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Exploitation of complement regulatory proteins by *Borrelia* and *Francisella*†

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Pathogens have developed sophisticated mechanisms of complement evasion such as binding to the host complement regulatory proteins (CRPs) on their surface or expression of CRP mimicking molecules. The ability of pathogens to evade the complement system has been correlated with pathogenesis and host selectivity. Hitherto, little work has been undertaken to determine whether *Borrelia* and *Francisella* exploit various CRPs to block complement attack. Seventeen *Borrelia* (twelve species) and six *Francisella* (three subspecies) strains were used to assess their ability to bind human, sheep and cattle CRPs or mimic membrane associated complement regulators. A series of experiments including affinity ligand binding experiments, pull-down assays and mass spectrometry based protein identification, revealed an array of CRP binding proteins of *Borrelia* and *Francisella*. Unlike *Francisella*, *Borrelia* strains were able to bind multiple human CRPs. Three strains of *Borrelia* (SKT-4, SKT-2 and HO14) showed the presence of a human CD46-homologous motif, indicating their ability to possess putative human CD46 mimicking molecules. Similarly, five strains of *Borrelia* and two strains of *Francisella* may have surface proteins with human CD59-homologous motifs. Among ovine and bovine CRPs, the only CRP bound by *Francisella* (LVS, Tul4 strain) was vitronectin, while ovine C4BP, ovine factor H and bovine factor H were bound to *Borrelia* strains SKT-2, DN127 and Co53. This study presents an array of proteins of *Borrelia* and *Francisella* that bind CRPs or may mimic membrane-CRPs, thus enabling multiphasic complement evasion strategies of these pathogens.

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Introduction

Borrelia and *Francisella* can infect a wide range of host species including wild and domestic animals, reptiles, birds, and humans. Both tick-borne pathogens can readily disseminate into various organs and persist for a longer period in the host. As with many other pathogens, *Borrelia* and *Francisella* have developed mechanisms of immune evasion, such as resistance to host's complement-mediated bacterial lysis (referred to as evasion of the complement system). The complement system is a crucial part of the innate immunity. One of the main goals of the complement system is to kill pathogenic microorganisms. Therefore, the ability to avoid or prevent being killed by the complement system is an important determinant of microbial pathogenicity. Some blood-borne pathogens are surrounded by a capsule, which is refractory to complement attack;[1] some express surface molecules that mimic the host's complement regulatory proteins (CRPs), while several others bind the host's soluble CRPs on their surface and block complement-mediated attack.[1]

Complement system can be activated through three different routes, the classical, the lectin and the alternative pathways. In normal conditions host cells are protected from complement-mediated lysis with the help of several soluble and cell-bound complement regulators.[1,2] Each CRP specifically degrades the complement activator and thus blocks further activation of the cascade. For example, C1 inhibitor (C1inh), a soluble serine protease, prevents spontaneous activation of the complement cascade *via* dissociation of C1r2s2 from the C1q molecule.[1,3] By binding the C1inh on the surface, a pathogen can degrade C1q and inhibit the activation of the classical pathway. Classical and

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lectin pathways of complement activation are also controlled by serum glycoprotein C4 binding protein (C4BP). C4BP bound on the bacterial cell surface inhibits activation of complement by multiple mechanisms; *e.g.* it blocks the insertion of C2 to C4b protein, accelerates decay of C3 convertase and acts as a cofactor for serine protease factor I (FI) which cleaves C4b and C3b.[4–6] In order to inhibit the classical and lectin pathways, many microbes have developed the ability to bind to host C4BP. Many pathogens including *Borrelia* exploit serum factor H (FH) to protect themselves from the alternative pathway. Factor H is another major complement regulatory protein that serves as a cofactor for the FI-mediated cleavage of C3b and accelerates decay of C3 convertase. Thus, FH bound to the host's cells exerts a protective action and prevents complement-mediated lysis.[7–9] Some pathogens use vitronectin (VTN, protein S). It is an abundant glycoprotein found in the serum and extracellular matrix, that blocks the membrane-damaging effect of the terminal cytolytic complement pathway by inhibiting the formation of the C5b–8 complex that acts as a catalyst in the polymerization of C9 molecules necessary to form a pore-like structure called the membrane attack complex.

Apart from soluble CRPs, membrane associated regulators also protect the self-cells from uncontrolled complement-mediated lysis. Most mammalian cells express surface molecules such as convertase regulators that act as decay accelerators (*e.g.* CR1 or decay-accelerating factor – DAF, also called as CD55), or as cofactors for fI, such as CR1 and membrane cofactor protein (MCP or CD46). CD35 and CD59 expressed on the cell surface degrades C4b or C3b and limits incorporation of multiple C9 into the membrane C5b–8 complex, respectively.[1] Several pathogens express cell surface proteins with structural motifs and function similar to membrane associated CRPs like CD35, CD46 or CD59. Expression of such proteins on the pathogen surface and their use in complement evasion has been reviewed comprehensively by various authors.[10–12]

It is noteworthy that pathogens have developed multiple strategies to evade complement. *Neisseria gonorrhoeae* effectively evades complement by exploiting CRPs like FH,[13] C4BP[14] and VTN.[15] *Candida albicans* also binds these CRPs to inhibit activation of complement.[16,17] Although it is known that some of the Lyme disease and relapsing fever borreliae bind FH,[18] C4BP[6] and express CD59-like protein (a mimicking molecule),[19] the binding ability of these CRPs by wide spectrum of *Borrelia* species is still unclear. It is also unclear whether *Borrelia* can exploit other CRPs such as C1inh and VTN, overexpressing surface molecules that mimic CD35 and CD46. It is predicted that *Francisella* may need CRPs to survive in the host. This facultative intracellular bacterium has a pathogenic extracellular form, which may exploits CRPs to avoid complement-mediated bacteriolysis.[20] Up to the present, the only one study has reported FH binding by *Francisella tularensis*.[21]

Complement resistant *Borrelia burgdorferi* sensu stricto (B.b.s.s.), *B. afzelii*, *B. hermsii* and some serotypes of *B. garinii* survive successfully in body fluids where the complement protein concentration is high. Subsequently they spread and establish infection in other niches, *e.g.*, brain and joints, where complement proteins are less abundant. There is a close correlation between complement evasion ability and the pathogenic nature of the strains. Likewise, complement sensitivity/resistance is one of the crucial components which govern the pathogen–host relationship and the reservoir competence of the host.[22] A competent reservoir host for tick-borne pathogens is a vertebrate animal species that becomes infected, harbours and subsequently acts as a source of infection for other vertebrates *via* the tick vector.[23] Very few genospecies of *Borrelia* can infect sheep. Lambs infected with *Borrelia burgdorferi* typically show an erythema at the site of a tick bite[24] and later show additional clinical signs including stumbling in the hind leg, poor appetite and high levels of IgG antibodies.[25] On the other hand, *Borrelia* genospecies, except *B. coriaceae*, are not able to infect cattle.[26]

With this background, a comparative study of complement–pathogen interaction was performed that included three different hosts (human as susceptible, cattle as resistant and sheep as intermediate susceptible host) and a repertoire of *Borrelia* and *Francisella* strains. In summary, the CRP binding/mimicking ability of *Borrelia* and *Francisella* was assessed; yet-unidentified CRP binding proteins of *Borrelia* and *Francisella* were revealed using mass spectrometry; and CRP binding/mimicking by pathogens was correlated with their susceptibility to complement-mediated killing.

