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„The interaction of fibrinogen with *Escherichia coli*“

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Abstract

Escherichia coli is part of the normal human bacterial flora within the intestinal tract. The organism can turn into an opportunistic pathogen when entering the blood stream. Like other pathogens, *E. coli* interacts with proteins of the blood coagulation systems. In my diploma thesis I investigated how certain strains of *E. coli* interact with fibrinogen. I could show that *E. coli* K12 which is lacking all known fibrinogen binding proteins and virulence factors is able to bind purified fibrinogen. *E. coli* K12 can also bind fibrinogen in human plasma if the concentration of plasma is low enough and does not kill the bacteria. In contrast, the pathogenic *E. coli* O18 failed to bind fibrinogen in human plasma but was able to bind to purified fibrinogen. The binding to fibrinogen has a bivalent character - I could demonstrate that the presence of purified fibrinogen slows down the proliferation rate of *E. coli* in liquid culture, but at the same time it can increase the viability of *E. coli* K12 which is or has been exposed to citrated human plasma.

Introduction

Escherichia coli (*E. coli*) are gram negative, facultative anaerobic, rod-shaped bacteria that prefer to reside in the lower intestine of mammals in a symbiotic manner [1]. The bacteria are part of the normal flora of the gut, and among other benefits help the host organism by providing Vitamin K2 [2]. Due to the fact that *E. coli* is very widespread within the gut, it prevents other harmful bacteria from colonization [2]. Despite this, *E. coli* is responsible for a large number of infectious diseases and is one of the most frequent perpetrators for infections [3].

The gram negative *E. coli* differs significantly from gram positive bacteria concerning the structural assembly of the bacterial cell walls [4]. The bacteria have a much thinner peptidoglycan layer but an additional outer membrane where lipopolysaccharide (LPS) and proteins are anchored [4].

The lipopolysaccharide is the major component of the outer membrane and consists basically of three LPS domains [5] (Fig.1): 1) The Lipid A which is embedded in the cell membrane 2) The core oliogosaccharide domain consisting of the inner core and its unusual monosaccharides including kdo (3-deoxy-D-Manno-oct-2-ulosonic-acid) and L-glyero-D-mannoheptose. 3) In wildtype *E. coli* strains there are additional core-oligosaccharides and a distal O-antigen polysaccharide [5]. LPS protects the cell from mechanical damage due to its contribution to the structural stability and helps stabilizing the membrane structure by increasing the negative charge [6]. Furthermore it protects the cell from chemical attacks [6]. The O-antigen may play a role in evading the complement system defense and in the resistance against some antibiotics [7, 8, 9]. It is suggested that the O antigen of coccobacillus *Francisella tularensis* (*ft*) is responsible for a faster conversion of C3b to C3bi or at least that the O antigen may hinder the MAC complex from penetrating the cell surface in a special manner since it was shown that *ft* strain lacking the O antigen are susceptible to complement mediated lysis [10]. Strains with a fully developed O-Antigen are considered as "smooth" shaped compared to the rendering of the LPS, whereas strains lacking the O-Antigen are considered as "rough" [11]. In rough strains the LPS is more hydrophobic which makes it easier for hydrophobic antibiotics to penetrate the cell [12].

Protecting the host from invading pathogen microorganism is a complex and elaborate task for the immune system, and involves various cells of the innate immune system and a cell communication framework consisting of many different proteins [13]. This general and unspecific defense against harmful particles and microorganisms relies on the overall recognition of those [13]. Basically the innate immune system comprises leukocytes which derive from multipotent hematopoietic stem cells in the bone marrow [13]. The family of leukocytes include: mast cells, natural killer cells, eosinophils, basophils and the phagocytic cells including macrophages, dendritic cells and neutrophils [13].

Polymorphonuclear neutrophils (PMNs) are professional phagocytes in humans and, along with other granulocytes, the first cells facing invading pathogens [14]. Generally the PMNs circulate inactive within the bloodstream until they get activated by a chemotactic signal (mediated by cytokines) which attracts them to a specific location and allows them to attach to the endothelium via a set of different adhesion molecules like integrins, selectins and ICAMs (intercellular adhesion molecules) [14].

Once they are activated, PMNs are able to engulf an invading pathogen followed by the uptake of the bacteria into the intracellular phagosomes where they are destroyed by antibacterial and digestive agents after the fusion of the phagosome with a lysosome, creating the phagolysosome [15]. The bacterial uptake is accompanied by an enormous oxygen consumption caused by the generation of reactive oxygen species like superoxide which are released into the extracellular space where they can kill many different bacteria [16, 17]. For this, a NADPH oxidase complex at the phagosomal membrane is assembled which transfers electrons from cytoplasmic NADPH to the oxygen molecules [18, 19, 20].

LPS triggers an innate immune response via TLR4 receptors present on macrophages and endothelial cells [21, 22]. This leads to the expression and release of cytokines and adhesion molecules, oxygen radical production and the activation of the immune system [21, 22]. In vitro, the lipopolysaccharide-binding protein (LBP) can bind LPS and transfers it to CD14, the co-receptor of TLR4 at the surface of macrophages [23, 24].

After activation of TLR4 by LPS [25], the synthesis of TNF-A and IL1-B in macrophages and the production of tissue factor in mononuclear and vascular endothelial cells [26] are - among other mediators of inflammation and co-stimulatory factors required for

the adaptive immune response - upregulated [25, 26, 27]. This is a necessary procedure to accomplish a systemic clearance of invading pathogens which can turn into life-threatening conditions like septicemia, septic shock, disseminated intravascular coagulation (DIC) and multiple organ failure [28, 29].

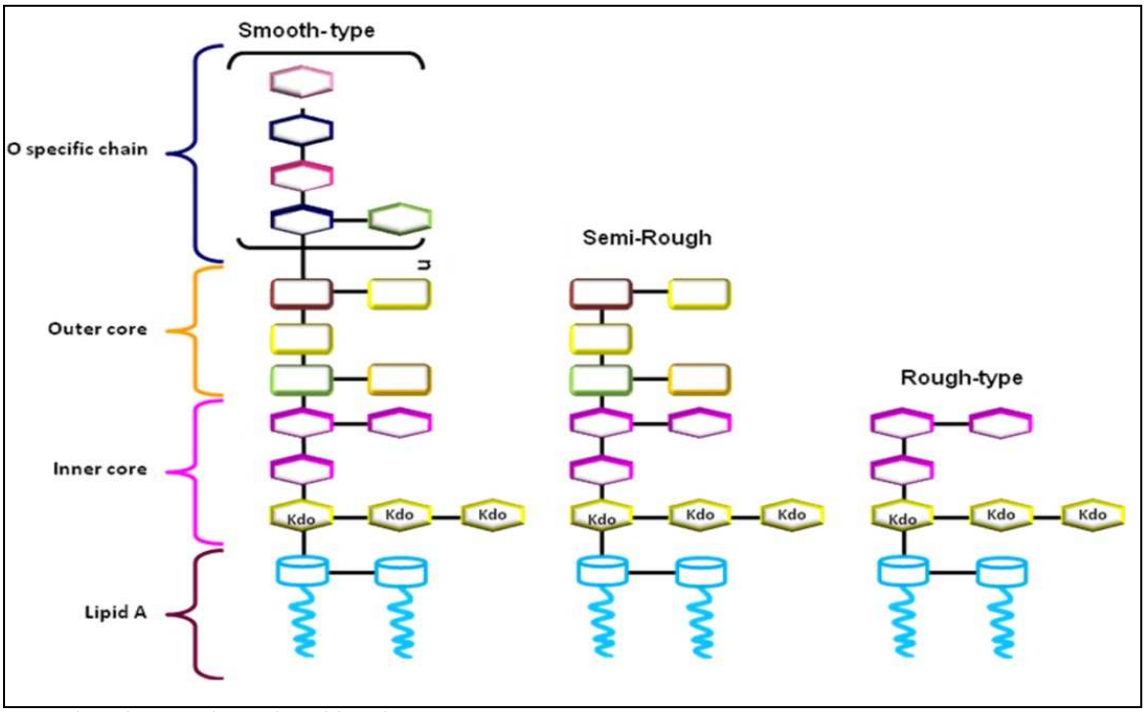

Fig. 1: The schematic lipopolysachharide structure:
(Image adopted from: Rosa Eugenia Reyes et al.; "Mechanisms of O-Antigen Structural Variation of Bacterial Lipopolysaccharide (LPS " Biochemistry, Genetics and Molecular biology: The complex world of polysachharides. Oct. 31, 2012 Chapter 3.)

Acute inflammation causes an activation of the coagulation system which is mediated by cytokines [30] and mostly initiated by the tissue factor pathway (extrinsic pathway) [31]. Tissue factor binds to and activates factor VII [31]. The resulting complex of tissue factor and factor VIIa is responsible for the generation of thrombin via the activation of factor X [31]. Factor X, in combination with factor V, can convert prothrombin into thrombin which cleaves fibrinogen and creates fibrin [30, 31].

The intravascular tissue factor expression and the expression of leukocyte adhesion molecules on the intravascular cell surfaces represents a first step for creating the cycle towards uncontrolled blood coagulation [32]. This leads to fibrin deposition and accumulation in organs resulting in vascular injury like ischemia and necrosis of organ tissue [32].

Besides, the function of the anticoagulation system which is comprised of protein C, antithrombin III and tissue factor pathway inhibitor is also impaired [33, 34]. The synthesis of antithrombin III, the most important thrombin inhibitor [35], and the level of protein S (protein C co-factor) are decreased [36]. Furthermore, antithrobin III is degraded through elastase which is released from activated neutrophiles [35].

Fibrinogen represents an important component of the coagulation system and plays a role in inflammation, thrombogenesis and atherogenesis [37] but is also associated with severe vascular disorders like stroke, thromboembolism, ischaemic heart disease shown by a number of epidemiological studies [38].

In case of inflammation it is interesting that fibrinogen as an acute-phase reactant seems to be able to trigger an increased activation of platelets when taken up by α-granules [39]. As response to adenosine-diphosphat the degranulation of platelets is augmented by elevated levels of fibrinogen and so it may operate in cardiovascular risk by increasing the reactivity of platelets [39].

It was shown that fibrinogen contains peptides with antibacterial activity like fibrinopeptide B, fragments D+E [40], and fragment GHR28 released from β-chain [41]. Fibrinogen is also able to entrap bacteria in a fibrin clot which acts as a physical barrier and prevents pathogens from spreading within the host [42]. Further it is assumed that activation of fibrinogen can lead to direct killing of the bacteria [42].

Besides fibrinogen other proteins of the coagulation system play an important role in supporting the immune system against bacterial pathogens [43]. Recent studies have shown that the domain 5 of the high molecular weight kininogen has antibacterial properties [44] as have peptides derived from thrombin [45]. From thrombin antimicrobial peptides can be released upon proteolysis mediated via neutrophil elastase [45]. Under physiological conditions, these peptides mediate membrane lysis of gram positive and gram negative bacteria [45].

