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Investigations on *Enterococcus faecalis* glycolipids and their role in bacterial virulence

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D. List of abbrevations

The following list contains all abbreviations used in this thesis.

AS	Aggregation substance
CD	Cluster of differentiation
cfu	Colony forming unit
CS	Chondroitin sulfate
d	Day
DAG	Diacylglycerol
DGlcDAG	Diglucosyldiacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	*Et alii*; and others
FBS	Fetal bovine serum
Fig.	Figure
GAG	Glycosaminoglycan
h	Hour
HS	Heparan sulfate
i.p.	Intraperitoneal
i.v.	Intravenous
IFN-γ	Interferon γ
IL	Interleukin
*i*NKT	Invariant natural killer T
kDa	Kilo Dalton
LB	Luria Bertani
LTA	Lipoteichoic acid
MGlcDAG	Monoglucosyldiacylglycerol
min	Minute
MOI	Multiplicity of infection
MSCRAMM	**M**icrobial **s**urface **c**omponents **r**ecognizing **a**dhesive **M**atrix **m**olecules
NK	Natural killer
OD	Optical density
PBS	Phosphate buffered saline
PLF	Peritoneal lavage fluid
PMN	Polymorphonuclear cell
Rf	Retention factor
RPMI	Roswell Park Memorial Institute Medium
RU	Response units
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SEM	Standard error of the mean
spp.	Subspecies
SPR	Surface plasmon resonance
TEER	Transepithelial electrical resistance
TLC	Thin-layer chromatography
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TSA	Tryptic soy agar

TSB	Tryptic soy broth
UTI	Urinary tract infection
VRE	Vancomycin resistant enterococci
WTA	Wall teichoic acid

E. List of publications

Printed publications

Free radical scavenging action of the natural polyamine spermine in rat liver mitochondria.
Sava I., Battaglia V., Rossi C.A., Salvi M., Toninello A.
Free Radic Biol Med. 2006 Oct 15;41(8):1272-81.

Glycolipids are involved in biofilm accumulation and prolonged bacteraemia in *Enterococcus faecalis.*
Theilacker C., Sanchez-Carballo P., Toma I., Fabretti F., Sava I., Kropec A., Holst O., Hübner J.
Mol Microbiol. 2009 Feb;71(4):1055-69.

Novel interactions of glycosaminoglycans and bacterial glycolipids mediate binding of enterococci to human cells.
Sava I., Zhang F., Toma I., Theilacker C., Li B., Baumert T.F., Holst O., Linhardt R.J., Hübner J.
J Biol Chem. 2009 Jul 3;284(27):18194-201.

Endodontic and salivary isolates of *Enterococcus faecalis* integrate into biofilm from human salivary bacteria cultivated in vitro.
Al-Ahmad A., Müller N., Wiedmann-Al-Ahmad M., Sava I., Hübner J., Follo M., Schirrmeister J., Hellwig E.
J Endod. 2009 Jul;35(7):986-91.

Poster presentations

Comparison of opsonic killing and titers of antibodies against LTA between healthy volunteers and patients with *Enterococcus faecalis* bacteremia
Sava I., Theilacker C., Toma I., Hammer F., Kropec A., Hübner J.
EUROPEAN WORKSHOP: Novel opportunities to develop VACcines to control antibiotic REsistant BActeria: from the trials back to the laboratory.
Siena, February 28th-March 2nd, 2007

Binding of enterococci to mammalian cells is mediated by glycosaminoglycans and by bacterial glycolipids
Sava I., Theilacker C., Toma I., Hammer F., Halima M.Z., Baumert T., Hübner J.
American Society of Microbiology 108th General Meeting
Boston, 1st-5th June, 2008

Enterococcal surface protein is a virulence factor in bacteremia but is not a target of opsonic antibodies in *E. faecium* infection
Sava I., Heikens E., Toma I., Kropec A., Willems R., Hübner J.
American Society of Microbiology 109th General Meeting
Philadelphia, 17th-21st May, 2009

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G. Curriculum vitae

1. INTRODUCTION

Enterococci are Gram-positive, facultative anaerobic cocci occurring ubiquitously in the gastrointestinal tract of humans and animals, in soil, food or water (1). Many traditional cheeses in Mediterranean countries contain enterococci, which contribute to their typical taste and flavour. Enterococci are also present in other fermented foods, or in probiotic products, although their role in such products is still a matter of debate, especially since these bacteria have emerged lately as an important opportunistic pathogen in humans.

Previously classified as Lancefield group D streptococci, enterococci became their own genus in 1984 (2). They are able to grow in a very wide range of pH, temperature (between 10°C and 45°C) and osmotic pressure (up to 6.5% NaCl and 40% bile salts) (3). The genus *Enterococcus* has more than 30 species, the most commonly encountered in humans being *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*). They have a predominant habitat in the gastrointestinal tract and, along with approximately 450 other aerobic and anaerobic bacterial species, they constitute the normal gut flora of healthy individuals, with usually between 10^5-10^8 colony forming units (cfu) per gram of feces (4).

Until recently enterococci were regarded as relatively harmless commensal bacteria associated only rarely with opportunistic infections in very ill patients. The ability of enterococci to accumulate and transfer genetic elements, leading to the rapid acquisition of new antibiotic resistance determinants, was one of the major reasons of their increased importance as nosocomial pathogens (5). Of particular concern was the isolation of the first enterococci with resistance to vancomycin (VRE) in 1986 (6). Nowadays many nosocomial strains resistant to vancomycin also show resistance to penicillins and high-level resistance to aminoglycosides. In recent years VRE emerged globally and the treatment of these infections is limited by the lack of effective antibiotics. Currently, enterococci are the third most common pathogen isolated from nosocomial bloodstream infections and the most common isolate from surgical-site infections, in the United States. Additionally, they are more frequently isolated from urinary tract infections (UTI), endocarditis and abdominal infections (7). About 30% of nosocomial enterococcal infections in the United States are caused by VRE (7), while the VRE prevalence in Europe has rapidely increased since the start of the third millennium, even in countries with relatively low antibiotics resistance levels (8).

Historically, *E. faecalis* accounted for 80-90% of the clinical isolates of enterococci, whereas *E. faecium* was found less frequently (5-10%) (9). However, in the last ten years, the proportion of clinical isolates of *E. faecium* increased to about 40%, replacing partially *E. faecalis* which today accounts usually 60-70% (10). The increase in number of enterococci isolated in the last 20 years seems to be predominantly due to changes in medical care (i.e. higher numbers of immunocompromised patients, increased use of indwelling medical devices).

Enterococci are associated with a variety of infections, being sometimes isolated from the same site together with other microorganisms. While the importance of enterococci in mixed infections is not always clear, they certainly play a major role in life-threatening infections such as subacute bacterial endocarditis.

In order to establish infection, enterococci first need to colonize the host. This occurs usually in the health care environment, where VRE can be isolated from virtually everywhere from monitoring devices to doors, floors or fabric seat cushions (11). It is believed that transmission occurs normally by direct contact through the hands of health care workers (12). Successful colonization leads to infection in susceptible hosts, such as older persons, transplant patients or oncologic patients or individuals receiving prolonged antimicrobial therapy. Invasive diseases often occur after initial (asymptomatic) colonization through translocation from the gastroinsteinal tract or through intravascular foreign devices such as intravenous catheters. In addion, enterococci frequently cause UTI, especially lower UTI in older men. They are the third most common pathogens isolated from patients in urological departments in Europe (13).

Enterococci are a leading cause of nosocomial bacteremia especially in immunocompromised patients with cancer, probably due to impaired gastrointestinal mucosa and subsequent translocation (14). Other risk groups include patients in intensive care units and patients receiving solid (especially abdominal) organ transplants (15). A major clinical concern in patients with enterococcal bacteremia is to assure that the source is not subacute bacterial endocarditis. Endocarditis is regarded as the most serious enterococcal infection, causing 10 to 15% of the cases (16). Prosthetic valve endocarditis is increasing due to a more frequent use of valvular prostheses. Although lower than for staphylococcal endocarditis, the mortality rate is of 15 to 20% (17). Enterococci are frequently involved in soft tissue, intra-abdominal and pelvic infections, but in these cases

infection is often polymicrobial. Uncommon infections caused by enterococci include meningitis, osteomyelitis, or septic arthritis (16).

1.1. Virulence factors

Because of the limitations of antimicrobial therapy in treating enterococcal infections, there is growing interest in a better understanding of the factors destabilizing the commensal relationship with the host and the apparent increase in virulence among clinical isolates in the last two decades. Several traits have been identified that contribute to the severity of enterococcal infections and lead to an increased fitness of these bacteria at the site of infection. Although (as an opportunistic pathogen) enterococci do not possess real virulence factors like toxins and hydrolytic enzymes there are several proteins that are commonly recognized as virulence factors contributing to the establishment and persistence of infections.

Table 1.1 The most studied proteinaceous virulence factors in *Enterococcus* subspecies (spp.) [modified after Creti et al. (18)]

Gene	Encoded protein
asa / asa373	Aggregation substance
cyl (operon)	Cytolysin
gelE	Gelatinase
esp	Enterococcal Surface Protein
ace	MSCRAMM
efaA	Antigen A

Aggregation substance is very well characterized, especially in *E. faecalis*, being a surface protein encoded by pheromone-inducible plasmids. Besides pAD1, other 17 pheromone-responsive plasmids with highly conserved sequences have been identified in *E. faecalis* encoding the protein aggregation substance (AS) (19). Secretion of sex pheromones by strains not carrying the above-mentioned plasmids (recipient strains) stimulates the donor strains to produce AS which mediates the physical contact between the donor and the recipient strain, allowing the transfer of the pheromone-inducible plasmid to the recipient

strain. After the transfer of the plasmid, the newly formed donor strain shuts off the synthesis of the sex pheromone (20). Several studies demonstrated the role of the AS as virulence factor of *E. faecalis*. This protein was shown to be involved in adhesion or invasion of eukaryotic cells (21) in binding of lipoteichoic acid (LTA) (22), and survival inside macrophages (23) or polymorphonuclear leukocytes (PMNs) (24). *In vivo,* AS has been shown to play a role in rabbit experimental endocarditis (25), but active immunization with the N-terminal domain of the protein did not confer protection in this model (26).

Many pathogenic enterococcus strains produce cytolysin, a protein encoded by an operon located on conjugative pheromone-responsive plasmids or on the chromosome within the pathogenicity island. Cytolytic *E. faecalis* strains express a bacteriocin with broad activity against Gram-positive, but not Gram-negative bacteria (27).

Fewer than 20% of humans are normally colonized with cytolytic enterococci, while several clinical studies reported an increased frequency of cytolytic enterococci among the strains causing infections (28). Using multiple animal models of enterococcal infection, cytolysin has been shown to be a major contributor to enterococcal pathogenicity. Ike and Clewell (29) showed a direct correlation between the presence of cytolysin and enterococcal virulence in a mouse peritonitis model. In a *Drosophila melanogaster* colonization model, the presence of cytolysin has been shown to be detrimental for the flies' survival (30) and the severity of endophthalmitis in a rabbit model was correlated with the expression of the cytolysin (31).

The enterococcal gelatinase is an extracellular zinc-metalloendopeptidase, which was discovered and characterized more than 40 years ago (32). It is co-transcribed with the serine protease SprE and regulated by the quorum-sensing *fsr* locus (33). Coque and colleagues found that 54% of the clinical isolates from patients with endocarditis produced gelatinase, while only 14% of the isolates from healthy volunteers produced this enzyme (34). In a very recent study, Gaspar *et alii* (et al.) showed that deletion of the gene encoding the gelatinase in an *E. faecalis* diary strain significantly attenuated its virulence in a wax worm (*Galleria mellonella*) model of infection, suggesting that probiotic strains bearing the *gelE* gene should be used with caution in diary products (35). Gelatinase was also shown to play a role in overcoming the innate immune response by inactivating antimicrobial peptides such as LL-37 produced by the host, leading to an enhanced survival of enterococci (36).

Enterococcal surface protein is a large [about 200 kilodaltons (kDa)] surface exposed protein present on pathogenicity islands which are different in *E. faecalis* and *E. faecium.* Shankar and colleagues first described an enterococcal surface protein in *E. faecalis* with homology to alpha-C and Rib proteins in group B streptococci (37). This protein has been shown to be associated with biofilm formation on polystyrene plates (38). However, since Kristich and colleagues (39) and Toledo-Arana and coworkers (40) were able to demonstrate that biofilm formation takes place also in the absence of *esp* gene, it is likely that this protein represents only one of a number of different mechanisms by which *E. faecalis* produces biofilm. In *E. faecium*, enterococcal surface protein is an important marker in epidemic strains since its presence seems to be limited to hospital acquired *E. faecium* clones (41). An insertion-deletion mutation of *esp* in a hospital-acquired *E. faecium* isolate confirmed the role of enterococcal surface protein in initial adherence and biofilm formation on polystyrene surfaces (42). The role of enterococcal surface protein in a mouse UTI model was demonstrated for both *E. faecalis* (43) and *E. faecium* (44) and together these two studies suggest that this protein is an important virulence determinant in UTI caused by either *E. faecium* or *E. faecalis*.