Materials and methods

Cultivation of bacteria and preparation of cell lysates

Seventeen Lyme disease and relapsing fever *Borrelia* strains (Table 1) were incubated in complete BSK-II medium containing 6% inactivated rabbit serum (Sigma-Aldrich, Madrid, Spain) at 33 °C. Six *Francisella* strains (Table 1) were cultured on enriched chocolate agar containing 1% (w/v) glucose and 0.1% (w/v) L-cystein at 37 °C with 2% (v/v) CO_2. *Borrelia* were harvested by centrifugation at 8000 × *g* for 10 min, while *Francisella* colonies were collected in phosphate buffered saline (PBS, pH 7.2). Cells were washed 5 times with PBS and resuspended in ultra-pure water containing 1% (v/v) of nuclease mix and 1% (v/v) of a protease inhibitor cocktail (GE Healthcare, Slovakia). Outer membrane proteins (OMPs) were extracted using a membrane protein extraction kit (Fermentas, Germany), and protein concentrations were measured with the Bradford method according to the manufacturer's instructions (Thermo Scientific, Slovakia).

Protein fractionation (SDS-PAGE) and electrotransfer

Outer membrane proteins (400 μg) were fractionated by SDS-PAGE in 15 cm long gels. The protein samples were mixed with lithium dodecyl sulfate sample buffer (LDS sample buffer, Invitrogen, Slovakia) as per the manufacturer's instructions and incubated at 72 °C for 15 min. Electrophoresis was carried out at 120 V, until the dye reached the bottom of the gel (4–12% Bis-Tris 2 well gel, Invitrogen), using a running buffer of 50 mM MOPS – (3-*N*-morpholino propanesulfonic acid), 50 mM Tris

Table 1 *Borrelia* and *Francisella* strains used in the study[a]

Species	Strain	Serotype
Francisella tularensis subsp. *tularensis*	FTT	NA
Francisella tularensis subsp. *novicida*	CRO-1	NA
Francisella tularensis subsp. *holarctica*	LVS	NA
Francisella tularensis subsp. *holarctica*	FT7	NA
Francisella tularensis subsp. *holarctica*	Tul4	NA
Francisella tularensis subsp. *holarctica*	Tul49	NA
Borrelia afzelii	SKT-4	ST2
Borrelia burgdorferi sensu stricto	SKT-2	ST1
Borrelia garinii	Rio2	ST3
Borrelia garinii (*B. bavariensis*)	PBi	ST4
Borrelia garinii	G117	ST5
Borrelia garinii	SKT-3	ST6
Borrelia garinii	T25	ST7
Borrelia garinii	CL-1	*ST8*
Borrelia valaisiana	VS116	NA
Borrelia andersonii	21123	NA
Borrelia lusitaniae	Poti B2	NA
Borrelia bissettii	DN127	NA
Borrelia japonica	HO14	NA
Borrelia hermsii	HS1	NA
Borrelia parkeri	M3001	NA
Borrelia anserina	ES-1	NA
Borrelia coriaceae	Co53	NA

[a] NA – not applicable. All *Borrelia* strains (except SKT-2, SKT-3 and SKT-4) and two *Francisella* strains (FTT and FT7) were provided by Dr Pedro Anda and Dr Raquel Escudero (Institute of Health Carlos III, Madrid, Spain). CRO-1 and LVS strains were kindly provided by Dr Marina Santic, University of Rijeka, Croatia. Tul4 and Tul49 strains were provided by Dr Miklos Gyuranecz, Szent Istvan University, Hungary.

base, 0.1% SDS and 1 mM EDTA (pH 7.7). Proteins were electrotransferred onto nitrocellulose membrane (30 V for 1 h) in Xcell Surelock-cell protein transfer system using NuPAGE transfer buffer (both Invitrogen). Membranes were cut into 3 mm vertical strips for further assays.

Preparation of r-C1inh, r-C4BP, r-FH and r-VTN and their use in affinity ligand binding (ALBI) assay

Total RNA isolated from liver (Purezol™, Bio-Rad, USA) and treated with DNase (Ambion, USA) was reverse transcribed. Human liver tissue was collected by biopsy and a written informed consent was obtained according to the regulations adopted by the ethical committee of University of Veterinary Medicine and Pharmacy, Kosice, Slovakia. cDNA was used to amplify the coding regions of C1inh, C4BP, FH and VTN. Primers are presented in Table 2. Cycling conditions for all gene fragments were 98 °C for 30 s; 35 cycles of [10 s at 95 °C, 15 s kb^{-1} at 72 °C min] followed by 72 °C for 5 min. Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland) was used to avoid any error in amplification. All amplicons were sequenced (ABI3100 Avant Applied Biosystem, USA), digested with restriction enzymes (restriction sites are depicted in primers with underlined letters) and then ligated into a PYEBME-1 shuttle expression vector (in a house-modified version of the pYES2 vector from Invitrogen; Fig. 1). Ligation mixes were purified with the standard phenol–chloroform method and transformed into electrocompetent *E. coli* M15 cells (Qiagen, USA). Transformed *E. coli* were selected based on their ampicillin resistance on LB agar and used for isolation of shuttle plasmids with the incorporated gene of interest. A total of 100 ng of isolated plasmid was transformed into the electrocompetent *Saccharomyces cerevisiae* strain YPH501 (Stratagene, USA). Transformed YPH501 strains were selected on SD agar with uracil auxotrophy (6.7 g yeast nitrogen base, 2% (w/v) dextrose, 1.3 g amino acid dropout powder without uracil and 2% (w/v) agar). Selected transformed colonies were cultured further in SD medium for 16 h at 30 °C. Expression of recombinant proteins in YPH501 was performed in SG medium (dextrose in SD was replaced by 2% (w/v) galactose) for 4–5 hours at 30 °C with constant shaking (300 rpm). Cells were lysed by vigorous shaking on a vortex with glass beads (Sigma, Slovakia) in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) containing 1% (v/v) of nuclease mix and 1% (v/v) of a protease inhibitor cocktail (GE Healthcare, Spain). Recombinant proteins were purified using Ni-NTA agarose columns (Qiagen, USA) and/or anti-Flag M2 agarose beads (Sigma, Slovakia) under native conditions as per the manufacturer's instructions. The presence of recombinant proteins in elutes was confirmed by western blotting. Briefly, proteins in elutes were fractionated by SDS-PAGE and electrotransferred onto nitrocellulose membranes as described above. Membranes were incubated in a nickel–HRP conjugate (Abcam, UK) diluted 1 : 5000 in (0.15 mol NaCl and 25 mmol Tris, 0.05% Tween20) for 1 h. After 7 times washing in TTBS, membranes were incubated for 5 min in Super-Signal West Pico ECL substrate (Pierce) and the signals were captured on X-ray film (Fig. S1, ESI†).