However, it is known that some bacterial enzymes, e.g. the *streptococcus gordonii F22S* challisin, can affect fibrin clot formation and can digest fibrinogen [46]. This may be a strategy of the bacteria to escape from this hazardous environment (as described below) [46].

Fibrinogen

Fibrinogen is a soluble glycoprotein with a molecular weight of 340kDa mainly synthesized in the liver with a biological half-life of around 100 hours [47]. It is secreted into the plasma at a level of 1.5 to 4.5 g/l [47]. Fibrinogen is an important component of the coagulation system and is the precursor of fibrin [47].

In plasma, the fibrinogen molecule exists as dimer. Each monomer is composed of 3 polypeptide chains [48]. The Aα-chain consists of 610 residues, the Bβ-chain has 461 residues and the γ-chain contains 411 residues [48]. The chains are connected by five symmetrical disulfide bridges at their N-terminal E-domain which builds the core domain of the molecule [49]. The C-terminal endings of the polypeptide chains form coiled-coil structures which stabilize the molecule [50, 51]. The C-terminal part of the molecule is known as the D-domain [50]. Overall, the fibrinogen molecule is connected via 29 disulfide bridges [52] (Fig.2).

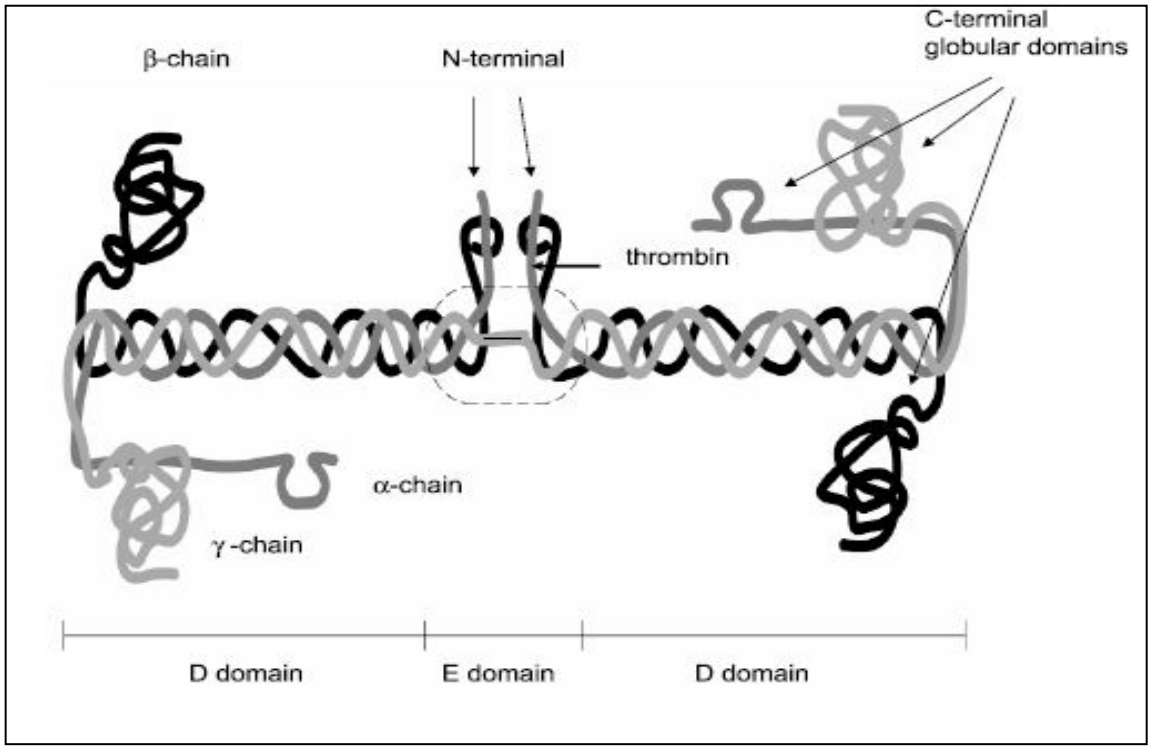

Fig. 2: Diagram of Fibrinogen.
(Image adopted from: Herrick S, Blanc-Brude O, Gray A, et al. Fibrinogen. Int J Biochem Cell Biol 1999; 31: 741‑746.)

The initial step for the conversion of fibrinogen to fibrin is the cleavage of the fibrinopeptides A and B from the amino-terminal ends of the α- and β-chains by thrombin [53]. For this, thrombin has to be generated from prothrombin by factor Xa which represents the end of a preceding activation cascade [53] (Fig.3). Thrombin can bind to fibrinogen via its fibrinogen recognition site exosite-I [53] and first cleaves the N-terminal fibrinopeptide A sequence (residues 11-13) located on each fibrinogen Aα-chain [54, 55]. This leads to the exposure of the polymerization site EA [56, 57]. EA consists of two parts located on the α-chain N-terminus (residues gly17-pro18-arg19-val20) [58, 59] and on the β -chain (residues 15-42) EA has a complementary binding partner located on the D-domain γ-chain (Da) [59] (residues 337-379) the prerequisite for the overlapping EA:Da complex between two adjacent fibrin molecules [57, 59, 60]. This leads to double-stranded twisting fibrils [61, 62] (Fig.4).

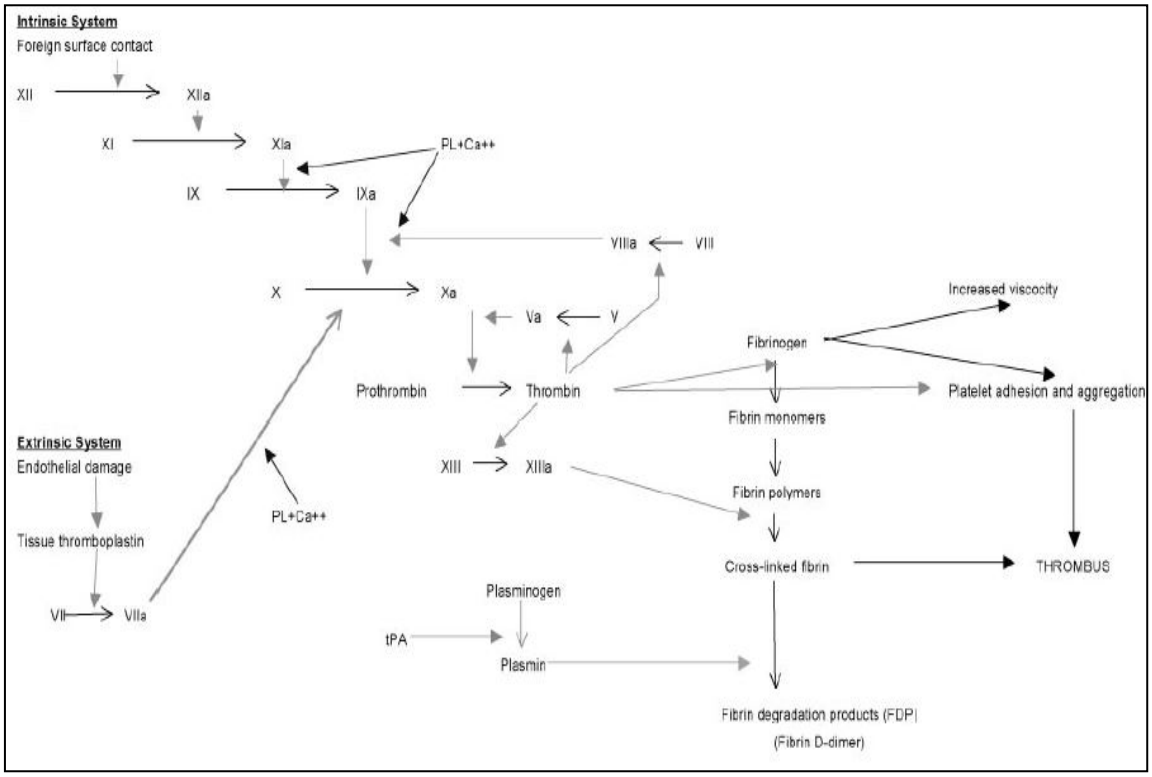

Fig. 3: Interactions between the fibrinolytic system coagulation system. PL, phospholipid
(image and text from S.Kamath et al. 2003, "Fibrinogen: biochemistry, epidemiology and determinants")

The cleavage of the fibrinopeptide B (residues 1-14) of the β-chain is performed slower than the release of fibrinopeptide A [63] and exposes the independent polymerization site EB (residues gly15-his16-arg17-pro18) [64]. EB binds to the complementary Db site

of the D-domain residing on the β-chain rearranging the β-c-region of the D-domain [65]. The fibrin network forms two types of branch junctions [66]. The so-called ´bilateral´ junction occurs when a double-stranded fibril is laterally converged with another fibril [66]. Addition of further fibril via lateral converges results in the formation of a multi-stranded molecule [66] (Fig.4). Formation of a tight cross-linked matrix is dependent on an additional 'equilateral' junction formed among three fibrin molecules creating a brunch resembling a limb [66]. The induced rearrangement of the β-c-regions through EB contributes to lateral association by promoting the intermolecular βc-βc contacts [64, 66]. For the fibrin assembly two sites are needed residing in the γ-chain of each D-domain named γXL-site and D:D-site [67]. They are self-associating sites and are necessary for the "end-to-end" alignment and cross-linking of fibrinogen to form the fibrin polymer [67]. This crosslinking is necessary to establish a branched and tight matrix of fibrin which can seal ruptured tissue [67]. The subsequent factor XIII or factor XIIIa-mediated transglutamination depends on intermolecular interactions between two connecting γXL –sites [68, 69, 70].

