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Surface proteins of *Finegoldia magna* interacting
with the human host

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Som med vederbörligt tillstånd från Medicinska Fakulteten vid Lunds Universitet för
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Abstract <p><i>Fingoldia magna</i> is a Gram-positive anaerobe and a member of the normal human microflora. This bacterium is also an opportunistic pathogen and isolated from ~10% of all anaerobic infections. Reoccurring taxonomical changes and the anaerobic growth have contributed to the neglect of <i>F. magna</i>. The present thesis describes the identification and characterization of two novel surface proteins of <i>F. magna</i>.</p> <p>One of the identified proteins, SufA, is a protease belonging to the subtilase family. This protease cleaves and inactivates the antimicrobial peptide LL-37 and the antibacterial chemokine MIG/CXCL9. Furthermore, the protease cleaves fibrinogen and thereby inhibits fibrin network formation. To our knowledge, the first example of directed mutagenesis of <i>F. magna</i> is presented with the disruption of the <i>sufA</i> gene.</p> <p>The other identified protein is FAF. This is a cell wall attached α-helical protein that forms hair-like projections on the bacterial surface. FAF is self-associating and contributes to bacterial clumping. FAF also mediates adhesion of the bacterium to basement membranes of human skin by interacting with BM-40. A further function of FAF is blocking of the activity of antimicrobial peptides. The genes encoding <i>faf</i> and <i>sufA</i> are present in a majority of investigated isolates indicating that the proteins have important functions.</p> <p>In conclusion, the findings presented in this thesis may help explain how <i>F. magna</i> colonizes the human host and causes opportunistic infections.</p>		
Key words: <i>Fingoldia magna</i> , Subtilase, LL-37, CXCL9/MIG, Fibrinogen, Adhesion, Basement membrane, Clumping		
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Surface proteins of *Finogoldia magna* interacting
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Cover image: Bacterial aggregates of *Finegoldia magna* strain ALB8, visualized by scanning electron microscopy (picture provided by courtesy of Dr. Matthias Mörgelin).

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List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-IV).

- I. SufA - a novel subtilisin-like serine proteinase of *Fingoldia magna*
Karlsson, C., Andersson, M-L., Collin, M., Schmidtchen, A., Björck, L., Frick, I-M.
Microbiology, 2007, 153: 4208-18
- II. SufA – a bacterial enzyme that cleaves fibrinogen and blocks fibrin network formation
Karlsson, C., Mörgelin, M., Collin, M., Lood, R., Andersson, M-L., Schmidtchen, A., Björck, L., Frick, I-M.
Microbiology Accepted
- III. Identification of a novel protein promoting the colonization and survival of *Fingoldia magna*, a bacterial commensal and opportunistic pathogen
Frick, I-M., Karlsson, C., Mörgelin, M., Olin, A., Janjusevic, R., Hammarström, C., Holst, E., de Chateau, M., Björck, L.
Mol Microbiol, 2008, 70: 695 – 708
- IV. SufA, a serine-protease of *Fingoldia magna*, enhances bacterial survival by modulating activities of the antibacterial chemokine MIG/CXCL9.
Karlsson, C.*, Eliasson, E.*, Olin, A, Mörgelin, M., Karlsson, A., Egesten, A., Frick, I-M.
Manuscript

* indicates these authors as equally contributing

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Paper II and III are Open Access articles.

Abbreviations

GPAC	Gram-positive anaerobic cocci
ORF	open reading frame
Ig	immunoglobulin
PAB	peptostreptococcal albumin binding
ECM	extracellular matrix
BM	basement membrane
AMP	antimicrobial peptide
MS	mass spectrometry
IFN- γ	interferon- γ
MIG/CXCL9	monokine induced by IFN- γ / chemokine CXC motif ligand 9
TF	tissue factor
α C	α C-chain of fibrinogen
TCT	thrombin clotting time
SufA	subtilase of <i>Fingoldia magna</i>
FAF	<i>Fingoldia magna</i> adhesion factor
kDa	kilodalton
EM	electron microscopy

Introduction

In utero all mammals are essentially germfree. Starting from a few hours from delivery, mainly bacteria, but also archaea and eukarya, colonize the newborn. Resident microorganisms will eventually outnumber the number of mammalian cells (116). The microflora colonizing the human is diverse and complex. It is believed that up to 1000 species inhabit the human body (1, 62) of which many are anaerobes and unculturable (56). The resident microflora is estimated to carry 3 million genes compared to the human 20,000–25,000 genes (62).

Interactions between bacteria and their host can be viewed as a gradual transition between symbiosis, commensalism and pathogenicity, with symbiosis and commensalism grouped in the general heading of mutualism. Symbiosis is a relationship between two species where at least one partner benefits without harming the other. Commensalism refers to partners that coexist without causing harm, but with no obvious benefit. Pathogenicity is when the bacteria causes damage to the host (62). Using the term 'commensal' for our normal flora is an inadequate term. Studies of germ-free (gnotobiotic) animals have revealed host symbiotic benefits on maturation of the immune system, susceptibility to pathogens, nutritional absorption and vitamin synthesis (119, 139). Members of the normal flora are also used as probiotics to support human health (113).

Transition from commensalism to pathogenicity may occur in a compromised host due to trauma or underlying diseases. Pathogenicity can also be dependent on the relative abundance of specific species. The differential growth of one microbe may lead to others causing infection. Commensals can acquire new traits and evolve to pathogens by transfer of horizontal gene transfer (38).