Adhesion of enterococci to host cells is believed to take place by recognition of ligands located on the extracellular matrix or on the surface of the eukaryotic cells. This recognition is mediated by **m**icrobial **s**urface **c**omponents **r**ecognizing **a**dhesive **m**atrix **m**olecules or MSCRAMM. After the publication of the genome sequence of *E. faecalis* V583 and *E. faecium* TX0016, seventeen (45) and fifteen (46), respectively, MSCRAMM have been identified. Three enterococcal MSCRAMM have been studied so far in more detail. The Ace (adhesion to collagen) protein was the first MSCRAMM described in *E. faecalis* and was shown to mediate binding to collagen, laminin and dentin. Acm and Scm are proteins present in *E. faecium* and also binding to different types of collagen and fibronectin [reviewed by Hendrickx et al. (47)].

E. faecalis antigen A is an lipoprotein adhesin with a high degree of similarity with streptococcal adhesins identified by Lowe et al. (48), which plays a role in enterococcal infection as suggested by data obtained in a mouse peritonitis model. An EfaA homolog with 73% of identity has also been identified in *E. faecium* (49).

In 2006, Nallapareddy and colleagues reported the presence of pili on the surface of *E. faecalis* and this proteinaceous fibers were shown to play an important role in biofilm formation and virulence in experimental murine UTI and rat endocarditis (50), (51). The

presence of pili has been recently documented also in *E. faecium*, but their role in establishment of infections was not elucidated so far (52).

1.2. Biofilm formation

The most important Gram-positive nosocomial pathogens, *Staphylococcus aureus*, *Staphylococcus epidermidis* and enterococci (*E. faecalis* and *E. faecium*) account for about 30% of all severe sepsis (53) and bacteremia cases (54). All these bacteria are well known for their ability to produce biofilm, although the mechanisms and factors driving these bacteria to form biofilm are not completely understood (55), (56).

Biofilms are communities of microorganisms living attached to biotic or abiotic surfaces in a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids (57). They represent a protected growth mode for bacteria in hostile environments (58). Biofilms are clinically relevant since a mature biofilm can tolerate antibiotics at a concentration of 10-1000 times higher than the one required to kill an equivalent population of planktonic bacteria; bacteria living in biofilms are more resistant to phagocytosis, making them very difficult to be eradicated from living hosts (59).

The capacity of enterococci to bind to various medical devices, such as uretheral stents, intravascular catheters, biliary stents, silicone gastrostomy devices, artificial heart valves or ocular lens materials has been demonstrated [reviewed by Mohamed et al. (60)]. Several putative enterococcal virulence factors previously discussed were also shown to be involved in biofilm formation.

Conflicting data have been published regarding the role of enterococcal surface protein in biofilm formation by *E. faecalis* and *E. faecium*, suggesting that this protein is not the only factor involved in biofilm development (60). The *fsr* locus has been characterized in *E. faecalis* and contains the *fsr*ABCD genes, which show similarity with the staphylococcal *agr* locus (33). It is a quorum sensing system, which induces and regulates the production of gelatinase and a serine protease, encoded by two genes downstream of this locus, *gel*E and *spr*E (61). The role of gelatinase in biofilm production by enterococci is still contradictory. Although genetic manipulation studies (62), (39), (63) have confirmed that gelatinase is essential for biofilm formation, epidemiological studies have not supported the link between gelatinase and biofilm production among the clinical *E. faecalis* isolates tested. A screening study of more than one hundred *E. faecalis* and *E. faecium* strains by

Di Rosa et al. showed no correlation between the ability to form biofilm and the presence of the *gelE* gene (64). However, Thomas and colleagues demonstrated recently that gelatinase in *E. faecalis* regulates biofilm formation by activating lysis of a bacterial cell subpopulation, which leads to release of extracellular genomic DNA, an important component of biofilms (65).

Bacterial cell wall polysaccharides have also been shown to play a role in formation of biofilm by enterococci. An *epa* (enterococcal polysaccharide antigen) gene cluster mutant of *E. faecalis* (66) formed significantly less biofilm (73% reduction) compared to the wild type bacteria, suggesting that this gene encoding a glycosyltransferase is involved in polysaccharide synthesis and biofilm production.

The sugar-binding transcriptional regulator, *BopD*, is a member of the *bop* (biofilm on plastic) operon. BopD is involved in biofilm formation by *E. faecalis* (67), (68) and recently, Bourgogne and coworkers, using a microarray-based system confirmed that genes of this locus are regulated by the same Fsr quorum sensing system regulating the activity of gelatinase and serine protease (69).

From the various cell wall-associated compounds of low GC Gram-positive bacteria, teichoic acids [LTA and wall teichoic acid (WTA)] represent the most abundant glycopolymers (70). Both these two molecules have been shown to participate in colonization and adherence to host cells. In *Staphylococcus aureus*, WTA has been shown to participate in biofilm formation to polystyrene plates and glass surfaces (71). The lack of a mutant which is devoid of WTA in enterococci made the investigation of the role of this molecule in biofilm formation of enterococci impossible. Mutants lacking LTA have been shown to be lethal (72), but several evidences indicating a role of LTA in biofilm formation have been obtained from mutants deficient in the Alanination of the LTA. Esterification of LTA with D-Alanine changes the net charge of the polymer and a defect in D-Alanination has been associated with a reduced ability of enterococci to adhere to plastic and also eukaryotic cells (73).

A **B**

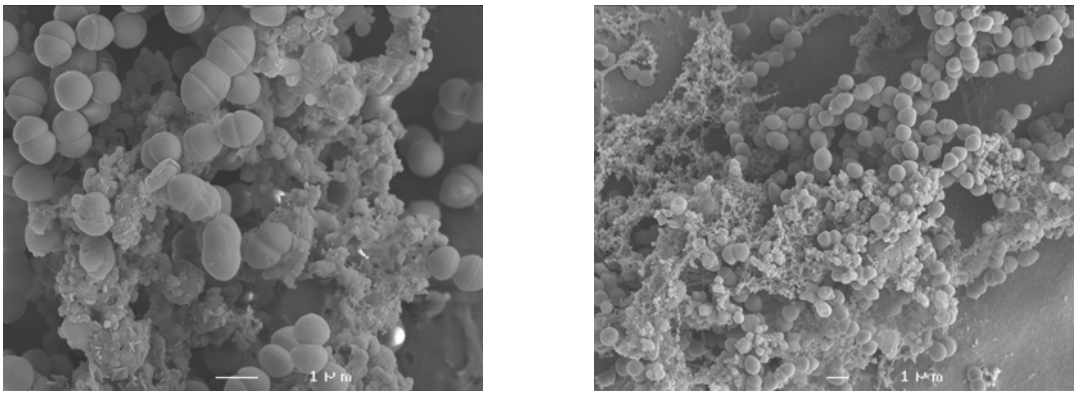


Fig. 1.1 Biofilm of *E. faecium*. Scanning electron microscopy (SEM) of 24 hours (h) old biofilm formed by *E. faecium* strain E1165 (A) and E470 (B). (Pictures courtesy of Dr. H. Leavis from University Medical Center Utrecht, The Netherlands)

1.3. Enterococcal cell wall and capsular polysaccharides

The bacterial cell wall represents a complex structure involved in several functions. It allows the bacteria to interact with their environment and provides the necessary factors needed for growth in its biological niche and in the infected host. It also separates the interior of the bacteria from harmful influences exerted by the surrounding milieu (74). The backbone of the Gram-positive bacterial cell wall is the peptidoglycan, organized like a fisherman's net and enclosing the plasma membrane. It consists of glycan chains of alternating β-(1→4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues cross-linked to each other by short peptides made of L- and D-amino acids. As in many other Gram-positive bacteria, the sequence of the stem peptide is L-Ala-D-Glu-L-Lys-D-Ala. Except for *E. faecalis,* the cross-bridge between the stem peptides is formed by a single D-Asp residue. In *E. faecalis*, this aminoacid is replaced with L-Ala oligopeptides (75).

Besides peptidoglycan, the enterococcal cell wall contains proteins, polysaccharides, lipids, lipoproteins, and glycoconjugates. Several studies have described distinct polysaccharides extracted from the *E. faecalis* cell wall (76), (77), (78), (79). The serological differences observed between different *E. faecalis* strains appear to be related to their carbohydrates composition. A study by Hufnagel et al. (80) grouped *E. faecalis* strains into four capsular serotypes (A-D) based on serospecificity. It was recently shown that the antibodies used to classify serotype A actually recognize the LTA molecule (77). The most abundant polysaccharides of the enterococcal cell wall are WTA and LTA. WTA

is a polymer usually consisting of a glycosylated and D-Alanine-substituted alditol phosphate-repeating unit covalently attached to the cell wall via a linkage unit [reviewed by Neuhaus et al. (81)]. LTA possesses a glycolipid anchor which is inserted in the cytoplasmic membrane. In a recent study, Theilacker and coworkers confirmed general aspects of the LTA structure (77) using nuclear magnetic resonance: LTA consist of (1 →3)-poly(glycerol phosphate) and (1 →3)-poly(glycerol phosphate) substituted with Alanine or kojibiose at position C-2 of glycerol residues and an Alanine substitution was identified at positions C-6 of glucoses in the kojibiose residue (Fig. 1.2).

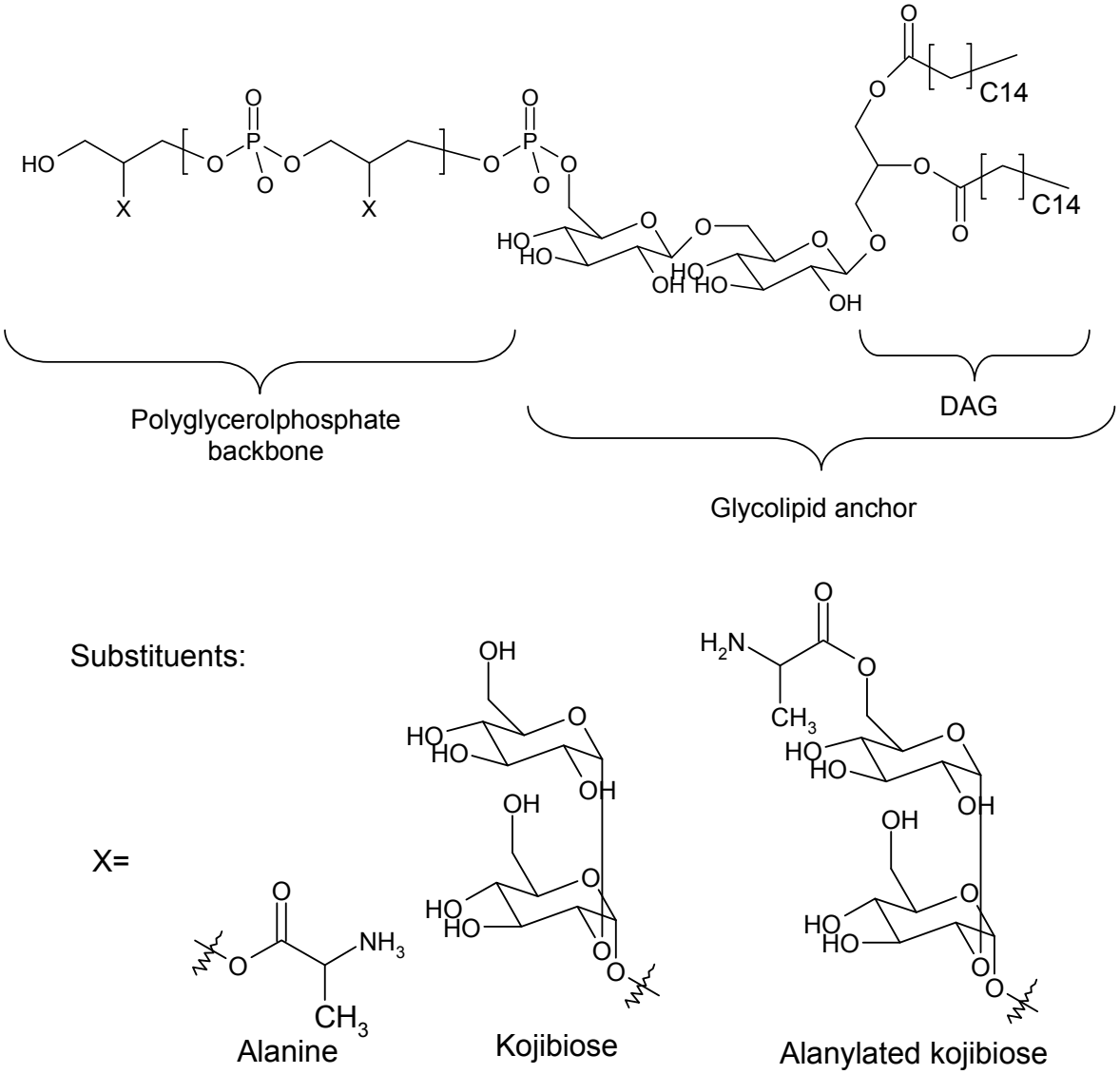


Fig. 1.2 Chemical structures of different repeating units of LTA from *E. faecalis* strain 12030.

Unpublished studies by our group that are based on cross-reactivity in enzyme-linked immunosorbent assays (ELISA) and opsonophagocytic assays showed that the predominant antigen of serotype B is also LTA (Fig 1.3). In the serotyping system

described by Hufnagel et al. it was shown that serotype C and D strains are resistant to opsonophagocytosis by antiserum directed against the serotype A strains, suggesting the presence of a capsule not expressed by serotype A and B strains that masks LTA from opsonisation by anti-LTA antibodies.