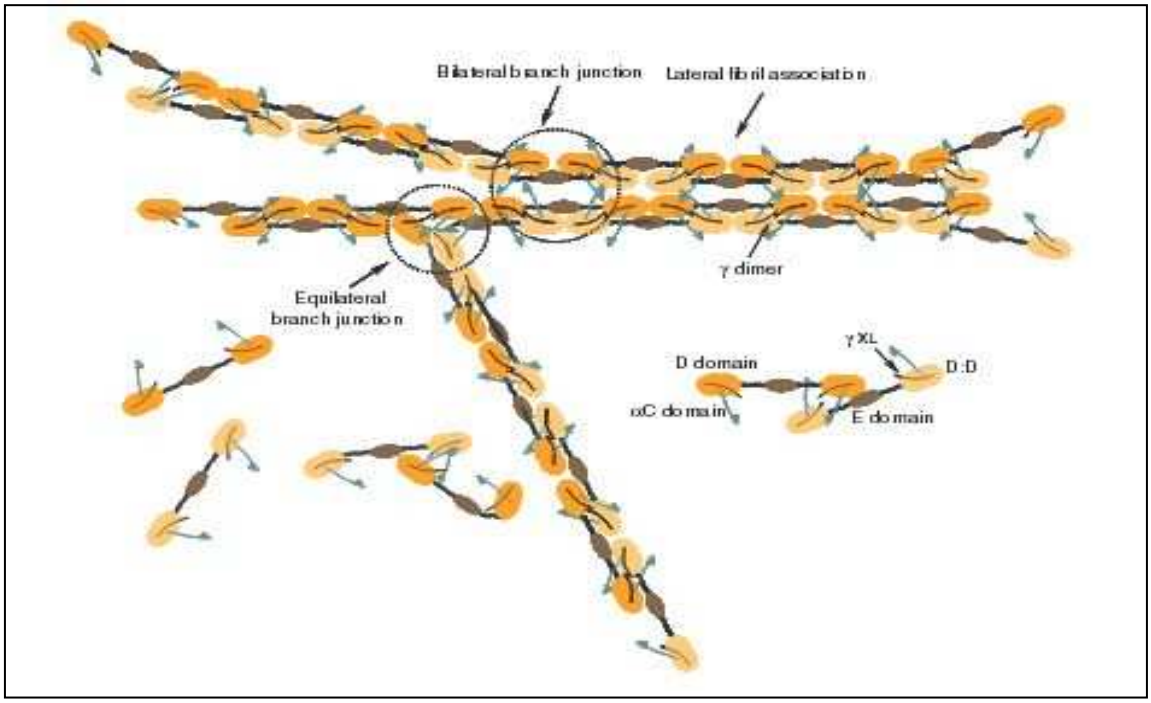

Fig. 4: Schamatic of fibrin assembly. Branching, the cross-linking of the γ-chain and , lateral fibril association are depicted in this image.
(Image and text adopted from: M. W. Mosesson; "Fibrinogen and fibrin structure and functions"; J Thromb Haemost 2005; 3: 1894-1904)

Intermolecular covalent ε-(γ-glutamyl)lysine bonds between the crosslinking sites of the γ-chains of two fibrinogen molecules (γ406lysine of one chain and γ398/399glutamin of the other chain) are produced by factor XIII (XIIIa) [71, 72, 73, 74].

An additional version of the γ-chain is derived from alternative processing of the mRNA transcript [75]. In this product a sequence substitution (γ408-411 Δ γ408-427) has been identified which comprises 2 sulfated tyrosins [75, 76]. This minor γ-chain variant is termed γ' and accounts for 8% of fibrinogen γ-chains in plasma [77]. Due to the fact that the variant form is usually found in heterodimeric molecules, the total amount of fibrinogen containing γ' is approximately 15% [77]. The γ'-chain containing fibrinogen (called fibrinogen 2) molecules play a role in regulation of activity of factor XIII by binding the A_2B_2 tetramer of plasma factor XIII at its β subunit [78]. In the bound state the FXIII cross-linking activity for fibrin is suppressed [78]. Presumably, this alternative form of fibrinogen plays an important role in clot-architecture because a binding site for platelets is missing [79]. Apparently, variant clots are more resistant to fibrinolysis because of thinner fibers and smaller pores. It is assumed that this raises the risk for thrombosis [79].

The degradation of fibrin represents an essential biological process and guarantees the dissolution of the fibrin clot [80]. Fibrin cleavage is brought about by plasmin during fibrinolysis [80]. Plasmin is formed from plasminogen by one of two activators known as tissue plasminogen activator (tPA) found in endothelial cells and urokinase located in the bloodstream and the extracellular matrix [80]. Both activators are serine proteases [80, 81].

The activation of plasminogen by tissue-type plasminogen activator (tPA) is initiated by the formation of a tPA-plasminogen-fibrin complex [82]. Thus, the presence of fibrin is an accelerating factor while fibrinogen has no direct effect on plasminogen activation [83]. A number of plasmin sensitive sites on the fibrinogen molecule were identified. When plasmin cleaves fibrin, lysine-binding sites are generated and they lead to an elevated plasminogen accumulation [84, 85].

Fibrinolysis generates various cleavage products known as X and Y fragments, D and E fragments, D-dimers and Bβ-15-42 fragments [86]. There are also smaller fragments derived from the α-chain [86] (Fig.5).

Inhibitors of fibrinolysis like α2-plasmin inhibitor (α2-PI) can bind to specific sites and prevent plasmin from degrading fibrin [87]. α2-PI can covalently bind to the fibrinogen α-chain and be incorporated into the fibrin clot by factor XIII [88]. The plasmin activation inhibitor 2 (PAI-2) can also be cross-linked to the αC-domain [80]. The binding site for PAI-2 lays distinct off the binding site of α2-PI and is able to inhibit fibrinolysis [81].

Lipopolysaccharide A (Lp(a)) is a LDL-like molecule capable of binding to the αC-domain of plasmin-degraded fibrin [89]. LP(a) competes with plasminogen for its binding site and thereby Lp(a) becomes an inhibitor of fibrinolysis [89].

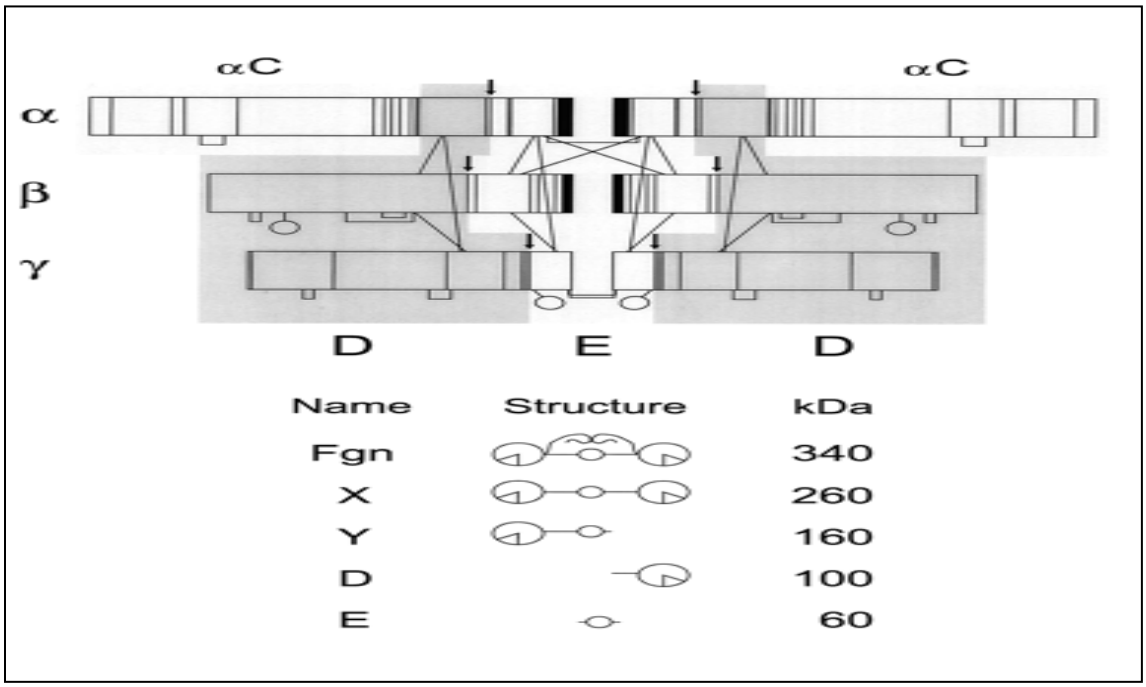

Fig. 5: Structure of fibrinogen and its cleavage fragments: The figure shows the arrangement of the fibrinogen molecule and its 6 chains. The lines between the chains indicate the disulfide bonds between the chains. Intrachain disulfide bindings are depicted as boxes. The black boxes on the α- and β-chains are fibrinopeptide A and B. D – E- and αC- regions are shaded. The vertical lines within the chains show the plasmin cleavage sites where the black arrows indicate the cleavage sites from which the major fragments D and E arise. These fragments are shown in the lower part of the picture where the E-fragment is depicted as a small circle, the D-fragment as a large circle and the αC- as a curved line. The D- and E- fragment are connected via the α-helical region with the plasmin-sensitive sides. The subsequent cleavage and the molecular weights of the derived fragments are shown.
(Photo and Text adopted from Walker JB, Nesheim ME. The molecular weights, mass distribution, chain composition, and structure of soluble fibrin degradation products released from a fibrin clot perfused with plasmin. J Biol Chem. 1999;274:5201–12)

Bacterial proteins capable of binding or processing human fibrinogen

The attachment of pathogens to host proteins is crucial for an infection [90]. More and more is known about the interaction of pathogens with human blood and their resulting effect on the human body [90]. Several putative candidates among blood plasma proteins have been identified, e.g. fibrinogen, plasminogen, fibronectin or enolase to name only a few [90].

Recognition and binding of fibrinogen by bacteria are associated with life threatening complications, well described for some gram positive bacteria [91]. Fibrinogen binding proteins of gram positive bacteria are divided into MSCRAMMS (microbial surface components recognizing adhesive matrix molecules) and SERAMs (secretable expanded repertoire adhesive molecules) [91].

The most common staphylococcal fibrinogen-binding MSCRAMMs contain ClfA (Clumping factor alpha), FnbpA (Fibrinogen binding protein a) and SdrG (serine-aspartate repeat protein G) [91]. The adherence of pathogenic bacteria to the extracellular matrix through these MSCRAMMs seems to be critical for the establishment of an infection [92]. Thus the capability of binding fibrinogen is an important virulence factor [92, 93].

The binding proteins of the bacteria are structurally related to a family of cell wall attached proteins which can be found in most gram positive bacteria [91]. The LPXTG motif at the C-terminus is important for the attachment to the cell wall. It ensures anchoring of the protein via a hydrophobic transmembrane domain which ends with a positively charged cytoplasmic end [94]. The A-domain at the N-terminus is exposed to the surface of the bacterial cells and is comprised of three subdomains (N1-N3) [94, 95], the fibrinogen-binding sites of FnbpA [96], ClfA [97] and SdrG [98] are located within N2 and N3 (320 residues). The secondary structure of N2 and N3 is highly conserved among those three proteins, but the amino acid sequences are very different and match only in 20% of bases [94, 95, 96, 97]. FnbpA and ClfA are important for endovascular infections like the pathogenic colonization of heart valves [99].

There are more than 10,000 individuals suffering from infective endocarditis (IE) every year with a mortality rate of around 35% [100]. The vast majority of bacteria causing IE are staphylococci and streptococci [101, 102]. *S. aureus* interacts with platelets via a fibrinogen bridge depending on FnbpA/ClfA, an FnbpA/ClfA directed IgG and the Fc receptor on platelets which causes the activation of the thrombocytes [103] (Fig.6).
The activation then leads to platelet aggregation and generation of thrombi on healthy as well as damaged heart valves [103].