Gram-positive anaerobic cocci (GPAC)

The GPAC constitute a heterogeneous group of bacteria and are a major part of the human normal flora. GPAC are present in the oral cavity, the upper respiratory and gastrointestinal tracts, the female genitourinary system and the skin. GPAC are also opportunistic pathogens and are isolated from human clinical material, constituting 20-40% of all anaerobic isolates (20, 85). They can be cultured from a wide variety of sites, of which the dominating are abscesses and infections of the mouth, skin and soft tissue, bone and joint, and upper respiratory and female genital tracts. Most infections with GPAC are polymicrobial, particularly abscesses and those developing from mucocutaneous surfaces. The virulence of the GPAC species is considered to vary, based on isolation rates and isolation of pure culture. There have been few attempts to develop selective media for GPAC. The relatively slow growth rate has likely led to an underestimation of infection rates since they are often isolated in polymicrobial infections, where the GPAC are overgrown by other bacteria in the microbiological identification (85).

Historically, the GPAC have undergone a considerable taxonomic revision. At least 40 species of GPAC have been described, but many were poorly defined and not approved. Other species have been recognized as synonyms or reclassified to the streptococcus genus. For GPAC in general, synonyms as “anaerobic streptococci”, “peptococci and peptostreptococci”, and “anaerobic Gram-positive coccus” have been used (85). The genera *Peptococcus* and *Peptostreptococcus* were first described by Kluver and van Niel in 1936 based on their morphological characteristics; *Peptococci* as the anaerobe equivalent of staphylococci and *Peptostreptococci* as the anaerobe equivalent of streptococci (69). This scheme lasted until the introduction of DNA hybridization techniques in 1983; Ezaki revealed that the genera were phylogenetically disordered and reclassified all GPAC except one species to the *Peptostreptococcus* genus (44). However, this revision was not supported by a later study using similar techniques (63). 16S rRNA DNA sequencing in 1994 confirmed the disorder (43, 76) and made Ezaki’s classification unsustainable. Proposals have been made that the genus *Peptostreptococcus* should be divided into several novel genera; *Peptostreptococcus*, *Fingoldia* (87), *Parvimonas* (138) (suggested first as *Micromonas* (87), but *Micromonas* are green algae), *Anaerococcus*, *Peptoniphilus* and *Gallicola* (42). These revisions are yet to be universally accepted as shown by recent publications using the 1983 classification. Most of the 15 recognized GPAC species are associated with humans, however some have been isolated from animals. All GPAC are classified in the order of Clostridiales, and form five distinct phylogenetic groups of which two are more closely related to the Clostridium family (61).

The GPAC have not been extensively studied. Inadequate classification, difficulties with laboratory identification, and the mixed nature of the infections from which they are usually isolated have contributed to the neglect of these bacteria (85).

Finegoldia magna

The initial descriptions of *F. magna* are unclear (87), but it might have been characterized in 1933 as *Diplococcus magnus* (107). *Peptococcus magnus* was described in 1974 (112). Following the 1983 GPAC taxonomic revision, the bacterium was reassigned to the genus *Peptostreptococcus* (44). The final reclassification was made in 1999 with the assignment to the current genus (87) (Table 1). The genus name *Finegoldia* is after the american microbiologist S.M. Finegold and the species name, *magna* (formerly *magnus*), derives from the relatively large cell size (87). *F. magna* and *Parvimonas micra* (formely *Peptostreptococcus micros*) are closely related and differentiation between the species has been problematic and time consuming. However, techniques such as 16S ribosomal PCR (110), multiplex PCR (130) and 16S rRNA-based probes (146) have been suggested for rapid identification.

TABLE 1. Changes in classification of *F. magna*

Year	Classification	Basis of description
1933	<i>Diplococcus magnus</i> ?*	Morphology (107)
1974	<i>Peptococcus magnus</i> *†	Morphology, fermentation (112)
1983	<i>Peptostreptococcus magnus</i>	DNA hybridization, fatty acid profiles, G+C content (44)
1999	<i>Finegoldia magna</i>	16S ribosomal RNA, pyrolysis mass spectrometry, cell wall structure, cellular carbohydrate composition & fatty acid profiles (87)

* The initial descriptions of *F. magna* are unclear

† Description in bacteriological textbook

Todo and co-workers published a physical and genetic map of *F. magna* in 2002. The size of the circular genome of strain ATCC29328 (abdominal wound isolate) was estimated to be 1.9 Mb. The studied strain also harbored a 200 kb mega-plasmid (140). The same research group later on also constructed a bacterial artificial chromosome (BAC) library of the same strain (52). The complete sequence of the genome and the mega-plasmid (pPEP1) was published in 2008. Totally 1813 open reading frames (ORFs) were found. This represented the first published genome of a GPAC (53).

F. magna has a coccoid cell shape which varies from 0.8 to 1.9 μm in diameter. Cells are arranged in clumps and tetraeds, but occasionally in pairs and short chains. *F. magna* has limited saccharolytic activity, fructose is generally weakly fermented and glucose occasionally. Genomic analysis revealed that *F. magna* only possesses a

complete glycolysis pathway for fructose. Instead peptones and amino acids can be used as a major energy source. The genome has many amino acid/oligopeptide transporters and aminopeptidases that may facilitate the uptake of amino acids from the environment (53, 87). *F. magna* is an anaerobic bacterium. Hence, culturing the bacterium requires an oxygen free environment. Even so, agar plates with *F. magna* can be exposed to air and viable cells are still present after a week. The bacterium is also recovered from specimens that appear to be oxygenated. Catalase activity has been reported (72). Also, detected in the genome of *F. magna* are genes for superoxide reductase, NADH oxidase and a putative NADH dehydrogenase (53) which may help the organism survive under aerobic conditions. The growth rate *in vitro* is relatively slow; colonies with diameters of 1-2 mm are formed after 2-5 days of incubation depending on the strain. Most strains have a generation time of about 10 h in liquid medium.

