Capnocytophaga canimorsus: Discovery of a deglycosylation mechanism that links metabolism to pathogenesis

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Cover: HeLa epithelial cells treated with *C. canimorsus* sialidase and stained with lectin PNA

Declaration:

I declare that I wrote this thesis

"*Capnocytophaga canimorsus:* Discovery of a deglycosylation mechanism that links metabolism to pathogenesis"

with the help indicated in the laboratory of Prof. Dr. Guy R. Cornelis and only handed it to the faculty of science of the University of Basel and to no other faculty and no other university.

Manuela Mally

Basel, 5th of February 2008

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Summary

We show that *C. canimorsus* (*Cc*) can serve as a recipient for RP4 mediated conjugation but there is neither replication of broad host range plasmid vectors nor expression of commonly used *E. coli* markers in *C. canimorsus*. We identified three selection markers, *ermF, tetQ* and *cfxA* leading to resistance against erythromycin, tetracycline and cefoxitin, respectively, that can be used in *C. canimorsus.* We engineered expression shuttle vectors using the replicon of a endogenous plasmid found in strain *Cc7* and the promoter of one of the selection markers for gene expression. We developed a transposon mutagenesis strategy based on Tn*4351* from *Bacteroides fragilis* and protocols for allelic exchange and electrotransformation. We carried out an extensive transposon mutagenesis and screened these mutants for different properties.

We demonstrate that presence of mammalian cells, including phagocytes, favors growth of *C. canimorsus 5* and this property was found to be dependent on direct cellular contacts. We isolated a Tn mutant unable to grow in presence of mammalian cells. The mutation occurred in a gene encoding a sialidase. The surface-exposed sialidase allows *Cc5* to feed on internal aminosugars of glycan chains from host cell glycans. In addition, sialidase confers resistance to complement by promoting the binding of factor H. We developed an experimental mouse infection in which the read-out is bacterial persistence. In this infection model, *Cc5*, but not the sialidase deficient mutant, grew and persisted, showing the importance of this metabolic pathway *in vivo*.

C. canimorsus by itself does not elicit the onset of an inflammatory response from macrophages. One strain, *Cc5* turned out to have a mechanism that actively blocks the pro-inflammatory signaling of macrophages upon stimulation with endotoxic LPS. We screened the Tn mutant library for clones of *Cc5* affected in this active mechanism. Isolated mutants have been mapped, characterized and complemented. The function of the mutated genes is presently under investigation as well as the mode of action of its gene product(s).

The prevalence of *C. canimorsus* in dogs has not been clarified at present. We therefore sampled dog swabs to isolate *C. canimorsus* strains in Swiss dogs. We could identify 61 *C. canimorsus* isolates from 103 dogs, which represents 59.22% of the dogs tested.

Besides this I also contributed to the analysis of LPS, to the study of resistance of *Cc5* to complement mediated lysis, to sequencing of the genome, the assembly of the reads and the annotation.

INTRODUCTION

Infections caused by *Capnocytophaga canimorsus*

It has been estimated that every second person is bitten by an animal or by another human once per lifetime in the US (Griego *et al.*, 1995). Although the majority of bite wounds are minor and do not need medical treatment, 1% of the total costs from emergency treatments result from bite wound cases. Infections of bite wounds are rare (5- 10%) and the overall mortality is around 6 out of 100 millions of cases annually in the US. Most of the infections are due to *Pasteurella*, *Streptococcus* and *Staphylococcus sp.* 80- 90% of all bite wounds are inflicted by dogs and this statistically accounts for 1 out of 20 dogs that will bite a human being during a dog's lifetime (Griego *et al.*, 1995). The second most common type of mammalian bite wounds are caused by cats, leading to an estimated 400,000 incidents per year (Griego *et al.*, 1995).

Capnocytophaga canimorsus (formerly Centers for Disease Control group DF-2) is rarely but regularly isolated from dog or cat bite infections since its discovery in 1976 (Bobo and Newton, 1976; Brenner *et al.*, 1989). *C. canimorsus* is a fastidious, thin, gram-negative rod, found as part of the normal oral flora of dogs and cats. Clinical infections by *C. canimorsus* generally appears as fulminant septicemia and peripheral gangrene (Pers *et al.*, 1996). The initial symptoms are fever, vomiting, diarrhea, malaise, myalgia, abdominal pain, dyspnea, confusion and headache. Symptoms of skin manifestations such as maculopapular rash and purpura are commonly associated to *C. canimorsus* infections (Hermann *et al.*, 1998; Lion *et al.*, 1996). Renal failure can be caused by disseminated intravascular coagulation or hypotension resulting from systemic infection (Mulder *et al.*, 2001). Meningitis is not as common as septicemia but well documented in the literature. Meningitis is accompanied by headache and meningism, but rarely by fever (Le Moal *et al.*, 2003). *C. canimorsus* can also, but less commonly, lead to endocarditis and myocarditis with a mortality rate of 25% (Sandoe, 2004). Fatality rate of systemic infections is as high as 30% (Lion *et al.*, 1996), while the mortality rate for meningitis is lower (5% [1 of 19]) (Le Moal *et al.*, 2003). *C. canimorsus* could be also identified from cultures of pleural fluid from a patient that had developed pneumonia (Chambers and Westblom, 1992).

Approximately 60% of the patients had a predisposing condition and the most prevalent was splenectomy (33% of systemic cases). Other cases have been associated with alcohol abuse (24%) or other immunosuppression (5%).

Trauma, Hodgkin's disease, idiopathic thrombocytopenic purpura, steroid therapy and chronic lung disease have been described as identifiable factors for increased risk. However, 40% of infections occurred in patients without any known risk factor. Although the majority of infections are associated to immunocompromised hosts, mortality is actually higher in patients without the predisposing conditions mentioned before (32% versus 28%). The reason for this phenomenon is unclear (Lion *et al.*, 1996). Thus, infections with *C. canimorsus* have to be considered not only as opportunistic infections. In one case, a patient died from a secondary infection by *Aspergillus niger*, suggesting that *C. canimorsus* may have induced some sort of immunosuppression (lePolain JB, personal communication).

Every year in Switzerland there are several cases of *C. canimorsus* infections (Trampuz A, personal communication) but no statistics are available for this disease. It has been estimated that every fourth dog carries *C. canimorsus* in its normal oral flora (Westwell *et al.*, 1989). There are more than 160 described cases of human patients infected with *C. canimorsus* and only one case of a dog infected with *C. canimorsus* followed by a dog bite has been reported recently (Meyers *et al.*, 2007).

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. At present, dramatic infections are well known by clinicians and are therefore less frequently reported in the literature. Nonetheless, there have been increasing reports of cases described in the past years (Janda *et al.*, 2006). In Denmark already between 1982 and 1995, the incidence was estimated to be 0.5 to 1 case annually per million (Pers *et al.*, 1996). This infers that a country like Switzerland faces around one fatal case per year.

What finally discriminates a virulent strain isolated from fatal cases from strains found in the dogs oral cavity needs to be elucidated. It is unclear how *C. canimorsus* can manipulate immune recognition after transmission and how bacteria can multiply up to a number, which finally results in fatal disease after silent entry without obvious symptoms. Taken together, this emphasizes the need for molecular studies on pathogenesis of *C. canimorsus*.

Taxonomic position of *Capnocytophaga canimorsus*

Capnocytophaga belongs to the phylum of *Bacteroidetes*. Taxonomically, the *Bacteroidetes* phylum is far remote from Proteobacteria and the common human pathogens. The phylum of *Bacteroidetes* includes *Porphyromonas gingivalis*, bacteria from human oral flora often associated to periodontal disease. The family of *Bacteroidaceae* contains many commensals of the mammalian intestinal system such as *Bacteroides fragilis, Bacteroides thetaiotaomicron* and *Bacteroides fragilis. Prevotella ruminicola* represents a ruminal inhabitant. The family of *Flavobacteriaceae* includes a variety of environmental and marine bacteria (Fig. 1) (Coyne and Comstock, 2008), among which *Flavobacterium johnsoniae* a common soil and freshwater bacterium is studied for gliding motility (McBride, 2004). There are only a few examples of pathogenic bacteria belonging to this family. These are *Flavobacterium psychrophilum* the causative agent of cold water disease in salmonid fish (Duchaud *et al.*, 2007), *Ornithobacterium rhinotracheale* a bacterial pathogen known for causing respiratory disease in poultry (Schuijffel *et al.*, 2005) and *Riemerella anatipestifer* leading to “duckling disease” in waterfowl and turkeys (Segers *et al.*, 1993; Subramaniam *et al.*, 2000). Finally, the family of *Flavobacteriaceae* includes the genus of *Capnocytophaga.* Nine species belong to *Capnocytophaga* and seven of them are found in normal human oral flora. *C. canimorsus* and *C. cynodegmi* are canine and feline commensals, but only *C. canimorsus* is associated with severe human infections.

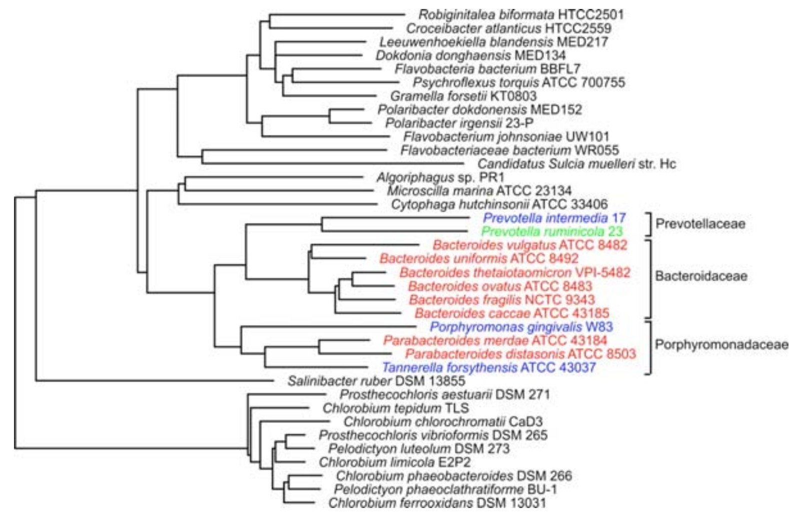

Fig. 1 16S rRNA gene cladogram of members of the *Bacteroidetes* phylum for which there is a partial or complete genome sequence. Members of the *Bacteroidales* order are shown in color. The three families of *Bacteroidales* present in this cladogram are indicated on the right. Within the *Bacteroidales* order, oral species are in blue, the ruminal species is in green, and the intestinal species are in red (Coyne and Comstock, 2008).

Studies on the pathogenesis of *Capnocytophaga canimorsus*

Few studies so far investigated the molecular basis underlying severe infections caused by *C. canimorsus* (Fischer *et al.*, 1995; Shin *et al.*, 2007). Since 2003, the group of G. Cornelis undertook elucidation of the molecular basis underlying *C. canimorsus* infections. The first finding was that *C. canimorsus* by itself does not elicit the onset of an inflammatory response. We showed that extracellular *C. canimorsus* did not lead to release of signals like IL1-alpha, IL1-beta, IL-6, IL-8, MIB-1beta, RANTES and TNF-alpha, from either naïve or activated murine macrophage cell line J774.1, bone-marrow derived macrophages, human macrophage cell line and human monocytes. This could be explained by the absence of Toll like receptor 4 (TLR4) recognition, presumably due to a hypo-reactive LPS structure. Even more, one strains, *Cc5* turned out to have a mechanism that actively blocks the pro-inflammatory signaling upon stimulation with endotoxic LPS. Live *Cc5* has been shown to down-regulate TLR4 expression and to dephosphorylate p38 mitogen-activated protein kinase (Shin *et al.*, 2007). The study on the pathogenesis of *C. canimorsus* could however not be done without efficient genetic tools. It was my task to develop the methods that allow genetic manipulation of *C. canimorsus* to be performed. This thesis describes all the necessary tools and the progress they allowed to do.

Chapter 1

Development of genetic tools for *Capnocytophaga canimorsus*

1.1. Manuscript in preparation

Genetic tools for *Capnocytophaga canimorsus*

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Abstract

Capnocytophaga canimorsus, commensal bacteria from canine oral flora, have been isolated throughout the world from severe human infections, caused by dog bites. Due to the low evolutionary relation to Proteobacteria, genetic methods suitable for the genus *Capnocytophaga* needed to be established. Here we show that Tn*4351* derived from *Bacteroides fragilis* could be introduced by conjugation in *C. canimorsus* and conferred resistance to erythromycin. By mapping and sequencing a naturally occurring plasmid isolated from a clinical isolate of *C. canimorsus*, we identified a *repA* gene, which allowed us to construct *E. coli* - *Capnocytophaga* shuttle vectors. Most commonly used antibiotic markers were not functional in *C. canimorsus* but cefoxitin (*cfxA*), tetracycline *(tetQ)* and erythromycin (*ermF)* resistances could be used as markers for plasmid maintenance in *Capnocytophaga*. Shuttle vectors were introduced into *C. canimorsus* either by conjugation using the origin of transfer (*oriT*) of RP4 or by electrotransformation. Taking advantage of the promoter of *ermF*, an expression vector was constructed. Finally, a method that allows site directed mutagenesis is described. All these genetic tools pave the way for molecular studies on the pathogenesis of *C. canimorsus*.

Introduction

Capnocytophaga canimorsus is a commensal bacterium found in the oral cavity of dogs and cats. Since its discovery in a patient that had developed septicemia and meningitis after a dog bite in 1976 (5), more than 160 cases of severe human infections by *Capnocytophaga canimorsus* have been reported (35). Human infections can result in septicemia or meningitis with mortality rates of 30% and 5%, respectively (19). Bacteria from the genus *Capnocytophaga* form part of the resident oral flora of humans and domestic animals (7). Seven species including *C. ochracea* and *C. gingivalis* are found in normal human oral flora, whereas the dog's oral flora contains *C. canimorsus* and *C. cynodegmi*. In spite of this diversity, *C. canimorsus* is the only *Capnocytophaga* that has been associated to severe human infections. Recently, we started to unravel the molecular mechanisms underlying *C. canimorsus* infections (23, 30), but we had to establish genetic methods adapted to this group of bacteria. *Capnocytophaga* belongs to the family of *Flavobacteriaceae* in the phylum of *Bacteroidetes*. Many genetic

methods that function in Proteobacteria have been shown to fail in *Bacteroidetes* (28) and commonly used broad host range plasmids did not result in ampicillin resistant (Ap^r), tetracycline resistant (Tc^r) or kanamycin resistant (Km^r) colonies of *Flavobacterium johnsoniae* (21). However, transposons and selectable markers identified and used in *Bacteroides sp.* (32, 34) have been successfully adapted for the family of *Flavobacteriaceae* (3, 20, 21).

In the present work, we describe the tools necessary to genetically manipulate *Capnocytophaga sp*. Taking advantage of genetic methods originating from *Bacteroides sp.,* we established ways to introduce DNA using functional selection markers and to perform transposon mutagenesis. Finally, we identified an endogenous plasmid in a clinical isolate of *C. canimorsus* and we generated the first shuttle vectors that allow plasmid replication in *Capnocytophaga sp*.

Materials and Methods

Bacterial strains, growth conditions and selective agents

The strains and plasmids used in this study are shown in Table 1. *E. coli* strains were routinely grown in LB broth at 37°C. *Capnocytophaga sp*. were grown on plates of Heart Infusion Agar (HIA, Difco) supplemented with 5% sheep blood (Oxoid) (SB plates) for 2 days at 37°C in presence of 5% CO_2. Bacteria were harvested by gently scraping colonies off the agar surface, washed and resuspended in PBS. *C. canimorsus* was alternatively grown in 50 ml Heart Infusion Broth (HIB, Difco) supplemented with 10 % (v/v) fetal bovine serum (FBS, Invitrogen) for approximately 24 h without shaking in an 37°C incubator with 5% CO_2 using Erlenmeyer flasks. To select for plasmids or transposons, antibiotics were added at the following concentrations: 10 µg/ ml erythromycin (Em); 10 µg/ ml cefoxitin (Cf); 20 µg/ ml gentamicin (Gm); 100 µg/ ml ampicillin (Ap); 5 µg/ ml tetracycline (Tc); 50 µg/ ml kanamycin (Km) and 10 µg/ ml chloramphenicol (Cm).

Conjugation

E. coli strains BW19581 and S17-1 used for conjugative transfer of mobilizable plasmids were grown without antibiotics to early exponential phase in LB. *C. canimorsus,* which are naturally resistant against Gm, were grown for 2 days on SB plates at 37°C and harvested by scraping. Bacteria were washed and resuspended in PBS. Donor and recipient were mixed in 1:10 ratio, centrifuged for 2 min at 8 000 x g, resuspended in 50 µl of PBS, and 2.2×10^8 cfu were spotted on 22 µm mesh nitrocellulose filters (Millipore) laid on the surface of a SB plate. The plates were incubated overnight in 5 % CO_2 at 37°C. Each filter was washed with 2 ml of HIB and 10% FBS containing Gm, kept for 1 h at room temperature and bacteria were diluted and plated on selective SB plates containing Gm and the appropriate antibiotic to select for plasmid or transposon transfer. Plates were incubated for 2 to 3 days.

Electroporation

C. canimorsus was grown in HIB and 10% FBS overnight to early or mid exponential phase without shaking, cooled to 4°C and harvested by centrifugation at 5 500 x g for 15 min at 4°C, washed 3 x in ice cold, double distilled (dd) H_2O and twice in dd H_2O plus 10% glycerol, and resuspended to a cell density of approximately 1x 10^{10}/ml in 10% glycerol. After shock freezing in liquid nitrogen, bacteria were either thawed and used for transformation or stored at -80°C. Plasmid DNA was added to 100 µl of bacterial suspension in BioRad Genepulser cuvettes with 0.2 cm electrodes and pulsed with 2.5 kV. After electroporation, bacteria were transferred to 900 µl prewarmed HIB and 10% FBS and incubated at 37°C for 2 to 3 h to allow expression of antibiotic resistance. Bacteria were plated on SB plates with the appropriate antibiotic and incubated for 2 to 3 days.

Analysis of Tn*4351* insertions

Tn*4351* was introduced into *C. canimorsus* by conjugation as described above. Genomic DNA from Em^r colonies was isolated with the GenElute™ Bacterial Genomic DNA kit (Sigma) following the manufacturer's instructions, digested with *Hind*III and analyzed by Southern hybridization using standard procedures (29). *IS4351* probes were prepared by PCR amplification using primers 3505 and 3506, plasmid pEP4351 DNA as a template and DIG -11- dUTP (Roche) according to the manufacturer's recommendations. To test for vector cointegration, the chloramphenicol acetyltransferase gene (*cat*), which is present on the Tn*4351* delivery vector pEP4351, was amplified as a 633-bp PCR product from genomic DNA using primers 3576 and 3577. All primers used are listed in Table 5.

Isolation and identification of naturally occurring plasmids in *C. canimorsus*

Plasmids were isolated from *Capnocytophaga sp.* by hot alkaline lysis (14) or alkaline lysis in combination with Qiagen columns (QIAprep® Spin Miniprep Kit, Quiagen). For analysis of pCC7, a 1.95-kb *Hind*III - *Eco*RI fragment was inserted into the corresponding restriction sites of the cloning vector pBSIIKS+ resulting in pMM7 that was subsequently sequenced. Based on the sequence information obtained, the native pCC7 plasmid was sequenced by primer walking (Fig. 1A) using BigDye Terminator Ready Reaction (PE Biosystems) and primers (3574, 3575, 3601, 3623, 3625, 3626, 3639, 3641, 3675, 3676, 3677 and 3678) described in Table 5. Results were analyzed using the Vector NTI 10.0 software (Invitrogen).

Directed gene replacement by allelic exchange

The replacement cassette with flanking regions spanning approximately 500 bp homologous to the *siaC* gene was constructed with a 3 fragment overlapping PCR strategy (Fig. 3A). First, two PCR reactions were performed on 100 ng genomic DNA of *C. canimorsus 5* with primers 4783 + 4784 for the upstream flanking region of *siaC* and with primers 4787 + 4788 for the downstream homologous regions to *siaC*. Primer 4784 for the upstream *siaC* region and primer 4787 for amplification of the downstream *siaC* region contained 20 bp homology sequence to the

ermF insertion cassette as 5' extension. The *ermF* resistance cassette was amplified from pEP4351 with primers 4785 + 4786, which contained as 5' extensions 30 bp of the *siaC* gene. All three PCR products were cleaned and then mixed in equal amounts for a PCR reaction using Phusion^{TM} polymerase (Finnzymes). The initial denaturation was at 98°C for 2 min, followed by 12 cycles without primers to allow annealing and elongation of the overlapping fragments (98°C 30 sec, 50°C 40 sec, 72°C 2 min). After addition of external primers (4783 and 4788), program continued with 20 cycles (98°C 30 sec, 50°C 40 sec, 72°C 2 min 30 sec) and final 10 min at 72°C. The final PCR product linking the three initial fragments led to the *siaC*::*ermF* insertion cassette and was then digested with the *Pst*I and *Spe*I for cloning into the appropriate sites of *C. canimorsus* suicide vector pMM25. The resulting plasmid pMM106 was transferred by RP4 mediated conjugative DNA transfer from *E. coli* S17-1 to *C. canimorsus 5* as previously described to allow integration of the insertion cassette by its homologous regions to *siaC*.

Immunoblotting

Total cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted according to standard procedures. Monoclonal antibody against C-terminal His was purchased from Invitrogen and polyclonal anti-SiaC serum is described elsewhere (Manuscript in preparation).

Nucleotide sequence accession number

The sequence of pCC7, which is reported here, has been deposited in the GenBank database under accession number EU741249.

Results

Conjugative DNA transfer

We first tried to introduce IncP and pBBR1 broad host range vectors into *C. canimorsus 5* (Table 1) by conjugative DNA transfer. Conjugation proficient *E. coli* strains (Table 1) were used to mobilize pMR20 (IncP; Tc^r), pBBR1MSC3 (Tc^r) or pBBR1MSC4 (Ap^r), but no *C. canimorsus* transconjugant could be isolated. Next we constructed pBBR1 derivatives (pMM2 and pMM3) with randomly cloned genomic DNA (500- 650 bp) of *C. canimorsus 5* in order to allow plasmid integration by cross over, but no Ap^r colonies were found, hinting that either conjugation did not occur or that the selection marker was not expressed in *C. canimorsus*. Since *C. canimorsus* belongs to the family of *Flavobacteriaceae*, we tested plasmid pCP29 derived from a natural plasmid of *Flavobacterium psychrophilum* and containing the Em^r gene *ermF*. This plasmid, which has been shown to replicate in *F. johnsoniae,* was transferred by a RP4 mediated system

from *E. coli* S17-1 to *E. coli* recipient strains and to *F. johnsoniae* but no Em^r *C. canimorsus* transconjugant colony could be obtained. To ensure that this failure was due to the replication origin and not to the selection marker or the DNA transfer itself, we turned to transposon Tn*4351*. Tn*4351* carrying the *ermF* gene was isolated in 1985 from pBF4, a self-transmissible plasmid from *Bacteroides fragilis* (32). As a delivery vector for Tn*4351* we used plasmid pEP4351, which can be mobilized from *E. coli* BW19851 by the chromosome encoded RP4 conjugation machinery. Em^r transconjugants of *C. canimorsus 5* could be isolated in this way, showing first that conjugation works as a method to transfer DNA into *C. canimorsus* and second, that *ermF* is expressed and can be used as a selection marker. This result also suggested that pCP29 from *F. psychrophilum* did not replicate in *C. canimorsus 5*. We then cloned the *ermF* gene including its own promoter into pBBR1MCS4 giving pMM5 and used *E. coli* S17-1 as a donor strain to transfer pMM5 to *C. canimorsus 5*. No Em^r colonies of *C. canimorsus* appeared after conjugation demonstrating that the pBBR replicon is not functional in *C. canimorsus*.

Generation of replicating shuttle vectors for *C. canimorsus*

In order to find a plasmid that can replicate in *C. canimorsus*, we screened eight *C. canimorsus* strains (Table 1) for the presence of endogenous plasmids. Two plasmids were identified in strain *C. canimorsus 7* (not shown). The smaller plasmid designated pCC7 was sequenced (4579 bp) (Fig. 1A). Blast homology search revealed a gene encoding a putative replication protein with homology to replicases of *C. ochracea*, *B. fragilis* and *B. vulgatus* (*repA*, 1074 bp) (Table 2). The gene product of a 1125-bp long open reading frame (designated orf CC7p_3) showed homology to ISPg1 transposase from *Porphyromonas gingivalis* (ref|NP_904520.1|).

We generated shuttle vectors by amplifying this *repA* gene including 408 bp of its upstream region and inserting into pLYL03 that contains *ermF* and the origin of transfer of RK2. The resulting vector pMM105.A could be mobilized by the RP4 mediated conjugation machinery from *E. coli* S17-1 to *C. canimorsus 5* with transfer frequencies of around 10^{-4} per recipient (Table 3). This plasmid could also be transferred to *C. canimorsus 12,* but the frequency of transfer was significantly lower than in *C. canimorsus 5* (Table 3). The replicase gene and upstream region

that we isolated were thus sufficient for autonomous plasmid replication in *C. canimorsus*.

Finally, we constructed similar shuttle vectors for *C. canimorsus* with a Tc selection marker (*tetQ* gene, pMM104.A) or a Cf^r marker (*cfxA* gene, pMM45.A) (Table 3).

Construction of an *E. coli* - *C. canimorsus* shuttle expression vector

To generate an expression vector for *C. canimorsus*, a 257-bp fragment upstream of *ermF* containing the canonical -33 and -7 boxes of *Bacteroides* promoters was amplified by PCR. Additionally, the primers used for amplification of the promoter region incorporated unique *Nco*I, *Xba*I and *Xho*I restriction sites for cloning purposes as well as 6 histidine codons, which allows the insertion of a coding sequence in or out of frame with a C-terminal His tag (Fig. 1B and 1C). The PCR product was digested with appropriate restriction enzymes and inserted in pMM41.A, creating the shuttle expression vector pMM47.A (Fig. 1B). To test this vector, we cloned the promoterless *siaC* gene encoding a sialidase from *C. canimorsus* 5 (manuscript in preparation) into pMM47.A resulting in pMM52. As shown in Fig. 3B, sialidase could be detected in crude extracts of a sialidase-deficient Tn*4351* mutant of *C. canimorsus 5* (Δ*siaC*) harboring pMM52 or of a site-directed mutant of *siaC* (*siaC::ermF*) complemented with pMM52, indicating the functionality of the expression vector pMM47.A.

Electrotransformation as method to transfer DNA

The shuttle vectors described above allowed us to test if electrotransformation could be applied as an alternative way of introducing DNA into *C. canimorsus*. Competent bacteria were prepared by washing in ice cold water and 10% glycerol and giving a final cold shock by freezing in liquid N_2. In this way, plasmid DNA (pMM47.A) isolated from an *E coli* host strain could be transformed into *C. canimorsus 5* with an efficiency of 2.1×10^3 clones per µg of DNA (Table 4). A 2 min heat treatment at 56°C before electroporation was tested in order to prevent degradation of DNA by intracellular restriction systems but this treatment turned out to reduce the transformation efficiency. The same decrease was observed when using $MgCl_2$ or NaCl during the washing steps (data not

shown). Hence, sufficient transformation efficiency could be reached using electrotransformation of DNA isolated from *E. coli.*

E. coli **-** ***C. canimorsus*** **shuttle vectors can be introduced to** ***C. cynodegmi, C. ochracea*** **and** ***C. gingivalis*** **by a RP4 mediated conjugation machinery of** ***E. coli***

E. coli S17-1 carrying plasmids pMM45.A (Cf^r), pMM104.A (Tc^r) or pMM105.A (Em^r) were mated with *C. cynodegmi, C. ochracea* or *C. gingivalis.* (Table 3). All three shuttle vectors (*ermF, tetQ* and *cfxA*) were functional in *C. cynodegmi*. In contrast, only *tetQ* and *ermF* were functional in *C. ochracea*, and *tetQ* and *cfxA* were functional in *C. gingivalis.* Although conjugation frequencies varied from 10^{-4} to 10^{-8} transconjugants per recipient cell depending on the species (Table 3), tools developed for *C. canimorsus* can thus be used for other species in this genus.

Tn*4351* transposition in *C. canimorsus*

As shown before, Tn*4351*, derived from *B. fragilis*, could be introduced to *C. canimorsus 5* using *E. coli* BW19851 to mobilize the delivery vector pEP4351 by conjugation. Em^r colonies of *C. canimorsus 5* appeared at a frequency of 10^{-6} to 10^{-8} per recipient. Genomic DNA was thereafter analyzed by Southern Blot after *Hind*III restriction (Fig. 2). For *C. canimorsus 5* mutants W2E9, X7B9 and Y2F12, two bands hybridized with DIG labeled *IS4351*, while for mutant X2E4 three bands hybridized (Fig. 2A). In mutant X2E4, the *cat* gene from the delivery vector could also be detected by PCR amplification indicating that a cointegration event took place (Fig. 2B). We conclude that clones W2E9, X7B9 and Y2F12 contained one copy of Tn*4351* flanked by the *IS4351* sequences, while X2E4 contained one copy of the Tn*4351,* but cointegrated with the vector resulting in 3 copies of the *IS4351* as schematically represented in Figure 2C.

Site-directed gene replacement using an antibiotic resistance cassette

Taking advantage of the DNA transfer procedures and selection markers that we had established, we next tried to perform site-directed gene replacement. For the proof of principle, we selected the *siaC* gene as a target. A replacement cassette consisting of *ermF* flanked by approximately 500 bp regions homologous

to *siaC* was constructed as schematically shown in Figure 3A. The resulting plasmid pMM106 lacking the replicon for *C. canimorsus* was introduced by *E. coli* S17-1 into *C. canimorsus 5.* Transconjugants selected on Em were assumed to have integrated pMM106 by a single recombination event at the homologous regions of *siaC* into the chromosome. Colonies were replicated on Cf and Em, and Cf sensitive and Em^r colonies assumed to have undergone an excising event of the vector backbone were picked. The disruption of the sialidase (*siaC::ermF*) was confirmed by PCR, sequencing and immunoblotting against SiaC (Fig. 3B) as well as by testing the loss of sialidase activity using 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN) as substrate (Fig. 3C). Activity and sialidase expression could be restored by introducing *in trans* the full length gene cloned into the expression shuttle vector pMM47.A (Fig. 3B and 3C).

Discussion

The availability of genetic methods is crucial for the study of molecular mechanisms associated with the pathogenesis of bacterial infections. In this study, techniques that allow the genetic manipulation of *C. canimorsus* were developed, opening the possibility of genetic analysis to bacteria of the genus *Capnocytophaga*. We show that *C. canimorsus* can serve as a recipient for RP4 mediated conjugation, but we found that the classical broad host range replicon pBBR1 is not functional in *C. canimorsus*. The replicon from a natural plasmid of the closely related *F. psychrophilum* did not lead to plasmid replication in *C. canimorsus* either. Therefore, we isolated and sequenced an endogenous plasmid from *C. canimorsus* 7 and identified a replication region that could be used to engineer shuttle vectors. These vectors could also be used in other species of the genus *Capnocytophaga*. Selection markers *ermF*, *cfxA* and *tetQ* originating from *Bacteroides sp.* could be successfully used in *C. canimorsus,* suggesting that the promoter region and the sigma factor resemble those found in *Bacteroides sp.* rather than those from *E. coli* (4). This is in line with the assumption that the classical selection markers used in *E. coli* could not be used in *C. canimorsus,* presumably due to the lack of promoter recognition. We thus engineered an expression vector for *C. canimorsus* using the promoter region of *IS4351* with the *Bacteroides* consensus for -33 and -7 boxes located upstream from the *ermF* gene in Tn*4351* (25).

For a Tn mutagenesis approach, we tested Tn*4351,* a transposon widely used in *Bacteroides sp.* (31), *Flavobacterium sp.* (20) and *P. gingivalis* (9-11). Southern Blot analysis showed that Tn*4351* integrated in the *C. canimorsus* genome, either alone or as a cointegrate with its vector. This vector coinsertion has been previously reported to occur in a strain dependent manner in bacteria of the *Bacteroidetes* phylum. It has been also reported that Tn*4351* does not integrate in a random manner (12). For these reasons, a *mariner*-based transposon for *Flavobacterium sp.* was constructed by Braun *et al*. using *ermF* as a selectable marker (6). Although *Himar* insertions are reported to occur at positions containing the target nucleotide sequence "TA" and are usually described as being otherwise random (38), *Himar* insertions were not completely random in *F. johnsoniae* (6). In spite of these limitations, the *mariner* Tn could be another approach for Tn mutagenesis of *C. canimorsus*.

A method for directed gene disruption by allelic exchange with a resistance marker cassette was also developed, demonstrating that homologous recombination occurs in *C. canimorsus*. Inserting a resistance marker cassette into the chromosome might influence expression of downstream genes located in an operon and thus limit this method in some instances. More work has to be performed to generate clean knock-outs without the availability of negative selections markers like the levan sucrase that have been widely used in *Proteobacteria* (15, 27).

Taken together, a collection of techniques allowing genetic manipulations in *C. canimorsus* has been established. This will provide the basis for new approaches to understand the mechanisms underlying pathogenesis of *C. canimorsus* infections.

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Figures

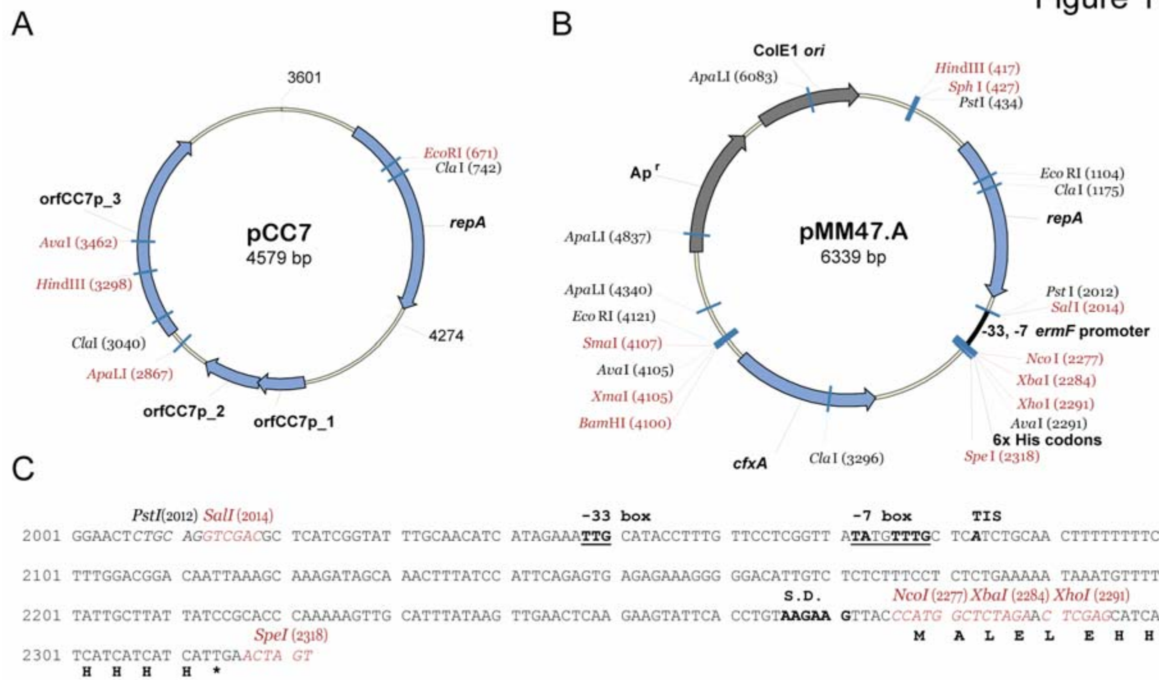
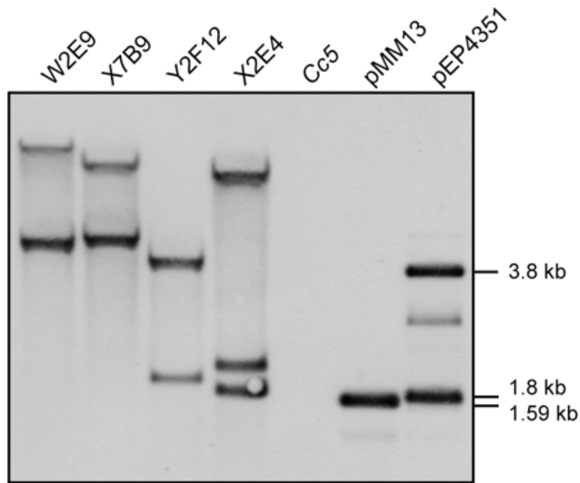
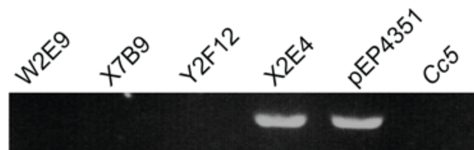


Figure 1. Engineering of an expression shuttle vector from a natural *C. canimorsus* plasmid. (A) Genetic and restriction map of the endogenous plasmid pCC7 showing the primer binding sites used for amplification of the replicon (3601 and 4274). The nucleotide sequence was deposited at GenBank under accession number EU741249. (B) Map of the shuttle expression vector pMM47.A containing the *cfxA* gene (Cf^r) for selection in *C. canimorsus*, the *repA* replicon of pCC7 and the promoter of *ermF* (-33, -7 boxes) upstream from the *Nco*I, *Xba*I and *Xho*I restriction sites that allow insertion of a coding sequence in frame or out of frame with 6 histidine codons. Unique restriction sites are shown in red. (C) Partial nucleotide sequence of pMM47.A showing the promoter with its -33 and -7 boxes (bold, underlined), the transcription initiation site (TIS, bold, italics) and the Shine Dalgarno (bold) (25). Restriction sites (italics) that are unique are shown in red. The ATG codon within the *Nco*I site can be used as the start codon giving the translation shown below the nucleotide sequence.





