Gram-positive anaerobic cocci

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Chapter 8

Finegoldia magna

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1. Introduction

1.1 Classification, morphology and epidemiology

Classification. The genus *Finegoldia* belongs to the class of clostridia, phylum Firmicutes. *Finegoldia magna*, the only species present in this genus, is the most commonly isolated gram-positive anaerobic coccus (GPAC) from human clinical specimens [44]. The taxonomy of this species has changed dramatically. In 1933 *F. magna* was first described by Prevot [52] and named *Diplococcus magna* based on the cellular appearance. Holdeman and Moore [25] added the species to the genus *Peptococcus*. In 1983 Ezaki et al. [15] transferred *Peptococcus magnus* to the genus *Peptostreptococcus*. This transfer was based on DNA base composition, DNA-DNA hybridization data and cellular fatty acid profiles. Classification of GPAC using pyrolysis mass spectrometry (PMS) showed that *P. magnus* was distinct from other GPAC species [41]. In addition, 16S ribosomal sequence analyses indicated that *Peptostreptococcus magnus* is phylogenetically distant from the other GPAC. Therefore, the genus *Finegoldia* was proposed to cover the species *F. magna* [43].

Morphology. Cells of different strains of *F. magna* can vary in size. Gram-staining shows clumps of cells, mostly in tetrad formation, with a diameter of 0.8 to 1.6 µm, which are stained gram-positive. A typical clump consists out of large cocci in the middle, surrounded by cells smaller in size. The largest cells have the strongest color, while smaller cells can be decolorized [42].

After 48 hours of incubation on blood agar plates, colonies are approximately 1 mm in diameter. After 5 days of incubation > 90 % of the strains had colonies > 2 mm in diameter. On one plate the colonies can vary in size and color. Some colonies are convex and whitish, while others are flat and translucent. This might give the impression that it is a mixed culture. However, colonies always have a perfect round shape, and are usually slightly convex [42].

Epidemiology. *F. magna* is one of the most frequently recovered anaerobes from clinical infectious material. In such infections approximately 30 % of the anaerobes are GPAC of which around 30 % is *F. magna* [69]. Remarkably, *F. magna* can be isolated from clinical specimens in pure culture and can be surpassed by *Bacteroides fragilis* in this aspect only.

Neut et al. [47] analyzed the presence of GPAC in normal oral, fecal and vaginal microbiota. *F. magna* was found in the vaginal and fecal microbiota (especially healthy adults), but not in the oral microbiota. Riggio et al. [54] determined the presence of *F. magna* in oral samples using a specific PCR. In total, 33 subgingival plaque samples of patients with adult periodontal disease and 60 pus aspirates from patients with acute dentoalveolar abscesses were analyzed.

In 2 subgingival plaque samples *F. magna* DNA was encountered. These results show that *F. magna* is not a major pathogen in adult periodontal disease and dentoalveolar abscesses. Gao et al. [19] analyzed the superficial skin bacterial biota of human forearm of 6 subjects. A total of 91 genera were found, of which 6 were observed in all subjects. One of these was *Finegoldia* AB109769, suggesting that *F. magna* is part of the normal skin microbiota. Higaki et al. [24] analyzed the anaerobes isolated from infectious skin diseases. The most commonly isolated anaerobes were GPAC (66 of 106 strains, 62 %), among these *F. magna* was the most frequently GPAC isolated (27 strains, 41 %). *F. magna* shows similar pathogenic features as *Staphylococcus aureus*. In patients with infected breast abscesses, *F. magna* can be isolated alone or together with *S. aureus* [24]. Since both organisms display synergism of pathogenicity, it is more difficult to cure such infections.

Brook [9] evaluated the recovery of anaerobes, among them *F. magna*, from clinical specimens during a twelve year period. Of all GPAC isolated, 18.4 % were identified as *F. magna*. The majority of these *F. magna* strains (65.7 %) were isolated from abscesses, obstetrical and gynecological infections, and wounds. The highest frequency of recovery of *F. magna* was shown in bone and chest infections. In pediatric patients 680 GPAC were recovered from 598 clinical specimens, from 554 patients [8]. From all these strains, 10.9 % were *F. magna*. The majority was isolated from abscesses. Bourgault et al. [4] evaluated the clinical significance of *F. magna* in 222 patients. Of these patients 183 had an infection in which *F. magna* played an important role. In 17.5 % of these cases *F. magna* was isolated as a pure culture, from infections of bone and joint (56.3 %), soft tissue (37.5 %) and vascular (6.3 %). In mixed infections in which *F. magna* involved, the most frequently isolated facultative bacteria were: group D *Streptococcus*, *Staphylococcus epidermis*, *Escherichia coli* and *S. aureus*. The most frequently found anaerobic bacteria were: *Prevotella melaninogenica*, *B. fragilis* and *Bacteroides* sp. These mixed infections were mainly infections of soft tissue (37.7 %), bone and joint (21.2 %) and foot ulcers (19.2 %). From this data it can be concluded that there is a strong association between *F. magna* found in pure culture and orthopedic procedures and post-operative wound infection.

