and killing by NETs but they became sensitive upon introduction of the functional *yadA* gene from *Y. enterocolitica* E40 (*yadA*$_{E40}$) or from *Y. pseudotuberculosis* (*yadA*$_{\Psi tb}$) (Fig. 1C). Since the scanning electron micrographs (Fig. 1A, B) suggested that sensitivity to NETs correlates with attachment, we investigated whether NETs contain substrates to which YadA is known to bind. We found that NETs contain collagen (Fig. 1D), and to test whether collagen was responsible for the attachment of YadA to NETs, we substituted from YadA$_{E40}$ six of the eight NSVAIG-S sites required for collagen binding (8). Substitution of these sites did not affect folding and insertion into the outer membrane since ΔYadA *Y. enterocolitica* expressing the modified YadA proteins were resistant to killing by human serum (Fig. S1D). Likewise, in an electrophoretic analysis, the modified YadA proteins behaved like the wt YadA multimers (not shown). However, none of the truncated adhesins conferred NET sensitivity, indicating a strong correlation between the ability of YadA to bind to collagen and to promote sensitivity to NETs (Fig. 1C).

In conclusion, these observations demonstrate that adherence to collagen has a cost, which may explain why *yadA* is a pseudogene in *Y. pestis*. They also show that virulence factors can be double-edged swords, illustrating the complexity of host-pathogen interactions.

References

1. K. A. Kline, S. Falker, S. Dahlberg, S. Normak, B. Henriques-Normak, *Cell host & microbe* 5, 580 (2009).
2. D. Linke, T. Riess, I. B. Autenrieth, A. Lupas, V. A. Kempf, *Trends Microbiol* 14, 264, (2006).

3. G. R. Cornelis, *Nat Rev Mol Cell Biol* 3, 742 (2002).
4. Y. El Tahir, M. Skurnik, *Int J Med Microbiol* 291, 209 (2001).
5. R. Rosqvist, M. Skurnik, H. Wolf-Watz, *Nature* 334, 522 (1988).
6. P. S. Chain *et al., PNAS* 101, 13826 (2004).
7. V. Brinkmann *et al., Science* 303, 1532 (2004).
8. Y. El Tahir, P. Kuusela, M. Skurnik, *Mol Microbiol* 37, 192 (2000).

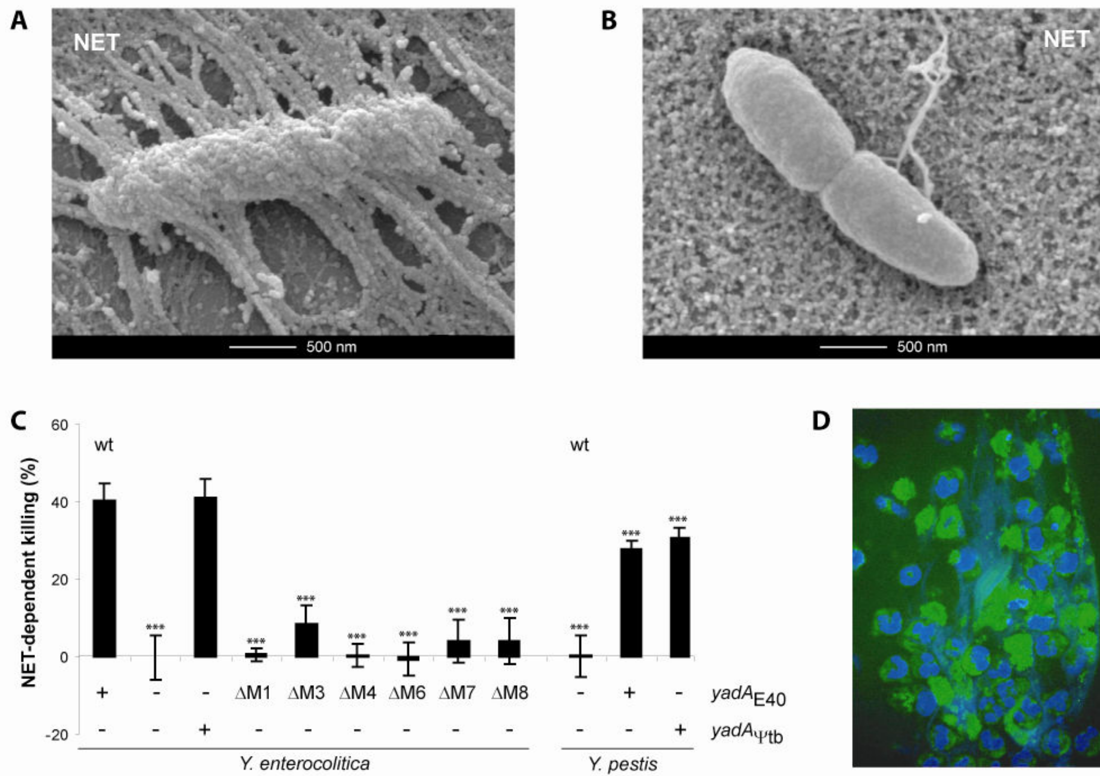
Acknowledgements

We thank C. Paroz for technical assistance, M. Amstutz for the generous gift of plasmid pMA1, E. Bieler and M. Düggelin for the scanning electron microscope analysis, J. Frey and P. Pilo (Swiss Federal Reference Center for anthrax, tularaemia, plague and brucellosis, NANT, Berne) for certification of the EV76 strain of *Y. pestis*, R. Landmann, N. Jann, A. Gupta, S. Ittig and C. Kasper for advice, U. Jenal and D. Bumann for critical reading of the MS. This work was supported by the Swiss National Science Foundation (grant 310000-113333).

Legend to Fig. 1

YadA renders *Y. enterocolitica* and *Y. pestis* sensitive to NET-dependent killing. **(A)** Scanning electron micrograph (SEM) of *Y. enterocolitica* E40 wt bacteria (expressing YadA) trapped in NETs after 120 min infection at an moi of 1. **(B)** *Y. enterocolitica* E40 ΔYadA bacteria induce NET formation but are not trapped (same conditions as in A) (SEM). **(C)** % of *Y. enterocolitica* E40 and *Y. pestis* EV76, expressing $YadA_{E40}$, $YadA_{E40}$ depleted from one of the eight NSVAIG-S motifs (M1, M3, M4, M6, M7, M8) or $YadA_{\Psi tb}$, killed by PMA-triggered NETs (120 min infection at an moi of 1). Phagocytosis was prevented by the addition of Cytochalasin D. Mean values from three or more experiments and standard deviations are shown. Statistical significance is shown with *** $p < 0.001$ using one-way ANOVA. Statistical significance is shown in comparison to wt *Y. enterocolitica* for all *Y. enterocolitica* strains and in comparison to wt *Y. pestis* for all *Y. pestis* strains. **(D)** Immunofluorescence analysis of NETs formed upon infection of PMNs with *Y. enterocolitica* E40 (120 min at an moi of 1). DNA (blue) and collagen (green).

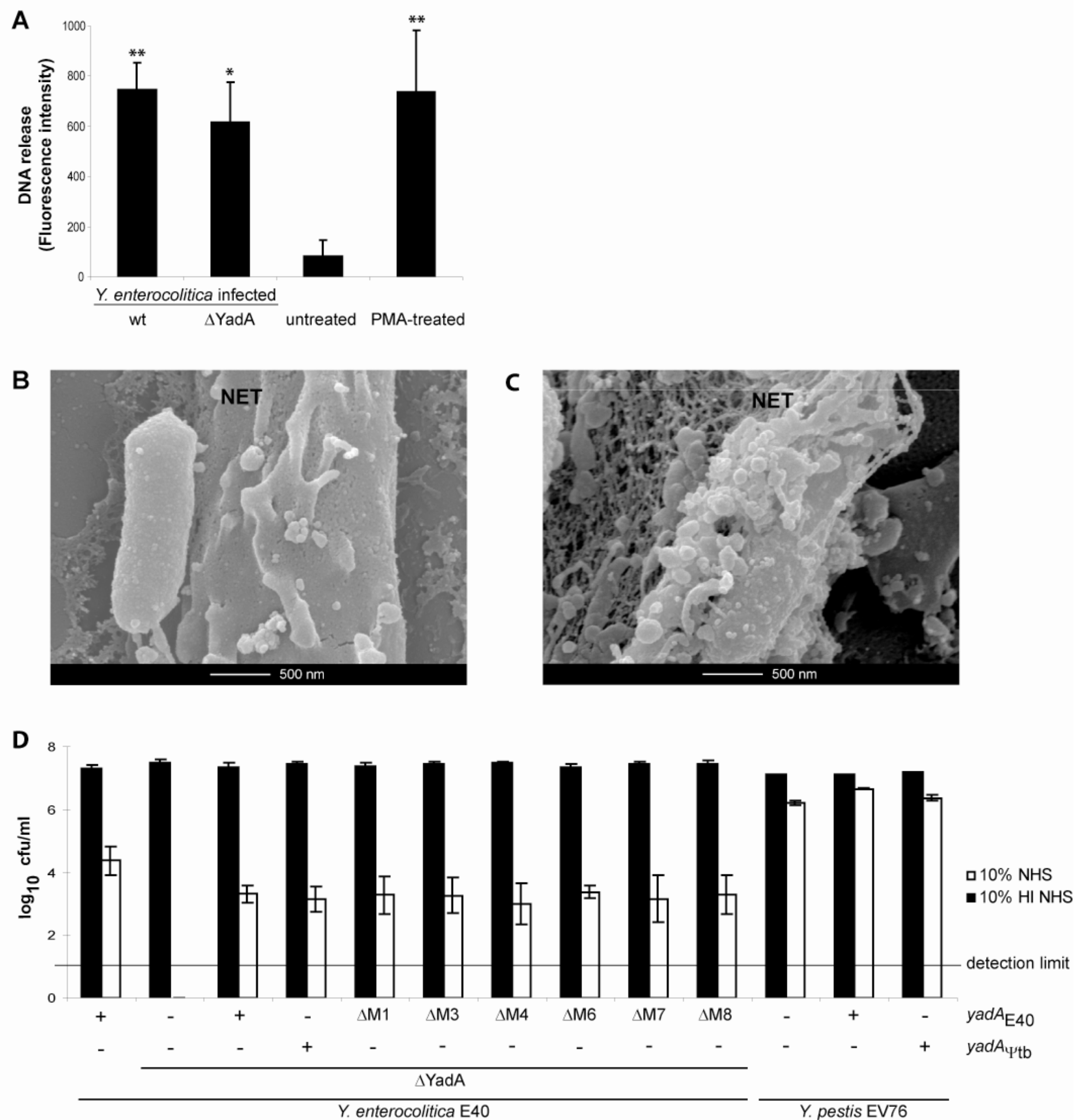
Figure 1



Supporting Online Material

Consists of:

- **Figure S1**
- **Legend to Figure S1**
- **Materials and Methods**
- **Table S1**



Legend to Fig. S1

(A) *Y. enterocolitica* induces DNA release upon infection of PMNs. PMNs were infected for 120 min with *Y. enterocolitica* E40 wt or ΔYadA at an moi of 1. DNA release was quantified by Sytox staining. Untreated PMNs were used as negative control and NET formation was induced by PMA as positive control. Mean values from three or more experiments and standard deviation are shown including statistical significance in comparison to untreated PMNs with ** $p < 0.01$ and * $p < 0.05$ using one-way ANOVA. **(B)** *Y. pestis* EV76 wt bacteria (do not express YadA) induce NET formation but are not trapped (SEM, same conditions as in A).