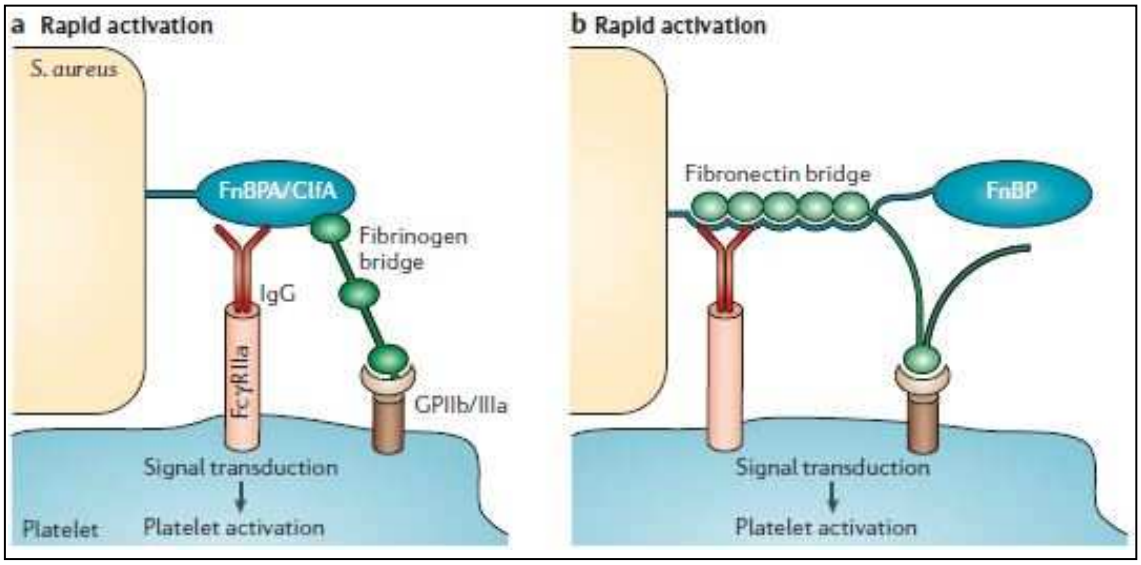

Fig. 6: Platelet adherence and activation by *S. aureus*. a: A fibrinogen bridge is formed between fibronectin binding protein A (FnBPa) or ClfA (clumping factor A) of *S. aureus* and low-affinity resting GPIIBb/IIa of a platelet. Additional an IgG (specific for FnBPA or ClfA) mediated connection of FnBPA/ClafA and the platelet FC receptor FcγRIIa. **B:** A Fibronectin bridge is formed between FnBPA and the platelet integrin GPIIb/IIa. The fibronectin resting on the FnBPA at the image indicates that this interaction creates neo-epitops necessary for a specific IgG which recognizes it and engages FcγRIIa.
(Photo and text adopted from: Fitzgerald J., Foster T., Cox Dermot; "The interaction of bacterial pathogens with platelets" Nature reviews microbiology, 2006 4:445-458)

Streptococcus pyogenes is a human pathogen causing many different kinds of diseases like the uncomplicated pharyngitis but also severe infections which can lead to the streptococcal toxic shock syndrome (STSS), ending up with multiple organ failure and death [104]. A classical pathogenicity factor expressed by *S. pyogenes* is the α-helical coiled-coil surface protein known as M protein [105].
This protein can bind fibrinogen with high affinity after it is cleaved off the cell membrane by a cysteine protease expressed by *S. pyogenes* itself [106, 107].

The M protein/fibrinogen complex can activate polymorphonuclear neutrophils (PMNs) by binding β_2 integrins and this leads to an enormous inflammatory response (STSS) [108]. In this process immobilized fibrinogen is bound by β_2 integrins at the surface of non-activated PMNs at a low affinity. As soon as the PMN get activated by an "inside-out-signaling" mechanism which increases the number of β_2 integrins at the cell surface due to the fusion and release of cytosolic stored granules and vesicles containing β_2 integrins [109].

The M-Protein in *S. pyogenes* can not only bind fibrinogen but is also able to bind the complement regulation proteins (RCA – regulators of complement activation) Factor H [110], FHL-1[111] and C4BP [112], and this may be the reason for the resistance of *S. pyogenes* to phagocytosis in human blood [112]. It was shown that the C3b deposition and the associated clearance of the pathogens by the alternative complement pathway is four fold higher and much more effective in mutant strains lacking the M-Protein [113].

It is hypnotized that fibrinogen bound to the M-protein is able to form a complex with factor H, which would lead to the protection against phagocytosis [114].

Staphylococcus epidermidis is responsible for serious foreign body infections via its property to adhere to the biomaterial. *S. epidermidis* can bind to fibrinogen deposited on plastics used for catheters as example [115].

Responsible for the binding of fibrinogen is the Fbe (also called SdrG) protein which has highly similar structures and organizations as the fibrinogen binding protein of *S. aureus* (ClfA) [116]. Interestingly, this strain does not clump in the presence of plasma, as S*. aureus* does although Fbe is very similar to ClfA [116].

The binding site of Fbe for Fibrinogen is at the N-Terminus of the Bβ-chain, proximal to the thrombin cleavage site [117]. This binding interferes with the release of fibrinopeptide B (FbB) and suppresses thrombin induced fibrin clotting. While FbB does recruit phagocytic neutrophils via chemotactic attraction [117]. This process is suppressed by the binding of Fbe to fibrinogen [117]. It is assumed that this conveys an advantage to the bacteria against the host immune system and may be a bacterial survival strategy [117].

***Finegoldia magna*, an anaerobic gram positive coccus** is a human commensal bacteria colonizing the skin and gastrointestinal and urogenital tracts [118]. It is also responsible for wound- bone- and joint-infections [119, 120]. *F. magna* expresses the serine protease SufA (Subtilase of *Finegoldia magna*) which can cleave antibacterial acting peptides (LL-37) [121] and chemokines (MIG/CXCL9) [122]. It was shown that sufA also cleaves fibrinogen at specific sites at the Aα-chain resulting in two degradation products [123]. The cleavage products have apparent molecular masses of 32 and 35 kD [123]. It is assumed that this results in an inhibition of the formation of a fibrin network because the clotting time of sufA incubated human plasma was significantly prolonged in vitro [123].

Other bacterial enzymes capable of digesting fibrinogen in human plasma: FSS2 challisin released from *streptococcus gordonii* [46]. Challisin can hydrolyse the Aα- and the Bβ chains of fibrinogen [46]. It doesn't inhibit fibrin formation but modifies the clot structure generated by thrombin and leads to increased density [46] and a fibrin network which is more resistant to fibrinolysis suggesting that FSS2 challisin is a pro-coagulant virulence factor [46].

Recognition and binding of plasma-proteins like fibrinogen is not only a feature of specific binding proteins [124]. For example, in several studies, it was found that some pathogens, mainly gram positive but also gram negative, present classical cytoplasmic (housekeeping-) enzymes at their surface which then deploy typical pathogen functions such as adhesion and virulence [124, 125]. Their usual task is to conduct essential metabolic functions but they also provide specific protein binding sequences which come into force as soon as they are expressed on the surface of the bacteria [125]. The mechanism by which this happens is currently not known, but a secreting and re-associating mechanism on the surface is suggested [125].
Glyceraldehyd-3-phosphat dehydrogenase (GAPDH; EC 1.2.1.12) is one of these surface housekeeping proteins [124] in prokaryotes which interact with different components of the host organism like plasminogen [126] and fibronectin [127] or cell surface receptors of host cells which enable or accelerate pathogenicity of these bacteria [126, 127, 128, 129, 130].

In enteropathogenic (EPEC) and enterohemorrhagic *E. coli* (EHEC) GAPDH is a secreted protein displayed at the cell surface which is able to bind plasminogen and fibrinogen [124]. The two genes gapA and gapC encoding for the GAPDH proteins are active in many pathogens, but due to different mutations leading to truncated and impractical versions of the protein most of the K-12 strains are not expressing a working GAPDH protein [131, 132].

There are also fibrinogen degrading proteins in gram negative bacteria. *Bacteroides fragilis* expresses several metallo- and cysteine-proteses which are able to degrade the Aα-chain primarily but also the Bβ- and γ-chain [133]. Furthermore, *B. fragilis* expresses a 54 kDa fibrinogen binding protein named „BF-FBP“ [133]. This protein interacts mainly with the Bβ-chain of fibrinogen and it is assumed that this is a strategy to avoid digestion of macrophages [133]. Apparently, *B. fragilis* interacts with fibrinogen in two very different ways. On one side *B. fragilis* can degrade fibrinogen and prevents abscess formation [133] and on the other hand the fibrinogen binding component increases the viability within the blood stream of the host [133].
This example shows that fibrinogen can act as a supportive or suppressive component, and the type of action depends on the pathogen. Furthermore, it shows that fibrinogen can be used in different ways by bacteria.

I took up this thought and investigated the interaction of non-pathogenic and pathogenic *E. coli* strains with fibrinogen in my master-thesis. I wanted to investigate whether the *E. coli* strain K-12(c600) which is lacking the common known fibrinogen binding proteins and virulence factors is able to bind fibrinogen as well as the pathogenic *E. coli* O18 strain which carries fibrinogen binding proteins. I also wanted to investigate if these strains are able to degrade fibrinogen. To verify that fibrinogen can inhibit growth of bacteria, I studied the effect of purified and plasma fibrinogen on the growth of these bacterial strains. I hypothesized that differences in the binding of fibrinogen may indicate that 1) additional factors are necessary for the binding of fibrinogen to bacteria. 2) bacteria can interact with fibrinogen (if purified fibrinogen is used), however, the interaction is prevented, amplified or modified in plasma. Furthermore, I wanted to analyze whether the subclone of *E. coli* K12, clone K12(c600)

- which is known to express an additional surface protein (Antigen 43, Ag43) - has the same fibrinogen binding ability as K12.

Material and treatment

1.5 ml tubes - Safelock tubes; *EPPENDORF*

15 ml tubes - *SARSTEDT*

2 ml tubes - Safelock tubes; *EPPENDORF*

50 ml tubes - *SARSTEDT*

6-well plates – Cellculture test plates 6; *TPP*

Acetic acid – ACS reagent, reag.150, reag. Ph. Eur., >99.8 %; *SIGMA ALDRICH*

Acrylamide/Bis solution – 30 % acrylamide/Bis solution, 29:1, 500ml; *BIORAD*

Agar – purified bacteriology grade; *APPLICHEM*

Antibody α-fbg from mouse –monoclonal antibody against human fibrinogen & fibrin No.311; *AMERICAN DIAGNOSTICA INC.* Used at a dilution of 1:1,500 (in PBS-T + 5% milk)

Antibody α-mouse hrp labelled – goat-anti-mouse IgG (H+L)-hrp conjugate; *BIORAD* used at a dilution of 1:25,000 (in PBS-T)

APS – Ammoniumpersulfate – 99 %, solid; *SIGMA ALDRICH*. For use it was solubilized in $d H_2 0$ and stored as 10 % solutions up to 4 weeks at -20°C.

Bacteria stocks for K12(c600) and O18 – stored at -80°C; for use a pipette tip of the frozen pellet was streaked on LB agar plates and incubated over night. Up to 5 colonies from this plate were picked and grown in liquid LB-medium at 37°C.

