Inhibition of the complement system is a characteristic trait of ecotin orthologs from opportunistic pathogens

36. OTDK Biology Section

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CCP	Complement control protein
***eco* KO**	Ecotin-knockout
EDTA	2,2',2",2"'-(Ethane-1,2-diyldinitrilo)tetraacetic acid
EGTA	Ethyleneglycol- bis(β-aminoethyl)-N,N,N′,N′-tetraacetic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K_I	Equilibrium inhibitory constant
LB	Luria-Berthani medium
MBL	Mannose binding lectin
MASP	MBL-associated serine protease
MAC	Membrane attack complex
NE	Neutrophil elastase
NHS	Normal human serum

1. INTRODUCTION

1.1 The complement system

The complement system was discovered over a century ago as a heat-sensitive serum component that ‘complemented‘ the heat-stable antibody–based component in essential antimicrobial and phagocytic processes (1). These days the complement system is known as a vital component of innate immunity, which is interconnected with the adaptive immune system. It is a cascade-structured surveillance system of around 40 fluid-phase and membrane-bound proteins acting in the blood, the lymph, on self and non-self cellular surfaces and in some cases inside the cells. Besides eliminating pathogens, its functions include clearance of apoptotic cells, mediation of inflammation and maintenance of immuno-homeostasis. It is connected and contributes to other vital processes such as blood coagulation, fibrinolysis, and embryonic development of the brain (2, 3).

The complement system activates through three routes (Fig. 1), the classical, the lectin and the alternative pathways. The classical and the lectin pathways follow similar activation scheme: one or more pathway-specific pattern recognition molecules carrying pathway-specific serine protease zymogens bind to i.e. ‘recognize’ a surface; the zymogens become partially active and activate each other through limited proteolysis; this step is often referred to as ‘auto-activation’. The fully active serine proteases activate the remaining zymogens and other complement system components in a cascade-like manner. Several complement proteins become cleaved in two parts: a larger, usually surface-bound ‘b’ fragment and a smaller, fluid-phase ‘a’ fragment.

1.1.1 The activation pathways

The classical pathway activation is initiated when its pattern recognition molecule, C1q simultaneously binds to multiple copies of antibodies densely packed either on cell surface or in fluid-phase immunocomplexes. C1q carries two copies of zymogen C1r and zymogen C1s. Upon target binding, C1q changes its conformation allowing the two C1r zymogens to activate each other. Active C1r molecules activate the C1s zymogens and active C1s cleaves C4 into C4a and C4b. The previously hidden thioester of C4 emerges on the surface of C4b, covalently binds to the surface and associates with C2, a zymogen serine protease. C4bC2 is cleaved by

C1s creating C4b2a, which is a C3 convertase that activates C3, the central component of the complement system (4).

The lectin pathway activation is antibody-independent: the lectin pathway has many diverse pattern recognition molecules that bind to various carbohydrates and acetylated compounds common on pathogens and altered self cells, but rare on healthy self cells. The pattern recognition molecules set includes mannose binding lectin (MBL), ficolins and collectins. The lectin pathway pattern recognition molecules carry zymogen MASP (MBL associated serine protease) enzymes, MASP-1 and MASP-2 that have similar functions to those of C1r and C1s. As our research group revealed, the binding of the lectin pathway specific pattern recognition molecules to a surface leads to MASP-1 auto-activation and active MASP-1 activates MASP-2 (5). MASP-1 cleaves C2, but it cannot cleave C4. MASP-2, on the other hand, cleaves both C2 and C4 generating the same C4b2a C3 convertase produced by the classical pathway (6). Although MASP-3 is also present in lectin pathway pattern recognition molecules, it is an alternative pathway component, the sole pro-factor D activator in resting blood (7).

Pattern recognition molecule binding of C1r, C1s, and the MASPs requires Ca^{2+} ions, while C4b2a formation needs Mg^{2+}, therefore classical and lectin pathway activation is both Ca^{2+}- and Mg^{2+}-dependent. Through their reactive thioester bonds, C3b and C4b bind covalently to the surface.

The alternative pathway constantly activates at a low rate by a unique mechanism called ‘tick-over’ (2, 8). Due to a rare conformational change, the otherwise buried thioester of C3 becomes accessible and becomes hydrolyzed. This results in a fluid-phase C3(H_2O) that has a C3b-like conformation, and binds factor B. In the C3(H_2O)B complex, factor B is cleaved by factor D generating C3(H_2O)Bb, a fluid-phase alternative pathway C3 convertase. C3(H_2O)Bb cleaves C3 to fluid-phase C3b, which binds factor B, and with factor D generates another fluid-phase alternative pathway C3 convertase, C3bBb. Once some of the C3b binds covalently to a nearby surface, a surface-bound C3bBb alternative pathway C3 convertase is formed that rapidly produces many surface-bound C3b, which serve as starting points for more C3bBb. The alternative pathway can use C3b generated by any of the three pathways providing a general positive feedback loop for the complement system (8).