(C) *Y. pestis* EV76 bacteria expressing $YadA_{E40}$ from *Y. enterocolitica* E40 trapped in NETs (same conditions as in B). **(D)** YadA confers serum resistance to *Y. enterocolitica* E40 even if one of the collagen-binding sites is removed. *Y. pestis* resists complement without YadA. Total cfu present after incubation of bacteria with either NHS (white) or heat-inactivated NHS (black) for 180 min.

Materials and Methods

Bacterial strains and media. Bacterial strains are listed in **Table S1a**. *Y. enterocolitica* and *Y. pestis* were grown as in ref (1). Expression of YadA was induced with 0.2% arabinose for 60 min at 37°C before infection of PMNs.

Site-directed mutagenesis of NSVAIG-S motifs of YadA from Y. enterocolitica was performed by PCR at sites indicated in ref (2). Primers are given in **Table S1b**.

Human PMNs were isolated from healthy volunteers using the Dextran-Percoll protocol, adapted with modifications from ref (3).

NET-dependent attachment and killing was adapted from ref (4).

Quantification of DNA released by activated neutrophils was adapted from ref (5).

Scanning electron microscopy was adapted from ref (5).

Immunofluorescence. Freshly isolated human PMNs were resuspended in RPMI 1640 (Gibco) supplemented with 2% Ab-depleted pooled human serum (Scipac Ltd.) and 2% L-glutamine (Gibco) and seeded on FalconTM culture slides (Becton Dickinson) coated with 0.001% poly-D-lysine (5×10^5 cells/well). PMNs were infected for 120 min with *Y. enterocolitica* wt bacteria at an moi of 1, washed with D-PBS (Gibco), fixed with 4% paraformaldehyde for 60 min at 37°C and then blocked overnight at 4°C with D-PBS containing 3% BSA. Collagen was labelled with mouse anti-human collagen type I antibody (1:1000; Sigma). FITC-conjugated secondary antibody (1:200; Southern Biotech) and Hoechst DNA staining dye (1:10000; Sigma) were added and slides were incubated for 30 min at RT, washed 4 times with D-PBS, mounted with antifade reagent (Vector Laboratories) and analyzed on an Olympus IX81F-3 microscope mounted with a high speed Yokogawa spinning head.

Serum sensitivity was determined as in ref (6).

References

1. N. Grosdent, I. Marridonneau-Parini, M. P. Sory, G. R. Cornelis, *Infection and Immunity* 70, 4165 (2002).
2. Y. El Tahir, P. Kuusela, M. Skurnik, *Mol Microbiol* 37, 192 (2000).
3. L. V. Jepsen, T. Skottun, Scandinavian journal of clinical and laboratory investigation 42, 235 (1982).
4. C. F. Urban, U. Reichard, V. Brinkmann, A. Zychlinsky, *Cell Microbiol* 8, 668, (2006).
5. A. K. Gupta, P. Hasler, W. Holzgreve, S. Gebhardt, S. Hahn, *Hum Immunol* 66, 1146 (2005).
6. G. Balligand, Y. Laroche, G. Cornelis, *Infection and immunity* 48, 782 (1985).

TABLE S1a: Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Description	Reference
Y. enterocolitica		
E40	Serotype O:9, wt	(Sory and Cornelis, 1994)
ΔYadA	isogenic knockout mutant (pLJM4029, *yadA::pLJM31*), Sm^R	(Mota *et al.*, 2005)
Y. pestis EV76	pla^+, $caf1^+$, $pCD1^+$ (70-kb virulence plasmid), Δpgm	(Rosqvist *et al.*, 1988)
Y. pseudotuberculosis	Serotype 1B wt, isolated from a hare	G Wauters 13215/72
Expression plasmids		
pMA1	Full-length *yadA* from *Y. enterocolitica* W227 cloned into pBAD digested with *Nco*I and *Eco*RI, Ara^I, Amp^R	This study
pSAM16	Full-length *yadA* from *Y. pseudotuberculosis*, cloned into pBAD digested with *Nco*I and *Eco*RI, Ara^I, Amp^R	This study
pSAM17	pBAD expressing full-length YadA from *Y. enterocolitica* W227 including mutation M1, Ara^I, Amp^R	This study
pSAM18	pBAD expressing full-length YadA from *Y. enterocolitica* W227 including mutation M3, Ara^I, Amp^R	This study
pSAM19	pBAD expressing full-length YadA from *Y. enterocolitica* W227 including mutation M4, Ara^I, Amp^R	This study
pSAM20	pBAD expressing full-length YadA from *Y. enterocolitica* W227 including mutation M6, Ara^I, Amp^R	This study
pSAM21	pBAD expressing full-length YadA from *Y. enterocolitica* W227 including mutation M7, Ara^I, Amp^R	This study
pSAM22	pBAD expressing full-length YadA from *Y. enterocolitica* W227 including mutation M8, Ara^I, Amp^R	This study

TABLE S1b: Oligonucleotides used in this study

Oligonucleotide	5'-3' sequence, mutated codons in bold	Purpose	Plasmid
5114	GGAATTCTTACCACTCGATATTAAATGATGCGT	PCR, cloning	pSAM16
5115	GACCATGGCCACTAAAGATTTTAAGATCAGTGTCTC	PCR, cloning	pMA1, pSAM16-pSAM22
5116	ACC**T**TC**G**TC**T**TCGTCATGGATACCCTTAGCGCTACA	mutator	M1
5117	CATGACGA**A**GA**C**GA**A**GGTGCTACTGCTGAAGCAGCG	mutator	M1
5118	ACC**C**TC**A**TCATCATCATTAACGCCTGTTGCAATTGAACC	mutator	M3
5119	AATGATGATGA**T**GA**A**GGTCCTTTAAGTAAGGCATTG	mutator	M3
5120	ACC**C**ACATC**A**TCTCCATCTTTCTGGGCGGTACT	mutator	M4
5121	GATGGAGA**T**GATGA**G**GGTGCGAGAGCATCAACTTCA	mutator	M4
5122	TCC**T**TCGTCATCAGAGTTTTTTGCATCAGCTTT	mutator	M6
5123	AACTCTGATGACGA**A**GGACACTCTAGTCACGTTGCG	mutator	M6
5124	CCC**T**TC**A**TC**T**TCTGAATAACCATGATTTGCCGC	mutator	M7
5125	TATTCAGA**A**GA**T**GAAGGGGATCGTTCTAAAACTGAC	mutator	M7
5126	ACC**T**TCGGA**A**TCACTATTTTCTCGGTCAGTTTT	mutator	M8
5127	AATAGTGA**T**TCCGA**A**GGTCATGAAAGCCTTAATCGC	mutator	M8
4561	GGAATTCTTACCACTCGATATTAAATGATGCAT	PCR, cloning	pMA1, pSAM17-pSAM22

Appendix

Abbreviations

Antibody	Ab
Antimicrobial peptide	AMP
Ampicillin resistance	Amp^R
Arabinose inducible	Ara^I
Bovine serum albumin	BSA
C4b-binding protein	C4BP
Capnocytophaga canimorsus	*C. canimorsus*
Capnocytophaga canimorsus 5	*Cc5*
Capnocytophaga canimorsus 5 ∆pitrilysin	*∆pit*
Capnocytophaga canimorsus 5 ∆pitrilysin complemented	c*∆pit*
Capnocytophaga canimorsus dog isolate	*CcD*
Capnocytophaga canimorsus human case isolate	*Cc*
Capnocytophaga cynodegmi	*C. cynodegmi*
Capnocytophaga gingivalis	*C. gingivalis*
Capnocytophaga ochracea	*C. ochracea*
Capnocytophaga sputigena	*C. sputigena*
Cefoxitine resistance	Cf^R
Chloramphenicol resistance	Cm^R
Colony forming unit	cfu
Complement receptor	CR
Conditioned	cond.
Cytochalasin D	Cyt. D
Dihydrorhodamine 1.2.3	DHR
Dysogenic fermenter	DF
Enhanced green fluorescence protein	eGFP
Erythromycin resistance	Em^R
Escherichia coli	*E. coli*
Factor H	fH
Fc receptor	FcR
Fetal calf serum	FCS
Gentamicin resistance	Gm^R

Glycosyltransferase	gtf
Group A *Streptococci*	GAS
Heat-inactivated	HI
Helicobacter pylori	*H. pylori*
Hour(s)	h
Hydrogen peroxide	H_2O_2
Hypochlorous acid	HOCl
Immunoglobulin	Ig
Interleukin	IL
Kanamycin resistant	Kan^R
Kilo Dalton	kDa
Lipopolysaccharide	LPS
Lysosome associated protein 1	LAMP-1
Mannose-binding lectin	MBL
Mass spectrometry	MS
Membrane attack complex	MAC
Minute(s)	min
Molecular oxygen	O_2
Multiplicity of infection	moi
Myeloperoxidase	MPO
Nalidixic acid resistance	Na^R
Neomycin resistant	Neo^R
Neutrophil extracellular trap	NET
Nicotinamide adenine dinucleotide phosphate (reduced)	NADPH
Open reading frame	ORF
Overnight	o/n
Paraformaldehyde	PFA
Pattern recognition receptor	PRR
Phorbol myristate acetate	PMA
Polymorphonuclear neutrophil	PMN
Pooled normal human serum	NHS
Porphyromonas gingivalis	*P. gingivalis*
Protein kinase C	PKC
Room temperature	RT