Two clusters of genes have been shown to contain putative genes for capsule production in *E. faecalis*: *epa* (78) and *cps* (79). The polysaccharide produced by the enzymes encoded by the *epa* locus is thought to be a rhamnopolysaccharide widespread among *E. faecalis* strains. The structure of the Epa polysaccharide has not been elucidated yet and preliminary (unpublished) information indicates that the localization is inside the bacterial cell wall (Fig. 1.4) (82). Unlike Epa, the products of the *cps* locus seem to be serotype specific polysaccharides. Only one gene of this locus, *cpsF*, seems to form the basis for differences in antigenicity between serotype C and D. CpsF is a putative glycosyltransferase modifying the ratio of glucose to galactose in serotype C strains (83). Since *E. faecalis* strains express only a limited number of capsular polysaccharides (unpublished observation), these molecules are attractive candidates for the development of immunotherapy regimen.

Regarding *E. faecium,* published information regarding capsular polysaccharides structures are completely lacking. About one third of the clinical isolates tested were efficiently killed by anti-LTA antibodies, suggesting that these strains do not express a capsule (76).

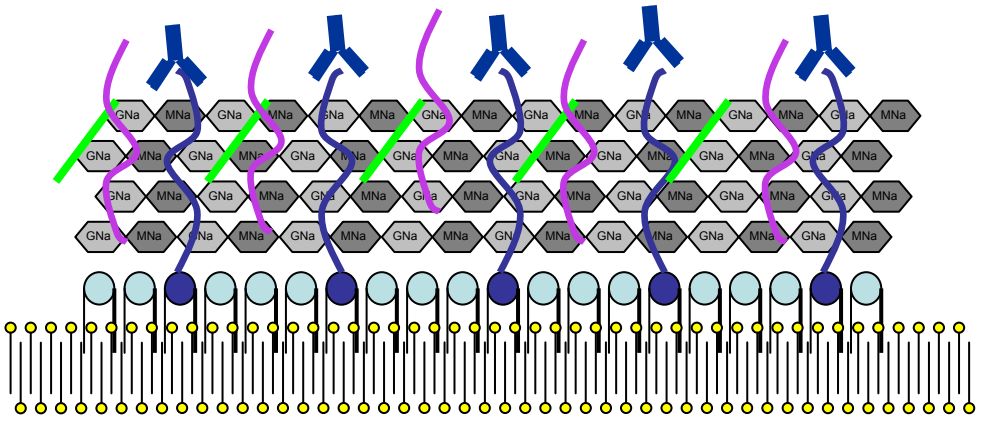


Fig. 1.3 Schematic representation of the cell wall of serotype A and B *E. faecalis* strains. The peptidoglycan is depicted in grey (GNa - *N*-acetylglucosamine, MNa - *N*-acetylmuramic acid) the LTA - in blue, the WTA - in purple, the rhamnopolysaccharides - in green and the antibodies - in blue.

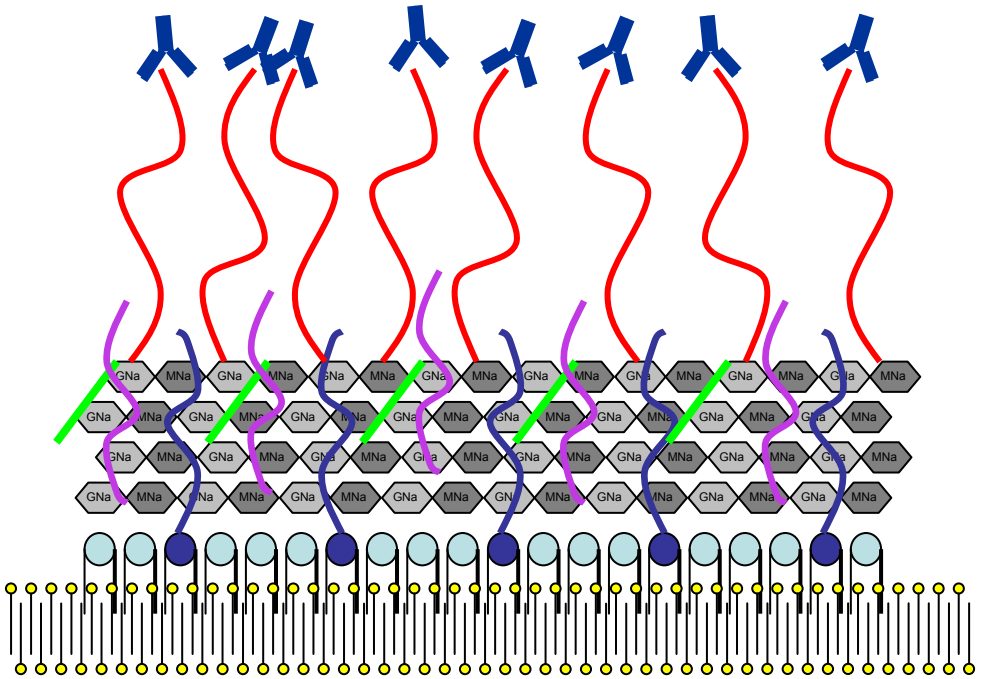


Fig. 1.4 Schematic representation of the cell wall of serotype C and D *E. faecalis* strains. The peptidoglycan is depicted in grey (GNa - *N*-acetylglucosamine, MNa - *N*-acetylmuramic acid), the LTA – in blue, the capsular polysaccharides - in red, the WTA - in purple, the rhamnopolysaccharides –in green and the antibodies - in blue.

1.4. Enterococcal adhesion to host cells

The attachment of microbial pathogens to the host cell is a principal ecological strategy and an important initial step of infection. For gastrointestinal commensals such as enterococci, binding to eukaryotic cells plays an important role in establishing and maintaining colonization. Without specific mechanisms for adhesion, enterococci would

likely be eliminated from the intestine. According to the translocation model, bacteria exit the apical side of enterocytes, migrate inside phagocytes to mesenteric nodes, proliferate and disseminate before causing systemic infections (84).

Interactions of bacteria with components of the host's extracellular matrix as well as with cell surface receptors are crucial factors in this process, and some of the mechanisms are similar among many pathogenic Gram-positive cocci. Host cells produce a large variety of macromolecules forming a matrix in the extracellular space. This network serves as a mechanical support for host cells, but can also be utilized by colonizing pathogenic bacteria. The initiation of infection requires bacterial surface-associated molecules to recognize structures on the eukaryotic cells surfaces. The identification of host cells receptors and complementary bacterial adhesins are important steps in understanding the molecular aspects of pathogenesis and designing alternative therapies against bacterial pathogens.

The proteins of the extracellular matrix and the glycosaminoglycans (GAGs) are the most common molecules used by bacteria as receptors on the host cells. Enterococcal MSCRAMM proteins bind with high affinity to various types of collagen, fibronectin and other components of the extracellular matrix (see Virulence factors).

Present on the surface of practically all the animal cells, the GAGs are among the first molecules encountered by the pathogens infecting the host. For almost two decades, it has been demonstrated that an increasing number of microorganisms bind sulfated GAGs on the eukaryotic cells [reviewed by Rostand and Menozzi (85), (86)]. Despite an apparent homogeneity, GAGs display a certain polymorphism due to chemical modifications. It has been speculated that these modifications might play a role in pathogens recognition at different sites, but this is still a hyopthesis requiring further investigations. Few studies have reported the involvement of GAGs in enterococcal binding to intestinal epithelial cell lines HT29 and Caco2 (87) or to professional and non-professional macrophages (88). While for the proteins of the extracellular matrix the binding partner on the enterococcal surface has been described, none of these studies investigated the bacterial components recognizing GAGs on the eukaryotic cells.

1.5. Host defense against enterococcal infections

The incidence of enterococcal infections increased substantially worldwide in the last decades. The limited choice of antimicrobial agents available against enterococci emphasizes the importance of understanding the normal immune response against these pathogens.

The first defense mechanisms encountered by invading enterococci belong to the innate immune system. The receptors of the innate immune system cells recognize highly conserved structures present exclusively on microbes (pathogens-associated molecular patterns). A major subset of these receptors are the Toll-like receptors (TLRs), recognizing microbial structures and subsequently initiating the production of cytokines and chemokines. So far, 11 human TLRs are known and among these, TLR2 and TLR4 are the best studied. The list of ligands for TLR2 is still controversial, whereas their common characteristic is a lipid moiety. Until recently, LTA was considered one of the most important TLR2 agonists but in the last years several studies demonstrated the existence of lipoproteins contaminating the LTA preparations that are able to stimulate TLR2 even in the absence of LTA [reviewed by Zähringer (89)].

The complement system is an important component of the immune system and efficient killing of enterococci by PMNs *in vitro* requires the presence of complement proteins and is enhanced by anti-enterococcal antibodies (unpublished observations). Studies by Leendertse et al. showed that PMNs and macrophages are important components involved in clearing E. *faecium* infection (90) and depletion of these cells lead to prolonged systemic infection and higher levels of inflammatory markers.

2. AIM OF THE STUDY

Intestinal colonization, followed by translocation is the first step in establishment of enterococcal infections. As described for other bacterial pathogens, eukaryotic cells express a variety of molecules recognized by microorganisms and used for adherence. Therefore, in the first part of this thesis GAGs, as prominent components of the extracellular matrix, were studied for their role to mediate *E. faecalis* adhesion to enteric epithelial cells. Different bacterial surface molecules were investigated for their role in binding to GAGs.

We wee able to show that bacterial membrane glycolipids mediate the interaction of enterococci with human enterocytes. Therefore, the focus of the second part of this thesis were bacterial glycolipids, as components of the bacterial cell membrane and anchors of the LTA molecule. The effects of replacement of the major enterococal glycolipid, diglucosyldiacylglycerol (DGlcDAG) with monoglucosyldiacylglycerol (MGlcDAG) were investigated in several *in vitro* and *in vivo* models using a deletion mutant. The role of glycolipids in bacterial cell shape, affinity to organic solvents, biofilm formation or adherence and translocation of eukaryotic cells were studied. Also, the susceptibility to killing by PMNs and macrophages *in vitro* and clearance from the blood stream in a mouse sepsis model of the wild type and the mutant was compared. The role of bacterial glycolipids as inflammatory mediators associated with sepsis and hyperinflammation was studied in a mouse peritonitis model and the host response was further investigated by measuring markers associated with inflammation and bacterial burden.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Equipment

- Autoclave (**Münchner Medizin Mechanik**, Planegg)
- Carbon dioxide (**Linde Gas**, Pullach)
- Cells disrupter BeadBeater™ (**BioSpec**, Bartlesville, Oklahoma, USA)
- Centrifuge Hearaeus Megafuge1,0R® (**Thermo Fisher Scientific**, Waltham, Massachusetts, USA)
- Electrophoresis chamber X-Cell SureLock™ (**Invitrogen**, Carlsbad, California, USA)
- ELISA Reader BioRad 550 (**BioRad**, Hercules, California, USA)
- ELISA Washer™ (**BioTek Instruments Inc.**, Winooski, Vermont, USA)
- Filter sterilization device SteriCups® 22µm (**Millipore**, Billerica, Massachusetts, USA)
- Filterpaper (**Whatman**, Maidstone, UK)
- Freezer -80°C Heraeus (**Thermo Fisher Scientific**, Waltham, Massachusetts, USA)
- Glassbeads 0.1 mm diameter (**Carl Roth**, Karlsruhe)
- Glasware (**Schott**, Mainz)
- Inverted microscope Axiovert 25 (**Carl Zeiss**, Jena)
- Laminar hood Heraeus HS12 Flowhood (**Thermo Fisher Scientific**, Waltham, Massachusetts, USA)
- Magnetic stirrer Combimag® (**IKA**, Staufen)
- Multichannel pipette (**Brand GmbH**, Wertheim)
- Needles and syringes (**B. Braun**, Melsungen)
- pH-Meter (**Brand Gmbh**, Wertheim)
- Pipette controler Akkupipette (**Hirschmann**, Eberstadt)
- Pipettes/Pipette tips (**Gilson**, Middleton, Wisconsin, USA)

- Polypropylene tubes (**BD Biosciences**, Franklin Lakes, NJ, USA)
- Power supply PowerPac 300 (**BioRad**, Hercules, California, USA)
- Precision weighing scale Sartorius CP225D (**Sartorius AG**, Göttingen)
- Scanning electron microscope Cambridge S360 (**Leika Microsystems GmbH**, Wetzlar)
- Sensor SA Chips (**GE Healthcare**, Sweden)
- Spectrophotometer Genesys 20™ (**Thermo Fisher Scientific**, Waltham, Massachusetts, USA)
- Surface plasmon resonance (SPR) - based system BIAcore 3000 (**GE Healthcare**, Sweden)
- Syringe filter for sterile filtration Acrodisc™ 22µm (**Pall Corporation**, New York, USA)
- Thermomixer Eppendorf 5436 (**Eppendorf AG**, Hamburg)
- Thin-layer chromatography syringe (**Hamilton**, Bonaduz, Switzerland)
- Tissues homogenator T10 basic ULTRA-TURRAX (**IKA**, Staufen)
- Ultrapure water purification system MilliQ A10 (**Millipore**, Billerica, Massachusetts, USA)
- Ultrasoundbath Sonorex™ (**Bandelin electronic**, Berlin)
- VortexGenie™ (**Scientific Industries, Bohemia**, New York, USA)
- Waterbath Lauda A100 (**Lauda**, Königshofen)
- Western blot chamber Blot-Modul (**Invitrogen**, Carlsbad, California, USA)