1.2 Clinical features and pathogenesis

F. magna is capable of producing several virulence factors (Table 1). In 1984, Brook et al. [7] examined the pathogenicity of GPAC in mixed infections. Abscesses caused by two organisms, including one strain of GPAC, were larger compared with abscesses caused by one organism. From their experiments it was concluded that *F. magna* and *Peptostreptococcus anaerobius* were equally

Table 1. Virulence factors of *F. magna.*

Virulence factor	Function	Reference
Collagenase	Breakdown of collagen	32
PAB	Binding to human serum albumin	46
Protein L	Immunoglobulin (Ig)-binding protein	3
	Release of de novo-synthesized mediators	51
SufA	Degradation of fibrinogen	28
	Degradation of antibacterial peptides LL37 and MIG/CXCL9	28, 29
	Release of FAF from bacterial cell wall	29
FAF	Mediation of bacterial aggregation through protein-protein interactions between FAF molecules on neighbouring *F. magna* bacteria	18
	Binding with BM-40, a non-collagenous glycoprotein, present on the skin	
	Blocking the activity of LL-37, an antibacterial peptide	
	Inactivation of the antibacterial peptide MIG/CXCL9	29

important or more important than the other bacteria in mixed infections. This supports the hypothesis that bacteria in mixed infections may have a synergistic nature. The collagenase production of *F. magna* is associated with the site of infection [32]. Collagen is abundantly present in the skin, tendons, and cartilage and is an organic component of bones, teeth, and the cornea. The breakdown of collagen will result in loss of tissue integrity and disease progression, hereby providing an environment suitable for growth of anaerobic bacteria. The production of collagenase may also be important for the growth of asaccharolytic bacteria such as *F. magna*, since during collagen breakdown amino acids are released which may be necessary for growth and survival [23]. Ng et al. [49] determined the aminopeptidase activities of some GPAC strains. *F. magna* together with *Parvimonas* (*Pa.*) *micra* were found to degrade most substrates. There was a correlation between gelatin hydrolysis and the number of aminopeptidases produced. The authors state that gelatin hydrolysis reflects the pathogenetic potential of a strain. The growth of the bacteria can be correlated to the amount of aminopeptidases produced, due to protein degradation.

Myhre [46] described that 42% of the *F. magna* strains is able to bind human serum albumin (HSA). This ability was originally described for different streptococcal species. In group C and G streptococci this is mediated by protein G, and in group A streptococci by protein M [45]. De Chateau et al. [11] demonstrated that some strains of *F. magna* express protein peptostreptococcal albumin binding (PAB) on their cell surface. Sequence analyses revealed homology with the HSA-binding domain of protein G and to the framework regions of protein L (described later). This suggests an interspecies exchange of an HSA-binding protein module.

In general, host binding cell wall proteins of gram-positive bacteria share a common structure, including a (from the distal NH_2 terminus) [56]:
- signal sequence
- variable NH_2-terminal region
- varying number of repeated domains that independently bind different plasma proteins
- proline-rich region supposedly intercalating the protein in the gram-positive cell wall
- COOH-terminal cell wall sorting signal, required to anchor the protein to the cell wall

PAB contains a GA module (protein G-related albumin binding module). This is a centrally located domain of 45 amino acid residues, which is responsible for the binding of HSA. This domain is subject to module shifting. The predecessor of the PAB protein is urPAB. This protein does not contain the shuffled GA module, but has a uGA domain in the NH_2 terminal region. This domain shows 38 % similarity with the GA module and binds HSA to a lesser extent. PAB also contains an analogous uGA domain, which indicates a second binding site for HSA. The affinity for HSA differs between the GA modules. A reason for bacteria to acquire the GA module is that the older uGA domain has lost its function due to the difference in affinity for HSA. The binding affinity for HSA is not only found on the cell surface, but also in the culture supernatant. The growth of HSA-binding strains is stimulated by the addition of HSA to the growth medium [12]. This selective advantage increases the virulence of HSA-binding *F. magna* strains.

Felten et al. [17] studied the binding of 14 *F. magna* strains isolated from bone joint infections to collagen fibrinogen, and fibronectin after implantation of a foreign body. From these strains, 81 % bound to collagen, 69 % to fibrinogen, and 46 % to fibronectin. When these results were compared to the binding abilities from *F. magna* strains from other infections, a correlation was found between fibrinogen binding and bone joint infections (69 % against 20 %). Krepel et al. [33] tried to elucidate the role of *F. magna* in three different polymicrobial environments: intra-abdominal infections, non-puerperal breast abscesses and diabetic foot ulcers. An association was made between phenotypic characteristics and the site of infection. In total 336 clinical specimens were examined, 222 intra-abdominal from which 11 *F. magna* strains were isolated, 58 nonpuerperal breast abscesses from which 21 *F. magna* strains were isolated and 56 diabetic foot ulcers from which 18 *F. magna* strains were isolated. From the *F. magna* strains the hippurate hydrolase, collagenase and gelatinase production was determined. Strains with the lowest enzymatic activity were isolated from intra-abdominal infections. The most

proteolytic strains were predominantly isolated from soft tissue infections. These are the kind of infections which tend to be chronic and healing slowly. Edmiston et al. [14] showed that *F. magna* is the most common anaerobe isolated from non-puerperal breast infections. *F. magna* strains isolated from non-puerperal breast abscesses and diabetic foot infections were shown to have a higher collagenase production compared to *F. magna* strains isolated from intra-abdominal infections [32]. Stephens et al. [62] determined the impact of the presence of GPAC present in deep tissues of chronic wounds. Clinical samples of 18 patients with chronic venous leg ulcers were cultured. Six of these patients had *F. magna*. None of these *F. magna* strains had any hydrolytic enzyme activity or affected the endothelial cell proliferation. All inhibited fibroblast proliferation and keratinocyte wound repopulation.