Bacteria stocks for K12A – This substrain of K12(c600) was identified when growing K12(c600) and was cultured in the lab by plating K12 cultures and picking those colonies which were morphologically distinct from K12(c600) colonies as described in the literature. The substrain of K12(c600) forms larger colonies without sharp borders and is likely to express the Antigen 43 gen.

BCA kit – BCA protein assay kit using bicinchoninic acid; *THERMO SCIENTIFIC*

Blood plasma – "Human acid-citrate dextrose (ACD) plasma of a healthy donor was obtained from the Department of Transfusion Medicine of the Medical University Vienna. The plasma was divided into aliquots which were kept at -20°C until use. For heat-inactivation the plasma was thawed at RT, gently mixed and briefly centrifuged (5sec). Following an incubation in a water bath at 56°C for 1 hour aliquots were stored at -20°C until use"

Centrifuge large – Allegro® X-12R centrifuge; *BECKMANNCOULTER®*. Used for tubes ≤ 2 ml.

Centrifuge small – Microfuge® 22R centrifuge; *BECKMANNCOULTER®*. Used for tubes ≤ 2 ml.

Chemiluminescent Substrate – supersiSignal ® West Pico / West femto - Luminol/Enhancer; *Thermo Scientific*

Coomassie-blue staining solution – Page Blue protein staining solution - *FERMENTAS*

Cuvettes for phomometrie – disposable cuvettes 1.5 ml semi-micro; *Brand GMBH*

Destaining solution for coomassie-blue – isopropanol 25 %, acetic acid 5 % in *d*H_20

dH_20 bottled – Aqua bidest.; *BRAUN*

DTT – Dithiothreitol – solid, >98 %; *SIGMA ALDRICH*. For use 1 M solutions were created and stored in 500 µl aliquots at -20°C up to 4 weeks.

EtoH – absolute; *MERCK KG*

Fibrinogen – purified fibrinogen from human plasma, 50-70 % protein (>80 % is clottable); *SIGMA ALDRICH*. The fibrinogen was reconstituted in NaCl solution (0.9 %) divided into 1ml aliquots (4 mg/ml) and stored at -20° C.

Glas pipettes for plating – disposable glas Pasteur pipettes 230 mm; *VWR*

HCL – hydrochloric acid, 37 % a.v.; *CHEM-LAB*

Imager – ChemiDoc™MP system; *BIORAD*

Imager software – ImageLab 4.1 build 16; *BIORAD*

Incubator for 37°C – 3031; *GFL*

Isopropanol – 2-propanol for molecular biology, >99 %; *SIGMA ALDRICH*

LB Broth Medium – powder; *SIGMA ALDRICH*

LB-plates – 80mm diameter, PET; *TPP*

Magnet stirrer – IKA®RET control / t

NaCl – sodium chloride, BioXtra > 99.5 %; *SIGMA ALDRICH*

NuPAGE Sample Buffer – LDS sample buffer (4x) 10 ml; *NOVEX*

PBS – Dulbeccos phosphate buffered saline 10x; *SIGMA ALDRICH*

PBS-T – PBS (1x) + tween 20® 0.1% (*SIGMA ALDRICH)*

Pipettes – Gilson pipettmen 20 µl, 200 µl and 1000 µl

Pippette electric. – Easypet 9 V ; *EPPENDORF.* Used serological pipettes 5 ml and 25 ml; *TPP*

Plate reader – EL808 - BIOTEK

Ponceau staining solution – *SIGMA ALDRICH*

Power device for electrophoresis and protein blotting – Power pac™ universal; *BIORAD*

Protein Standart – precision plus protein™ dual color standard; *BIORAD*

Rotator mixer – MultiRS 60 programmable rotator mixer; *LAB4YOU.* Used for incubation of *E .coli* with fibrinogen, plasma fractions, plasma and heat inactivated plasma

Sarkosyl – N-Lauroylsarcosyl sodium salt – BioXtra, >97 % (TLC); *SIGMA ALDRICH*

SDS – sodium dodecyl sulfate – Bioreagent suitable for electrophoresis for molecular biology > 98.5% (GC); *SIGMA ALDRICH*

Sds page chamber – Mini protean® Tetra cell, 600 V 30 W powerlimit; *BIORAD*

SDS-Page Running Buffer – Made as 10 x stock solutions: 288 g Glycine (*SIGMA ALDRICH) + 60.4 g TrizmaBase™ (SIGMA ALDRICH) + 20 g SDS (SIGMA ALDRICH) in 2 L dH2. For use, 100 ml of the 10 x stock solution was added to 900 ml dH2O.*

Small sterilizer – Certoclav tish autoclave. Multicontrol 12C

Spectrophotometer – Nanodrop 2000C; *PEQLAB*

Table shaker – 3015 orbital shaker; *GFL*. Used for washing of Western Blot membranes

Table wipe shaker – Duomax 1030; *HEIDOLPH.* Used for incubation of nitrocellulose membranes with primary antibody over night at 4°C.

TEMED – N,N,N',N'-Tetramethylethylendiamine for electrophoresis –99 %; *SIGMA ALDRICH*

Thermoblock – Thermomixer comfort - EPPENDORF

Transfer Buffer for Western blotting- Made as 10 x stock solutions: 288 g Glycine (*SIGMA ALDRICH) + 60.4 g TrizmaBase™ (SIGMA ALDRICH) in 2L dH2. For use, 100 ml of the 10 x stock solution was added to 900 ml dH2O.*

TRIS-HCL 0.5M ph=6.8 – 0.5 mol TrizmaBase™; *SIGMA ALDRICH,* in 1 L *d*H_20 + 37 % HCl to final pH=8.8

TRIS-HCL 1,5M pH=8.8 – 1.5 mol TrizmaBase™; *SIGMA ALDRICH,* in 1 L *d*H_20 + 37 % HCl to final pH=8.8

Vortex – Vortex genius 3; IKA

Waving shaker – Polymax 1040; *HEIDOLPH*. Used for incubation of nitrocellulose membranes with secondary Antibody. It is also used for blocking and staining of nitrocellulose (ponseau) and acrylamide gels (coomassie blue). Briefly washing and destaining of membranes and gels were also performed by this device.

Westernblot chamber – Mini protean® II Cell, 600 V 30 W powerlimit; *BIORAD*

Westernblot filter paper – Extra thick blot paper (100 % cotton) filter paper; *BIORAD*

Westernblot membrane – Nitrocellulose membrane 0.45 µm*; BIORAD*

Methods

All described Incubation experiments were performed with citrated human plasma diluted with saline (NaCl solution 0.9 %) to a final plasma concentration of 5 %, heat inactivated citrated human plasma - final concentration of plasma of 5 %, and ethanol precipitate prepared from human plasma and dissolved and diluted with saline 1 : 10. As negative controls, 900 µl bacterial suspensions were incubated with 100 µl saline.

EtOH precipitation of plasma proteins

Fifty ml of undiluted citrated human plasma were diluted with 50 ml aqua bidest and stored on ice until the solution had a temperature of 5° C. Ethanol abs. (EtOH) was cooled in a mixture of crushed Ice plus salt until it reached a temperature of -5° C. Then, the plasma was mixed with cold ethanol to a final concentration 8% EtOH and stirred at low speed at -5° C for 1 hour. The pH was set to 7.2 with HCl. The temperature was monitored with two thermometers, one placed in the ice and one in the solution to ensure a constant temperature. After 1 hour the plasma was centrifuged at 0°C at 800 rpm for 10 min in 50 ml tubes. The supernatant was removed and each pellet was reconstituted in 22.5 ml 20 mM Tris-HCl pH 7.5, 150 mM NaCl and stored at -20°C. It was assumed that 90 % of the fibrinogen could be precipitated from plasma by this technique, so the pellet was resuspended in 90% of the original plasma-volume to provide (nearly) the same fibrinogen concentration as it was in the plasma. After all proteins were dissolved, 1 ml aliquots were prepared and stored at -20° C. Each aliquot was only thawed once. Control samples for gel-electrophoreses and western blots always came from the same starting material used for the respective experiment.

Clumping assay

The *E. coli* strains K12, K12A (substrain) and O18 were grown in liquid LB-medium at 37° C and 140 rpm in an incubator until an OD_{600} of 1.4 was reached. An aliquot of 2.7 ml of the culture was transferred into a 6-well plate and incubated with either 300 µl purified fibrinogen, 5% citrated human plasma, 5% heat inactivated citrated human plasma or appropriately diluted ethanol precipitate from human plasma. As control 300 µl saline were added to 2.7 ml of the bacterial culture. The specimens were incubated in liquid solution for 2 hours at the Polymax 1040 shaking table at 20 rpm and RT. After incubation, photos of the plates were taken.

Viability assay of fibrinogen incubated *E. coli* in plasma

The bacteria were prepared as described previously. 900 µl of each bacterial strain were incubated with 100 µl purified fibrinogen (4 mg / ml, final concentration 0.4 mg / ml) for 2 hours at RT at 5 rpm on a rotator mixer. Control samples were 900 µl of each strain + 100 µl saline. 50 ml aliquots of all specimen were taken and diluted 1:($4*10^8$) and plated on LB-Agar plates to calculate the number of colony forming units per ml (cfu/ml) at this point. It was assumed that the bacteria have bound fibrinogen on their surface after the incubation with purified fibrinogen.

The remaining 950 µl of these cultures were transferred into new 1.5 ml Eppendorf tubes and were incubated with 50 µl citrated human plasma to see if the putative binding of fibrinogen to the cell surface of *E. coli* is a survival advantage if exposed to human plasma. The tubes were placed on a rotator mixer and incubated for 2 hours at 5 rpm at room temperature RT. A mixture of 50 µl NaCl-solution (0.9 %) plus 950 µl of the culture which was also pre-incubated with fibrinogen served as control. After incubation the specimen were vortexed and diluted to a final concentration of 1:($4*10^6$). 100 µl of this dilution were plated onto LB-Agar plates and incubated over night at 37° C. The colonies were counted the next day and the cfu/ml of every specimen were calculated.

Viability assay of *E. coli* incubated with purified fibrinogen

The bacteria were prepared as described above. An aliquot (1 ml) of the bacterial culture was taken and mixed with 8 ml fresh liquid LB-medium and the OD_{600} was measured. One ml of purified fibrinogen (final concentration 0.4 mg / ml) was added to the specimen and incubated at 37°C and 140 rpm to check if the presence of fibrinogen has an impact of the growth of *E. coli*. The OD_{600} of the specimens and the control were measured every 30 minutes.

Fibrinogen binding analysis and protein-sample preparation

The bacteria were prepared as described previously. 900 µl of this solution were mixed with 100 µl of a fibrinogen stock solution (final concentration 0.4 mg / ml) and incubated for 2 hours within a 1.5 ml tube using a rotator mixer at 7 rpm.