Affinity ligand binding assays were performed to assess binding affinities of *Francisella* and *Borrelia* OMPs to r-C1inh, r-C4BP, r-FH and r-VTN, as described previously.[27] Briefly, nitrocellulose membrane strips with OMPs of *Borrelia* and *Francisella* were blocked for one hour in TTBS containing 3% (w/v) non-fatty skimmed milk. After two washings with TTBS, strips were incubated with purified recombinant CRPs (1500 μg ml^{-1} in TTBS containing 1% (w/v) non-fatty skimmed milk) for 2 h at 37 °C. Unbound proteins were washed off with TTBS and recombinant CRPs bound to *Francisella* or *Borrelia* proteins were detected using the nickel–HRP conjugate and West Pico ECL substrate as described above. For negative controls no recombinant proteins were included in the assay, instead membrane was incubated in TTBS containing 3% (w/v) non-fatty skimmed milk. The assay was repeated at least three times to confirm the protein interactions.

Affinity ligand binding (ALBI) assay to assess binding of human serum CRPs (hCRPs) to *Borrelia* and *Francisella* OMPs

No antibodies against bovine or ovine CRPs were readily available, therefore this ALBI assay was performed only with human serum as described earlier.[18] Human serum was a pool obtained from healthy individuals. A written consent was prepared before blood collection. Nitrocellulose membrane strips with *Borrelia* and *Francisella* OMPs were blocked for 1 h in TTBS containing 3% (w/v) non-fatty skimmed milk. After two times washing with TTBS, strips were incubated for 3 h with diluted human serum (1 : 2 in TTBS with 1% (w/v) skimmed milk). Unbound proteins were washed with TTBS and strips

Table 2 Primers used to amplify the coding sequence of C1inh, C4BP, FH and VTN of human, sheep and cattle[a]

Protein	Primer sequence	Genebank accession no.
Human		
C1inh	F-5′ATAACAAGCTTAATCCAAATGCTACCAGCTCC	NM_000062.2
	R-5′TCTTTCAAGACCTGGGGTCATATACTCGCC	
C4BP	F-5′ATAACAAGCTTCAATTGTGGTCCTCCA	AK313164
	R-5′TCTTTCAAGACCACTCACACTTGGGCAC	
FH	F-5′AAAGAGCTCGGGTTATGAATACAGTGAAAG	Y00716.1
	R-5′TTGCTCAAGACACAAGTTGGATACTCCAG	
VTN	F-5′AAAAAGCTTGCCCTGCTGGCATGGGTTTCT	X03168.1
	R-5′GTTTCTAGAAAGCAATGGAGCGTGGGTAGGG	
Sheep		
C1inh	F-5′ATAACAAGCTTGCCTCAGATATGATCGTCGGCCCA	XM_004016808.1
	R-5′TCTTTCAAGACTTGGGGTCATACACCCGAC	
C4BP	F-5′ATAACAAGCTTAAGTTGTGGTATTCCA	XM_004013579.1
	R-5′TCTTTCAAGACCACTCACACTTGGGTGC	
FH	F-5′AAAGAGCTCCGGTTTTGAATATGGTCAGAG	GU991526.1
	R-5′TTGCTCAAGACTATTGGTGGAGGAGGCCCAC	
VTN	F-5′AAAAAGCTTGCCCTGCTGGCGTGGGTTGTT	XM_004013222.1
	R-5′GTTTCTAGAGGCGATGGAGCGTGGGTAGGGA	
Cattle		
C1inh	F-5′ATAACAAGCTTACCTCAGATATGATCGTCGGCCCA	U30332.1
	R-5′TCTTTCAAGACTTGGGGTCGTACACCCGAC	
C4BP	F-5′ATAACAAGCTTCAGTTGTGGTATTCCA	NM_174252.3
	R-5′TCTTTCAAGACCACTCACACTTGGGCAC	
FH	F-5′AAAGAGCTCCGGTTTTGAATATGGTCAGAG	NM_001033936
	R-5′TTGCTCAAGACACATCGAGGATACACCAC	
VTN	F-5′AAAAAGCTTGCCCTGCTGGCGTGGGTTGTT	NM_001035050.2
	R-5′GTTTCTAGAGGCGATGGAGCGTGGGTAGGGA	

[a] Underlined sequence indicates restriction enzyme site.

were incubated overnight at 4 °C with antibodies (in TTBS with 1% skimmed milk) against various hCRPs: anti-C1inh (1 : 500 dilution, Santa-Cruz, UK); anti-C4BP (1 : 500 dilution, Santa-Cruz, UK); anti-FH (1 : 1000 dilution, Abcam, UK); and anti-VTN (1 : 100 dilution, Abcam, UK). After three times washing with TTBS, the strips were incubated with secondary antibodies conjugated with HRP at 37 °C for 1 h. The strips were washed 7 times with TTBS and incubated for 5 min in Super-Signal West Pico ECL substrate. Signals were captured on X-ray films. For negative controls either serum or primary antibodies or both were excluded from the affinity ligand binding experiments. The assay was repeated at least three times.

Assessment of possible mimicry of human CD35, CD46 and CD59 by *Borrelia* and *Francisella*

Nitrocellulose membrane strips with OMPs were incubated overnight at 4 °C with monoclonal anti-human CD35 or anti-human CD46 (both Abcam, UK; 1 : 300 dilution) or anti-human CD59 (1 : 200 dilution, Santa-Cruz, UK) diluted in TTBS containing 1% (w/v) skimmed milk. After washing three times with TTBS, strips were incubated for 1 h at 37 °C with secondary antibodies conjugated with HRP and subjected to chemiluminescence as described above.

All antibodies were tested for their specific affinity. Briefly, recombinant human CD35, CD46 and CD59 (produced in-house) were immobilized on the nitrocellulose membrane and hybridized with the antibodies used above. For a negative control primary antibodies were excluded from the affinity ligand binding experiment. The assay was repeated at least three times to confirm the protein interactions. Possible mimicry of bovine and ovine CD35, CD46, and CD59 was not performed due to the lack of suitable commercial antibodies.

Pull-down assay with rCRPs and OMPs

In short, nickel affinity beads (50 μl per reaction) were washed twice with one ml of RIPA buffer (both Sigma-Aldrich, Slovakia) and incubated with purified ovine or bovine or human r-C4BP, r-FH or r-VTN diluted in 500 μl of RIPA buffer (600 μg ml^{-1}) for 1 h at 8 °C with constant shaking. To remove unbound proteins, affinity beads were washed once with RIPA buffer and two times with TTBS (pH 7.5). A 1000 μl aliquot of OMP of *Borrelia* or *Francisella* diluted in TTBS (600 μg ml^{-1}) was added to affinity beads and incubated for 1 h at room temperature with constant shaking. Affinity beads were centrifuged at 6000 × *g* for 1 min, the supernatant was removed and unbound proteins were washed twice with 1 ml of TTBS. Finally, affinity beads were centrifuged at 13 000 × *g* for 2 min and beads were incubated in 60 μl LDS sample buffer (Invitrogen) at 72 °C for 15 min. Electrophoresis was carried out at 120 V, until the dye reached the bottom of the gel (NuPAGE 4–12% Bis-Tris 12 well gel, Invitrogen). Proteins were visualized with Coomassie blue staining. Gels were compared with the results of ALBI assay and the corresponding protein bands were excised from the gel for identification. The assay was repeated at least three times.