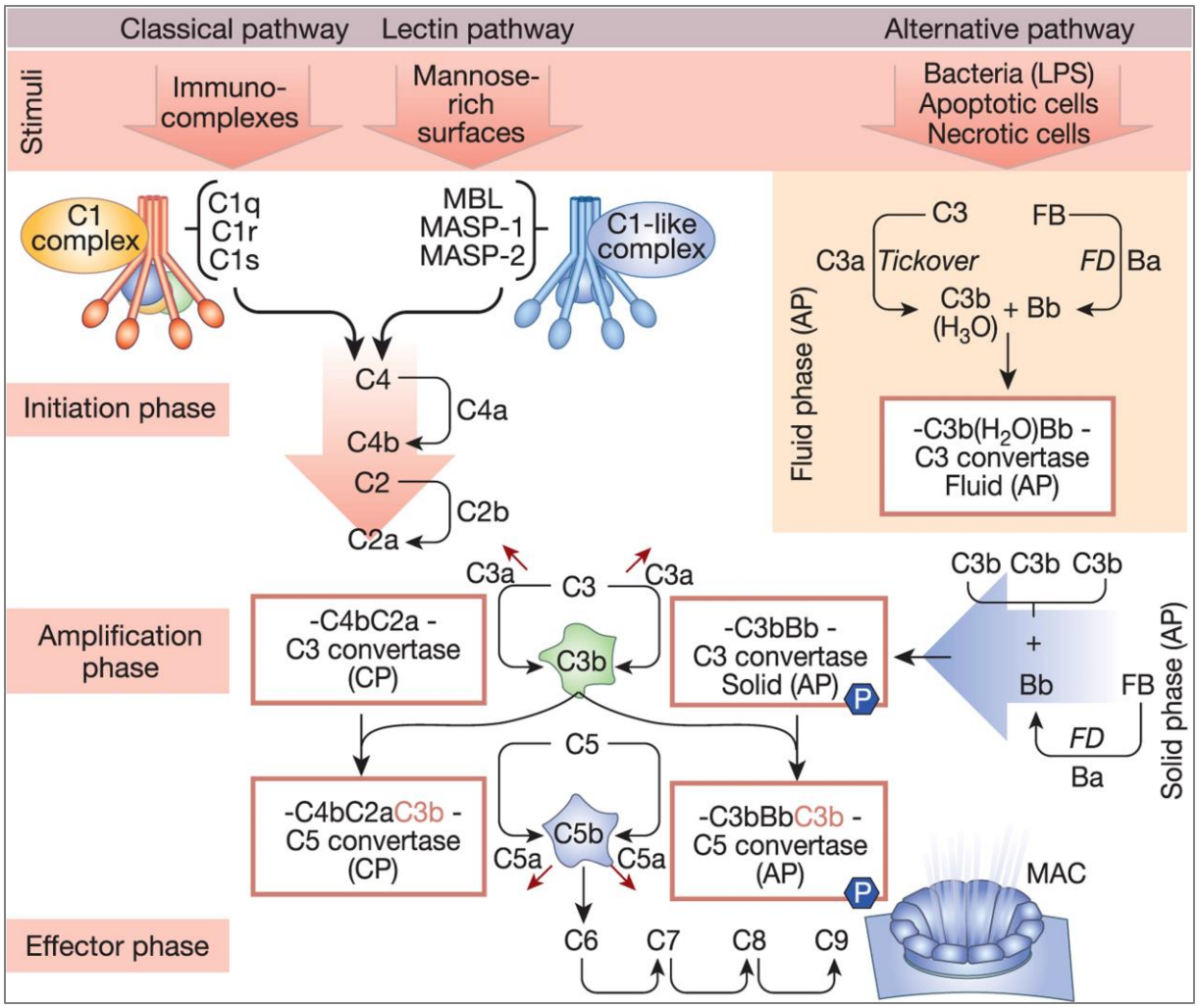

Figure 1. Activation pathways of the complement system

Activation of the classical and the lectin pathways is initiated when their dedicated pattern recognition molecules bind to a surface. The alternative pathway does not have a pattern recognition molecule. Sequential activation of complement proteins of the three cascades converge in the terminal pathway (9).

High level complement system activation leads to high C3b surface density yielding C4b2aC3b and C3bBbC3b, which are C5 convertases that preferentially cleave C5 instead of C3 (10).

C5 convertases mark the initiation of the terminal complement pathway. C5 is cleaved into C5a, an anaphylatoxin, and C5b. The latter binds C6, C7, C8 and C9 forming a C5b-9 ring, the membrane attack complex (MAC), which leads to cell lysis (10).

1.1.2 Complement proteases

Complement proteases are serine proteases with trypsin-like specificity. The classical and the lectin pathway proteases share a homologous six-domain composition. The three N-terminal domains govern dimerization and pattern recognition molecule binding. The C-terminal CCP1-CCP2-SP (Fig. 3) part (CCP, 'complement control protein') carries the substrate recognition and catalytic functions. The corresponding recombinant protein is called catalytic fragment (11).

1.2 Complement protease inhibition: ways of complement system regulation and immune evasion

Complement system activation occurs through sequential serine protease activation and can be kept at bay by serine protease inhibitors. Both the host and the invading pathogens produce complement system regulatory proteins: the host to maintain immune homeostasis, the pathogen to evade the immune response (12, 13). Microbial complement system regulators essential for the survival of the pathogen are identified as virulence factors (13, 14). As rapid spreading of multidrug-resistant pathogens raises the global death toll, better understanding of these complement system regulators is critically important. The simplest pathogen strategy to cease complement system activation is based on serine protease inhibitors.

1.2.1 Classification of protease inhibitors

For substrates and substrate-like inhibitors Schechter and Berger (15) introduced the following nomenclature: residues N-terminal from the scissile peptide bond are numbered P1-P2-P3 etc., while those toward the C-terminus P1'-P2'-P3' and so on. The scissile bond is between P1 and P1'. P residues of the substrate/inhibitor fit into various S ('S' stands for subsite) binding sites of the protease having characteristic geometries and electrostatics based on the enzyme residues that form them. The P1 of the substrate or inhibitor fits into a usually deep S1 pocket (Fig. 2).

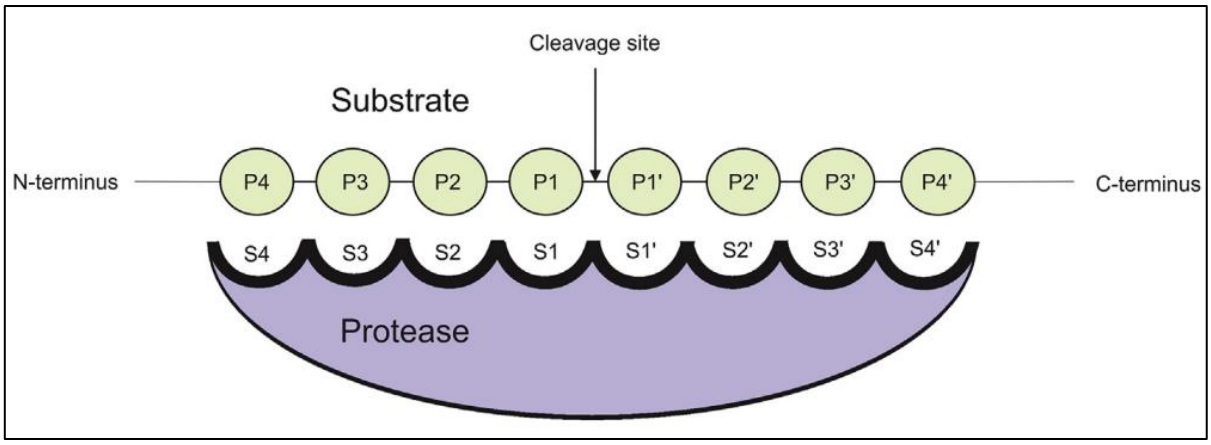

Figure 2. The Schechter-Berger nomenclature

The residue positions (P) in the substrate or inhibitor are numbered upstream and downstream in relation to the scissile bond. The complementary substrate binding sites (S) of the enzyme are numbered according to the P residues they interact with (15).

Based on their molecular mechanism of action protease inhibitors are canonical, non-canonical, or serpin type (16). Canonical inhibitors bind in a substrate-like manner reversibly to the enzyme. The inhibitor:protease complex resembles a protease-substrate Michaelis complex. High target specificity can be achieved through a proper inhibitory binding loop sequence that binds tightly only a few proteases. Usually, the energetically most important inhibitory loop residue is the P1 (Fig. 2). Ecotin, the main subject of my investigation, is a canonical inhibitor (16).