C

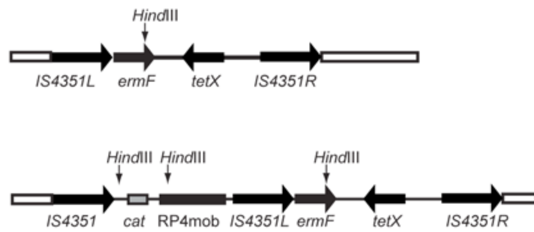


Figure 2. Integration of Tn*4351* into the chromosome of *C. canimorsus 5*.

(A) Southern blot hybridization. Genomic DNA from wt (*Cc5*) and four insertion mutants (W2E9, X7B9, Y2F12, X2E4) as well as DNA from pMM13 and pEP4351 were digested with *Hind*III and hybridized with DIG-labeled *IS4351.* Plasmids pMM13, containing one copy of *IS4351* and pEP4351, containing Tn*4351* (2 copies of *IS4351*) served as positive controls. (B) PCR amplification of the 633-bp *cat* gene from the vector pEP4351, to identify vector cointegration events. (C) Top: schematic representation of Tn*4351* integrated into the chromosome (open bars) with the *IS4351* insertion sequences flanking *ermF* and *tetX*. Bottom: schematic representation of a cointegrate with the *cat* gene and the mobilization (*mob*) site of the vector.

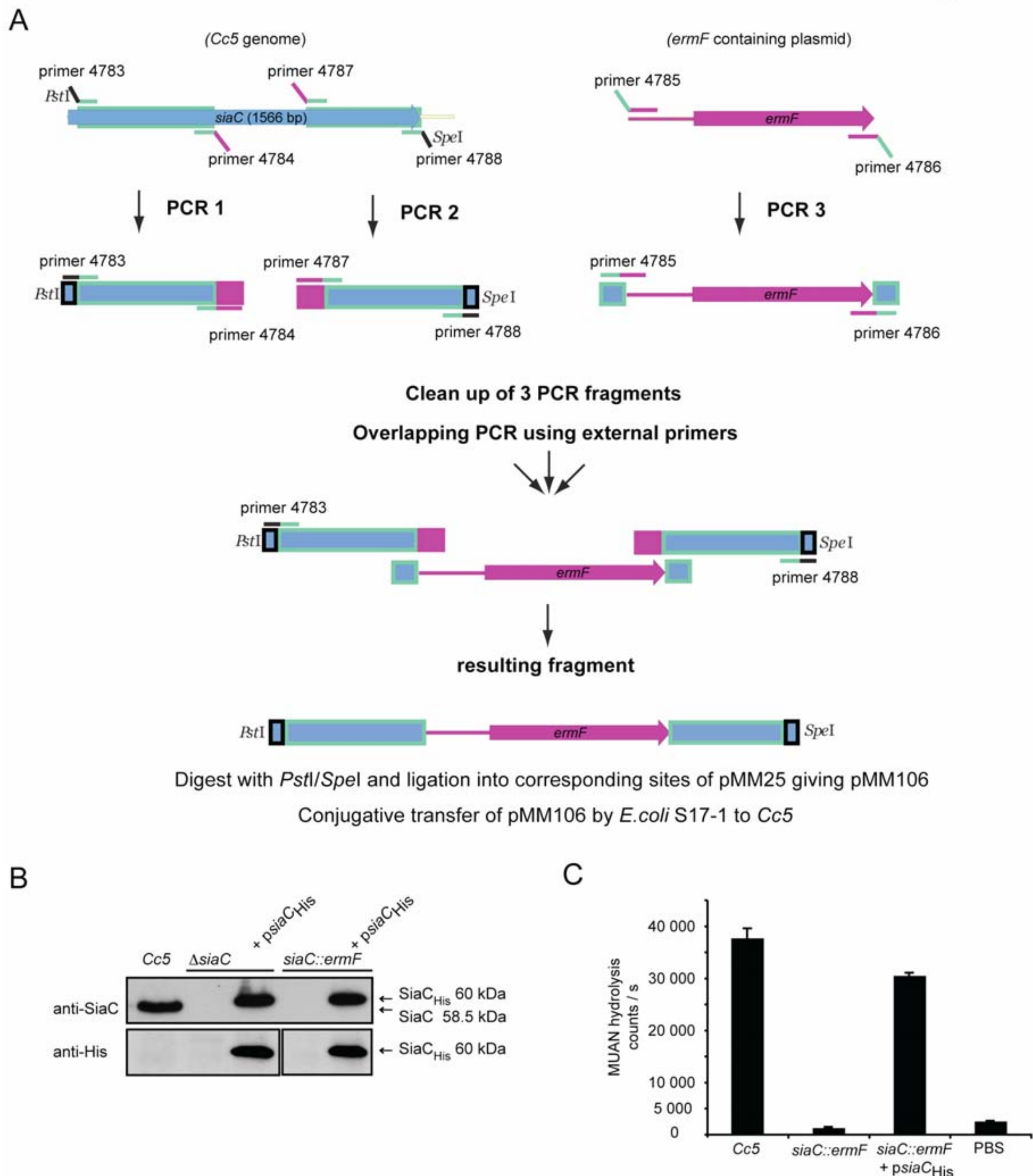


Figure 3. Generation of a *C. canimorsus 5* sialidase knock-out and complementation *in trans* by *siaC*$_{His}$ cloned in expression shuttle vector pMM47.A.

(A) Schematic representation of the deletion strategy. The upstream flanking region was amplified from genomic *C. canimorsus 5* DNA with primers 4783 (*Pst*I, black) and 4784 containing an additional 5' 20 nt extension homologous to the resistance cassette *ermF* (magenta) (PCR 1). The same was done for the downstream flanking region with primers 4787 including 20 nt complementary to the resistance cassette in 5' (magenta) and 4788 including a *Spe*I restriction site (black) (PCR 2). The *ermF* resistance cassette (magenta) was amplified with primer 4785 which

included the 30 bp homology to the end of the upstream flanking region at 5' (green) and primer 4786 with 30 bp homology to the downstream flanking region (green)(PCR 3). All three PCR products were subjected in equal amounts to another PCR reaction after addition of external primers (4783 and 4788). The final PCR product linking the three initial fragments was digested with *Pst*I and *Spe*I and cloned into the suicide vector pMM25, giving pMM106. (B) Immunoblot analysis of crude cell extracts of wt (*Cc5*), *siaC* deficient Tn*4351* mutant (Δ*siaC*), the site-directed mutant of *siaC* (*siaC::ermF*) and both mutants (Δ*siaC* and *siaC::ermF)* complemented *in trans* with pMM52, using a polyclonal serum against SiaC (top) and a monoclonal antibody against the C-terminal His tag encoded by pMM52 (p$siaC_{His}$) (bottom). (C) Sialidase activity was measured by monitoring the fluorescence at 445 nm generated by the cleavage of 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN) (mean +/- SD of a representative experiment).

Tables

Table 1. Strains and Plasmids

Strain or Plasmid	Genotype or Description	Reference or Source
Bacterial strains		
E. coli		
BW19851	S17-1 derivative, RP4-2-*tet*:Mu-1*kan*::Tn*7*, *recA1 creC510 hsdR17 endA1 zbf-5 uidA*::*pir*+ *thi.* Sm^r	ATCC 47083 (22)
S17-1	*hsdR17 recA1* RP4-2-*tet*::Mu-1*kan*::Tn7 . Sm^r	(33)
Top10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L, *end*A1 *nup*G. Sm^r	Invitrogen
C. canimorsus		
C. canimorsus 2	Human septicemia 1989.	(30)
C. canimorsus 3	Human septicemia 1990.	(30)
C. canimorsus 5	Human septicemia 1995.	(30)
C. canimorsus 5 ΔsiaC	*C. canimorsus 5 siaC*::Tn*4351*; Em^r	Manuscript in preparation
C. canimorsus 5 siaC::ermF	Site directed mutation of *siaC* by replacement of an internal part by *ermF*; Em^r	This study
C. canimorsus 7	Human septicemia 1998.	(30)
C. canimorsus 9	Human septicemia 1965	(30)
C. canimorsus 10	Human septicemia	(30)
C. canimorsus 11	Human septicemia	(30)
C. canimorsus 12	Human septicemia ATCC 35979	(30)
C. cynodegmi	Dog's mouth; USA, Virginia 1979	ATCC 49044

C. cynodegmi 2	Hand wound; United States	LMG 11538. (36)
C. gingivalis	Human isolate	G. Wauters, University of Louvain, Belgium
C. ochracea	Human isolate	G. Wauters, University of Louvain, Belgium

Plasmids[a]	Description	
pBBR1MCS3	Broad host range *ori* from *Bordetella bronchiseptica* S87, Tc^r	(17)
pBBR1MCS4	Broad host range *ori* from *Bordetella bronchiseptica* S87, Ap^r	(17)
pBSIIKS (+)	ColE1 *ori*, Ap^r	Stratagene
pCC7	Endogenous plasmid of *C. canimorsus 7*	This study
pCP23	ColE1 *ori*; (pCP1 *ori*); Ap^r (Tc^r); *E. coli - F. johnsoniae* shuttle plasmid	(1)
pCP29	ColE1 *ori* (pCP1 *ori*); Ap^r (Cf^r, Em^r); *E. coli - F. johnsoniae* shuttle plasmid	(16)
pEP4351	*pir* requiring R6K *oriV*; RP4 *oriT*; Cm^r Tc^r (Em^r); vector used for Tn*4351* mutagenesis	(8)
pK18	ColE1 *ori*, Km^r	(24)
pLYL001	ColE1 *ori*; Ap^r (Tc^r)	(26)
pLYL03	ColE1 *ori*; Ap^r (Em^r)	(18)
pMM2	pBBR *ori*; Ap^r; Random 650-bp *Sau*3A chromosomal fragment of *C. canimorsus 5* inserted in *Bam*HI site of pBBR1MCS4	This study
pMM3	pBBR *ori*; Ap^r; Random 500-bp *Sau*3A chromosomal fragment of *C. canimorsus 5* inserted in *Bam*HI site of pBBR1MCS4	This study
pMM5	pBBR *ori* ; Ap^r, (Em^r); *ermF* from pEP4351 amplified by PCR using primers 3505 and 3506 cut with *Eco*RI/*Pst*I and inserted into the corresponding sites of pBBR1MCS4	This study
pMM7	ColE1 *ori*; Ap^r; 1.95-kb *Eco*RI/*Hind*III fragment of pCC7 inserted into corresponding sites of pBSIIKS(+)	This study
pMM12	ColE1 *ori*; Km^r (Cf^r); *cfxA* gene from pCP29 cloned as a *Bam*HI/*Spe*I fragment into corresponding sites of pK18	This study
pMM13	ColE1 *ori*; Ap^r (Em^r); *ermF* from pEP4351 amplified by PCR as a 1.95-kb fragment using primers 3505 and 3506, cut with *Eco*RI/*Pst*I and inserted into the corresponding sites of pBSIIKS(+)	This study

pMM25	ColE1 *ori*; Km^r (Cf^r); Suicide vector for *C. canimorsus*. RP4 *oriT* amplified by PCR using primers 4416 and 4417 inserted into *Bam*HI site of pMM12.	This study
pMM40.A	ColE1 *ori* (pCC7 *ori*); Km^r (Cf^r); *E. coli - C. canimorsus* shuttle plasmid. The *repA* gene from pCC7 was amplified by PCR using primers 3601 + 4274, digested with *Pst*I and inserted into the corresponding site of pMM12.	This study
pMM41.A	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); *E. coli - C. canimorsus* shuttle plasmid. The *cfxA* and *repA* genes as a *Bam*HI/*Sph*I fragment from pMM40.A inserted into corresponding sites of pUC19.	This study
pMM45.A	ColE1 *ori* (pCC7 *ori*); Km^r (Cf^r); *E. coli - C. canimorsus* shuttle plasmid, RP4 *oriT.* The 1.58-kb *Pst*I fragment of pMM47.A containing *repA* inserted into *Pst*I site of pMM25.	This study
pMM47.A	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); *E. coli - C. canimorsus* expression shuttle plasmid. -33 and -7 of the *ermF* promoter was amplified from pEP4351 as a 257-bp fragment by PCR using 3868 and 4128. Unique *Nco*I, *Xho*I, *Xba*I sites and 6 histidine codons were incorporated by reverse primer 4128. *Sal*I/*Spe*I digested PCR fragment inserted into corresponding sites of pMM41.A.	This study
pMM52	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); *siaC* full length inserted in *Nco*I/*Xba*I sites of pMM47.A in frame with a C-terminal His tag.	Manuscript in preparation
pMM104.A	ColE1 *ori* (pCC7 *ori*); Ap^r (Tc^r); *E. coli - C. canimorsus* shuttle plasmid, RP4 *oriT.* *Pst*I fragment of pMM47.A containing *repA* inserted into *Pst*I site of pLYL001.	This study
pMM105.A	ColE1 *ori* (pCC7 *ori*); Ap^r (Em^r); *E. coli - C. canimorsus* shuttle plasmid, RP4 *oriT.* *Pst*I fragment of pMM47.A containing *repA* inserted into *Pst*I site of pLYL03.	This study

pMM106	ColE1 *ori*; Km^r (Cf^r); To create *siaC::ermF* three initial PCR products were amplified with 4783 + 4784 and 4787+ 4788 from *Cc5* chromosomal DNA and 4785 + 4786 from pEP4351. *siaC::ermF* was then amplified by overlapping PCR using external primers 4783 and 4788, cut with *Pst*I/*Spe*I and inserted into corresponding sites of pMM25.	This study
pMR20	Tc^r derivative of pGLIO, RK2-based broad host-range vector; IncP	(13) (Chris Mohr and Rick Roberts)
pUC19	ColE1 *ori*, Ap^r	(37)

[a] Antibiotic resistance phenotypes: ampicillin, Ap^r; cefoxitin, Cf^r; chloramphenicol, Cm^r; erythromycin, Em^r; streptomycin, Sm^r; tetracycline, Tc^r. Antibiotic resistance phenotypes and other features listed in parentheses are those expressed by secondary host (*F. johnsoniae* or *C. canimorsus*) but not by *E. coli.*

Table 2. Best matches of RepA of *C. canimorsus 7* to known protein sequences of non redundant database (June 2008) using a blast algorithm (2)

Species	Accession	Score	E value
Capnocytophaga ochracea	gb|AAY78540.1|	304	5e-81
Bacteroides vulgatus	emb|CAA60389.1|	300	1e-79
Bacteroides fragilis	emb|CAA60390.1|	299	3e-79
Bifidobacterium bifidum	gb|AAZ79481.1|	292	2e-77
Ornithobacterium rhinotracheale	gb|AAT09350.1|	266	6e-69
Prevotella intermedia	gb|AAL73041.1|AF454701_2	263	5e-68

Table 3. Frequencies of Transfer of *E. coli - Capnocytophaga* shuttle vectors, in matings with *E. coli* S17-1 (transconjugants / recipient)

Strain	Frequency of transfer					
	Plasmid pMM45.A		**pMM104.A**		**pMM105.A**	
	Mean[a]	SD	Mean[a]	SD	Mean[a]	SD
C. canimorsus 5	4.8×10^{-4}	4.1×10^{-4}	1.1×10^{-3}	9.6×10^{-4}	5.9×10^{-4}	4.3×10^{-4}
C. canimorsus 12	2.7×10^{-7}	2.5×10^{-7}	2.0×10^{-6}	1.8×10^{-6}	4.4×10^{-8}	6.8×10^{-10}
C. cynodegmi	1.7×10^{-4}	2.7×10^{-4}	9.1×10^{-5}	6.2×10^{-5}	5.8×10^{-5}	8.0×10^{-5}
C. cynodegmi 2	3.8×10^{-5}	1.0×10^{-5}	5.4×10^{-6}	2.8×10^{-6}	2.3×10^{-5}	3.2×10^{-5}
C. ochracea	($< 2.7 \times 10^{-7}$)		1.9×10^{-4}	2.7×10^{-4}	1.4×10^{-1}	1.8×10^{-1}
C. gingivalis	3.9×10^{-6}	3.3×10^{-6}	1.4×10^{-5}	8.4×10^{-6}	($< 4.5 \times 10^{-8}$)	

[a] mean values from at least 3 independent experiments

Table 4. Quantification of DNA transfer into *C. canimorsus* by electroporation

Strain	Plasmid	Transformants / µg DNA		Transformants / viable *Cc5*	
		Mean[a]	SD	Mean[a]	SD
C. canimorsus 5	pMM47.A	2.1×10^{3}	+/- 2.3×10^{3}	4.4×10^{-7}	+/- 4.0×10^{-7}

[a] mean values from at least 3 independent experiments

Table 5. Oligonucleotides used in this study

Collection number	Sequence
3505	GCAACAGAATTCTGATTAATAA
3506	TTTTCTGCAGCTACGAAGGATGAA
3574	TTCAAATCTCTTAAAACCCCAG
3575	TCTAAGGCGAATAGGGAATATC
3576	CACTGGATATACCACCG
3577	TGCCACTCATCGCAGTA
3601	TTTTCTGCAGGTTAAAATCGGCCGCC
3623	ATGTAGATATACAAATGCCTG
3625	ACCCACCATTTCCTTTCCCTAAC
3626	CAGCCACTTCCTTGAAGAAATG
3639	GAAGTATTTTTGTTCGATACCAAGG
3641	TAATACTGGCATCGACCTTTACGCC
3675	CATTTCGGTTACATCCCATAATAGC
3676	AATTTCTAATGTCAAGGAAAAACCG
3677	TTACCTTCTTGTTGGTTTTAACTG
3678	TTTATCGTGCACAGGTCTCATTAG
3868	TCATGTCGACGCTCATCGGTATTTGCAACA
4128	TTACTAGTTCAATGATGATGATGATGATGCTCGAGTTCTAGAGCCATGGGG
4274	ATGGCTGCAGAGTTCCTACGATTGCCATA
4416	CCGGATCCCTTGGTTTCATCAGCCATC
4417	GCGGATCCATCAGTAATTTCCTGCATTTG
4783	CCCTGCAGATTTGTCGGCTTGTGGAAGCC
4784	GAGTAGATAAAAGCACTGTTGTGCTTCGACTCATTCCTAC
4785	AGATGTAAACGTAGGAATGAGTCGAAGCACAACAGTGCTTTTATCTACTCCGA TAGCTTC
4786	AGCTCCCGTTCCACAATGCCACGTTTTTCCCTACGAAGGATGAAATTTTTCAGG GACAAC
4787	AAAAATTTCATCCTTCGTAGGGAAAAACGTGGCATTGTGG
4788	CCACTAGTTTAGTTCTTGATAAATTCCTCAACTGG

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1.2. Additional results

1.2.1. Reporters for *C. canimorsus*

There have only been few publications addressing use of fluorescent proteins for markers in the *Bacteroidetes* phylum. Only one report described a successful use of GFP in this phylum. In this recent study, a promoter-trap system was successfully used to isolate and analyze several strong promoters, and GFP_{mut3} was shown to be functional as a reporter in *Flavobacterium hibernum* (Chen et al., 2007).

We earlier used different approaches to introduce reporters to the collection of genetic tools for *C. canimorsus*. First, we used the *Bacteroides ermF* promoter of the *IS4351* ("IS-33") to transcribe *egfp* with the mammalian codon usage or gfp_{mut2} using codon usage from *Aequorea victoria* but resulting in an optimized GFP in *E. coli*. Transcript analysis by reverse transcription confirmed presence of the mRNA of the corresponding constructs (Fig 4A). However, no GFP protein could be detected by immunoblotting against GFP (Fig. 4B). By fluorescence microscopy or FACS analysis, no fluorescent *C. canimorsus* could be identified (not shown). IS-33*egfp in trans* of *E. coli* was not expressed (Fig. 4B), therefore we suggest that the IS-33 promoter is not recognized by the *E. coli* sigma factors.

Notably, one study addressing reporters in *Porphyromonas gingivalis* showed that luciferase was functional in this species, while GFP could not be expressed (Liu *et al.*, 2000). We therefore constructed plasmids with luciferase genes (*luxAB*) transcribed by the IS-33 promoter. While all the plasmids containing *luxAB* could be transcribed (Fig. 4C), no luminescence could be detected in a luciferase assay, while the positive control *Y. enterocolitica* KNG22703 (Kaniga *et al.*, 1992) was functional (Fig. 4D).

To test for promoter activity we next used the chloramphenicol acetyl transferase (*cat*) from *E. coli* transcribed by the IS-33 promoter in *C. canimorsus* (Fig. 4E). We assessed the specific activity of Cat and found that it was about 3-fold enhanced in *Cc5* harboring the reporter construct *in trans* as compared to the empty vector control (Fig 4F). However, the positive control of plasmid encoded Cat in *E. coli* showed about 30-fold higher activity. This suggests that the IS-33 promoter is weak in *Cc5*. The Shine Dalgarno site might also result in poor expression.

It is conceivable that the promoter from the IS sequence identified in *Bacteroides sp.* (Shoemaker *et al.*, 1985; Shoemaker *et al.*, 1986) used in this study is sufficient for use in *C. canimorsus* but a strong promoter might improve certain applications. The results from the publication aforementioned (Chen *et al.*, 2007) suggests that promoter strength might be a limiting factor for GFP expression in *Cc5.* Shine Dalgarno sites as well as codon usage suitable for use in *C. canimorsus* further need to be carefully evaluated, and the completion of the *Cc5* genome will provide access to this information.

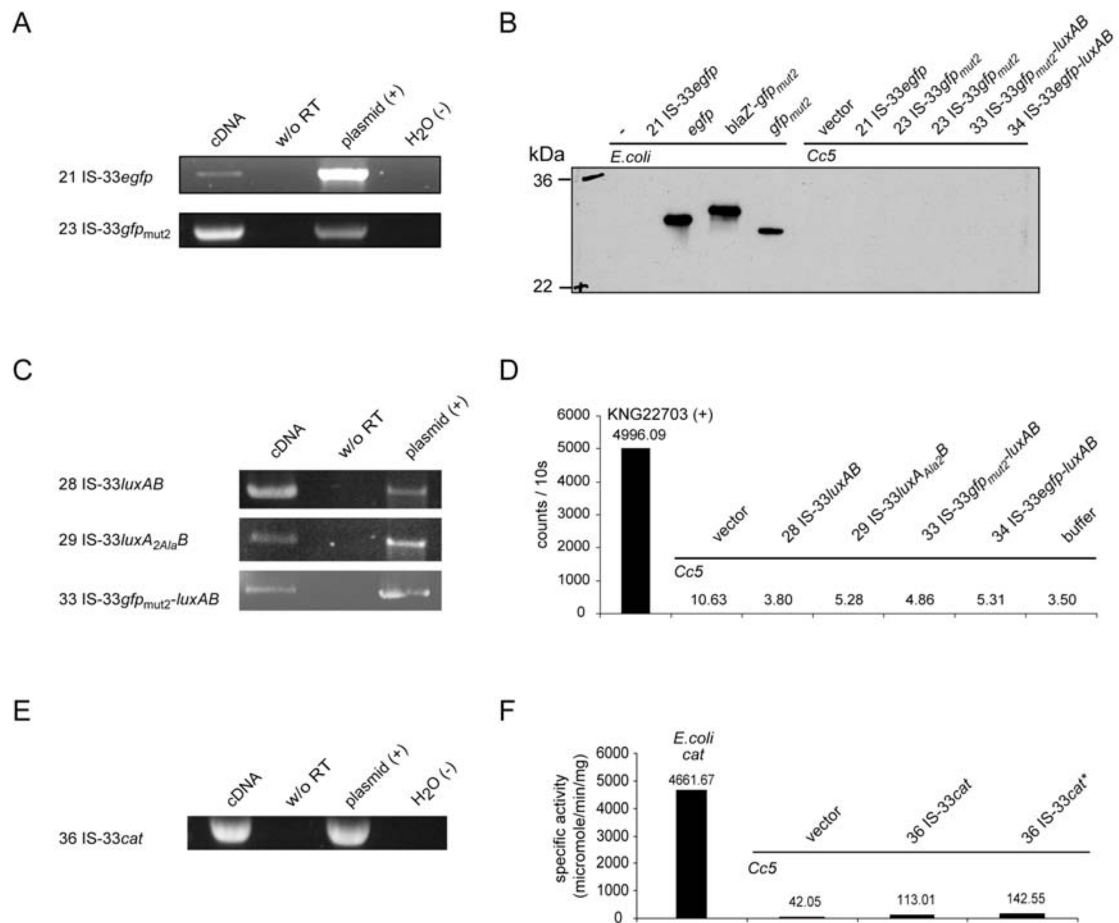

Fig. 4 GFP and Luciferase are transcribed by the IS-33 promoter in *Cc5*, but were not functional, only Cat could be used as reporter.

Transcript analysis was performed by RT PCR with (cDNA) or without (w/o RT) reverse transcriptase or on control plasmid DNA (+) using primer 3578 + 3852 (IS-33egfp) or 3578 + 3869 (IS-33gfpmut2) (A) and immunoblotting using anti-GFP (Invitrogen) (B). RT PCR was used to assess transcript levels in luciferase constructs using primers 3578 + 3952 (C) and luciferase activity was determined as luminescence including as a positive control *Y. enterocolitica* strain KNG22703 (D). Transcript was analyzed for *cat* using primers 3578 and 3972 (E) and the specific

activity of Cat in *Cc5* was assessed by the Chloramphenicol acetyl transferase assay in comparison to plasmid encoded Cat of *E. coli* (F).

1.2.2. Methods section

RNA isolation and reverse transcription (RT) PCR

RNA was isolated from bacteria grown for 2 days on HIA blood plates by a hot phenol/ chloroform extraction method followed by DNase I (Amersham Pharmacia) treatment (0.5 U / µg RNA) for 2 h at 37°C. RNA was further cleaned by using a RNeasy kit (Quiagen) and stored at -80°C until use. An additional DNase I digest was introduced with 0.25 U / µg RNA for 15 min at 37°C and stopped by addition of final 2.5 mM EDTA and heat inactivation at 75°C for 10 min. Subsequent reverse transcription was performed with 50 U Superscript II / µg RNA in RT buffer (Invitrogen), 10 mM DTT and 50 µM specific primer for 60 min at 42°C and stopped at 70°C for 10 min.

Luciferase assay luciferase NADH/ FMN oxidoreductase coupled assay

Bacteria were resuspended in PBS, lysed by sonication (*Yersinia* and *C. canimorsus* strains) or with triton 0.5% (*C. canimorsus* strains). N-decanal was added at 0.1%. The reaction mix contained 0.0005% mercaptoethanol, 1.3 mM NADH, 0.042 mM FMN (flavin mononucleotide) in 0.065 M final sodium phosphate buffer pH 6.8. Samples were normalized against protein content of the lysates determined by Bradford. All chemicals were purchased from Sigma Aldrich unless otherwise stated.

Chloramphenicol acetyl transferase assay

Cc5 bacteria were lysed with 0.5% triton for 5 min at RT and *E coli* strains were sonicated. 10 µl lysate was mixed with 100 µM 5, 5′-Dithiobis 2-nitrobenzoic acid (DTNB) and 5 mM acetyl coenzyme A in 1 M Tris HCl pH 8. Background reading was recorded for 2 min. After addition of chloramphenicol to 0.1 mM final concentration, absorbance at 412 nm was measured for 5 min in 30 sec intervals. Specific activity of cat (µmol/min/mg) was calculated by ΔA_{412} / (min x 0.0136 x mg protein). As positive control, *E.coli* BW19851 [pEP4351] was included in the assay resulting in plasmid encoded Cat.

GFP expression

GFP expression was tested by immunoblotting against GFP (Invitrogen) according to manufacturer's suggestions. Fluorescence was tested by microscopy and FACS.

Plasmids

All plasmids are described in Table 12.

Chapter 2

C. canimorsus 5 sialidase links metabolism and pathogenesis

2.1. Manuscript in preparation

Author contributions. MM, HS and GC conceived and designed the experiments. MM, HS and CP performed the experiments. MM, HS, RL and GC analyzed the data. MM, GC, RL and HS wrote the paper.

Statement of my work. My contribution was the data of figures: Fig. 1c, Fig. 2, Fig. 3, Fig. 4 and Fig. 5; mutant identification, plasmids for complementation and genetic system for *C. canimorsus*.

Capnocytophaga canimorsus: a human pathogen feeding at the surface of phagocytes

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Abstract

Capnocytophaga canimorsus, commensal bacteria from canine oral flora, have been repeatedly isolated since 1976 from severe human infections transmitted by dog bites. Here we show that *C. canimorsus* grows better when it is in direct contact with animal cells, including phagocytes. This unique property was dependent on a surface-exposed sialidase allowing *C. canimorsus* to feed on internal aminosugars of glycan chains from host cell glycoproteins. In addition, sialidase conferred resistance to complement by promoting the binding of factor H. In a murine infection model, the wild type, but not the sialidase deficient mutant, grew and persisted, both when infected singly or in competition. This study unravels a unique example of pathogenic bacteria feeding on phagocytes and it illustrates how the adaptation of a commensal to its ecological niche in the host, here the dog's oral cavity, inevitably contributes to being a potential pathogen.

Introduction

C. canimorsus, a Gram-negative commensal of dogs and cats mouth, has been reported since its discovery in 1976 to cause peripheral gangrene, fulminant septicemia or meningitis in humans that have been bitten, scratched or simply licked by a dog, less commonly by a cat (Brenner *et al.*, 1989). Splenectomy, alcohol abuse and immunosuppression history have been associated with a number of cases, but more than 40% of the patients had no obvious risk factor (Lion *et al.*, 1996). More than 160 cases of *C. canimorsus* infections have been reported (Tierney *et al.*, 2006) but so far very few studies have addressed the molecular mechanisms of *C. canimorsus* pathogenesis.

Recently, we showed that murine or human macrophage cells infected with *C. canimorsus* remain viable and do not release pro-inflammatory cytokines. This lack of response results from an absence of Toll like receptor 4 (TLR4) stimulation and one strain isolated from a human fatal septicemia, turned out to even actively block the onset of the inflammatory response (Shin *et al.*, 2007). Most interestingly, in the experimental set up of that study, *C. canimorsus 5 (Cc5)* could only grow in the presence of cultured macrophages and contact to cells was a prerequisite to sustain bacterial replication during the 24 h infection assay (Shin *et al.*, 2007). In the present study, we aimed at identifying the essential nutrients from cells that are used by *C. canimorsus*. To address this question, we

engineered and screened a transposon mutant library. A Tn mutant unable to multiply *in vitro* in the presence of cells but fully proficient for growth on blood agar was isolated. The mutant turned out to be affected in a surface-exposed sialidase and could be rescued by exogenously added sialidase but surprisingly not by sialic acids. However, addition of N- acetyl glucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) rescued growth, showing that sialidase allows *C. canimorsus* to feed on glycans from the host cell surface glycoproteins. Furthermore, the surface-exposed sialidase conferred resistance to killing by human complement by binding factor H. In agreement with these two observations, the sialidase deficient mutant turned out to be hypo-virulent in a mouse model and we provide evidence that *C. canimorsus* also feeds on phagocytes *in vivo*.

Results

Growth of *C. canimorsus 5* (*Cc5*) requires serum and direct contact with cells

When inoculated at a multiplicity of infection (moi) of 20 to J774.1 macrophages cultured in complete RPMI (cRPMI), which includes 10% fetal bovine serum (FBS), *Cc5* multiplied about 100-fold during the 24 h of infection. This bacterial growth was reduced when FBS was omitted during infection but more surprisingly, it was abolished when J774.1 macrophages were omitted (Fig. 1A). Using a transwell system, we next tested whether direct contact between *Cc5* and J774.1 macrophages is required for bacterial growth. *Cc5* was unable to grow in this experimental setup where a membrane prevented physical contact between bacteria and J774.1, while the culture medium (cRPMI) remained the same (Fig. 1A). These data imply that *Cc5* may take advantage of some nutrient that is present at the cell's surface. In order to investigate this intriguing property, we generated a transposon (Tn) mutant library using transposon Tn*4351* from *Bacteroides fragilis* (Cooper *et al.*, 1997). We screened 6700 mutants and isolated a clone that was unable to grow in the presence of J774.1 cells, but grew normally on blood agar plates. The impaired growth of this Tn mutant was not due to an increased phagocytic uptake by J774.1 since addition of cytochalasin D had little effect on bacterial growth. Moreover, the observation could be repeated with non-phagocytic cells: wt *Cc5* could also grow in the presence of HeLa cells while

the mutant could not (Fig. 1B). In contrast, growth of *Cc5* and the Tn mutant was comparable in serum enriched heart infusion medium (Fig. 1C), indicating that the mutant had no central metabolic deficiency.

Surface localized sialidase is required for the growth of *Cc5*

Arbitrarily primed PCR analysis of the mutant showed that the transposon inserted within a gene encoding a protein with similarity to bacterial sialidases, glycosylhydrolases that cleave a terminal sialic acid from glycoconjugates (Fig. 2A). This hypothetical sialidase (designated SiaC) contains Asp box motifs known to be conserved among sialidases of microbial origin and a putative catalytic site (Fig. 2A) (Roggentin *et al.*, 1989). The sequence starts with a putative N-terminal signal peptide (Sigrist *et al.*, 2002), indicating that SiaC could be either periplasmic or surface exposed.

Sialidase activity was tested by incubating wt or mutant *Cc5* bacteria with 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN). While intact *Cc5* bacteria cleaved MUAN, the Tn mutant could not, indicating that the mutated gene does indeed encode a sialidase (Fig. 2B).

In order to complement the mutation, we engineered an expression shuttle vector by taking advantage of the replicon from a endogenous plasmid isolated in another strain of *C. canimorsus* and the promoter of an insertion sequence from *B. fragilis* (Manuscript in preparation). We constructed plasmids encoding full length (FL) SiaC, a variant deprived of its predicted signal sequence (Δ1-21) and a catalytic mutant (Y488C) (Fig. 2B). Three constructs were expressed in comparable amounts in *C. canimorsus* as assessed by immunoblotting of crude extracts with a polyclonal serum directed against recombinant SiaC (Fig. 2C). Sialidase activity of intact Tn mutant (Δ*siaC)* bacteria could be restored by introducing *siaC*$_{FL}$, but not *siaC*$_{\Delta 1\text{-}21}$ or *siaC*$_{Y488C}$ *in trans* (Fig. 2B). Impaired growth of Δ*siaC* in the presence of J774.1 cells was also complemented by introducing *siaC*$_{FL}$, but not *siaC*$_{\Delta 1\text{-}21}$ or *siaC*$_{Y488C}$ (Fig. 2C).

We next determined the localization of SiaC in *Cc5* and in Δ*siaC* complemented with three *siaC* genes. SiaC$_{FL}$ and SiaC$_{Y488C}$ were found in the outer membrane fraction (Fig. 2D), whereas SiaC$_{\Delta 1\text{-}21}$ was only detected in total cells (Fig. 2C). Analysis of fixed but unpermeabilized bacteria by indirect

immunofluorescence using polyclonal anti-SiaC serum confirmed that SiaC is indeed exposed on the bacterial surface. Deletion of the predicted signal prevented surface exposure (Fig. 2E). Although it is surface exposed, no SiaC could be detected in the supernatant of cultured J774.1 that have been infected with *Cc5* for 24 h (Fig. 2D). Hence, surface-anchored sialidase is required for the growth of *Cc5* at the expenses of J774.1 macrophages or HeLa cells.