Björk [3] was the first to describe a novel bacterial cell wall protein which is able to bind with Ig light chains (L chains), therefore this protein was named protein L. L chains are shared between the different Ig classes. Protein L was found to have affinity with IgG, IgM, IgA, $F(ab')_2$, Fab fragments, and with κ and λ L chains. The reaction with λ L chains is very weak compared with κ L chains. Nilson et al. [50] described that protein L binds exclusively to the V_L domain of Ig and not to the C_L domain. This binding strongly depends on the three-dimensional structure of the V_L domain, indicating that several sites of V_L are involved. It requires the spatial proximity of the κI, κIII and κIV light chain molecules. κ L chains represent 65 % of human immonoglobulins, and of the entire κ chain population, κI, κIII and κIV proteins represent 60, 28, and 2 %, respectively [58]. Protein L has five highly homologous domains which are involved in the binding of Ig. These domains interact with the framework regions of the V_L domain [67]. The strength of the binding of protein L with κ chains is less when compared with the binding of the complete Ig. The conformation resulting from the interaction between heavy and light chains in the Ig gives a more favorable binding site for protein L [1]. The binding site of Ig is close to the antigen-binding site, but the interaction between protein L and Ig was not obstructed by occupation of the antigen-binding site. [1, 26]. Åkerström et al. [1] showed that protein L has no disulfide bond or a subunit structure, and that protein L has two non-Ig-binding fragments which were found to be unique. This was confirmed by Graille et al. [22]. A single protein L domain can react with the variable regions of κ L chains of two Fab molecules, in a sandwich fashion. The contact residues in the variable region are remote from the hypervariable loops. It was suggested that the two binding sites on protein L have a different affinity for Ig. In vivo experiments by Smith et al. [57] showed that protein L prefers to target B cells. This is due to the interaction with Ig on the surface of these cells. This interaction strongly activates the B cells which results in

an upregulation of MHC-II and CD86. These surface molecules are important in initiating an antibody response. No specific binding of protein L with other splenocytes, like T cells and certain dendritic cell subsets, was observed. The activation of B cells also results in an increased expression of the target immunoglobulin. When mature B cells are exposed to protein L, a reduction of splenic marginal zone B cells and peritoneal B1 cells was observed [66]. These two B cell subsets are involved in the first-line immune response against foreign invaders. They have a high antigen presenting capacity and secrete preferentially potentially protective natural IgM. B1 cells are located in the cavities of the body and important in contributing to the production of natural antibodies and T cell independent immune responses. Marginal zone B cells are located at the periphery of the splenic periarteriolar lymphoid sheath at the border of white and red pulp, and they are the first to encounter blood born antigens.

The overall design of protein L is similar to that of protein A from staphylococci and protein G from streptococci, but the primary structure is different. When the amino acid compositions of these proteins are compared, protein L has a higher amount of glycine and a lower amount of lysine. No amino acid sequence homology was demonstrated between these three proteins, apart from the carboxyl-terminal transmembrane region. Some similarity was seen between the W-region of protein G and the amino acid sequence of one of the tryptic peptides of protein L. This W-region is proposed to anchor protein G to the cell wall. Protein L is much smaller in size than protein G, which facilitates tissue penetration [26]. One common feature between the three proteins is that they all possess multiple copies of Ig-binding domains. In each protein these domains are highly conserved [22].

Since protein L is able to bind with all human Ig, it is also able to bind with the κ L chain of IgE. Since the binding of anti-IgE with the Fc portion of IgE stimulates the release of histamine from human basophils, it is possible that the binding of protein L with IgE also stimulates the release of histamine, which will trigger an inflammatory response. Histamine and tryptase are both involved in allergic reactions. This stimulation of basophils was described by Patella et al. [51] The release of histamine is dependent on the concentration of protein L. The interaction with IgE present on the surface of basophils mediates the release of protein L. The stimulation by protein L on the basophils is greater than the stimulation by anti-IgE. Patella et al. [51] also described the release of the preformed de novo-synthesized mediators leukotriene C4 (LTC4) from basophils, or PGD2 from human skin mast cells, both chemical mediators of human inflammatory cells. Genovese et al. [20] described the release of histamine from human heart mast cells. They found a significant correlation between histamine release and tryptase release, and the

release of LTC_4. It is interesting to note that mast cells tend to accumulate at the site of a chronic infection [36]. The release of de novo-synthesized mediators may contribute to the pathogenesis of the infecting strain. It is hypothesized that this may cause mycocardial damage in patients with bacterial infections [20]. Protein L helps the bacteria to adhere to the wound surfaces. The covering of the bacterial cell wall by host proteins allows the bacteria to evade the immune response of the host [26].