3.1.2. Chemicals and reagents

- 1-Butanol (**Merck**, Darmstadt)
- 2,4,6-Tris(dimethylaminometyl)phenol (**Serva**, Heidelberg)
- 2-*O*-Desulfated heparin (**Neoparin**, San Leandro, California, USA)
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (**Sigma Chemicals**, St. Louis, Missouri, USA)
- 4-Nitrophenyl-dinatriumphosphat (**Sigma Chemicals**, St. Louis, Missouri, USA)
- 6-*O*-Desulfated heparin (**Neoparin**, San Leandro, California, USA)
- Acetic acid (**Merck**, Darmstadt)

- Alkaline Phosphatase Colour Development Kit (**BioRad**, Hercules, California, USA)
- Alpha-naphtol (**Merck**, Darmstadt)
- Amine-PEO3-Biotin (**Pierce, Termo Scientific**, Rockford, USA)
- Ammonium acetate (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Ammonium chloride (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Ammonium oxalate (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Aqua dest. (MilliQ®**, Millipore**, Billerica, Massachusetts, USA)
- Araldite Cy 212 (**Serva**, Heidelberg)
- Araldite Hardener Hy 964 (**Serva**, Heidelberg)
- Baby Rabbit Complement (**Cedarlane Laboratories**, Hornby, Ontario, Canada)
- Bovines Serum Albumin (BSA) (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Chloroform (**J.T. Baker**, Deventer, Holland)
- Chondroitin sulfate (CS) B sodium salt from porcine intestinal mucosa (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Chondroitinase ABC from *Proteus vulgaris* (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Citric acid (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Crystal violet (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Cyprofloxacin (**Sigma Chemicals**, St. Louis, Missouri, USA)
- *N*-Desulfated-heparin (**Neoparin**, San Leandro, California, USA)
- Dextran MW 400000-500000 (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Dimethyl sulfoxide (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Disodium hydrogenphosphate (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Dodecane (**Fluka Chemie**, Buchs)
- Dulbecco's Modified Eagle Medium (DMEM) (**Pan^{TM}-Biotech GmbH**, Aidenbach)
- Endotoxin-free water (**B.Braun**, Melsungen)
- Epon 812 (**Serva**, Heidelberg)

- Ethanol (**Merck**, Darmstadt)
- Ethylene diamine tetraacetic acid (EDTA) (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Ethylene glycol (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Fetal Bovine Serum (FBS) (**GIBCO Invitrogen**, Carlsbad, California, USA / **PAA Laboratoires**, Pasching, Austria)
- Fluorescein-5-isothiocyanate (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Formaldehyde (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Glucose (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Glutaraldehyde (**Merck**, Darmstadt)
- Glycerol (**Merck**, Darmstadt)
- Goat-Anti Rabbit IgG Alkaline Phosphatase Conjugate (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Heparan sulfate (HS) fast-moving fraction sodium salt from porcine intestinal mucosa (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Heparin sodium salt from porcine intestinal mucosa (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Heparinase I from *Flavobacterium heparinum* (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Hog gastric mucin (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Hydrochloric acid (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Hydrogen peroxide (**J.T. Baker**, Deventer, Holland)
- LB broth (**Carl Roth**, Karlsruhe)
- Low melting point agarose (**Biozym**, Oldendorf)
- LTA (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Luria Bertani (LB) agar (**Carl Roth**, Karlsruhe)
- Lysine acetate (**Merck**, Darmstadt)
- MES- Buffer™ (**Invitrogen**, Carlsbad, California, USA)
- Methanol (**Merck**, Darmstadt)
- Molecular sieve (**Serva**, Heidelberg)

- MOPS-Buffer™ (**Invitrogen**, Carlsbad, California, USA)
- Nonessential aminoacids (**GIBCO Invitrogen**, Carlsbad, California, USA)
- NuPAGE 10% Bis-Tris Gel (**Invitrogen**, Carlsbad, California, USA)
- NuPAGE SDS Sample Buffer™ **(Invitrogen**, Carlsbad, California, USA)
- NuPAGE Transfer Buffer™ (**Invitrogen,** Carlsbad, California, USA)
- Osmium (**ChemPur**, Karlsruhe)
- Penicillin G potassium salt (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Phosphate buffered saline (PBS) low endotoxin (**Biochrome AG**, Berlin)
- Phthalic acid dibutylester (**Serva**, Heidelberg)
- Physiological solution (**B.Braun**, Melsungen)
- PLC silica gel 60 F_{254} plates (**Merck**, Darmstadt)
- Polystyrene plates with polycarbonate (3µm pore size) membrane inserts (**Corning Inc.**, New York, USA)
- Polytetrafluoroethylene filters (**Carl Roth**, Karlsruhe)
- Porcine intestinal heparin for SPR (**Celsus Laboratories**, Cincinnati, Ohio, USA)
- Porcine intestinal HS for SPR (**Celsus Laboratories**, Cincinnati, Ohio, USA)
- Potassium chloride (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Potassium dihydrogenphosphate (**Merck**, Darmstadt)
- Proteinase K (**Sigma Chemicals**, St. Louis, Missouri, USA)
- PVDF membrane (**Invitrogen**, Carlsbad, California, USA)
- Roswell Park Memorial Institute medium (RPMI)+L-Glutamin® (**GIBCO Invitrogen**, Carlsbad, California, USA)
- Ruthenium red (**Merck**, Darmstadt)
- SeeBlue Plus2 Prestained Standard (**Invitrogen**, Carlsbad, California, USA)
- SeeBlue™Plus2 PreStained Standard (**Invitrogen**, Carlsbad, California, USA)
- Skim milk (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Sodium azide (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Sodium cacodilate (**Electron Microscopy Science**, Hartfield, Pennsylvania, USA)

- Sodium chlorate (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Sodium chloride (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Sodium citrate (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Sodium cyanoborohydride (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Sodium di-hydrogenphosphate (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Sodium heparin (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Sodium hydroxide (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Sodium meta-periodate (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Sulfuric acid (**Merck**, Darmstadt)
- Thin-layer cromatography (TLC) silica gel 60 F_{254} plates (**Merck**, Darmstadt)
- Trypan blue (**Biochrome AG**, Berlin)
- Trypsin EDTA (**GIBCO Invitrogen**, Carlsbad, California, USA)
- Tryptic Soy Agar (TSA) (**Carl Roth**, Karlsruhe)
- Tryptic Soy Broth (TSB) (**Merck**, Darmstadt)
- Tryton X-100 (**Sigma Chemicals**, St. Louis, Missouri, USA)
- Tween 20™ (**Sigma Chemicals**, St. Louis, Missouri, USA)

3.1.3. Buffers and media

All buffers and solutions were prepared using deionized, sterile water (Milli Q, MILLIPORE) or sterile, endotoxin-free water (B.BRAUN). All glass recipients were autoclaved at 121°C for 15 minutes (min) and all buffers were filter sterilized or autoclaved. Preparation of special buffers is described in the respective methods section.

LB agar	Tryptone 10 g/l
	Yeast extract 5 g/l
	Sodium chloride 10 g/l
	Agar 15 g/l
LB broth	Tryptone 10 g/l
	Yeast extract 5 g/l
	Sodium chloride 10 g/l
PBS	140 mM NaCl,
	2.7 mM KCl
	7.4 mM Na_2HPO_4
	1.5 mM KH_2PO_4, pH 7.4
TSA	Casein peptone (pancreatic digest.) 15 g/l
	Soya peptone (papainic digest.) 5 g/l
	Sodium chloride 5 g/l
	Agar 15 g/l
TSB	Casein peptone (pancreatic digest.) 17 g/l
	Soya peptone (papain digest.) 3 g/l
	Sodium chloride 5 g/l
	Dipotassium hydrogen phosphate 2.5 g/l
	Glucose 2.5 g/l

3.2. METHODS

3.2.1. Preparation of bacteria

Table 3.1 Bacterial strains

The bacterial strains used in this study are listed below.

Bacterium	Reference
E. faecalis 12030	(76)
E. faecalis 12030Δ*bgsA*	(91)
E. faecalis V583	(92)
E. faecalis OG1-RF	(93)
E. faecalis FA2-2	(79)
E. faecalis T5	(94)
E. faecalis T9	(94)
Salmonella enterica serovar Oranienburg	From Prof. D. Jonas, Uniklink Freiburg

3.2.1.1. Preparation of bacteria for cell culture experiments

E. faecalis strains were grown for 18 h on TSA plates at 37°C. Prior to the experiment, one colony was picked, inoculated in TSB and cultivated 2 to 4 h to mid-log phase [optical density at 650 nm (OD_{650}) of 0.4]. Bacteria were then centrifuged, washed once with PBS and resuspended in DMEM with 10% FBS.

Salmonella enterica serovar Oranienburg was grown for 18 h on LB agar plates at 37°C. Before the experiment, one colony was picked, inoculated in LB medium and cultivated under agitation to reach the mid-log phase (OD_{650}=0.4). Afterwards, bacteria were centrifuged, washed once with PBS and resuspended in DMEM containing 10% FBS. The number of bacteria in the inoculum was extrapolated from previously derived growth curves and confirmed by viable counts.

In some experiments, enterococci were treated with sodium-meta-periodate or with proteinase K. Bacteria were treated with various amounts of fresh sodium meta-periodate (0.125 mM - 1mM) for 60 min at 4°C on a rotor rack in the dark [modified after Ardiuno et al.(95)]. Sodium meta-periodate was neutralized using ethylene glycol (20 µL/mL inoculum) for 10 min at 20-22°C, on a rotor rack, in the dark. Bacteria were washed five times with PBS to remove the sodium meta-periodate.

Bacteria were treated with proteinase K as described (96) by incubation with 0.1 mg/mL enzyme at 37°C during cultivation to mid-log phase. After reaching an OD_{650} of 0.4 bacteria were washed five times with PBS to remove the enzyme. Bacterial viability after these treatments was confirmed by enumation of cfu on TSA plates.

3.2.1.2. Preparation of bacteria for opsonophagocytic assay

The bacterial strains to be tested for killing by opsonophagocytosis were taken from a fresh TSA plate, adjusted to OD_{650} of 0.1 in 10 mL fresh TSB and allowed to grow to an OD_{650} of 0.4. Then, bacteria were washed and a 1:100 dilution was made in RPMI with 15% FBS for use in the opsonic killing assay.

3.2.1.3. Preparation of bacteria for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

For preparing different enterococcal strains for gel electrophoresis and immunoblotting, 8 mL stationary-phase culture was centrifuged at maximum speed for 30 min at 4°C. The supernatant was collected and the pellet was washed twice with PBS. Samples (bacterial pellet and supernatant) were boiled for 10 min, mixed with dH_2O and loading buffer, and boiled again before loading them on the gel.

3.2.1.4. Preparation of bacteria for extraction of glycolipids

To extract bacterial glycolipids, enterococccci were grown in large batches (1-7 L) for 18 h to stationary phase, centrifuged at maximum speed for 30 min at 4°C and washed once with citrate-buffer (see 3.2.7.1). The pellets were frozen at - 80 C until used.

3.2.1.5. Preparation of bacteria for mouse experiments

Bacterial strains were grown for 18 h on TSA plates at 37°C. One colony was inoculated in 500-1000 mL TSB and cultivated at 37°C without shaking for 18 h. The following day, bacteria were centrifuged, washed once and resuspended in saline solution, shock-frozen

and stored at -80°C. The concentration of the stocks was verified by serial dilutions and viable counts. Pilot experiments confirmed that of the inoculum is not changed significantly by this procedure.

3.2.1.6. Preparation of bacteria for infection of *Drosophila melanogaster*

From a fresh TSA plate with bacteria few colonies were inoculated in 10 mL TSB to obtain an OD_{650}=0.1. Bacteria were grown to OD_{650}=0.3 centrifuged at maximum speed and diluted or concentrated to obtain $5x10^8$ cfu to $5x10^9$ cfu/mL.

3.2.1.7. Preparation of bacteria for scanning electon microscopy

Scanning electron microscopy (SEM) experiments presented in this study were performed by Dr. L. Baldassarri (National Health Institute, Rome, Italy). Bacterial cells were prepared for visualization using the method described by Hufnagel et al. (67). Bacteria grown at 37ºC to exponential phase in 10 mL TSB were centrifuged for 5 min at 2000g and fixed for 20 min at 25°C using fixation solution I (see below). After the first fixation step, bacteria were centrifuged for 3 min at 2000 *g*, washed once with washing buffer and fixed for 2 h at 25°C with fixation solution II. Next, bacteria were centrifuged for 3 min at 2000 *g* and washed once with washing buffer. The third fixation step was performed using fixation solution III for 1 h at 25°C. For SEM samples were dehydrated at 4ºC using a graded series of ethanol (30, 50, 70, 85 and 95%) and at 25°C with ethanol 100%. Dehydrated specimens were critical point dried, to retain the original three-dimensional shape of the bacterial cells, gold sputtered and examined by a Cambridge SE 360 SEM.