F. magna is the most common species of GPAC in human clinical specimens and probably the most pathogenic. Alone, the bacterium accounts for 5-12% of all anaerobic infections (85). The bacterium has been isolated from cases of endocarditis (8, 106, 142), and pneumonia (96) of which some have been fatal. *F. magna* is most commonly associated with infections of skin, soft tissue, bone, and joints (12), but also prosthetic implants (27, 47), breast abscesses (24, 36), diabetic feet (66, 144), the vagina (3, 67) and the upper respiratory tract (19, 86). As mentioned earlier the infection rates may be underestimated because of the anaerobic nature of the bacterium. This is exemplified by a study of prosthetic valve endocarditis caused by *F. magna*, where it was shown that the bacterial identification depended on the blood culture system used (8). *F. magna* is usually susceptible to the antibiotics used to treat anaerobic infections. However, lower antibiotic resistance rates (<10%) of clindamycin, metronidazole, penicillin and higher resistance rates (>20%) of erythromycin and tetracyclin have been reported (4, 14, 15).

Surface proteins

Surface appendages are critical in determining the unique properties of individual bacterial species and their interaction with the environment. Most Gram-positive cell walls have an extensive meshwork of peptidoglycan surrounding the single bilayered membrane. The cell wall is a physical barrier providing protection from the environment and a scaffold for the attachment of secondary cell wall polymers and surface proteins. Proteins present on Gram-positive surfaces are attached to the cytoplasmic membrane or cell wall components. Two types of cell wall association are currently known; LPXTG-like proteins that are covalently attached to the cell wall by membrane-associated transpeptidases called sortases, and cell wall binding proteins (32). *In silico* analysis of the *F. magna* genome predicts the presence of 26 genes encoding surface proteins with LPXTG-like motifs, 11 genes encoding sortase homologues and 22 genes encoding proteins with cell wall binding repeats. The

putative functions are albumin or collagen binding, proteolysis, amidase activity or unknown (53). Some of the predicted proteins are similar to studied proteins.

The most well characterized surface protein of *F. magna* is Protein L. This protein binds all classes of immunoglobulins (Ig) by interacting with κ -light chains (9). Protein L is associated with strains isolated from bacterial vaginosis (67). The mature protein is 76 kDa and attached to the peptidoglycan layer with an LPXTG anchor. Five recurring regions, named B repeats, are responsible for the Ig interaction (68). When expressed on the surface of *Streptococcus gordonii*, Protein L enhances their ability to colonize the vaginal mucosa of mice (109). Protein L has been identified as a human B-cell superantigen *in vitro* (6) and also in mice expressing human Ig (145). The Ig binding properties also mediates the activation of basophils and mast cells *in vitro* (99). Protein L is widely used to purify, immobilize or detect Ig.

Some isolates of *F. magna* bind human serum albumin to their surface (88). The gene encoding the albumin binding protein PAB (peptostreptococcal albumin-binding) was cloned from the strain ALB8, isolated from an abscess. The 43 kDa protein is LPXTG anchored and has regions similar to Protein L. The functional domain (GA module) is also found in albumin binding proteins of group G and C streptococci, suggesting horizontal gene transfer (10, 29). Short directed repeats, called recer sequences, were identified as nucleotide sequences facilitating gene transfer (28). *F. magna* albumin binding is linked to suppurative infections and also has positive effects on the bacterial growth rate (30). In the genome of *F. magna* ATCC 29328 four genes encoding the GA-module could be identified (53), indicating that encoded putative proteins may also contribute to albumin binding.

The subtilisin-like serine protease SufA and the adhesin FAF are two novel proteins also identified in the ALB8 strain. These proteins will be discussed later on.

Other studies on properties of *F. magna* have not focused on isolation of proteins or genes responsible for the studied interactions. Strains isolated from non-puerperal breast abscesses and diabetic foot infections have significantly greater collagenase, gelatinase and hippurate hydrolase activity compared to strains from abdominal infections (71, 72). Supernatant from strains isolated from chronic leg ulcers inhibits both keratinocyte wound repopulation and endothelial tubule formation *in vitro* (132). In studies of abscess induction in mice by a mixture of two bacterial species, synergistic effects between GPAC and aerobic species were seen (21). GPAC strains that synthesized a polysaccharide capsule were more virulent compared to non-encapsulated in the mouse abscess model (18). Listed in Table 2 are identified properties and proteins of *F. magna*.

Table 2. Summary of surface proteins and properties of *F. magna*.

Protein(s)	Function	Biological activity	Association	References
Protein L	Ig binding	Promotes vaginal colonization Superantigen	Bacterial vaginosis	(20, 67, 99, 109, 145)
PAB	Albumin binding	Increase growth rate	Suppurative infections	(29, 30)
FAF	BM-40 binding Self-associating AMP binding	BM binding, blocks AMP activity, bacterial clumping	none	Paper III, IV
SufA	Serine protease	Cleaves AMPs, gelatin, fibrinogen, FAF	none	Paper I, II, IV
nd	Capsule*		Abscess formation	(18)
nd	Hippurate hydrolysis	Aminopeptidase activity?	Nonpuerperal breast and diabetic foot infections	(72)
nd	Collagenase		Nonpuerperal breast and diabetic foot infections	(72)
nd	Catalase	Oxygen tolerance?	nd	(72)

nd = not determined

* Unidentified GPAC species

Gene targeting

Directed mutagenesis is a crucial tool for analysis of genes contributing to pathogenesis in bacteria. These techniques are used for constructing a defined mutation to inactivate a specific gene for isogenic mutant analysis. One of the most commonly used techniques is directed insertional inactivation. For this purpose, shuttle vectors for gene disruption in Gram-positives have been developed. These plasmids are restricted to replication in *Escherichia coli* and have selective markers. An internal segment of the target gene is cloned into the vector. The resulting plasmid is transformed into the Gram-positive bacterium with selection for the introduced marker. Resistant transformants arise as a result of a single homologous recombination event. The resulting chromosomal structure consists of a partial duplication of the target gene, which now flanks the integrated plasmid. This results in that one of the duplicated copies will lack the 5' end and the other the 3' end (46, 105). We successfully used this method for disruption of the *sufA* gene (Paper II).