Protein L is expressed at the surface of ± 10 % of the *F. magna* strains [26, 31]. Some protein L molecules are released into the growth medium, but most molecules are associated with the cell wall. The protein L from the growth medium shows a considerable heterogeneity in size. This indicates proteolytic degradation of the released protein [1]. Kastern et al. [31] found that only *F. magna* strains which express protein L possess the protein L encoding gene. In non-expressing protein L strains, this gene is not present rather than being down-regulated. The features described for protein L may explain why protein L expressing *F. magna* strains are more often associated with clinical infections than non-expressing strains.

Kastern et al. [31] determined the presence of protein L in 30 *F. magna* strains, all derived from clinical specimens. Four of these strains expressed protein L, and all of these four strains were isolated from women with bacterial vaginosis. The negative strains were from healthy women (n=19), men (n=4) and women with bacterial vaginosis (n=3). These results indicate that there is a correlation between protein L expressing *F. magna* strains and bacterial vaginosis. This was confirmed by de Château et al. [12]. They determined the presence of protein L and HSA-binding protein in 48 *F. magna* strains. Thirty of these strains were isolated from suppurative infections. One of them expressed protein L and 16 strains (53 %) were binding HSA. Eight strains of the 48 strains were isolated from patients with bacterial vaginosis. None of these strains showed HSA binding and five were expressing protein L. The remaining ten strains of the originally 48 were commensal strains, and none of them was expressing protein L or binding HSA. These results confirm the correlation between protein L expressing *F. magna* strains and bacterial vaginosis. Furthermore, it also shows that *F. magna* strains isolated from localized suppurative infections preferentially express HSA binding protein. It is striking to notice that no strains were found which expressed protein L and HSA-binding protein in combination. This was also noticed by Ng et al. [48]. A total of 32 *F. magna* strains, from different origins and countries, were analyzed. Strains were found to be protein L expressing, HSA-binding protein expressing, or expressing neither of them. No strains were found to express both proteins.

Molecular typing of these strains showed that protein L and HSA-binding strains are associated with genotypic clusters.

Recently two other proteins which enhance the virulence of *F. magna* have been described, i.e. a subtilisin-like proteinase (SufA) and a *F. magna* adhesion factor (FAF). FAF is expressed by more than 90% of the *F. magna* strains. This protein is cell wall bound and can be released in the growth medium. The soluble form of FAF causes large protein aggregates and the cell wall bound FAF induces bacterial aggregation through protein-protein interactions between the FAF proteins of the different *F. magna* cells [18]. FAF has the typical features of surface proteins of gram-positive bacteria; a C-terminal part with a cell wall spanning region, a membrane anchor and an intracellular charged tail. *F. magna* strains which express FAF interact with BM40 and colonize the skin in the same way as SufA expressing *F. magna* strains. Another feature of FAF is that it protects *F. magna* against LL-37, an antimicrobial peptide [18]. BM40 is able to stimulate wound healing and it increases the albumin transport across the endothelium. The increase of albumin will stimulate PAB expressing *F. magna* strains in their growth.

SufA is the first described proteinase for *F. magna* [28]. It is associated with the cell wall, but is also released in the growth medium. Most *F. magna* strains possess homologs of SufA. SufA is able to degrade the antibacterial peptides LL-37 and MIG/CXCL9. Hereby *F. magna* enhances its own growth and can spread to commensal areas where it is not present under normal conditions. Since SufA is a subtilisin, it first has to undergo auto-catalytic maturation before it can be active. For full enzymatic activity, dimer formation of SufA is required. The antibacterial peptide MIG/CXCL9 binds with the CXCR3 receptor which activates the G-protein [35]. This receptor is expressed on eosinophils, NK cells, activated T cells, and endothelial cells. Remarkably, MIG/CXCL9 degraded by SufA is still able to bind to the CXCR3 receptor [29]. The fragments of MIG/CXCL9 are still able to kill *Streptococcus pyogenes*, but *F. magna* remains unaffected. This may be explained by the fact that the dimer formation is affected due to the fragmentation of MIG/CXCL9. This may result in a reduced antibacterial activity against *F. magna*. During infections caused by *S. pyogenes* an ecological niche can be created for *F. magna*. SufA is also able to release the FAF protein from the cell wall of *F. magna*. Karlsson et al. [29] described that FAF is able to bind with MIG/CXCL9 with a high affinity. The release of FAF from the cell wall results in a decrease of the antibacterial activity of MIG/CXCL9, hereby promoting the growth of *F. magna* during inflammation. In human plasma SufA degrades fibrinogen, a major clotting enzyme [30]. It increases the thrombin-induced coagulation time in a dose-dependent manner. Fibrinogen is cleaved by thrombin to create fibrin, which forms a temporary matrix in which cells can proliferate during wound repair. In its soluble