Fixation solution I
- 0.270 mL formaldehyde 2%
- 0.5 mL glutaraldehyde 2.5%
- 0.5 mL ruthenium red 0.075%
- 0.5 mL lysine acetate 0.075%
- 0.5 mL sodium cacodylate

Fixation solution II	0.270 mL formaldehyde 2%
	0.5 mL glutaraldehyde 2.5%
	0.5 mL ruthenium red 0.075%
	0.5 mL sodium cacodylate
	3.23 mL dH_2O
Fixation solution III	2.5 mL osmium 1%
	0.5 mL ruthenium red 0.075%
	0.5 mL sodium cacodylate
	1.5 mL dH_2O
Washing buffer	5 mL sodium cacodylate buffer
	5 mL ruthenium red 0.075%
	40 mL dH_2O

3.2.2. Cultivation of eukaryotic cells

3.2.2.1. Cultivation of Caco2 cell line

Caco2, a human cell line derived from colon carcinoma, was a gift from Prof. Dr. O. Opitz (University Hospital Freiburg). Cells were cultivated in 75cm^2 flasks in DMEM supplemented with 10% heat-inactivated FBS and 1% non-essential amino acids at 37°C, in a 5% CO_2 humid atmosphere, as previously described (96). Cells were examined macroscopically and microscopically using an inverted microscope and were passaged once per week in typsin buffer (0.05% trypsin in 0.02% EDTA), diluted to 10^6 cells/flask. Before each experiment, cells were seeded in 24-wells plates to a density of 1-5x10^5 cells/well and cultivated for 13-15 d to allow them to differentiate. All experiments were performed on cells between the 15th and 25th passage and viability of the cells was confirmed during each passage using Trypan blue exclusion.

3.2.2.2. Cultivation of Hep2 cell line

Hep2, a cell line derived from a human larynx carcinoma was used in some experiments. Cells were grown in RPMI supplemented with 10% heat-inactivated FBS. Cells were passaged every 5 d, and prior the experiments they were seeded in 24-wells plates to a

density of 1-5x10^5 cells/well, and cultivated for 24 h as described by Fabretti et al. (73). Experiments were performed with cells below the 20th passage.

3.2.2.3. Cultivation of RAW 264.7 cell line

The RAW 264.7 macrophage cell line was established from ascites of a tumour induced in a mouse. Cells were maintained as previously described (97). Macrophages were cultivated in DMEM supplemented with 10% heat-inactivated FBS and 10 µg of ciprofloxacin/mL and passaged once/week. Only cells below passage 20th were used.

3.2.2.4. Isolation of polymorphonuclear cells

Polymorphonuclear cells (PMNs) were prepared from fresh human blood collected from healthy adult donors as previously described (91). Twenty-five mL blood was mixed with an equal volume of heparin-dextran buffer (see below) and incubated at 37°C for 45-60 min. The upper layer containing leukocytes was collected, the cells were pelleted by centrifugation and washed with RPMI. Hypotonic lysis of the remaining erythrocytes was done by resuspension of the pellet in NH_4Cl hypotonic solution (see below) and incubation for 15 min at 25°C. PMNs were then washed twice and resuspended with RPMI with 15% FBS. The final number of PMNs was adjusted to 2.5 x 10^7 PMNs/mL using the Trypan blue exclusion method.

Heparin-dextran buffer	4.5 g NaCl, 10 g dextran and 32.8 mg heparin-sulfate sodium salt in 500 mL dH_2O sterilized by autoclaving
NH_4Cl hypotonic solution	1 g NH_4Cl in 100 mL dH_2O sterilized by autoclaving

3.2.3. Cell culture methods

3.2.3.1. Bacterial adhesion to Caco2 cells

Caco2 cells were cultivated in 24-well plates as described (3.2.2.1). Prior to the experiment cells were washed once with warm PBS and infected with 1 mL bacterial inoculum/well prepared as described in 3.2.1.1. To infect the monolayers, bacteria were used at a bacteria-to-cell ratio [multiplicity of infection (MOI)] of 100:1 and 1000:1 as indicated. Epithelial cells were incubated with bacteria for 2-4 h at 37°C in 5% CO_2 atmosphere. The monolayers were then gently washed five times with warm PBS and lysed with DMEM-Triton-X buffer for 15 min at 37°C. Lysis of cells was confirmed microscopically and lysates were serially diluted in TSB and plated on TSA plates incubated for 18 h at 37°C.

The number of cfu corresponds to the number of bacteria attached and internalized by the cells. The last washes were plated in pilot experiments to insure that any remaining non-adherent bacteria would be <1% of the total adherent cfu.

DMEM-Triton-X buffer	DMEM supplemented with 10% FBS, 0.25% Triton-X 100 and filter-sterilized

3.2.3.2. Bacterial invasion of Caco2 cells

To quantify the number of bacteria invading the cells, an antibiotic protection assay modified after Baldassarri et al. was used (88). Epithelial cells were infected for 2 h at 37°C using two different MOI, 100:1 and 1000:1. Monolayers were washed three times, and incubated subsequently for 2 h in medium supplemented with 10% FBS plus penicillin G (1000 μg/mL). After 2 h, the monolayers were gently washed five times with PBS and lysed with DMEM-Triton-X buffer for 15 min at 37°C. To confirm that extracellular bacteria were killed by the antibiotic treatment, 100 μL DMEM were taken from one well during the last washing step and plated. The number of cfu counted the next day from the cell lysates plated on TSA plates correspond to the number of bacteria internalized by the Caco2 cells.

3.2.3.3. Bacterial translocation through Caco2 cells monolayers

Translocation of enterococci across the gut epithelial cell line Caco2 was studied in a two-chamber system to mimic the transcytosis across the intestinal epithelial cells as previously described (98, 99), with minor modifications. Caco2 cells maintained as described were seeded at a density of 10^5 cells/well in a 12-wells transwell system containing 3 μm-pore size membranes. These membranes are sufficiently large to permit bacteria but not eukaryotic cells to translocate. Five hundred μl DMEM with 10% FBS were added to the upper chamber and 1500 μl were added to the lower chamber. Cell culture media was changed every three d. Transepithelial electrical resistance (TEER) was measured by Milicell-ERS to assess formation of tight junctions. Monolayers developing a TEER value of 220±5Ω/cm^2 were used in the experiments and this usually occurred after 12 to 14 d (99). To calculate the TEER value, the following formula was used: TEER/cm^2 = ($TEER_{well}$-$TEER_{blank}$) x membrane surface (cm^2), where $TEER_{well}$ is the resistance measured in each well and $TEER_{blank}$ is the resistance measured in a well containing no cells. On the day of the experiment, the filters were washed once with PBS, 1500 μl fresh

cell culture media was added to the lower chamber and bacteria (MOI of 100:1) prepared as described (3.2.1.1), resuspended in 500 µl DMEM with 10% FBS were added to the upper chamber containing the cells. The resistance was measured at the beginning and during the experiment until the end. Bacterial translocation was quantified by removing 100 µl aliquots from the lower chambers after 0, 2, 4 and 6 h, plating serial dilutions on TSA plates and by counting the colonies the next day.

3.2.3.4. Bacterial adhesion and invasion of Hep2 cells

Hep2 cells were passaged in 24-well plates at a densitiy of 1-5x10^5 cells/well one day prior the experiment. Before infecting them, cells were examined microscopically to confirm confluence of the monolayers. Bacterial adherence and invasion were studied as described in 3.2.3.1 and 3.2.3.2

3.2.3.5. Phagocytic killing by mouse macrophages

A bacterial killing assay was performed as described by Blander et al. (100). RAW 264.7 macrophages were seeded at a density of 10^5 cells/well in 24-well plates for 24 h. The following day, confluent monolayers were washed with warm PBS and incubated with serum-free medium for at least 2 h. Bacterial inoculum prepared as previously described (3.2.1.1) was added to the cell monolayers at a MOI of 100:1. Entercocci were centrifuged onto macrophage monolayers at 1000 *g* for 5 min at 4°C. Plates were incubated at 37°C for 10 min and then each well was washed 5 times with ice-cold PBS to remove extracellular bacteria. For measuring bacterial internalization after 10 min, triplicate wells were lysed with 1 mL DMEM-Triton-X buffer/well. This was designated as point 0. Warm serum-free medium was added in the remaining wells and the cells were incubated at 37°C for 10 to 60 min. At each time point, triplicate wells were washed 5 times with ice-cold PBS and the cells were lysed subsequently. Viable bacterial counts were determined by plating serial dilutions onto TSA plates and bacterial killing was determined with the following formula: % killing = 100 - [(cfu at time X / cfu at time 0) x 100].

3.2.4. Inhibition of bacterial binding to Caco2 cells

3.2.4.1. Inhibition of bacterial binding to Caco2 cells using GAGs

GAGs (heparin, chemically modified heparins, HS and CS) were diluted in DMEM and added to the epithelial cells 30 min prior to addition of the bacterial inoculum. Various concentrations of these polysulfated agents were used (ranging from 10 to 1000 µg/mL).

Samples containing only DMEM were used as negative controls. The adhesion assay was then performed as described above (3.2.3.1). All experiments were performed at least twice.

3.2.4.2. Inhibition of bacterial binding to Caco2 cells after enzymatic treatment of the cells

Epithelial cells were depleted of some of the surface GAG chains by using heparinase and chondroitin lyase ABC. The lyophilized enzymes were resuspended in PBS, diluted in DMEM and added to the cells 10 min prior the bacterial inoculum in concentrations between 0.125 and 2 U/mL (101). Additionally, 50 min after infection, the same amount of enzyme was added to the cells and the incubation with bacteria was continued for an additional hour. Then, cells were lysed, the lysates were plated and the cfu were enumerated after 18 h.

3.2.4.3. Sodium chlorate treatment of the cells

Sodium chlorate (an inhibitor of GAG chains sulfation) was added to the cells 48 h prior to the experiments in a concentration between 12.5 and 50 mM as described elsewhere (102), (103). For screening of different enterococcal strains a concentration of 50 mM was used and the adhesion assay was performed as usual.

3.2.4.4. Inhibition of enterococcal binding to Caco2 cells using cell wall carbohydrates

Unfractionated polar lipids (67.5-250 µg/mL), polar lipids treated with sodium meta-periodate (250 µg/mL), and purified MGlcDAG (250 µg/mL), DGlcDAG (250 µg/mL), LTA (62.5-250 µg/mL) or WTA (62.5-250 µg/mL) isolated from enterococcal cell walls were added to the cells 30 min before infection. The assays were then performed as described above.

3.2.5. Immunological and enzymatic techniques

3.2.5.1. ELISA for cytokine detection

3.2.5.1.1. ELISA for detection of Tumor Necrosis Factor-α (TNF-α) and Interferon-γ (IFN-γ)

To detect TNF-α or IFN-γ**,** 96-well plates were coated with capture antibody (100 µL/well). The sealed plates were incubated for 2 h at 25°C shaking or for 18 h at 4°C. Uncoated surfaces of the wells were blocked with assay diluent (200 µL/well) and plates

were incubated for 1 h at 25°C. After removing the blocking solution, the peritoneal lavage fluid (PLF) and standards were diluted in assay diluent, added to the plates in duplicate or triplicate and incubated for 2 h at 25°C. Unbound samples or standards were removed by washing followed by incubation for 1 h at 25°C with 100 µL/well biotin-conjugate anti-mouse TNF-α or IFN-γ detection antibody. Excess of detection antibody was removed by washing and 100 µL/well of avidin-horse radish peroxidase (HRP) was added to each well. Plates were incubated for 30 min at 25°C, liquid was discarded by washing, and 100 µL/well of substrate solution was added and incubated for 15 min at 25°C. After the HRP-catalyzed reaction occurred, the reaction was stopped by addition of 50 µL of stop solution. The quantification of converted substrate was measured at λ = 450 nm using an ELISA reader and compared to the standard curve. After each incubation step liquid was discarded and plates were washed 5 times with at least 250 µL washing buffer allowing 1-2 min for soaking during each washing step.

Capture antibody	Monoclonal hamster anti-mouse TNF-α or IFN-γ diluted 1:250 in coating buffer
Assay diluent	Working solution in dH_2O
Standards	Recombinant mouse TNF-α or IFN-γ diluted in assay diluent
Detection antibody	Biotin-conjugate rabbit polyclonal anti-mouse TNF-α or IFN-γ diluted 1:250 in assay diluent
Enzyme	Avidin-horse radish peroxidase (HRP) diluted 1:250 in assay diluent
Substrate solution	Tetramethylbenzidine (TMB)
Stop solution	2 N H_2SO_4
Washing buffer	PBS + 0.05% Tween20

3.2.5.1.2. ELISA for detection of Interleukin 4 (IL-4)

For the detection of IL-4, 96 wells plates were coated with capture antibody (100 µL/well), sealed and incubated for 18 h at 25°C. Liquid was discarded, plates were washed and blocked with 300 µL/well reagent diluent for 1 h at 25°C. After removing the blocking solution, samples and standards were diluted in reagent diluent, added to the plates and incubated for 2 h at 25°C. Unbound samples were removed by washing and 100 µL/well of detection antibody was added to the wells. Each sample was tested in duplicate or

triplicate. After 2 h at 25°C, plates were washed and incubated with streptavidin-HRP for 20 min at 25°C in the dark. The excess of enzyme was removed by washing, 100 µL/well substrate was added for 20 min and the reaction was stopped with 50 µL/well of stop solution. Quantification of converted substrate was measured at λ = 450 nm using an ELISA reader and calculated according to a standard curve. After each incubation step plates were washed 3 times with at least 250 µL washing buffer allowing 1-2 min for soaking during each washing step.