After the incubation the specimens were centrifuged at 5000 rpm for 10 min at 4° C and the supernatants were collected. The pellets were either used directly or washed 4 times. For washing, the pellets were resuspended in 500 µl PBS, mixed by pipetting up and down, and centrifuged at 5000 rpm for 10 min at 4° C. The supernatants of the washing steps were also collected for further analyses (washing solutions).

The pellets were resuspended in 125 µl sarkosyl (1 % sarkosyl in aqua bidest at 4° C) and incubated for 10 min at the rotator mixer at 45 rpm. Then the specimens were centrifuged at 10.000 rpm for 15 min at 4° C and the supernatants were collected, aliquoted (25 µl) and stored at -20° C until use.

SDS-Polyacrylamide Gel electrophoresis of protein samples and Western Blot

The proteins were analyzed on 9 % (w/v) polyacrylamide gels in the presence of 1 % (w/v) SDS. The samples were mixed with SDS-sample buffer (1x) and reduced by DTT (final conc. 100mM).

For electrophoretic separation 3 µg proteins per lane were applied from supernatants and controls, 20 µg proteins per lane were applied from pellet samples.

The blotting transfers of the proteins onto 0.2 µm nitrocellulose membranes were performed for 3 hours at 125 mA. After the transfers the membranes were stained with Ponceau staining solution and then blocked with PBS containing 5 % (w/v) dry milk powder and 0.1 % (w/v) Tween-20 (PBS-T). Then the membranes were probed with a specific polyclonal antibody to epitopes on human fibrinogen (primary antibody) over night at +4°C. The membranes were washed (5 x 5 min) with PBS-T and incubated for 2 hours with a peroxidase-conjugated secondary antibody and developed with a chemiluminescence detection method to visualize the results.

Coomassie blue staining of Gel-electrophoresis gels

Samples were mixed with SDS-sample buffer (1x) and reduced with DTT (final conc. 100mM). The separation of the proteins was performed on 9 % (w/v) polyacrylamide gels as described above. Afterwards the gels were washed three times in aqua bidest. for 10 min, and fixed with 25 % isopropanol/10 % acetic acid solution for 15 min. Then the gels were incubated with 25 ml of PageBlue Protein Staining Solution at RT and 25 rpm (wave shaker) for 1 hour. The staining-solution was removed, the gels were washed twice with aqua bidest for 10 min at RT and 35 rpm. Complete destaining was achieved by washing with a 10 % Methanol/10 % acetic acid de-staining solution over night at RT and 25 rpm.

Results

Binding of fibrinogen

Clumping assay

I could show that *E. coli* K12 and O18 form aggregates when exposed to citrated human plasma, heat inactivated citrated human plasma and ethanol precipitated fibrinogen isolated from citrated human plasma (EPfib) (Fig.7). The formation of clumps was observed for K12 and O18 in the presence of 1%, 2% and 5% plasma.

It seems that a concentration of 1% plasma is sufficient to induce aggregation. Interestingly, the shape and the size of the aggregates are not changed by higher plasma concentrations. For heat inactivated plasma and for EPfib, concentrations of 1% were not sufficient to induce aggregate formation of the bacteria. Aggregates became only visible at concentrations of 5% heat inactivated plasma and EPfib. At a concentration of 10% aggregates of approximately the same size were generated. Exposure to purified fibrinogen (0.4 mg / ml) lead to very little or no aggregation of K12 and O18 (Fig.7). The bacteria didn´t form aggregates even when the purified fibrinogen concentration was increased to 2 mg/ml.

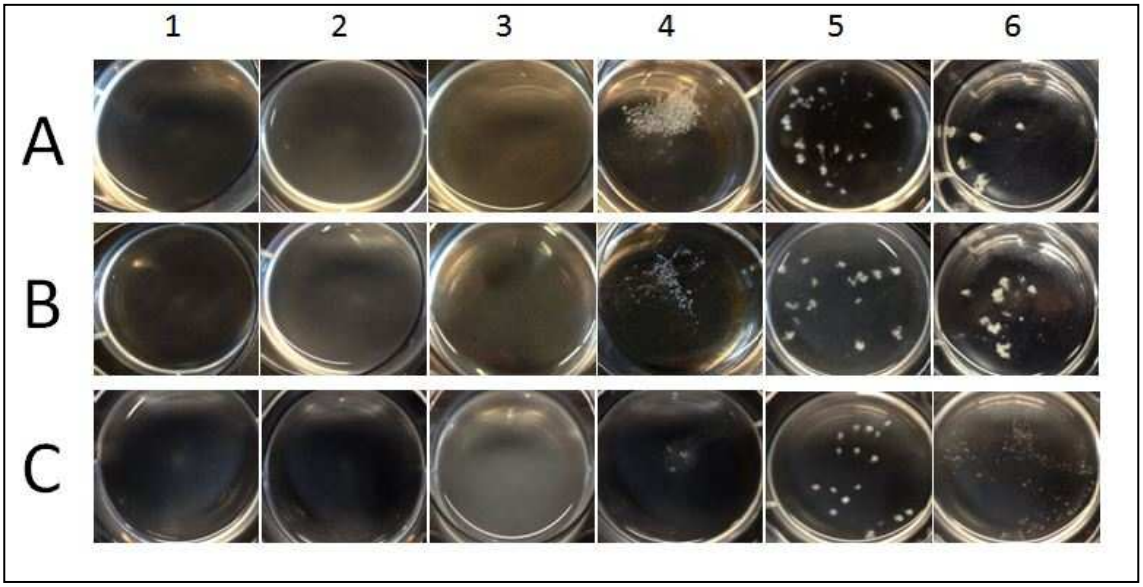

Fig. 7: Clumping assay for *E. coli*. A) *E. coli* K12/c600 incubated with 1) Saline 2) purified fibrinogen (0.4 mg/ml) 3) purified fibrinogen (2 mg/ml) 4) EPfip (10%) 5) heat inactivated plasma (10%) and 6) plasma (5%). **B)** *E. coli* K12/c600 A (subclone) incubated with 1) Saline 2) purified fibrinogen (0.4 mg/ml) 3) purified fibrinogen (2 mg/ml) 4) EPfip (10%) 5) heat inactivated plasma (10%) and 6) plasma (5%). **C)** *E. coli* O18 incubated with 1) Saline 2) purified fibrinogen (0.4 mg/ml) 3) purified fibrinogen (2 mg/ml) 4) EPfip (10%) 5) heat inactivated plasma (10%) and 6) plasma (5%)

Western blot analysis

Western blot analysis of the bacteria and the supernatants after incubation with purified fibrinogen (0.4 mg/ml), plasma (5%), heat inactivated plasma (HIplasma) (5%) and ethanol-precipitated fibrinogen from human plasma (EPFib) (10%) was performed to see if fibrinogen binds to the bacteria. All three chains of fibrinogen could be detected in the bacterial pellet after incubation of *E. coli* K12 and O18 with purified fibrinogen. Fibrinogen was still present in the bacterial pellet after 4 washing steps (Fig.8).

Three major bands with apparent molecular weights (MW_{app}) between 60 and 65 kDa migrating with the same mobility as the α-chain of fibrinogen were detected in all K12 cell lysates following incubation with fibrinogen, EPFib, HIplasma or plasma. Additionally two faint bands with lower molecular weight were detected, one with a MW_{app} of 56 kDa migrating at the same position as the fibrinogen β-chain and the second with a MW_{app} of 47 kDa, corresponding to the fibrinogen γ-chain. A similar band pattern was observed for the control samples of fibrinogen, EPFib, HIplasma and plasma.

O18 cell lysates showed a corresponding band pattern if the bacteria were incubated with fibrinogen or EPFib. There were no bands detectable in O18 cell lysates following incubation with heat inactivated plasma or plasma (Fig.8). Western blot analysis of the bacterial culture supernatants showed similar band patterns as control samples (Fig.9). Here, the blot for plasma shows an unusual strong band slightly below the 50 kDa marker. In general this band gave a much weaker signal and needed a very long exposure time during developing to become visible. In a few experiments there was also a slightly shift of the K12/c600 bands visible in the supernatant (Fig.10). This shift seems to affect the α- and γ-bands, occurs randomly and was not reproducible.

The supernatants of bacteria incubated with purified fibrinogen were also analyzed on gels that were stained with coomassie blue. The results obtained on stained gels corresponded to those observed on Western blots but were much sharper (Fig.11).

To exclude the possibility of unspecific binding of the antibody to bacterial proteins, *E. coli* K12 and O18 were incubated with saline and also analyzed by western blots. I could show that the antibodies did not bind unspecifically, and no signals were obtained (data not shown).

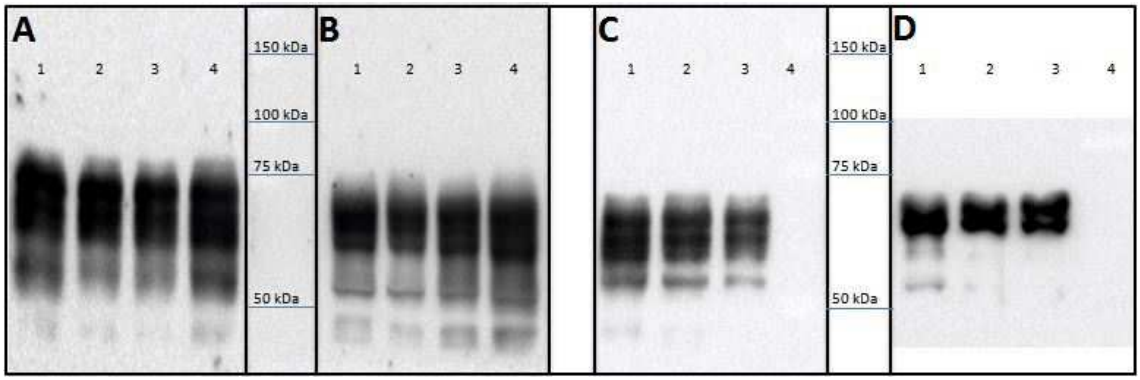

Fig. 8: Western Blot analysis of cell-lysates after incubation with: A) purified fibrinogen (0.4 mg/ml), lanes: 1)fibrinogen control, 2)*E. coli* K12/c600, 3)*E. coli* K12a(subclone) and 4) *E. coli* O18; **B)** EPfib (10%), lanes: 1)EPfib control, 2)*E. coli* K12/c600, 3)*E. coli* K12a(subclone) and 4) *E. coli* O18; **C)** heat inactivated plasma (5%), lanes: 1)heat inactivated plasma control, 2)*E .coli* K12/c600, 3)*E. coli* K12a(subclone) and 4) *E. coli* O18; **D)** Plasma (5%), lanes: 1)plasma control, 2)*E .coli* K12/c600, 3) *E .coli* K12a(subclone) and 4) *E .coli* O18;