form SufA forms dimers and/or multimers, which are proteolytically more active when compared with the monomers [30]. Fibrinogen consists out of 3 pairs of non-identical chains Aα, Bβ and γ [40]. Firstly, SufA removes the C-terminal portion of fibrinogen Aα chains. Secondary, the NH_2-terminal part of the Bβ chains are attacked. At higher concentrations of SufA the Aα chains are further processed. Hereby, the central polymerization sites are removed. SufA associated with the cell wall prevents the formation of fibrin networks by binding to keratinocytes [30]. When the skin is damaged or infected, *F. magna* SufA expressing bacterial cells will be in contact with plasma proteins. The fibrinogen present in the plasma will be broken down by SufA present on the cell wall. The formation of a fibrin network is delayed. The fibrinopeptides (FPA and FPB) which are released during the cleavage of fibrinogen are chemotactic agents for neurophils, macrophages and fibroblasts. They also exert antibacterial properties against gram-negative and gram-positive bacteria [63]. It seems that besides the clotting also other fibrinogen mediated processes are disturbed by SufA. FAF and SufA expressing strains might impair wound healing, as has been described by Stephens et al. [62].

1.3 Genome organization

Recently, the genome of *F. magna* ATCC29328 was assessed by Goto et al. [21]. It consists of a circular chromosome (1.797.577 bp, average G+C content 32.3 %) and a plasmid pPEP1 (189.163 bp, average G+C content 29.7 %). Complete gene sets for the biosynthesis of glycine, serine, threonine, aspartate, and asparagine were present. There were no carbohydrate phosphotransferase system (PTS) genes present for glucose, maltose, mannose, glucitol, cellobiose, and lactose. PTS genes for mannitol, galactitol, and sucrose were incomplete. A lot of genes encoding aminoacid/oligopeptide transporters were found on the genome. This enables *F. magna* to take up amino acids from the environment for growth and survival. Genes for superoxide reductase, NADH oxidase, and putative NADH dehydrogenase were also present. They probably are important for the survival of *F. magna* in intermediate aerobic conditions, such as mucosa and the skin. Virulence factors for antiphagocytosis were encoded by genes present on the chromosome and the plasmid, one on each. In total 4 genes encoding albumin-binding protein homologs were present, 3 on the chromosome and 1 on the plasmid. In total, 10 genes encoding collagen binding proteins were found, 5 on the chromosome and 5 on the plasmid. In total 20 genes encoding N-acetylmuramoyl-L-alanine amidase homologs (Cwp66) were encountered, most of them located on the chromosome. These proteins play a role in the adherence of bacteria to host cells. The presence of genes encoding sortase was assessed on the chromosome and plasmid. On the chromosome 4 genes encoding sortase homologs were

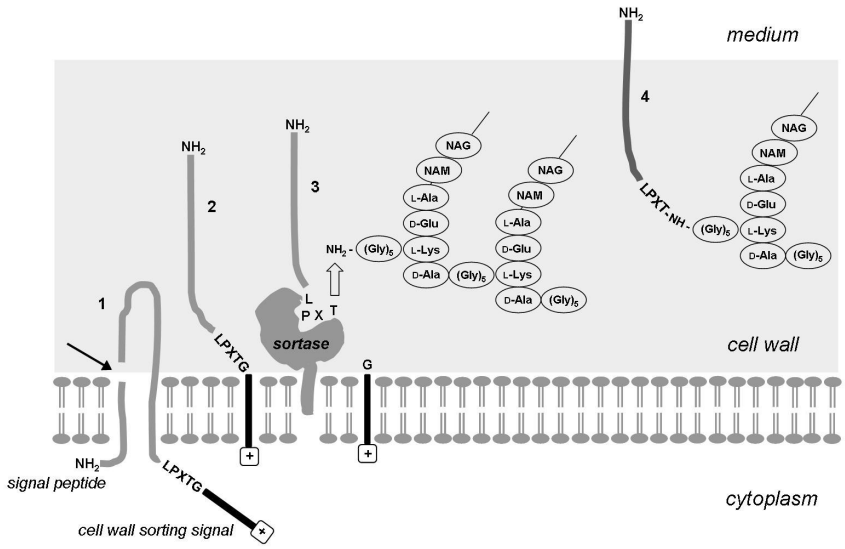

Fig. 1 A schematic overview of the mechanism of covalent binding of surface proteins. (Adapted from: Cossart, P. and Jonquières, R., *Proc. Natl. Acad. Sci. USA,* 97, 5013, 2000)

1. The precursor protein is directed by the N-terminal region of the signal peptide to the cell membrane, after which it is cleaved by a signal peptidase (arrow).
2. The precursor retains within the secretory pathway, probably due to the positively charged tail and the hydrophobic region.
3. The pentapeptide motif LPXTG is cleaved by sortase between the threonine and glycine residues. An amide-bond is formed between the produced carboxyl and peptidoglycan in the cell wall.
4. The surface protein is anchored in the cell wall.