Rounds per minute	rpm
Salmonella enterica	*S. enterica*
Scanning electron microscopy	SEM
Staphylococcus aureus	*S. aureus*
Streptococcus pneumoniae	*S. pneumoniae*
Streptococcus pyogenes	*S. pyogenes*
Streptomycin resistance	Sm^R
Superoxide	O_2^-
Superoxide dismutase	SOD
Tetracycline resistance	Tet^R
Toll-like receptor	TLR
Transposon	Tn
Wild-type	wt
Yersinia adhesin A	YadA
Yersinia enterocolitica	*Y. enterocolitica*
Yersinia pestis	*Y. pestis*
Yersinia pseudotuberculosis	*Y. pseudotuberculosis*

Strains and Plasmids

Table 1. Strains

Bacterial strain	Genotype or description	Reference or source
E. coli		
Top10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*)7697 *gal*U *gal*K *rps*L (Sm^R) *end*A1 *nup*G	Invitrogen
C. canimorsus		
Cc2	Human fatal septicaemia after dog bite 1989	(Hantson *et al.*, 1991)
Cc3	Human septicaemia	(Vanhonsebrouck *et al.*, 1991)
Cc5	Human fatal septicaemia after dog bite 1995	(Shin *et al.*, 2007)
Cc5 Y1C12	*Cc5* Tn mutant, Tn inserted into *gtf* gene	(Shin *et al.*, 2009)
Cc5 Δ1980	*Cc5* knockout mutant of ORF #1890	This study
Cc5 Δpit	*Cc5* knockout mutant of ORF #958 (pitrilysin)	This study
Cc7	Human septicaemia 1998	(Shin *et al.*, 2007)
Cc9	Human septicaemia 1965	(Vandamme *et al.*, 1996)
Cc10	Human septicaemia after dog bite	(Vandamme *et al.*, 1996)
Cc11	Human septicaemia (BCCM/LMG 11551	A. von Graevenitz, Univ.

	MCCM 01373)	Zurich, Switzerland
Cc12	Human septicaemia after dog bite	ATCC 35979, CDC 7120
CcD1	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD3	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD4	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD5	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD6	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD7	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD10	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD11	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD13	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD16	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD18	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD20	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD25	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD33	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD34	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD35	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD36	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD37	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD38	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD39	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD40	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD43	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD44	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD46	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD47	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD50	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD51	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD52	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD53	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD54	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD57	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD58	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD63	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD64	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD66	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD68	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD69	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD71	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD73	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD75	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD76	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD77	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD79	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)

CcD80	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD81	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD82	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD84	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD85	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD88	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD89	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD93	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD94	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD95	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD96	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD97	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD101	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD103	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
CcD104	Isolate from healthy dog's saliva; formerly known as *Cc13*	(Shin *et al.*, 2007)
CcD105	Isolate from healthy dog's saliva; formerly known as *Cc14*	(Shin *et al.*, 2007)
CcD106	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
C. cynodegmi		
Ccy19	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
Ccy46	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
Ccy74	Isolate form healthy dog's saliva	(Mally *et al.*, 2009)
S. enterica		
wt	Serovar Typhimurium, strain SL1344, *aroA*	(Hoiseth and Stocker, 1981)
∆rfaG	Serovar Typhimurium, strain SL1344, *aroA, rfaG*	(Shin *et al.*, 2009)
Y. enterocolitica		
E40	Serotype O:9, wt	(Sory and Cornelis, 1994)
∆YadA	Isogenic knockout mutant (pLJM4029, *yadA::pLJM31*), Sm^R	(Mota *et al.*, 2005)
∆YscN	Deletion mutant $YscN^{\Delta 169\text{-}177}$	(Woestyn *et al.*, 1994)
Y. pestis		
EV76	pla^+, $caf1^+$, $pCCD1^+$ (70-kb virulence plasmid), ∆*pgm*	(Rosqvist *et al.*, 1988)
Y. pseudotuberculosis		
Clinical isolate	Serotype 1B wt, isolated from a hare	G. Wauters 13215/72

Table 2. Plasmids

Plasmid	Description	Reference or source
pBAD	Expression vector; Ara^I, Amp^R; Myc and HisA tags	Invitrogen
peGFP-N1	Expression vector for eGFP; Kan^R, Neo^R	Clonetech
pEP4351	*pir* requiring R6K *oriV*; RP4 *oriT*; Cm^R, Tet^R, Em^R	(Cooper *et al*., 1997)
pYVe227	Wt virulence plasmid from strain *Y. enterocolitica* W22703	NC_002120
pMA1	YadA from *Y. enterocolitica* pYVe227; 4560 + 4561 amplified gene cut with *Nco*I and *Eco*RI and inserted into the corresponding sites of pBAD	This study
pMM13	ColE1 *ori*; Amp^R (Em^R)	(Mally and Cornelis, 2008)
pMM25	ColE1 *ori*; Km^R (Cf^R); Suicide vector for *C. canimorsus*.	(Mally and Cornelis, 2008)
pMM47	ColE1 *ori* (pCC7 *ori*); Amp^R (Cf^R); *E. coli – C. canimorsus* expression shuttle plasmid	(Mally and Cornelis, 2008)
pSAM16	YadA from *Y. pseudotuberculosis*; 5113 + 5114 amplified gene cut with *Nco*I and *Eco*RI and inserted into the corresponding sites of pBAD	This study
pSAM17	YadA from *Y. enterocolitica* pYVe227 including mutation M1; amplified by overlapping PCR using primer pairs 5115 + 5116 and 5117 + 4561, followed by 5115 + 4561; amplified gene cut with *Nco*I and *Eco*RI and inserted into the corresponding sites of pBAD	This study
pSAM18	YadA from *Y. enterocolitica* pYVe227 including mutation M3; amplified by overlapping PCR using primer pairs 5115 + 5118 and 5119 + 4561, followed by 5115 + 4561; amplified gene cut with *Nco*I and *Eco*RI and inserted into the corresponding sites of pBAD	This study
pSAM19	YadA from *Y. enterocolitica* pYVe227 including mutation M4; amplified by overlapping PCR using primer pairs 5115 + 5120 and 5121 + 4561, followed by 5115 + 4561; amplified gene cut with *Nco*I and *Eco*RI and inserted into the corresponding sites of pBAD	This study
pSAM20	YadA from *Y. enterocolitica* pYVe227 including mutation M6; amplified by overlapping PCR using primer pairs 5115 + 5122 and 5123 + 4561, followed by 5115 + 4561; amplified gene cut with *Nco*I and *Eco*RI and inserted into the corresponding sites of pBAD	This study
pSAM21	YadA from *Y. enterocolitica* pYVe227 including mutation M7; amplified by overlapping PCR using primer pairs 5115 + 5124 and 5125 + 4561, followed by 5115 + 4561; amplified gene cut with *Nco*I and *Eco*RI and inserted into the corresponding sites of pBAD	This study
pSAM22	YadA from *Y. enterocolitica* pYVe227 including mutation M8; amplified by overlapping PCR using primer pairs 5115 + 5126 and 5127 + 4561, followed by 5115 + 4561; amplified gene cut with *Nco*I and *Eco*RI and inserted into the corresponding sites of pBAD	This study

pSI02	ColE1 *ori*; Km^R (Cf^R); To create a *pit::ermF* three initial PCR products were amplified with 5100+5101 and 5102+5103 from *Cc5* chromosomal DNA and 5104+5105 from pMM13. *pit::ermF* was then amplified by overlapping PCR using external primers 5100+5105, cut with *Pst*I and *Spe*I and inserted into corresponding sites of pMM25	This study
pSI03	ColE1 *ori*; Km^R (Cf^R); To create a *1980::ermF* three initial PCR products were amplified with 5094+5095 and 5096+5097 from *Cc5* chromosomal DNA and 5098+5099 from pMM13. *1980::ermF* was then amplified by overlapping PCR using external primers 5094+5099, cut with *Pst*I and *Spe*I and inserted into corresponding sites of pMM25	This study
pSI04	Pitrilysin gene from *Cc5* (ORF #958) amplified using primer pair 5213 + 5214 and cut with *Nco*I and *Xba*I, inserted into the corresponding sites of pMM47	This study

Oligonucleotides

Table 2. Oligonucleotides

MIPA #	Sequence 5'-3' sequence, mutated codons in bold	Reference
5094	CACTGCAGGCTTCTGCGGTAACAGTAGG	This study
5095	GAGTAGATAAAAGCACTGTTCAATGCTTGCGTAACCTAC	This study
5096	CACAAGCAGGTGTAGGTTACGCAAGCATTGAACAGTGCTTTTATCTACTCCGATAGCTTC	This study
5097	CATCATTGTCTCTGTCTCTGTGAGGACAACCCTACGAAGGATGAAATTTTTCAGGGACAAC	This study
5098	AAAAATTTCATCCTTCGTAGGGTTGTCCTCACAGAGACAG	This study
5099	CAACTAGTCAAGTTGATTTCAACACGACGG	This study
5100	CACTGCAGGAAGGGACATTTATTAACTGC	This study
5101	GAGTAGATAAAAGCACTGTTGTACGTGTAGGGTCTTCGTC	This study
5102	TGGGGCAAAAGACGAAGACCCTACACGTACAACAGTGCTTTTATCTACTCCGATAGCTTC	This study
5103	GTTTTATAGGCTCTTCTTGAATATCCACTCCTACGAAGGATGAAATTTTTCAGGGACAAC	This study
5104	AAAAATTTCATCCTTCGTAGGAGTGGATATTCAAGAAGAG	This study
5105	CAACTAGTCTGGCAAGTAATCCAAATCC	This study
5114	GGAATTCTTACCACTCGATATTAAATGATGCGT	This study
5115	GACCATGGCCACTAAAGATTTTAAGATCAGTGTCTC	This study
5116	ACC**T**TC**G**TC**T**TCGTCATGGATACCCTTAGCGCTACA	This study
5117	CATGACGA**A**GA**C**GA**A**GGTGCTACTGCTGAAGCAGCG	This study
5118	ACC**C**TC**A**TCATCATCATTAACGCCTGTTGCAATTGAACC	This study
5119	AATGATGATGA**T**GA**A**GGTCCTTTAAGTAAGGCATTG	This study

5120	ACC**C**ACATC**A**TCTCCATCTTTCTGGGCGGTACT	This study
5121	GATGGAGA**T**GATGA**G**GGTGCGAGAGCATCAACTTCA	This study
5122	TCC**T**TCGTCATCAGAGTTTTTTGCATCAGCTTT	This study
5123	AACTCTGATGACGA**A**GGACACTCTAGTCACGTTGCG	This study
5124	CCC**T**TC**A**TC**T**TCTGAATAACCATGATTTGCCGC	This study
5125	TATTCAGA**A**GA**T**GAAGGGGATCGTTCTAAAACTGAC	This study
5126	ACC**T**TCGGA**A**TCACTATTTTCTCGGTCAGTTTT	This study
5127	AATAGTGA**T**TCCGA**A**GGTCATGAAAGCCTTAATCGC	This study
4560	GATCATGGCCACTAAAGATTTTAAGATCAGTGTCTC	This study
4561	GGAATTCTTACCACTCGATATTAAATGATGCAT	This study