Human skin and basement membranes

The skin is both a physical and chemical barrier vital for preventing invasion of pathogens. Skin consists of 2 layers, the epidermis and dermis, which rest on a fatty subcutaneous layer. Located between the dermal layers are thin sheets of specialized extracellular matrix (ECM) called basement membranes (BMs).

Epidermis

Epidermis is a stratified squamous epithelium consisting mainly of keratinocytes at various differential stages. Keratinocytes located at the basal layer are anchored to components in the BM by integrins. Most of the defensive properties are located in the outermost layer of the epidermis (Stratum corneum). The tightly packed and riveted corneocytes (terminally differentiated keratinocytes) located here provide mechanical strength and are subsequently shed. The ECM of Stratum corneum contains antimicrobial peptides and lipids, has low water content and acidic pH. Together this limits microbial growth (39).

Basement membranes

BMs separate the dermal layers and act as barriers to cell penetration. BMs are constituted of more than 25 components of proteins and proteoglycans. Major structural components are two independent networks of collagen type IV and laminins. The two networks are connected by nidogen, which together with other components stabilizes the structure and adds special properties. Collagen IV consists of heterotrimers of two different chains in a 550 kDa complex and is the most widespread BM component and also considered to be responsible for the mechanical stability. The laminins consist of three different chains with a molecular mass of 140-400 kDa. There are 7 isoforms of laminins that form T-shaped molecules. Proteoglycans are another important BM component of which perlecan is the most abundant. Perlecan consists of a core protein of 450 kDa with long arms of glycosaminoglycans and interact with several other BM components. Nidogen is a 150 kDa protein that is crucial for BM assembly. Nidogen forms stable complexes with both collagen IV and the laminins (137, 149). BM-40, (also known as osteonectin and SPARC) is a 35 kDa glycoprotein found in BMs. The COOH-terminal half harbors a binding site for collagen IV (82). BM-40 does not contribute to the BM structure; instead BM-40 is a so-called matricellular protein (secreted macromolecules that regulate cell-matrix interactions). The exact function of the protein is not known. BM-40 is capable of sequestering growth factors, inhibiting proteases and activating cytokines (11). A suggested function is facilitation of collagen protein folding (80). Mice deficient in BM-40 develops cataracts, have immature collagen fibrils and accelerated wound healing (13).

Bacterial adhesion to skin and BMs

Adherence is the initial important step in the infectious process. Membrane attached lipoteichoic acid or cell wall-bound teichoic acid of Gram-positive bacteria function as adhesive factors that mediate non-specific binding to host cells (90). Bacterial hyaluronic acid capsules can mediate binding to collagens in host tissue (33). Extracellular matrix such as BMs may also serve as substrates for the attachment of microorganisms by specific interactions. Gram-positives express a multitude of different specific adhesins or so called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that bind ECM components including BM (101). These include collagen binding proteins such as CNA (*Staphylococcus aureus*) (102) and FOG (Group G *streptococcus*) (89), and Laminin binding protein PLBP (Group G *streptococcus*) (5). Fibronectin is another ECM protein important for bacterial adhesion to host cells (46). An example is SfbI of *Streptococcus pyogenes*, that mediates adhesion to epithelial cells and is important for host colonization (54, 135). We identify the *F. magna* adhesin FAF that binds BM-40 and mediates binding to BMs (Paper III).

Antimicrobial peptides and chemokines

Antimicrobial peptides (AMPs) are an evolutionary conserved component of the innate immune response and are present in species from bacteria to mammals. They were first identified on the skin of frogs, in granules of human neutrophils and in lymph of insects (55), and today more than 880 have been discovered (141). These peptides have a broad spectrum of antimicrobial activity, including bacteria, eukaryotic parasites, viruses, and fungi. The peptides vary considerably in sequence and structure, with a few common features. Peptides are generally 12-50 amino acids in length, have a positive net charge and contain about 50% of hydrophobic residues. The peptides folds into amphiphilic structures, where the charged / hydrophilic regions are separated from the hydrophobic. However, AMP secondary structure varies widely. There are four major structural classes: β -sheet molecules stabilized by disulphide bonds, amphipatic α -helices, extended molecules, and loops stabilized by a single disulphide bond (55). The amphiphilic property of AMPs is well suited for interactions with membranes and especially bacterial membranes with their negatively charged phospholipids. The exact mechanism of killing is not known. AMPs targeting the membrane are assembled on the membrane surface, inserted to the membrane bilayer and pores or channels are formed disrupting the membrane integrity. Other peptides are translocated over the membrane, entering the bacterial cytoplasm and inhibit metabolism or other enzymatic activities (16). Molecules previously not regarded as AMPs have been found to have antimicrobial activities. These include chemokines (37, 92), neuropeptides (17, 70, 147) and peptide hormones (70, 83). Also activation of the complement (94, 98) and the contact systems (48) generate AMPs from fragments of protein components of these pathways. The holoproteins (C3 respectively high molecular weight kininogen) in these cases are not antibacterial. Apart from their antibacterial effects, AMPs exert additional biological functions, including stimulation of growth and angiogenesis, protease inhibition and chemotaxis (91, 117, 148).

Cathelicidins

Cathelicidins are a family of AMPs found in mammals. The only characterized human member is LL-37 that derives from proteolytic cleavage of the CAP18 protein and is expressed in leukocytes and in epithelial cells of the testis, skin and the gastrointestinal and respiratory tracts. LL-37 is active against both Gram-positive and Gram-negative bacteria. Inflammatory mediators such as IL-6 induce the expression and the concentration can reach up to 5 $\mu\text{g/ml}$. Apart from the antibacterial activity, LL-37 is chemotactic for leukocytes and can neutralize LPS. LL-37 is 37 amino acid residues long and has a linear α -helical structure (7, 31). Mice deficient in the mouse ortholog of LL-37, called CRAMP, exhibit increased susceptibility to group A streptococcus infection (93).