present and on the plasmid 7. Especially the presence of 7 sortase homologs on the plasmid is interesting. It is the highest number of sortase homologs present on a plasmid, for as far as genome sequences are determined for gram-positive bacteria. This feature might be unique for *F. magna*. Since plasmids are considered to be of foreign origin, the amount of sortase homologs present on a plasmid may play an important role in the pathogenesis of *F. magna*. Sortases, are extracellular transpeptidases, which catalyze the cell anchoring of cell wall proteins. Sortases can be grouped into 4 or 5 different classes [10, 13]. Each subgroup has his own preference for substrates, depending on the amino acids present in the cell wall sorting signal pentapeptide motif. Sortase A is the most important one and catalyzes the highest number of substrates. The precursor of a cell wall bound protein is synthesized in the cytoplasm with an N-terminal signal

peptide and a C-terminal sorting signal (Figure 1). The cell wall sorting signal consists out of a pentapeptide motif (for sortase A: LPXGT motif) a hydrophobic region, and a tail of charged residues [55]. The N-terminal signal peptide directs the precursor to the membrane for translocation. It is assumed that, after cleavage by a signal peptidase, the hydrophobic region and the positively charged tail retain the precursor within the secretory pathway, until the sortase has recognized the substrate [56]. The membrane-anchored sortase A cleaves between the threonine and glycine residues of the LPXTG-like motif [38]. An amide-bond is formed between the carboxyl group produced by the cleavage of the LPXGT motif and peptidoglycan of the cell wall. Since most virulence factors are displayed on the cell wall, sortases play an important role in the virulence of bacteria. The amount of cell wall anchored protein will be enriched and more varied. This may result in an enhancement of interaction between host tissue and other bacteria in mixed infections. Therefore, sortases are a possible target for the development of new therapeutic drugs against bacterial infections [37].

1.4 Diagnosis

Phenotypical techniques. F. magna strains are difficult to identify, since the strains do not show any saccharolytic activity and only produce acetate as volatile fatty acid (VFA). Identification is therefore based on negative reactions. Microscopic appearance may be rather variable and is therefore not suitable for identification. Ezaki et al. [16] showed that GPAC can easily be identified by their amidase and oligopeptidase activies. Murdoch et al. [42] characterized the described species more fully, among them *F. magna*. In total 9 reference strains of *F. magna* and 78 clinical isolates were analyzed. All strains showed similar proteolytic activity. Strongly positive reactions were obtained for: arginine, leucine, and pyroglutamyl aminopeptidase. Negative reactions were obtained for: proline, phenylalanine, and glutamylglutamyl aminopeptidases. Variations were observed for other proteolytic enzymes. No acid production from mannose, raffinose, glucose, and trehalose was found. A minor part of the strains (22 %) was able to produce a small amount of acid from fructose. Most of the strains were negative for alkaline phosphatase, but 7 % was found to be positive, including the type strain. 19 % of the strains were able to produce catalase. All strains were resistant to sodium polyanethol sulfonate (SPS). If a metronidazole disk is used to exclude anaerobic staphylococci or capnophilic streptococci, it should be noted that *F. magna* is able to produce colonies in the inhibition zone after 48 hours of incubation. Recently, Song et al. [59] developed a flow chart which made the identification of GPAC easier. In this flow chart *Pa. micra* and *F. magna* are differentiated from the other GPAC by their inability to produce β-glucuronidase,

and to ferment glucose and their ability to produce pyroglutamyl arylamidase. *Pa. micra* and *F. magna* are differentiated from each other by the production of proline arylamidase, *Pa. micra* is a producer and *F. magna* is not [59].

Two current methods to phenotypically identify anaerobes are the Vitek system and Matrix-Assisted Laser Desorption Ionisation Time of Flight Mass Spectometry (MALDITOF-MS). Recently, BioMérieux (Marcy, France) has developed a new colorimetric identification card (ANC card), which can be used in the Vitek 2 system, for the identification of anaerobes, including *F. magna*. Evaluation of this card showed that *F. magna* can be reliably identified using this method [39, 53]. MALDITOF-MS is a promising new method to identify bacteria, however no evaluation studies have been published yet, which describe the suitability of the method for the identification of GPAC, including *F. magna*.

Genotypical techniques. Nowadays, nucleic acid based techniques are available to improve the identification of bacteria. Song et al. [60] evaluated if 16S rDNA sequencing is suitable for the identification of GPAC. They established that the quality of the sequences in the public databases can be poor and might lead to misidentification. They sequenced the type strain of *F. magna* and 36 clinical isolates. When the sequences of the clinical isolates were compared with the sequenced type strain, the homology was >99 %. However, when they were compared with the sequences available in the public databases homology was <98 %. When analyzing sequences of GPAC it is best to compare them with the sequences published by Song et al. [60].

PCR methods to identify *F. magna* were developed by Song et al. [61] and by Riggio et al. [54]. Primers specific for *F. magna* were designed and validated. Song et al. [61] validated the primer set on the type strain of *F. magna* and 60 sequenced clinical isolates. The primers were shown to be specific. Riggio et al. [54] validated the specificity of their primers with 5 *F. magna* strains. Furthermore, they applied their PCR method on subgingival plaque samples from patients with adult periodontal disease and on 60 pus aspirates from subjects with acute dentoalveolar abscesses. No nonspecific reactions were encountered.