Antibodies

Table 3. Antisera

MIPA #	Name	Antigen	Origin	Source
	α-1D4B	LAMP-1	Rat	Homemade, gift from J. Pieters
	AffiniPure α-rat IgG/Cy3 conjugated	Rat IgG	Goat	Jackson Immuno Research, gift from Ch. Dehio
	Lysotracker red	Acidic regions		Invitrogen, gift from J. Pieters
161	α-rabbit IgG/FITC conjugated	Rabbit IgG	Goat	SBA
164	α-goat IgG/HRP conjugated	Goat IgG	Rabbit	DAKO
168	α-rabbit IgG/HRP conjugated	Rabbit IgG	Swine	DAKO
215	α-*Cc5*	HK Cc5	Rabbit	Homemade
243	α-goat IgG/FITC conjugated	Goat IgG	Rabbit	Santa Cruz
246	α-p22^{phox}	p22^{phox}	Rabbit	Santa Cruz
247	α-p47^{phox}	p47^{phox}	Goat	Santa Cruz
248	α-p67^{phox}	p67^{phox}	Goat	Santa Cruz
249	α-gp91^{phox}	gp91^{phox}	Goat	Santa Cruz
640	Hoechst	DNA		Sigma, 10 mg/ml

References

Abo, A., Webb, M.R., Grogan, A., and Segal, A.W. (1994) Activation of NADPH oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane. *Biochem J* **298 Pt 3**: 585-591.

Allen, L.-A.H., DeLeo, F.R., Gallois, A., Toyoshima, S., Suzuki, K., and Nauseef, W.M. (1999) Transient association of the nicotinamide adenine dinucleotide phosphate oxidase subunits $p47^{phox}$ and $p67^{phox}$ with phagosomes in neutrophils from patients with X-linked chronic granulomatous disease. *Blood* **93**: 3521-3530.

Allen, L.-A.H. (2003) Mechanisms of pathogenesis: evasion of killing by polymorphonuclear leukocytes. *Microbes Infect* **5**: 1329-1335.

Allen, L.-A.H., Beecher, B.R., Lynch, J.T., Rohner, O.V., and Wittine, L.M. (2005) *Helicobacter pylori* disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release. *J Immunol* **174**: 3658-3667.

Allen, L.-A.H. (2007) Immunofluorescence and confocal microscopy of neutrophils. *Methods Mol Biol* **412**: 273-287.

Alper, B.J., Nienow, T.E., and Schmidt, W.K. (2006) A common genetic system for functional studies of pitrilysin and related M16A proteases. *Biochem J* **398**: 145-152.

Babior, B.M. (1995) The respiratory burst oxidase. *Curr Opin Hematol* **2**: 55-60.

Bailie, W.E., Stowe, E.C., and Schmitt, A.M. (1978) Aerobic bacterial flora of oral and nasal fluids of canines with reference to bacteria associated with bites. *J Clin Microbiol* **7**: 223-231.

Beachey, E.H., Dale, J.B., Simpson, W.A., Evans, J.D., Knox, K.W., Ofek, I., and Wicken, A.J. (1979) Erythrocyte binding properties of streptococcal lipoteichoic acids. *Infect Immun* **23**: 618-625.

Becker, A.B., and Roth, R.A. (1992) An unusual active site identified in a family of zinc metalloendopeptidases. *PNAS* **89**: 3835-3839.

Beebe, J.L., and Koneman, E.W. (1995) Recovery of uncommon bacteria from blood: association with neoplastic disease. *Clin Microbiol Rev* **8**: 336-356.

Beiter, K., Wartha, F., Albiger, B., Normark, S., Zychlinsky, A., and Henriques-Normark, B. (2006) An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. *Current Biology* **16**: 401-407.

Bennouna, S., Bliss, S.K., Curiel, T.J., and Denkers, E.Y. (2003) Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection. *J Immunol* **171**: 6052-6058.

Blanche, P., Bloch, E., and Sicard, D. (1998) *Capnocytophaga canimorsus* in the oral flora of dogs and cats. *J Infection* **36**: 134.

Bobo, R.A., and Newton, E.J. (1976) A previously undescribed gram-negative bacillus causing septicemia and meningitis. *Am J Clin Pathol* **65**: 564-569.

Born, J., and Bhakdi, S. (1986) Does complement kill *E. coli* by producing transmural pores? *Immunology* **59**: 139-145.

Borregaard, N., Heiple, J.M., Simons, E.R., and Clark, R.A. (1983) Subcellular localization of the *b*-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J Cell Biol* **97**: 52-61.

Borregaard, N., and Cowland, J.B. (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89**: 3503-3521.

Brenner, D.J., Hollis, D.G., Fanning, G.R., and Weaver, R.E. (1989) *Capnocytophaga canimorsus sp. nov*. (formerly CDC group DF-2), a cause of septicemia following dog bite, and *C. cynodegmi sp. nov*., a cause of localized wound infection following dog bite. *J Clin Microbiol* **27**: 231-235.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004) Neutrophil extracellular traps kill bacteria. *Science* **303**: 1532-1535.

Buchanan, J.T., Simpson, A.J., Aziz, R.K., Liu, G.Y., Kristian, S.A., Kotb, M., Feramisco, J., and Nizet, V. (2006) DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. *Curr Biol* **16**: 396-400.

Buu-Hoi, A.Y., Joundy, S., and Acar, J.F. (1988) Endocarditis caused by *Capnocytophaga ochracea*. *J Clin Microbiol* **26**: 1061-1062.

Campanelli, D., Melchior, M., Fu, Y., Nakata, M., Shuman, H., Nathan, C., and Gabay, J.E. (1990) Cloning of cDNA for proteinase 3: a serine protease, antibiotic, and autoantigen from human neutrophils. *J Exp Med* **172**: 1709-1715.

Campbell, J.R., and Edwards, M.S. (1991) *Capnocytophaga* species infections in children. *Pediatr Infect Dis J* **10**: 944-948.

Carlisle, M.D., Srikantha, R.N., and Brogden, K.A. (2009) Degradation of α- and β-defensins by culture supernatants of *Porphyromonas gingivalis* strain 381. *Antimicrob Agents Ch* **52**: 638-642.

Chambers, G.W., and Westblom, T.U. (1992) Pleural infection caused by *Capnocytophaga canimorsus*, formerly CDC group DF-2. *Clin Infect Dis* **15**: 325-326.

Chen, Z., Potempa, J., Polanowski, A., Wikstrom, M., and Travis, J. (1992) Purification and characterization of a 50-kDa cysteine proteinase (gingipain) from *Porphyromonas gingivalis*. *J Biol Chem* **267**: 18896-18901.

Cheng, N., He, R., Tian, J., Dinauer, M.C., and Ye, R.D. (2007) A critical role of protein kinase C delta activation loop phosphorylation in formyl-methionyl-leucyl-phenylalanine-induced phosphorylation of p47(phox) and rapid activation of nicotinamide adenine dinucleotide phosphate oxidase. *J Immunol* **179**: 7720-7728.

Clark, R.A., Volpp, B.D., Leidal, K.G., and Nauseef, W.M. (1990) Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. *J Clin Invest* **85**: 714-721.

Clark, S.R., Ma, A.C., Tavener, S.A., McDonald, B., Zahra, G., Kelly, M.M., Patel, K.D., Chakrabarti, S., McAvoy, E., Sinclair, G.D., Keys, E.M., Allen-Vercoe, E., DeVinney, R., Doig, C.J., Green, F.H., and Kubes, P. (2007) Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* **13**: 463-469.

Coleman, J.E. (1998) Zinc enzymes. *Curr Opin Chem Biol* **2**: 222-234.

Conrads, G., Mutters, R., Seyfarth, I., and Pelz, K. (1997) DNA-probes for the differentiation of *Capnocytophaga* species. *Mol Cell Probe* **11**: 323-328.

Cooper, A.J., Kalinowski, A.P., Shoemaker, N.B., and Salyers, A.A. (1997) Construction and characterization of a *Bacteroides thetaiotaomicron recA* mutant: transfer of *Bacteroides* integrated conjugative elements is RecA independent. *J Bacteriol* **179**: 6221-6227.

Cornista, J., Ikeuchi, S., Haruki, M., Kohara, A., Takano, K., Morikawa, M., and Kanaya, S. (2004) Cleavage of various peptides with pitrilysin from *Escherichia coli*: kinetic analyses using beta-endorphin and its derivatives. *Biosci Biotechnol Biochem* **68**: 2128-2137.

Cramer, E., Pryzwansky, K.B., Villeval, J.L., Testa, U., and Breton-Gorius, J. (1985) Ultrastructural localization of lactoferrin and myeloperoxidase in human neutrophils by immunogold. *Blood* **65**: 423-432.

Criss, A.K., and Seifert, H.S. (2008) *Neisseria gonorrhoeae* suppresses the oxidative burst of human polymorphonuclear leukocytes. *Cell Microbiol*.

Curnutte, J.T. (1993) Chronic granulomatous disease: the solving of a clinical riddle at the molecular level. *Clin Immunol Immunopathol* **67**: S2-15.

de Boer, M.G., Lambregts, P.C., van Dam, A.P., and van't Wout, J.W. (2007) Meningitis caused by *Capnocytophaga canimorsus*: when to expect the unexpected. *Clin Neurol Neurosurg* **109**: 393-398.

de Haas, C.J., Veldkamp, K.E., Peschel, A., Weerkamp, F., Van Wamel, W.J., Heezius, E.C., Poppelier, M.J., Van Kessel, K.P., and van Strijp, J.A. (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J Exp Med* **199**: 687-695.

DeCoursey, T.E., and Ligeti, E. (2005) Regulation and termination of NADPH oxidase activity. *Cell Mol Life Sci* **62**: 2173-2193.

Dinauer, M.C. (1993) The respiratory burst oxidase and the molecular genetics of chronic granulomatous disease. *Crit Rev Clin Lab Sci* **30**: 329-369.

Ding, J., Badwey, J.A., Erickson, R.W., Balazovich, K.J., and Curnutte, J.T. (1993) Protein kinases potentially capable of catalyzing the phosphorylation of p47-phox in normal neutrophils and neutrophils of patients with chronic granulomatous disease. *Blood* **82**: 940-947.