Capture antibody	Rat anti-mouse IL-4 (4 µg/mL) reconstituted with PBS
Reagent diluent	1% BSA in PBS, pH 7.2-7.4, 0.2 mm filtered
Standards	Recombinant IL-4 reconstituted with reagent diluent
Detection antibody	Biotinylated goat anti-mouse IL-4 (600ng/mL) reconstituted with reagent diluent
Enzyme	Streptavidin-HRP diluted 1:200 in reagent diluent
Substrate solution	1:1 Mixture of color reagent A (H_2O_2) and color reagent B (TMB)
Stop solution	2 N H_2SO_4
Washing buffer	PBS + 0.05% Tween 20, pH 7.2-7.4

Table 3.2 Cytokines and chemokines

The cytokines and chemokines measured in this study are listed below.

Cytokines/Chemokines	Test Kit Manifacturer
TNF-α	eBIOSCIENCE
IFN-γ	eBIOSCIENCE
IL-4	R&D Systems

3.2.5.2. Measurement of the bacterial opsonophagoyctosis

To measure the bacterial killing *in vitro* by PMNs, an opsonopagocytic assay was performed as previously described (91). Human PMNs were isolated and quantified as described in section 3.2.2.4. Lyophilizied baby rabbit serum was used as a complement source and was resuspended in ice-cold sterile water and diluted 1:15 in RPMI with 15% FBS. This serum was absorbed for 60 min at 4°C on a rotor rack with the target bacterial strain taken from a freshly prepared plate. Bacteria were removed by centrifugation and filter-sterilization, and the final solution was used as complement source. The bacterial strains to be evaluated for phagocyte-dependent killing were prepared as described in 3.2.1.2**.** The opsonophagocytic assay was performed with 100 µL of leukocytes ($2x10^7$/mL), 100 µL of bacteria ($2x10^7$cfu/mL), 100 µL of the complement solution, and 100 µL of heat-inactivated rabbit immune serum raised against whole-bacteria as a source of antibodies at various dilutions as specified in the RESULTS section. Controls were prepared by replacing the antibody, complement, or PMNs with RPMI plus 15% FBS. The reaction mixture was incubated on a rotor rack at 37°C for 90 min. The tubes were vortexed for 30 s, diluted in TSB with 0.25% Tween to prevent bacterial aggregation, and samples were plated on TSA plates. The percent killing was calculated by comparing the mean of the negative controls with the colony counts obtained after incubation for 90 min at 37°C (T_{90}) on a rotor rack by use of the following formula: [(mean cfu controls – mean cfu at T_{90})/(mean cfu controls)] x 100.

3.2.5.3. SDS-PAGE and Western blotting

Electrophoretic separation of bacterial cell components was performed in a NuPAGE 10% Bis-Tris Gel. The gel chamber was filled with 500 mL of running buffer prior to loading of the samples. A volume of 15µL sample prepared as described in 3.2.1.3 was loaded in each well and the gel was run at 170-175 V. Separation of different bacterial components according the molecular weight took place after 55-60 min.

From the gel, proteins and other cellular components were transferred on a polyvinylidene fluoride (PVDF) membrane. Prior to the transfer, the PVDF membrane was soaked in methanol for 30 s, washed briefly in dH_2O, and then soaked in transfer buffer, together with the blotting pads and the filter paper. The "sandwich" was assembled in the following order, from the cathode to the anode: two pads, filter paper, gel, PVDF membrane, filter paper, two pads. All air bubbles were removed and the "sandwich" was placed into the blotting chamber. Transfer was performed at 30 V in transfer buffer for 90 min. After the

transfer, the residual binding capacity of the membrane was blocked by incubation with skim milk for 18 h at 4°C. After washing, the membrane was incubated with the primary antibody diluted 1:500 in skim milk for 1 h at 25°C. Excess of primary antibody was washed away and incubation with the secondary antibody conjugated with alkaline phosphatase (diluted 1:1000 in skim milk) was performed for 1 h at 25°C. After removing unbound secondary antibody, the substrate was added and the reaction was carried out until color development. The reaction was stopped by washing the membrane with dH_2O. A molecular mass size marker composed of different proteins of known size was used to identify the approximate size of the molecules blotted on the membrane. After each incubation step, the PVDF membrane was washed three times for 10 min with washing buffer.

Sample buffer	NuPAGE LDS Sample Buffer
Running buffer	NuPAGE MES SDS runnig buffer or NuPAGE MOPS SDS Running Buffer
Transfer buffer	NuPAGE Transfer Buffer with methanol added (100 mL methanol at 1000 mL transfer buffer
PVDF membrane	Invitrolon PVDF 0.45 µm pore size with filter paper sandwich
Skim milk	Skim milk 3% in PBS with NaN_3 0.02% as preservative
Washing buffer	PBS with 0.05% Tween 20
Primary antibody	Anti-whole bacteria antibodies raised in rabbit
Secondary antibody	Anti-rabbit IgG raised in goat conjugated with alkaline phosphatase
Substrate	1 mL 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) and 1 mL nitro blue tetrazolium (NBT) to 100 mL alkaline phosphatase color development buffer
Molecular mass standard	SeeBlue Plus2 Prestained Standard

3.2.6. Biofilm formation analysis and bacterial hydrophobicity

3.2.6.1. Biofilm formation

Bacteria were compared with regard to their ability to form biofilm in polystyrene microtiter plates using the protocol described by Baldassarri et al. (104). Bacteria were

grown for 18 h at 37°C in TSB. Polystyrene tissue culture plates were filled with 180 µl of TSB supplemented with 1% glucose and 20 µl of stationary phase culture, and were incubated at 37°C for 18 h. The plates were read in a microtiter plate reader at OD_{630} to confirm equivalent growth rates, the culture medium was then discarded and the wells were washed three times with 250 µl of PBS. Special care was taken not to disturb the biofilm on the bottom of the wells. The plates were dried at 42°C for 2 h or at 60°C for 1 h and then stained with 2% Hucker´s crystal violet for 2 min. Excess stain was removed by rinsing the plates thoroughly under tap water. The plates were then dried for 30 min at 42°C or for 10 min at 60°C. OD was determined at 630 nm and biofilm formation was normalized to growth with the biofilm index, which was calculated as $OD_{biofilm}$ x $(0.5/OD_{growth})$ (105).

TSB with 1% glucose	25 mL Glucose 20% mixed with 475 mL TSB and filter sterilized
Hucker´s crystal violet	A: 2 g of Crystal violet in 20 mL ethanol 95%
	B: 0.8 g Ammonium oxalate in 80 mL dH_2O
	20 mL A mixed with 80 mL B and filter sterilized

3.2.6.2. Initial adherence and biofilm accumulation

Biofilm production by different strains over time was monitored as described by Heikens et al. (42) by measuring initial adherence and the amount of biofilm formed after 2, 4, 6, 8 and 24 h. The biofilm was quantified as described above (3.2.6.1).

3.2.6.3. Bacterial affinity to organic solvents

The hydrophobicity of bacterial cells was analysed by comparing distribution in hydrophilic and hydrophobic solvents as described previously (106). Bacteria were grown to logarithmic phase in TBS, washed two times in sodium phosphate buffer (10 mM, pH 7), and resuspended in the same buffer to an OD_{600} between 0.44 and 0.62. Subsequently, 3 mL of bacterial suspension was mixed with 150µL dodecane and the two phases were mixed vigorously for 1 min. The mixture was allowed to separate for 10 min at 25°C. After the separation of the two phases was complete, absorbance of the water-phase was measured at OD_{600}. The proportion of cells in the dodecane phase was calculated according to the formula: % hydrophobicity = [1-(A/A0)] x 100.

Sodium phosphate buffer	A: 500mL sodium dihydrogenphosphate (13.2g/500 mL)
	B: 500 mL disodium hydrogenphosphate (19.2g/500 mL)
	39 mL A and 61 mL B in 500 mL dH_2O

3.2.7. Isolation of bacterial glycolipids

3.2.7.1. Butanol and Bligh andDyer extractions

Bacteria prepared as described (3.2.1.4**)** were resuspended in citric-buffer, the same volume of glass-beads was added and the cells were disrupted with a Beadbeater three times for 1 min. The supernatant was carefully collected and stirred with an equal volume of butanol for 30 min as described previously (77). The organic phase (upper phase) containing all products soluble in butanol was collected, dried by evaporation and extracted according to the method of Bligh and Dyer (107). Dry butanol extracts were resuspended in sodium acetate buffer and vortexed or sonicated for 15 min to obtain a suspension of lipids. Chloroform ($CHCl_3$) and methanol were added to the suspension to get a ratio $CHCl_3$:methanol:buffer of 1:2:0.8 and the whole mixture was vortexed for 20 min. For phase separation, more $CHCl_3$ was added to obtain a ratio $CHCl_3$:methanol:buffer of 2:2:0.8 and the mixture was vortexed again for 30 s. Finally, sodium acetate buffer was added to a ratio $CHCl_3$:methanol:buffer of 2:2:1.8, the mixture was centrifuged at 10000 *g* for 10 min and the organic phase was then collected and dried under a stream of nitrogen.

Citrate buffer	A: 9.8 g citric acid in 500 mL dH_2O
	B: 15.56 g sodium citrate in 500 mL dH_2O
	Mix 500 mL A and 500 mL B to have a 0.1 M solution with pH 4.6
Sodium acetate buffer	4.1 g Sodium acetate in 500 mL dH_2O to have a 0.1 M solution with pH 4.6

3.2.7.2. Glycolipid analysis by TLC

The mixture of polar lipids extracted as described above was separated by TLC according to the affinity of its different components for organic solvents. Prior the separation, the silica gel plates (10 x 10 cm) were dried for 2 h at 100°C and the atmosphere in the chromatography chamber was equilibrated for 30 min with running buffer using Whatman paper. Samples resuspended in chloroform (10-30µg) were loaded on plates using a

microsyringe and separated in TLC glass chamber using running buffer. For visualizing of glycolipids, the plates were sprayed with α-naphtol buffer and heated for 5-10 min at 110°C.

Running buffer	65 mL Chloroform mixed slowly with 25 mL methanol and then with 4 mL dH_2O added in drops, while stirring
α-Naphtol buffer	6 mL H_2SO_4 (98%) added slowly in 50 mL methanol and then 4 mL dH_2O added in drops; 1.92 g α-naphtol added to the solution α-naphtol

3.2.7.3. Glycolipid extraction by preparative TLC (PLC)

Glycolipids were purified by PLC. Silica gel plates (20 x 20 cm) were equilibrated with running buffer (3.2.7.2**)** for 2-3 h in the chromatography chamber. Next, the silica gel plates were let to evaporate for 18 h at 25°C and the following day they were gradually dried in an oven (40 to 70°C). After drying the plates, they were loaded with Bligh-Dyer extracts prepared as described (3.2.7.1). For detection of the bands, the margin of the plate was cut off and glycolipids were visualized by staining with α-naphtol buffer as described above. The fractions of interest were detected, scraped off the portion of the plates that were not treated with α-naphtol, and eluted with $CHCl_3$/MeOH in different ratios (1:1; 2:1, 3:1, v/v). The eluates were combined, filtered with a 0.2 μm PTFE filter and dried under a stream of nitrogen. Purity of the fraction was assesed by TLC as described above.

3.2.7.4. Glycolipids oxidation using sodium-meta-periodate

In some experiments, unfractionated polar lipids (250 μg/mL) extracted using the Bligh-Dyer method as described above (3.2.7.1**)** were treated for 1 h with fresh solution of sodium meta-periodate (1 mM) at 4°C on a rotor rack in the dark followed by neutralization with ethylene glycol (20 μL/mg polar lipids).

3.2.8. Surface Plasmon resonance measurements using the BIAcore system

Surface Plasmon resonance (SPR) measurements presented in this study were performed by Prof. F. Zhang and Prof. R. Linhardt (Chemistry and Chemical Biology, Biology, Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York, United States). In brief, the

interaction between different GAGs and enterococcal MGlcDAG or DGlcDAG was measured using SPR. Although there are several SPR-based systems, by far the most widely used one is the Biacore (GE Healthcare). SPR-based instruments measure the refractive index near a sensor surface. In order to detect an interaction one molecule (the ligand) is immobilised onto the sensor (chip) surface. Its binding partner (the analyte) is injected in aqueous solution (HBS running buffer) through the flow cell, also under continuous flow. As the analyte binds to the ligand, the accumulation of mass on the surface of the chip results in an increase in the refractive index. This change in the refractive index is measured in real time, and the result is plotted as response or resonance units (RUs) versus time (sensorgrams).

3.2.8.1. Biotinylation of GAGs

The biotinylated GAGs were prepared by using the reaction of sulfo-*N*-hydroxysuccinimide long-chain biotin with free amino groups of unsubstituted glucosamine residues in the polysaccharide chain. Mixtures of 5 mg GAGs and 5 mg Amine-PEO3-Biotin were dissolved in 200 µl H_2O and 10 mg sodium cyanoborohydride (NaCNBH3) was added. The reaction mixture was heated at 70°C for 24 h; further 10 mg NaCNBH3 were added and the solution was heated at 70°C for 24 h. After cooling, the mixture was desalted using a spin column (3000 Da). The biotinylated polysaccharides were collected and dry frozen until they were used for streptavidin (SA) chips preparation.

3.2.8.2. Preparation of GAGs biochips

The biotinylated GAGs were immobilized to streptavidin chips using the manufacturer's protocol, by non-covalent capture (binding to streptavidin). The streptavidin chip was pre-conditioned for 1 min with injection of 1 M NaCl in 50 mM NaOH before the biotinylated GAGs were immobilized. Different biotinylated GAGs (0.3 mg/mL) in HBS-EP buffer were injected to different flow cells using a 10 µL /min flow rate. The successful immobilization of GAG was confirmed by the observation of a ~300 resonance unit (RU) increase in the sensor chip except for heparin immobilization with ~ 70 resonance unit (RU) increase.