Growth is sustained by GlcNAc and GalNAc but not by sialic acids

Since sialidases cleave terminal sialic acid from glycoconjugates, we first investigated whether the addition of sialic acids could restore growth of Δ*siaC*. Surprisingly, the addition of neither Neu5Ac nor CMP-Neu5Ac restored growth of Δ*siaC* in the presence of J774.1. In contrast, addition of purified recombinant sialidase SiaC, but not the catalytic mutant $SiaC_{Y488C}$, to the culture medium restored growth. Even addition of neuraminidase NanH from *Clostridium perfringens* could restore growth of Δ*siaC* mutant bacteria (Fig. 3A). This suggests that removal of terminal sialic acids from glycoconjugates could be the key element. Since this removal is expected to make other carbohydrates accessible, we next tested whether GlcNAc and GalNAc, common carbohydrate moieties of glycoconjugates, would not allow growth of Δ*siaC* in the presence of macrophages. As shown in Fig. 3B, these aminosugars indeed rescued Δ*siaC* bacteria. We also tested addition of 0.1% glucose (Glc), galactose (Gal) or mannose (Man), which are known to be utilized by *Cc* (Brenner *et al.*, 1989) and commonly found in glycoconjugates but none of these carbohydrates could restore growth of Δ*siaC*. Supplementation with Gal even decreased the number of viable counts of Δ*siaC* (Fig. 3B).

Sialidase desialylates macrophage surfaces

We next investigated the effect of SiaC on the cells. We treated J774.1 cells with recombinant SiaC and analyzed them using *Sambucus nigra* agglutinin (SNA) and a fluorescent labeling. This lectin, which recognizes terminal sialic acids (2- 6 or 2- 3) linked to Gal or to GalNAc (Fig. 4A), did not bind to SiaC treated cells, indicating that SiaC desialylates the surface of J774.1 (Fig. 4B). We then tested cells that were infected with *Cc5* and observed that the signal for terminal sialic

acids was reduced after 15 h of infection with wt *Cc5* but not with Δ*siaC* (Fig. 4B). We next tested binding of peanut agglutinin (PNA), a lectin specific for Gal (β 1-3) GalNAc (Fig. 4A), a disaccharide often forming the core unit in glycoconjugates. Binding of PNA to J774.1 was only detected after adding SiaC to J774.1, confirming that the core of glycoconjugates needed to be unmasked by the action of sialidase (Fig. 4B). In contrast, no PNA binding could be detected after incubation of J774.1 with live Δ*siaC* or *Cc5* at any time point. This shows first that Δ*siaC* is deficient in cleaving terminal sialic acids on glycoconjugates and therefore unable to reveal the disaccharide Gal (β 1-3) GalNAc. Secondly, the absence of a PNA signal after *Cc5* infection suggests that live *Cc5* bacteria remove sialic acids and further deglycosylate the surface glycoconjugates. To test this hypothesis, macrophages were infected with Δ*siaC* in the presence of recombinant SiaC at a concentration 10 ng/ml, giving the same activity as 2x 10^6 *Cc5* bacteria. As expected, cell surfaces were desialylated by the recombinant enzyme as indicated by a decrease of the SNA signal after 15 h. In addition, no PNA fluorescence was observed after 15 h meaning that unmasked Gal (β 1-3) GalNAc was not detectable. This suggests that Δ*siaC* is still proficient in extensive deglycosylation of unprotected glycans chains. We also tested HeLa cells as an example for epithelial cells and monitored the same deglycosylation of cell surfaces as observed for J774.1 macrophages (Fig. 4C).

Sialidase confers resistance to complement-mediated killing

Our preliminary experiments have shown that *Cc5* resists the bactericidal action of human complement (unpublished data and Fig. 5A). Since complement resistance can be due to surface exposed proteins, we tested in parallel wt *Cc5* and Δ*siaC*. While *Cc5* survived 3 h of incubation in 10% human serum, Δ*siaC* viable counts went down by 3 logs. There was no significant difference in the survival of both strains in heat-inactivated (HI) serum (Fig. 5A). The ability to resist complement was restored in Δ*siaC* by introducing $siaC_{FL}$, but not by $siaC_{\Delta 1\text{-}21}$. In agreement with our previous result, addition of Neu5Ac did not restore complement resistance in Δ*siaC.* Hence, surface-exposed sialidase contributes to the very high resistance of *C. canimorsus* to killing by complement.

One mechanism that contributes to complement resistance is the binding of the complement regulatory protein factor H (fH) to the cell surface. *Cc5* or Δ*siaC*

bacteria were mixed with HI serum as a source of fH, and fH binding was measured by immunoblotting using anti-fH. As shown in Figure 5B, *Cc5* recruited fH to its surface but Δ*siaC* did not. Binding of fH by Δ*siaC* was however restored by introducing *in trans* $siaC_{FL}$, but not by $siaC_{\Delta 1\text{-}21}$. We next asked whether SiaC alone is competent to confer complement resistance by recruiting fH. To test this idea, we took advantage of *E. coli* expressing FL SiaC (Fig. 5C). *E. coli* with surface localized SiaC was then assayed for complement killing and fH binding (Fig. 5D and 5E). There was no increase in complement resistance or fH binding after SiaC expression in *E. coli*, indicating that SiaC alone is not sufficient to confer fH binding and complement resistance.

Δ*siaC* is attenuated in a mouse localized infection model

We next tested whether SiaC is essential for survival during animal infection. No animal model has been developed so far for *C. canimorsus*. We selected a murine tissue cage model, which is commonly used with *Staphylococci* and mimics a localized infection (Kristian *et al.*, 2003). Teflon tissue cages were subcutaneously implanted in C57BL/6 mice; two weeks later, 10^7 wt *Cc5* or Δ*siaC* mutant bacteria were injected directly into the cages of five mice each. Prior to infection, the extracellular fluid accumulating in tissue cages was analyzed for its leukocyte content (1.8×10^4 +/- 1.3×10^4 leukocytes / µl). The cell population consisted of 68% +/- 4.8% polymorphonuclear neutrophils (PMNs), 18% +/- 3.2 % monocytes and 9.1% +/- 3.7 % macrophages.

Bacterial growth was monitored over a time period of 27 days. Cfu numbers of wt *Cc5* decreased on day 2 and 5. However, on day 9 they increased by 1 to 3 logs in 4 out of 5 mice, and were able to persist in 3 of 5 mice after 27 days post infection with more than 10^7 bacteria per ml of tissue cage fluid. Δ*siaC* bacteria were already undetectable after the second day (<20 bacteria /ml) in 5 out of 5 infected mice (Fig. 6A). After infection, the total number of leukocytes in tissue cage fluid did not significantly increase and was not related to the bacterial load, suggesting that *Cc5* infection did not lead to strong leukocyte recruitment.

In a competition experiment where 10^7 cfu of *Cc5* and Δ*siaC* were inoculated at a 1:1 ratio, Δ*siaC* was out-competed by *Cc5*. Δ*siaC* bacteria could be detected in only 1 out of 5 mice with 20 to 140 cfu/ml on days 5 to 14, while the corresponding wt *Cc5* cfu numbers rose to 10^8/ml. This represents a competitive

index of 9.7 x 10^{-4}, 5.8 x 10^{-7} and 4.7 x 10^{-7} on day 5, 9 and 14, respectively. As observed during infection with wt *Cc5* alone, 3 mice out of 5 developed a persistent infection (Fig. 6B).

To test whether *Cc5 in vivo* feeds on cells, we collected leukocytes from uninfected tissue cages, suspended them in RPMI and added 2x 10^6 or 2x 10^4 bacteria. We monitored bacterial growth *in vitro* and observed that *Cc5* also grew in presence of *ex vivo* isolated cells while Δ*siaC* did not (Fig. 6C). This experiment suggests that growth in the presence of cells may represent an essential feature during infection.

Both strains grew equally well in HIB supplemented with 10% FBS, indicating a similar fitness *in vitro* (Fig. 1C). When tested in a competition experiment *in vitro* using 100 cfu/ml of wt *Cc5* and Δ*siaC*, their growth curves were comparable measured at 2, 6, 10 and 24 h. Both strains reached 10^6 cfu/ml after 24 h (Fig. 6D). This clearly demonstrates that SiaC plays an essential role in establishing infection by *Cc5* and that clearance of Δ*siaC* is not due to a growth defect *per se* but to an altered host interaction of the mutant.

Discussion

In the present study we showed that extracellular *C. canimorsus* replicate very efficiently when they are in direct contact with macrophages or epithelial cells. A surface-exposed sialidase is a key feature for this behaviour and in good agreement with this, *C. canimorsus* desialylates glycoproteins from the cellular surface. Bacterial sialidases have been thought since a long time to contribute to virulence in pathogenic bacteria that colonize mucosal surfaces such as *Vibrio cholerae, Streptococcus pneumoniae,* group B *streptococci*, *Clostridium perfringens* and *Bacteroides fragilis* but the exact impact of sialidase on virulence has been difficult to assess (Corfield, 1992). Recently it was shown that a sialidase is involved in the formation of *Pseudomonas aeruginosa* biofilms and hence contributes to colonization of the lungs during the initial stages of infection in cystic fibrosis patients (Soong *et al.*, 2006). Besides a role of sialidase as a direct virulence factor, there is evidence that sialidase could play a nutritional role for the pathogen during infection. King et al. demonstrated that exoglycosidases are responsible for growth of *S. pneumoniae* on human α-1 acid glycoprotein and that *S. pneumoniae* causes an extensive deglycosylation of different host proteins

including IgA1 and human secretory component (Burnaugh *et al.*, 2007) (King *et al.*, 2006).

While the impact of sialidase in microbial pathogenesis is still debated, the role of sialic acids is very well documented (Vimr *et al.*, 2004). Several pathogens have evolved ways to expose sialic acid on their surface and hence to escape complement killing and opsonisation by mimicry. Sialic acids are incorporated into capsules by *E. coli* K1 (Barry, 1959), Group B *Streptococcus* (Wessels *et al.*, 1989), Serotype B and C *Neisseria meningitidis* (Bhattacharjee *et al.*, 1975). The lipooligosaccharide of *Neisseria gonorrhoeae, Neisseria meningitidis* and *Haemophilus influenzae* are also sialylated. In this case, the key enzyme is not a sialidase but a sialyltransferase using as a substrate CMP-Neu5Ac from the host (Mandrell and Apicella, 1993). Alternatively, sialic acids can be synthesized by *Neisseria* itself, from lactate, demonstrating a close link between metabolism and evading innate immune defenses (Exley *et al.*, 2005). Here, we provide evidence, that sialic acids are not used by *C. canimorsus* to replicate or to contribute to complement resistance by mimicry. In agreement with this, the LPS and capsular polysaccharide of *C. canimorsus* cultivated on blood agar plates do not contain sialic acids (manuscript in preparation). Thus, the role of sialidase is not to supply sialic acid for growth or mimicry but to provide access to masked carbohydrates of surface exposed glycoproteins.

Taking into account the ecology of *C. canimorsus*, this observation suggests that this commensal bacterium feeds on live buccal cells and/or on saliva which is rich in glycopeptides (Larsen *et al.*, 2007). This observation of extracellular bacteria specifically feeding on the surface of host cells is uncommon but not unprecedented. Somehow, this reminds of *Bacteroides thetaiotaomicron*, another major commensal, but from the intestine, which feeds on fucosylated intestinal cells. Colonization by *B. thetaiotaomicron* even triggers the appearance of fucosyltransferase and fucosylated glycan expression (Bry *et al.*, 1996). Recent studies showed that host acquired fucose is incorporated by *B. fragilis,* another intestinal commensal into capsular polysaccharide or glycoproteins, which in turn provides a survival advantage in the mammalian intestinal ecosystem (Coyne *et al.*, 2005). However, in the present study, we observed that *C. canimorsus* feed on epithelial cells and even on phagocytes, and this to our knowledge has never been shown before.

It is remarkable that the sialidase from *C. canimorsus* is anchored at the bacterial surface. The N-terminal signal sequence suggests that it crosses the plasma membrane by the Sec pathway but we have at present no explanation on how it crosses the outer-membrane and remains anchored. It is probably not by a *C. canimorsus* specific mechanism since SiaC appeared to be also surface-exposed when expressed in *E .coli*. This observation somehow evokes the surface-anchored auto-transporter proteins like the *Y. enterocolitica* YadA (Koretke *et al.*, 2006) and it raises an important question which will be addressed at some stage. Whatever the mechanism by which this protein is anchored, our data indicate that extremely little is released into the culture supernatant and this fits with the fact that *C. canimorsus* needs to be in direct contact with cells to feed on them. This makes sense in the context of the mouth commensal microflora. Indeed, the oral cavity is occupied by some 500 different bacterial strains (Kroes *et al.*, 1999; Paster *et al.*, 2001), creating a fierce competition for nutrition. The fact that *C. canimorsus* does not release this enzyme suggests that *C. canimorsus* maximizes the benefit of sialidase by not sharing this fitness factor with competing bacteria. Since sialic acid itself is not the main resource, one would predict that other glycan-hydrolyzing enzymes must also be surface-associated. One would even envision that they form a surface-anchored multi-enzyme complex arranged in such a way that they can process cellular glycans in a sequential manner as it is proposed for *S. pneumoniae* (King *et al.*, 2006). Ongoing work in the laboratory is testing this hypothesis.

C. canimorsus is resistant to killing by complement and this resistance results, at least to some extent, from binding human factor H. Interestingly, sialidase contributes to this binding but other proteins are also required, possibly the other components of the hypothetical complex digesting cellular glycan chains. Complement resistance in a mouth commensal is not surprising. Indeed, some complement components like C3 have been detected in human saliva (Andoh *et al.*, 1997) and we infer that the same might be true for dog's saliva. In terms of evolution, it is very interesting to observe that metabolic enzymes of commensals have evolved to also confer protection against components of the innate immunity system. The observation the *C. canimorsus* binds fH strongly suggests that there is fH in saliva but, to our knowledge, this has not been reported so far.

Since our *in vitro* experiments showed the importance of sialidase for growth in the presence of cells, we tested whether sialidase would not also behave as a "virulence" factor in patients infected after a dog bite. We used a mouse tissue cage model in which the readout is bacterial persistence and we observed a dramatic persistence difference between wt and sialidase-deficient *C. canimorsus*. Even more, we gained evidence that *in vivo C. canimorsus* also feeds on phagocytes. Thus, sialidase represents a virulence factor. To our knowledge, this is one of the very first cases where it appears so clearly that one metabolic pathway is the key to persistence *in vivo*. It is also interesting to observe that nutrition *in vivo* may be quite specific in spite of a very rich nutritional environment. Indeed, only GlcNAc and GalNAc could rescue growth while Glc had no effect and Gal was even deleterious. This specialization is probably the hallmark of a bacterium which is primarily a commensal and only rarely a pathogen. The situation appears to be different for bacteria which essentially multiply and evolve as pathogens. A recent analysis of *Salmonella* infections using an *in vivo* proteomics approach showed that the large majority of metabolic enzymes are critical but non essential and hence *Salmonella* is remarkably robust during host colonization (Becker *et al.*, 2006). Using direct microinjection of intracellular pathogens into the host cytosol, bacterial growth was dependent on metabolic enzymes (Goetz *et al.*, 2001). Finally, *C. canimorsus* represents one more example illustrating that the distinction between commensals and pathogens is illusive. Commensalism and pathogenicity are two facets of host-bacteria interaction. Most factors that allow a commensal to adapt to its niche by colonizing, feeding and resisting local immune defences inevitably represent potential virulence factors if the commensal breaches the barrier from its host or maybe more likely from another host.

Material and Methods

Bacterial strains and growth conditions

C. canimorsus 5 (Shin *et al.*, 2007) was routinely grown on Heart Infusion Agar (Difco) supplemented with 5% sheep blood (Oxoid) for 2 days at 37°C in presence of 5% CO_2. Bacteria were harvested by gently scraping colonies off the agar surface, washed and resuspended in PBS. *C. canimorsus* was also grown in Heart Infusion Broth (Difco) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen) for approximately 24 h without shaking in an 37°C incubator with 5% CO_2. Selective agents were added at the following concentrations: erythromycin, 10 µg/ml; cefoxitin, 10 µg/ml; gentamicin, 20 µg/ml; ampicillin, 100 µg/ml.

Cell Culture and Infection

Murine monocyte-macrophage J774A.1 cells (ATCC TIB-67) were cultured in RPMI 1640 (Invitrogen) supplemented with 10 % (v/v) fetal bovine serum (Invitrogen), 2 mM L-glutamine and 1 mM sodium pyruvate. HeLa cells (ATCC CCL-2) were grown in DMEM (Invitrogen) with 10% (v/v) fetal bovine serum. Cells were seeded in medium without antibiotics at a density of 10^5/cm^2 cultured at 37°C in humidified atmosphere containing 5% CO_2. Unless otherwise indicated, infection was performed after 15h at a moi of 20 at 37°C.

Monosaccharides (Sigma Aldrich) were added to 0.1% (w/v) final concentration. Neu5Ac and CMP- NeuAc were added to 0.01% final concentration.

Arbitrarily Primed PCR

Primers specific to the ends of the transposon and primers of random sequence that may anneal to chromosomal DNA sequences in close proximity to the transposon insertions were used in two rounds of PCR before sequencing. The first round of amplification was carried out in 50 µl containing 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 200 µM of primers 5' CAGAATTCTGTTGCATTTGCAAGTTG 3' complementary to Tn*4351* and 5'ggccacgcgtcgactagtacNNNNNNNNNNacgcc3', 2.5 U of DNA polymerase (DyNAzymeII, Finnzymes), 200 µM of each dNTP, in 10 mM Tris HCl (pH 8.3) for 6 cycles (94°C for 1 min, 30°C for 1 min, 72°C for 2 min) and 30 cycles (94°C for 1 min, 45°C for 1 min, 72°C for 2 min) and final 10 min at 72°C. 10 µl of PCR product containing random fragments was used as template in a second round of 30 cycles of amplification (94°C for 30 sec, 45°C for 30 sec, 72°C for 1 min) using primers 5' CAGAATTCTGTTGCATTTGCAAGTTG 3' and 5' GGCCACGCGTCGACTAGTAC 3', from the 5' of the random primer. PCR products were purified using NucleoSpin® from Machery Nagel. 20- 50 ng of random sized products were sequenced using ABI sequencer. The Tn integration site was further confirmed by using primers on chromosomal DNA by sequencing towards the Tn integration site. Primers used were 5' AATTGTTGTAACGATTGTCG 3' or 5' GCGAAGCGTTATCCCAAAGC 3' complementary to the *siaC* sequence in a sequencing reaction containing 2 µg genomic DNA of Δ*siaC*, betaine 0.25 M and BigDye Terminator Ready Reaction (PE Biosystems) with an initial denaturation step for 5 min and subsequent 99 cycles (95°C for 30 sec, 50 °C for 20 sec, 60°C for 4 min).

Construction of complementation and expression plasmids

Full length *siaC* was amplified with 5'CATACCATGGGAAATCGAATTTTTATCTT3' and 5'GTTCTAGAGAGTTCTTGATAAATTCCTCAACTG3' primers and cloned into the *E. coli- C. canimorsus* shuttle vector pMM47.A with *Nco*I and *Xba*I, leading to the insertion of a glycin at position 2 and a C- terminal histidine 6x tag (plasmid) pMM52 ($siaC_{FL}$). Forward primer 5'AAAGCCATGGGAAACGTAATCGGCGGAGGCG 3' was used with the same reverse primer to construct pMM50 ($siaC_{\Delta 1\text{-}21}$), deleting the first 63 bp of *siaC*, but still including methionin and glycin at position 1 and 2, respectively, and using a C- terminal His 6x tag. The catalytic mutation in *siaC* of was introduced by site directed mutagenesis with an inverse PCR on pMM52, using primers 5'GAAGGATTTGGGTGTTCGTGTATGTCG3'
and 5'CGACATACACGAACACCCAAATCCTTC3' leading to pMM59 ($siaC_{Y488C}$). The cDNAs encoding $SiaC_{FL}$ (pHS1) and $SiaC_{\Delta 1\text{-}21}$ (pHS2) were subsequently amplified using 5'GGAATTCCATATGAATCGAATTTTTATC3'
and 5'CGCGGATCCCTAGTTCTTGATAAATTCCTC3'
and 5'GGAATTCCATATGAACGTAATCGGCGGAGGC3'
plus 5'CGCGGATCCCTAGTTCTTGATAAATTCCTC3', respectively and cloned into the expression vector pET15b(+) (Novagen). Plasmid pHS3 encoding $SiaC_{\Delta 1\text{-}21,Y488C}$ was constructed by site directed mutagenesis on template pHS2 using the same primers as described for pMM59. All constructs were sequenced with ABI sequencer. The sequence of SiaC was deposited at GenBank (accession number: EU329392).

Purification of recombinant SiaC and immunoblotting

Expression of *siaC* constructs in *E.coli* BL21(DE3) was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside at A_{600} = 0.5 for 3 h. Proteins were purified by affinity chromatography using chelating Sepharose (Pharmacia) charged with $NiSO_4$ according to the manufacturer's instructions. Samples were analyzed by SDS-PAGE by the system of Laemmli, and immunoblotted. Antibody against C-terminal His was purchased from Invitrogen. Polyclonal serum from rabbit was generated against recombinant $SiaC_{\Delta 1\text{-}21}$.

MUAN hydrolysis

10^7 bacteria were incubated with 0.006% 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN) in 0.25 M sodium acetate pH 7.5 at 37°C for 3 min. Reactions were stopped with 50mM Na_2CO_3 pH 9.6 and fluorescence was determined at 445 nm was detected with a Wallac $Victor^2$ 1420 Multilabel counter (Perkin Elmer).

Outer Membrane Preparation

Bacterial cells resuspended in PBS containing DNase and RNase (10 µg/ml), were sonicated on ice, unbroken cells were removed at 3000 x g for 15 min, and total membranes were collected at 20 000 x g for 30 min at 4°C. The membranes were suspended in PBS and sarcosyl (N-Lauroylsarcosine sodium salt, Sigma) was added to a final concentration of 1% (v/v). After

incubation on ice for 1 h, membranes were collected at 20 000 x *g* for 30 min and resuspended in electrophoresis sample buffer and analyzed by SDS-PAGE by the system of Laemmli.

Immunofluorescence of bacteria

10^7 bacteria were incubated on poly-D-lysine (BD) coated glass slides for 1 h at 37°C and subsequently fixed with 3% paraformaldehyde for 15 min. Anti- SiaC polyclonal serum (1:500) and a FITC conjugated secondary antibody (Goat Anti- Rabbit IgG, Southern Biotech) was used at 1 µg/ ml and fluorescence was measured with a Leica DMIRE2 microscope. Pictures were taken with a digital camera (Hamamatsu Photonics) and OpenLab software (version 3.1.2).

Lectin Staining

10^5 J774.1 macrophages were seeded on poly-D-lysine coated slides. Infection was carried out with 2 x 10^6 bacteria at indicated time points. Macrophages were alternatively treated with recombinant SiaC for 15 h at 10 ng/ ml. Cells were fixed with 3% paraformaldehyde for 15 min. Biotinylated lectins SNA (Vector Laboratories) and PNA (kindly provided by Daniela Finke) were incubated with cells at 5 µg/ ml for 1 h. After washing with PBS, cells were treated with 1 µg/ ml fluorescein conjugated streptavidin (Vector Laboratories) and fluorescence was determined on mounted slides.

Sensitivity to human Complement and fH binding

10^7 bacteria were incubated with normal human serum (10% final) for 3 h at 37°C. The number of cfu in the inoculum and after incubation with serum was determined by plating on sheep blood agar plates. Heat-inactivated serum (56°C for 1 h) was used in control assays. For fH binding assays, 7 x 10^8 bacteria were mixed with heat-inactivated serum for 1 h at room temperature. Bacteria were washed five times with PBS/0.05% Tween-20 and bound proteins were eluted with 0.1 M glycine-HCl, pH 2.2 for 15 min at room temperature. After removal of bacteria by centrifugation, eluates were neutralized with 1 M Tris, pH 8.0 and analyzed by SDS-PAGE followed by immunoblotting with anti-factor H antibody (Calbiochem).

Mice and tissue cage infection model

12 week-old male C57BL/6 mice were maintained under pathogen-free conditions in the Animal Facility of the Department of Research, University Hospital Basel. Animal experiments were performed in accordance with the guidelines of the Swiss veterinary law. Teflon tissue cages were implanted subcutaneously in the back of anesthetized mice as previously described (Kristian *et al.*, 2003). The cages consisted of closed Teflon cylinders (10 mm diameter, 30mm length, internal volume 1.84 ml) with 130 regularly spaced 0.2 mm holes. 2 weeks after surgery, 200 µl of bacterial suspension was injected percutaneously into the cage. Prior to infection, sterility of the tissue cage was verified. Tissue cage fluid (TCF) was sampled at day 2, 5, 9, 14 and 27 and examined for leukocytes and bacterial viable counts. Leukocytes from TCF were quantified with a Coulter counter (Coulter Electronics) and differentiated by Diff-Quick (Medion Diagnostics) Wright

staining of cytospins and examined under light microscopy. The percentage of viable leukocytes was assessed by trypan blue exclusion.

The survival of the Δ*siaC* mutant in the competition experiment was compared directly with *Cc5* in individual animals giving a 1:1 ratio of wild-type to mutant bacteria. The number of mutant (Em^r) and wild-type bacteria recovered from the TCF of animals was established by plating to media with and without erythromycin. The competitive index was calculated as the (number of mutant/ wild-type bacteria recovered from animals) / (number of mutant/ wild-type bacteria in the inoculum).

Statistical analysis

For growth experiments, means and standard deviations were calculated and statistical significance was evaluated by using a two- tailed, unpaired Student's *t* test. Differences were determined to be significant when $p < 0.05$. For *in vivo* experiments, individual mouse values are shown including the median value of each group. Mann Whitney test with the post hoc Bonferroni correction was used for comparison between *Cc5* and Δ*siaC* CFU numbers during infection.

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Figures

Figure 1

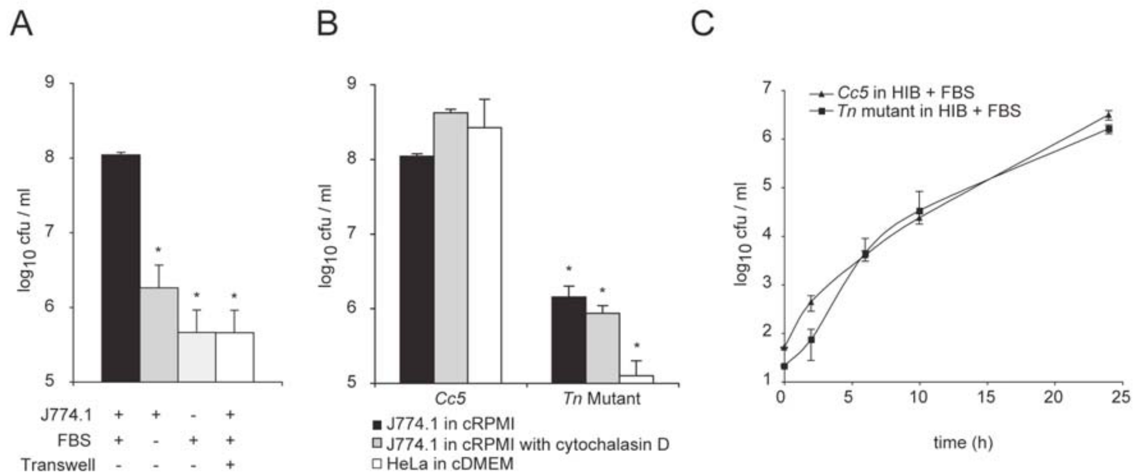


Fig. 1 Growth of *Cc5* is dependent on serum and contact to cells

(A) Viable counts of 2 x 10^6 *Cc5* monitored after 24 h of culture in the in presence of J774.1 macrophages in RPMI supplemented with 10% FBS (moi = 20) (black); in the same condition without FBS (dark grey), in RPMI with FBS but no cells (light grey); in a transwell system preventing physical contact between bacteria and macrophages in RPMI with FBS. (B) Viable counts of wt *Cc5* and Tn mutant after 24 h culture with macrophages in RPMI and FBS (black), with macrophages in RPMI and FBS in addition of cytochalasin D (light grey) and with HeLa cells in DMEM and FBS (white). The difference in the growth of the strains is statistically significant between wt and Tn mutant (Student's t test $p < 0.05$) in 3 or more experiments. (C) Growth curve of wt *Cc5* (triangles) and Tn mutant (squares) in heart infusion broth supplemented with 10% FBS, represented as the mean of 3 or more experiments with the error bars showing the standard deviation (SD).

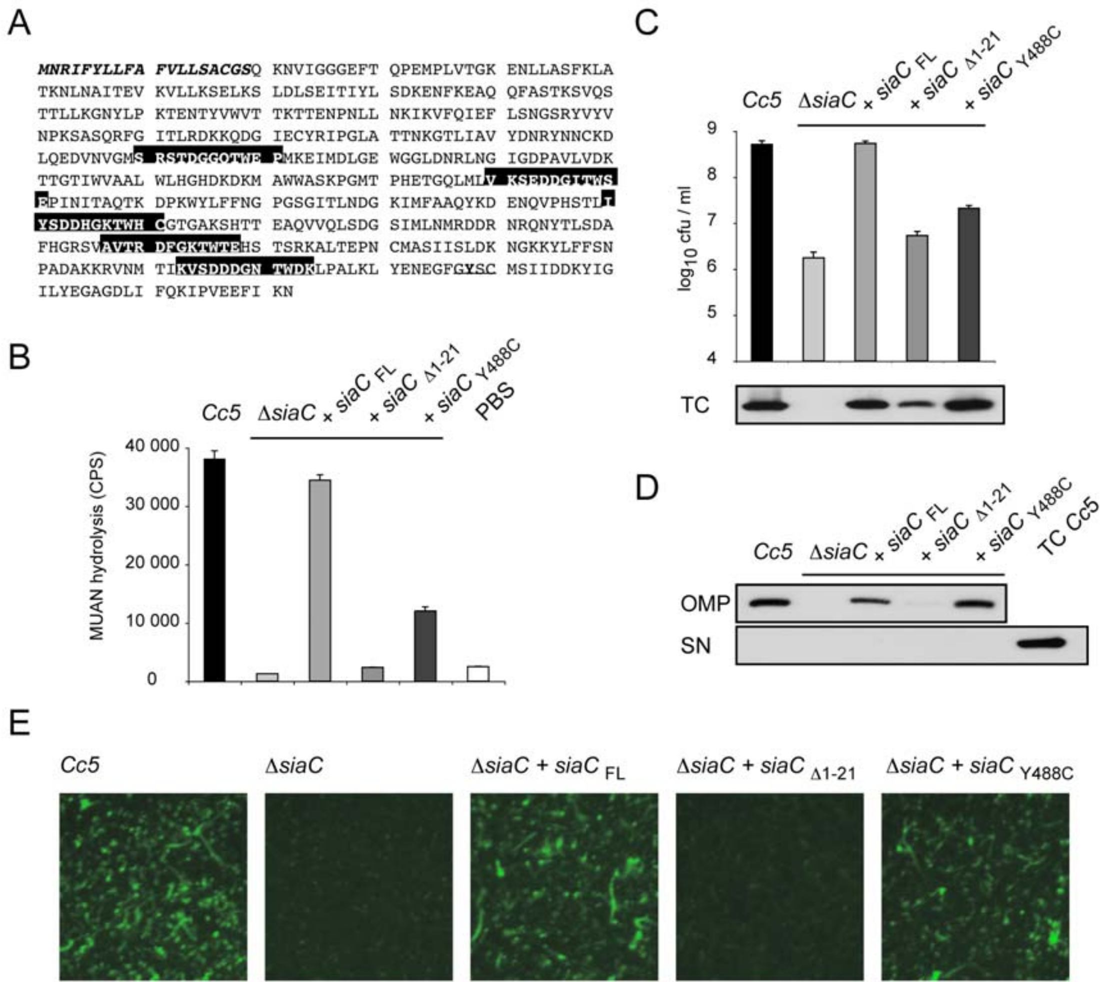


Fig. 2 Surface localized sialidase is required for growth

(A) Amino acid sequence of the *C. canimorsus* sialidase showing the signal peptide (italics) and the BNR/asp repeats (Ser/Thr-X-Asp-X-Gly-X-Thr-Trp/Phe) of bacterial sialidases (boxed). Domain predictions were analyzed by InterProScan. The residues conserved in sialidases at the C terminus are underlined and the tyrosine 488 is bold (Roggentin *et al.*, 1989). (B) Sialidase activity of intact bacteria was measured with the substrate 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN). Data show the mean of triplicate measurements and SD of a representative experiment. (C) Viable counts of 2x 10^6 *Cc5* (black), Δ*siaC* mutant (light grey) or Δ*siaC* mutant complemented with plasmids containing $siaC_{FL}$, $siaC_{\Delta 1\text{-}21}$ and $siaC_{Y488C}$ after 24 h in the presence of J774.1 infection. Sialidase was detected by immunoblotting with a polyclonal antibody against SiaC in total cells (TC). (D) Outer membrane protein fractions (OMP) and cell free supernatants of the J774.1 cultures shown in (B) were analyzed by immunoblotting for the presence of SiaC. (E) Surface localization of SiaC was tested by immunofluorescence on paraformaldehyde fixed but not permeabilized bacteria using anti-SiaC followed by anti- rabbit IgG conjugated to FITC.

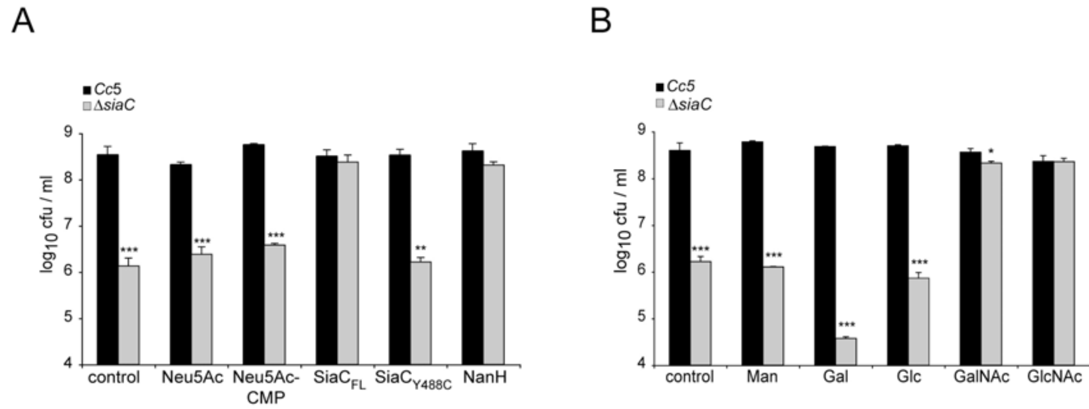


Fig. 3 Addition of GlcNAc and GalNAc but not Neu5Ac rescues the growth defect of Δ*siaC*
(A) Viable counts of 2x 10^6 *Cc5* (black) or Δ*siaC* (grey) grown for 24h with J774.1 in cRPMI and FBS 24 h (control) or in the same condition with the addition of Neu5Ac, Neu5Ac- CMP or 12.5 ng/ml enzyme $SiaC_{FL}$, $SiaC_{Y488C}$ or NanH from *C. perfringens.* (B) Viable counts of 2x 10^6 *Cc5* (black) or Δ*siaC* (grey) after culturing in the presence of J774.1, RPMI, FBS (control) and supplemented with Man, Gal, Glc, GalNAc or GlcNAc. Mean values from 3 or more experiments and SD are shown. Unpaired Student's t test was used to show statistical difference between wt *Cc5* and Δ*siaC* with * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$ for each pair of columns.