Dire, D.J., Hogan, D.E., and Riggs, M.W. (1994) A prospective evaluation of risk factors for infections from dog-bite wounds. *Acad Emerg Med* **1**: 258-266.

Dusi, S., and Rossi, F. (1993) Activation of NADPH oxidase of human neutrophils involves the phosphorylation and the translocation of cytosolic $p67^{phox}$. *Biochem J* **296 (Pt 2)**: 367-371.

El Benna, J., Faust, L.P., and Babior, B.M. (1994) The phosphorylation of the respiratory burst oxidase component $p47^{phox}$ during neutrophil activation. Phosphorylation of sites recognized by protein kinase C and by proline-directed kinases. *J Biol Chem* **269**: 23431-23436.

El Benna, J., Dang, P.M.-C., Gaudry, M., Fay, M., Morel, F., Hakim, J., and Gougerot-Pocidalo, M.-A. (1997) Phosphorylation of the respiratory burst

oxidase subunit p67phox during human neutrophil activation. *J Biol Chem* **272**: 17204-17208.

Engels, W., Endert, J., and Van Boven, C.P. (1985) A quantitative method for assessing the third complement factor (C3) attached to the surface of opsonized *Pseudomonas aeruginosa*: interrelationship between C3 fixation, phagocytosis and complement consumption. *J Immunol Methods* **81**: 43-53.

Esteban, J., Albalate, M., Caramelo, C., Reyero, A., Carriazo, M.A., Hernandez, J., Ortiz, A., and Soriano, F. (1995) Peritonitis involving a *Capnocytophaga* sp. in a patient undergoing continuous ambulatory peritoneal dialysis. *J Clin Microbiol* **33**: 2471-2472.

Fischer, L.J., Weyant, R.S., White, E.H., and Quinn, F.D. (1995) Intracellular multiplication and toxic destruction of cultured macrophages by *Capnocytophaga canimorsus*. *Infect Immun* **63**: 3484-3490.

Font, R.L., Jay, V., Misra, R.P., Jones, D.B., and Wilhelmus, K.R. (1994) *Capnocytophaga* keratitis. A clinicopathologic study of three patients, including electron microscopic observations. *Ophthalmology* **101**: 1929-1934.

Frank, M.M., Joiner, K., and Hammer, C. (1987) The function of antibody and complement in the lysis of bacteria. *Rev Infect Dis* **9 Suppl 5**: S537-545.

Frey, E., Pressler, B., Guy, J., Pitulle, C., and Breitschwerdt, E. (2003) *Capnocytophaga sp*. isolated from a cat with chronic sinusitis and rhinitis. *J Clin Microbiol* **41**: 5321-5324.

Frieling, J.T.M., Mulder, J.A., Hedriks, T., Curfs, J.H., van der Linden, C. J., and Sauerwein, R.W. (1997) Differential induction of pro- and anti-inflammatory cytokines in whole blood by bacteria: effects on antibiotic treatment. *Antimicrobial Agents Ch* **41**: 1439-1443.

Fuchs, T.A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V., and Zychlinsky, A. (2007) Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* **176**: 231-241.

Gaastra, W., and Lipman, L.J. (2009) *Capnocytophaga canimorsus*. *Vet Microbiol*.

Gaillard, J.L., Berche, P., Frehel, C., Gouin, E., and Cossart, P. (1991) Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. *Cell* **65**: 1127-1141.

Ganz, T., Selsted, M.E., Szklarek, D., Harwig, S.S., Daher, K., Bainton, D.F., and Lehrer, R.I. (1985) Defensins. Natural peptide antibiotics of human neutrophils. *J Clin Invest* **76**: 1427-1435.

Goldsby, R.A., Kindt, T.J., Osborne, B.A., and Kuby, J. (2003) *Immunology, 5th edition.* New York.

Griego, R.D., Rosen, T., Orengo, I.F., and Wolf, J.E. (1995) Dog, cat, and human bites: a review. *J Am Acad Dermatol* **33**: 1019-1029.

Grosdent, N., Maridonneau-Parini, I., Sory, M.-P., and Cornelis, G.R. (2002) Role of Yops and adhesins in resistance of *Yersinia enterocolitica* to phagocytosis. *Infect Immun* **70**: 4165-4176.

Gupta, A.K., Hasler, P., Holzgreve, W., Gebhardt, S., and Hahn, S. (2005) Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. *Human Immunology* **66**: 1146-1154.

Hagblom, P., Segal, E., Billyard, E., and So, M. (1985) Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. *Nature* **315**: 156-158.

Hantson, P., Gautier, P.E., Vekemans, M.C., Fievez, P., Evrard, P., Wauters, G., and Mahieu, P. (1991) Fatal *Capnocytophaga canimorsus* septicemia in a previously healthy woman. *Ann Emerg Med* **20**: 93-94.

Hermann, C.K., Hansen, P.B., Bangsborg, J.M., and Pers, C. (1998) Bacterial infections as complications of dog bites. *Ugeskr Laeger* **160**: 4860-4863.

Heyworth, P.G., Bohl, B.P., Bokoch, G.M., and Curnutte, J.T. (1994) Rac translocates independently of the neutrophil NADPH oxidase components $p47^{phox}$ and $p67^{phox}$. Evidence for its interaction with flavocytochrome b_{558}. *J Biol Chem* **269**: 30749-30752.

Hoiseth, S.K., and Stocker, B.A. (1981) Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**: 238-239.

Hong, Y.Q., and Ghebrehiwet, B. (1992) Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components C1q and C3. *Clin Immunol Immunopathol* **62**: 133-138.

Horstmann, R.D., Sievertsen, H.J., Knobloch, J., and Fischetti, V.A. (1988) Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. *PNAS* **85**: 1657-1661.

Hoyal, C.R., Gutierrez, A., Young, B.M., Catz, S.D., Lin, J.-H., Tsichlis, P.N., and Babior, B.M. (2003) Modulation of $p47^{phox}$ activity by site-specific phosphorylation: Akt-dependent activation of the NADPH oxidase. *PNAS* **100**: 5130-5135.

Hummell, D.S., and Winkelstein, J.A. (1986) Bacterial lipoteichoic acid sensitizes host cells for destruction by autologous complement. *J Clin Invest* **77**: 1533-1538.

Inanami, O., Johnson, J.L., McAdara, J.K., Benna, J.E., Faust, L.R.P., Newburger, P.E., and Babior, B.M. (1998) Activation of the leukocyte NADPH oxidase by phorbol ester requires the phosphorylation of $p47^{phox}$ on serine 303 or 304. *J Biol Chem* **273**: 9539-9543.

Iyer, S.S., Pearson, D.W., Nauseef, W.M., and Clark, R.A. (1994) Evidence for a readily dissociable complex of $p47^{phox}$ and $p67^{phox}$ in cytosol of unstimulated human neutrophils. *J Biol Chem* **269**: 22405-22411.

Janda, J.M., Graves, M.H., Lindquist, D., and Probert, W.S. (2006) Diagnosing *Capnocytophaga canimorsus* infections. *Emerg Infect Dis* **12**: 340-342.

Jepsen, L.V., and Skottun, T. (1982) A rapid one-step method for the isolation of human granulocytes from whole blood. *Scand J Clin Lab Inv* **42**: 325-328.

Jesaitis, A.J., Buescher, E.S., Harrison, D., Quinn, M.T., Parkos, C.A., Livesey, S., and Linner, J. (1990) Ultrastructural localization of cytochrome *b* in the membranes of resting and phagocytosing human granulocytes. *J Clin Invest* **85**: 821-835.

Johnsson, E., Thern, A., Dahlback, B., Heden, L.O., Wikstrom, M., and Lindahl, G. (1996) A highly variable region in members of the streptococcal M protein family binds the human complement regulator C4BP. *J Immunol* **157**: 3021-3029.

Jolivet-Gougeon, A., Sixou, J.-L., Tamanai-Shacoori, Z., and Bonnaure-Mallet, M. (2007) Antimicrobial treatment of *Capnocytophaga* infections. *Int J Antimicrobial Ag* **29**: 367-373.

Kang, T., Yi, J., Guo, A., Wang, X., Overall, C.M., Jiang, W., Elde, R., Borregaard, N., and Pei, D. (2001) Subcellular distribution and cytokine- and

chemokine-regulated secretion of leukolysin/MT6-MMP/MMP-25 in neutrophils. *J Biol Chem* **276**: 21960-21968.

Kanthack, A., and Hardy, W.B. (1895) The morphology and distribution of the wandering cells of mammalia. *Journal of Physiology* **18**: 80.81-119.

Keppie, J., Harris-Smith, P.W., and Smith, H. (1963) The chemical basis of the virulence of *Bacillus anthracis*. Ix. Its aggressins and their mode of action. *Br J Exp Pathol* **44**: 446-453.

Kjeldsen, L., Bjerrum, O.W., Askaa, J., and Borregaard, N. (1992) Subcellular localization and release of human neutrophil gelatinase, confirming the existence of separate gelatinase-containing granules. *Biochem J* **287 (Pt 2)**: 603-610.

Klebanoff, S.J. (1999) Myeloperoxidase. *Proc Assoc Am Physicians* **111**: 383-389.

Lazarus, G.S., Brown, R.S., Daniels, J.R., and Fullmer, H.M. (1968) Human granulocyte collagenase. *Science* **159**: 1483-1485.

le Moal, G., Landron, C., Grollier, G., Robert, R., and Burucoa, C. (2003) Meningitis due to *Capnocytophaga canimorsus* after receipt of a dog bite: case report and review of the literature. *Clin Infect Dis* **36**: e42-46.

Lee, W.L., Harrison, R.E., and Grinstein, S. (2003) Phagocytosis by neutrophils. *Microbes Infect* **5**: 1299-1306.

Lion, C., Escande, F., and Burdin, J.C. (1996) *Capnocytophaga canimorsus* infections in human: review of the literature and cases report. *Eur J Epidemiol* **12**: 521-533.

Macrea, M.M., McNamee, M., and Martin, T.J. (2008) Acute onset of fever, chills, and lethargy in a 36-year-old woman. *Chest* **133**: 1505-1507.

Majumdar, S., Kane, L.H., Rossi, M.W., Volpp, B.D., Nauseef, W.M., and Korchak, H.M. (1993) Protein kinase C isotypes and signal-transduction in human neutrophils: selective substrate specificity of calcium-dependent beta-PKC and novel calcium-independent nPKC. *Biochim Biophys Acta* **1176**: 276-286.