3.2.8.3. Measurement of glycolipid interaction with GAGs using Biacore

The glycolipids suspension was prepared in HBS-EP buffer by heating to 65ºC for 15 min in water bath, then sonicated for 15 min. Glycolipid samples with different concentrations diluted in HBS running buffer were injected at a flow rate of 30 µL/min. At the end of the

sample injection (90 s), the same HBS running buffer was flowed over the sensor surface to facilitate dissociation for 180 s. After a 3 min dissociation time, the sensor surface was regenerated with injections of 30 µl of 2 M NaCl. The response was monitored as RUs at 25°C. The SPR data were processed using the BIAevaluation software (version 4.0.1).

HBS-EP Buffer	0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA and 0.005% polysorbate 20 (v/v) mixed together and the final solution pH adjusted at 7.4
HBS running buffer	0.01 M HEPES and 0.15 M NaCl mixed together and the final solution pH adjusted at 7.4.

3.2.9. Animal experiments

3.2.9.1. Mouse bacteremia model

The virulence of different *E. faecalis* strains was evaluated in a mouse bacteremia model as described previously (67). Female 6-8 weeks old BALB/c mice (obtained from **Charles River GmbH**, Sulzfeld) were inoculated intravenously (i.v.) via the tail vein with bacterial inoculum prepared as previously described (3.2.1.5**)**. The inoculum was plated on TSA plates immediately after injection to determine viable counts. The challenge dose used is indicated in the Results section and mice were sacrificed after 72 h.

3.2.9.2. Mouse peritonitis model

The virulence of different bacterial strains was compared in a mouse peritonitis model as described (108). Mucin from porcine stomach was used when indicated. Groups of six or eight BALB/c mice were injected intraperitoneally (i.p.) with 200 µL mucin per animal and after 2 h each animal was infected also by i.p. injection with bacteria prepared as described (3.2.1.5**).** The inoculum was plated immediately after inoculation on TSA plates to determine viable counts. Animals were sacrificed at different time points, as indicated in the Results section.

In some experiments, survival of mice infected with different bacterial strains was studied. In these cases, the mice were observed daily for 5 d up to one week. All moribund mice (as determined by an inability to move upon repeated stimulus, inability to right itself after being placed on its side, piloerection, rapid respiration, and overall appearance of illness

associated with imminent death) were sacrificed and counted as dead. Mice that expired in between observations were also counted as lethal.

Hog gastric mucin	Autoclaved 5% hog gastric mucin in dH_2O at pH 7

3.2.9.3. Samples collection

To quantify bacterial burden and to examine the host responses in different conditions, mice were sacrificed at different time points, as indicated in the RESULTS section. Mice were euthanized by inhalation of CO_2 and a peritoneal lavage was performed with 4 mL sterile PBS using an 18-gauge needle; PLF was collected in sterile tubes. Blood was collected by cardiac puncture, with a sterile syringe and transferred to tubes containing heparin. Next, the abdomen was opened and the kidneys were harvested and all collected samples were immediately placed on ice.

For quantification of bacterial numbers in PLF and blood, serial dilutions were made in TSB and plated on agar plates. For quantification of bacterial numbers in the kidneys, the organs were homogenized on ice with TSB (five times the weight of the kidney) using a tissue homogenizer. The homogenizer was cleaned with ethanol and flamed after each homogenization. Serial dilutions of the samples were made in TSB and plated. Cell counts in the PLF were performed using a hemocytometer.

Relevant cytokines for the investigation of inflammation produced by bacteria were measured by ELISA in PLF and mouse blood. In this thesis TNF-α, IFN-γ and IL-4 levels were measured using a sandwich ELISA (3.2.5.1).

3.2.9.4. Bacterial infection of *Drosophila melanogaster*

The *Drosophila* infection model was performed together with Dr. Miriam Baron, Channing Laboratory, Harvard Medical School, Boston, United States. To study the enterococcal virulence in a *Drosophila* model, only wild type male adult flies (strain *OreR)* 2-3 d old were used. Flies were maintained on standard fly culture medium (maize molasses medium) at 30°C and 60% humidity as described (109). Flies were anaesthetized with CO_2 for only few seconds until they were falling in the culture tubes and were infected by pricking the dorsal thorax at the base of the wing with a 10 μm tungsten needle dipped in the bacterial suspension prepared as described in 3.2.1.6**.** This procedure assures that each fly is infected with a volume of 25-50 nl bacterial inoculum (M. Baron personal

observations). After infection, flies were returned to standard fly culture vials with food and incubated as described. After 2 h, flies were checked and the dead ones were eliminated, assuming that death was due to the trauma incurred at inoculation. The remaining flies were checked for viability twice every day for 3 d, as described by Baron et al. (110).

3.2.10. Statistical analisys

All data obtained are shown as means ± SEM. Significance was analyzed using GraphPad Prism software. Significant differences were considered at * $p < 0.05$.

4. RESULTS

4.1. Enterococci bind matrix components on the colonic cells

4.1.1. Adherence of *E. faecalis* strain 12030 and invasion of human colonic cell line Caco2

When confluent monolayers of Caco2 cells were incubated for 1 or 2 h at 37°C with *E. faecalis* strain 12030 at a MOI of 100:1, the adherence of bacteria correlated directly with the time of incubation (Fig. 4.1).

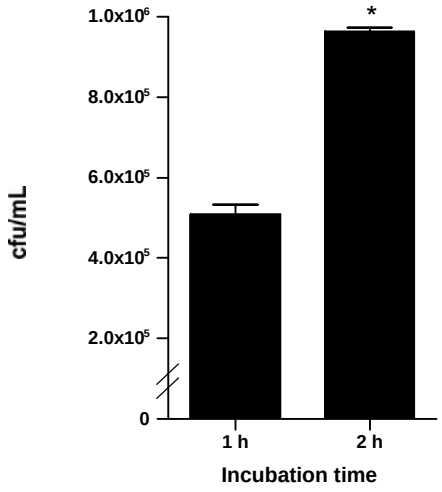


Fig. 4.1 Enterococcal adhesion to Caco2 cell line. Differentiated Caco2 monolayers were incubated for 1 or 2 h with E. faecalis strain 12030 at a MOI of 100:1. Significantly more bacteria adhered to cells after 2 h compared with 1 h (*$p<0.0001$ with two-tailed t test). Bars represent average plus SEM.

For the invasion assay a very high MOI (1000:1) was used, since at a MOI of 100:1 only few bacteria could be detected inside the cells. The number of internalized bacteria was approximately 4 logs smaller then the number of adherent bacteria (Fig. 4.2).

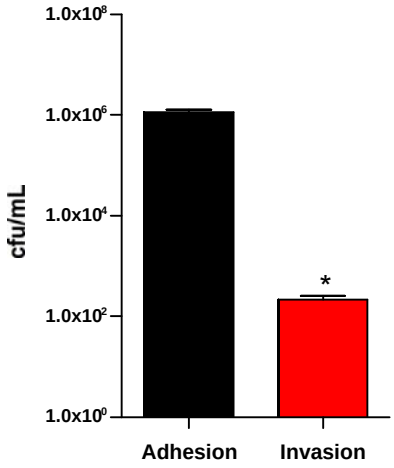


Fig. 4.2 Enterococcal adhesion/invasion to/into Caco2 cells. Bacterial invasion into Caco2 cells was studied using an antibiotic protection assay to kill the extracellular bacteria. To infect the monolayers, a high MOI (1000:1) was used, since at MOI 100:1 no bacteria were detected inside the cells after 2 h. The number of bacteria invading the cells was significantly lower than the number of bacteria adhering to the cells (*p=0.0002 using two-tailed t test). Bars represent average plus SEM.

4.1.2. Inhibition of bacterial binding to Caco2 cells using GAGs

The involvement of GAGs in the adherence of bacteria to the host cells was investigated using heparin, HS and CS as inhibitors of binding to eukaryotic cells, as described (3.2.4**)**. These polysulfates significantly decreased (up to 56%) the adherence of the *E. faecalis* strain 12030 to the epithelial cells. The highest inhibition (56% at a concentration of 500 µg/mL) was obtained by using the GAG heparin (Fig 4.3). HS decreased also significantly the level of adherence of the bacteria (Fig. 4.4, 50% inhibition at a concentration of 1000 µg/mL) while the binding of the bacteria to the eukaryotic cells was not affected (11% inhibition) by CSB (Fig. 4.5). The inhibition of bacterial binding was dose-dependent when HS was used (Fig. 4.4). For heparin, a dose-dependent inhibition was observed for all but the highest concentration (Fig .4.3). This latter observation may be explained by the unphysiologically high concentration of the inhibitor.

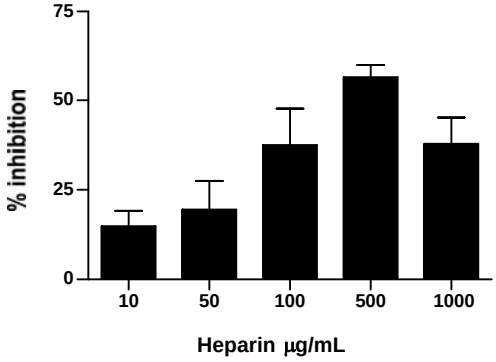


Fig. 4.3 Inhibition of enterococcal attachment to Caco2 cells using heparin. Adhesion of *E. faecalis* strain 12030 to Caco2 cells was tested in presence of various concentrations (10-1000 µg/mL) of heparin sodium salt from porcine intestinal mucosa. Monolayers were incubated with heparin for 30 min prior adding the bacterial inoculum. Heparin reduced bacterial adhesion to cells in a significant linear trend ($p<0.0001$ using ANOVA with Test for linear trend). Bars represent average with SEM.

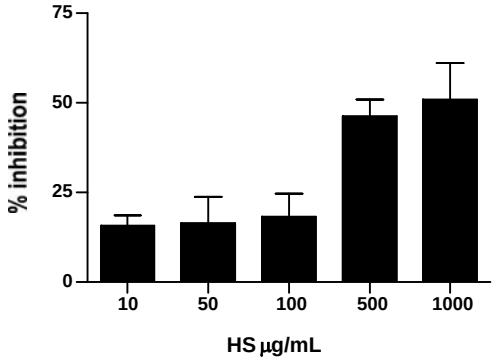


Fig. 4.4 Inhibition of enterococcal attachment to Caco2 cells using HS. Adhesion of *E. faecalis* strain 12030 to Caco2 cells was tested in presence of various concentrations (10-1000 µg/mL) of HS sodium salt from porcine intestinal mucosa. Monolayers were incubated with HS for 30 min prior adding the bacterial inoculum. HS reduced bacterial adhesion to cells in a significant linear trend ($p=0.0003$ using ANOVA with Test for linear trend). Bars represent average with SEM.

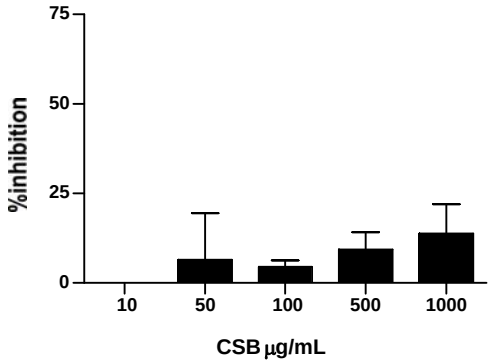


Fig. 4.5 Inhibition of enterococcal attachment to Caco2 cells using CSB. Adhesion of *E. faecalis* strain 12030 to Caco2 cells was tested in presence of various concentrations (10-1000 µg/mL) of CSB sodium salt from porcine intestinal mucosa. Monolayers were incubated with CSB for 30 min prior adding the bacterial inoculum. CSB had no significant effect on bacterial adhesion to cells (p=0.14 using ANOVA with Test for linear trend). Bars represent average with SEM.

4.1.3. Inhibition of bacterial binding to Caco2 cells using chemically modified GAGs

The structural complexity of heparan sulfate proteoglycans is determined among other things by the degree of sulfation of the polysaccharide chain. To identify the importance of these modifications for bacterial binding, inhibition assays were performed using several chemically modified heparins as inhibitors. For these assays, heparin from porcine intestinal mucosa served as a positive control. As shown in Fig. 4.6, preincubation of cell monolayers with 500 µg/mL of normal heparin and 6-*O*-desulfated heparin decreased bacterial binding with 58% and 56%, respectively. In contrast, both 2-*O*-desulfated heparin and *N*-desulfated heparin were less potent inhibitors (21% and 23% inhibition, respectively). These findings indicate that specific sulfate groups on cellular heparin sulfate rather than the total level of sulfation may be important for mediating enterococcal-host cell interaction.