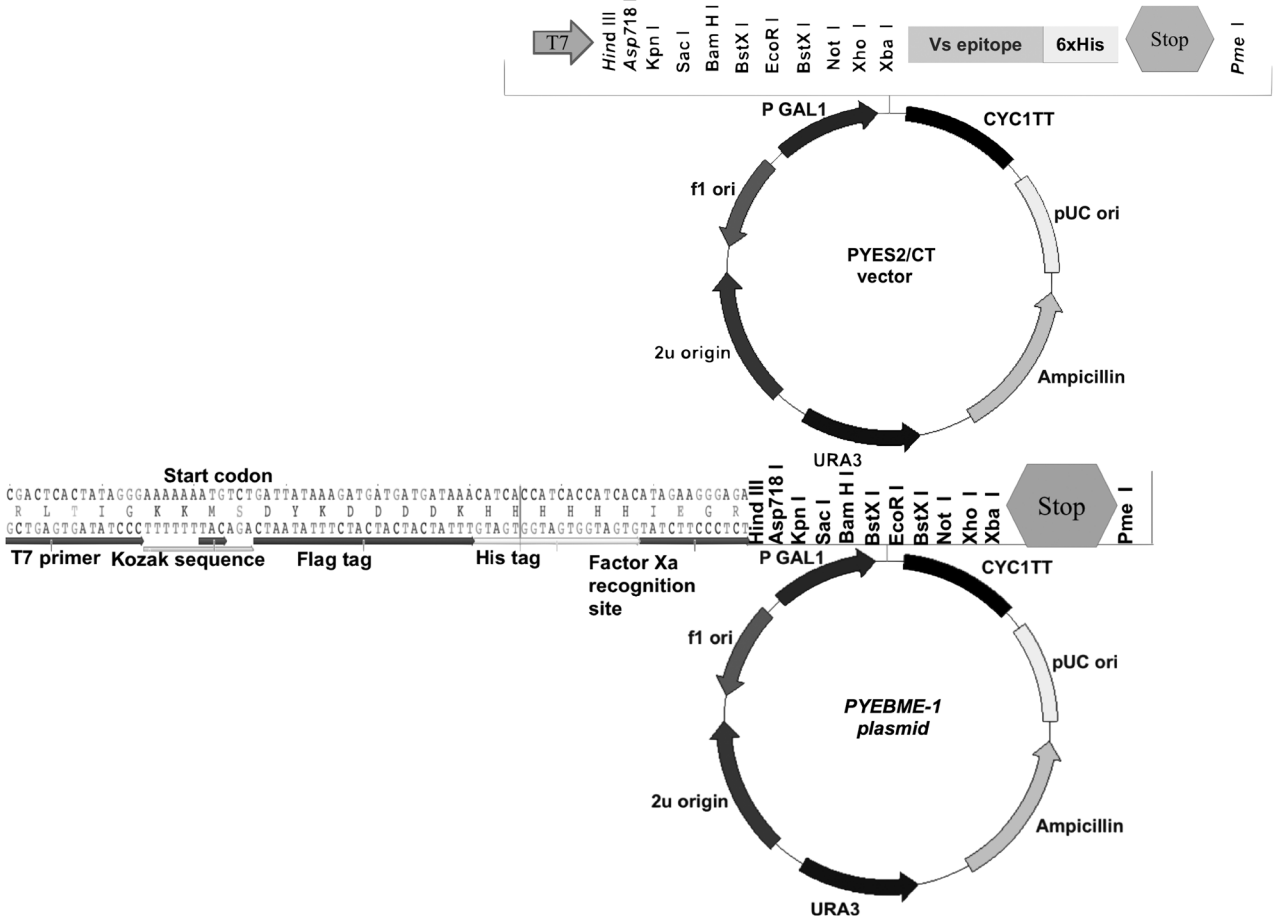

Fig. 1 PYEBME-1 plasmid. In-house-modified version of the PYES2/CT plasmid. The PYEBME-1 plasmid consisted of N-terminal double tags, Flag, and hexa-histidine, used in effective purification of target recombinant proteins as well as in ALBI assay to avoid non-specific signals. The Kozak consensus sequence was also incorporated around the start codon to ensure consistent translation. Another feature of PYEBME-1 is the incorporated factor Xa cleavage site to cleave tags from rest of the protein, which can be used elsewhere in functional assays. Sequence of PYES2/CT can be found at Invitrogen: http://tools.invitrogen.com/content/sfs/vectors/pyes2ct_seq.txt, while other features of this plasmid are here: http://tools.invitrogen.com/content/sfs/vectors/pyes2ct_map.pdf.

Protein identification

Protein bands were destained, reduced with dithiothreitol, alkylated with iodoacetamide, digested with trypsin and acidified with formic acid as described earlier.[28] Digests were subsequently used for protein identification with MALDI-MS/MS, following either one of two procedures previously described: procedure one[29] or alternatively, procedure two.[28]

For procedure one, peptides were desalted and concentrated with commercial Zip-tips (Millipore) and mixed with α-cyano-4-hydroxycinnamic acid in 33% (v/v) aqueous acetonitrile and 0.25% (v/v) trifluoroacetic acid. This mixture was then deposited onto a 600 μm AnchorChip prestructured MALDI probe (Bruker-Daltonics, Billerica, MS, USA) and allowed to dry. Protein identification was performed by MALDI TOF/TOF (Ultraflex, Bruker-Daltonics, Billerica, MS, USA). Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100 to 1000 individual spectra were averaged. Automated analysis of mass data was performed (version 3.0, FlexAnalysis software; Bruker-Daltonics, Billerica, MS, USA). MS and MS/MS data were combined (version 3.0, BioTools, Bruker-Daltonics, Billerica, MS, USA) to search the non-redundant protein database (NCBInr) using Mascot software (version 2.1.0, Matrix Science). The major parameters for the search were: taxonomy – bacteria (eubacteria); fixed modifications – carbamidomethyl; variable modification – methionine oxidation; peptide tolerance – 1.2 Da (for MS); automatic decoy database search on; missed-cleavage 1; peptide tolerance 50 ppm and fragment mass tolerance 0.25 Da.