1.2.2 Ecotin, a canonical, but non-conventional serine protease inhibitor

Ecotin (*Escherichia coli* trypsin inhibitor) is a homodimeric serine protease inhibitor first isolated from *E. coli* (17). It has unprecedentedly wide protease specificity: besides pancreatic trypsin, chymotrypsin and elastase it inhibits proteases of the contact, coagulation, fibrinolytic and complement systems of the human plasma (17–20).

This broad specificity is due to several structural features of ecotin. Ecotin has a P1 Met that is not optimal for most target proteases but appears to be a great compromise. The Met side chain has exceptional flexibility and accommodates to very different S1 pockets. Moreover, besides the primary binding site, Site1 comprising the 80s (canonical inhibitory loop) and the 50s loops, ecotin also has a secondary binding site (Site2), comprising the 60s and 100s loops. In the ecotin homodimer stabilized by long overlapping C-terminal arms, Site1 and Site2 from two protomers (Fig. 3) constitute a structurally preformed combined binding site. This way an

imperfect fit at Site1 can be compensated by a perfect fit at Site2 and vice-versa (21). Relative roles of Site1, Site2 and their pre-formed spatial arrangement are highly protease dependent (22, 23).

By observing that ecotin inhibits all major pancreatic proteases while does not inhibit any *E. coli* proteases, Chung and colleagues suggested that ecotin protects the bacteria from host proteases present in the gut (17). Later on, many additional proteases were shown to be inhibited by ecotin including neutrophil elastase (NE), blood clotting factors Xa and XIIa, plasma kallikrein and the three MASP enzymes (18–20, 24). Ecotin orthologs are present in many Gram-negative bacteria and are more common in pathogenic than other microbes (25). It has become widely accepted that ecotin is a virulence factor of several opportunistic pathogenic microbes (26–28).

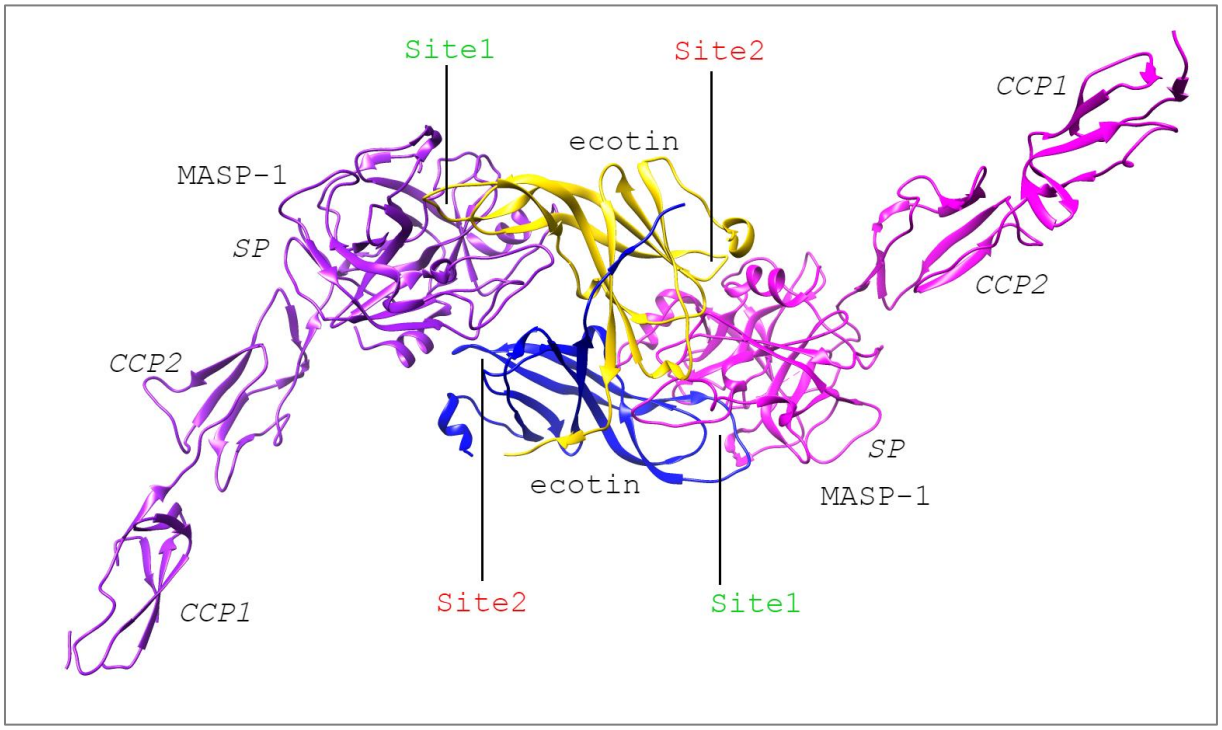

Figure 3. The molecular structure of Site1R ecotin:MASP-1 complex

Ecotin protomers (yellow, blue) contact the catalytic fragment (CCP1-CCP2-SP) of MASP-1 (magenta, purple) through their Site1 and Site2 binding surfaces (PBD ID: 7PQO).

1.2.3 Ecotin-expressing pathogens

As I pointed out, many Gram-negative bacteria express ecotin. Orthologs from the following eight opportunistic Gram-negative pathogens were chosen for investigation and are briefly introduced:

Burkholderia pseudomallei is an opportunistic intracellular pathogen causing melioidosis. Its ecotin has a P1 Leu. Using *eco* KO cell lines, Ireland and his colleagues demonstrated that *B. pseudomallei* ecotin contributes to the growth rate of *B. pseudomallei* in infected macrophages, and lack of ecotin leads to attenuated infection and melioidosis in mice (27).

Escherichia coli ecotin is the most extensively studied ortholog (17). There are many opportunistic pathogen *E. coli* strains.

Klebsiella pneumoniae is normally found in the mouth, the intestine and the skin. Due to aspiration the pathogen occupies organs outside its natural *niche* causing severe infections. It is part of the ESKAPE group of pathogens, which are known for rapidly developing antibiotic-resistance (29).

Pseudomonas aeruginosa is also an ESKAPE member. It causes ~10% of nosocomial infections, such as pneumonia and sepsis (29).

Pseudomonas fluorescens can cause acute infections in the bloodstream, in the respiratory system and may be associated with inflammatory bowel disease (30). *P. fluorescens* ecotin has a P1 Arg. Since the P1 residue is a major specificity and affinity-determinant for most protease-canonical inhibitor interactions, and since complement serine proteases prefer a basic residue at the P1 position, we hypothesized that *P. fluorescens* ecotin might be a superior efficiency complement inhibitor.

Salmonella paratyphii and ***Shigella flexneri*** are intracellular pathogens. *S. flexneri* can infect the intestinal wall-lining epithelial cells causing diarrhea. *S. paratyphii* is a nontyphoidal serotype causing paratyphoid fever. Their ecotin is highly similar to that of *E. coli*.

Yersinia pestis is the causative agent of the most devastating pandemic in history, the plague. Clark and her colleagues reported *Y. pestis* ecotin as a virulence-associated protein (26).