A species-specific 16S rRNA based probe was developed by Wildeboer-Veloo et al. [68]. The fluorescently labeled probe was used to rapidly and reliably identify *F. magna*, using fluorescence *in situ* hybridization (FISH). The probe was designed and validated using sequences of reference strains of *F. magna* and 26 clinical isolates and was shown to be specific. The probe was applied on 100 unknown clinical isolates of GPAC, of which 29 strains were identified as *F. magna*. Permeabilization of the cell wall of *F. magna* strains, using proteinase-K, was

necessary prior to hybridization. The probe has the potential to be suitable for direct application on clinical material.

2. Methods

2.1 Sample preparation

No special sample preparation is necessary for the isolation of *F. magna.* The usual sample preparation conditions for the isolation of anaerobic bacteria will be sufficient [27]. Clinical specimens should be collected, avoiding contamination with commensal skin or mucus surface microbiota. Transportation of specimens should take place in an appropriate transport medium for anaerobic bacteria.There is no selective medium available for the isolation of *F. magna*. *F. magna*, like most GPAC, will grow on a standard medium, like Brucella Blood Agar (BBA) and Schaedler blood agar. Mostly, clinical specimens suspected to contain anaerobic bacteria are cultured on a set of plates. A universal medium (for example BBA), phenyl alcohol blood agar (PEA), which prevents swarming of bacteria, and media selective for gram-negative anaerobes like Bacteroides bile esculin agar (BBE) and kanamycin vancomycin laked blood agar (KVLB). The latter promotes also the pigmentation of bacteria. *F. magna* will grow on the universal medium and PEA [27]. Strains suspected to be *F. magna*, after being tested for aerotolerance, can be identified as described in section 1.3.

2.2 Detection procedures

F. magna can be recognized by its colony form and cell morphology, as described above. However, this is not sufficient for identification. Other biochemical features which can be used are its slow growth, the odor, resistance to SPS, nitrate reduction, coagulase production, no indole production, and enzyme profile [27, 34, 59].

Bassetti et al. [2] reported a case of endocarditis caused by *F. magna*. However, in this case the blood cultures were initially negative. They showed that the detection of *F. magna* in blood depends on the type of blood culture systems used. Bact/ALERT FA and FN bottles were still negative after 4 weeks of incubation. SEPTI-CHEK BHI-S bottles and the ISOLATOR system already showed growth after 2 days of incubation. This may account for the fact that bacteremia caused by *F. magna* is rare [64]. Microbiologists should be aware of the fact that a bacteremia caused by *F. magna* can be missed when the blood culture system Bact/ALERT is used.

We present below three molecular-based techniques for identification and confirmation of *F. magna.*

(i) Protocol of Song et al. [61]

Principle. A multiplex PCR assay was developed to rapidly identify GPAC. Genus- and species-specific primers were used. Since *F. magna* is the only species present in the genus *Finegoldia*, it can be identified directly to species level.

Procedure:

(1) Suspend one or two colonies of the bacterial strain in 50 μl Tris-HCl/EDTA/saline (pH 8.0), and incubate for 10 min at 95 °C.
(2) Centrifuge for 2 min at 18 600 g, and decant the supernatant.
(3) Resuspend the pellet.
(4) Prepare the PCR mixture (50 μl) containing: 1.25 U *Taq* polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1 % Triton, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 μl of bacterial lysate, 0.25 μM of the universal primer 1392B and 0.5 μM of the genus/species-specific primer FIGD.
(5) Perform the PCR for 35 cycles; denaturation at 95 °C for 20 s, annealing at 50 °C for 1 min, and extension at 72 °C for 30 s. To the final extension add a cycle of 72 °C for 5 min.
(6) Analyze PCR products on a 2 % agarose gel, followed by ethidium-bromide staining.
(7) Visualize under UV illumination.

Note. *F. magna* strains will yield an amplicon size of 1200 bp.

(ii) Protocol of Riggio et al. [54]

Principle. This PCR assay was developed for the direct detection of *F. magna* in subgingival plaques samples.

Procedure:

(1) Take a subgingival plaque sample using a sterile curette and put it in 0.5 ml freshly prepared fastidious anaerobe broth (Bioconnections). Mix for 30 s.
(2) Add 3 μl achromopeptidase (20 U/μl in 10 mM Tris-HCl, 1 mM EDTA, pH 7.0) to 100 μl of plaque sample. Incubate at 56 °C for 30 min, boil for 5 min and store at -70 °C.
(3) Prepare the PCR mixture (50 μl) containing: 45 μl 1xPCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100), 1 U *Taq* DNA polymerase (Promega), 0.2 mM dNTPs, 0.2 μM of each primer, and 5 μl lysed plaque sample.
(4) Perform the PCR, starting with a denaturation step of 94 °C of 5 min followed by 35 cycles of denaturation of 1 min at 94 °C, annealing of 1 min at 60 °C, and extension of 1 min at 72 °C. To the final extension add a cycle of 72 °C for 10 min.

(5) Analyze the PCR products on a 2 % agarose gel containing ethidium bromide, by adding 2 μl gel loading dye [0.25 % (w/v) bromophenol blue, 50 % (v/v) glycerol, 100 mM EDTA, pH 8.0] to 10 μl PCR product.
(6) Visualize under UV illumination.