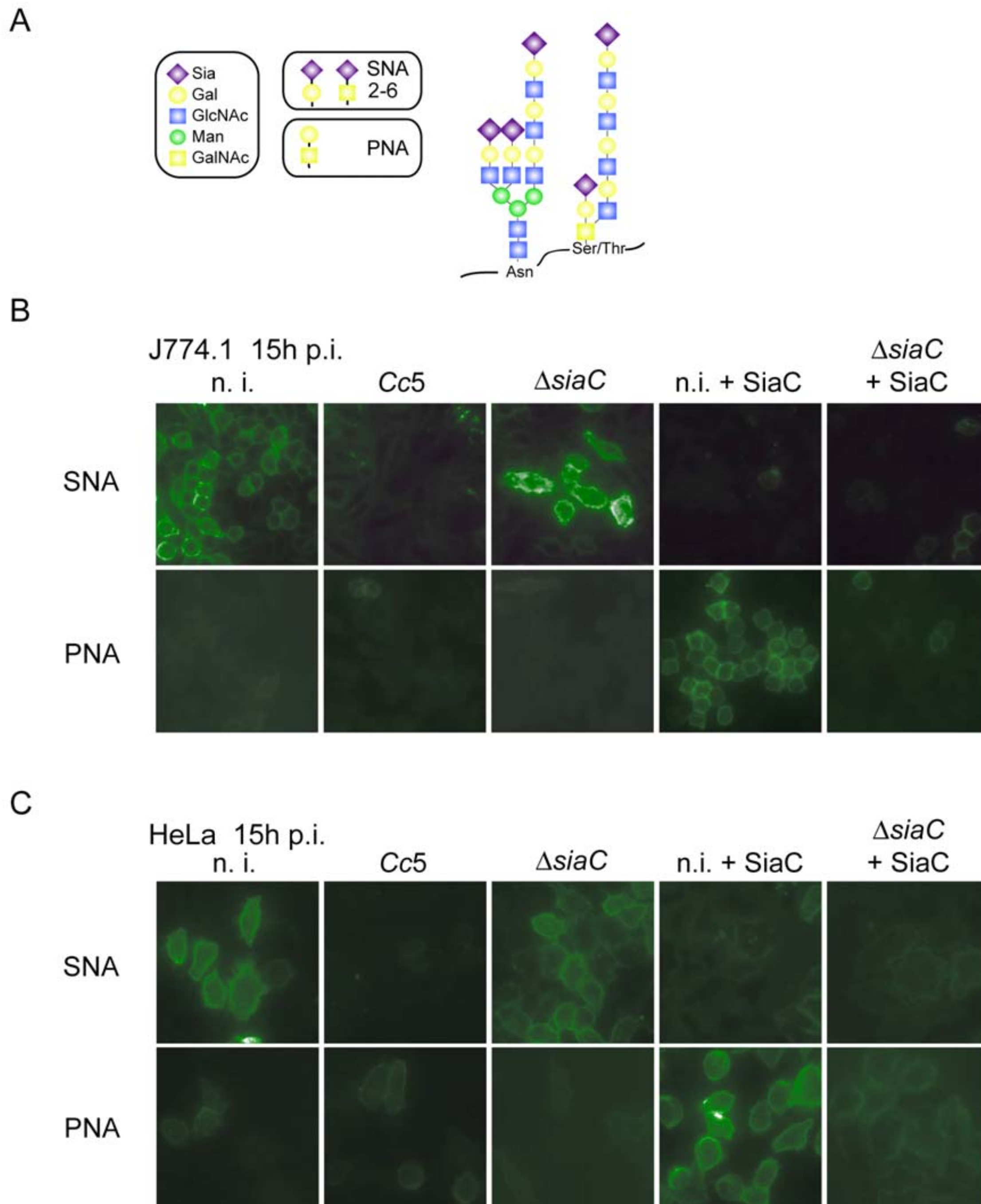


Fig. 4 *Cc5* desialylates macrophage and epithelial cells surfaces

(A) The targets of the lectins used in this study are schematically represented (adapted from (Varki, 2007)). Surface carbohydrates of (B) J774.1 macrophages and (C) HeLa cells were analyzed by lectin binding after 15 h of infection with wt *Cc5* or Δ*siaC* mutant. Cells were fixed with paraformaldehyde and incubated for 1h with SNA, which recognizes terminal sialic acids or PNA that binds to the oligosaccharide Gal 1-3 linked to GalNAc. Biotinylated lectins were then visualized by FITC conjugated streptavidin.

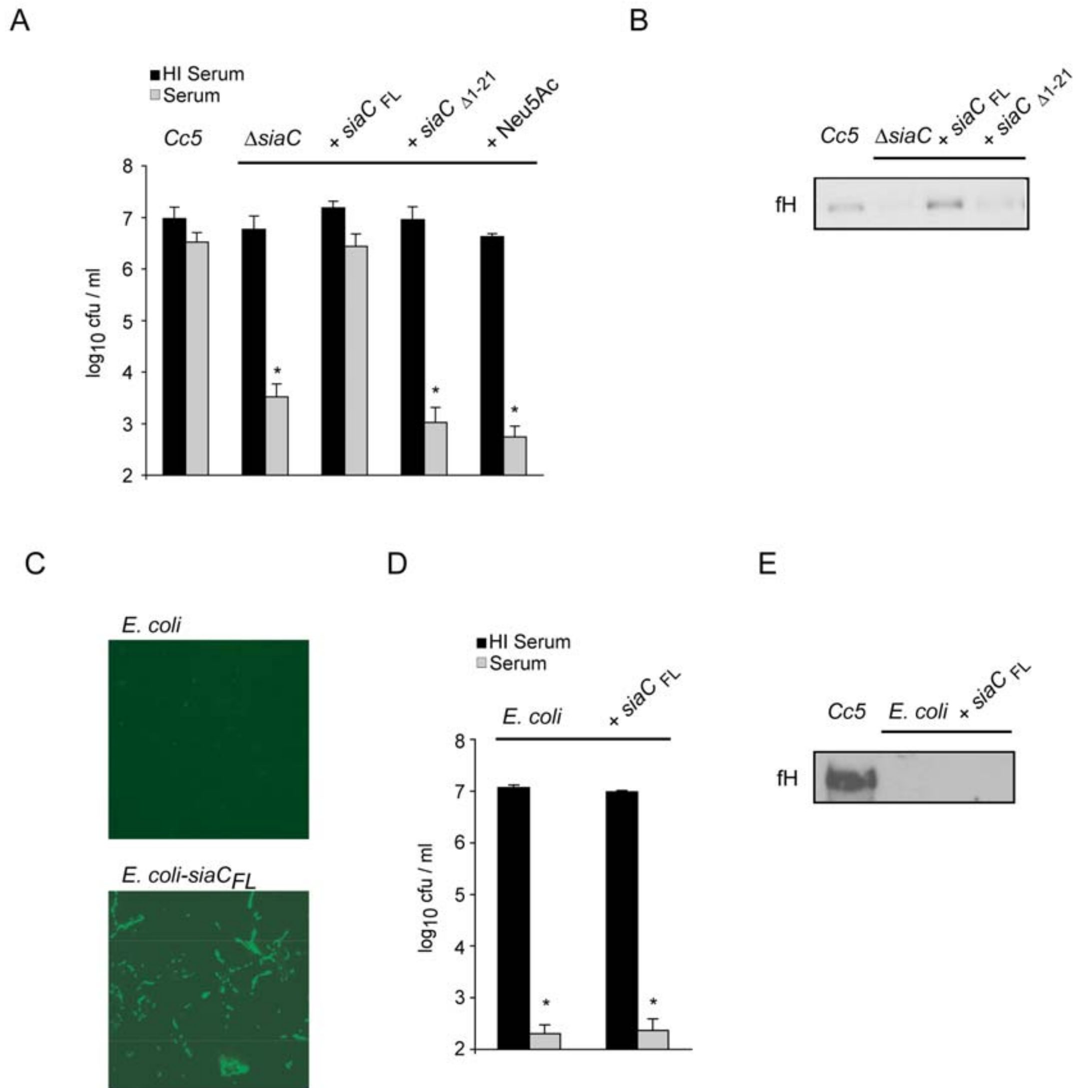


Fig. 5 Sialidase confers resistance to complement-mediated killing

(A) Viable counts of each bacterial strains after incubation with 10% normal (grey) or heat-inactivated serum (black) for 3 h at 37°C. Mean values from 3 or more experiments and SD are shown. Unpaired Student's t test was used to show statistical difference between wt *Cc5* and different Δ*siaC* strains with $p < 0.05$. (B) Bacteria were mixed with heat-inactivated serum for 1 h at 37°C. Bound proteins were eluted and the eluates were subjected to immunoblot analysis using anti-factor H antibody. (C) Immunofluorescence analysis of fixed but unpermeabilized *E. coli* expressing SiaC with anti-SiaC followed by anti- rabbit IgG conjugated to FITC. Viable count assay (D) and factor H binding assay (E) were performed as in (A and B).

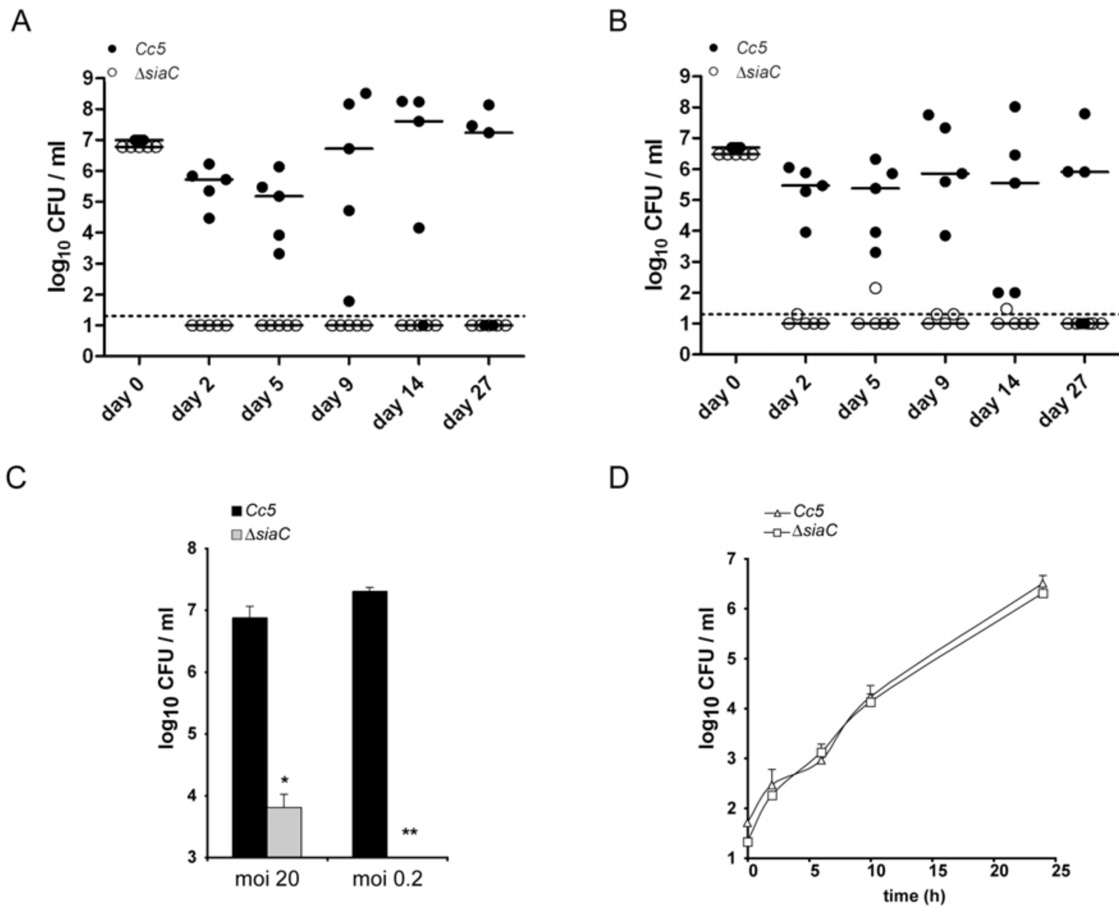


Fig. 6 The sialidase mutant is hypo-virulent in a tissue cage mouse infection model

Infection of tissue cages in C57BL/6 mice with *Cc5* and Δ*siaC* singly (A) or in competition (B), analysis of tissue cage fluid for bacterial growth. (A) Bacterial load (cfu/ml) after infection with 10^7 *Cc5* bacteria (n = 5), black circles, or Δ*siaC* bacteria (open circles) during 27 days. Individual values are shown; horizontal lines indicate the median value of each group. The dotted line is the detection limit of 20 bacteria per ml fluid examined. Cfu numbers between groups were significantly different on days 2, 5 and 9 with $p < 0.01$ and on days 14 and 27 with $p < 0.05$. (B) 10^7 cfu wt *Cc5* and erythromycin resistant mutant Δ*siaC* bacteria were inoculated at a 1:1 ratio. Bacterial numbers were analyzed for 27 days (n = 5). Viable counts between *Cc5* and Δ*siaC* were significantly different on day 2, 5 and 9 with $p < 0.01$ and on day 14 $p < 0.05$. (C) *Ex vivo* isolated total leukocytes were resuspended in serum free RPMI and inoculated at a moi of 20 (2x 10^6 bacteria) or 0.2 ($2x10^4$ bacteria) and bacterial viable count was monitored after 24h. Values are represented as the mean from three independent experiments using TCF cells from 3 uninfected mice. *Cc5* and Δ*siaC* numbers were significantly different with $p < 0.05$ (*) and $p < 0.001$ (**). (D) *In vitro*, *Cc5* and Δ*siaC* were tested in HIB with FBS inoculated at a 1:1 ratio with approximately 100 bacteria total and bacterial growth was monitored for 2, 6, 10 and 24 h.

2.2. Additional Results

2.2.1. Sialidase desialylates serum proteins

In order to extend our observations and see whether sialidase would favor multiplication of *C. canimorsus* during the septic phase of infection, we investigated the effect of *Cc5* on human serum proteins. We therefore tested if serum proteins are desialylated by *Cc5*. Serum was incubated with *Cc5* for 90 min and the sialylation pattern of proteins was analyzed by SDS PAGE followed by lectin detection. Lectins SNA and *Maackia amurensis* agglutinin (MAA), both recognizing sialic acid were used (Fig. 7A). The glycosylation pattern of serum proteins incubated with Δ*siaC* was identical to that of untreated serum control. In contrast, *Cc5* and complemented Δ*siaC* mutant bacteria desialylated total serum proteins. Removal of terminal sialic acids by *Cc5* was further demonstrated by unmasking internal carbohydrate Gal (β 1- 3) GalNAc and carbohydrate Gal-(1-4) GlcNAc recognized by PNA and *Datura stramonium* agglutinin (DSA), respectively, exposed after removal of terminal sialic acids (Fig. 7A).

We previously provided evidence for sequential deglycosylation of host glycans by *Cc5*. We therefore examined deglycosylation of purified serum glycoproteins, human transferrin and bovine fetuin. Recombinant SiaC but not SiaC_{Y488C} desialylated both human transferrin and bovine fetuin after 5 and 18 h of incubation, respectively, whereas live *Cc5* desialylated these proteins after 18 h (Fig. 7B). The appearance of a signal using lectin PNA for fetuin and DSA for transferrin after incubation with recombinant SiaC demonstrates removal of terminal sialic acids. The lack of signal with the same lectins after treatment of transferrin or fetuin by *Cc5* suggests a more extensive deglycosylation process. These results showing a different outcome from PNA staining after incubation with recombinant SiaC or *Cc5* are consistent with the results observed on macrophage surfaces (Fig. 4B).

We then tested whether deglycosylation of serum proteins by *Cc5* could in turn promote growth of Δ*siaC.* Live *Cc5* and Δ*siaC* bacteria were therefore incubated with heat inactivated human serum (Fig. 7C) or bovine serum (data not shown) for 1 h and the resulting conditioned sera were added during infection of J774.1 in serum free RPMI. Interestingly, addition of serum conditioned by incubation with *Cc5* enabled Δ*siaC* to grow in the presence of cells. Serum conditioned by incubation with Δ*siaC* bacteria on the other hand could not restore

growth of Δ*siaC* (Fig. 7C). Taken together, we conclude that SiaC plays a crucial role by revealing oligosaccharides usually masked by terminal sialic acids present on serum proteins as well as in glycoconjugates present on cell surfaces. Thus, deglycosylation results in release of available GlcNAc and GalNAc necessary for replication and survival of *Cc5*.

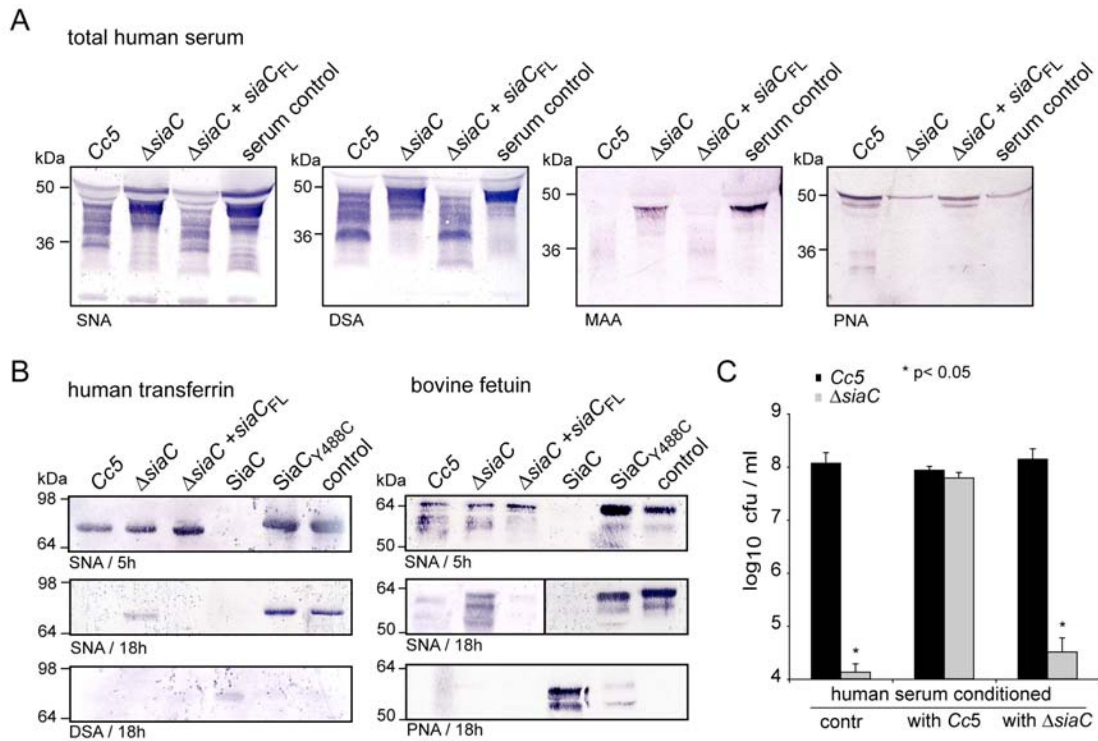


Fig. 7 *Cc5* desialylates and deglycosylates serum proteins

(A) HI serum was incubated with bacteria, separated by SDS PAGE, blotted and protein glycosylation pattern was subsequently examined by DIG labeled lectin binding. Lectins (Roche) used were SNA that recognizes terminal sialic acids (2- 6 or 2- 3) linked to Gal or to GalNAc; DSA, that binds to Gal-(1-4) GlcNAc in N- or O-glycans and/or GlcNAc (O-glycans); MAA which binds to sialic acid linked (2- 3) to Gal and PNA that is specific for Gal (β 1- 3) GalNAc. (B) Lectin detection was used to determine changes in glycosylation state of bovine fetuin or human transferrin upon treatment with bacteria or purified SiaC. (C) Bacteria were incubated with HI serum for 1 h and the resulting conditioned sera were filter sterilized and added during infection of J774.1 in serum free RPMI. Bacterial growth was monitored after 24 h.

We next analyzed growth behavior of *Cc5* and Δ*siaC* in heat inactivated serum and found that *Cc5* but not Δ*siaC* grew well on heat-inactivated human serum (Fig. 8).

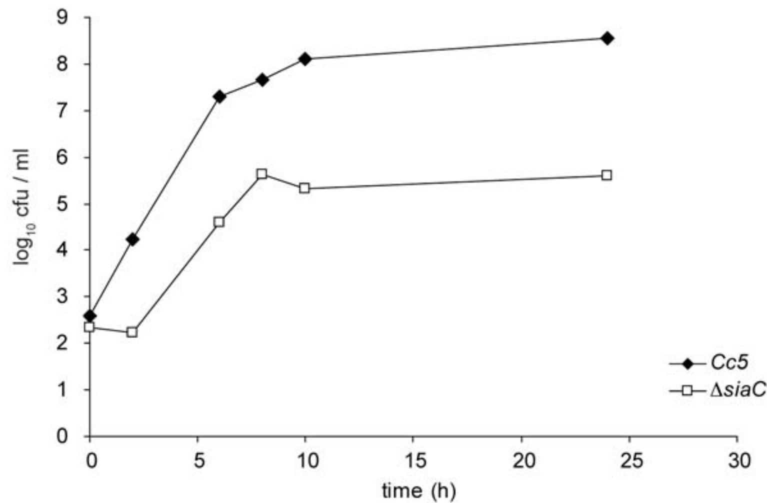


Fig. 8 Growth behavior of *Cc5* and Δ*siaC* in HI serum

Cc5 and Δ*siaC* were inoculated with approximately 150 cfu/ ml in 100% HI serum and bacterial growth was monitored for 24 h.

2.2.2. Analysis of sugars on the bacterial surface reveals no difference between Δ*siaC* and *Cc5*

Molecular mimicry, such as sialylation of LPS or CPS, is a way for several bacterial pathogens to evade complement killing and opsonisation. Therefore, we wanted to exclude that sialic acid is used by *C. canimorsus* for surface decoration. An extensive biochemical analysis of *C. canimorsus* LPS and capsular polysaccharide did not identify sialic acids on wt *Cc5* grown on blood plates (U. Zähringer). In addition to these observations, we analyzed the proteins for glycosylation. We tested in parallel crude extracts or proteinase K digests of *Cc5* and Δ*siaC* bacteria by SDS PAGE followed by stainings with silver periodic acid (Fig. 9A), Alcian Blue/Coomassie (Fig. 9B) or Stains-all/silver (Fig. 9C). No difference in the pattern could be observed. We also stained proteinase K digested extracts of bacteria that were pre-incubated with fetal bovine serum (FBS) in order to see if incubation with FBS would result in glycosylation. There

was no difference visible in the pattern of the samples stained with by Alcian blue/Coomassie or Stains-all/silver stain (Fig. 9D and 9E). Next, outer membrane preparations from bacteria that were treated before with FBS were analyzed and no clear difference could be observed (Fig. 9F and 9G). Additionally, outer membrane proteins or crude extracts derived from bacteria that had been incubated with FBS were analyzed with lectins SNA, GNA, DSA or PNA and no binding could be observed (data not shown). Immunoblotting with an antibody that was generated against heat killed *Cc5* bacteria did not reveal any difference between *Cc5* and Δ*siaC* either, indicating that no epitope is missing in Δ*siaC* (Hwain Shin, data not shown). It would be interesting to perform experiments chasing incorporation of tritium labeled sialic acids. However, such experiments are hampered by the unavailability of a minimal medium suitable for *C. canimorsus*.

Taken together, we conclude that sialidase provides access to internal aminosugars of eukaryotic glycans by cleaving terminal sialic acids, whereas sialic acids are not used for bacterial replication or for molecular mimicry.

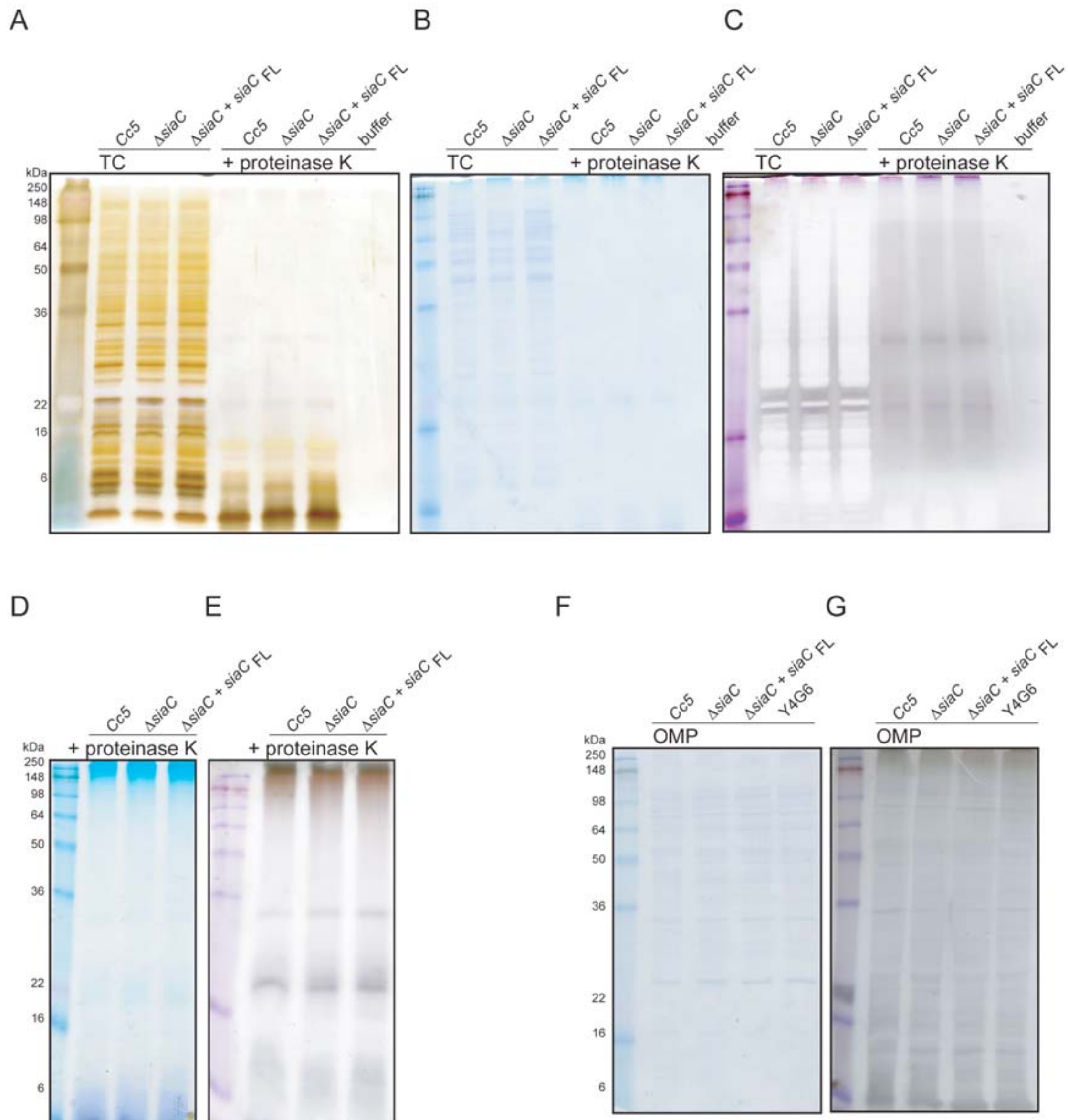


Fig. 9 Analysis of bacterial carbohydrates reveals no difference between Δ*siaC* and *Cc5*

Bacterial carbohydrates of total cells (TC) or proteinase K digests were analyzed by (A) periodic acid, (B) Alcian blue/Coomassie or (C) Stains-all/silver staining. Bacteria were incubated with HI serum prior to analysis of bacterial extracts digested with proteinase K using (D) Alcian blue/Coomassie or (E) Stains-all/silver staining. Outer membrane proteins (OMP) prepared from bacteria incubated with serum were analyzed with (F) Alcian blue/Coomassie or (G) Stains-all/silver staining. Bacteria used were *Cc5*, Δ*siaC* or Δ*siaC* complemented with plasmids encoding SiaC *in trans*, or Tn mutant Y4G6 (G and H) (Chapter 2).

2.2.3. Dog's saliva can complement impaired growth of Δ*siaC* in presence of cells

We next wanted to test, if growth of *Cc5* in saliva, which represents a part of the ecological niche of *C. canimorsus,* is dependent on the action of sialidase or if nutrients present could complement impaired growth of Δ*siaC.* Saliva was sampled from a dog that contained *C. canimorsus* in its normal flora (Chapter 4, dog 018). Saliva was prepared by removing aggregates and cells. Filter-sterilized saliva was added to cultured J774.1 at 5 or 10% final concentration. Growth of Δ*siaC* after 24 h in this experimental set up, which included cells and FBS, was restored in a dose dependent manner. Saliva samples derived from different preparations led to differences when added at 5%, but 10% was enough to sustain bacterial growth. When cells were cultured without serum, the addition of saliva also rescued impaired growth of Δ*siaC*. We suspect that free aminosugars are present in sufficient amounts in dog's saliva for *C. canimorsus* to multiply. Hence sialidase would be required to grow at the expenses of cells but not on saliva.

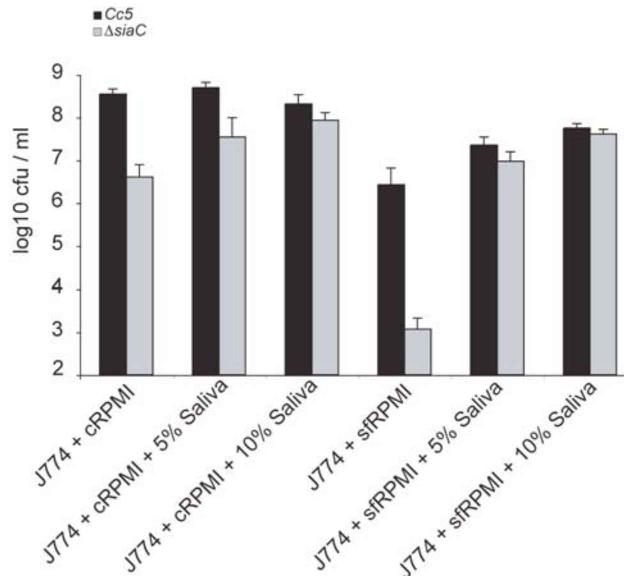


Fig. 10 Dog's saliva can rescue impaired growth of Δ*siaC* in presence of J774.1

Filter sterilized and cell free dog's saliva was added to cultured J774.1 at 5 or 10%. Bacteria were inoculated at a moi 20 (2x 10^6 cfu/ ml) and viable counts were determined after 24 h.

2.2.4. A second mutant displaying impaired growth in presence of macrophages can be complemented by aminosugars

A Tn mutant screen led to the identification of two mutants that were defective in growth in the presence of J774.1 macrophages but proficient in growth on blood agar. One of these mutants turned out to be affected in the surface-exposed sialidase. Here, we analyzed Y4G6, the second mutant.

We tested whether the addition of aminosugars would restore growth of mutant Y4G6 in the presence of macrophages. GlcNAc and GalNAc restored growth of mutant Y4G6 (Fig. 11) as demonstrated for Δ*siaC* (Fig. 3B). This suggests that the gene disrupted in mutant Y4G6 plays a role in the acquisition of the aminosugars GlcNAc and GalNAc. Surprisingly, both mutants Δ*siaC* and mutant Y4G6, could grow in presence of cells and ManNAc, which is not present in eukaryotic glycans.

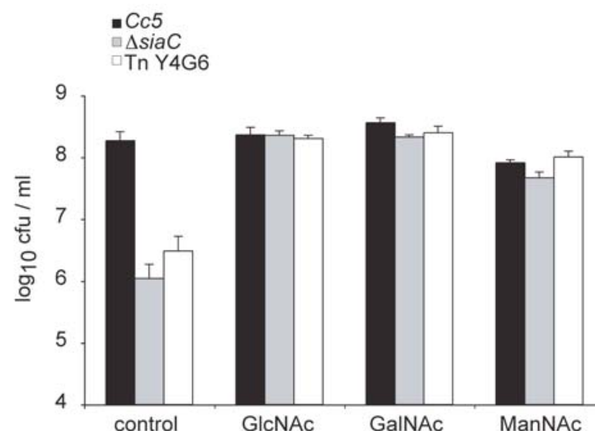


Fig. 11 Mutant Y4G6 has a growth defect in presence of macrophages and aminosugars can rescue impaired growth of Δ*siaC* or Tn Y4G6.

Viable counts of 2 x 10^6 *Cc5* (black), Δ*siaC* (grey) or TnY4G6 (white), monitored after 24 h of culture in the in presence of J774.1 macrophages in cRPMI (moi = 20); and in the same condition supplemented with 0.1% GlcNAc, GalNAc or ManNAc. Mean values from 3 or more experiments and SD are shown.

2.2.5. Identification of the mutant Y4G6

Mapping of the transposon integration site identified a gene ("*yfg*" for Y four G six, gene "A") encoding for a protein with homology to the hypothetical protein BF3612 of *B. fragilis* (Table 5). Using domain predictions by InterProScan (Quevillon *et al.*, 2005) there was no further hint on its function except for a predicted signal sequence. The operon contains one predicted outer membrane protein ("*ompY*") upstream of *yfgA*. InterProScan predicted the gene product of *yfgB* with a glycoside hydrolase domain (IPR001579). The downstream genes called *yfgCD* encode for proteins with unknown function.

In order to exclude polar effects of the Tn integration, several plasmids were constructed to complement the growth defect (Fig. 12). The gene *yfgA* was cloned with and without C-terminal His tag into the shuttle expression plasmid pMM47.A, leading to constructs pMM57 and pMM56, respectively. Introduction of both constructs *in trans* of mutant Y4G6 did not complement the growth defect in presence of J774.1, suggesting a polar effect of the transposon insertion. Therefore, we cloned *yfgA* with its downstream gene *yfgB* (pMM76), but this construct introduced *in trans* did not rescue the Tn mutant for growth. YfgB$_{His}$ (pMM76) was however expressed as assessed by immunoblotting against its C-terminal His tag (not shown). In addition, we constructed plasmids including *ompY* (upstream gene), *yfgA* with or without *yfgB* (Fig. 12). The plasmid pMMP98 contained *ompY* and *yfgA* without any modification, while in pMMP99 *yfgA* was in frame with a C-terminal His tag. Plasmid pMMP100 contained the upstream gene *ompY, yfgA* and the downstream gene *yfgB*. Introduction of pMMP98 and pMMP100 *in trans* of Y4G6 rescued the growth defect in presence of cultured cells, while pMMP99 did not, suggesting the C-terminal modification disturbed function of *yfgA.* Growth of Y4G6 could be rescued by introducing pMMP98 (*ompYyfgA*) but not by pMMP99 (*ompYyfgA*$_{His}$) (Fig. 13). This indicates that there is no or only little polar effect in Y4G6. We therefore conclude that the *yfgA* gene without modification of the Shine Dalgarno site N-terminus or C-terminus is necessary for growth with cells.

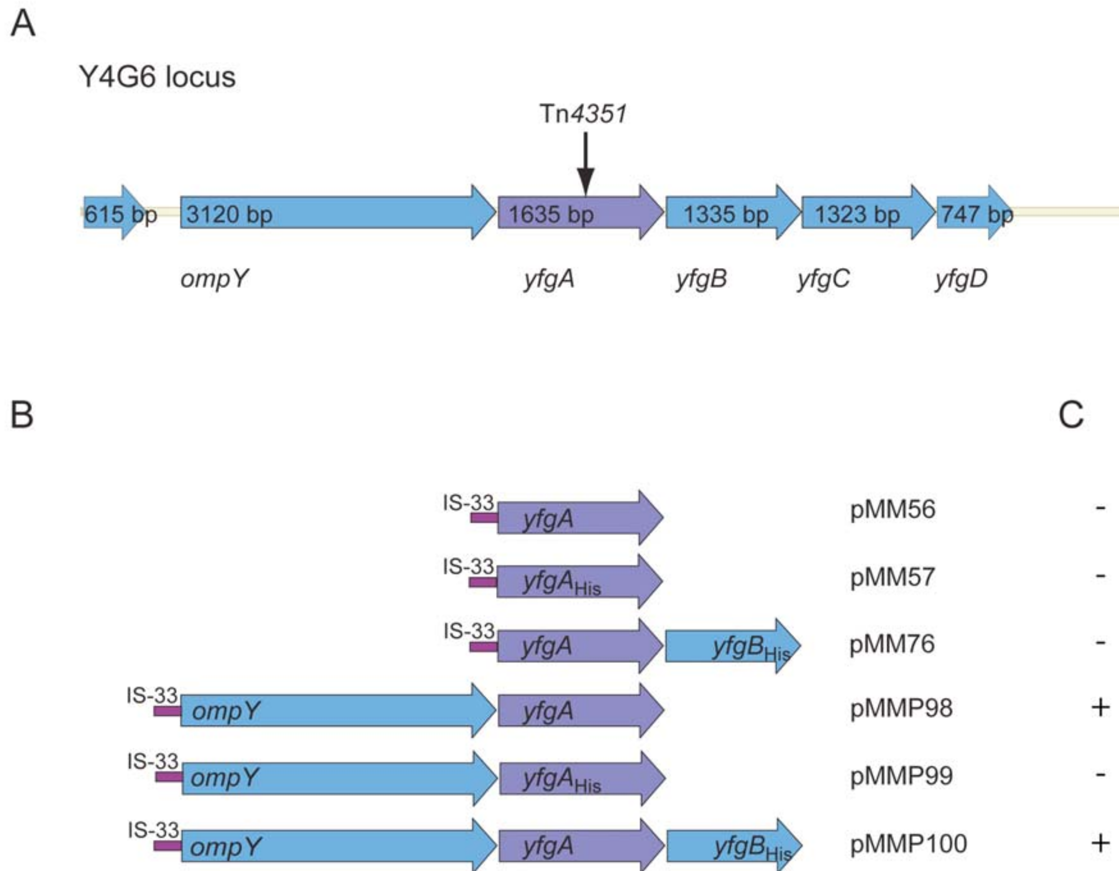


Fig. 12 Schematic representation of the Y4G6 locus and the genes cloned for complementation.