1.2.4 Multiple sequence alignment

Multiple sequence alignment of ecotin orthologs reveals a considerable level of sequence heterogeneity. Nevertheless, the regions corresponding to the known primary and secondary binding sites of *E. coli* are rather conserved, suggesting that those segments have similar functional importance in the orthologs (Fig. 4). Eggers and colleagues reported, that 28 amino acid residues out of 142 are conserved among 16 proteobacterial ecotin orthologs (19).

Figure 4. Conserved positions of ecotin orthologs

After multiple sequence alignment using ClustalW, amino acid residues of ecotin orthologs identical with their *E. coli* homolog were plotted as dots using BioEdit. The highly conserved positions are overrepresented at the primary (indicated by green frames) and secondary binding site (indicated by magenta frames) regions. Interestingly, *B. pseudomallei* ecotin lacks a part of its secondary binding site, the 100s loop.

2. AIMS AND OBJECTIVES

Based on our previous observations related to *Escherichia coli*, *Pseudomonas aeruginosa* and *Yersinia pestis* ecotin, we hypothesized that complement inhibitory capacity might be a common trait of ecotin orthologs from pathogenic bacteria. To test this hypothesis, we extended our studies to ecotin orthologs from *Burkholderia pseudomallei*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens*, *Salmonella paratyphii* and *Shigella flexneri*, all being opportunistic pathogens.

I aimed to answer the following questions: (i) Are all the above ecotin orthologs potent complement inhibitors? (ii) Do these orthologs show different pathway inhibitory patterns? (iii) What is the affinity of ecotin orthologs to various complement proteases?

I aimed to express and purify recombinant forms of these ecotins, assess their protease inhibitory efficacy in *in vitro* enzyme inhibition assays on purified proteases and test their inhibitory potency on the three complement pathways in human serum.

3. MATERIALS AND METHODS

3.1 Reagents

All common reagents were from Sigma-Aldrich, Thermo Fisher Scientific, Molar Chemicals, VWR, and Bachem. Ecotin-knockout (*eco* KO) *E. coli* BL21 Star (DE3) was made as described (24).

Buffers and media:

- Luria-Berthani (LB) medium: for 1 L: 10 g Tryptone N1, 5 g Yeast Extract, 5 g NaCl
- MASP kinetic buffer: 20 mM HEPES, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 150 mM NaCl, 0.1% Triton X-100, pH 7.5
- ELISA buffers:
    - Coating buffer: 50 mM $NaHCO_3/Na_2CO_3$, pH 9.6
    - NHS buffer: 20 mM HEPES, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 150 mM NaCl, 0.1% Tween 20, pH 7.5
    - TBS: 50 mM Tris-HCl, 150 mM NaCl, pH 7.5
    - Wash buffer: TBS with 5 mM $CaCl_2$ and 0.1% Tween 20
    - Antibody buffer: Wash buffer with 1% bovine serum albumin (BSA)
    - Reagent buffer: 50 mM K-citrate, pH 5.0

3.2 Recombinant complement proteases

MASP-1, MASP-2, MASP-3 and C1s catalytic fragments were from Prof. Péter Gál (31–34).

3.3 Expression and purification of the ecotin orthologs

Ecotins were expressed and purified as described (23), with some modifications. Briefly, synthetic genes of the ecotin orthologs were subcloned into the pET25b(+) expression vector enabling periplasmic expression of ecotins due to a built-in PelB periplasmic export signal. *E. coli* BL21 Star (DE3) *eco* KO cells were transformed with the pET25b(+) vector constructs and grown in LB media supplemented with 100 µg/ml ampicillin at 37 °C until reaching an optical density (OD_{600nm}) of 4. Expression of the ecotins was induced with 0.5 mM IPTG, and the cells were further incubated for 4 hours at 37 °C with vigorous shaking. After 4 hours of

expression, the cells were chilled on ice and centrifuged (4,500 x g, 4°C, 10 min). The cell pellet was resuspended in 1 mM ice-cold $MgCl_2$ and frozen at -20 °C. The hypo-osmotic *milieu* disrupts the periplasmic membrane, but the cytoplasmic membrane remains intact, allowing the isolation of the periplasmic fraction that contains the ecotin. The frozen cell suspension was gently thawed, the cell debris was sedimented (48,000 x g, 25 °C, 20 min) and ecotin was salted out from the supernatant (the periplasmic faction) by gradually adding 65% (w/v %) ammonium sulfate. The samples were centrifuged (48,000 x g, 25 °C, 20 min). The pellet was resuspended in 2.5 mM HCl and dialyzed against the same solution. After 3 hours the HCl solution was replaced by 150 mM ammonium acetate and the dialysis ran overnight at 4 °C. Aggregates were removed by centrifugation (48,000 x g, 4 °C, 20 min). The supernatant was frozen at -80 °C and lyophilized. The lyophilized protein was resuspended in 150 mM ammonium acetate and purified by size-exclusion chromatography on a Superdex 200 Increase 10/300 GL column, on an ÄKTA Pure system with 0.8 ml/min flow rate. The purified samples were lyophilized again and stored at 4 °C. Quality of the inhibitors was verified by mass spectrometry. Before use, ecotin concentration was determined by spectrophotometry using the following molar extinction coefficients (Table 1) calculated by the amino-acid sequence based ProtParam tool of the Expasy website:

Table 1. Extinction coefficient values (280 nm)

Ecotin orthologs	**Extinction coefficient**
E. coli	23045 $M^{-1}cm^{-1}$
Y. pestis	18575 $M^{-1}cm^{-1}$
P. aeruginosa	21555 $M^{-1}cm^{-1}$
B. pseudomallei	28545 $M^{-1}cm^{-1}$
S. flexneri	23045 $M^{-1}cm^{-1}$
S. paratyphii	27055 $M^{-1}cm^{-1}$
K. pneumoniae	20065 $M^{-1}cm^{-1}$
P. fluorescens	23045 $M^{-1}cm^{-1}$

3.4 Equilibrium binding assay

Equilibrium inhibitory constant (K_I) values of ecotins for C1s, MASP-1, MASP-2, and MASP-3 were determined according to Green and Work, with the modifications of Empie and

Laskowski (35, 36). Briefly, in each sample, the enzyme was applied at a constant concentration, while the inhibitor was added at different concentrations in MASP kinetics buffer and the mixture was incubated until reaching equilibrium. The concentration of the free enzyme [E] in inhibitor containing samples and that of in samples containing no inhibitor ($[E]_0$) were determined based on the initial rate of the enzyme-catalyzed chemical reaction using 2.5 mM Z-L-Lys-SBzl hydrochloride thioester substrate with 5 mM 5,5′-dithiobis(2-nitrobenzoic acid) co-substrate dissolved in a 1:1 ratio of H_2O and dimethyl sulfoxide. For the high-affinity *P. fluorescens* ecotin - MASP-1 interaction, Thiol Fluorescent Probe IV co-substrate was used (37).