Note. *F. magna* strains will yield an amplicon size of 553 bp. A positive and negative control were added to the assay, consisting of genomic *F. magna* DNA and water, respectively. An appropriate DNA ladder should be included during electrophoresis.

(iii) Protocol of Wildeboer-Veloo et al. [68]

Principle. *F. magna* strains are identified by fluorescent *in situ* hybridisation, using a fluorescently labeled species-specific 16S rRNA-based probe.

Procedure:

(1) Take some colonies of bacterial cells and suspend them in phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4/L, pH 7.4). Add an equal volume of 96 % v/v ethanol. Mix and store at -20 °C.
(2) Spot the ethanol-fixed cells on three gelatin-coated glass-slides and dry at room temperature.
(3) Immerse the slides in 96 % v/v ethanol for 10 min and allow them to dry.
(4) Add 100 μl proteinase K (500 mg/L in 50 mM Tris-HCl, pH 7.6) to each slide and cover it with a coverslip. Incubate for 10 min at room temperature.
(5) Stop the enzymatic reaction by incubating the slides in 96 % v/v ethanol for 2 min. Allow the slides to dry.
(6) Prepare three different hybridisation mixtures; one for the positive control (probe EUB338), one for the specific probe (Fmag1250) and one for the negative control (probe non-EUB338). Each mixture contains: 10 μl probe (100 ng/μl) and 110 μl hybridisation buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, SDS 0.1 % w/v).
(7) Add to each slide approx. 100 μl of a probe-hybridisation mixture and cover the slide with a coverslip.
(8) Incubate overnight at 50.0 °C in a dark humid chamber/box.
(9) Wash the slides in washing buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2) for 15 min at 50.0 °C. Rinse the slides quickly in milli-Q and dry with compressed air.
(10) Add approx. 10 μl vectashield® (Vector laboratories, Burlingame, USA) and a coverslip. Fix the coverslip with nailpolish.
(11) Examine the slides under an epifluorescence microscope and compare the signal of the specific probe with the positive and negative control.

Note. The proteinase K treatment is necessary to permeabilize the cell wall of *F. magna* to enable the probe to access the bacterial cell. However, this treatment

also increases loss of bacterial cells from the slide. It is therefore recommended to put sufficient bacterial cells on the slide. This technique has the potential for direct application on clinical material.

3. Conclusion and future perspectives

F. magna is part of the commensal microbiota and clearly an important pathogen. *F. magna* is able to express different virulence factors; collagenase, PAB, protein L, SufA, and FAF. The sequencing of the genome of *F. magna* ATCC29328 revealed a total of 4 albumin-binding proteins, 10 collagen-binding proteins, 20 Cwp66 homologs, and 11 sortase homologs. Beside these proteins, some other putative virulence factors may have been revealed, e.g. hemolysin and bacteriocin. The high number of sortase homologs enables *F. magna* to enrich and vary cell wall anchored proteins, hereby increasing its virulence.

Each of these virulence factors increases the pathogenicity of *F. magna*. Until now, it is unclear how many virulence factors one *F. magna* strain can express at the same time. Especially the high number of sortase homologs enables *F. magna* to acquire several virulence factors in one strain. Ng et al. [48] studied 32 *F. magna* strains and found none of the strains expressed both protein L and HSA-binding protein. Molecular typing of these *F. magna* strains expressing protein L or protein PAB revealed that these strains form their own genotypic cluster. More studies should be performed whether *F. magna* strains expressing certain virulence factors, e.g. collagenase etc. are genotypically different from each other. It is also interesting to know if more expressed virulence factors are associated with genotypic clusters of *F. magna*.

Several authors [11, 12, 14, 17, 32, 62] have shown that there is a relationship between certain virulence factors of *F. magna* and the site of infection. For example, *F. magna* strains isolated from non-puerperal breast abscesses and diabetic foot infections have a higher collagenase production when compared with strains isolated from intra-abdominal infections [32]. This site-specific infection and expression of virulence factors have also been shown for protein L and HSA-binding protein [31]. However, these conclusions are based on a small number of studies, and therefore on a relatively small number of strains. Whether there is a site-specific infection for collagenase, SufA, and FAF expressing *F. magna* strains is still unknown. Further studies are necessary to eludicate all virulence factors of *F. magna* and their role in certain infections.

It is also shown that *F. magna* has a negative influence on healing of chronic wounds [62]. It is not clear whether these strains express only one virulence factor or several, and if there is a coherence between the different virulence factors, as has been described by Karlsson et al. [29] between SufA and FAF. In order to

eludicate the role of *F. magna* and the expression of virulence factors, microbiologists should pay more attention to the identification of *F. magna* from clinical specimens. In general, GPAC are susceptible to the antibiotics used to treat an anaerobic infection [44]. However, microbiologists should realize that *F. magna* has one of the highest resistance rates of the GPAC [6, 65], and that there seem to be geographical differences in resistance [5, 44]. To get a coherent picture of *F. magna* and its pathogenicity, studies with larger groups of strains should be performed. Hereby, studying all the specific virulence factors of *F. magna* at the same time and taking the site of infection into account.

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