Mally, M., and Cornelis, G.R. (2008) Genetic tools for studying *Capnocytophaga canimorsus*. *Appl Environ Microbiol* **74**: 6369-6377.

Mally, M., Shin, H., Paroz, C., Landmann, R., and Cornelis, G.R. (2008) *Capnocytophaga canimorsus*: a human pathogen feeding at the surface of epithelial cells and phagocytes. *PLoS Pathog* **4**: e1000164.

Mally, M., Paroz, C., Shin, H., Meyer, S., Soussoula, L.V., Schmiediger, U., Saillen-Paroz, C., and Cornelis, G.R. (2009) Prevalence of *Capnocytophaga canimorsus* in dogs and occurrence of potential virulence factors. *Microbes Infect* **11**: 509-514.

Mandrell, R.E., Lesse, A.J., Sugai, J.V., Shero, M., Griffiss, J.M., Cole, J.A., Parsons, N.J., Smith, H., Morse, S.A., and Apicella, M.A. (1990) *In vitro* and *in vivo* modification of *Neisseria gonorrhoeae* lipooligosaccharide epitope structure by sialylation. *J Exp Med* **171**: 1649-1664.

Maskos, K., Jozic, D., and Fernandez-Catalan, C. (2005) Cyrstal structure of pitrilysin, the prototype of insulin-degrading enzymes. *RCSB Protein Data Bank, Accession no. 1Q2L*.

McCaffrey, R.L., and Allen, L.-A.H. (2006) *Francisella tularensis* LVS evades killing by human neutrophils via inhibition of the respiratory burst and phagosome escape. *J Leukoc Biol* **80**: 1224-1230.

Medina, E., Rohde, M., and Chhatwal, G.S. (2003) Intracellular survival of *Streptococcus pyogenes* in polymorphonuclear cells results in increased bacterial virulence. *Infect Immun* **71**: 5376-5380.

Menestrina, G., Serra, M.D., and Prevost, G. (2001) Mode of action of beta-barrel pore-forming toxins of the staphylococcal alpha-hemolysin family. *Toxicon* **39**: 1661-1672.

Meri, S., and Pangburn, M.K. (1990) A mechanism of activation of the alternative complement pathway by the classical pathway: protection of C3b from inactivation by covalent attachment to C4b. *Eur J Immunol* **20**: 2555-2561.

Meybeck, A., Aoun, N., Granados, D., Pease, S., and Yeni, P. (2006) Meningitis due to *Capnocytophaga canimorsus*: contribution of 16S RNA ribosomal sequencing for species identification. *Scand J Infect Dis* **38**: 375-377.

Meyer, S., Shin, H., and Cornelis, G.R. (2008) *Capnocytophaga canimorsus* resists phagocytosis by macrophages and blocks the ability of macrophages to kill other bacteria. *Immunobiology* **213**: 805-814.

Meyers, B., Schoeman, J.P., Goddard, A., and Picard, J. (2007) The bacteriology and antimicrobial susceptibility of infected and non-infected dog bite wounds: fifty cases. *Vet Microbiol* **127**: 360-368.

Mirza, I., Wolk, J., Toth, L., Rostenberg, P., Kranwinkel, R., and Sieber, S.C. (2000) Waterhouse-Friderichsen syndrome secondary to *Capnocytophaga canimorsus* septicemia and demonstration of bacteremia by peripheral blood smear. *Arch Pathol Lab Med* **124**: 859-864.

Miyoshi, S., and Shinoda, S. (2000) Microbial metalloproteases and pathogenesis. *Microbes Infect* **2**: 91-98.

Mota, L.J., Journet, L., Sorg, I., Agrain, C., and Cornelis, G.R. (2005) Bacterial injectisomes: needle length does matter. *Science* **307**: 1278.

Mulder, A.H., Gerlag, P.G., Verhoef, L.H., and van den Wall Bake, A.W. (2001) Hemolytic uremic syndrome after *Capnocytophaga canimorsus* (DF-2) septicemia. *Clin Nephrol* **55**: 167-170.

Muller-Eberhard, H.J. (1986) The membrane attack complex of complement. *Annu Rev Immunol* **4**: 503-528.

Muller-Eberhard, H.J. (1988) Molecular organization and function of the complement system. *Annu Rev Biochem* **57**: 321-347.

Nathan, C. (2003) Specificity of a third kind: reactive oxygen and nitrogen intermediates in cell signaling. *J Clin Invest* **111**: 769-778.

Nathan, C. (2006) Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* **6**: 173-182.

Nixon, J.B., and McPhail, L.C. (1999) Protein kinase C (PKC) isoforms translocate to Triton-insoluble fractions in stimulated human neutrophils: correlation of conventional PKC with activation of NADPH oxidase. *J Immunol* **163**: 4574-4582.

Owen, C.A., and Campbell, E.J. (1999) The cell biology of leukocyte-mediated proteolysis. *J Leukoc Biol* **65**: 137-150.

Parenti, D.M., and Snydman, D.R. (1985) *Capnocytophaga* species: infections in nonimmunocompromised and immunocompromised hosts. *J Infect Dis* **151**: 140-147.

Park, J.W., Ma, M., Ruedi, J.M., Smith, R.M., and Babior, B.M. (1992) The cytosolic components of the respiratory burst oxidase exist as a M(r)

approximately 240,000 complex that acquires a membrane-binding site during activation of the oxidase in a cell-free system. *J Biol Chem* **267**: 17327-17332.

Pers, C., Gahrn-Hansen, B., and Frederiksen, W. (1996) *Capnocytophaga canimorsus* septicemia in Denmark, 1982-1995: review of 39 cases. *Clin Infect Dis* **23**: 71-75.

Persson, C., Nordfelth, R., Andersson, K., Forsberg, A., Wolf-Watz, H., and Fällman, M. (2002) Localization of the *Yersinia* PTPase to focal complexes in an important virulence mechanism. *Mol Microbiol* **33**: 828-838.

Quinn, M.T., Evans, T., Loetterle, L.R., Jesaitis, A.J., and Bokoch, G.M. (1993) Translocation of Rac correlates with NADPH oxidase activation. Evidence for equimolar translocation of oxidase components. *J Biol Chem* **268**: 20983-20987.

Rabinovitch, M. (1995) Professional and non-professional phagocytes: an introduction. *Trends Cell Biol* **5**: 85-87.

Rautemaa, R., Jarvis, G.A., Marnila, P., and Meri, S. (1998) Acquired resistance of *Escherichia coli* to complement lysis by binding of glycophosphoinositol-anchored protectin (CD59). *Infect Immun* **66**: 1928-1933.

Rautemaa, R., and Meri, S. (1999) Complement-resistance mechanisms of bacteria. *Microbes Infect* **1**: 785-794.

Reeves, E.P., Dekker, L.V., Forbes, L.V., Wientjes, F.B., Grogan, A., Pappin, D.J., and Segal, A.W. (1999) Direct interaction between $p47^{phox}$ and protein kinase C: evidence for targeting of protein kinase C by $p47^{phox}$ in neutrophils. *Biochem J* **344 Pt 3**: 859-866.

Reinholdt, J., Tomana, M., Mortensen, S.B., and Kilian, M. (1990) Molecular aspects of immunoglobulin A1 degradation by oral Streptococci. *Infect Immun* **58**: 1186-1194.

Risi, G.F., Jr., and Spangler, C.A. (2006) *Capnocytophaga canimorsus* meningitis after routine myelography: a sentinel event identifies multiple opportunities for improvement of standard practices in radiology. *Am J Infect Control* **34**: 540-542.

Rosqvist, R., Skurnik, M., and Wolf-Watz, H. (1988) Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. *Nature* **334**: 522-524.

Roth, R.A. (2004) Insulysin. In *Handbook of Proteolytic Enzymes*. Barret, A.J., Rawlings, N.D. and Woessner, J.F. (eds): Elsevier Academic Press, London, pp. 871-876.

Rubsamen, P.E., McLeish, W.M., Pflugfelder, S., and Miller, D. (1993) *Capnocytophaga* endophthalmitis. *Ophthalmology* **100**: 456-459.

Ruckdeschel, K., Harb, S., Roggenkamp, A., Hornef, M.W., Zumbihl, R., Köhler, S., Heesemann, J., and Rouot, B. (1998) *Yersinia enterocolitica* impairs activation of transcription factor NF-κB: Involvement in the induction of programmed cell death and in the suppression of the macrophage tumor necrosis factor α production. *J Exp Med* **187**: 1069-1079.

Salvesen, G., Farley, D., Shuman, J., Przybyla, A., Reilly, C., and Travis, J. (1987) Molecular cloning of human cathepsin G: structural similarity to mast cell and cytotoxic T lymphocyte proteinases. *Biochemistry* **26**: 2289-2293.

Sandoe, J.A.T. (2004) *Capnocytophaga canimorsus* endocarditis. *J Med Microbiol* **53**: 245-248.

Schenkein, H.A. (1989) Failure of *Bacteroides gingivalis* W83 to accumulate bound C3 following opsonization with serum. *J Periodontal Res* **24**: 20-27.

Schiller, N.L., and Joiner, K.A. (1986) Interaction of complement with serum-sensitive and serum-resistant strains of *Pseudomonas aeruginosa*. *Infect Immun* **54**: 689-694.

Schmidtchen, A., Holst, E., Tapper, H., and Bjorck, L. (2003) Elastase-producing *Pseudomonas aeruginosa* degrade plasma proteins and extracellular products of human skin and fibroblasts, and inhibit fibroblast growth. *Microb Pathog* **34**: 47-55.

Shin, H., Mally, M., Kuhn, M., Paroz, C., and Cornelis, G.R. (2007) Escape from immune surveillance by *Capnocytophaga canimorsus*. *J Infect Dis* **195**: 375-386.

Shin, H., Mally, M., Meyer, S., Fiechter, C., Paroz, C., Zaehringer, U., and Cornelis, G.R. (2009) Resistance of *Capnocytophaga canimorsus* to killing by human complement and polymorphonuclear leukocytes. *Infect Immun* **77**: 2262-2271.

Sinha, S., Watorek, W., Karr, S., Giles, J., Bode, W., and Travis, J. (1987) Primary structure of human neutrophil elastase. *PNAS* **84**: 2228-2232.

Slaney, J.M., Gallagher, A., Aduse-Opoku, J., Pell, K., and Curtis, M.A. (2006) Mechanisms of resistance of *Porphyromonas gingivalis* to killing by serum complement. *Infect Immun* **74**: 5352-5361.

Sory, M.P., and Cornelis, G.R. (1994) Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. *Mol Microbiol* **14**: 583-594.