The K_I values were calculated using the OriginPro Software with the following equation (38):

$$[E] = [E]_0 - \frac{[E]_0 + [I]_0 + K_I - \sqrt{([E]_0 + [I]_0 + K_I)^2 - 4[E]_0[I]_0}}{2}$$

where [E] and $[E]_0$ are the free and the total enzyme concentration, respectively, and $[I]_0$ is the total inhibitor concentration. In this assay, the rate of the chromogenic reaction (V_0) depends on the concentration of the free enzyme ([E]); hence, the $[E]/[E]_0$ ratios can be calculated from the ratios of the corresponding V_0 values.

Based on the literature, for enzyme-inhibitor pairs with K_I values higher than 2 nM the competition of the substrate with the inhibitor was also taken into account, the determined K_I was regarded as apparent K_I (K_I*) and was corrected with the K_M of the substrate – enzyme pair to calculate the genuine K_I according to Eggers et al. (19). The K_M values were determined previously (24, 39).

3.5 Normal human serum (NHS) preparation

Blood samples were collected from 10 informed healthy individuals after written consent, and serum was prepared from the pooled samples as described elsewhere (24).

3.6 ELISA

3.6.1 Classical and lectin pathway inhibition ELISA

For all three pathways, activation level of the complement system was determined by measuring the level of C3 fragment (C3c) deposition. C3c deposition was measured by ELISA

with the method of Kocsis et al. (38), with modifications. Greiner high-binding microtiter plates were coated with 10 µg/ml aggregated IgG (for classical pathway activation) or 10 µg/ml mannan (for lectin pathway activation) in coating buffer overnight at 4 °C. Free surfaces were blocked with 1% BSA in TBS for 1 h at 37 °C. NHS was diluted 50-fold in NHS buffer and incubated in loose tubes with serial dilutions of the ecotins for 30 min at 25 °C. The preincubated samples were applied to the wells and incubated for 30 min at 37 °C, followed by thorough washing. For C3c detection the primary antibody (rabbit polyclonal anti-human C3c Ab; A0062 DakoCytomation) was diluted 2,000-fold in antibody buffer, and was added in the wells and incubated for 1 h at 37 °C. After washing the wells, the secondary antibody (HRP-conjugated goat anti-rabbit Ab; A1949 Sigma) diluted 40,000-fold in antibody buffer was added and incubated for 30 min at 37 °C.

The plate was washed again, and the signal was developed with 100 µl/well of 1 mg/ml o-phenylenediamine dihydrochloride and 0.1% H_2O_2 in reagent buffer. The chromogenic reaction was stopped by adding 50 µl/well 1M HCl.

The absorbance of the samples was measured at 490 nm. Signal produced by NHS samples containing no inhibitor was considered 100%, while that corresponding to 20 mM EDTA containing samples was considered 0% (negative control). The inhibitor concentration providing 50% inhibition (IC_{50}) was calculated using the OriginPro Software.

3.6.2 Alternative pathway inhibition ELISA

C3c deposition was measured as described for the classical pathway and lectin pathway ELISA with the following modifications: (i) The wells were coated using 100 µg/ml zymosan. (ii) The NHS was diluted 9-fold in NHS buffer supplemented with 20 mM EGTA and devoid of $CaCl_2$ to prevent classical and lectin pathway activation.

4. RESULTS AND DISCUSSION

4.1 Recombinant protein expression and purification

I expressed and purified all ecotin orthologs using the same recombinant protein expression system. Purity and identity of the samples was verified with mass spectrometry on a Phenomenex Kinetex C8 (100A, 1.7 u, 100 × 2.1 mm) - Q-Exactive Focus (Thermo Scientific) LC-MS system. The measured molecular weight of ecotins matched the expected theoretical values and all samples showed at least 95% purity.

4.2 Binding affinities of the ecotins to complement proteases

I tested the inhibitory capacity of ecotin orthologs against MASP-1, MASP-2, MAPS-3, and C1s (Table 2, Fig. 5). Even though most ecotins inhibit all tested proteases, they also show characteristic differences regarding their affinities. K_I values of *E. coli*, *P. aeruginosa* and *Y. pestis* ecotins towards the three MASP enzymes and their inhibitory properties against the three complement pathways were previously published and discussed (24), but I included those results here for comparison.

Table 2. Comparison of K_I values of ecotins against complement serine proteases

Ecotin orthologs	C1s	MASP-1	MASP-2	MASP-3
	K_I (nM)			
E. coli	25150	4200	11.0	0.5
P. aeruginosa	1171	34	76	9.6
Y. pestis	38.5	35000	2.2	800
S. flexneri	4865	1819	9.6	2.3
S. paratyphii	2217	2008	4.6	28.0
B. pseudomallei	NI	NI	1243	25.5
P. fluorescens	308	0.1	6.1	2.6
K. pneumoniae	NI	1411	2787	27.7

NI: no inhibition

K_I values are also visualized in Figure 5:

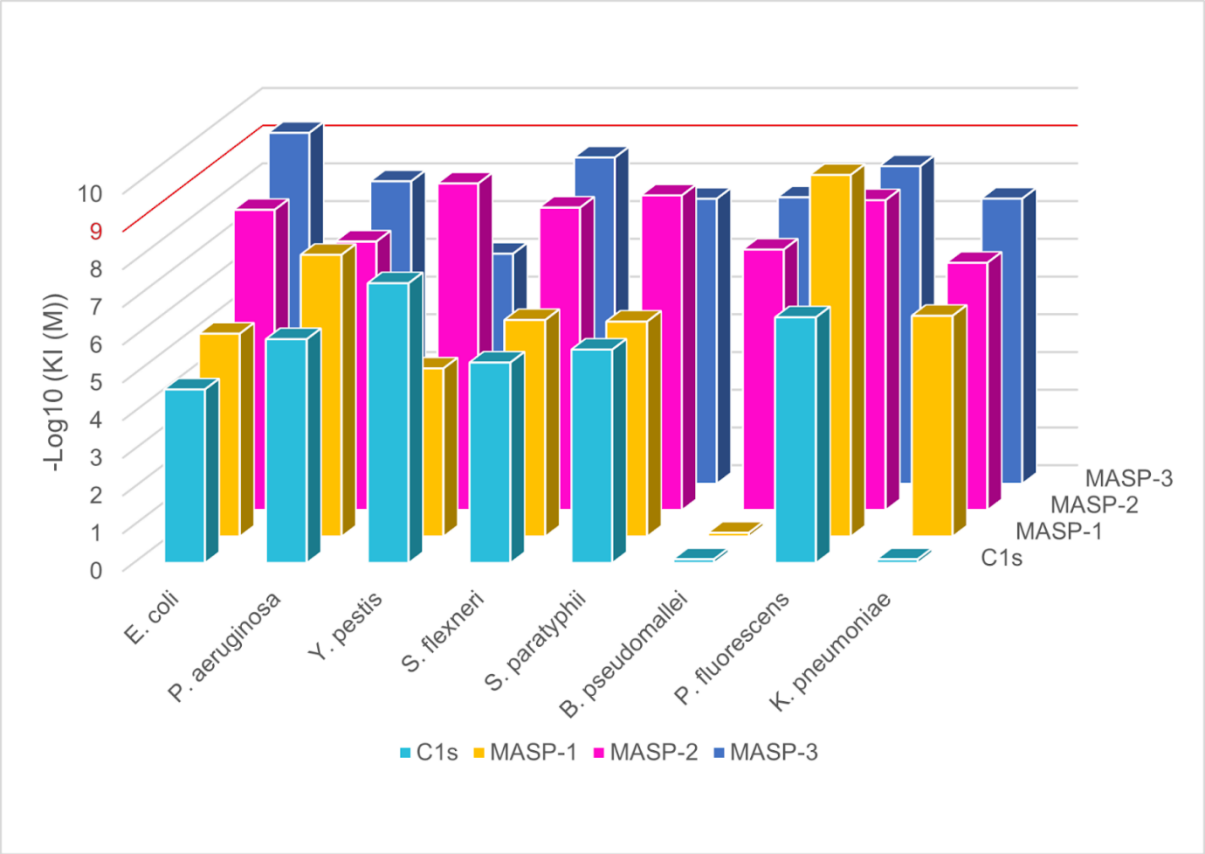

Figure 5. Equilibrium inhibitory constants of ecotins

The negative logarithm of the measured K_I values of ecotin orthologs towards the catalytic fragments of C1s, MASP-1, MASP-2, and MASP-3 are shown. The stronger the inhibition, the higher the columns are. The region above the red line corresponds to subnanomolar inhibition values.

4.2.1 *E. coli* ecotin is the most potent MASP-3 inhibitor

Most ecotins are strong inhibitors of MASP-3 (Table 2, Fig. 5), with the only exception of *Y. pestis* ecotin having a near-micromolar K_I. The strongest MASP-3 inhibitor is the *E. coli* ecotin with a K_I of 0.5 nM (24), closely followed by *S. flexneri* and *P. fluorescens* ecotins. High similarity of the *E. coli* and *S. flexneri* ecotin sequences explain their similar affinities. Potent inhibition of MASP-3 mainly relies on the interactions through the Site2 of ecotin (23), so it is not surprising that *P. fluorescens* ecotin is not the strongest MASP-3 inhibitor in spite of having a P1 Arg at its Site1.

4.2.2 Potent MASP-2 inhibition is a common trait of ecotins

Similarly, to the case of MASP-3, most ecotin orthologs have high affinity to MASP-2 (Table 2, Fig. 5), with K_I values in the nanomolar range. Again, the highly similar *E. coli* and *S. flexneri* ecotin orthologs have similar affinities. Interestingly, while *Y. pestis* ecotin is a weak inhibitor of MASP-3, it is a strong inhibitor of MASP-2, showing the highest affinity of all ecotins tested.

4.2.3 MASP-1 inhibition – a great challenge for ecotins

MASP-1 appears to be a challenging target, as most ecotins show low affinity towards MASP-1 (Table 2, Fig. 5) and *B. pseudomallei* ecotin does not inhibit it at all. The only two potent inhibitors of this protease are *P. aeruginosa* ecotin (K_I = 34 nM) and *P. fluorescens* ecotin (K_I = 0.1 nM). These traits can be explained through the structural data on MASP-1. The MASP-1 S1 pocket contains a self-inhibitory salt bridge between Asp640 and Arg677 enzyme residues, which hinders entry of the P1 residue of the inhibitor. Apparently, only a P1 Arg can efficiently compete with Arg677 (visualized in Fig. 6) to disrupt that blockade. This phenomenon was already demonstrated by our group with a P1 Arg *E. coli* ecotin mutant having a 7000-fold increased affinity on MASP-1 (23). Hence, my results are congruent with previous observations.

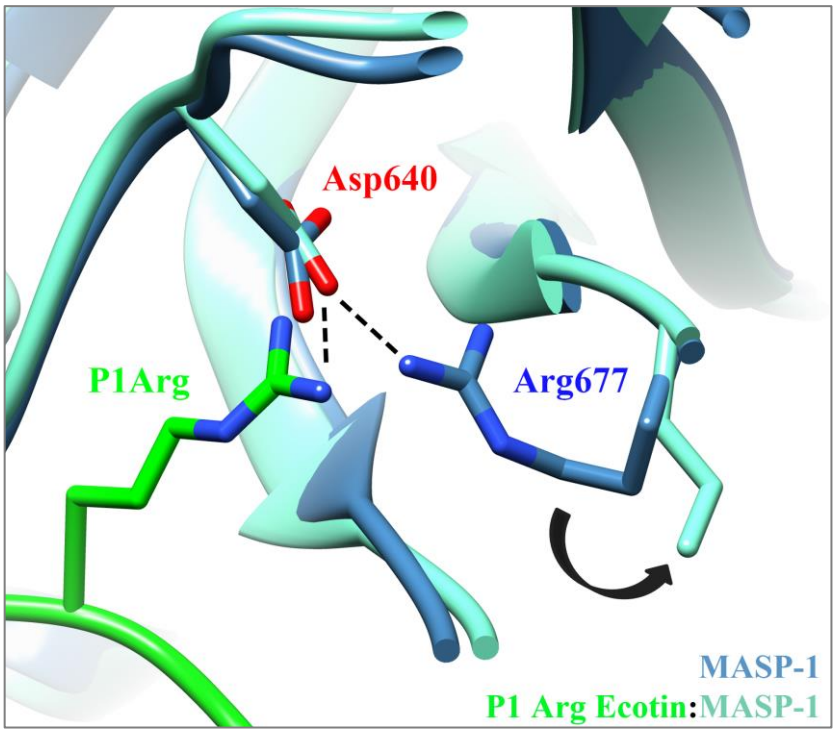

Figure 6. P1 Arg interaction with MASP-1 in the MASP-1:P1 Arg ecotin complex

Asp640 and Arg677 forms an intramolecular self-inhibiting salt bridge in the S1 pocket of MASP-1. Arginine mutant P1 *E. coli* ecotin can disrupt this by forming a new salt bridge with Asp640 (MASP-1, PDB ID: 3GOV; P1 Arg ecotin:MASP-1, PBD ID: 7PQO).

4.2.4 Most ecotins are weak C1s inhibitors

Most ecotin orthologs only weakly inhibit C1s (Table 2, Fig. 5). The two exceptions are the *Y. pestis* and *P. fluorescens* ecotins, which inhibit C1s with a strong and moderate affinity, respectively.