Defensins

Defensins are another AMP family found in both vertebrates and invertebrates. Human defensins contain 6 conserved cysteine residues that form three disulphide bridges and have β -sheet structures. The defensins are subdivided based on the location of the cysteins and the disulphide bonds. The two human subgroups include the α - and β -defensins. α -defensins are mainly found in granules of neutrophils (HNP-1-4) or intestinal Paneth cells (HD-5-6). β -defensins (HBD-1-4) are mainly produced by epithelial cells where the main expression is constitutive (HBD1) or induced and constitutive (HBD-2-4). Several other putative or not well characterized β -defensins are also found. The defensins kill a broad spectrum of bacteria, but the efficiency between Gram-positive or Gram-negative bacteria varies (31, 103).

Antibacterial chemokines

Recognition of bacterial antigens by cells in epithelial tissues results in production of cytokines, including interferon γ (IFN- γ) and tumor necrosis factor α (TNF- α) (114). These pro-inflammatory cytokines cause epithelial cells to produce host defense molecules, including chemokines (115). The CXC ELR⁻ group is one of the chemokine subfamilies based on conserved cysteins in the NH₂-terminal and the absence of a Glu-Leu-Arg sequence preceding the cysteine motif. (78) The CXC ELR⁻ chemokines signals through CXC chemokine receptor 3 (CXCR3) which is present on T cells, NK cells and endothelium. Ligand binding to the receptor results in activation and recruitment of the leukocytes or respectively inhibition of angiogenesis (79, 133). In addition CXC ELR⁻ chemokines have antibacterial activity analogous to human defensins *in vitro*, where Monokine induced by IFN- γ / Chemokine CXC motif ligand 9 (MIG/CXCL9) is the most potent (25, 37). Inflamed pharyngeal epithelial cells produce bactericidal amounts of MIG/CXCL9 (37, 40). The antibacterial effect in MIG/CXCL9 is mapped to a α -helical, cationic and amphiphatic region in the COOH-terminal (37). There seems to be a partial overlap between chemokine and AMP function since both groups can exert similar functions (35).

Bacterial AMP resistance mechanisms

The susceptibility to AMPs among different bacterial species varies. For instance, AMPs contribute to the rapid killing of certain bacterial species on the surface of human skin, whereas other species are left unaffected (23, 51). Numerous and diverse bacterial mechanisms of resistance to AMPs have been demonstrated. One mechanism is production of proteases and peptidases that degrade these peptides. Linear peptides are especially sensitive to degradation and such activity has been shown for several bacterial proteases. *S. pyogenes* utilizes the cysteine protease SpeB in complex with the human host protein α -2 macroglobulin to cleave LL-37 (95), and proteases of

human pathogens, such as *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterococcus faecalis*, *S. aureus* also cleave the same peptide (118, 123). The subtilase SufA from *F. magna* cleaves and inactivates both LL-37 and MIG/CXCL9 (Paper I, IV). Another adaptation to avoid killing involves binding of AMPs to secreted or cell wall attached bacterial proteins, thus hindering the AMP activity. SIC is a secreted protein of invasive *S. pyogenes* strains that inactivates HBD-1-3 (45), HNP-1, LL-37 (49) and MIG/CXCL9 (37). Cell wall bound and released protein FAF of *F. magna* binds LL-37 and MIG/CXCL9 and can rescue the bacterium from AMP killing (Paper III, IV). Reducing the charge or hydrophobicity of bacterial cell surface structures are other mechanisms; this lowers the affinity for AMPs to the bacterial membrane. Inactivation of enzymes modifying teichoic acids of Gram-positive cell walls (2, 73) or modifying LPS hydrophobicity of Gram-negative bacteria (121) both results in increased AMP susceptibility. Membrane AMP efflux pumps (122), and downregulation of host AMP production (65) are other demonstrated mechanisms of resistance in Gram-negative bacteria.

The Coagulation System

The primary function of the coagulation system is to prevent bleeding at the site of an injury until the wound is repaired. The coagulation system includes a number of serine proteases, or coagulation factors, that circulate in the blood in zymogen forms. Clotting is initiated by the intrinsic or the extrinsic pathways of coagulation, that both result in activation of the common pathway. The common pathway is initiated by activation of factor X to Xa, which catalyses the conversion of pro-thrombin to thrombin. Active thrombin cleaves fibrinogen to fibrin that polymerizes and forms the clot.

The tissue factor (extrinsic) pathway is the most important initiator of coagulation. The pathway is activated by Tissue factor (TF), which is a transmembrane glycoprotein that is constitutively expressed at high levels in all tissues, except cells that are in direct contact with blood (22). Disruption of endothelial boundaries exposes TF to plasma proteins and TF forms a complex with factor VII resulting in activation of factor X. Thereafter the coagulation cascade propagates along the common pathway until a blood clot is formed. The contact activation (intrinsic) pathway is initiated by factors XI and XII, plasma kallikrein and high molecular weight kininogen. The contact system assembles on negatively charged surfaces and leads to activation of factor XII and prekallekrin. Activated factor XII triggers the sequential activation of factors XI, IX and X and ends in induction of the common pathway (26).