Staali, L., Bauer, S., Morgelin, M., Bjorck, L., and Tapper, H. (2006) *Streptococcus pyogenes* bacteria modulate membrane traffic in human neutrophils and selectively inhibit azurophilic granule fusion with phagosomes. *Cell Microbiol* **8**: 690-703.

Stuart, L.M., and Ezekowitz, R.A.B. (2005) Phagocytosis: elegant complexity. *Immunity* **22**: 539-550.

Sundqvist, G., Figdor, D., Hänström, L., Sörlin, S., and Sandström, G. (1991) Phagocytosis and virulence of different strains of *Porphyromonas gingivalis*. *Scand J Dent Res* **99**: 117-129.

Swamy, K.H., and Goldberg, A.L. (1982) Subcellular distribution of various proteases in *Escherichia coli*. *J Bacteriol* **149**: 1027-1033.

Takahashi, S., Nagano, Y., Nagano, N., Hayashi, O., Taguchi, F., and Okuwaki, Y. (1995) Role of C5a-ase in group B streptococcal resistance to opsonophagocytic killing. *Infect Immun* **63**: 4764-4769.

Tan, B.H., Meinken, C., Bastian, M., Bruns, H., Legaspi, A., Ochoa, M.T., Krutzik, S.R., Bloom, B.R., Ganz, T., Modlin, R.L., and Stenger, S. (2006) Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens. *J Immunol* **177**: 1864-1871.

Tanaka, D., Miyasaki, K.T., and Lehrer, R.I. (2000) Sensitivity of *Actinobacillus actinomycetemcomitans* and *Capnocytophaga* spp. to the bactericidal action of LL-37: a cathelicidin found in human leukocytes and epithelium. *Oral Microbiol Immunol* **15**: 226-231.

Tapper, H., Furuya, W., and Grinstein, S. (2002) Localized exocytosis of primary (lysosomal) granules during phagocytosis: role of Ca^{2+}-dependent tyrosine phosphorylation and microtubules. *J Immunol* **168**: 5287-5296.

Thern, A., Stenberg, L., Dahlback, B., and Lindahl, G. (1995) Ig-binding surface proteins of *Streptococcus pyogenes* also bind human C4b-binding protein

(C4BP), a regulatory component of the complement system. *J Immunol* **154**: 375-386.

Tierney, D.M., Strauss, L.P., and Sanchez, J.L. (2006) *Capnocytophaga canimorsus* mycotic abdominal aortic aneurysm: why the mailman is afraid of dogs. *J Clin Microbiol* **44**: 649-651.

Tobé, T.J., Franssen, C.F., Zijlstra, J.G., de Jong, P.E., and Stegeman, C.A. (1999) Hemolytic uremic syndrome due to *Capnocytophaga canimorsus* bacteremia after a dog bite. *Am J Kidney Dis* **33**: e5.

Tsai, C.M., and Frasch, C.E. (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* **119**: 115-119.

Urban, C.F., Reichard, U., Brinkmann, V., and Zychlinsky, A. (2006) Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cell Microbiol* **8**: 668-676.

van Duijkeren, E., van Mourik, C., Broekhuizen, M., Leuven, M., Gaastra, W., and Houwers, D. (2006) First documented *Capnocytophaga canimorsus* infection in a species other than humans. *Vet Microbiol* **118**: 148-150.

Vandamme, P., Vancanneyt, M., van Belkum, A., Segers, P., Quint, W.G.V., Kersters, K., Paster, B.J., and Dewhirst, F.E. (1996) Polyphasic analysis of strains of the genus *Capnocytophaga* and Centers for Disease Control group DF-3. *Int J Syst Bacteriol* **46**: 782-791.

Vanhonsebrouck, A.Y., Gordts, B., Wauters, G., and van Landhuyt, H.W. (1991) Fatal septicemia with *Capnocytophaga canimorsus* in a compromised host. A case report with review of the literature. *Acta Clin Belg* **46**: 364-370.

Voyich, J.M., Sturdevant, D.E., Braughton, K.R., Kobayashi, S.D., Lei, B., Virtaneva, K., Dorward, D.W., Musser, J.M., and DeLeo, F.R. (2003) Genome-wide protective response used by group A *Streptococcus* to evade destruction by human polymorphonuclear leukocytes. *PNAS* **100**: 1996-2001.

Walport, M.J. (2001a) Complement. First of two parts. *N Engl J Med* **344**: 1058-1066.

Walport, M.J. (2001b) Complement. Second of two parts. *N Engl J Med* **344**: 1140-1144.

Ward, C.K., and Inzana, T.J. (1994) Resistance of *Actinobacillus pleuropneumoniae* to bactericidal antibody and complement is mediated by capsular polysaccharide and blocking antibody specific for lipopolysaccharide. *J Immunol* **153**: 2110-2121.

Wartha, F., Beiter, K., Albiger, B., Normark, S., Zychlinsky, A., and Henriques-Normark, B. (2007a) Capsule and D-alanylated lipoteichoic acids protect Streptococcus pneumoniae against neutrophil extracellular traps. *Cell Microbiol* **9**: 1162-1171.

Wartha, F., Beiter, K., Normark, S., and Henriques-Normark, B. (2007b) Neutrophil extracellular traps: casting NET over pathogenesis. *Curr Opin Microbiol* **10**: 52-56.

Weinberg, E.D. (2000) Microbial pathogens with impaired ability to acquire host iron. *Biometals* **13**: 85-89.

Westwell, A.J., Kerr, K., Spencer, M.B., and Hutchinson, D.N. (1989) DF-2 infection. *Bmj* **298**: 116-117.

Wilkinson, B.J., Sisson, S.P., Kim, Y., and Peterson, P.K. (1979) Localization of the third component of complement on the cell wall of encapsulated *Staphylococcus aureus* M: implications for the mechanism of resistance to phagocytosis. *Infect Immun* **26**: 1159-1163.

Wilson, M.E., Burstein, R., Jonak-Urbanczyk, J.T., and Genco, R.J. (1985) Sensitivity of *Capnocytophaga* species to bactericidal properties of human serum. *Infect Immun* **50**: 123-129.

Wilson, M.E., Jonak-Urbanczyk, J.T., Bronson, P.M., Dudas, K.C., Apicella, M.A., and Genco, R.J. (1987) *Capnocytophaga* species: increased resistance of clinical isolates to serum bactericidal action. *J Infect Dis* **156**: 99-106.

Wimley, W.C., Selsted, M.E., and White, S.H. (1994) Interactions between human defensins and lipid bilayers: evidence for formation of multimeric pores. *Protein Sci* **3**: 1362-1373.

Wittamer, V., Bondue, B., Guillabert, A., Vassart, G., Parmentier, M., and Communi, D. (2005) Neutrophil-mediated maturation of chemerin: a link between innate and adaptive immunity. *J Immunol* **175**: 487-493.

Woestyn, S., Allaoui, A., Wattiau, P., and Cornelis, G.R. (1994) YscN, the putative energizer of the *Yersinia* Yop secretion machinery. *J Bacteriol* **176**: 1561-1569.

Yaqub, S., Bjørnholt, J.V., Hellum, K.B., Steinbakk, M., and Enger, A.E. (2004) Bite wound infections. *Tidsskr Norske Laege* **124**: 3194-3196.

Acknowledgements

First of all, I am extremely grateful to my Ph.D. mentor **Prof. Dr. Guy R. Cornelis**, who supported me throughout my time here with guidance, input, criticism, advice and faith in my capabilities. Thank you very much for your professional and personal support, no matter whether it was about my projects, my concerns, or my future career.

I am especially grateful to **Simon Ittig**, who was joining the *C. canimorsus* team two years ago and will go on with the pitrilysin story. Thank you for our discussions (the professional ones as well as the private ones), your suggestions, your creativity and your commitment. Working with you was a pleasure and I wish you all the best for your future!

I am greatly thankful to **Cécile Pfaff-Paroz** who helped me with her outstanding technical assistance on many projects. Without you, I would have never managed…

I would like to thank **Dr. Hwain K. Shin** who was not only a pioneer in the *C. canimorsus* project, but raised my interest in and introduced me to *Cc*. You were always an invaluable source for all kind of information on *Cc*. I thank you for your advice, your tips and tricks and your support!

I would also like to thank **Dr. Manuela Mally** for many discussions and uncounted suggestions for my projects, technically as well as theoretically. You were my office neighbour for such a long time… I enjoyed discussing with you, not only *Cc* but also our hopes and wishes. Playing squash with you was an excellent break to clear my mind and was lots of fun!

I thank **Andreas Diepold**, my office and lab neighbour, for so many hours of more or less scientific discussions. You kept on putting the finger on the weak side of a story, which re-opened my eyes for missing controls or unanswered questions.

I am greatly thankful to **Marina Kuhn**, my good spirit. For your tips and help on so many technical aspects, but most of all for your personal support in all situations!

A big “thank you” goes to the whole **Cornelis lab**! Thanks for all your suggestions, support, assistance and the enjoyable time we had together. First of all, I’d like to thank the *Cc* team: **Pablo Manfredi**, for taking care of the *Cc* genome and for discussions; **Chantal Fiechter**, for the discovery of Y1C12 and the fun we had together; **Dr. Francesco Renzi**, for your curiosity and your humor; **Giacomo Golfieri**, for purifying pitrilysin and for taking the efforts needed; **Lavinia V. Soussoula**, for screening the library and for introducing me to the fun of supervision. I’d also like to thank the other members of the team: **Dr. Catherine Müller**, for being a friend in and out of the lab and for many discussions; **Dr. Isabel Sorg**, for advice, protocols and coffee chats; **Stefanie Wagner**, for company; **Marlise Amstutz**, for organising and ordering many things; **Dr. Caroline Montagner**, for suggestions; **Lisa Metzger**, for office discussions; **Cédric Cattin**, for your curiosity; **Dr. Petr Broz**, for starting help; **Dr. Gottfried Eisner**, for lab chats; **Dr. Michel Letzelter**, for the work we did together; **Yaniv Cohen**, for your interest.

I am especially thankful to **Prof. Dr. Regine Landmann**, for her constant support and input; **Dr. Mathias Schmaler** for discussions and help on animal experiments and **Dr. Naja Jann** for so many invaluable lunch discussions, support and help on animal experiments, and mostly for your friendship.