4.3 Complement pathway inhibition ELISA

I investigated the inhibitory effects of ecotin orthologs on the three complement pathways. As the pathways are driven by sequential activation of serine proteases, inhibiting even only one protease could result in overall inhibition of complement pathway activity. In the ELISA tests, I determined the inhibitor concentration needed for 50% inhibition of pathway activity (IC_{50}). The assays were based on detecting deposited C3c.

4.3.1 Only *P. fluorescens* ecotin is capable of classical pathway inhibition

Out of the eight ecotin orthologs only *P. fluorescens* ecotin inhibits the classical pathway, showing a ~ 6 μM IC_{50} value (Table 3, Fig. 7). These results are congruent with the serine protease inhibitory measurements, as *P. fluorescens* ecotin could moderately inhibit isolated C1s. An intriguing finding is that the high inhibitory potency of *Y. pestis* ecotin towards isolated C1s (Table 2) is not reflected in classical pathway inhibition, as demonstrated by the ELISA results (Fig. 7). This *ex vivo* result could be explained by two main mechanisms: (i) Other serum components may capture *Y. pestis* ecotin and decrease its 'effective concentration' against the classical pathway. (ii) Access to C1s is sterically blocked by other C1 complex components, such as C1r or C1q. In case of the other ecotins, lack of classical pathway inhibition is congruent with the K_I values, as the remaining six ecotin orthologs were either low affinity C1s inhibitors or at the applied concentration did not inhibit C1s at all.

Table 3. IC_{50} values on the classical pathway

Ecotin orthologs	**IC_{50} values**
E. coli	NI
Y. pestis	NI
P. aeruginosa	NI
B. pseudomallei	NI
S. flexneri	NI
S. paratyphii	NI
K. pneumoniae	NI
P. fluorescens	5847 nM

NI: no inhibition

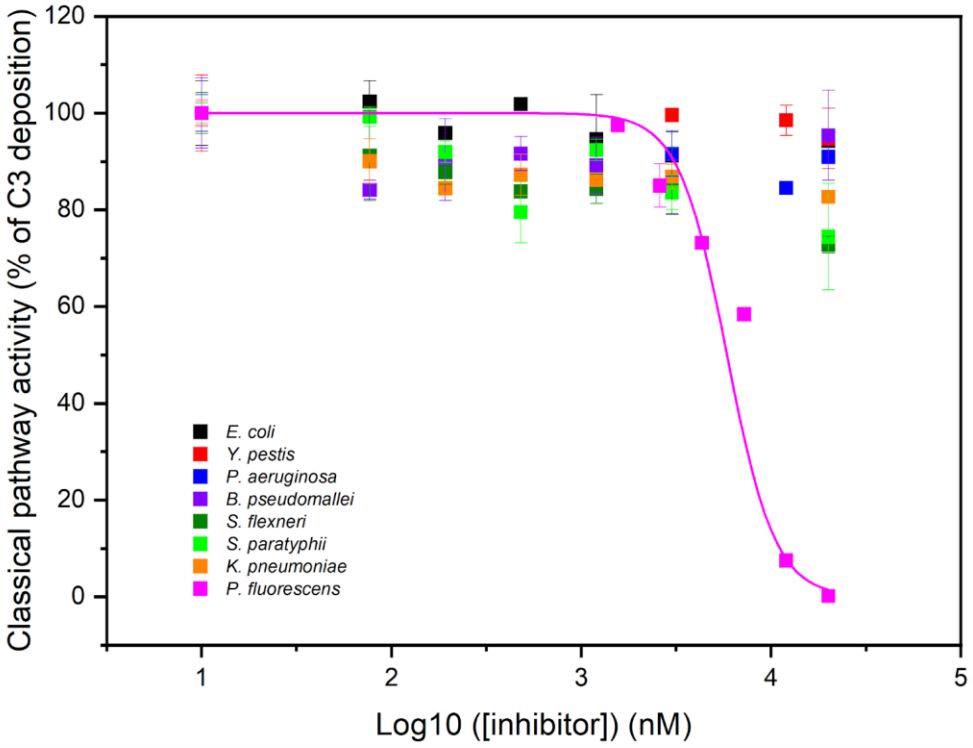

Figure 7. Classical pathway inhibition ELISA

Inhibitory effect of ecotin orthologs on classical pathway activation. The classical pathway was activated in 50-fold diluted NHS with 10 μg/ml aggregated IgG immobilized in the wells. Classical pathway activity as a function of inhibitor concentration was detected in the form of C3c deposition. While *P. fluorescens* ecotin could inhibit the classical pathway, the other ecotin orthologs could not exert any inhibitory effect even at high micromolar concentrations.

4.3.2 All ecotins are potent lectin pathway inhibitors

As expected, all ecotin orthologs are from potent to moderate lectin pathway inhibitors. The trend is as follows, stronger MASP inhibitors tend to be more potent lectin pathway inhibitors. The most potent lectin pathway inhibitor is the *P. fluorescens* ecotin (Table 4, Fig. 8), which potently inhibits both MASP-1 and MASP-2, followed by the *P. aeruginosa* ecotin, which is moderately efficient on both MASP-1 and MASP-2. While *Y. pestis* ecotin is one of the strongest MASP-2 inhibitors, it is one of the weakest MASP-1 inhibitor, which results in only a moderately potent lectin pathway inhibition, in line with our previous finding that MASP-1 inhibition can greatly contribute to lectin pathway inhibition (38). *B. pseudomallei* ecotin provides the same example: lack of MASP-1 inhibition cannot be fully compensated by a potent MAPS-2 inhibition. These results are congruent with previous findings, namely, that lectin pathway activation can be blocked by inhibiting either MASP-1 or MASP-2, but simultaneous inhibition of both MASP enzymes results in more potent lectin pathway inhibition (38).

Table 4. IC_{50} values on the lectin pathway

Ecotin orthologs	**IC_{50} values**
E. coli	282 nM
Y. pestis	380 nM
P. aeruginosa	78 nM
B. pseudomallei	851 nM
S. flexneri	316 nM
S. paratyphii	490 nM
K. pneumoniae	186 nM
P. fluorescens	42 nM