For procedure two, peptides were desalted and concentrated using homemade reversed phase microcolumns (POROS R2, ABI, Foster City, CA, USA), and eluted onto a MALDI plate using a matrix solution that contained 5 mg ml^{-1} α-cyano-4-hydroxycinnamic acid dissolved in 70% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid.[28] The mixture was allowed to air-dry. Protein identification was performed by MALDI-TOF–TOF with a 4800 Proteomics Analyser (ABI, Foster City, CA, USA) in both MS and MS/MS mode. Each MS spectrum was obtained in a result independent acquisition mode with a total of 800 laser shots per spectra and a fixed laser intensity of 3500 V and

externally calibrated using the calibration mix from ABI (Foster City, CA, USA). Ten s/n best precursors from each MS spectrum were selected for MS/MS analysis. Raw data were generated by the 4000 Series Explorer Software v 3.0 RC1 (ABI, Foster City, CA, USA) and contaminant *m*/*z* peaks (human keratin, trypsin autodigestion and matrix) were excluded. The database search was conducted with MASCOT-demon 2.1.0 Software (Matrix-Science) with the same parameters described in procedure 1. The interpretation of the combined MS + MS/MS data was carried out using the GPS Explorer Software (version 3.5, ABI, Foster City, CA, USA) using earlier described parameters.[28]

Protein identifications were accepted if the protein score was above a threshold of 95% ($p < 0.05$) for Mowse. For proteins identified as hypothetical and with an unclear or uncharacterized biological function, a BLAST search was conducted using Uniprot (http://www.uniprot.org/blast/). Proteins submitted for BLAST analysis were considered to be homologous to the obtained results when at least 95% of the sequence matched.

Complement sensitivity assay (CSA)

CSA for *Borrelia* was performed as described.[30] Serum was separated from the blood collected from healthy persons, sheep and cattle. Each serum sample was tested with ELISA for the absence of antibodies against *Borrelia* or *Francisella*. Negative sera were pooled and used in CSA. *Borrelia* suspensions (10^7 cells) in BSK-II medium were added to equal volume of either fresh or inactivated serum (56 °C for 30 min) to make a final volume of 1000 μl in 24 well plates. Plates were carefully sealed and incubated at 33 °C for 6 h. A live cell control was run without addition of serum. After incubation, 5–7 μl of suspension from each well was observed under a dark field microscope for cell immobilization, bleb formation and bacteriolysis. Simultaneously, flow-cytometry was used to enumerate live and dead *Borrelia*.[26] In short, 50 μl of suspension was diluted 1 : 5 in phosphate buffered saline (PBS 0.01 M, pH 7.2; filtered through 0.2 μl filter) and freshly prepared acridine orange (1 μg ml^{-1}) was added. The suspension was incubated at room temperature for 5 min and subjected for flow-cytometry (FACS single-laser flow cytometer, Becton-Dickinson). Events were acquired in the list mode for 2 min (each event approximately corresponding to one bacterium). The sample fluid flow rate was kept low to reduce signal variability. *Borrelia* were differentiated from BSK-II and serum particles by adjusting the fluorescence intensity and side scatter parameters. Non-rectangular gates were defined first using side-scatter *vs.* FL-1 fluorescence (logarithmically amplified) using live and dead (heat killed) *Borrelia*. These gates (live cells in gate-L and dead cells in gate-D) were then used to differentiate and enumerate live and dead spirochetes in the samples. Data were analyzed using the WinMDI software (version 2.8).

To assess the complement sensitivity of different *Francisella* strains, bacteria were cultivated in brain heart infusion (BHI) broth enriched with 1% (w/v) glucose and 0.1% (w/v) L-cystein. Cells were harvested in the mid log phase and resuspended in fresh enriched BHI broth to obtain a cell concentration of 10^5 ml^{-1}. Cells were diluted serially (10 fold dilutions) with sterile BHI up to 10^3 ml^{-1} concentration. One thousand microliters of the bacterial cell suspension from each serial dilution (10^5 to 10^3) was mixed with 1000 μl of sterile filtered (0.2 μm) active or inactive (inactivated at 56 °C for 30 min) serum. Culture without addition of serum was kept as live control. Suspensions were incubated at 37 °C for 4 h with constant shaking. One-hundred microliters from each cell suspension was inoculated on the enriched chocolate agar (1% (w/v) glucose and 0.1% (w/v) L-cystein) in duplicate. Plates were incubated for a week at 37 °C with 2% (v/v) CO_2 and colonies were counted.

Statistical analysis

CSA for both *Borrelia* and *Francisella* were repeated three times and mean values were taken into account for evaluation of complement sensitivity. The percent sensitivity of *Francisella* strains was calculated as follows: 100 × (1 − (sum of the number of colonies in case of active serum)/sum of the number of colonies in case of inactive serum). The percent sensitivity of *Borrelia* was calculated as follows: 100 × (1 − (average number of events observed in gate-L in case of active serum)/average number of events observed in gate L in case of inactive serum). The paired *t*-test was also used to compare significant complement resistance and sensitivity ($p < 0.05$).

Results

As a result of co-evolution, several pathogens have evolved functional surface proteins that interact with the host complement regulatory factors guaranteeing their survival. Here we present a repertoire of surface proteins of *Borrelia* and *Francisella* that interact with FH, C4BP or VTN or mimic membrane associated complement regulatory molecules. However, we found that none of the OMP of *Francisella* and *Borrelia* could bind C1inh.

Protein interactions obtained in the ALBI assay performed with the recombinant form of hCRPs and human sera were identical. The results presented in Fig. 2 and compiled in Table S1 (ESI†), are cross-validated by those obtained after incubation with human sera. In order to avoid duplication, only the first results will be shown. Some of the OMPs interacting with CRPs might not be expressed abundantly in *Francisella* or *Borrelia*. Therefore we were unable to detect them in the gel in the pull-down assay. A total of 26 bands were submitted for MS identification but due to the low amount of protein, the identification could not be achieved in 35% of the bands.

Francisella tularensis subsp. *holarctica* strains bind human and sheep VTN

Among six *Francisella* strains, only Tul4 was able to bind human vitronectin (hVTN) with its 160 kDa and 58 kDa surface proteins (Table S1, ESI†). A 64 kDa protein of the LVS and 58 kDa protein of Tul4 showed binding affinity to ovine VTN (oVTN). None of the outer surface proteins of *Francisella* showed affinity to recombinant bovine vitronectin (bVTN). Meanwhile, none of the *Francisella* proteins showed affinity to