(A) Tn*4351* inserted in a locus resembling an operon, called Y4G6 according to the Tn mutant. (B) Genes cloned for complementation are schematically represented for the corresponding plasmids. (C) + or - indicates positive or negative results of complementation during growth in presence of macrophages.

Table 5 Blast comparisons of the gene products of locus Y4G6 (February 2008, nr database)

ID	Annotation	Species	score	E value
OmpY				
ref|YP_100888.1|	putative outer membrane protein	*Bacteroides fragilis*	916	0.0
ref|YP_213018.1|	putative outer membrane protein Omp117	*Bacteroides fragilis*	916	0.0
ref|NP_813315.1|	putative outer membrane protein	*Bacteroides thetaiotaomicron*	890	0.0
(Tn hit) YfgA				
ref|YP_100889.1|	hypothetical protein BF3612	*Bacteroides fragilis*	347	1e-93
ref|YP_213019.1|	hypothetical protein BF3413	*Bacteroides fragilis*	346	3e-93
ref|NP_813316.1|	hypothetical protein BT_4405	*Bacteroides thetaiotaomicron*	308	5e-82
YfgB				
ref|NP_813317.1|	hypothetical protein BT_4406	*Bacteroides thetaiotaomicron*	92.4	5e-17
gb|EDO10923.1|	hypothetical protein BACOVA_03558	*Bacteroides ovatus*	77.0	2e-12
ref|NP_812664.1|	endo-beta-N-acetylglucosaminidase	*Bacteroides thetaiotaomicron*	73.6	3e-11
YfgC				
gb|EDO10657.1|	hypothetical protein BACOVA_04106	*Bacteroides ovatus*	68.9	7e-10
ref|YP_100891.1|	hypothetical protein BF3614	*Bacteroides fragilis*	67.8	1e-09
ref|YP_213021.1|	putative lipoprotein	*Bacteroides fragilis*	65.1	8e-09
YfgD				
Emb|CAG38650.1|	hypothetical protein	*Ornithobacterium rhinotracheale*	42.7	0.022
ref|ZP_01926445.1|	hypothetical protein LMHG_01920	*Listeria monocytogenes*	40.4	0.11

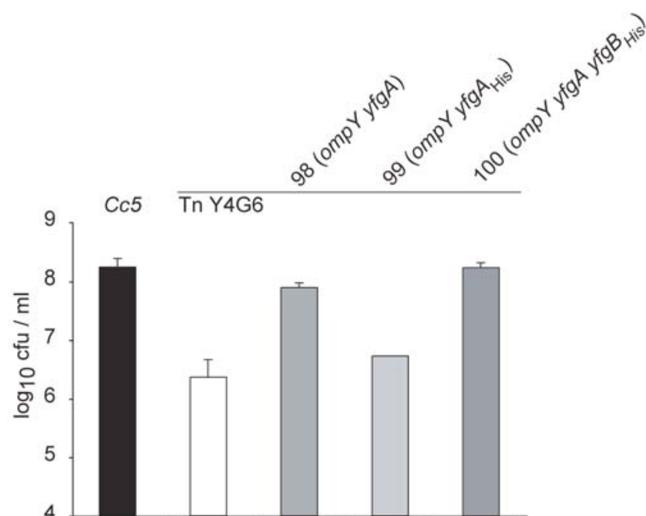


Fig. 13 Gene *yfgA in trans* of Tn mutant Y4G6 is required to restore impaired growth in presence of macrophages

Bacterial viable counts of *Cc5*, Tn mutant Y4G6 (Tn Y4G6) alone or harboring different plasmids *in trans* inoculated at a moi of 20 (2x 10^6 bacteria) were monitored after 24 h in presence of J774.1 in cRPMI. Mean values from 3 experiments and SD are shown, except for pMMP99.

2.2.6. Serum sensitivity

We next tested the bactericidal action of human serum by incubating 10^7 bacteria in normal or heat inactivated (HI) human serum for 3 h. Y4G6 was more sensitive to complement mediated lysis than wt *Cc5* (Fig. 14A). As shown for Δ*siaC* (Fig. 5A), complement sensitivity correlated to a lack of factor H binding (Hwain Shin, not shown). Serum sensitivity was restored by introducing pMMP100 and to a lesser extent by pMMP98 *in trans* of Y4G6. This suggests that expression of the downstream gene *yfgB* enhances the ability of Y4G6 to resist complement (Fig. 14A).

We next tested if addition of aminosugars changed surface properties of the serum sensitive mutants Δ*siaC* and Y4G6 (Fig. 14B). We observed that addition of aminosugars did not confer protection against human serum when added at 1% to Δ*siaC* or Y4G6. However, GlcNAc or ManNAc did increase the total viable counts of Δ*siaC* or Y4G6 in heat-inactivated serum, indicating that even in conditions in which serum is the only nutritional source, addition of aminosugars

sustains bacterial growth of mutants, at least to some extent comparable to wt levels.

To summarize, aminosugars added to Δ*siaC* or Y4G6 did not confer protection against the bactericidal action of human complement. This suggests that aminosugars are not used by the bacteria to modify the carbohydrate surface structures.

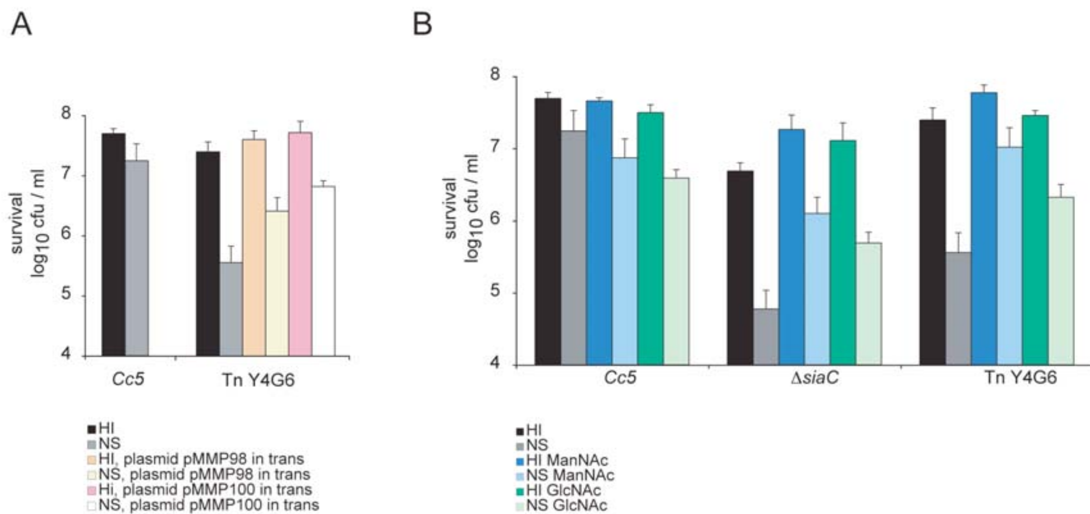


Fig. 14 Analysis of serum sensitivity and effects of aminosugars

(A) Viable counts of each bacterial strain after incubation with 10% normal (grey) or HI serum (black) for 3 h at 37°C. Mean values from 3 or more experiments and SD are shown. (B) Effects of aminosugars was tested by monitoring viable counts as described in (A) and in addition of 1% ManNAc or GlcNAc

2.2.7. Serum proteins are bound by *Cc5* and desialylated

We showed previously that bacterial growth was dependent on contact to host cells. However, serum also influenced growth, at least to some extent (Fig. 1A). In addition, we showed that *Cc5* bacteria not only desialylated but also deglycosylated serum proteins (Fig. 7). We next tested whether serum derived host glycoproteins are recruited to the bacterial surface of *Cc5*.

With immunofluorescence on fixed but unpermeabilized bacteria that were pre-treated with HI serum, we tested binding of lectin SNA that recognizes terminal sialic acids. We observed the presence of sialic acids on the surface Δ*siaC* but not on the wt *Cc5* bacterial surface or on mutant Y4G6 (Fig. 15.). The signal from SNA binding disappeared after the addition of SiaC, confirming that terminal sialic acids can be removed by sialidase treatment. This observation suggests that *Cc5,* Δ*siaC* + $siaC_{FL}$ and mutant Y4G6 might bind the same glycosylated components as Δ*siaC* or Δ*siaC* + $siaC_{\Delta 1\text{-}21}$ after serum treatment but desialylation by a functional sialidase in those strains leads to a lack of signal. Immunofluorescence indicates that the surface of Δ*siaC* becomes sialylated after incubation with HI serum. No fluorescence was observed when bacteria were not incubated with HI serum but with buffer instead (data not shown).

We next wanted to identify the source for the sialic acid signal on Δ*siaC*. We asked if host derived factors could be bound and subsequently deglycosylated by *C. canimorsus*. We therefore performed a serum absorption experiment, detached bound serum proteins from the bacterial surface by low pH and analyzed eluates by lectin binding. Lectin SNA recognized terminal sialic acids, while lectins GNA (*Galanthus nivalis* agglutinin, recognizing mannose), PNA (specific for Gal-GalNAc); or DSA (Gal-GlcNAc / GlcNAc) can only bind to these internal carbohydrates if they are exposed after removal of terminal sialic acids. We could detect signals for lectins GNA (Fig. 16B) and PNA (Fig. 16C) in eluted protein supernatants from *Cc5*. This shows that bound serum proteins were desialylated by wt *Cc5,* or, even subsequently deglycosylated as indicated by a decrease of DSA signal (Fig. 16D). Notably, serum proteins eluted off from mutant Y4G6 clearly showed more binding to DSA (Fig. 16D) and GNA (Fig. 16B) (n=3), as compared to proteins detached from wt *Cc5.* This shows that sialic acids are removed by mutant Y4G6. More interestingly, this suggests that sugars Gal-

GlcNAc / GlcNAc (DSA) and mannose (GNA) on the glycoproteins bound by the mutant Y4G6 can not be deglycosylated.

Taken together, mutant Y4G6 is clearly affected in the deglycosylation process as shown by the inability to further hydrolyze internal Gal-GlcNAc / GlcNAc and/or mannose.

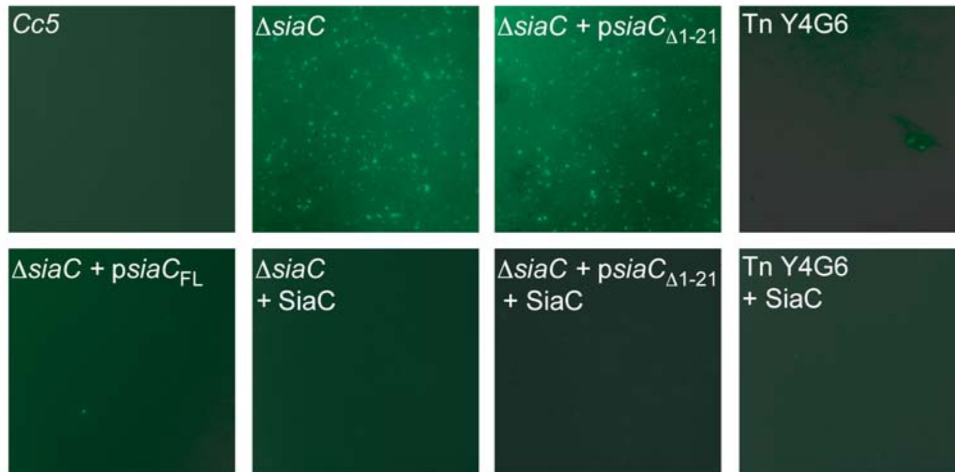


Fig. 15 Sialic acids can be detected after serum treatment on sialidase-deficient bacteria
Lectin SNA was used on fixed but unpermeabilized bacteria that were pretreated with HI serum to detect terminal sialic acids. Biotinylated lectin was visualized by fluorescein conjugated streptavidin and examined by microscopy.

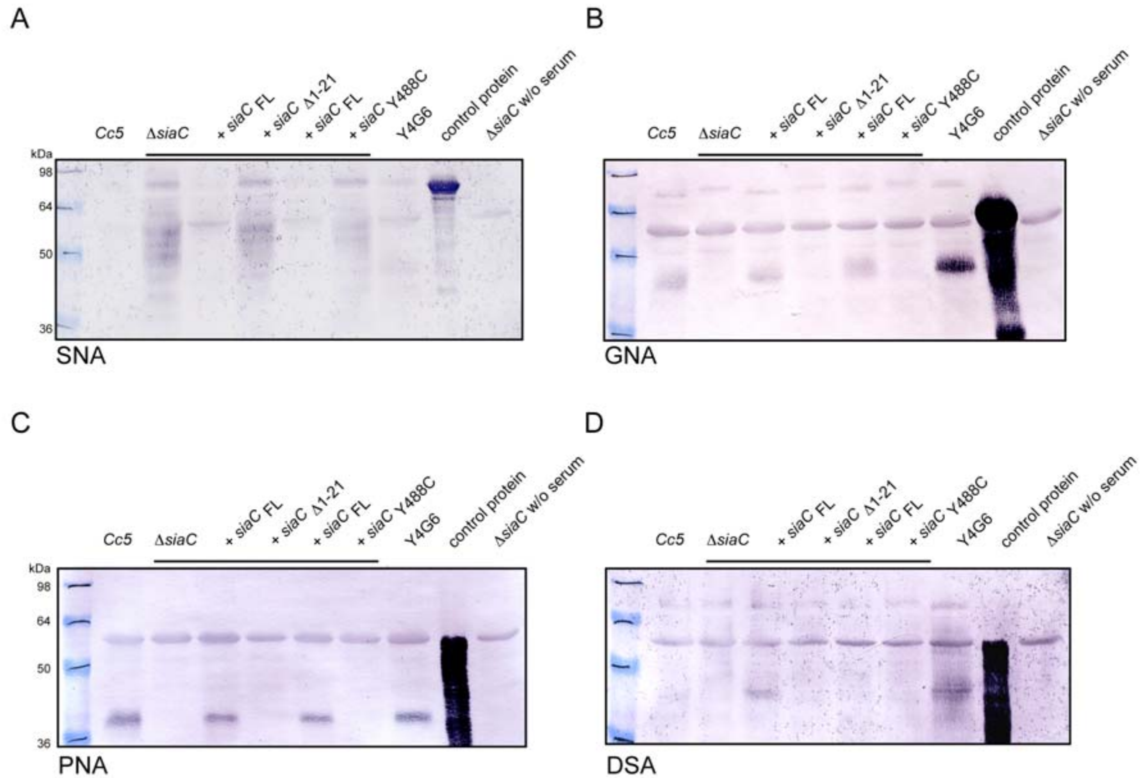


Fig. 16 Bacteria bind serum proteins and subsequent deglycosylation is dependent on the action of sialidase.

5x 10^9 bacteria were incubated with HI serum and bound serum proteins were detached from the bacteria by glycine pH 2.2. Eluted proteins were separated by SDS PAGE and analyzed by lectin staining. Lectins (Roche) used were SNA that recognizes terminal sialic acids (2- 6 or 2- 3) linked to Gal or to GalNAc (A); GNA (*Galanthus nivalis* agglutinin) recognizing terminal mannose, (1-3), (1-6) or (1-2) linked to mannose (B); PNA that is specific for Gal (β 1- 3) GalNAc (C); or DSA, that binds to Gal-(1-4) GlcNAc in N- or O-glycans and/or GlcNAc (O-glycans).

We next asked which serum proteins are recruited to the bacterial surface of *C. canimorsus*. As shown in Fig. 15 and Fig. 16, Δ*siaC* is able to bind serum derived proteins and due to the inability of Δ*siaC* to remove terminal sialic acids, the bound glycoproteins can not be deglycosylated. We therefore used lectin SNA bound to agarose beads to affinity purify fully sialylated glycoproteins bound on Δ*siaC* bacteria. By mass spectrometry, a 50 kDa could be identified as the human beta-2 glycoprotein I (β2GPI) from plasma (Fig. 17) and a 64 kDa band was identified as the heavy chain from IgA (Fig. 17B).

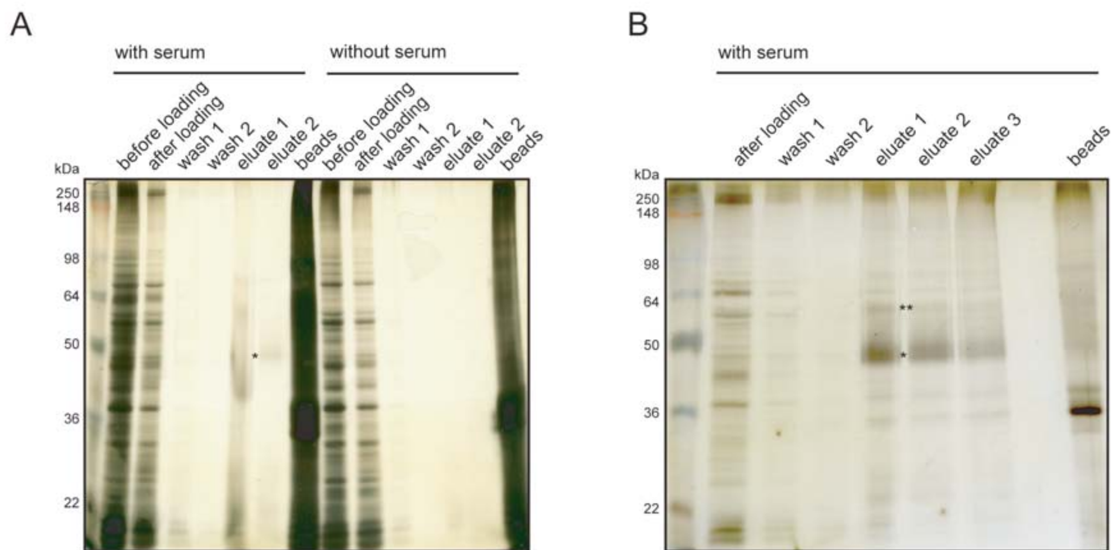


Fig. 17 Affinity chromatography using lectin SNA reveals sialylated serum proteins bound by Δ*siaC*

(A) Δ*siaC* was pretreated with HI serum ("with serum") or with buffer only ("without serum") and bound proteins were detached from bacteria by glycine pH 2.2. Eluates were incubated with SNA bound on agarose that was used to bind sialylated proteins which were then eluted by 500 mM lactose ("eluate 1") followed by 500 mM lactose in acetic acid ("eluate 2") and separated by SDS PAGE and stained with silver. (B) The same conditions were applied as shown in (A) but with increased sample size. Asterisks indicate (*) human beta-2 glycoprotein I and (**) heavy chain from IgA1.

A deglycosylation process of bound host proteins was dependent on presence of sialidase and our experiments strongly suggest that mutant Y4G6 is also involved in the deglycosylation process on unprotected carbohydrates after the removal of terminal sialic acids.

2.2.8. Methods Section

Deglycosylation of serum proteins and lectin blotting

Total pooled human serum was heat inactivated at 55°C for 60 min, and incubated with 5x 10^9 bacteria in PBS for 90 min at 37°C. Bacteria were removed by centrifugation at 20 000 x g for 5 min and serum supernatant was cleared by filtering though a 0.22 µm filter (Sarstedt) and diluted 100 x. Serum proteins were precipitated by trichloroacetic acid for 1 h and pellets were washed with acetone and dissolved in electrophoresis sample buffer. 1 µg transferrin or fetuin (Roche) were incubated with 10^7 bacteria or with 1 µg purified sialidase at 37°C for 5 or 18 h. Reactions were stopped by adding sample buffer and samples were boiled for 10 min. To examine deglycosylation of serum proteins, samples with a protein concentration of approximately 0.2 µg were electrophoresed on an SDS-PAGE gel (12.5%), transferred onto nylon C+ membranes (Nucleobond), and detected using GNA, SNA, MAA, DSA or PNA lectins from the DIG glycan differentiation kit (Roche), according to manufacturers instructions.

Silver periodic staining

SDS gels were fixed in 40% EtOH, 5% acetic acid for at least 4 h. 0.7% periodic acid in 40% EtOH and 5% acetic acid was used to oxidize sugars for 5 min. Gels were washed extensively for 3x 15 min with H_2O and subsequently stained for 10 min with a reagent containing 1.34% concentrated ammonium hydroxide, 18.67% 0.1 M NaOH and 0.67% $AgNO_3$ followed by three washing steps in H_2O for 10 min. Developer containing 0.05% formaldehyde and 0.005% citric acid, stained sugars as LPS dark brown in 2 to 5 min. Reaction was terminated by 10% acetic acid.

Stains-all staining and combined Stains-all/silver

SDS gels were fixed for Stains-all (Fluka) over night in 10% acetic acid and 25% isopropanol to remove residual SDS. The gels were stained for up to 48 h in the dark at room temperature with freshly prepared working solution from Stains-all stock solution with final 0.01% Stains-all in 7.5% formamide, 25% isopropanol, 45 mM Tris- HCl, pH 9.2. The background stain was removed by several changes of 25% isopropanol in the dark and scanned for documentation. The gels were finally destained in 25% isopropanol in the dark. For the combined Stains-all/ silver staining, Stains-all destained gels were washed 3 x in H_2O and subsequently stained with 0.1% $AgNO_3$ for 20 min. Developer contained 2.5 % sodium carbonate and 0.04 % formaldehyde and reaction was stopped by 7 % acetic acid.

Alcian Blue 8GX staining

After staining with Coomassie Blue (0.25 % Coomassie Blue R-250 8% acetic acid and 46% EtOH), gels were extensively destained with 5% acetic acid, 35% EtOH to remove SDS and unspecific bindings. Gels were shaken in 7% acetic acid for 1 h with changes of the solution several times. 0.5% w/v Alcian Blue 8GX (Sigma) in 7% acetic acid was used for 30 min to stain

glycans and gels were washed in 7% acetic acid for 12- 24 h. After destaining, gels were kept 1- 2 h in 5% acetic acid, 35% EtOH and stored in water (Wardi and Michos, 1972).

Preparation of dog's saliva

Saliva was sampled from a healthy dog, immediately stirred with 2.5 mM DTT for at least 20 min or up to 1 h at RT to remove aggregates. Cells and aggregates were removed by high speed centrifugation at 40 000 x g for 10 min at 4°C and supernatants were filtered though 0.20 µm. Sterility was verified in infection assays.

Serum absorption

After incubating 5x 10^9 bacterial strains *Cc5*, Δ*siaC* or mutant Y4G6 with HI human serum for 90 min, serum supernatant was removed and loosely attached serum proteins were washed off with 0.05% PBS Tween-20. Proteins bound by the bacteria were eluted using glycine pH 2.2 and the neutralized supernatants were analyzed by SDS PAGE by lectin binding.

SNA Affinity Chromatography

C. canimorsus Δ*siaC* was grown on HIA plates, harvested by scraping and resuspended in PBS. HI human serum was incubated with 5x 10^9 bacteria in PBS for 90 min at 37°C. Bacteria were washed five times with PBS/0.05% Tween-20 and bound proteins were eluted with 0.1 M glycine-HCl, pH 2.2 for 5 min at room temperature. After removal of bacteria by centrifugation, eluates were neutralized with 1 M Tris, pH 8.0 and mixed with SNA-agarose (Vector Laboratories). Beads were incubated on a stirring wheel over night at 4°C followed by extensive washes and bound molecules were eluted with 500 mM lactose in buffered saline followed by 500 mM lactose in acetic acid. Eluates were separated on SDS PAGE and subsequently stained with silver. Alternatively, Coomassie stained proteins contained in gel slices were digested with trypsin and subjected to mass spectroscopy analysis.

2.3. Discussion

We could demonstrate that unmasking of carbohydrates by SiaC results in production of accessible aminosugars necessary for multiplication of *C. canimorsus*. Addition of GlcNAc and GalNAc could restore the growth defect of Y4G6 and Δ*siaC*. We therefore expect a sequential deglycosylation process of host glycans either present on cell surfaces or on circulating host glycoproteins by wt *Cc5*. A sequential action of exoglycosidases was previously described for *S. pneumoniae* and a similar mode of action may be also involved during *C. canimorsus* growth *in vivo*. We demonstrated that *C. canimorsus* bound and deglycosylated serum derived host proteins. This deglycosylation process was altered in Δ*siaC* at the first step, which is cleavage of terminal sialic acids, and we suggest that the gene disrupted in mutant Y4G6 encodes an enzyme involved in the subsequent steps of deglycosylation. This hypothesis is supported by lectin stainings on serum proteins bound on the bacterial surface. More biochemical analysis on the protein needs to be performed to understand its properties *in vitro*. Even though we excluded polar effects on *yfgB*, more genetic and biochemical analysis on *yfgB* should be performed in addition. YfgB was predicted to have a glycoside hydrolase domain by using InterProScan.

It also has to be addressed in the future if alteration in the glycosylation state of host clearance proteins affects their function. Many proteins of the adaptive and innate immune systems are glycosylated and it has been shown that altering the glycosylation of a protein modifies its functions (Schauer, 2000). It has already been proposed that pathogenic bacteria modulate the activity of host clearance proteins by deglycosylation. EndoS, *S. pyogenes* extracellular enzyme, hydrolyzes conserved N- linked oligosaccharides from the heavy chain of IgG. This deglycosylation in turn alters the binding of IgG to FcR II and EndoS treated IgG were shown to significantly reduce activation of the classical pathway of complement (Collin *et al.*, 2002). Similarly, in *S. pneumoniae,* sialidase (NanA), β-galactosidase (BgaA) and β-N-acetylglucosaminidase (StrH) have been shown to sequentially deglycosylate human secretory component hSC, human lactoferrin and hIgA1 (King *et al.*, 2006). However, there is no proof that this contributes to pathogenesis. In the case of *C. canimorsus,* we could demonstrate that the lack of one metabolic pathway leads to increased sensitivity to human complement. One mechanism leading to complement resistance is the recruitment of fH, but if the

deglycosylation process itself contributes to serum resistance or to fH binding remains an open question. One could envision that a surface localized multi-enzyme complex that deglycosylates host glycans itself binds heavily glycosylated fH. Experiments testing this hypothesis are currently ongoing.

Interestingly, we found during this study that Y4G6 and Δ*siaC* could be rescued for growth in presence of cells by addition of ManNAc, which is not found in eukaryotic glycoproteins. This raises several questions about metabolism of *Cc5*. ManNAc and pyruvate are the products of sialic acid catalyzed by the neuraminiate lyase NanA from *E. coli*, *Salmonella sp.* or *Haemophilus influenza*, all known for sialic acid catabolism (Vimr *et al.*, 2004). In case of *C. canimorsus,* we provide strong evidence against sialic acid catabolism: First, sialic acid is not the source for growth in *C. canimorsus*. Neither Neu5Ac nor CMP-Neu5Ac could restore the growth defect of Δ*siaC*. Moreover, biochemical analysis by U. Zähringer did not show any sialic acid in CPS, LPS or glycans on *C. canimorsus* grown on blood plates. This furthermore excludes that serum resistance is due to molecular mimicry by acquisition on sialic acids in surface structures of *C. canimorsus*. A possible reason for the usage of ManNAc may be explained from the sialidase locus in the genome. We could identify a gene with domain prediction of an N- acyl epimerase in a putative operon with the sialidase. Looking at the metabolic pathways, we could therefore suggest that this enzyme epimerizes GlcNAc to ManNAc and *vice versa*. This would not involve a neuraminiate lyase converting pyruvate and ManNAc from Neu5Ac (Vimr *et al.*, 2004; Walters *et al.*, 1999) in *C. canimorsus* for generating ManNAc or GlcNAc. However, the question whether GlcNAc, GalNAc or ManNAc are incorporated in LPS or CPS has to be addressed in the future. GlcNAc may be the main source to be involved in LPS, CPS and peptidoglycan structure or biosynthesis and therefore renders *C. canimorsus* dependent on the uptake of host derived aminosugars. Again, this would clearly indicate the close link between host adaptation and bacterial metabolism. If this deglycosylation process is related to resistance against complement and fH binding remains poorly understood and requires more experiments to be performed. One possible theory as discussed before would be that changes in glycosylation of host proteins results in altered functions. On the other hand, one can exclude that alteration of the capsular

structure or LPS on *C. canimorsus* mutants, Δ*siaC* and Y4G6, leads to serum sensitivity, for instance by the inability to recruit fH.

In a recent study, peptides from human beta-2 glycoprotein I upon cleavage by PMN-derived proteinases have been shown to have a broad antibacterial activity. *S. pyogenes* was able to counteract this activity by binding human beta-2 glycoprotein I via the M1 and H protein (Nilsson *et al.*, 2008). It has to be addressed in the future what role the recruitment of human beta-2 glycoprotein I to the surface of *C. canimorsus* plays in pathogenesis or in serum resistance.

To summarize, we provide evidence that the gene *yfgA* encodes a protein involved in deglycosylation of host-derived glycans on cell surfaces or soluble glycoproteins. This contributes to the efficient replication of *C. canimorsus 5* in presence of phagocytes. Moreover, serum proteins, presumably IgA or human beta-2 glycoprotein I, are recruited to the bacterial surface and subsequently deglycosylated. This deglycosylation required the action of the sialidase and *yfgA.*

Chapter 3

Isolation of mutants affected in the anti-inflammatory mechanism of *C. canimorsus 5*

Chapter 3 Isolation of mutants affected in the anti-inflammatory mechanism of *C. canimorsus 5*

Author contributions. HS, MM and GC designed the experiments and HS, MM, and CP performed the experiments.

Statement of my work. My contribution was the development of the genetic methodology; I contributed to the Tn mutant library and the screen with HS and CP. I mapped and characterized the mutants. I generated the tools such as complementation plasmids and antibodies. Data from HS is indicated.

3.1. Summary

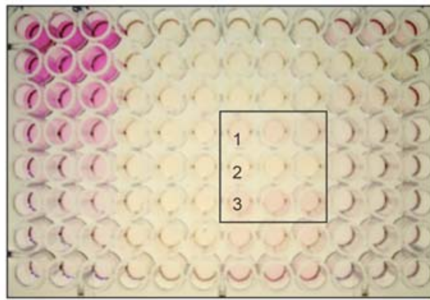
We applied an extensive Tn mutagenesis approach to screen for mutants of *Cc5* that are defective in the active anti-inflammatory mechanism that we observed during infections of stimulated J774.1 macrophages. Four mutants were associated to a defect in blocking NO release during co-infection of J774.1 macrophages. Of these mutants, three were deficient in blocking tumor necrosis factor (TNF) -alpha release upon LPS stimulation of J774.1, whereas one could still inhibit TNF-alpha release as wt *Cc5*. We identified the disrupted genes in three Tn mutants. We further analyzed one locus in more detail and found that at least two other genes in the same operon are involved in the active mechanism of blocking LPS induced NO release of macrophages. Despite exhaustive genetic analysis, the functions of the gene products involved remain to be understood.

3.2. Screening the Tn*4351* library for defects in the anti-inflammatory mechanism

Infection of murine or human macrophages with *C. canimorsus* does not lead to proinflammatory response (Shin *et al.*, 2007). First, there is a lack of TLR 4 stimulation by live or heat-killed (hk) *C. canimorsus,* which could be explained by a hypo-reactive LPS structure (Shin *et al.*, 2007). Secondly, when macrophages were stimulated with a potent LPS derived from *E. coli*, two strains, *Cc5* (Shin *et al.*, 2007) and *Cc11*, actively down-regulated release of NO and TNF-alpha. To investigate this intriguing property of manipulating host cell inflammatory signals, we screened the Tn*4351* mutant library. We infected J774.1 macrophages in 96-well plates with either wt or the Tn mutants adjusted to a moi of 20. Simultaneously a source of endotoxic LPS was added to stimulate pro-inflammatory response of J774.1. We used in parallel two different stimuli either hk *E. coli* or hk *Y. enterocolitica* as a source of LPS to validate the results of the screen. After 24 h of co-infection with either hk *E. coli* or from hk *Y. enterocolitica*, cell free supernatants were then analyzed with a colorimetric assay using Griess reagent for measuring the amount of NO release. We isolated mutants that lost the ability to actively inhibit proinflammatory signals (Fig. 18A).

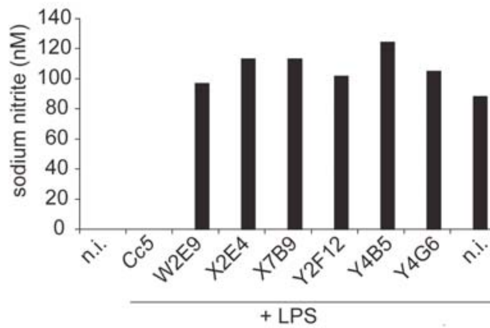
Of 6 719 mutants screened, we isolated six mutants that were unable to block NO release after stimulation of J774.1 macrophages with hk *E. coli* or hk *Y. enterocolitica*. Four out of six mutants (Y2F12, X7B9, Y4B5 and X2E4) displayed normal growth in presence of macrophages 24 h post infection but had a defect in the active anti-inflammatory mechanism (Fig. 18B). We then tested their ability to inhibit TNF-alpha release by macrophages upon LPS stimulation and found that three of four mutants (Y2F12, X7B9 and X2E4) were also unable to block TNF-alpha release, meaning that only one mutant (Y4B5) was still proficient in inhibiting TNF-alpha (Fig. 18C).

A



1. hk *Y. enterocolitica E40*
2. hk *Y. enterocolitica E40* + *Cc5*
3. hk *Y. enterocolitica E40* + Tn

B



C

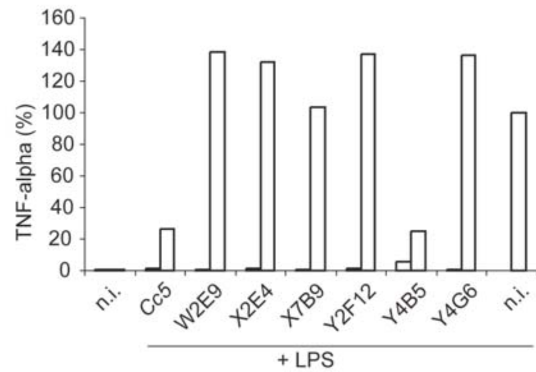


Fig. 18 Screening a Tn*4351* mutant library based on co-infection of macrophages identified mutants defective in inhibition of LPS-induced NO release

(A) J774.1 cells were co-infected with Tn mutants by simultaneous addition of a source of stimulatory LPS (hk *Y. enterocolitica*) for 24 h in a 96 well format. Cell free supernatants were analyzed for NO in form of stable nitrite using Griess reagent. Cells were stimulated with hk *Y. enterocolitica* only (1) or infected in addition with wt *Cc5* (2) or a Tn mutant defected (3). (B) Isolated mutants could not inhibit hk *Y. enterocolitica* induced NO release in one representative experiment (Hwain Shin). (C) TNF-alpha release was tested after co-infection with hk *Y. enterocolitica* for the same mutants as indicated by one representative experiment shown (Hwain Shin).

3.3. Mutant Y2F12 maps in an operon of 4 genes

By an arbitrarily primed PCR, we located the Tn disruption in front of the stop codon of an ORF in a locus that resembled an operon. To finalize the mapping of the region, we first needed to sequence gaps in the draft sequence of a shotgun library of the *Cc5* genome. Gaps were closed by primer walking on genomic DNA to find the upstream genes and the putative promoter region.