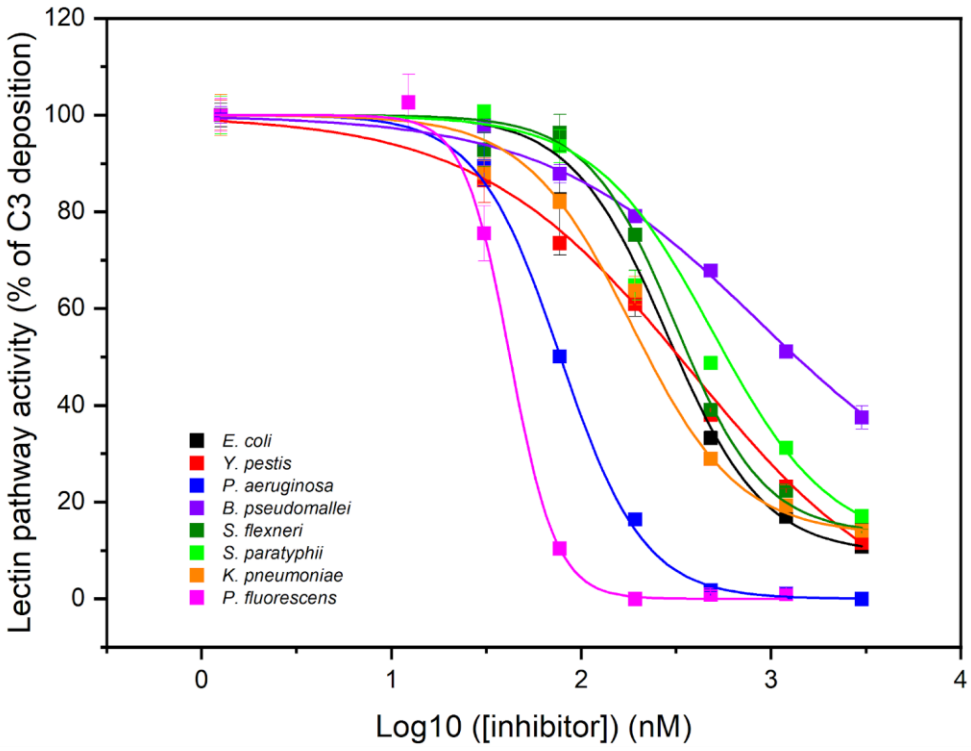

Figure 8. Lectin pathway inhibition ELISA

Inhibitory effect of ecotin orthologs on lectin pathway activation. The lectin pathway was activated in 50-fold diluted NHS with 10 µg/ml mannan immobilized in the wells. Lectin pathway activity as a function of inhibitor concentration was detected in the form of C3c deposition. The most potent MASP-1 and MASP-2 inhibitors, *P. fluorescens* and *P. aeruginosa* ecotins, are the most potent lectin pathway inhibitors, while ecotins with moderate to weak MASP-1 inhibitory potency are in overall weaker inhibitors of the lectin pathway.

4.3.3 All ecotins inhibit the alternative pathway

All ecotins block the alternative pathway (Table 5, Fig. 9), which can be done by two mechanisms, either by inhibiting the factor B component of the C3bBb convertase or by inhibiting factor D. Based on my preliminary non-included results, *P. fluorescens* and *B. pseudomallei* ecotins are the most effective factor D inhibitors and, accordingly, the most effective against the alternative pathway as well. The factor D inhibitory results are not included because the assay requires further optimization.

Table 5. IC_{50} values on the alternative pathway

Ecotin orthologs	**IC_{50} values**
E. coli	1911 nM
Y. pestis	6866 nM
P. aeruginosa	906 nM
B. pseudomallei	840 nM
S. flexneri	2034 nM
S. paratyphii	3643 nM
K. pneumoniae	1584 nM
P. fluorescens	264 nM

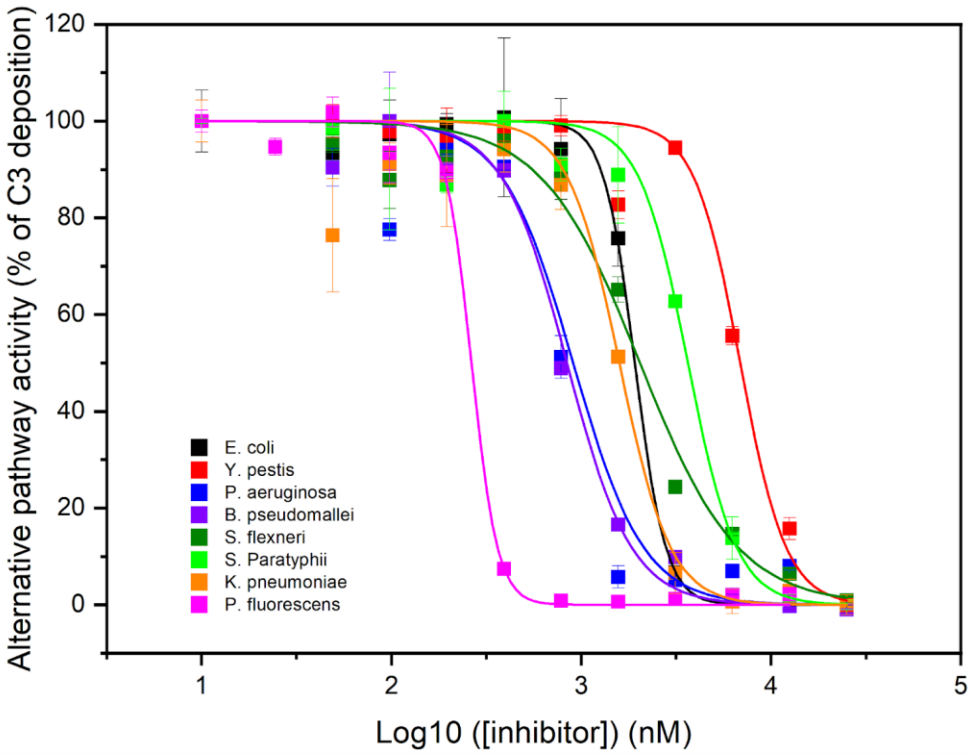

Figure 9. Alternative pathway inhibition ELISA

Inhibitory effect of ecotin orthologs on alternative pathway activation. The alternative pathway was activated in 9-fold diluted NHS with 100 μg/ml zymosan immobilized in the wells. Alternative pathway activity as a function of inhibitor concentration was detected in the form of C3c deposition. *P. fluorescens* ecotin is the most potent alternative pathway inhibitor as well as the most potent factor D inhibitor. Nevertheless, all ecotins inhibit the alternative pathway at least with a moderate potency.

5. CONCLUSIONS

During colonization of the host, the invading pathogens face a powerful first line defense arm of the immune system, the complement system. Based on my presented results, complement inhibition is an essential trait of most if not all Gram-negative pathogen bacteria, and ecotin is a key component in this immune evading mechanism. I found that it is the lectin and the alternative pathways that are the major targets of ecotin. Notably, these are the two complement system pathways that can immediately attack the invading pathogen even in the absence of preexisting specific antibodies. Therefore, protection against these pathways should be vital for the microbe. Moreover, if a pathogen can block the lectin pathway and the alternative pathway, it can avoid opsonization-based antigen presentation and could postpone or even avoid an antibody response. Nevertheless, pathogens that colonize the host for a prolonged time might benefit from classical pathway inhibition and *P. fluorescens* might be such an example. In terms of K_I and IC_{50} values, the 8 ecotin orthologs represent a diverse population, which might be a sign of slightly different immune evading strategies of the corresponding pathogens. As ecotin is a virulence factor for several opportunistic pathogenic microbes, ecotin-blocking compounds could be developed into novel antibiotics to render these invading species susceptible to complement mediated attacks.

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