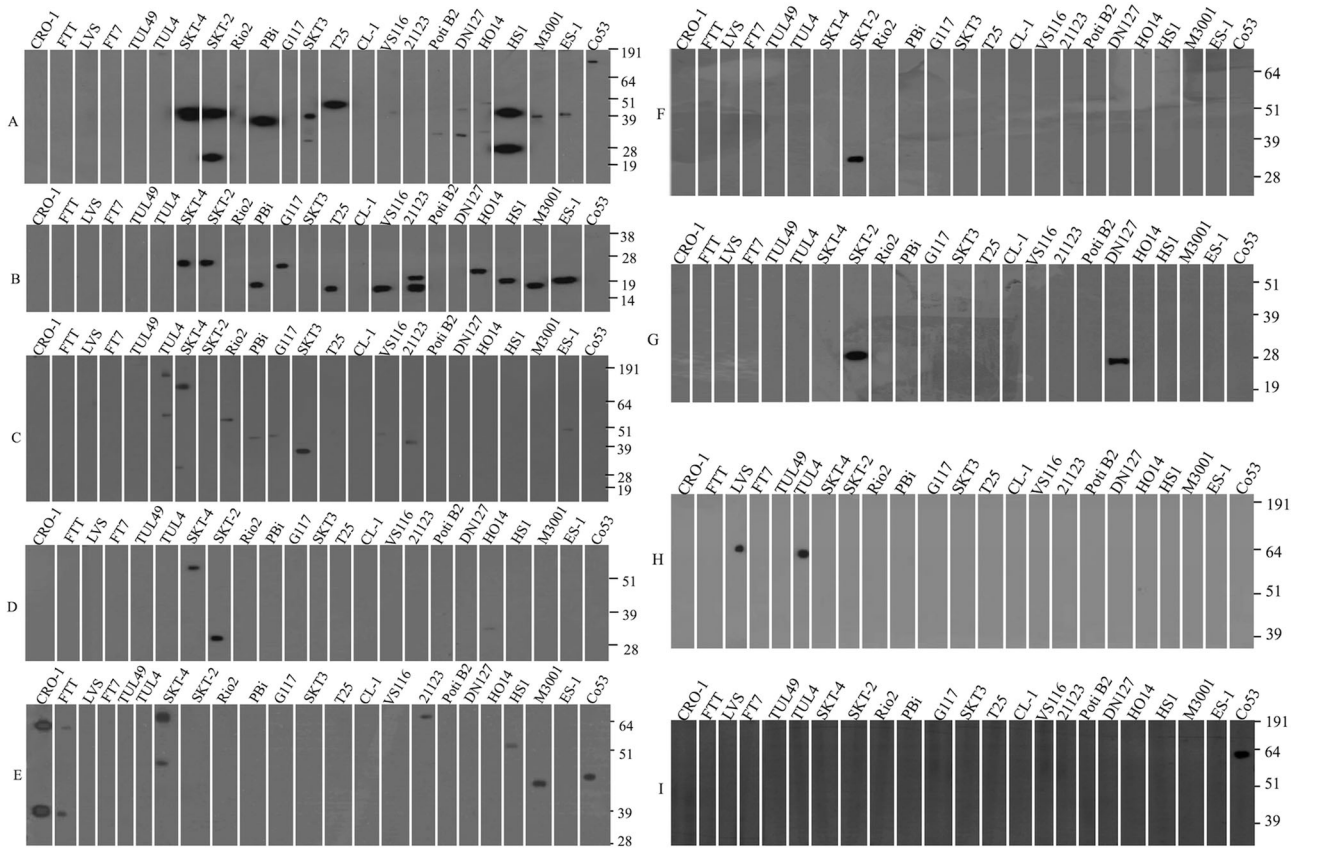

Fig. 2 Affinity ligand binding assays to unfold binding and mimicry of CRPs by *Francisella and Borrelia*. ALBI assays for detection of *Francisella* and *Borrelia* OMPs showing affinity to recombinant hC4BP (panel A); hFH (panel B); hVTN (panel C). Results of ALBI assay performed with human serum are not presented in the manuscript to avoid repetition. Putative human CD46 and CD59 mimicking ligands on *Borrelia* or *Francisella* are presented in panels D and E, respectively. Interaction between bacterial surface proteins and animal CRPs are presented in panel F (oC4BP), panel G (oFH), panel H (oVTN) and panel I (bFH). The molecular masses of the markers for each assay are presented in the right side of panels.

other recombinant bovine or ovine or human C4BP and FH (Fig. 2).

Mass spectrometry identification performed for oVTN binding 64 kDa protein of LVS and hVTN binding 58 kDa protein of Tul4 gave the most relevant match with a lipoprotein (GenBank – gi|56708240, Table S2, ESI†). A BLAST search performed for this protein showed homology with tetratricopeptide repeat domain protein from *Francisella holarctica* (Uniprot – A7NBF2_FRATF). Tetratricopeptide repeat domains take part in several protein–protein interactions, which may suggest a novel potential candidate in the repertoire of CRP binding proteins. Mass spectrometry identification of 160 kDa protein of Tul4 failed to give any confident protein match.

Ability of *Borrelia* to bind C4BP, FH and VTN

Unlike *Francisella*, several *Borrelia* strains possessed surface proteins that showed affinity for soluble CRPs (Fig. 2 and Table S1, ESI†). Surface proteins of some of the *Borrelia* strains (SKT-2, SKT-4, PBi, SKT-3, T25, and HS1) showed evident binding with hC4BP. On the other hand, proteins of *B. valaisiana* (VS116), *B. lusitaniae* (Poti B2), *B. bissettii* (DN127), and *B. japonica* (HO14) showed weaker binding to hC4BP. Most of the hC4BP binding OMPs ranged between 20 to 49 kDa whereas OMP of *Borrelia coriaceae* (Co53) was the only high molecular mass protein (140 kDa). Proteins showing evident binding with hC4BP were subjected to pull down assays and identified as: Vlp (variable large protein, gi|426202330, 39 kDa of SKT-4), Vlp (gi|106534293, 41 kDa of SKT-2), OspA (outer surface protein A, gi|1507705, 35 kDa of PBi), Vlp (gi|75344820, 39 kDa of SKT-3), VMP (variable major protein, gi|1507653, 28 kDa of HS1) and Vlp (gi|106534293, 41 kDa of HS1) (Tables S1 and S2, ESI†).

An array of hFH binding proteins was observed among the *Borrelia* strains. All hFH binding candidates were low molecular mass proteins ranging between 17 kDa and 26 kDa. Mass spectrometry revealed that the identified proteins either belonged to the complement regulator-acquiring surface protein (CRASP) family (*e.g.* BaCRASP-1 of SKT-4 and BbCRASP-1 of SKT-2) or the hFH binding protein family of relapsing fever borreliae (FhbA) (Table S2, ESI†). The binding affinity of hVTN to surface proteins of *Borrelia* also differed from strain to strain, for example binding hVTN with 37 kDa protein of SKT-3 or 130 kDa protein of SKT-4 was documented with stronger signals than with proteins of other *Borrelia* (Fig. 2 and Table S1, ESI†). Most of the hVTN binding proteins of *Borrelia* were of low molecular weight (ranging between 30 to 55 kDa). The only high

molecular weight candidate which bound hVTN was a 130 kDa protein in SKT-4 strain. The 37 kDa protein of SKT-3 was identified as VlpA protein (gi|426202330). Surprisingly, we observed that SKT-4, PBi and VS116 strains could bind hC4BP, hVTN and hFH (Table S1 and Fig. S2, ESI†), while other *Borrelia* strains (SKT-2, T25, DN127, HO14, HS1 and M3001) showed hC4BP and hFH binding ability. G117 and 21123 strains bound both hVTN and hFH, while SKT-3 and ES-1 strains showed affinity to hC4BP and hVTN.