We refer to the genes in this locus as "*cam*" for canimorsus active mechanism N, O, A and B (Fig. 19). A blast analysis of gene products of the locus is shown in Table 6. Protein CamN encoded by the first gene in the operon shows high similarities to outer membrane proteins of the *Bacteroides* genus. Using InterProScan (Quevillon *et al.*, 2005), CamN showed a signature of a TonB-dependent receptor with the conserved part of the beta-barrel structure (IPR000531). Domain prediction using InterProScan indicated signal peptides in the other Cam proteins. However, other predictions only located a zinc binding motif (IPR006025) in CamA. Using blast algorithms, CamO, CamA and CamB appear to have no homologues with known functions. However, they share many homologues to conserved hypothetical proteins in the *Bacteroides* genus even in the same operon organization. We found one locus in *B. thetaiotaomicron* with a putative outer membrane protein followed by hypothetical proteins BT_3241, BT_3242 and BT_3243 with similarities to CamOAB. Another homologues locus of *B. thetaiotaomicron* consisted of a gene encoding an outer membrane protein followed by BT_ 3238, BT_3237 and BT_3236. Interestingly, three loci in *B.ovatus* shared a similar organization as the *cam* genes: (1) hypothetical proteins BACOVA_01405 to _01409; (2) hypothetical proteins BACOVA_2729 to _2732 and (3) hypothetical proteins BACOVA_2817 to _2820. In *B. fragilis* only CamO shares 2 homologues (BF_1925 and BF_1933) and CamA only one (BF_1926).

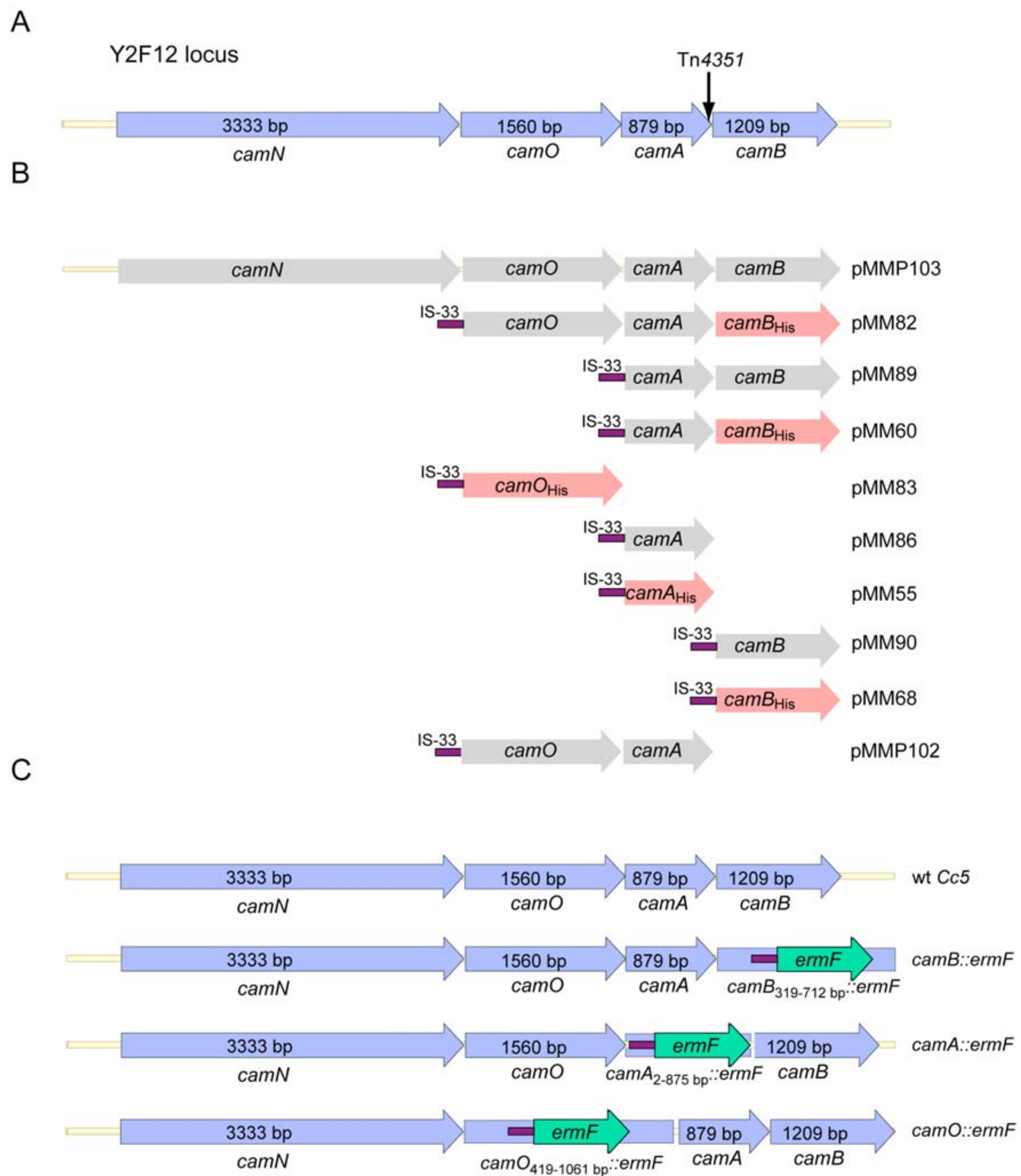


Fig. 19 Schematic representation of the locus Y2F12 and genes cloned for complementation
(A) Tn integration is indicated in locus identified in mutant Y2F12 with the genes designated "*cam*". (B) *cam* genes used for construction of complementation vectors are represented with IS-33 (*IS4351) ermF* promoter in expression shuttle vector pMM47.A. (C) Site directed mutations at positions indicated were constructed by replacing an internal part by an *ermF* cassette.

Table 6 Blast result of gene products of locus Y2F12 (February 2008, nr database)

ID	Annotation	species	score	E value
CamN				
ref|ZP_02065743.1|	hypothetical protein BACOVA_02729	*Bacteroides ovatus* ATCC 8483	1258	0
ref|NP_812151.1|	putative outer membrane protein, probably involved in nutrient binding	*Bacteroides thetaiotaomicron* VPI-5482	1257	0
ref|NP_812152.1|	putative outer membrane protein, probably involved in nutrient binding	*Bacteroides thetaiotaomicron* VPI-5482	1245	0
ref|ZP_02064439.1|	hypothetical protein BACOVA_01405	*Bacteroides ovatus* ATCC 8483	1181	0
ref|ZP_01959819.1|	hypothetical protein BACCAC_01429	*Bacteroides caccae* ATCC 43185	1149	0
ref|ZP_02065830.1|	hypothetical protein BACOVA_02817	*Bacteroides ovatus* ATCC 8483	859	0
ref|YP_001195234.1|	TonB-dependent receptor, plug	*Flavobacterium johnsoniae* UW101	829	0
ref|YP_211624.1|	putative outer membrane protein	*Bacteroides fragilis* NCTC 9343	791	0
ref|YP_099206.1|	putative outer membrane protein probably involved in nutrient binding	*Bacteroides fragilis* YCH46	790	0
ref|NP_812191.1|	putative outer membrane protein, probably involved in nutrient binding	*Bacteroides thetaiotaomicron* VPI-5482	414	2e-113
CamO				
ref|NP_812150.1|	hypothetical protein BT_3238	*Bacteroides thetaiotaomicron* VPI-5482	380	2e-103
ref|ZP_02065744.1|	hypothetical protein BACOVA_02730	*Bacteroides ovatus* ATCC 8483	379	2e-103
ref|NP_812153.1|	hypothetical protein BT_3241	*Bacteroides thetaiotaomicron* VPI-5482	371	7e-101
ref|ZP_02064440.1|	hypothetical protein BACOVA_01406	*Bacteroides ovatus* ATCC 8483	357	1e-96
ref|ZP_01959820.1|	hypothetical protein BACCAC_01430	*Bacteroides caccae* ATCC 43185	310	2e-82
ref|YP_001195233.1|	hypothetical protein Fjoh_2893	*Flavobacterium johnsoniae* UW101	266	2e-69
ref|YP_211625.1|	hypothetical protein BF1993	*Bacteroides fragilis* NCTC 9343	220	2e-55
ref|YP_099207.1|	hypothetical protein BF1925	*Bacteroides fragilis* YCH46	220	2e-55
ref|ZP_02065831.1|	hypothetical protein BACOVA_02818	*Bacteroides ovatus* ATCC 8483	201	9e-50
ref|YP_001196990.1|	RagB/SusD domain protein	*Flavobacterium johnsoniae* UW101	96.7	4e-18
CamA				
ref|ZP_02065745.1|	hypothetical protein BACOVA_02731	*Bacteroides ovatus* ATCC 8483	236	2e-60
ref|NP_812154.1|	hypothetical protein BT_3242	*Bacteroides thetaiotaomicron* VPI-5482	228	5e-58
ref|NP_812149.1|	hypothetical protein BT_3237	*Bacteroides thetaiotaomicron* VPI-5482	208	3e-52
ref|ZP_01959821.1|	hypothetical protein BACCAC_01431	*Bacteroides caccae* ATCC 43185	206	2e-51
ref|ZP_02064441.1|	hypothetical protein BACOVA_01407	*Bacteroides ovatus* ATCC 8483	179	2e-43
ref|ZP_02065832.1|	hypothetical protein BACOVA_02819	*Bacteroides ovatus* ATCC 8483	133	1e-29
ref|YP_001195232.1|	hypothetical protein Fjoh_2892	*Flavobacterium johnsoniae* UW101	114	8e-24
ref|YP_099208.1|	hypothetical protein BF1926	*Bacteroides fragilis* YCH46	69.7	2e-10
CamB				
emb|CAG38649.1|	hypothetical protein	*Ornithobacterium rhinotracheale*	152	6e-35
ref|NP_812155.1|	hypothetical protein BT_3243	*Bacteroides thetaiotaomicron* VPI-5482	103	2e-20
ref|ZP_01959822.1|	hypothetical protein BACCAC_01432	*Bacteroides caccae* ATCC 43185	95.1	8e-18
ref|NP_812148.1|	hypothetical protein BT_3236	*Bacteroides thetaiotaomicron* VPI-5482	93.6	2e-17
ref|ZP_02064443.1|	hypothetical protein BACOVA_01409	*Bacteroides ovatus* ATCC 8483	88.6	8e-16
ref|ZP_02065746.1|	hypothetical protein BACOVA_02732	*Bacteroides ovatus* ATCC 8483	77.8	1e-12
ref|ZP_02065833.1|	hypothetical protein BACOVA_02820	*Bacteroides ovatus* ATCC 8483	52.8	5e-05

We first wanted to test whether the Tn insertion affected *camA* or its downstream gene *camB*. Tn*4351* integration occurred at nucleotide position 879 of *camA* after the last codon (Asn) and before the stop codon. This suggested a modification on the C-terminus of CamA due to a frameshift, stopping translation only after a 12 additional amino-acids. We constructed the complementation plasmids, schematically shown in Fig. 19B, to analyze the genes responsible for blocking NO release in response to LPS. We then generated antibodies against the gene products involved in the active mechanism. To this end, CamO, CamA and CamB were cloned in *E. coli* expression vectors including a C-terminal His tag (CamO and CamB) or a N-terminal His tag (CamA, performed by Hwain Shin) to purify the recombinant proteins. The proteins were excised from polyacrylamide gels and used for immunization of rabbits (Table 14).

We first analyzed expression of the proteins in the mutant Y2F12 and observed that CamA and CamB were both expressed (Fig. 20). However, the amount of CamA in Y2F12 as compared to wt *Cc5* was reduced and we speculated that this could account for a defect in blocking NO release (Fig. 21A). We could observe that both genes, *camA* and *camB,* were required to restore the phenotype of Y2F12. Indeed, pMM60 ($camAB_{His}$), pMM82 ($camOAB_{His}$), pMM89 (*camAB*) or pMM103 (*camNOAB*) could complement the mutation, while pMM55 ($camA_{His}$), pMM68 ($camB_{His}$), pMM86 (*camA*), pMM90 (*camB*) or pMM102 (*camOA*) could not (Fig. 21A). A polar effect on CamB would explain the requirement of *camB* on the complementation plasmid. However, as shown in Fig. 20B, CamB was expressed in the mutant Y2F12, indicating only little polar effects. To clarify the role of CamB, we also analyzed a site-directed knock-out of *camB* which is described in the following section.

The Tn insertion at the C-terminus of CamA presumably leads to reduced stability but the mutation may also alter the functionality of CamA. We propose that the modification of CamA leads to the defect in inhibition of LPS induced NO release.

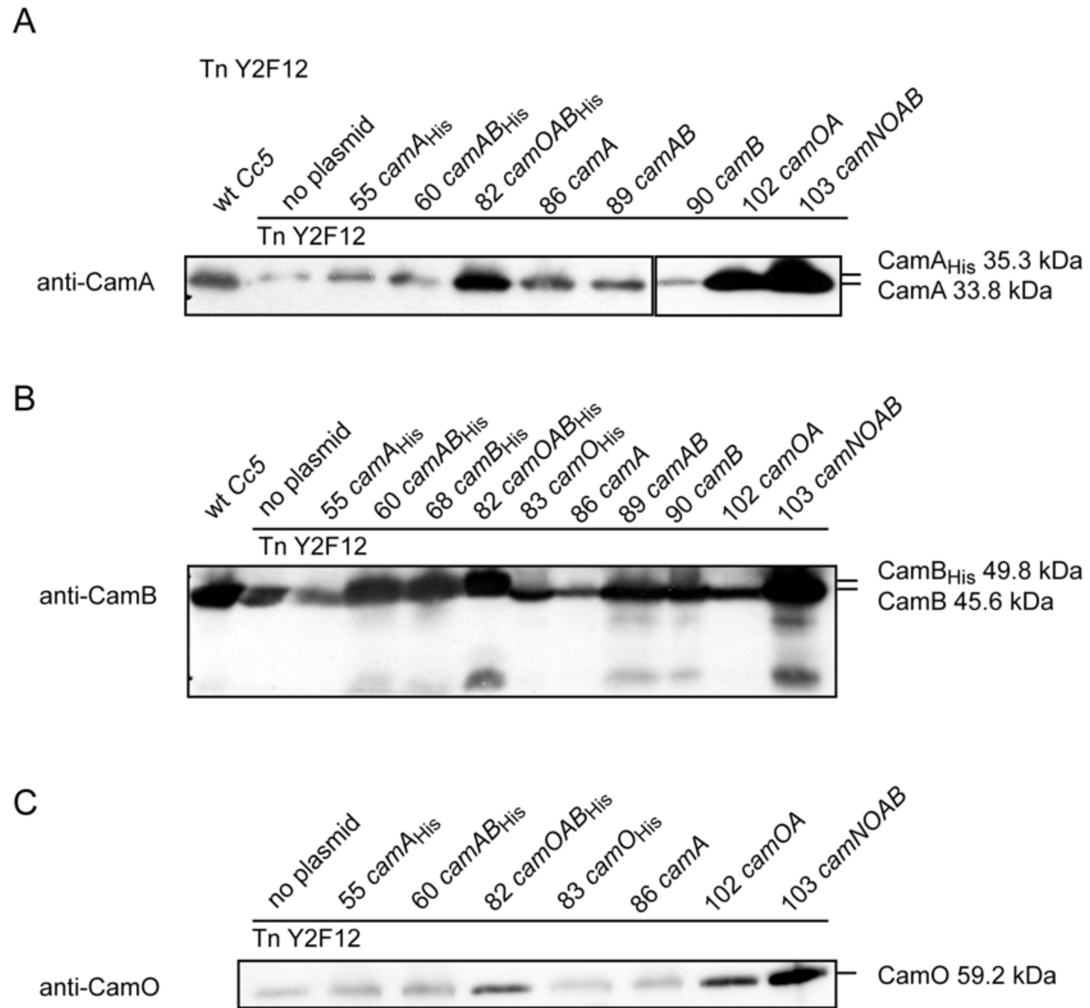


Fig. 20 Expression Cam A, CamB and CamO in mutant Y2F12

Immunoblotting using polyclonal antisera generated against CamA (A), CamB (B) and CamO (C) to test expression of Y2F12 harboring different complementation plasmids.

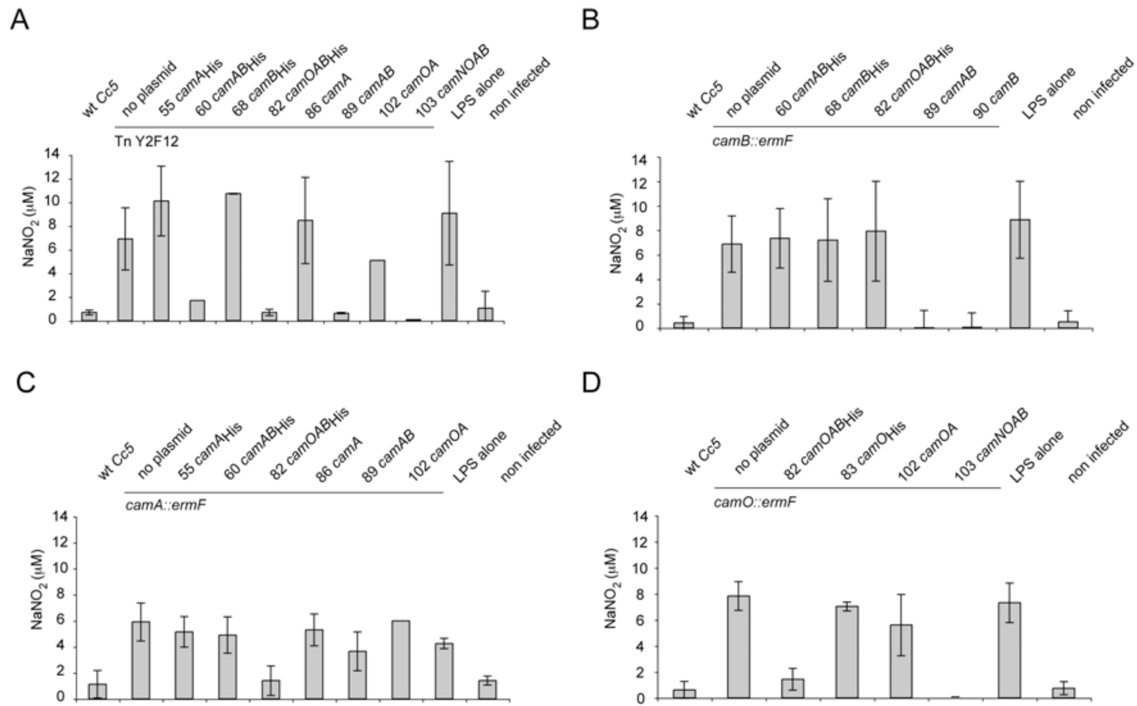


Fig. 21 Analysis of Y2F12 locus by NO release after LPS induced co-infection of macrophages

J774.1 were co-infected with different strains by simultaneous addition of stimulatory LPS (hk *Y. enterocolitica*) for 24 h. Cell free supernatants were analyzed for NO as nitrite using Griess reagent. (A) Mutant Y2F12 and complemented mutant, (B) *camB::ermF* including complemented strains, (C) *camA::ermF* including complemented strains and (D) *camO::ermF* including complemented strains are analyzed for blocking NO release. Mean values from 3 experiments are shown with the SD.

To determine whether other *cam* genes are involved, we next generated a site directed knock-out by replacing an internal part of the last gene of the operon *camB* with *ermF* (methodology is described in Chapter 1) resulting in a deletion of amino-acids 107 to 237 in CamB (Fig. 19C). The mutant *camB::ermF* was not able to block NO release during co-infection of macrophages and we could restore the phenotype by introducing the gene *camB in trans* using pMM90 (Fig. 21B). We also found that the addition of a His tag at the C-terminus of CamB prevents complementation (pMM60, pMM68, pMM82), even though the recombinant protein was expressed in crude cell extracts (Fig. 22). As shown in Fig. 21B, all constructs that insert a C-terminal His tag on CamB were not functional in blocking NO release during LPS co-infection.

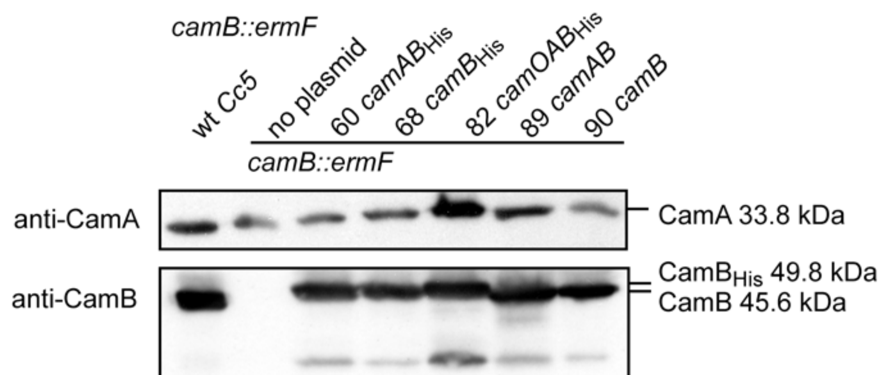


Fig. 22 Expression of CamA and CamB in mutant *camB::ermF*

Immunoblotting using polyclonal antisera anti-CamA and anti-CamB was used to test expression of *camB::ermF* harboring different complementation plasmids.

As CamB was predicted to have a N- terminal signal peptide, we therefore tested whether CamB was exposed at the bacterial surface. Indirect immunofluorescence on fixed but unpermeabilized bacteria demonstrated that CamB was surface associated in wt *Cc5* and in *Cc5 camB::ermF* harboring pMM90 (*camB*) (Fig. 23). Interestingly, the non-functional versions of CamB$_{His}$ could also be detected on the surface as summarized in Table 7.

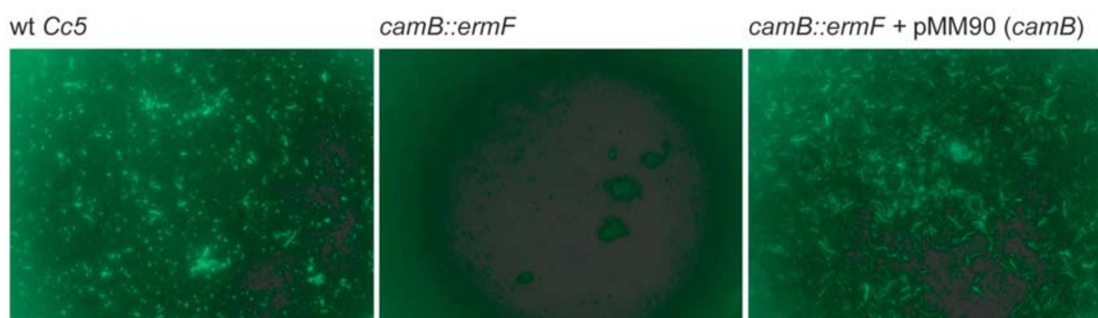


Fig. 23 CamB is surface localized

Indirect immunofluorescence was used to detect CamB on the surface of fixed but unpermeabilized bacteria using anti-CamB with a FITC conjugated secondary antibody.

We next analyzed the site-directed mutant *camA::ermF,* which had a complete deletion of CamA (Fig. 19C). First we observed that this mutant was unable to block NO release like mutant Y2F12 and *camB::ermF*. The mutant *camA::ermF* could be complemented in blocking NO release by introducing pMM82 that contains *camOAB*$_{His}$*,* but not by introducing *camA* alone (pMM86) (Fig. 21C). In contrast, the construct pMM82 leading to overexpression of CamA (Fig. 24) complemented the mutation (Fig. 21C). The non-functional CamB$_{His}$ of pMM82 had no dominant negative effect. A polar effect on CamB could be excluded since introducing pMM89 (*camAB*) did not rescue the mutant. Analysis of strains harboring different constructs *in trans* (pMM60, 82 or 89, Fig. 24) suggests that the amount of CamA is crucial to the outcome during co-infection. For instance, CamA was severely reduced in *camA::ermF* harboring pMM89 (*camAB*), even though CamB was well expressed (Fig. 24). Taken together, if CamA was encoded on pMM82 that contains *camOAB*$_{His}$, it was well expressed. In contrast, *camA* downstream from the IS-33 promoter resulted in reduced amounts. Therefore we suggest that the amount of CamA is critical to rescue the phenotype in inhibition of induced NO release (Fig. 21C).

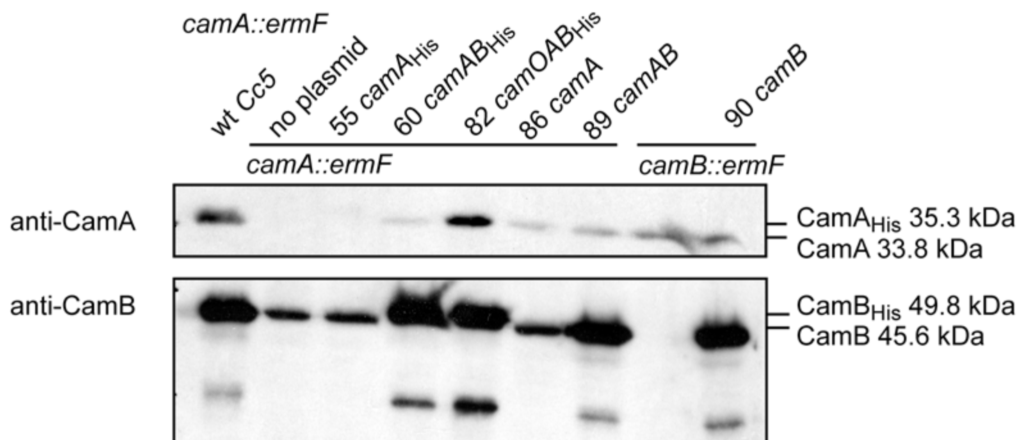


Fig. 24 Expression analysis of CamA and CamB in mutant *camA::ermF*
Immunoblotting directed against CamA or CamB was used to test expression of *camA::ermF* harboring different complementation plasmids.

Taken together, we showed that deletion of *camA* or *camB* results in the same phenotype as mutant Y2F12 which is presumably deficient in CamA.

We next wanted to test if *camO*, the gene upstream from the Tn integration site of mutant Y2F12, plays a role in the active mechanism. Therefore, we mutated *camO* leading to an internal deletion of amino-acids 146- 354 leaving the C-terminus out of frame (Fig. 19C). The deletion of *camO* resulted in a defect of blocking NO release during co-infection (Fig. 21D). If the mutant was complemented *in trans* with pMM82 (*camOAB*$_{\text{His}}$) or pMM103 (*camNOAB*), the phenotype was restored (Fig. 21D). Amounts of CamO in *camO::ermF* harboring pMM82, pMM83 or pMM102 were much lower compared to CamO encoded on pMM103 *in trans* (Fig. 25A), which contains the native promoter instead of the IS-33 promoter (Fig. 19C). CamA and CamB were expressed in *camO::ermF*, however, we can not exclude polar effects (Fig. 25A). A plasmid containing *camNO* under the native promoter should be constructed to clarify this question.

To summarize, CamO could be involved in inhibiting LPS induced NO release of macrophages, but this is not formally shown yet.

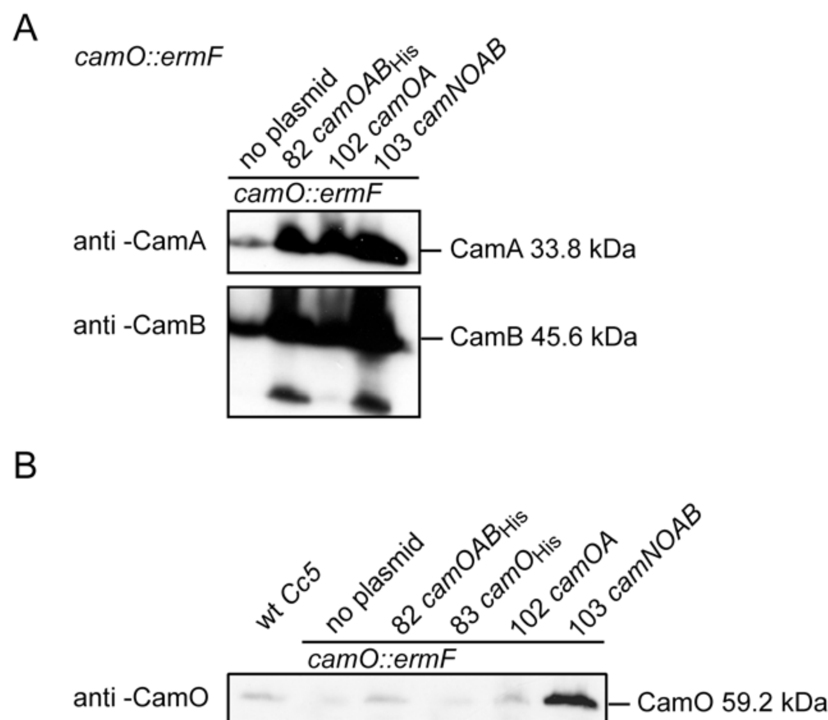


Fig. 25 Expression analysis of CamA, CamB and CamO in mutant *camO::ermF*

Immunoblotting against CamA or CamB (A) or against CamO (B) was used to analyze expression of *camO::ermF* and complemented strains

We therefore summarize that the genes of the locus identified in Tn Y2F12 are organized in an operon and are required for inhibition of NO release during co-infection of macrophages.

Table 7 Summary of the phenotypes from the analysis of locus Y2F12

Designation	Compl. genes	Expression CamA	Expression CamB	block NO release	Surface CamA[a]	Surface CamB[b]
wt *Cc5*	NOAB	Yes	yes	yes	yes	yes
camA::ermF		No	yes	no	no	no
camA::ermF 55	*camA*$_{His}$	No	yes	no	no	no
camA::ermF 60	*camAB*$_{His}$	Low expression	yes	no	no	yes
camA::ermF 82	*camOAB*$_{His}$	Yes	yes	yes	yes	yes
camA::ermF 86	*camA*	Low expression	yes	no	no	no
camA::ermF 89	*camAB*	Low expression	yes	no	nd.	nd.
camA::ermF 102	*camOA*	Yes	yes	no	yes	no
camB::ermF		Yes	no	no	yes	no
camB::ermF 60	*camAB*$_{His}$	Yes	yes	no	yes	yes
camB::ermF 68	*camB*$_{His}$	Yes	yes	no	yes	yes
camB::ermF 82	*camOAB*$_{His}$	Yes	yes	no	yes	yes
camB::ermF 89	*camAB*	Yes	yes	yes	yes	yes
camB::ermF 90	*camB*	Yes	yes	yes	yes	yes
Y2F12	Tn integration	Low expression	yes	no	no	no
Y2F12 55	*camA*$_{His}$	Low expression	yes	no	nd.	nd.
Y2F12 60	*camAB*$_{His}$	Yes	yes	yes	yes	yes
Y2F12 68	*camB*$_{His}$	Low expression	yes	no	nd.	nd.
Y2F12 82	*camOAB*$_{His}$	Yes	yes	yes	yes	yes
Y2F12 83	*camO*$_{His}$	low expression	yes	nd.	nd.	nd.
Y2F12 86	*camA*	Yes	yes	no	no	not clear
Y2F12 89	*camAB*	Yes	yes	yes	yes	yes
Y2F12 90	*camB*	low expression	yes	no (HS)	nd.	nd.
Y2F12 102	*camOA*	Yes	yes	no	yes	no
Y2F12 103	*camNOAB*	Yes	yes	yes	yes	yes
camO::ermF		low expression	yes	no	no	yes
camO::ermF 82	*camOAB*$_{His}$	Yes	yes	nd.	nd.	nd.
camO::ermF 83	*camO*$_{His}$	Yes	yes	nd.	nd.	nd.
camO::ermF 102	*camOA*	Yes	yes	nd.	nd.	nd.
camO::ermF 103	*camNOAB*	Yes	yes	nd.	nd.	nd.

[a, b] tested by indirect immunofluorescence on fixed but unpermeabilized bacteria using anti-CamA ([a]) or anti-CamB ([b]). nd. not determined.

3.4. CamA and CamB are only present in strains *Cc5* and *Cc11* which actively inhibit the onset of pro-inflammatory response

We analyzed the presence of proteins CamA and CamB in all *C. canimorsus* strains by immunoblotting analysis (Fig. 26A and 26B). CamA and CamB were only observed in strains *Cc5* and *Cc11*. However, CamO was present in all strains tested (Fig. 26C). We also tested the presence of the genes *camA*, *camB* and *camA+B* by PCR on genomic DNA of the strains available in the collection (Fig. 26D). In agreement with the presence of proteins, only strains *Cc5* and *Cc11* contained the genes *camA* and *camB*. Notably, only these two strains are able to block pro-inflammatory signals in these two strains, while none of the other strains can (Hwain Shin). All strains except *Cc14* contained *camO* as analyzed by PCR (Fig. 26D).

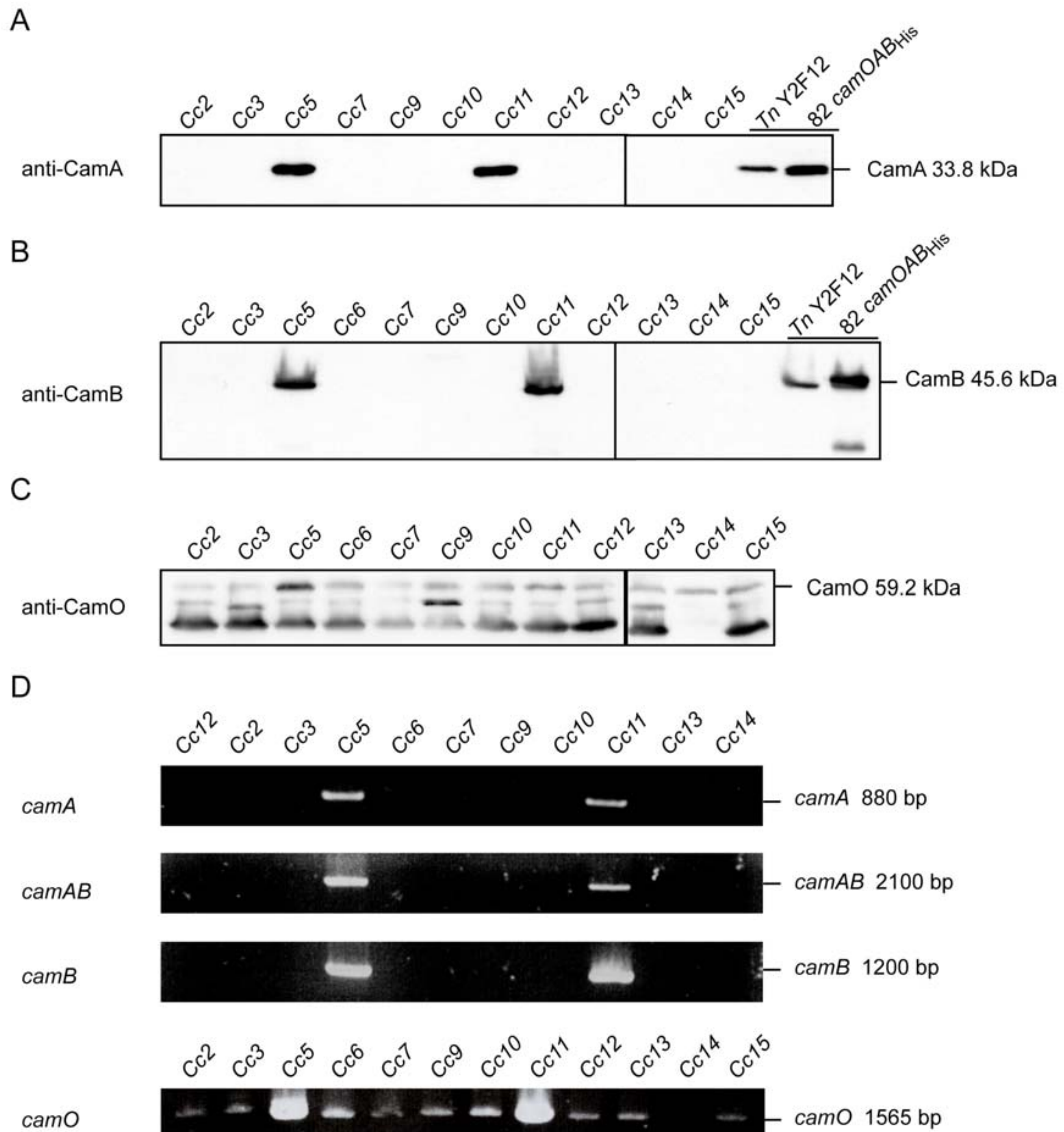


Fig. 26 Detection of genes and gene products of the Y2F12 locus in *C. canimorsus* strains
Immunoblotting against CamA (A) or CamB (B) or against CamO (C) was used to analyze expression of gene products present locus Y2F12 in different *C. canimorsus* strains. (D) PCR amplification of *camA, camAB, camB* or *camO* using primers 4200 + 4201, 4200 + 4254, 4332 + 4254 or 4571 + 4572, respectively.