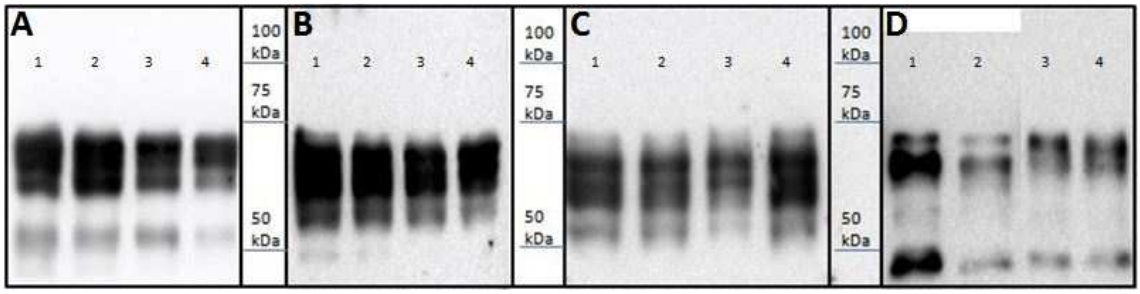

Fig. 9: Western Blot analysis of supernatants after incubation with: A) purified fibrinogen (0.4 mg/ml);
lanes: 1)fibrinogen control, 2) *E .coli* K12/c600, 3) *E .coli* K12a(subclone) and 4) *E .coli* O18; **B)** EPfib (10%), lanes: 1)EPfib control, 2) *E .coli* K12/c600, 3) *E .coli* K12a(subclone) and 4) *E .coli* O18; **C)** heat inactivated plasma (5%), lanes: 1)heat inactivated plasma control, 2) *E .coli* K12/c600, 3) *E .coli* K12a(subclone) and 4) *E .coli* O18; **D)** Plasma (5%), lanes: 1)plasma control, 2) *E .coli* K12/c600, 3) *E .coli* K12a(subclone) and 4) *E .coli* O18;

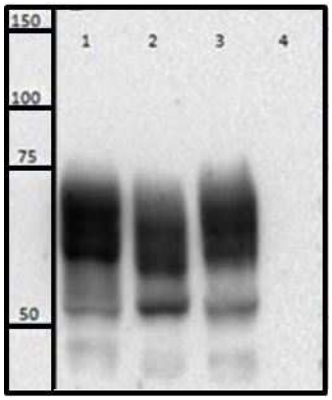

Fig. 10: Western Blot analysis of supernatants after incubation with heat inactivated plasma: lanes: 1) control, 2) *E .coli* K12/c600, 3) *E .coli* K12a (subclone) and 4) *E .coli* O18; There is a shift of the upper bands in lane 2 (K12/c600) visible. This effect occurred randomly in a few samples and was not reproducible. The smaller bands (< 50kDa) seem also slightly shifted.

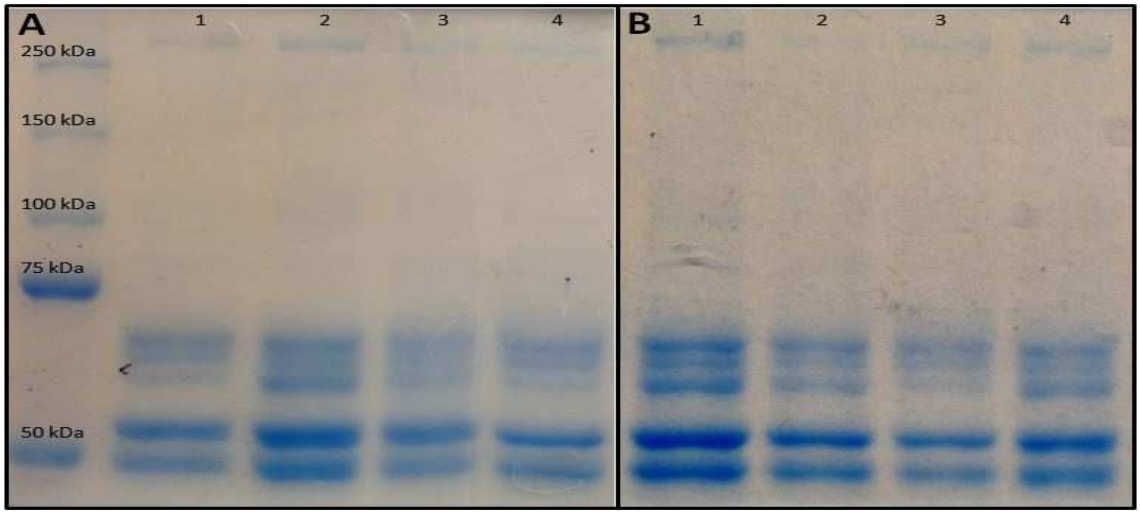

Fig. 11: Coomassie-blue stained electrophoresis-gels of supernatants from *E. coli* after incubation with purified fibrinogen: A) Lanes: 1) purified fibrinogen control 2) *E .coli* K12/c600 + fibrinogen 3) *E .coli* K12A + fibrinogen 4) *E .coli* O18 + fibrinogen **B)** Lanes: 1) purified fibrinogen control 2) *E .coli* K12/c600 + fibrinogen 3) *E .coli* K12A + fibrinogen 4) *E .coli* O18 + fibrinogen; The two gels represent two different experiments made under the same conditions.

Growth of *E. coli* in the presence of fibrinogen

As discussed before, it is assumed that fibrinogen can have supportive and repressive impacts onto the viability of bacteria. When *E. coli* K12 and O18 were incubated with fibrinogen (0.4 mg / ml) and grown in liquid LB-medium the proliferation rate was suppressed by nearly 20% (K12) respectively 10% (O18) (Fig.12). The OD_{600} values of the specimens before the incubation with fibrinogen was 0.150 (STD=0.03). This was accomplished by growing a liquid culture, splitting and diluting it into aliquots, adding fibrinogen (4mg/ml) and measuring the OD_{600} value every hour. After 3 hours of incubation the approximate OD_{600} value of the control culture of K12 was 2.55 (STD=0.12) and of the fibrinogen incubated K12 culture the OD_{600} value was 2.06 (STD=0.1) which corresponded to a decrease of 19%. For O18 the control reached an OD_{600} value of 2.71 (STD=0.06) after 3 hours and the fibrinogen incubated O18 bacteria had an OD_{600} value of 2.48 (STD=0.09) which means a reduction of growth by 8.5% after 3 hours of incubation. Data were calculated from 6 specimens of each.

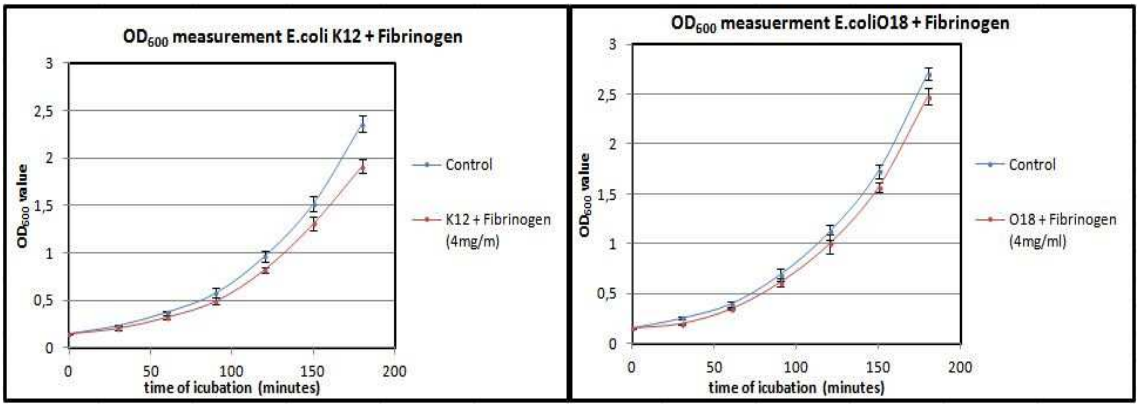

Fig. 12: Growing of *E .coli* in liquid LB-medium after incubation with purified fibrinogen (4mg/ml). The left chart shows the growth of *E .coli* K12/c600 whereas the right chart depicts the growth of *E .coli* O18. The OD_{600} values of the cultures were measured every 30 minutes for 3 hours. The graphs show the growth of each culture under incubated conditions (red graphs) compared to a control culture (blue graphs).

Growth of "fibrinogen-covert" *E. coli* in the presence of plasma

Pre-incubation of *E. coli* with fibrinogen seems to convey an advantage for the survival of bacteria in plasma. If *E. coli* K12 was pre-incubated with fibrinogen, the bacteria interacted and bound fibrinogen. Interestingly, the "fibrinogen coated" *E. coli* K12 survived exposure to 5% plasma for 2 hours much better than uncoated bacteria (Fig.13). The cfu/ml (Colony forming units per ml) of the "fibrinogen coated" specimens were significantly higher than the control specimen which haven't been incubated with fibrinogen. After an exposure to 5% plasma the control *E. coli* K12 had a cfu/ml of $2.26*10^7$ whereas the "fibrinogen coated" K12 specimens had a cfu/ml of $4.75*10^7$. Data were gathered from nine specimens of each (control and incubated specimen) within three experiments.

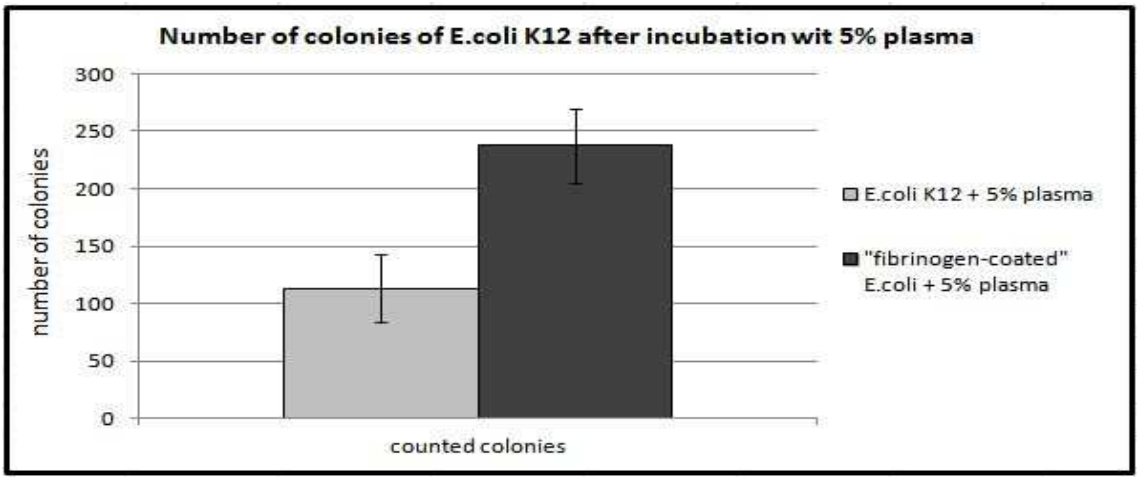

Fig. 13: Number of colonies formed on Agar-plates by *E. coli* K12 after incubation with 5% plasma for 2 hours: After incubation with 5% plasma, the bacteria were plated at a dilution of 1:200,000. The untreated *E .coli* K12 (left) formed app. 113 colonies on the plates which means a cfu/ml of $2.26*10^7$, whereas the fibrinogen-coated *E .coli* K12 (right) formed app. 237 colonies which indicates a cfu/ml of $4.75*10^7$.