Very few proteins of *Borrelia* could bind ovine or bovine CRPs. A 34 kDa protein of SKT-2 interacted with oC4BP and was identified as outer surface protein A (gi|239835984). Only two strains, SKT-2 and DN127, could bind oFH through the same size of the proteins (26 kDa). Both proteins were identified as members of the complement factor H binding protein family (BbCRASP-1 and its ortholog, Table S2, ESI†). None of the *Borrelia* proteins showed affinity to oVTN or oC1inh. The only protein candidate, that showed affinity to bovine CRPs, was 60 kDa bFH binding protein of Co53 (*B. coriaceae*), which was identified as bacterial extracellular solute binding protein (BESBP, gi|195942064). None of the outer surface proteins of *Borrelia* showed affinity to bVTN or bC4BP.

Possible mimicry of CD59 and CD46 by *Francisella* and *Borrelia*

To assess whether *Francisella* and *Borrelia* strains express human CD35 or CD46 or CD59 mimicking molecules, western blotting with monoclonal antibodies was performed. The monoclonal anti-CD46 antibody showed affinity to *B. afzelii* and B.b.s.s. proteins (Fig. 2), which indicates that these strains might possess CD46 mimicking molecule or homologous motif. Anti-CD46 antibody also reacted with *B. japonica* protein; however the signal captured on the X-ray film was very weak (Fig. 2). Affinity of anti-CD59 antibody to proteins of *F. t. novicida* and *F. t. tularensis* also suggests the presence of CD59 like moiety among their surface proteins (Fig. 2). Among the several *Borrelia* species, *B. afzelii*, *B. andersonii*, *B. hermsii*, *B. parkerii* and *B. coriaceae* possessed proteins that may mimic CD59. No CD35 mimicking molecule was found in the surface proteome of *Borrelia* and *Francisella* species.

Complement sensitivity of *Borrelia* and *Francisella* strains

Complement sensitivity of *Borrelia* and *Francisella* strains was performed to find a correlation between CRP binding and bacterial resistance to complement-mediated lysis. Complement sensitivities of *Borrelia* and *Francisella* strains varied significantly, the least sensitive *Francisella* strain to human serum was Tul4 followed by LVS and Tul49 (all *F. t. holarctica*; Fig. 3 panel A). On the other hand, *Borrelia* strains SKT4, SKT-2, PBi, VS116, and HS1 were significantly resistant to human complement-mediated lysis (Fig. 3 panel A). The complement sensitivity of *Borrelia* and *Francisella* was also tested to ovine and bovine complement. We found significant resistance of LVS (11% bacteriolysis), Tul4 (8% bacteriolysis), SKT-2 (19% bacteriolysis) and DN127 (13% bacteriolysis) (Fig. 3 panel B) to sheep complement. However none of the *Francisella* and *Borrelia* strains, except *B. coriaceae*, was able to survive in the bovine complement (Fig. 3 panel C).

Francisella and *Borrelia* strains incubated in inactivated serum were alive even after 6 h of incubation, which indicates that bacterial death/lysis observed in the case of normal serum was due to complement-mediated killing, and no other non-specific bactericidal activities were involved.

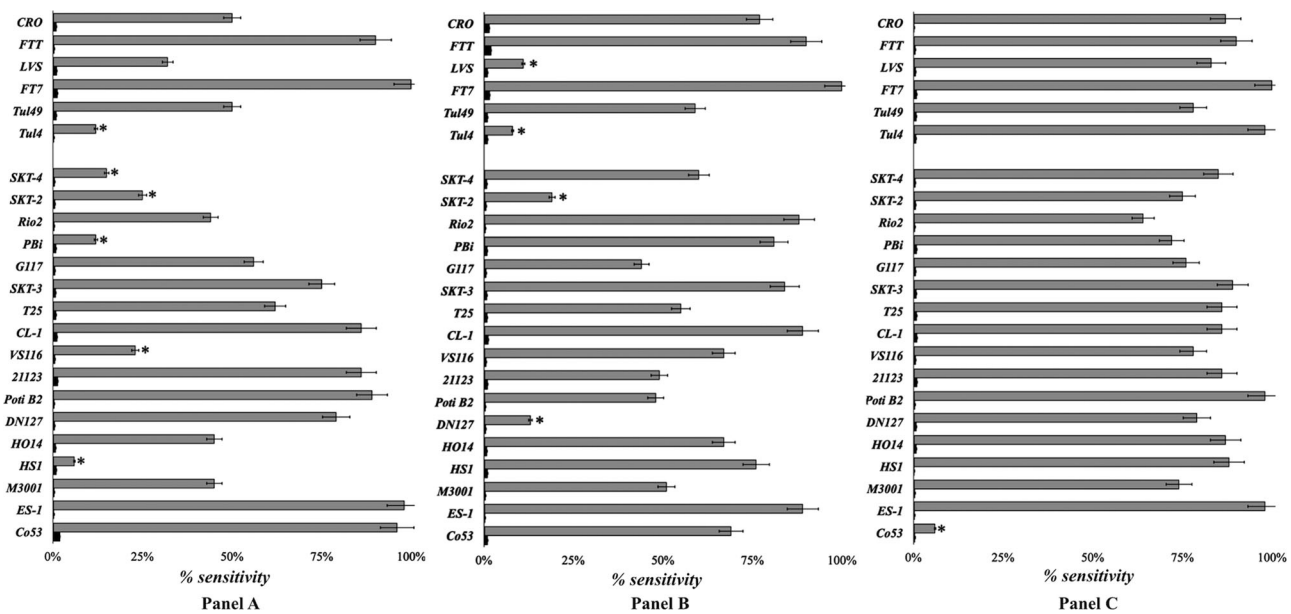

Fig. 3 Complement sensitivity of *Francisella* and *Borrelia* strains. Sensitivity of *Francisella* and *Borrelia* to human (panel A) sheep (panel B) and cattle (panel C) complement. Results are the average values from triplicate experiment (grey columns). * indicates significant ($p < 0.05$) resistance of *Francisella* and *Borrelia* strains to complement-mediated lysis. Black columns present bacteriolysis in inactivated serum.

Discussion

Borrelia possess multiple complement evasion strategies. Hitherto, binding of hFH and hC4BP, and mimicry of CD59 by *Borrelia* has been reported.[6,19,31,32] In the last decade, a plethora of reports has been published, presenting details of FH mediated complement evasion by *Borrelia*. However, very little is known about its ability to exploit other CRPs (like C4BP, C1inh, vitronectin, CD46, CD35 *etc.*). Up to the present, no report is available that describes a binding affinity between surface proteins of *Borrelia* or *Francisella* and various CRPs of human and animal origin.

In the present study, we showed that *Borrelia* could bind at least three soluble hCRPs: hC4BP, hVTN and hFH. *Borrelia* species, which are pathogenic to humans, such as *B. afzelii* (strain SKT-4) and *B. garinii* (strain PBi), showed evident binding of all three CRPs, while other pathogenic species like B.b.s.s. (strain SKT-2) and *B. hermsii* (strain HS1) could only bind hC4BP and hFH. Binding of hC4BP on the surface of Lyme disease related borreliae was reported earlier, in a study where a 41 kDa protein was the prominent hC4BP binding candidate and a 34 kDa protein showed weak affinity.[32] Here we present a wide repertoire of hC4BP binding proteins of *Borrelia*, including the earlier described 41-kDa protein, which was identified as Vlp protein. VMP and OspA were two more hC4BP binding proteins identified by mass spectrometry. Interestingly, OspA showed affinity to human as well as ovine C4BP (Table S2, ESI†). Although many of the *Borrelia* strains expressed surface proteins which could bind hC4BP, proteins of pathogenic borreliae like *B. afzelii*, *B.b.s.s.*, *B. garinii* (PBi) and *B. hermsii* showed stronger affinity to hC4BP than proteins from less pathogenic species such as *B. andersonii*, *B. bissettii*, *B. lusitaniae* and *B. japonica* (Fig. 2).