There are numerous people from the Biozentrum I would like to acknowledge: **Prof. Dr. Christoph Dehio**, for support during my Ph.D.; **Michaela Hanisch**, for helping out in every situation with a smile; **Roger Sauder**, for your endless pool of useful things; **Roland Gut**, for preparing uncounted amounts of plates, which made my lab life so much easier; **Prof. Dr. Cécile Arrieumerlou** and her team, especially **Harry Wischnewski**, **Christoph Kasper** and **Dr. Gregory Melroe** for help, suggestions and tips for handling different microscopes; **Evi Bieler** for the excellent technical support on scanning electron microscopy; **Maxime Québatte** for FACS help; **Dr. Rajesh Jayachandran** for protocols and discussions; **Dr. Nadine Cybulski**, for company and chats; **Samuel Steiner**, **Dr. Jake Malone** and **Dr. Tina Jäger**, for playing badminton; **Prof. Dr. Dirk Buman**, for tips and *Salmonella* strains; **Dr. Alain Mazé** for help with frozen spleens; **Dr. Alex Böhm**, for tips, **Vesna Olivieri** and **Ursula Sauder** for transmission electron microscopy; all past and present members of the **4th floor**, for the excellent atmosphere, their help, the barbecues and the film evenings.

I would like to thank **Prof. Dr. Joachim Frey** and **Dr. Paola Pilo** for putting so much effort in the verification of EV76.

I am deeply grateful to my (WIN-) mentor **Janice Branson**. Thank you for accompanying me with care, passion and friendship!

Ein besonderer Dank gilt meinen Eltern, **Heidi und Fritz Meyer**. Eure Unterstützung und Eure liebevolle Begleitung haben mir die Kraft und die Zuversicht gegeben meine Träume zu verwirklichen.

Auch meinen Schwiegereltern, **Myrtha und Otto Casutt**, möchte ich von Herzen für Ihre Unterstützung und Ihr Verständnis danken.

Words cannot express how grateful I am to my husband, **Marco Casutt**. It is for knowing you at my side that I can spread my wings.

Curriculum Vitae

Curriculum Vitae Salome Casutt-Meyer

Salome Casutt-Meyer

Private address:
In den Ziegelhöfen 181
CH-4054 Basel
Phone: +41 (0)79 378 51 69

Work address:
Klingelbergstrasse 70
CH-4056 Basel
Phone: +41 (0)61 267 21 27

E-mail: Salome.Meyer@unibas.ch

Date of Birth: August 7th 1981
Nationality: Swiss

Education & Experience

Biozentrum, Infectious Diseases,
University of Basel, CH
12/05 – 11/09

PhD with honours (Magna cum laude) in Microbiology
Group of Prof. Guy R. Cornelis
"*Capnocytophaga canimorsus*: Interaction with the innate immune system"
Analysis of host - pathogen interactions with a focus on the innate immune system using *in vitro* infection, microscopy, FACS, and molecular biological, biochemical, and immunological techniques.

ETH Zurich
Zurich, CH
12/00-11/05

Diploma in Microbiology
Dipl. Natw. ETH, grade 5.75 (6 = best, 1= worst)
Majors: microbiology, cell biology, virology, parasitology, immunology, gene technology, and enzyme technology.
Minors: organic chemistry, anorganic chemistry, physical chemistry, analytical chemistry, plant biology, and biochemistry.

Institute of Virology
University of Zurich, CH
03/04 – 04/05

Diploma Thesis & Semester Work
grade 6 (6 = best, 1 = worst)
Group of Prof. Mathias Ackermann
"The ovine herpesvirus-2 ORF68 protein targets the Golgi complex and is incorporated into heterologous herpesvirus particles"
Analysis of protein function by cloning, expression, and purification of recombinant proteins by *in vitro* transfections and microscopic analysis of protein localization and function.

Gymnasium Leonhard
Basel, CH
08/92 – 07/00

School leaving examination
Matura Type "B" (Latin)

Training

Women into Industries
Novartis and University of Basel, CH
04/06 – 04/07

Selected mentee and member of the mentee-organizing committee. Mentor: Janice Branson (CIS – BIOS ECD, Novartis Pharma AG, janice.branson@novartis.com).

Microscopy Course
University of Basel, CH
08/06

Introduction and knowledge broadening in light microscopy, fluorescence microscopy, and electron microscopy.

Didactics Seminar
Institute of Virology,
University of Zurich, CH
07/05

Introduction into scientific presentation, held in Rathen, GE.

Social Practical Course
Kindergarten Montessori, Basel, CH
06/99

Care of physically and mentally disabled children, as well as foreign-language children (English).

Awards

BioValley Industry Poster Award 10/08	For my poster *Capnocytophaga canimorsus* interferes with macrophages" presented at the Roche BioValley Symposium, Basel, CH.
Young Investigator Award	From the European Macrophage and Dendritic Cell Society for my speech 09/07 "*Capnocytophaga canimorsus* prevents phagocytosis & killing by macrophages" held at the society meeting in Innsbruck, AU.

Scientific Publications

2009	Casutt-Meyer S. and G. R. Cornelis "*Yersinia* adhesin YadA is a double-edged sword" Manuscript in preparation. My contribution to this paper includes the design, performance and evaluation of all experiments demonstrating a new function of the *Yersinia* adhesin A in host interaction and I am contributing to the writing of the paper.
06/09	Shin H.*, Mally M.*, Meyer S.*, Fiechter C.*, Paroz C., Zaehringer U., and G. R. Cornelis (* contributed equally to this work) "Resistance of *Capnocytophaga canimorsus* to killing by human complement and polymorphonuclear leukocytes" Infection and Immunity, 77, 2262 – 2271 (2009). My contribution to this publication included the design, performance and evaluation of the experiments shown in fig.1A, 1B, 1D, 2, 3, 4C, and 5 and I contributed to the writing of the paper.
04/09	Mally M.*, Paroz C.*, Shin H.*, Meyer S.*, Soussoula L. V., Schmiediger U., Saillen-Paroz C., and G. R. Cornelis (* contributed equally to this work) "Prevalence of *Capnocytophaga canimorsus* in dogs and occurrence of potential virulence factors" Microbes and Infection, 11, 509 – 514 (2009). My contributions to this publication included the design, performance and evaluation of the experiments on the capability of *C. canimorsus* to block the killing ability of macrophages and I contributed to the writing of the paper.
03/08	Meyer S., Shin H, and G. R. Cornelis "*Capnocytophaga canimorsus* resists phagocytosis by macrophages and blocks the ability of macrophages to kill other bacteria" Immunobiology, 213, 805 – 814 (2008). My contribution to this publication included the design, performance and evaluation of all experiments except fig. 7 and I contributed to the writing of the paper.
07/06	Letzelter M., Sorg I., Mota L. J., Meyer S., Stalder J., Feldman M., Kuhn M., Callebaut I., and G. R. Cornelis "The discovery of SycO highlights a new function for type III secretion effector chaperones" EMBO, 25, 3223 – 3233 (2006). My contribution to this publication included construction of expression vectors, *in vitro* transfection of human cell line and protein localization by confocal microscopy (especially fig.7).

Scientific Presentations

Poster 09/09	Casutt-Meyer S. and G. R. Cornelis "YadA is a double-edged sword rendering *Yersinia* sensitive to neutrophil extracellular trap-dependent killing" Microbial Pathogens & Host Interactions Meeting, Cold Spring Harbor Laboratory, New York, United States of America.
Speech 06/09	Meyer S. and G R. Cornelis "Why binding can be obstructive to survival: the *Yersinia* Yad story" 1st Biozentrum PhD Retreat, Engelberg, Switzerland.
Poster 10/08	Meyer S.*, Ittig S.*, and G. R. Cornelis (* contributed equally to this work) "*Capnocytophaga canimorsus* interferes with the oxidative burst in macrophages" Roche BioValley Symposium, Basel, Switzerland.
Poster 09/08	Meyer S.*, Ittig S.*, and G. R. Cornelis (* contributed equally to this work) "*Capnocytophaga canimorsus* impairs the ability of macrophages to clear bacterial infections" European Macrophage and Dendritic Cell Society Meeting, Brescia, Italy.
Poster 06/08	Meyer S., Shin H., Landmann R., and G. R. Cornelis "*Capnocytophaga canimorsus* interferes with the ability of macrophages to kill bacteria after phagocytosis" 67th Annual Assembly of the Swiss Society for Microbiology, Interlaken, Switzerland.
Poster 10/07	Meyer S., Shin H., Landmann R., and G. R. Cornelis "*Capnocytophaga canimorsus* prevents phagocytosis and killing by macrophages" Roche BioValley Symposium, Basel, Switzerland.
Speech 09/07	Meyer S., Shin H., Landmann R., and G. R. Cornelis "*Capnocytophaga canimorsus* prevents phagocytosis and killing by macrophages" European Macrophage and Dendritic Cell Society Meeting, Innsbruck, Austria.
Poster 06/07	Meyer S., Shin H., Landmann R., and G. R. Cornelis "*Capnocytophaga canimorsus* prevents phagocytosis and killing by macrophages" Swiss Molecular Microbiology Meeting, Villars-sur-Ollon, Switzerland.

Languages

German	Native tongue
English	Fluent (spoken and written) // company language at the Biozentrum
French	Intermediate (spoken and written)
Italian	Basic (spoken and written)

Curriculum Vitae Salome Casutt-Meyer

Other Skills

Safety	Four years experience working in a biosafety level 2 laboratory.
Software	PC and Mac, Microsoft Word/Excel/Power Point, CorelDraw, Adobe Acrobat Reader, Adobe Illustrator CS, EndNote, Cell Quest and FlowJo (FACS analysis programs), Openlab/Andor IQ and ImageJ (microscopy analysis programs).
Teaching	Supervision of a master student, an internship student, and a research assistant on laboratory work, project design, data evaluation and protocol writing. Biozentrum, University of Basel, CH. Instruction of a PhD colleague on laboratory work with *Capnocytophaga canimorsus*. Biozentrum, University of Basel, CH. Assistance and supervision of experiments and evaluation of protocols in the annual student's laboratory course "Microbiology". Biozentrum, University of Basel, CH.

References

Prof. Dr. Guy R. Cornelis	Biozentrum University of Basel Klingelbergstrasse 70 CH-4056 Basel Tel. +41 (0)61 267 21 10 guy.cornelis@unibas.ch
Dr. Hwain K. Shin	Novartis Pharma AG WSJ-340.3.18.5 Novartis Campus CH-4056 Basel Tel. +41 (0)61 324 71 31 hwain_kathy.shin@novartis.com
Prof. Dr. Mathias Ackermann	Institute of Virology University of Zurich Winterthurerstrasse 266a CH-8057 Zurich Tel. +41 (0)44 635 87 01 mathias.ackermann@access.unizh.ch

Basel, November 2009

Salome Casutt-Meyer