3.5. Identification of other mutants affected in blocking NO release

Mutant X7B9 that was defective in inhibition of NO and TNF-alpha release was disrupted in a putative glutamine synthetase (Fig. 27A). We tested if we could rescue the mutant by complementation (Fig. 27B). The complementation plasmids contained the gene, which we further called *glnA*, either with its own promoter region (pMM44) or transcribed from the *IS4351* ("IS-33") *ermF* promoter (pMM42). Both fully restored the ability to block NO release during co-infection of J774.1 (Hwain Shin, not shown). We also constructed *glnA* with a C-terminal His tag (pMM74). This construct *in trans* did not complement the mutation of X7B9, even though it was expressed as assessed by immunoblotting against the C-terminal His (Fig. 27D). Using a polyclonal serum directed against GlnA (protein purification was performed by Hwain Shin), a significantly lower amount was detected as compared to *Cc5* wt or X7B9 harboring pMM42 or pMM44 (Fig. 27C). This observation suggests that the amount of GlnA is critical for *C. canimorsus* to inhibit NO release during co-infection. As the *Bacteroides sp.* -33 and -10 promoter consensus was located upstream of *glnA* in pMM42, one can assume equal amounts of transcript in both cases, IS-33 *glnA* (pMM42) and IS-33 $glnA_{His}$ (pMM74). In contrast, immunoblotting against GlnA showed that there is less protein, which might have resulted from instability or reduced expression levels.

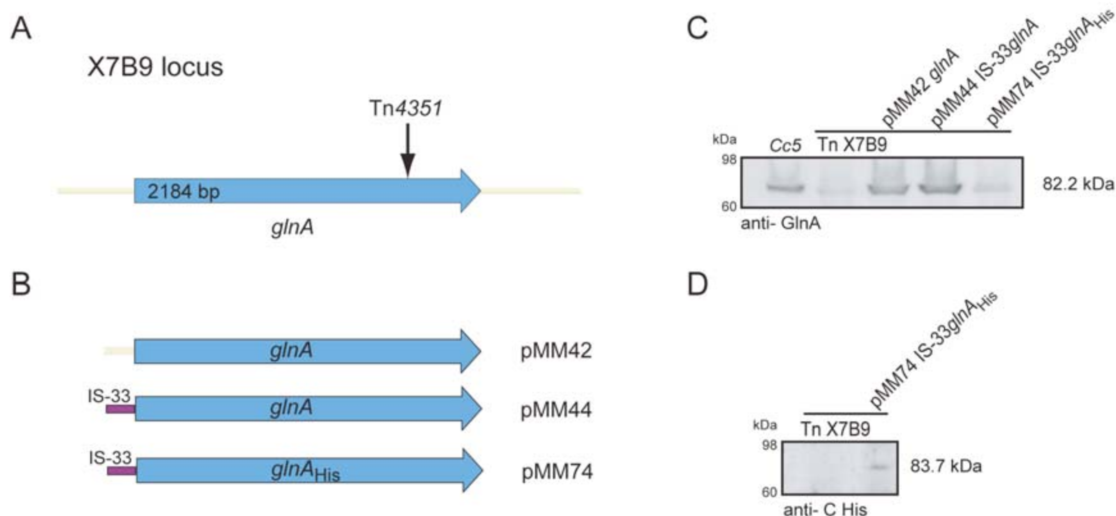


Fig. 27 Identification of the glutamine synthetase gene (*glnA*) disrupted by Tn*4351* in mutant X7B9

Tn integration (A) and gene cloned for complementation plasmids (B) are schematically depicted. Immunoblot analysis of different strains was performed using a polyclonal antiserum generated against GlnA (C) or using anti-C terminal His (D).

Tn integration in mutant Y4B5, localized in a gene "*yfbA*" (=Y4B5) (Fig. 28A), lead to a different phenotype (Fig. 18C). This mutant showed no defect in inhibiting TNF-alpha release. The gene product of *yfbA* showed similarities to a hypothetical conserved protein of a *Flavobacteriales* bacterium (ref|ZP_01107615.1|). When we introduced a plasmid containing *yfbA in trans* (pMM63) (Fig. 28B), we could not complement the mutant Y4B5 (Hwain Shin, data not shown). Expression of the *yfbA* with C-terminal His tag showed a protein with a molecular weight lower than the calculated weight (Fig. 28C). pMM63 was constructed by inserting *yfbA* into the shuttle expression vector which used the *IS4351* ("IS-33") *ermF* promoter, however in pMM71 the native promoter of *yfbA* was used (Fig. 28B). We have not been able to restore the ability to inhibit NO release of Y4B5 using plasmids pMM63 and pMM71 *in trans*. Possible reasons are the C-terminal His tag that influences stability and/or function or polar effects of the Tn integration on expression of the downstream gene *yfbB.* Therefore more plasmids containing the downstream gene need to be constructed and C-terminal modifications should be avoided.

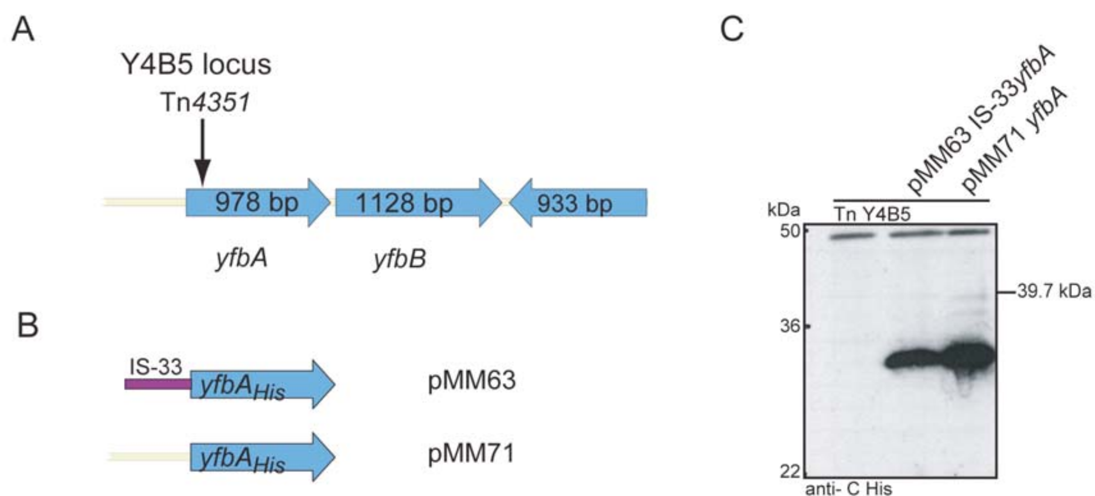


Fig. 28 Identification of the gene (*yfbA*) disrupted by Tn*4351* in mutant Y4B5

Tn integration (A) and gene cloned for complementation plasmids (B) are schematically depicted. (C) Immunoblot analysis of different strains was performed using anti- C terminal His.

Mutant X2E4 was deficient in blocking both, NO and TNF-alpha release upon macrophage co-infection. The mutation in X2E4 consisted of a vector co-integration along with the transposon (Chapter 1, Fig. 2C). It could not be mapped by arbitrarily primed PCR and remains to be identified.

3.6. Methods section

Screening of Tn mutants for defect in anti-inflammatory mechanism

Tn*4351* mutagenesis of *Cc5* was carried out as described (Chapter 1). Obtained Tn mutants were replicated after conjugation on HIA plates containing selective agents 20 µg/ml Gm and 10 µg/ml Em. After one passage, clones were inoculated in HIB + 10% FBS in 96-well plates and incubated at 37°C in humidified atmosphere containing 5% CO2 for 48 h without shaking. J774.1 macrophages were then infected with Tn mutants at a moi adjusted to approximately 20. Co-infection was based on simultaneous addition of either hk *E. coli* or hk *Y. enterocolitica* as a source for stimulatory LPS. After 24 h of infection, cell free supernatants were analyzed using Griess reagent as previously described (Shin *et al.*, 2007).

Plasmids

All plasmids are described in Table 12.

3.7. Discussion

Four mutants of *Cc5* were associated with the inability to block LPS induced NO release of macrophages. The presence of the locus disrupted in mutant Y2F12 was detected only in strains *Cc5* and *Cc11*, hinting an important role during active inhibition of NO release. Interestingly, Blast analysis indicated that there are some homologues in the *Bacteroides* genus which are located in the same operon organization. Notably, either CamB is the protein with the lowest homologies or the homologue of CamB is not present at all (*B. fragilis*).

Despite our extensive genetic characterization of mutants, we are still not able to understand the function of the gene products involved in inhibition of LPS induced NO release of macrophages. The question arises if the inhibition of LPS induced NO release of macrophages by *Cc5* requires direct contact. The cell free supernatant of macrophages that have been infected with *Cc5* should be tested for activity on stimulated macrophages. If the supernatant on fresh but stimulated cells allowed inhibition of the induced pro-inflammatory response measured by NO release, this would indicate a factor secreted by *Cc5* responsible for the observed effect. On the other hand if *Cc5* bacteria need to be in contact with macrophages to block induced NO release, one should try to identify interaction partners from the host, for instance by two hybrid analysis or co-immunoprecipitation. Independent of this, biochemical analysis could be performed to investigate protein-protein interactions between the Cam proteins. Approaches using domain predictions like InterProScan gave only little indication about the function of gene products identified in locus Y2F12. Other algorithms could be applied to extend current predictions. Further experiments could also address the presence of the genes identified in our screen and the gene products in more strains and in the *C. canimorsus* strains isolated during the dog survey (Chapter 4). More genetic analysis could be performed, ideally with a comparison between the clinical isolates of *C. canimorsus* and isolates from healthy dog's saliva using comparative genome analysis. However, the mechanism(s) and bacterial factors involved in inhibition of pro-inflammatory response remain to be identified.

Chapter 4

Prevalence of *C. canimorsus* in dogs in Switzerland

Author contributions. MM and GC designed the experiments, CP and MM performed the characterization. Swabs were isolated by Dr. med. vet. Caroline Saillen-Paroz and Ueli Schmidiger.

Statement of my work. My contribution was the culturing of the isolates, data and sequence analysis, the supervision of CP who sequenced 16S RNA genes and evaluated the primary sequences.

4.1. Introduction

At present, only two studies addressed the prevalence of *C. canimorsus* in dog mouths (Bailie *et al.*, 1978; Westwell *et al.*, 1989). Westwell and colleagues demonstrated in the U.K. that out of 180 dogs, 44 (24%) contained DF-2 (*C. canimorsus*) and 20 (11%) contained DF-2 like (*C. cynodegmi*). *C. canimorsus* was found in 42 (17%) of 249 tested cats, and 19 cats (8%) had *C. cynodegmi.* None of those species were detected in 13 pigs, whereas out of 12 sheep tested, 3 (25%) contained *C. canimorsus*. Furthermore, 5 (33%) *C. canimorsus* was isolated from 15 cattle tested. The identity of suspected isolates was established by sugar fermentation previously used to identify *C. canimorsus* and *C. cynodegmi* (Brenner *et al.*, 1989), including additional rapid enzyme tests (Westwell *et al.*, 1989). According to this study, every fourth dog carries *C. canimorsus* in its oral flora. This number was actually higher than what had been described previously (Bailie *et al.*, 1978). In this earlier study, only 5 *C. canimorsus* strains could be detected in 50 dogs tested. However, since then, identification methods have dramatically improved by introducing 16S rRNA sequence analysis.

4.2. Isolation and identification of *C. canimorsus* and *C. cynodegmi* of Swiss dogs

Oral swabs of 103 dogs were examined during our survey. Isolation was performed using a medium optimal for *C. canimorsus* growth (HIA and 5% sheep blood) containing gentamicin to select for aminoglycoside resistant *Capnocytophaga sp.* Colonies with morphology resembling *Capnocytophaga sp.* were then selected for 16S RNA sequencing.

By using the Vector NTI software package, raw data was aligned using ContigExpress and AlignX and consensus sequence was compared to 16S RNA

genes from either *C. canimorsus* ATCC 35979 (*Cc12)* with its accession number L14637 or *C. cynodegmi* ATCC 49044 using accession number X97245. In cases where neither of those sequences was close, blast algorithms were applied to identify other bacterial species. The Ribosomal Database Project II- Release 9 tool "Sequence match" (http://rdp.cme.msu.edu/) was further used for *C. canimorsus* strains to compare 16S RNA gene sequences to the deposited sequences (Wang *et al.*, 2007).

We could identify 61 *C. canimorsus* isolates from 103 dogs, which represents 59.22 % (Table 8). 62 *C. cynodegmi* strains (60.19 %) were isolated and 33 % of the dogs contained both species in their oral cavity. We observed more dogs from urban area carrying *C. canimorsus* than dogs from a rural environment (39.81% vs. 18.45%). However, this result is not representative as we tested about 2 times more dogs from city areas. The percentage of healthy dogs, which contained *C. canimorsus* in their oral flora was 2.46 fold higher than what was previously reported by Westwell *et al.* This raises the question about the incidence of *C. canimorsus* related infections in Switzerland, where no statistics has been available yet. However, a compulsory registration of dog bites (http://www.bs.ch/mm/2006-05-02-sd-001.htm) could support studies in the future.

Table 8 Summary of canine isolates after dog survey

Description	Number	Percent	
C. canimorsus	61	59.22	%
C. cynodegmi	62	60.19	%
C. canimorsus alone	27	26.21	%
C. cynodegmi alone	28	27.18	%
C. canimorsus and *C. cynodegmi*	34	33.01	%
C. canimorsus from dogs in city	41	39.81	%
C. canimorsus from dogs in country	19	18.45	%
Male dogs	45	43.69	%
C. canimorsus in male dogs	26	57.78	%
Female dogs	56	55.37	%
C. canimorsus in female dogs	33	58.93	%
Dogs with *C. canimorsus, C. cyndegmi* or both	89	86.41	%

4.3. Methods section

Isolation and identification of *C. canimorsus* sampled from dog mouths

Cotton pads were swabbed from dog mouths and subsequently streaked on HIA containing 5% sheep blood with 20 µg/ ml gentamicin and allowed for growth at 37°C in presence of 5% CO_2 for 2 to 3 days. Several single colonies were then passaged once on the same medium. Isolates were frozen for collection linked to the dog number and isolate number. A single colony per isolate was resuspended in 100 µl H_20 and incubated 12- 15 min at 95- 98°C. One µl was then used as a template for PCR using primers 27F (3451) and 1100R (3454) at 0.4 µM concentration, including 200 µM dNTP and 1U Taq polymerase (NEB) in the corresponding buffer conditions (1x). PCR was carried out after denaturation step for 5 cycles (94°C for 30 sec, 60°C- 1.5°C /cycle for 2 min, 72°C for 3 min) followed by 30 cycles (94°C for 30 sec, 52°C 1 min 30 sec, 72°C for 3 min) and final elongation for 10 min at 72°C. The PCR product was loaded on a 1.2% agarose gel and the 1.5 kb band was excised and cleaned by a NucleoSpin® from Machery Nagel. 20- 50 ng DNA was used for sequencing using BigDye Terminator Ready Reaction (PE Biosystems) with primers 27F (3451), 1100R (3454) and 685R (3455) for the ABI sequencer.

The extended Table of the survey is included as appendix (asterisk symbolize sterilized individuals.)

4.4. Appendix

Table 9 *C. canimorsus* isolates from Swiss dogs

Cc Strain	** RDP HIT*	*RDP score*	Dog Nr.	Race
1.4	*Capnocytophaga canimorsus*; 24231; AY643078N.d.	0.833	001	n.d.
3.4	*Capnocytophaga canimorsus*; 24231; AY643077	0.952	003	Belgian German Sheppard
4.3	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.795	004	Coton de Tulear
5.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.95	005	Labrador
6.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.919	006	Hound dog mix
7.4	*Capnocytophaga canimorsus*; 24231; AY643078	0.87	007	Collie mix
10.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.939	010	Bischon frisé
11.3	*Capnocytophaga canimorsus*; 24231; AY643078	0.839	011	Hound dog mix
13.2	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.952	013	Pekinese
15.2	*Capnocytophaga canimorsus*; 24231; AY643078	0.802	015	Yorkshire-Jack Russell
16.1	*Capnocytophaga canimorsus*; ATCC 35979; X97246	0.76	016	Yorkshire-Jack Russell
18.5	*Capnocytophaga canimorsus*; 24231; AY643077	0.933	018	Labrador
19.1	*Capnocytophaga canimorsus*; 24231; AY643078	0.817	019	Shih-Tzu
20.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.963	020	Yorkshire
25.2	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.922	025	Labrador
33.3	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.945	033	Hound dog mix
34.2	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.981	034	Tivet-Terrier mix
35.3	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.95	035	Berger Pyrenée
36.1	*Capnocytophaga canimorsus*; 24231; AY643078	0.848	036	Terrier mix

37.4	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.977	037	Terrier mix
38.2	*Capnocytophaga canimorsus*; 24231; AY643078	0.846	038	Labrador
39.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.94	039	Border Collie
40.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.925	040	Husky
43.1	*Capnocytophaga canimorsus*; 24231; AY643077	0.91	043	Cane corso
44.2	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.93	044	Bong Nhat
46.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.718	046	Carlin
47.2	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.886	047	Border Collie
50.1	*Capnocytophaga canimorsus*; 24231; AY643078	0.801	050	Cavalier King Charles
51.3	*Capnocytophaga canimorsus*; 24231; AY643077	0.907	051	Golden Retriever
52.3	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.84	052	Espagnol tibetain
53.2	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.932	053	Golden Retriever mix
54.2	*Capnocytophaga canimorsus*; 24231; AY643078	0.635	054	Bouvier mix
57.1	*Capnocytophaga canimorsus*; 24231; AY643077	0.856	057	Appenzeller mix
58.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075; and *Capnocytophaga canimorsus*; 24231; AY643077	0.898 both	058	Shetland mini
63.3	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.943	063	Terrier mix
64.1	*Capnocytophaga canimorsus*; 24231; AY643078	0.684	064	Westie
66.2	*Capnocytophaga canimorsus*; 24231; AY643078	0.839	066	Cavalier King Charles
68	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.923	068	Golden Retriever
69.2	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.914	069	German Sheppard
71.2	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.933	071	Labrador mix
73.2	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.914	073	German Sheppard
74.3	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.95	074	Border Terrier
75.1	*Capnocytophaga canimorsus*; 24231; AY643078	0.797	075	German Sheppard
76.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.833	076	French Bulldog
77.3	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.859	077	German Sheppard
79.2	*Capnocytophaga canimorsus*; 24231; AY643077	0.702	079	Spaniel mix
80.2	*Capnocytophaga canimorsus*; 24231; AY643077	0.91	080	Golden Retriever
81.1	*Capnocytophaga canimorsus*; 24231; AY643077	0.894	081	Malinois
82.1	*Capnocytophaga canimorsus*; 24231; AY643078	0.774	082	Pinscher mix
84.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.831	084	BSH
85.1	*Capnocytophaga canimorsus*; 24231; AY643078	0.758	085	Collie mix
88.2	*Capnocytophaga canimorsus*; 24231; AY643078	0.831	088	Jack Russell
89.2	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.956	089	Yorkshire-Malteser
93.1	*Capnocytophaga canimorsus*; 24231; AY643078	0.794	093	Golden Retriever
94.2	*Capnocytophaga canimorsus*; 24231; AY643078	0.81	094	Hawanesian
95.1	*Capnocytophaga canimorsus*; 24231; AY643078	0.83	095	Mittelschnauzer
96.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.947	096	Terrier
97.1	*Capnocytophaga canimorsus*; 24231; AY643078	0.758	097	Terrier
101.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.928	101	English Cocker Spaniel
102.1	*Capnocytophaga canimorsus*; CIP 103936; AY643075	0.887	102	Malamut Husky
103.3	*Capnocytophaga canimorsus*; 24231; AY643078	0.822	103	n.d.

* RDP, Ribosomal Database Project II- Release 9 tool “Sequence match” (http://rdp.cme.msu.edu/)

grey box: presence of CamA and CamB tested b immunoblotting.

Chapter 5

The LPS and/or capsular polysaccharide protects *C. canimorsus* against the bactericidal action of complement

Chapter 5 The LPS and/or capsular polysaccharide protects *C. canimorsus* against the bactericidal action of complement.

Author contributions. HS, MM and CF designed the experiments and CF and HS performed the experiments. UZ and co-workers analyzed LPS, CPS and carbohydrate structures of *Cc5*, Y1C12 and M1C12.

Statement of my work. My contribution was the supervision and analysis of the genetic experiments performed by Chantal Fiechter for her master thesis (Tn mutant library, mapping, sequence analysis, cloning and complementation) and I provided protocols and advice for carbohydrate staining procedures.

5.1. Study of the resistance of *Capnocytophaga canimorsus* to the killing action of complement

We observed that *Cc5* was highly resistant against human complement (Chapter 2). We therefore screened our Tn*4351* mutant library on survival in 10% human serum and twenty serum sensitive mutants could be isolated. Mapping of one highly serum sensitive mutant, called Y1C12, identified a gene with homology to glycosyltransferase (Table 10). The mutation could be complemented by introducing the glycosyltransferase gene *in trans*. Y1C12 showed increased surface deposition of C3b, hinting increased opsonization. In contrast to Δ*siaC* and Y4G6, mutant Y1C12 still recruited fH to its surface as the wt *Cc5*. Addition of Ca^{2+} chelators to inhibit the classical and/or lectin pathway led to a less sensitive phenotype of Y1C12, suggesting that the classical and/or lectin pathway are responsible for complement activation in case of Y1C12. During infections of macrophages, the mutant was readily phagocytosed, which could be inhibited by the addition of cytochalasin D, suggesting that the increased phagocytosis of Y1C12 could be dependent on antibody opsonization. Analysis of the carbohydrate surface structures was performed with an antibody generated against heat-killed (hk) *Cc5* and the result showed an antigenic determinant missing in Y1C12. The antiserum was absorbed against the mutant Y1C12, providing an antiserum that specifically recognized the antigenic determinant present on *Cc5* that was missing in Y1C12. In addition, proteinase K digests of crude extracts were analyzed by different stainings including silver periodic acid staining and confirmed the same determinant missing in Y1C12. To summarize,

these data imply that the antigenic determinant missing in Y1C12 was part of a carbohydrate structure on *C. canimorsus*.

Analysis of carbohydrates from *Cc5* carried out by U. Zähringer demonstrated that wt *Cc5* contained two different LPS molecules, in addition one polymeric glucan in high concentration and one capsular polysaccharide. Biochemical analysis and comparison of LPS derived from *Cc5* and Y1C12 cultivated on blood plates showed that part of the O-antigen was missing in the mutant Y1C12. Indeed, the same carbohydrate structure was also part of the wt *Cc5* extracellular capsular polysaccharide (CPS) and this extracellular carbohydrate structure was missing in mutant Y1C12. Another mutant, designated M1C12, was isolated from the serum sensitivity Tn screen. M1C12 showed the same LPS structure as wt *Cc5*. In contrast to Y1C12, the O-chain of M1C12 was still present but the structural identical capsule was lacking. Interestingly, in this mutant the third carbohydrate glucan found in *Cc5* was also lacking. Increased phagocytosis and complement opsonization presumably resulted from a missing capsular structure and/or altered O-antigen composition. In respect to this, we suggest that the surface carbohydrates are responsible for the very high resistance against human complement.

Taken together, we hypothesize that at least 2 mechanisms contribute to the high resistance of *Cc5* against bactericidal action of complement. fH recruitment to the bacterial surface is one mechanism of *Cc5* to resist complement. In addition, bacterial surface carbohydrates protect *Cc5* from complement mediated killing.

Table 10 Blast result of gene disrupted in Tn mutant Y1C12

ID	Annotation	species	score	E value
Gene product of Y1C12				
ref|YP_099128.1|	putative glycosyltransferase	*Bacteroides fragilis*	382	2e-104
ref|YP_001301973.1|	glycosyltransferase family 4	*Parabacteroides distasonis*	386	3e-100
ref|ZP_01774118.1|	glycosyl transferase, group 1	*Geobacter bemidjiensis*	359	2e-97
ref|YP_001353950.1|	glycosyltransferase	*Janthinobacterium sp.*	320	2e-85
ref|ZP_02165391.1|	glycosyltransferase, group 1	*Hoeflea phototrophica*	314	6e-84
ref|ZP_01713950.1|	glycosyl transferase, group 1	*Pseudomonas putida*	307	7e-82

Appendix

Strains and Plasmids

Table 11 Strains

Bacterial Strains	Genotype or Description	Reference or Source
E. coli		
BW19851	S17-1 derivative, *pir*+ RP4-2-*tet*:Mu-1*kan*::Tn*7* (Sm^r)*recA1 creC510 hsdR17 endA1 zbf-5 uidA*(D*Mlu*I):*pir*/ *thi*	ATCC 47083 (Metcalf *et al.*, 1996)
β2155	*thrB1004 pro thi strA hsdS lacZ*D*M15* (F9 *lacZ*D*M15 lacI*q *traD36 proA*1 *proB*1) D*dapA*::*erm* (Em^r) *pir*::RP4 [::*kan* (Km^r) from SM10]	(Dehio and Meyer, 1997)
S17-1	*hsdR17 recA* RP4-2-*tet*::Mu-1*kan*::Tn7 (Sm^r)	(Simon *et al.*, 1983)
SM10λ*pir*	*thi-1, thr, leu, tonA, lacY, supE, recA::*RP4-2-*tet::Mu1 kan* (Km^r)	(Miller and Mekalanos, 1988)
Top10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*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		
C. canimorsus 2	Human fatal septicemia after dog bite 1989.	(Hantson *et al.*, 1991)
C. canimorsus 3	Human septicemia 1990.	(Vanhonsebrouck *et al.*, 1991)
C. canimorsus 5	Human fatal septicemia after dog bite1995.	(Shin *et al.*, 2007)
C. canimorsus 6	Human isolate 1996	KUL Leuven
C. canimorsus 7	Human septicemia 1998.	(Shin *et al.*, 2007)
C. canimorsus 9	Human septicemia 1965	(Vandamme *et al.*, 1996)
C. canimorsus 10	Human septicemia after dog bite	(Vandamme *et al.*, 1996)
C. canimorsus 11	Human septicemia (BCCM/LMG 11551 MCCM 01373)	A. von Graevenitz, Univ. Zürich, Switzerland
C. canimorsus 12	Human septicemia after dog bite	ATCC 35979, CDC 7120
C. canimorsus 13	Isolate from healthy dog's saliva (Jackie) 2005	(Shin *et al.*, 2007)
C. canimorsus 14	Isolate from healthy dog's saliva (Pouchka) 2005	(Shin *et al.*, 2007)
C. cynodegmi		
C. cynodegmi	Isolate of a dog's mouth; USA, Virginia 1979	ATCC 49044
C. cynodegmi 2	Human hand wound (LMG 11538; BCCM/LMG; CCUG30624; CDC E679; MCCM 00262)	(Vandamme *et al.*, 1996)
C. gingivalis		
C. gingivalis	Human isolate	B. Wauters, Belgium
C. ochracea		
C. ochracea	Human isolate	B. Wauters, Belgium

Table 12 Plasmids

Plasmids	Description[a]	Reference or Source
Plasmids		
pBBR1MCS3	Broad host range *ori* from *Bordetella bronchiseptica* S87, Tc^r	(Kovach *et al.*, 1995)
pBBR1MCS4	Broad host range *ori* from *Bordetella bronchiseptica* S87, Ap^r	(Kovach *et al.*, 1995)
pBSIIKS (+)	ColE1 *ori*, Ap^r	Stratagene
pCC7	Endogenous plasmid of *Cc7*	This study
pCP23	ColE1 *ori*; (pCP1 *ori*); Ap^r (Tc^r); *E.coli-F.johnsoniae* shuttle plasmid	(Agarwal *et al.*, 1997)
pCP29	ColE1 *ori* (pCP1 *ori*); Ap^r (Cf^r Em^r); *E.coli-F.johnsoniae* shuttle plasmid	(Kempf and McBride, 2000)
pEP4351	*pir* requiring R6K *oriV*; RP4 *oriT*; Cm^r Tc^r (Em^r); vector used for Tn*4351* mutagenesis	(Cooper *et al.*, 1997)
pK18	ColE1 *ori*, Km^r	(Pridmore, 1987)
pLYL001	ColE1 *ori*; Ap^r (Tc^r); not replicating in *Bacteroides sp.*, *Flavobacterium sp.* and *Capnocytophaga sp.*	(Reeves *et al.*, 1996)
pLYL03	ColE1 *ori*; Ap^r (Em^r); not replicating in *Bacteroides sp.*, *Flavobacterium sp.* and *Capnocytophaga sp.*	(Li *et al.*, 1995)
pMMB206	Cm^r Tra− *mob*+ *ori*RSF1010 (IncQ), p_{tac}-$_{lac}$, $lacI^Q$	(Morales *et al.*, 1991)
pMR20	Tc^r derivative of pGLIO, RK2 based broad host-range vector (IncP)	(Jenal and Shapiro, 1996) (Chris Mohr and Rick Roberts)
pUC19	ColE1 *ori*, Ap^r	(Yanisch-Perron *et al.*, 1985)
Chapter 1		
pMM2	pBBR *ori*; Ap^r; Random 650-bp *Sau*3A chromosomal fragment of *Cc5* inserted in *Bam*HI site of pBBR1MCS4	This study, 1
pMM3	pBBR *ori*; Ap^r; Random 500-bp *Sau*3A chromosomal fragment of *Cc5* inserted in *Bam*HI site of pBBR1MCS4	This study, 1
pMM5	pBBR *ori* ; Ap^r, (Em^r); *ermF* from pEP4351 amplified by PCR using primers 3505 and 3506 cut with *Eco*RI/*Pst*I and inserted into the corresponding sites of pBBR1MCS4	This study, 1
pMM7	ColE1 *ori*; Ap^r; 1.95-kb *Eco*RI/*Hind*III fragment of pCC7 inserted into corresponding sites of pBSIIKS(+)	This study, 1
pMM12	ColE1 *ori*; Km^r (Cf^r); *cfxA* gene from pCP29 cloned as a *Bam*HI/*Spe*I fragment into corresponding sites of pK18	This study, 1
pMM13	ColE1 *ori*; Ap^r (Em^r); *ermF* from pEP4351 amplified by PCR as a 1.95-kb fragment using primers 3505 and 3506, cut with *Eco*RI/*Pst*I and inserted into the corresponding sites of pBSIIKS(+)	This study, 1
pMM25	ColE1 *ori*; Km^r (Cf^r); Suicide vector for *C. canimorsus.* RP4 *oriT* amplified by PCR using primers 4416 and 4417 inserted into *Bam*HI site of pMM12.	This study, 1
pMM40.A	ColE1 *ori* (pCC7 *ori*); Km^r (Cf^r); *E. coli - C. canimorsus* shuttle plasmid. The *repA* gene from pCC7 was amplified by PCR using primers 3601 + 4274, digested with *Pst*I and inserted into the corresponding site of pMM12.	This study, 1
pMM41.A	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); *E. coli - C. canimorsus* shuttle plasmid. The *cfxA* and *repA* genes as a *Bam*HI/*Sph*I fragment from pMM40.A inserted into corresponding sites of pUC19.	This study, 1
pMM45.A	ColE1 *ori* (pCC7 *ori*); Km^r (Cf^r); *E. coli - C. canimorsus* shuttle plasmid, RP4 *oriT.* The 1.58-kb *Pst*I fragment of pMM47.A containing *repA* inserted into *Pst*I site of pMM25.	This study, 1

pMM47.A	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); *E. coli - C. canimorsus* expression shuttle plasmid. -33 and -7 of the *ermF* promoter was amplified from pEP4351 as a 257-bp fragment by PCR using 3868 and 4128. Unique *Nco*I, *Xho*I, *Xba*I sites and 6 histidine codons were incorporated by reverse primer 4128. *Sal*I/*Spe*I digested PCR fragment inserted into corresponding sites of pMM41.A.	This study, 1
pMM104.A	ColE1 *ori* (pCC7 *ori*); Ap^r (Tc^r); *E. coli - C. canimorsus* shuttle plasmid, RP4 *oriT*. *Pst*I fragment of pMM47.A containing *repA* inserted into *Pst*I site of pLYL001.	This study, 1
pMM105.A	ColE1 *ori* (pCC7 *ori*); Ap^r (Em^r); *E. coli - C. canimorsus* shuttle plasmid, RP4 *oriT*. *Pst*I fragment of pMM47.A containing *repA* inserted into *Pst*I site of pLYL03.	This study, 1
pMM106	ColE1 *ori*; Km^r (Cf^r); To create *siaC::ermF* three initial PCR products were amplified with 4783 + 4784 and 4787+ 4788 from *Cc5* chromosomal DNA and 4785 + 4786 from pEP4351. *siaC::ermF* was then amplified by overlapping PCR using external primers 4783 and 4788, cut with *Pst*I/*Spe*I and inserted into corresponding sites of pMM25.	This study, 1
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pMM16	ColE1 *ori*, Ap^r; *IS4351* cut with *Xma*I/*Nco*I and inserted in front of *egfp* into the corresponding sites of pEGFP using 3755 + 3756	This study, 1
pMM19.a	ColE1 *ori*, (pCC7 *ori*); Km^r (Cf^r); First shuttle vector based on pMM12 (Cf^r), replicating in *C. canimorsus* but has a frame shift in *repA;* amplified with primers 3640 + 3601, cut with *Pst*I and inserted into the corresponding sites of pMM12, resulting in truncated RepA	This study, 1
pMM21	ColE1 *ori*, (pCC7 *ori*); Km^r (Cf^r); "IS-33*egfp"*: *egfp* with IS-33 promoter of pMM16 inserted into shuttle pMM19.a using *Sal*I/*Spe*I restriction	This study, 1
pMM22	ColE1 *ori*, Ap^r; IS-33 promoter amplification using 3851 + 3756, cut with *Xba*I/*Nco*I an insertion into the corresponding sites upstream of gfp_{mut2} in pGS-GFP3	This study, 1
pMM23	ColE1 *ori*, (pCC7 *ori*); Km^r (Cf^r); "IS-33gfp_{mut2}": Fragment IS-33gfp_{mut2} from pMM22 was amplified by 3868 + 3869, cut with *Sal*I/*Spe*I and inserted into the corresponding sites of shuttle pMM19.a	This study, 1
pMM24	ColE1 *ori*, Ap^r; Amplification of *luxAB* by 3872 an 3873 from pHSK728 inserted into pMM13 downstream *ermF*	This study, 1
pMM26	ColE1 *ori*, Ap^r; "$luxA_{Ala2}B$": Amplification of *luxAB* by 3875 + 3873 from pHSK728 inserted downstream of the IS-33 promoter in pMM16	This study, 1
pMM27	ColE1 *ori*, (pCC7 *ori*); Km^r (Cf^r); *Sal*I/*Spe*I IS-33*ermF* $luxA_{Ala2}B$ fragment of pMM24 inserted into the corresponding sites of shuttle pMM19.a	This study, 1
pMM28	ColE1 *ori*, (pCC7 *ori*); Km^r (Cf^r); IS-33*luxAB* cloned by overlapping PCR with primers 3868 + 3952 on products 3868 + 4041 and 4042 + 3952; inserted by *Sal*I/*Spe*I into shuttle pMM19.a	This study, 1
pMM29	ColE1 *ori*, (pCC7 *ori*); Km^r (Cf^r); *Sal*I/*Spe*I IS-33$luxA_{Ala2}B$ fragment of pMM26 inserted into the corresponding sites of shuttle pMM19.a	This study, 1
pMM32	ColE1 *ori*, (pCC7 *ori*); Km^r (Cf^r); "IS-33 *ermF-luxA luxB":* fusion of *ermF* and *luxAB* cloned by overlapping PCR (3868 + 3909; 3910 + 3873) and introduced into pMM19.a using *Sal*I/*Spe*I restriction	This study, 1
pMM33	ColE1 *ori*, (pCC7 *ori*); Km^r (Cf^r); "IS-33gfp_{mut2}-*luxAB*": gfp_{mut2}-*luxAB* fusion cloned by overlapping PCR (3868 + 3911; 3912 + 3873) into shuttle pMM19.a using *Sal*I/*Spe*I restriction	This study, 1
pMM34	ColE1 *ori*, (pCC7 *ori*); Km^r (Cf^r); "IS-33*egfp-luxAB*": *egfp-luxAB* fusion amplified by overlapping PCR (3950 + 3952; 3951 + 3952) digested with *Sal*I/*Spe*I and inserted into the corresponding sites of shuttle pMM19.a	This study, 1