Fibrinogen and fibrin

Fibrinogen is a 340 kDa glycoprotein with three pairs of polypeptide chains ($A\alpha$, $B\beta$ and γ) linked together by 27 disulphide bonds. The central region (E domain) contains the so-called fibrinopeptides and binds thrombin. Two outer regions (D domains) are connected to the E domain by a coiled-coil segment. The COOH-terminal of the two $A\alpha$ -chains (αC) extend from the D domains and interact with each other in the E domain. Thrombin cleaves the $A\alpha$ -chains between residues 16-17 and the $B\beta$ -chains between residues 14-15 leading to the release of the fibrinopeptides A and B (Figure 1). The removal of the fibrinopeptides exposes four residues on the NH_2 -termi of the α - and β -chains that fit into complementary pockets in the D domains of nearby molecules. This association causes fibrin molecules to align in overlapping E and D domain arrangements forming twisted fibrils. Following thrombin cleavage the αC chains dissociate from the central region and are available for intermolecular interactions. This association is important for lateral aggregation during fibrin polymerization. The fibrin network is further stabilized by factor XIII forming covalent bonds that cross-link the α - and γ -chains of the fibrin network (84, 143). The degradation of fibrin involves activation of plasminogen to the active protease plasmin by tissue-type plasminogen-activator (tPA). Plasmin cleaves fibrin at the NH_2 -terminal

of the β -chain, α C-chain and the coil-coiled sequence joining the D and E domains (50).

Fibrin(ogen) is important in wound healing, where the fibrin network forms a temporary matrix in which cells can proliferate during wound repair. Fibrin also promotes the proliferation of fibroblasts and endothelia (34, 75). Fibrinogen and some of the coagulation factors are upregulated in response to inflammation. The fibrinopeptides are chemotatic for neutrophils, macrophages and fibroblasts (120, 128), and also exert antibacterial activities (136).

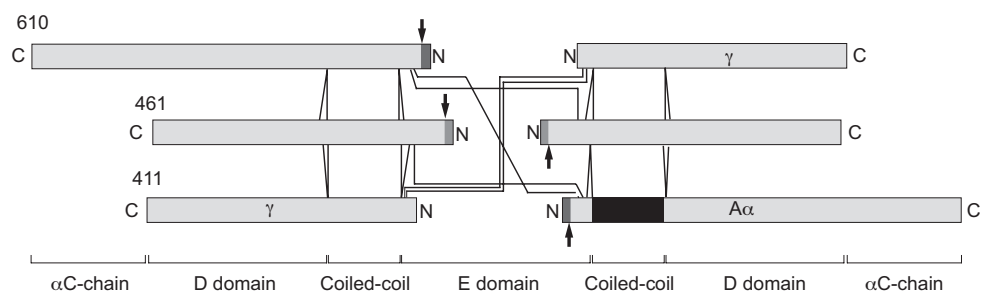


Fig 1 Primary structure of fibrinogen The α C, β and γ chains are represented by bars proportional to the numbers of amino acid residues in each chain without the signal peptide. Intrachain disulfide bonds are indicated with solid lines. Interchain disulfide bonds are not shown. Thrombin cleavage sites are indicated with arrows.

Interactions between pathogens and the coagulation system

An important role for host coagulation is to limit bacterial invasion and spread at the site of a wound. Formation of fibrin clots walls off the injury and contains present organisms locally. However, some bacterial species are able to activate the host's fibrinolytic system. *S. pyogenes*, *S. aureus* and *Yersinia pestis* all have plasminogen activating proteins (41, 77, 129), thought to enable bacteria to dissolve local clots and spread systemically (134). The streptococcal protein, streptokinase, has been used clinically for therapeutic fibrinolysis (74). *S. aureus* can also activate fibrin network formation by producing the prothrombin-binding protein staphylocoagulase. It is unclear if this property is an important virulence factor (97). Many pathogens synthesize fibrin(ogen)-binding proteins. This binding may serve as substrate for microbial adhesion (101) or contribute to survival in human blood (58, 111). Several bacterial proteases are capable of cleaving fibrinogen. *Porphyromonas gingivalis* express the potent fibrinogenolytic proteases gingipain K and R (104) that in nanomolar concentrations makes plasma unclottable (64). Similar effects on plasma are also seen with SufA from *F. magna* (paper II). Proteolytic cleavage of fibrinogen with the subtilase CspA from Group B streptococcus contributes to evasion from opsonophagocytosis (58).

Present Investigation

SufA – a novel subtilisin-like serine proteinase of *Fingoldia magna* (Paper I)

Subtilisin-like proteases (or subtilases) are a large and very diverse family of serine proteases found in all kingdoms of life. In prokaryotes subtilases are generally secreted outside the cell and play a role either in nutrition or in host invasion (108, 127). Several subtilases with high specificity have been implicated as virulence factors; Streptococcal C5a peptidase (ScpA) inactivates the complement chemotaxin C5a (60), Streptococcal chemokine protease (ScpC) degrades the neutrophil attractant CXCL8/IL-8 (59), AB₅ subtilase cytotoxin from Shiga toxigenic *E. coli* inactivates the endoplasmic reticulum chaperone BiP (100). Some subtilases from the *Bacillus* species with low specificity are utilized commercially (81). Most prokaryotic subtilases have a multi-domain structure, and are synthesized as pre-pro enzymes. The domain structure consists of a signal peptide facilitating membrane translocation, a pro-peptide for enzymatic activation, a protease domain, and frequently additional domains. Secreted subtilases can remain attached to the cell wall if a cell wall anchoring motif is present (124-126).

In paper I we describe the purification and characterization of a novel subtilase in *F. magna* denoted SufA (Subtilase of *F. magna*). The project starting point was a gelatinase activity found on the cell surface and in the medium of clinical isolates of *F. magna*. Gelatinase activity of *F. magna* strains have earlier been reported (57, 72, 131), but the responsible gene(s) were not identified. Zymographic technique identified two zones of gelatin degrading activity in both growth medium, and cell surface protein preparations from the ALB8 strain. A protein band corresponding to the major gelatinase activity in a partially purified enzyme preparation from the cell surface was subjected to MS/MS analysis. *De novo* peptide sequences generated from the analysis were used for designing degenerate PCR primers. Combination of the PCR primers resulted in amplification of target regions flanked by the *de novo* peptide coding sequences. Flanking regions were amplified with nested suppression PCR. The generated PCR fragments were DNA sequenced and the assembled gene structure revealed an ORF encoding a 127 kDa protein with a putative promoter region and a ribosome binding site.