No reports have presented possible exploitation of VTN by *Borrelia* or *Francisella*. Tul4 showed hVTN binding with its 160 kDa and 58 kDa proteins, whereas other *F. t. holartica* strains (Tul49, LVS and FT7) failed to bind vitronectin. The 58 kDa protein of Tul4, identified as functionally unknown hypothetical lipoprotein (gi|56708240), was able to bind VTN from human as well as an ovine origin; however its ortholog in LVS interacted only with ovine vitronectin. *Francisella* infects preferentially phagocytic cells, however recent evidence suggests that during active infection a significant number of bacteria can be found in the blood plasma fraction.[33] It is also known that some *Francisella* strains are relatively resistant to complement-mediated lysis.[34] Vitronectin binding may explain how some *Francisella* strains survive extracellularly and effectively evade complement attack. *Borrelia*, *B. afzelii* and *B. garinii* strains showed binding ability to hVTN but none of the strains expressed proteins that could bind ovine or bovine VTN. Binding of VTN by other complement resistant pathogens was demonstrated earlier with *Haemophilus* and *Neisseria* using protein E and Opa50, respectively.[15,35]

Recent studies have shown that StcE, a metalloprotease secreted by *E. coli* O157:H7, binds and localizes functional C1inh to the cell membrane and enhances the ability of C1inh to regulate complement effectors at sites of lytic complex formation.[36] It has been proposed that some pathogens, such as *Bordetella pertussis*, may upregulate C1ihn binding proteins exclusively in an active infection and/or a virulent phase to avoid activation of the classical complement cascade.[3] Binding of C1inh on the bacterial surface might be crucial to maintain the infection. Surprisingly, none of the proteins of *Francisella* and *Borrelia* showed affinity to human or bovine or ovine C1inh.

In order to inhibit complement, some pathogens can also take advantage of their endogenously expressed complement-like molecules mimicking the host complement. Well known examples are the parasitic (*Trypanosoma*) or viral (vaccinia virus, *Herpes simplex* virus) proteins related to mammalian DAF (decay accelerating factor), C4BP or CD59.[37–39] The mimicry of CD59 is quite remarkable, including functional and structural similarities of CD59 to ORF-15 of *Herpesvirus saimiri*,[39] 170 kDa adhesion of *Entamoeba histolytica*[40] or the SCIP-1 protein of *Schistosomamansoni*.[41] Mimicry of CD59 by *Borrelia* has also been described previously.[19] In the present study, we found additional putative protein candidates in *Borrelia* (SKT-4, 21123, HS1, M3001 and Co53) and *Francisella* strains (CRO-1 and Tul4) that might mimic CD59. Another membrane associated CRP, CD46 or membrane cofactor protein (MCP) is widely expressed in human cells and plays a complex role in pathogen invasion. It helps internalization of pathogens and affects cytokine production by macrophages and T-cell activation. Interaction between pathogen ligands and CD46 results in its downregulation from the cell surface, increasing susceptibility to complement-mediated lysis.[42] Molecular mimicry of the MCP by *Vaccinia virus* (gp35), human herpesvirus 8 (ORF-4) and *Herpesvirus saimiri* (ORF-4) has been reviewed earlier.[43] We found two CD46 like proteins in *Borrelia* (SKT-4 – 60 kDa and SKT-2 – 30 kDa); however it seems that *Francisella* strains do not possess a CD46 mimicking molecule.

Many microorganisms have an elaborate arsenal of multiple defence strategies, which are required at different stages within the complement cascade. For example, *Schistosoma mansoni* can bind to C1q, exploits DAF to accelerate decay of surface bound C3, cleaves C9 and encodes a protein mimicking CD59. Similarly, *Streptococcus pyogenes* expresses M protein that binds FH, FHL-1 and C4BP. Thus exploitation of multiple complement regulators might be an efficient way to ensure successful evasion of complement-mediated lysis. It seems that a sophisticated and elaborate arsenal for successful complement evasion has also been evolved in *Borrelia*. *B. afzelii* (SKT-4) and *B. garinii* (PBi) are the model candidates among *Borrelia*, which exploit multiple hCRPs and resist complement-mediated lysis (Table S1, Fig. S2 and S3, ESI†).

The pattern of serum complement sensitivity of *Borrelia* matches the known reservoir status of many vertebrate species for *B. burgdorferi* sensu lato. Several studies have indicated that *B. garinii* and *B. valaisiana* can infect birds and are transmitted to ticks by avian hosts, whereas, *B. afzelii* is transmitted to ticks *via* rodents.[44,45] Significant resistance of SKT-2 to ovine complement (only 19% sensitivity) observed in this study suggests that B.b.s.s. can infect sheep. Resistance of B.b.s.s. to sheep serum

complement might be mediated through BbCRASP-1:FH and OspA:C4BP dyad formations on the bacterial surface. Although the Lyme disease symptoms are not seen commonly in sheep, some researchers have reported localized infection of sheep skin with evident erythema migrans and arthritis.[24,25,46] Resistance of *B. bissettii* to sheep complement (only 13% sensitivity) was also observed in this study. This serum resistance might be mediated by 26 kDa FHBP (ortholog of BgCRASP-1) (Table S2, ESI†). The pathogenic nature of the *B. bissettii* has not been clear, however, recent reports have shown its pathogenic nature.[47–49] Based on the complement sensitivity (Fig. 3, panel B) and oCRPs binding ability of different *Borrelia* and *Francisella* strains, it can be supposed that B.b.s.s. (SKT-2), *B. bissettii* (DN-127), and some of the *F. t. holarctica* strains (Tul4 and LVS) may have the potential to infect sheep. None of the *Borrelia* and *Francisella* strain, except Co53 (*B. coriaceae*), was able to exploit bovine CRPs to resist to complement-mediated lysis. This explains why *B. coriaceae* can establish infection in cattle while other *Borrelia* species fail.

In summary, this study revealed the ability of *Francisella* and *Borrelia* to exploit CRPs from three different hosts. Our study also revealed novel putative protein candidates of *Francisella* and *Borrelia* that bind CRPs. Identification and characterization of the molecules circumventing the host complement system is a challenging and rapidly developing field. Practically every invading pathogen must take measures to evade the complex complement system, which explains why multiple strategies of complement evasion have evolved. Basic understanding of complement evasion mechanisms and characterization of protein candidates involved in the evasion are important aspects to develop therapeutic or preventive approaches.

Conflicts of interest

All authors declare that they do not have any conflict of interest.

Acknowledgements

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