pMM36	ColE1 *ori*, (pCC7 *ori*); Km^r (Cf^r); "IS-33*cat*": amplified by overlapping PCR (3868 + 3970; 3971 + 3972), cut with *Sal*I/*Spe*I and introduced in the corresponding sites of shuttle pMM19.a	This study, 1
Chapter 2		
pMM46	ColE1 *ori* (pCC7 *ori*); Km^r (Cf^r); 4078 + 4052 amplified fragment cut with *Sal*I and inserted into the corresponding sites of pMM19.a; contains the putative transcription regulator and the N- acyl glucosamine epimerase upstream of *siaC*	This study, 2
pMM50	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); $siaC_{\Delta 1\text{-}21}$ was amplified using primers 4156 + 4158, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A, deleting the first 63 bp of *siaC*, but including codons for methionine and glycine at position 1 and 2, in frame with a C-terminal His tag.	This study, 2
pMM52	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); $siaC_{FL}$ was amplified with primers 4159 + 4158 and cut with *Nco*I/*Xba*I and inserted into the corresponding sites of shuttle pMM47.A, leading to the insertion of a glycine at position 2 and a C- terminal His tag ($siaC_{FL}$)	This study, 2
pMM59	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); The catalytic mutation in *siaC* of was introduced by site directed mutagenesis with an inverse PCR on pMM52, using primers 4171 + 4172 ($siaC_{Y488C}$)	This study, 2
pMM56	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); Y4G6 *yfgA* amplified with 4202 + 4203, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A	This study, 2
pMM57	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); Y4G6 *yfgA* amplified with 4202 + 4204, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A in frame with a C-terminal His, not expressed.	This study, 2
pMM76	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); Y4G6 *yfgAB* amplified with 4202 + 4438, digested with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A in frame with a C-terminal His in *yfgB*	This study, 2
pMMP98	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); Y4G6 *ompYyfgA* amplified with 4396 + 4203, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A	This study, 2
pMMP99	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); Y4G6 *ompYyfgA* amplified with 4396 + 4202, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A in frame with a C-terminal His	This study, 2
pMMP100	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); Y4G6 *ompYyfgAB* amplified with 4396 + 4438, digested with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A in frame with a C-terminal His on YfgB	This study, 2
Chapter 3		
Y2F12		
pMM55	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); *camA* amplified with primers 4200 + 4201, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A in frame with C-terminal His.	This study, 3
pMM60	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); *camA camB* amplified with primers 4200 + 4254, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A in frame with C-terminal His in *camB*	This study, 3
pMM67	ColE1 *ori*; Ap^r; $camB_{\Delta 1\text{-}25}$ amplified with 4333 + 4334 and inserted into pET22b+ using *Nde*I/*Xho*I restriction; for expression of $CamB_{\Delta 1\text{-}25\ His}$	This study, 3
pMM68	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); *camB* amplified with primers 4339 + 4254, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A in frame with C-terminal His.	This study, 3

pMM81	ColE1 *ori*; Km^{r} (Cf^{r}, Em^{r}); To create *camB::ermF* three initial PCR products were amplified with 4551 + 4552 and 4555 + 4556 from *Cc5* chromosomal DNA and 4553 + 4554 from pEP4351. *camB::ermF* was then amplified by overlapping PCR using external primers 4551+ 4556, cut with *Pst*I/*Spe*I and inserted into corresponding sites of pMM25. Leading to deletion of amino-acids 107-237.	This study, 3
pMM82	ColE1 *ori* (pCC7 *ori*); Ap^{r} (Cf^{r}); *camO camA camB* amplified with primers 4571 + 4254, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A in frame with C-terminal His on *camB*	This study, 3
pMM83	ColE1 *ori* (pCC7 *ori*); Ap^{r} (Cf^{r}); *camO* amplified with primers 4571 + 4572, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A in frame with C-terminal His	This study, 3
pMM86	ColE1 *ori* (pCC7 *ori*); Ap^{r} (Cf^{r}); *camA* amplified with primers 4200 + 4661, digested with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A	This study, 3
pMM89	ColE1 *ori* (pCC7 *ori*); Ap^{r} (Cf^{r}); *camO camA camB* amplified with primers 4200 + 4662 cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A	This study, 3
pMM90	ColE1 *ori* (pCC7 *ori*); Ap^{r} (Cf^{r}); *camB* amplified with 4339 +4662, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A	This study, 3
pMM93	ColE1 *ori*; Km^{r} (Cf^{r}, Em^{r}); To create *camA::ermF* three initial PCR products were amplified with 4664 + 4665 and 4668 + 4669 from *Cc5* chromosomal DNA and 4666 + 4667 from pEP4351. *camA::ermF* was then amplified by overlapping PCR using external primers 4664 + 4669, cut with *Pst*I/*Spe*I and inserted into corresponding sites of pMM25. Leading to complete deletion of *camA*	This study, 3
pMMP94	ColE1 *ori*; Km^{r} (Cf^{r}, Em^{r}); To create *camO::ermF* three initial PCR products were amplified with 4731 + 4732 and 4735 + 4736 from *Cc5* chromosomal DNA and 4733 + 4734 from pEP4351. *camO::ermF* was then amplified by overlapping PCR using external primers 4731+ 4736, cut with *Pst*I/*Spe*I and inserted into corresponding sites of pMM25. Leading to deletion of amino-acids 146-354.	This study, 3
pMMP102	ColE1 *ori* (pCC7 *ori*); Ap^{r} (Cf^{r}); *camO camA* amplified with 4361 + 4661, cut with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47.A	This study, 3
pMMP103	ColE1 *ori* (pCC7 *ori*); Ap^{r} (Cf^{r}); *camN camO camA camB* amplified with 4487 + 4662, cut with *Sal*I/*Xba*I and inserted into the corresponding sites of pMM47.A. Contains native promoter upstream *camN*	This study, 3
pMM107	ColE1 *ori*, Ap^{r}; Y2F12 *camO* delta 1-25 amplified with 4790 + 4791, cut with *Nde*I/*Xho*I and inserted into the corresponding sites of pET22b+	This study, 3
X7B9		
pMM39	ColE1 *ori*, Ap^{r}; *glnA* amplified with 4053 + 4054, cut with *Eco*RI and inserted into the corresponding sites of pUC19	This study, 3
pMM42	ColE1 *ori*, (pCC7 *ori*); Km^{r} (Cf^{r}); IS-33 *glnA* amplified by overlapping PCR (3868 + 4063; 4064 + 4065), digested with *Sal*I/*Spe*I and inserted into the corresponding sites of pMM19.a	This study, 3
pMM44	ColE1 *ori*, (pCC7 *ori*); Ap^{r} (Cf^{r}); *cfxA* and *repA* containing *Bam*HI/*Sph*I fragment from pMM19.a and inserted into the corresponding sites of pMM39	This study, 3
pMM74	ColE1 *ori*, (pCC7 *ori*); Ap^{r} (Cf^{r}); *glnA* amplified by 4444 + 4445, cut with *NcoI*/*Xba*I and inserted into the corresponding sites of pMM47.A, including a C-terminal His tag	This study, 3
Y4B5		
pMM63	ColE1 *ori* (pCC7 *ori*); Ap^{r} (Cf^{r}); Y4B5 *yfbA* amplified with 4272 + 4273, digested with *Nco*I/*Xba*I and inserted into the corresponding sites of pMM47, including a C-terminal His tag	This study, 3

pMM71	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); Y4B5 *yfbA* amplified with 4154 + 4155 containing its native promoter, cut with *Sal*I/*Spe*I and inserted into the corresponding sites of pMM47.A, leading to a C-terminal His tag.	This study, 3

[a] Antibiotic resistance phenotypes: ampicillin, Ap^r; cefoxitin, Cf^r; chloramphenicol, Cm^r; erythromycin, Em^r; gentamicin, Gm^r; kanamycin, Km^r; streptomycin, Sm^r; tetracycline, Tc^r. Antibiotic resistance phenotypes and other features listed in parentheses are those expressed by *Capnocytophaga sp.* but not by *E. coli.*

Oligonucleotides

Table 13 Oligonucleotides

Number	Sequence
3402	AATTAACCCTCACTAAAGGG
3288	TAATACGACTCACTATAGGG
3574	TTCAAATCTCTTAAAACCCCAG
3575	TCTAAGGCGAATAGGGAATATC
3576	CACTGGATATACCACCG
3577	TGCCACTCATCGCAGTA
3578	GCTCTAGAGCCGCACCCAAAAAG
3579	CAGAATTCTGTTGCATTTGCAAGTTG
3580	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
3581	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
3582	GGCCACGCGTCGACTAGTAC
3601	TTTTCTGCAGGTTAAAATCGGCCGCC
3623	ATGTAGATATACAAATGCCTG
3625	ACCCACCATTTCCTTTCCCTAAC
3626	CAGCCACTTCCTTGAAGAAATG
3639	GAAGTATTTTTGTTCGATACCAAGG
3640	TTTCTGCAGGACATAGAAATTCAGGAGTG
3641	TAATACTGGCATCGACCTTTACGCC
3675	CATTTCGGTTACATCCCATAATAGC
3676	AATTTCTAATGTCAAGGAAAAACCG
3677	TTACCTTCTTGTTGGTTTTAACTGC
3678	TTTATCGTGCACAGGTCTCATTAG
3755	ATAACCCGGGAACTTGCAAATGCAACA
3756	GGTGCCATGGGTAACTTCTTACAGGTG
3815	TGCTTATTATCCGCACCCAA
3818	GTTTTCCCAGTCACGAC
3819	CAGGAAACAGCTATGAC
3851	GCTCTAGAGCTCATCGGTATTTGCAACA
3868	TCATGTCGACGCTCATCGGTATTTGCAACA
3869	GACTAGTAGGGTTTTCCCAGTCACGACGTT
3872	AACTGCAGAAGAAGTAGAGTATGAAGTTTGGA
3873	GGACTAGTTTGCCTTTAATTTTATTATGGTA
3875	AGGTCCATGGCTAAGTTTGGAAATATTTG
3909	ATATTTCCAAACTTAGCCATCGAAGGATGAAAT
3910	CTGAAAAATTTCATCCTTCGATGGCTAAGTTTG
3911	TATTTCCAAACTTAGCCATTTTGTATAGTTCA
3912	GCATGGATGAACTATACAAAATGGCTAAGTTTG
3913	GTAAATGCGACCAATATCTTTAATG
3914	TTGGATAATATGGTAAAGACTGTC
3915	AAGTGGGAGGATAATTTAGAAACC
3950	CTGAAAAATTTCATCCTTCGATGAAGTTTGGAAATAT
3951	GCATGGATGAACTATACAAAATGAAGTTTGGAAATAT
3952	GGACTAGTTTATGGTAAATTCATTTCGATTTTTTGGTTC
3953	ATATTTGTTTTTCGTATCAAC
3954	ATTAGCTCTGATAGTGATTAC
3970	CCAGTGATTTTTTTCTCCATTAGTAACTTCTTAC
3971	TCACCTGTAAGAAGTTACTAATGGAGAAAAAAATC
3972	ATACTAGTTTACGCCCCGCCCTGCC
4010	TCACACATTATGCACCAAAC
4011	TTGGATAAAGGATTTGTAAG

4012	AATTGTTGTAACGATTGTCG
4013	GCGAAGCGTTATCCCAAAGC
4029	TACCATTATGGCAAAACAAC
4030	AACCGAACCTCATCTTTCGC
4041	ATATTTCCAAACTTCATTAGTAACTTCTTAC
4042	ATTCACCTGTAAGAAGTTACTAATGAAGTTTGG
4047	TTGTTGCGCTTGTTGAAATTTTC
4048	CCAAATATTACCAATATAACAAC
4049	CGCCCATCAGTAGACATTATAG
4050	ATTGAACCGTTACAGCAGAAG
4051	GATAGGGTTTCAACTCCATTGG
4052	GGCGGTCGACGATTGGTTTAGTTCTTG
4053	CAAGGAATTCTGCAAAATCTGATTTAGTAG
4054	CGGCGAATTCTATATTACATATGAAATGC
4063	TGAAATCTTAAAGTGGGCATTAGTAACTTCTTAC
4064	ACCTGTAAGAAGTTACTAATGCCCACTTTAAG
4065	GCACTAGTATATGAAATGCTTTTTAGTGTG
4075	AATACCAGTTGAGGAATTTATC
4076	GTTCAATGGAACAAGAAGCA
4078	AACGGTCGACGCTCAAAAACACTCCCTAAA
4128	TTACTAGTTCAATGATGATGATGATGATGCTCGAGTTCTAGAGCCATGGGG
4130	GGGTAACAACAAAAACCACTG
4132	TATAAGAATAATTGGTGGGC
4133	TCTCTGCCTAATGAGAATAAC
4151	CCCCACAGGAAGTAATAAAC
4152	ATCAATAATATAGGCGTACC
4153	GCAACTTAGCGTTAGTATAG
4154	AACGGTCGACGGTATATTTGGCATTTGCG
4155	CGACTAGTGTATTTCTTTGACGGTCAACATC
4156	AAAGCCATGGGAAACGTAATCGGCGGAGGCG
4157	GTTCTAGAGTTAGTTCTTGATAAATTCCTCAACTG
4158	GTTCTAGAGAGTTCTTGATAAATTCCTCAACTG
4159	CATACCATGGGAAATCGAATTTTTTATCTT
4171	GAAGGATTTGGGTGTTCGTGTATGTCG
4172	CGACATACACGAACACCCAAATCCTTC
4200	CATGCCATGGTGATGAAAAAATTTAG
4201	GTTCTAGATTGTTAATGTTATCTAAATCTAC
4202	CGTCCCATGGTGAAAAAATACTTTATG
4203	CGTCTAGATTATCTTGTATTAGGATTCAC
4254	GTTCTAGATTTTTGTTGAGAATAATCC
4272	ATCCATGGGAGTGTTATATTTGCCAAAGGC
4273	AGTCTAGAAATTTTTTCTCTTGAGGAAGGAATTTG
4274	ATGGCTGCAGAGTTCCTACGATTGCCATA
4275	ATTCTATAAAGTTAAGAATG
4276	TCAGGGTACATCAATAATAC
4277	ATCAAACGGGTCACGATACG
4278	AAATGAGAGAAGTAATGGAG
4279	TTGAAGGTAAAACAACAGAG
4280	ATGGAAATTGATGGAGCAGA
4332	GGGAATTGCATATGAAAAAAATAAAACAAC
4333	GGGAATTGCATATGGACAAAGAGTCCGTTTTTG
4334	CCGCTCGAGTTTGTTGAGAATAATCCAAAAATC
4339	CATACCATGGGAAAAAAAAATAAAACAACTAATAG
4361	CATACCATGGGAAAACATAAAATTTTAACATA
4362	ACTCTAGAACGGATTATTTGTTGAGAATAATC
4363	GTTCTAGATTTTTTGAATCCAATTCTTTTC
4364	GTTCTAGATTTTACCCCCTCTTACAAGTTTG
4395	AGAAAGGTACTTCGCCCAAAG
4396	CATACCATGGGAATAGATTTGCTAAGTAGATC
4416	CCGGATCCCTTGGTTTCATCAGCCATC
4417	GCGGATCCATCAGTAATTTCCTGCATTTG
4436	CTCCGGCTTGGCATAGGGGT
4437	CTTGAAAACGAACATCTACC
4438	TGTCTAGAGCTTTTTTAGGTAATCTGATAA
4439	TGTCTAGAGCAAATTCTACTTTGGTATTAA
4440	TGTCTAGAGCCAAGTTTGGTTCAGAGAAAG
4444	CATACCATGGGACCCACTTTAAGATTTCAC
4445	TGTCTAGAGCGTGTGTAAAAAGCATTTC
4472	CCTGCATACACATTCACAATA
4473	GATGATCTATATTGGAGTTT
4474	TACCTGGTGCTCTTTGTTTTC
4475	TGGGTCCACCAAGGCACTAA
4484	GTCGCGTTAGCAAAGAATGC
4485	ATCGGCGCTTATTTACAGGA
4486	AACTATTCCAACGAAACGAC

4487 ACTTGTCGACGATAATATAACTTTGCTGC
4546 TATTGTGAATGTGTATGCAGG
4547 CGACCTAATTTATTCGTTC
4548 GTAGTACAAACTGTTGTCTT
4549 AGCAATTTTTTGGTTATTGA
4550 ATCAAAACCATTATGGGTTG
4551 ATCTGCAGTCACACATTATGCACCAAAC
4552 GAGTAGATAAAAGCACTGTT/AAGAGTAGGACCTCCGTTTG
4553 GATATTATTCCAAACGGAGGTCCTACTCTT/AACAGTGCTTTTATCTACTCCGATAGCTTC
4554 AGGTTCCTTCACTGGTATTTAAAACCATCT/CTACGAAGGATGAAATTTTTCAGGGACAAC
4555 AAAAATTTCATCCTTCGTAG/AGATGGTTTTAAATACCAGT
4556 CCACTAGTAGAATAATCCAAAAATCTGCATCG
4571 CATACCATGGGAAAACATAAAATTTTA
4572 GATCTAGATATCTTGGGTTAGGGGTTA
4573 TTGGATACCTCACGCCAAAC
4574 GGACATTGTCTCTCTTTCC
4661 GGTCTAGAGCCACCAATACGCCTATTAG
4662 GGTCTAGAAAGTGTAAATGATGTTATCTTC
4664 CCCTGCAGATATAGTAGCTCAAATTGGTTAC
4665 GAGTAGATAAAAGCACTGTT/CTATCTTGGGTTAGGGGTTA
4666 AAAGCAGGTTTAACCCCTAACCCAAGATAG/AACAGTGCTTTTATCTACTCCGATAGCTTC
4667 GTTTTATTTTTTTCATAGGTATTTTAGTTA/CTACGAAGGATGAAATTTTTCAGGGACAAC
4668 AAAAATTTCATCCTTCGTAG/TAACTAAAATACCTATGAAA
4669 CCACTAGTAAGTAATTGGCATTTTGGAGTTTTAC
4730 GGCACGTTCCAGTTCTTTCAG
4731 CCCTGCAGAAACTCAGGAGGAGTAGCCGTAC
4732 GAGTAGATAAAAGCACTGTT /TGTACGCTCGCGCTACCAAG
4733 GAGGTGAAGGCTTGGTAGCGCGAGCGTACA/ AACAGTGCTTTTATCTACTCCGATAGCTTC
4734 TGTCGGTAACCAATTTGAGCTACTATATCA/CTACGAAGGATGAAATTTTTCAGGGACAAC
4735 AAAAATTTCATCCTTCGTAG/TGATATAGTAGCTCAAATTG
4736 CCACTAGTTCTTGGGTTAGGGGTTAAACCTGC
4783 CCCTGCAGATTTGTCGGCTTGTGGAAGCC
4784 GAGTAGATAAAAGCACTGTT / GTGCTTCGACTCATTCCTAC
4785 AGATGTAAACGTAGGAATGAGTCGAAGCAC/AACAGTGCTTTTATCTACTCCGATAGCTTC
4786 AGCTCCCGTTCCACAATGCCACGTTTTTCC /CTACGAAGGATGAAATTTTTCAGGGACAAC
4787 AAAAATTTCATCCTTCGTAG /GGAAAAACGTGGCATTGTGG
4788 CCACTAGTTTAGTTCTTGATAAATTCCTCAACTGG
4789 GGGAATTGCATATGAAACATAAAATTTTAACATATAGT
4790 GGGAATTGCATATGGATGAATTACCTGACAACCGC
4791 CCGCTCGAGTCTTGGGTTAGGGGTTAAACCTG
4856 CAGCGGTGGCAGCAGCCAAC

Antisera

Table 14 Polyclonal antisera

MIPA Number	Name	Antigen	Source	
226	Anti-SiaC	SiaCΔ1-21 (58 kDa)	Rabbit	Laboratoire d'Hormonologie, Marloie, Belgium
228	Anti-GlnA	GlnA (82 kDa)	Rabbit	Laboratoire d'Hormonologie, Marloie, Belgium
233	Anti-CamB	CamBΔ1-22 (44.5 kDa)	Rabbit	Laboratoire d'Hormonologie, Marloie, Belgium
234	Anti-CamA	CamA (34 kDa)	Rabbit	Laboratoire d'Hormonologie, Marloie, Belgium
240	Anti-CamO	CamOΔ1-25 (57.6 kDa)	Rabbit	Laboratoire d'Hormonologie, Marloie, Belgium

Dog survey

Table 15 Survey of *C. canimorsus* and *C. cynodegmi* in Swiss dogs

Nr.	Race	Sex	Born on	Isolation date	Location	Area	Results
001	n.d.	n.d.	n.d.	15.01.2007	Zürich	country	*C.c, C.cyno*
002	Belgian Sheppard	male	24.10.2006	23.01.2007	St- Gallen, BL	country	*C.cyno*

003	Belgian German Sheppard	male	30.03.2005	23.01.2007	Lucern, BL	country	*C.c*
004	Coton de Tulear	male	14.09.2002	23.01.2007	France, CH-BL	country	*C.c, C.cyno*
005	Labrador	male	03.12.1995	23.01.2007	Lucern, BL	country	*C.c, C.cyno*
006	Hound dog mix	female	2005	29.01.2007	BL	country	*C.c, C.cyno*
007	Collie mix	male	2005	29.01.2007	BL	country	*C.c*
008	Appenzeller mix	male*	5.2004	30.01.2007	BL	city	*C.cyno*
009	Labrador mix	female	27.11.1999	30.01.2007	BL	city	other
010	Bischon frisé	female*	2003	30.01.2007	BS	city	*C.c*
011	Hound dog mix	female*	2002	30.01.2007	BS	city	*C.c, C.cyno*
012	Jack Russell Terrier	male	25.10.2006	30.01.2007	BS	city	*C.cyno*
013	Pekinese	female	1999	30.01.2007	BS	city	*C.c*
014	middle Poodle	male	2006	31.01.2007	BL	city	other
015	Yorkshire-Jack Russell Terrier	female*	2001	31.01.2007	BS	city	*C.c*
016	Yorkshire-Jack Russell Terrier	female*	2001	31.01.2007	BS	city	*C.c*
017	Pekinese	female	2006	31.01.2007	BS	city	*C.cyno*
018	Labrador	male*		30.01.2007	DE/CH-BL	country	*C.c, C.cyno*
019	Shih-Tzu	male	1995	05.02.2007	BS	city	*C.cyno*
020	Yorkshire	female*	2004	05.02.2007	BS	city	*C.c*
021	French bulldog	male	2005	05.02.2007	BS	city	other
022	Labrador	female*	1994	13.02.2007	BL	city	other
023	Pekinese mix	female	01.05.2001	13.02.2007	JU	country	*C.cyno*
024	German Sheppard	female	01.11.2001	13.02.2007	BS	city	*C.cyno*
025	Labrador	male	01.01.2003	13.02.2007	BS	city	*C.c, C.cyno*
026	Bischon frisé mix	male	26.10.2006	14.02.2007	BL	country	other
027	West High-Land-Terrier	female*	1996	14.02.2007	BL	country	*C.cyno*
028	Sennen-Newfoundland mix	male	2006	14.02.2007	BL	country	*C.cyno*
029	Schipperke	female*	1992	14.02.2007	BL	country	*C.cyno*
030	Schipperke	male*	1986	14.02.2007	BL	country	*C.cyno*
031	Pekinese mix	female	1995	14.02.2007	BS	city	other
032	Dingo	female*	20.11.1999	14.02.2007	BL	country	*C.cyno*
033	Hound mix	male	2004	14.02.2007	BL	country	*C.c, C.cyno*
034	Tivet-Terrier mix	male	2004	19.02.2007	BL	city	*C.c, C.cyno*
035	Berger Pyrenée	male*	1994	20.02.2007	BS	city	*C.c*
036	Terrier mix	female	2001	20.02.2007	BS	city	*C.c*
037	Terrier mix	female*	01.09.2004	20.02.2007	BS	city	*C.c, C.cyno*
038	Labrador	female*	14.10.1999	23.02.2007	VS	mountain	*C.c*
039	Border Collie	male*	30.05.2005	23.02.2007	VS	country	*C.c*
040	Husky	male	01.09.2006	23.02.2007	VD	city	*C.c*
041	Labrador	male	03.11.2006	23.02.2007	VD	mountain	other
042	English Cocker	female*	21.12.2002	23.02.2007	VD	city	*C.cyno*
043	Cane corso	female	11.06.2002	23.02.2007	VD	country	*C.c*

044	Bong Nhat	female*	01.09.2000	23.02.2007	VD	mountain	*C.c, C.cyno*
045	Carlin	female*	23.04.1998	23.02.2007	VD	mountain	*C.c, C.cyno*
046	Carlin	female*	01.03.1998	23.02.2007	VD	mountain	*C.c*
047	Border Collie	male*	13.09.2004	23.02.2007	VD	mountain	*C.c, C.cyno*
048	Labrador mix	male*	1995	24.02.2007	VD	city	*C.cyno*
049	Malinois	male	10.05.2004	24.02.2007	VD	mountain	*C.c, C.cyno*
050	Cavalier King Charles	male*	24.05.1994	24.02.2007	VS	country	*C.c*
051	Golden retriever	male	04.08.2002	24.02.2007	VD	city	*C.c, C.cyno*
052	Espagnol tibetain	male	01.06.2001	24.02.2007	VS	country	*C.c, C.cyno*
053	Golden Retriever mix	female	12.11.2006	24.02.2007	VD	city	*C.c, C.cyno*
054	Bouvier mix	female	13.10.2006	24.02.2007	VD	city	*C.c, C.cyno*
055	Samoyede	male	01.06.2003	24.02.2007	VD	country	*C.cyno*
056	Shi-Tzu	male	02.12.1999	24.02.2007	VD	country	*C.c*
057	Appenzeller mix	female*	22.07.1998	24.02.2007	VD	city	*C.c, C.cyno*
058	Shetland mini	male	07.06.2006	27.02.2007	VD	city	*C.c, C.cyno*
059	Podenco	female	01.01.2003	27.02.2007	VD	mountain	*C.cyno*
060	Yorkshire	male	25.10.2003	27.02.2007	VD	city	*C.cyno*
061	Westie	female*	12.11.2000	27.02.2007	VS	country	*C.cyno*
062	Bouvier Bernois	male	02.06.2000	27.02.2007	VS	city	*C.cyno*
063	Terrier mix	female	29.11.1998	27.02.2007	VD	city	*C.c, C.cyno*
064	Westie	female*	02.01.2001	27.02.2007	VD	city	*C.c, C.cyno*
065	Border Appenzeller mix	female*	06.10.2001	27.02.2007	VD	city	other
066	Cavalier King Charles	male	26.08.2004	27.02.2007	France, Paris	city	*C.c, C.cyno*
067	St-Bernard	male	01.08.1997	27.02.2007	VS	country	other
068	Golden retriever	female	09.09.2005	02.03.2007	BS	city	C.c
069	German Sheppard	female*	10.07.2000	02.03.2007	BS	city	*C.c*
070	Belgian& German Sheppard	female	31.07.2000	02.03.2007	BS	city	*C.c*
071	Labrador mix	female*	6.1997	05.03.2007	BS	city	*C.c*
072	German Sheppard	female*	1996	05.03.2007	BS	city	*C.cyno*
073	German Sheppard	female*	24.09.2005	05.03.2007	BS	city	*C.c*
074	Border Terrier	female*	2003	05.03.2007	BL	country	*C.c, C.cyno*
075	German Sheppard	female	1.2003	05.03.2007	BL	country	*C.c, C.cyno*
076	Bulldog	female	2006	06.03.2007	BS	city	*C.c*
077	German Sheppard	male	19.09.2002	06.03.2007	BS	city	*C.c, C.cyno*
078	Golden retriever	female*	1.06	09.03.2007	BL	country	other
079	Spaniel mix	female*	1993	09.03.2007	BL	city	*C.c, C.cyno*
080	Golden retriever	female*	26.11.2003	10.03.2007	BS	city	*C.c*
081	Malinois	female*	2003	11.03.2007	BL	country	*C.c, C.cyno*
082	Lappi-BSH-Pinscher mix	female*	2004	12.03.2007	BS	city	*C.c, C.cyno*
083	Lhasa-Apso	female	1994	12.03.2007	BL	city	*C.cyno*

084	BSH	male	1999	13.03.2007	BS	city	*C.c, C.cyno*
085	Collie mix	male*	01.08.2002	14.03.2007	BL	city	*C.c*
086	Terrier	female*	23.10.2000	16.03.2007	BL	city	other
087	Greek hound	male*	8.2003	16.03.2007	BL	city	*C.cyno*
088	Jack Russell Terrier	female*	15.06.1993	16.03.2007	BS	city	*C.c, C.cyno*
089	Yorkshire-Malteser	male	01.05.2002	17.03.2007	BS	city	*C.c ?*
090	Poodle	male	1998	18.03.2007	BS	city	*C.cyno*
091	Chinese	female	02.02.1996	19.03.2007	BS	city	other
092	Malinois	female*	8.1995	20.03.2007	BS	city	*C.cyno*
093	Golden retriever	female*	1998	20.03.2007	BL	city	*C.c, C.cyno*
094	Hawaneser	female*	2005	20.03.2007	BS	city	*C.c, C.cyno*
095	Schnauzer	male	2000	20.03.2007	BS	city	*C.c*
096	Terrier	female*	03.01.1999	20.03.2007	BL	city	*C.c*
097	Terrier	male	7.1998	20.03.2007	BS	city	*C.c*
098	Tibet Terrier	female*	1991	21.03.2007	BS	city	*C.cyno*
099	Poodle	male*	15.08.2006	21.03.2007	BL	city	*C.cyno*
100	Bichon frisé	female*	03.05.1995	21.03.2007	BS	city	*C.cyno*
101	English Cocker Spaniel	male	17.01.1999	21.03.2007	BL	city	*C.c, C.cyno*
102	Malamut Husky	male*	04.11.2004	23.03.2007	BL	city	*C.c, C.cyno*
103	n.d.	n.d.	n.d.	02.04.2007	DE	n.d.	*C.c, C.cyno*

* indicates sterile individuals; *C.c., C. canimorsus* ; *C. cyno, C. cynodegmi.*

Abbreviations.

Ap^r, ampicillin resistance; *Cc*, *C. canimorsus*; cfu, colony forming units; Cf^r, cefoxitin resistance; CMP-Neu5Ac, Cytidine-5′-monophospho-N-acetylneuraminic acid; Cm^r, chloramphenicol resistance, DSA, *Datura stramonium* agglutinin; Em^r, erythromycin resistance; EtOH, ethanol; FBS, fetal bovine serum; fH, factor H; Gal, galactose; Glc, glucose; GlcNAc, N-acetyl glucosamine; GalNAc, N-acetyl galactosamine; GNA, *Galanthus nivalis* agglutinin; HI, heat-inactivated; hk, heat-killed; Neu5Ac, N-acetylneuraminic acid; Man, mannose; moi, multiplicity of infection, MUAN, 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid; nr, non redundant; SNA, *Sambucus nigra* agglutinin; PNA, peanut agglutinin; Tc^r, tetracycline resistance; Tn, transposon.

CONCLUSION AND OUTLOOKS

Conclusion and Outlooks

The development of a gene transfer system, selectable markers, a replicating shuttle vector and a transposition mutagenesis system allowed to perform the very first genetic manipulations of *C. canimorsus*. Establishment of the genetic system was the basis for molecular studies on *C. canimorsus* -host interactions.

We found that *C. canimorsus* multiplies efficiently in presence of mammalian cells including phagocytes. We isolated two mutants unable to grow in contact to cells but fully proficient for growth on blood agar. One mutant turned out to be affected in a surface-exposed sialidase and could be rescued by exogenously added sialidase but surprisingly not by sialic acids. However, addition of GlcNAc or GalNAc rescued growth, showing that sialidase allows *C. canimorsus* to feed on glycans from host cell surface glycoproteins. The sialidase deficient mutant turned out to be hypo-virulent in a mouse model and we provided evidence that *C. canimorsus* also feeds on phagocytes *in vivo*. Another Tn integration was localized in a gene encoding for a hypothetical conserved protein. Notably, we could rescue growth of this second mutant (Y4G6) in presence of macrophages by introducing the gene *in trans* or by adding GlcNAc or GalNAc. Mutant Y4G6 is presumably deficient in the deglycosylation process of host glycan structures. Using lectin stainings we demonstrated that Δ*siaC* is deficient in the first step of hydrolyzing terminal sialic acids to expose internal carbohydrates, while the mutant Y4G6 is blocked in the subsequent deglycosylation process. The role of *yfgA* disrupted in mutant Y4G6 needs to be clarified for instance by using enzyme substrates. *Cc5* uses its surface-exposed sialidase to reveal internal GalNAc and GlcNAc residues on host glycoconjugates, which are then presumably released by hexoseaminidase(s). We propose that glycosylhydrolases are part of an extracellular "degradosome." However, we further need direct evidence that supports the existence of this complex and the extracellular localization of the enzymes.

We observed that *Cc5* was resistant against human complement which resulted at least to some extent, from binding fH. The sialidase-deficient mutant and mutant Y4G6 were sensitive to killing by human complement. This sensitivity correlated to a lack of fH binding. We also showed that SiaC alone does not promote binding of fH therefore we are investigating if other proteins are involved

in the recruitment of fH. It remains to be determined if the hypothetical "degradasome" complex of the deglycosylation process is involved in binding fH. Serum sensitivity of both mutants could however not be restored by the addition of aminosugars. This indicates that there is no surface modification upon addition of GlcNAc or GalNAc. It has to be addressed if the deglycosylation *per se* is related to resistance against complement and/or fH binding.

Cc5 evades and even down-regulates the onset of pro-inflammatory signaling. In a Tn mutagenesis approach, we screened for *Cc5* mutants that were unable to inhibit the pro-inflammatory response of macrophage upon co-infection by heat-killed *E. coli*. Mutants have been mapped, characterized and complemented but despite our extensive analysis of the genes, their function remains to be understood. Interestingly, many of the genes identified are also found in commensal bacteria of the *Bacteroides* genus. Little is known how commensal bacteria highly adapted to their niche are influencing the host. However, a few examples nicely highlight that commensals not only induce or suppress immune responses but also modulate immune response. The obligate anaerobe *B. fragilis,* which is present in all mammals, has an extensively variable surface structure by expressing many different capsular polysaccharides (Cerdeno-Tarraga *et al.*, 2005; Kuwahara *et al.*, 2004). Remarkably, polysaccharide A (PSA) of *B. fragilis* was shown to stimulate the balance between T_H cells during gut homeostasis. This zwitterionic polysaccharide was not only able to stimulate T cells, but also played a role in the development and maturation of the immune system (Mazmanian *et al.*, 2005). *B. fragilis* clearly influences the immune response that is aimed at neutralizing most, if not all, other microorganisms (Mazmanian and Kasper, 2006). Another intestinal commensal *B. thetaiotaiomicron* induces the expression of antimicrobial molecules that directly bind and eliminate potentially pathogenic bacteria (Cash *et al.*, 2006). Other studies using zebrafish- commensal interactions showed that LPS is necessary and sufficient to trigger expression and activity of alkaline phosphatase. This host enzyme played a crucial role in promoting mucosal tolerance to resident intestinal bacteria which prevented inflammatory responses (Bates *et al.*, 2007). *C. canimorsus* primarily evolved as a commensal in canine and feline oral cavities but it can turn into a pathogen if it is accidentally introduced into the tissues of another host. It is not known if commensal *C. canimorsus* carry the same "virulence

factors” as those isolated from septic patients. Somehow this resembles the situation of *Neisseria meningitidis* which is present in the naso-pharynx of 5- 30% of the human population but causes only a few cases of fatal infections. The functional analysis and presence of genes involved in the active inhibition of pro-inflammatory signals in different isolates of *C. canimorsus* could provide more explanation.

Commensalism and pathogenesis represent different facets of microbial-host interactions. Our results show how a commensal can turn into a pathogen by using features presumably evolved for host adaptation. Our results will provide the basis for further molecular studies to be performed on the pathogenesis of *C. canimorsus*, a bacterium, which is of great medical interest.

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