The ORF sequence was homologous to subtilases with the closest relationship to the genus of Firmicutes, and displayed the typical multidomain structure of subtilases. Furthermore, a putative catalytic triad in the order of Asp, His and Ser was predicted. Based on sequence homology, domain structure and the catalytic triad order the protein was denoted SufA.

Recombinant expression of SufA in fusion with GST resulted in an inactive enzyme. Antibodies raised against recombinant SufA reacted against multiple products of purified enzyme preparations and also blocked the gelatinase activity.

SufA efficiently cleaved the AMP LL-37 and the antibacterial chemokine MIG/CXCL9 and the resulting fragments were not active against *F. magna*. Furthermore, the *sufA* gene and gelatinase activity was present in the majority of investigated strains.

In conclusion, SufA is a novel protease of *F. magna*. The enzyme may contribute to the resistance to the innate immune system of humans through cleavage of LL-37 and MIG/CXCL9.

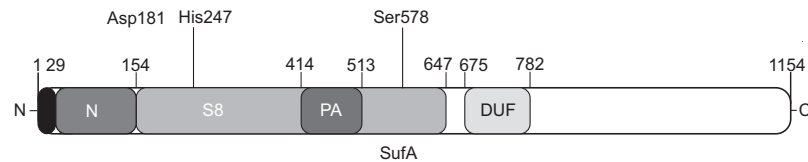


Figure 2 Primary structure of SufA. Predicted catalytic triad sites are indicated at the top. Putative domains, S: signal peptide, N: Subtilisin pro-peptide, S8: catalytic domain of peptidase S8 family (subtilase), PA: Protease associated domain, DUF: domain of unknown function 1034.

SufA – a bacterial enzyme that cleaves fibrinogen and blocks fibrin network formation (Paper II)

In paper II we studied the interaction between SufA and fibrinogen. Purified SufA rapidly cleaved fibrinogen in plasma and both A α - chains and B β -chains were degraded by the enzyme. Possible cleavage sites, identified with MS, were located at the NH₂-termini of the A α - and B β -chains and also in the COOH-terminal of the A α -chain. SufA delayed plasma thrombin clotting time (TCT) in a dose dependant manner and at the highest SufA concentration no detectable clot was formed. This indicating that SufA-cleaved fibrinogen is not available for forming fibrin networks probably by removal of the central polymerization sites.

To directly study the role of SufA on the bacterial surface and the interaction with fibrinogen we generated an isogenic mutant lacking SufA activity. The *sufA* gene in the ALB8 strain was disrupted by insertional duplication. The generated strain lacked gelatinase and fibrinolytic activities. TCT of plasma incubated with the wild-type strain displayed an increasing delay and no detectable clot was formed at the highest bacterial concentration. In contrast, CK05 bacteria did not influence TCT of plasma. In a plasma environment with wild-type and mutant bacteria adhering to keratinocytes, only wild-type bacteria blocked the formation of fibrin networks. Blocking of fibrin network formation might be advantageous for *F. magna* at the site of infection.

Identification of a novel protein promoting the colonization and survival of *Finegoldia magna*, a bacterial commensal and opportunistic pathogen (Paper III)

Paper III describes the identification and characterization of a novel *F. magna* protein FAF (*F. magna* adhesion factor). Some strains of *F. magna* form large aggregates when cultured *in vitro*. The aggregating strain ALB8 displays hair like projections protruding from the cell wall. These molecules were solubilized with CnBr and subjected to Edman degradation. With basis of the peptide sequence, the *faf* gene was cloned and sequenced. The mature 64.7 kDa protein is anchored to the cell wall with a LPXTG-like motif and the segment protruding from the surface is predominantly α -helical and probably arranged in a coiled-coil structure. A fragment of FAF is also shed from the bacterial surface. The mechanism of release is not known in detail, but SufA may contribute to this by proteolytic cleavage (Paper IV). Recombinant FAF was found to self-associate and the interaction was mapped to the COOH-terminal half of the protein. In contrast to the ALB8 strain, another isolate, 505, lacking FAF did not display the hair like projections and did not form aggregates.



Figure 3 Primary structure of mature FAF. A: alanine rich region Putative domains include CNA: *Cna* protein B-type domain, W: wall spanning domain, M: membrane spanning domain.

FAF-expressing bacteria were found to adhere to BMs of human skin biopsies. BM components were tested for recombinant FAF binding, and BM-40 was found to interact with FAF with high affinity in Surface plasmon resonance spectroscopy. This association was also mapped to the COOH-terminal half of FAF. Self-association and BM-40 binding are probably located at different sites in FAF since the associations do not compete. In electron microscopy of human skin biopsies incubated with ALB8 bacteria, co-localization of BM-40 and FAF was detected by using specific antibodies. In addition, in human skin biopsies from a healthy donor, endogenous *F. magna* expressing FAF were detected with FAF-specific antibodies. The bacteria were located adjacent to BMs at the epidermal/dermal junction. Recombinant FAF also binds LL-37 and ALB8 (FAF⁺) bacteria were more resistant to LL-37 killing compared to 505 (FAF⁻) bacteria. Furthermore, soluble FAF was able to rescue 505 bacteria from killing with the AMP.