Discussion

Binding of fibrinogen

Many bacteria have the ability of binding fibrinogen regardless of their way and place of infection. It was shown in different studies of *Y. enterocolitica*, *L. monocytogenes*, *Y. pestis* and group A streptococci, that mice which are lacking fibrinogen are significantly more sensitive to microbial infections [134, 135, 136]. In contrast, two studies of *S. aureus* have shown that fibrinogen can either be a benefit for the microbe (eg septicemia) [137] or supporting the immune system by eliminating the pathogen (e.g. *S. aureus* within the peritoneal cavity in peritonitis) [138]. It is suggested that different functional properties of fibrinogen (e.g. platelet activation, binding to bacterial proteins) are responsible for the effects onto microbes [137].

All three fibrinogen chains were detected in the cell pellet by western blot following incubation of *E. coli* K12 and O18 with purified fibrinogen (Fig.8). This indicates that both strains can bind fibrinogen, but the binding did not induce clump formation because the result of the clumping assay showed no visible aggregates when the bacteria were incubated with purified fibrinogen.

Presumably, purified fibrinogen is not sufficient to induce clump formation and other proteins present in plasma, are necessary. However, we cannot exclude that purified fibrinogen is slightly altered during the purification process and can no longer be properly bound to the *E. coli* bacteria.

However, if we consider that *E. coli* O18 survives within the bloodstream whereas *E. coli* K12 is rapidly destroyed by human plasma, it seems that the ability of forming aggregates is not a marker for pathogenicity of *E. coli*. Currently, it is still unclear whether the aggregation ability in human plasma is a benefit or a disadvantage for the survival of *E. coli*. Besides this, *E. coli* is affected differently when exposed to purified fibrinogen or human plasma. This shows that the results from experiments obtained with purified fibrinogen may not reflect the behavior of the bacteria under physiological conditions. Presumably, an additional protein is present in human plasma which contributes to the formation of aggregates with *E. coli*.

Surprisingly, *E. coli* O18 is able to bind purified fibrinogen but does not seem to interact with fibrinogen in human plasma. The differences in binding of *E. coli* O18 to purified fibrinogen and fibrinogen in plasma raise the question why *E. coli* O18 can´t bind fibrinogen in human plasma. Possibly, another protein with a higher affinity to the putative fibrinogen binding site competes with fibrinogen. This protein/proteins may occupy the putative fibrinogen binding sites so that they are no longer accessible for fibrinogen. Alternatively, the exposure of *E. coli* O18 to human plasma is a stress factor which prevents the expression of the fibrinogen-binding protein and supports the viability of the bacteria within the bloodstream.

As described previously *E. coli* K12 is lacking all known virulence factors and does not express GAPDH which can also bind to fibrinogen. Recent studies have revealed that *E. coli* K12/c600 and a subclone (K12/c600-LK2) are also deficient in type 1 fimbriae expression [138]. These pili are encoded by the fimE gene [139] and are a virulence factor of *E. coli* and play a role in pathogen-host cell attachment [140, 141]. Considering that *E. coli* K12 is lacking all known putative fibrinogen binding proteins it seems possible that there is at least one unknown protein on the surface of *E. coli* which has an affinity to fibrinogen. An identification of this protein was beyond the scope of the diploma thesis. However, the characterization of this ligand would be very interesting.

In *S. aureus* fibrinogen can be beneficial as well as harmful. This strain expresses fibrinogen-binding proteins and fibrinolytic proteins (staphylokinase) [137] which can process fibrinogen. For *E. coli* K12 and O18 there is no evidence that fibrinogen is cleaved. The western blots show the same band pattern for the fibrinogen-control samples and the *E. coli*-exposed fibrinogen samples in all experiments except few exemptions (Fig.9). In some cases, the α-bands migrated with smaller molecular weight in some K12/c600 samples compared to the controls. This could be due to a modification of the molecule by the bacteria. However, the observation was not reproducible in every experiment. I cannot exclude small differences during the procedure of the western blot or in the sample preparation. To exclude the effect of

western blot conditions, supernatants of different incubation mixes of bacteria and purified fibrinogen were tested on an electrophoresis gel stained with Coomassie brilliant blue. The stained gels revealed that there are no differences in the migration behavior of the bands of the control samples and fibrinogen which was exposed to bacteria (Fig 12). This indicates that the fibrinogen molecule was not cleaved to a major extend by the bacteria. In contrast to the western blot results, the β- and γ-chain were clearly visible on the stained gel. From these results we assume that the antibody we used in the western blot had a relatively low affinity to the β- and γ-chains.

Another interesting observation was the occasional presence of a strong signal for a low molecular weight band in plasma samples (Fig 9). Normally, this signal was weak. As this band was only present occasionally, I assume that the product was specific for this individual plasma. Interestingly, this product did not interact with the bacteria to a significant extent. Currently, I do not know the source for this product – possibly, it corresponds to a fibrinogen cleavage product which does, however, not bind to the *E. coli* bacteria.

Impact of fibrinogen onto the viability and growth of *E. coli* K12 and *E. coli* O18

It is interesting that on the one side, the growth of *E. coli* is suppressed if incubated with purified fibrinogen but on the other hand, the binding of fibrinogen to the bacteria is an advantage for viability of *E. coli* if incubated with human plasma.

It is still unclear how fibrinogen can achieve the growth suppression. However, it seems possible that the binding of fibrinogen is a stress factor for *E. coli* and slows down the proliferation rate. Alternatively, interaction of bacteria with fibrinogen can increase the viability of the bacteria as we have been able to show. We observed a doubling of cfu/ml after 2 hours of incubation (Fig.13). The binding of fibrinogen to the surface of *E. coli* seems to confer a survival advantage as shown in experiments with fibrinogen coated bacteria which survived better in the presence of plasma. An important part of the human immune system is the complement activated membrane attack complex which destroys intruding pathogens. Binding of complement factor C3 to the pathogen plays a critical role for the formation of the membrane attack complex. It seems possible that the surface bound fibrinogen limits the deposition of C3 the same way as it was reported for the O-antigen [8].

Interestingly, the growth of the pathogenic *E. coli* O18 is slightly depressed following incubation with purified fibrinogen, which indicates that this suppression of proliferation is a general mechanism regardless of the strain of *E. coli*. Thus, the presence of fibrinogen may inhibit the spreading of *E. coli* but can also be used by the bacteria to escape from the immune system. *E. coli* K12 is defenseless against the immune components in human plasma, so binding of fibrinogen would be an advantage for the survival. In contrast, *E. coli* O18 does not bind fibrinogen from human plasma. Therefore, one may hypothesize that this theoretically could help the pathogen to overcome the depressing effect of fibrinogen on proliferation. However, due to other protection mechanisms (different surface and membrane proteins) *E. coli* O18 doesn't need the supportive feature of fibrinogen to survive in the presence of human plasma.

The putative role of Ag43 expression in the sub-clone of K12/c600 in fibrinogen binding

There was no difference between *E. coli* K12/c600 and its sub-clone detected in any experiment. Both strains showed the same aggregate formation and the same growth inhibition in media containing fibrinogen. Previous experiments which showed a higher sensitivity against plasma (data not shown) do not seem to be linked to an altered interaction to fibrinogen.

Conclusion

Fibrinogen plays an important and complex role in pathogenic infection processes. It can support or prevent pathogens in infections. However, it is still difficult to predict whether fibrinogen binding is an advantage or disadvantage for the bacterial growth. Furthermore, it is still unclear how much fibrinogen can contribute to the formation of aggregates when bacteria are incubated with plasma. In fact, *E. coli* didn't form clumps when incubated with purified fibrinogen but formed aggregates when exposed to

human plasma. One has to consider that fibrinogen has many functions. Besides its role in coagulation and clot formation, it also acts as chemo-attractant for neutrophiles and has antibacterial properties. Fibrinogen and its cleavage products interact with many proteins in plasma and thus the effect of fibrinogen on bacteria can be influenced by a number of plasma proteins. There is still a lot of research to do, to understand what role fibrinogen plays in gram negative bacterial infections (e.g. bacteraemia and septicemia)

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Appendix

Zusammenfassung

Escherichia coli ist Teil der gewöhnlichen Darmflora innerhalb des humanen Verdauungstraktes. Wenn *E. coli* in den Blutkreislauf gelangt kann er jedoch zu einem opportunistischen pathogenen Mikroorganismus werden der das Leben des Wirts bedroht. *E. coli* interagiert wie viele andere Bakterien mit den Proteinen des Bluts, insbesondere jenen des Blutgerinnungssystems. In meiner Diplomarbeit habe ich die Interaktion einiger *E. coli* Stämme mit Fibrinogen untersucht. Ich konnte zeigen, dass *E. coli* K12, welcher keine der bekannten Fibrinogen-Bindeproteine oder Virulenz-Faktoren exprimiert, gereinigtes Fibrinogen binden kann. Weiter kann *E. coli* K12 Fibrinogen aus humanem Plasma binden wenn die Konzentration des Plasmas so gering gewählt wird, dass es zu keiner vollständigen Lyse der eingesetzten Bakterien kommt. Im Gegensatz zu *E. coli* K12 konnte der humane pathogene *E. coli* O18 Stamm kein Fibrinogen aus humanem Plasma binden wenngleich *E. coli* O18 fähig ist gereinigtes Fibrinogen zu binden. Die Bindung von Fibrinogen an die bakterielle Oberfläche hat einen bivalenten Charakter - Ich konnte zeigen, dass die Proliferationsrate von *E. coli* in flüssiger Kultur durch Fibrinogen gebremst wird aber zur gleichen Zeit die Überlebensrate der Bakterien in humanem Plasma erhöht wird.

Abstract

Escheriachia coli is part of the normal human bacterial flora within the intestinal tract. The organism can turn into an opportunistic pathogen when entering the blood stream. Like other pathogens, *E. coli* interacts with proteins of the blood coagulation systems. In my diploma thesis I investigated how certain strains of *E. coli* interact with fibrinogen. I could show that *E. coli* K12 which is lacking all known fibrinogen binding proteins and virulence factors is able to bind purified fibrinogen. *E. coli* K12 can also bind fibrinogen in human plasma if the concentration of plasma is low enough and does not kill the bacteria. In contrast, the pathogenic *E. coli* O18 failed to bind fibrinogen in human plasma but was able to bind to purified fibrinogen. The binding to fibrinogen has a bivalent character - I could demonstrate that the presence of purified fibrinogen slows down the proliferation rate of E. coli in liquid culture, but at the same time it can increase the viability of *E. coli* K12 which is or has been exposed to citrated human plasma.

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