SufA, a serine-protease of *Fingoldia magna*, enhances bacterial survival by modulating activities of the antibacterial chemokine MIG/CXCL9 (Paper IV)

In paper IV we further study the interaction between *F. magna* and MIG/CXCL9. Earlier studies have shown that IFN- γ induces the expression of the chemokine in pharyngeal epithelial cells and that the M protein of *S. pyogenes* has an enhancing effect (37). Similar results were seen in this study with a skin-derived keratinocyte cell line. In contrast to *S. pyogenes*, *F. magna* had no enhancing effect on the MIG/CXCL9 expression. MIG/CXCL9 secreted by the keratinocytes was cleaved by SufA-expressing bacteria, but not by the SufA mutant strain CK05. MS identified that SufA trims MIG/CXCL9 in the antibacterial COOH-terminal region. The remaining fragments were not active against *F. magna* but retained their activity against *S. pyogenes*. Transmission electron microscopy showed differences in cell wall architectures between the species and this might contribute to the variable AMP susceptibility. Finally, we found that MIG/CXCL9, in analogy to LL-37, binds to FAF and that this blocks the antimicrobial activity.

Conclusions

- SufA is a novel subtilisin-like protease of *F. magna* that hydrolyses gelatin and inactivates the AMP LL-37 and the antibacterial chemokine MIG/CXCL9.
- SufA cleaves fibrinogen and thereby blocks fibrin network formation.
- The novel protein FAF forms hair-like projections on *F. magna* cell surfaces.
- FAF is important for bacterial clumping, adhesion to BMs and resistance to LL-37 and MIG/CXCL9 killing.
- The genes for *sufA* and *faf* are present in a majority of investigated clinical isolates of *F. magna*.
- *F. magna* is transformable with recombinant plasmids

Populärvetenskaplig sammanfattning

Människans normalflora uppskattas bestå av närmare tusen olika bakteriearter varav de flesta är anonyma anaeroba. Gruppen Grampositiva anaeroba kocker (peptostreptokocker), en heterogen del av människans normalflora, bär sällan ansvar för kliniskt viktiga infektioner hos friska individer, men orsakar led-, hud- och djupa sårinfektioner samt vaginoser hos människor med nedsatt immunförsvar. Trots att bakterierna utgör ett medicinskt problem är de dåligt karakteriserade avseende virulens- och kolonisationsfaktorer vilket sannolikt beror på den långsamma anaeroba tillväxten, återkommande ändringar i klassificeringen samt att de ofta isoleras tillsammans med andra bakterier.

Fingoldia magna (tidigare *Peptostreptococcus magnus*) återfinns i 1/10 av alla anaeroba infektioner och är troligen den mest patogena grampositiva anaeroba kocken som isoleras från mänskliga infektioner. Avhandlingsarbetet har inriktats på att identifiera och karakterisera ytbundna och extracellulära protein hos *F. magna*, samt att studera molekylära interaktioner mellan dessa proteiner och den humana värdorganismen. Målsättningen är en ökad förståelse, och insikt i, denna normalflorbakteries sjukdomsalstrande egenskaper.

Proteolys (sönderdelning av proteiner) är en viktig faktor för patogena bakterier. Det finns många exempel på bakterieproteaser som inaktiverar proteinkomponenter i värdens immunförsvar eller degraderar mänsklig vävnad.

Delarbete I identifierar ett proteas benämnt SufA (Subtilase of *E. magna*); proteaset återfinns i de flesta kliniska isolaten av *F. magna* som undersökts. Den proteolytiska aktiviteten framrenades och genen som kodar för proteaset sekvenserades. SufA verkar genom att klyva två av det medfödda immunförsvarets bakteriedödande komponenter, LL-37 och MIG/CXCL9. Hotande bakterier dödas vanligtvis snabbt av dessa, men genom sin förmåga att uttrycka SufA verkar *F. magna* delvis kunna undvika detta.

I delarbete II karakteriseras SufA-klyvningen av fibrinogen, nyckelproteinet i koagelbildning. Vid aktivering av koagulationssystemet omvandlas fibrinogen till fibrin, vilket polymeriserar och bildar ett fibrinätverk. Fibrinogen som inkuberats med SufA visar sig inte kunna bilda fibrin. En mutantstam av *F. magna* utan proteolytiskt aktivt SufA konstruerades för att studera hela bakteriens interaktion med fibrinogen och koagulationen. Mutantstammen saknar, till skillnad från ursprungsstammen, förmågan att förhindra fibrinbildning.

Delarbete III identifierar ett ytprotein kallat FAF (*E. magna* adhesion factor). Proteinet syns i elektronmikroskop som hårliknande strukturer dominerande på bakterieytan. FAF är självassocierande och bidrar till att *F. magna* bildar aggregat. FAF binder även starkt till ett basalmembranprotein (BM-40), som finns i gränsområdet mellan den mänskliga över- och läderhuden, interaktionen bidrar till att bakterien kan fästa sig i huden. FAF visar sig även ha förmåga att binda LL-37 och förhindrar därmed, tillsammans med SufA, peptidens avdödning av bakterien.

I delarbete IV studeras interaktionen mellan MIG/CXCL9 och *F. magna* närmare. Förutom sina bakteriedödande egenskaper är MIG/CXCL9 en kemokin som attraherar vita blodkroppar och motverkar blodkärlsbildning. Kemokinen utsöndras bland annat från hudceller vid stimulering av cytokinen IFN- γ , en del i den inflammatoriska processen och kroppens svar på infektioner. MIG/CXCL9 produceras i mindre mängd av hudceller som inkuberats med *F. magna* jämfört med hudceller inkuberade med grupp A streptokocker. SufA klyver det MIG/CXCL9 som utsöndras från hudceller inkuberade med *F. magna*, vilket leder till att molekylen förlorar sina antibakteriella egenskaper, men behåller sin förmåga att verka som kemokin.

Avhandlingsarbetet identifierar två nya ytprotein vars egenskaper bidrar till adherens av *F. magna* och resistens mot antibakteriella peptiders aktivitet. *F. magna* kan även hindra fibrinbildning genom klyvning av fibrinogen med SufA.

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