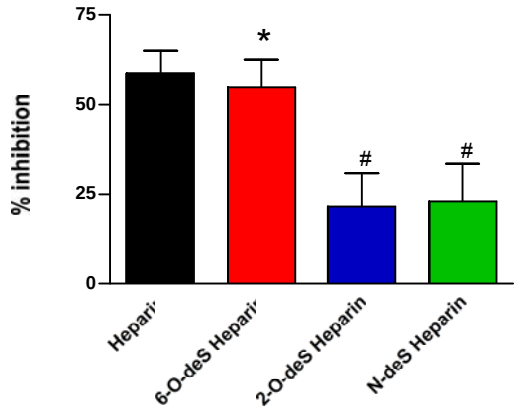


Fig. 4.6 Inhibition of bacterial binding to epithelial cells using chemically modified heparins. Normal heparin, 2-*O*-desulfated heparin, 6-*O*-desulfated heparin, and *N*-desulfated heparin (all at 500 µg/mL concentration) were used as inhibitors of bacterial binding. Heparin and 6-*O*-desulfated heparin significantly reduced bacterial binding to cells (*p>0.05, against heparin, using ANOVA with Dunnett's Multiple Comparison Test), while 2-*O*-desulfated heparin and *N*-desulfated heparin partially lost their inhibitory activity (#p<0.05 against heparin). Bars represent average plus SEM.

4.1.4. Effect of GAG lyases treatment of Caco2 cells on *E. faecalis* strain 12030 adherence

Treatment with GAG lyases was performed on the intestinal epithelial cells before and during infection with bacteria to investigate the role of different GAGs as receptors for pathogens. Target cells were treated with heparin lyase I (which cleaves heparin and HS chains) and chondroitin lyase ABC (which degrades CS chains). Heparin lyase I reduced bacterial adherence to cells in a dose dependent manner (25 through 52.5%), while enzymatic degradation of the glycocalix with chondroitin lyase ABC had no significant effect on enterococcal adherence to cells (Fig. 4.7). These results confirm the role of heparan sulfate proteoglycans as cellular receptors for enterococci.

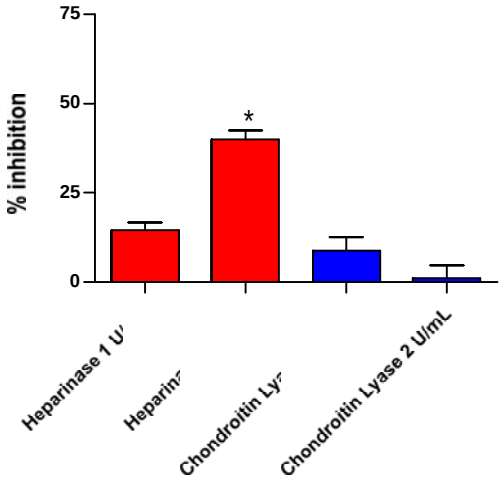


Fig. 4.7 Reduction of bacterial attachment to the epithelial cells using GAG lyases. Heparin lyase I (1 and 2 U/mL) and chondroitin lyase ABC (1 and 2 U/mL) were used to digest the GAG chains and were added to the cells 10 min prior and 50 min after the bacterial inoculum (* $p<0.001$, against the control containing no enzymes using ANOVA with Bonferroni's Multiple Comparison Test). Bars represent average plus SEM.

4.1.5. Inhibition of *E. faecalis* adhesion by sodium chlorate treatment of the cells

Overall sulfation of the GAG chains was decreased by treating the cells for 48 h with various concentrations of sodium chlorate as described (3.2.4.3).

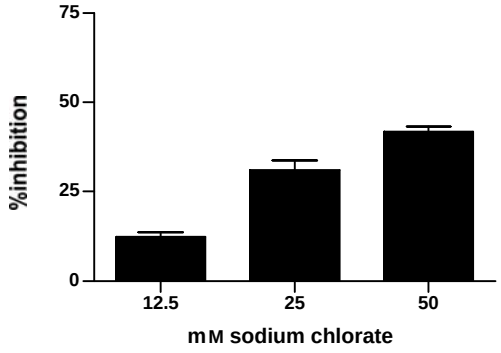


Fig. 4.8 Decrease of bacterial binding to Caco2 cells using sodium chlorate as an inhibitor of the sulfation for the GAG chains. Sodium chlorate in various concentrations (12.5-50 mM) was added to the adhesion assay 48 h prior to the experiment. Untreated cells were used as a negative control. All concentrations reduced binding significantly ($p<0.001$ using ANOVA with Bonferroni's Multiple Comparison Test) as compared to untreated cells. Bars represent average plus SEM.

Sodium chlorate is known to inhibit the ATP sulfurylase, the first enzyme of the sulfation pathway of the cellular GAGs. Preincubation with sodium chlorate reduced bacterial adhesion to Caco2 cells in a dose-dependent manner up to 42% (Fig. 4.8), which emphasizes the role of GAGs sulfation in bacterial interaction with the intestinal cells.

To determine whether the interaction between GAGs and enterococci is specific only for one tested strain, five additional strains (see **Table 3.1**) were tested on Caco2 cells treated with sodium chlorate. Pretreatment with sodium chlorate significantly reduced adhesion of two of the strains (FA2-2 and T9) compared to untreated Caco2 cells (Fig. 4.9). These inhibition values were comparable to the value obtained when reference strain *E. faecalis* strain 12030 was used (41% inhibition for FA2-2 and 68% inhibition for T9). No significant difference between treated and untreated cells was detected for the other strains tested (T5, V583 or OG1-RF).

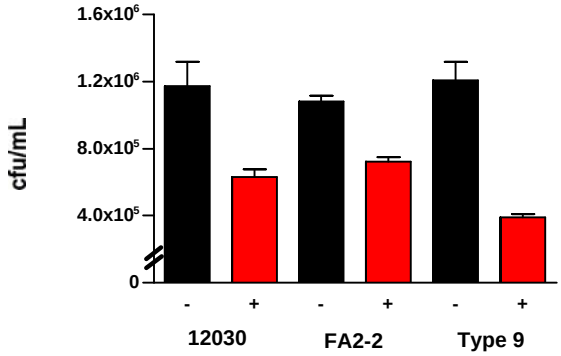


Fig. 4.9 Inhibition of different *E. faecalis* strains binding to Caco2 cells using sodium chlorate treatment. Reduction of GAG chain sulfation by sodium chlorate treatment (50 mM) decreased the ability of *E. faecalis* strains 12030, FA2-2, and type 9 to adhere (using ANOVA with Bonferroni's Multiple Comparison Test). For the other three strains tested, there was no significant difference in binding to Caco2 cells (data not shown). Bars represent average plus SEM. + with sodium chlorate, - without sodium chlorate.

4.2. Enterococcal specific surface structures mediate binding to colonic cells

4.2.1. Digestion of bacterial surface proteins with proteinase K

Involvement of bacterial surface proteins in adherence to epithelial intestinal cells was tested by treating bacteria with proteinase K as described in 3.2.1.1**.** This enzymatic treatment did not affect bacterial viability, as confirmed by viable counts. Untreated bacteria were used as controls and the adhesion assay was performed as usual. By using bacteria that were treated with proteinase K, adhesion to eukaryotic cells was increased by up to 250% compared with the untreated control (Fig. 4.10)

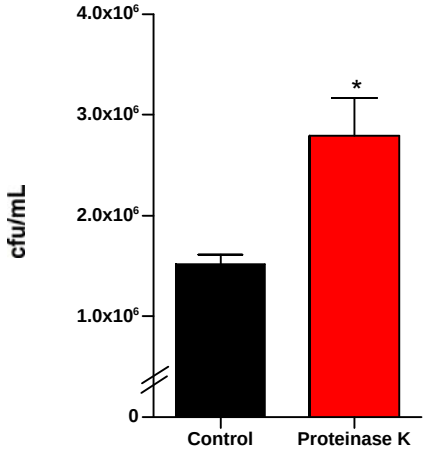


Fig. 4.10 Effect of proteinase K treatment of *E. faecalis* 12030 on attachment to Caco2 cells. Bacterial cells treated with proteinase K (0.1 mg/mL, at 37°C during cultivation to mid-log phase) adhere better to Caco2 monolayers. Bacteria grown under the same conditions, but not treated with enzyme were used as a negative control (*p=0.025, using two-tailed t test). Bars represent the average with SEM.

4.2.2. Oxidation of bacterial cell-wall glycoconjugates using sodium meta-periodate

The role of bacterial surface carbohydrates in adhesion to Caco2 cells was evaluated by oxidation of polysaccharide-containing cell-wall polymers with sodium meta-periodate (0.125 to 1 mM).

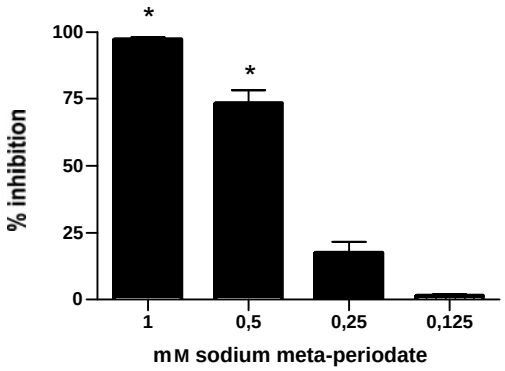


Fig. 4.11 Effect of sodium meta-periodate-treatment of *E. faecalis* 12030 on attachment to Caco2 cells. Bacterial cells treated with sodium meta-periodate (0.125-1 mM) partially lost their ability to adhere to Caco2 monolayers. Bacteria grown in the same conditions, but not treated with sodium meta-periodate, were used as a control (*p<0.001-ANOVA with Bonferroni´s Multiple Comparison Test using sodium meta-periodate and untreated bacteria). Bars represent the average with SEM.

This treatment resulted in a dose-dependent reduction of the enterococcal binding to the epithelial cells from 2% to 100% (Fig. 4.11). These results indicate that bacterial surface carbohydrates play an important role in binding to Caco2 cells.

4.2.3. Treatment of Caco2 cells with LTA and WTA

LTA and WTA isolated from *E. faecalis* strain 12030 as described (77), (111) were provided by Dr. Christian Theilacker (University Hospital Freiburg). Since depleting bacteria of cell-wall carbohydrates resulted in abolished binding to Caco2 cells, LTA and WTA were evaluated regarding their inhibition of bacterial binding. Pre-treatment of cell monolayers with various amounts of WTA (62.5-250 µg/mL) or LTA (62.5-250 µg/mL) did not have any effect on bacterial adhesion (Fig. 4.12).

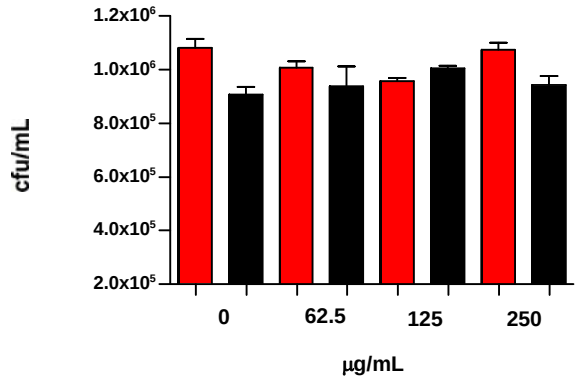


Fig. 4.12 LTA and WTA isolated from *E. faecalis* strain 12030 do not inhibit bacterial binding to Caco2 cells. Confluent monolayers of Caco2 cells were incubated with WTA (red bars) or LTA (black bars) (67.5-250 µg/mL) from *E. faecalis* strain 12030 for 30 min before addition of bacteria. Cells incubated only with DMEM were used as negative control. None of the components was able to reduce significantly bacterial binding ($p>0.05$ using ANOVA with Bonferroni´s Multiple Comparison Test). Bars represent the average plus SEM.

4.2.4. Inhibition of enterococcal binding to Caco2 cells using bacterial polar lipids

To examine the involvement of bacterial membrane lipids on adhesion, these were first isolated as described in 3.2.7.1. The dried preparations were resuspended in dH_2O and sonicated for 15 min in a water bath to obtain a homogenous suspension. By incubating the cells with various concentrations of extracts of unfractionated membrane lipids (62.5-250 µg/mL), bacterial adherence to cells was impaired by up to 59%, and maximum inhibition was observed at a concentration of 125 µg/mL (Fig. 4.13).

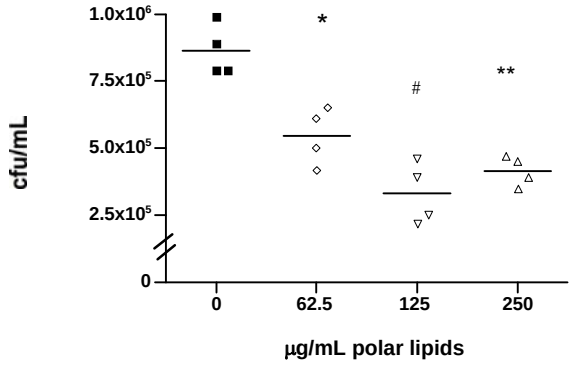


Fig. 4.13 Polar lipids isolated from enterococci inhibit bacterial attachment to Caco2 epithelial cells. Confluent monolayers of Caco2 cells were incubated with glycolipids (67.5-250 µg/mL) from E. faecalis strain 12030 for 30 min before addition of bacteria in order to measure adherence. Cells incubated only with DMEM were used as negative control. All concentrations of polar lipids significantly reduced bacterial binding to Caco2 cells (*$p<0.01$; #$p<0.001$ and **$p<0.001$). Data points represent individual replicates and lines represent the mean.

4.2.5. Oxidation of bacterial polar lipids with sodium meta-periodate

As mentioned before, oxidation of bacterial cell-wall glycoconjugates using sodium meta-periodate abolished bacterial binding to Caco2 cells (4.2.2) and treatment of cells with bacterial polar lipids also impaired adhesion (4.2.4). These findings indicate that a sugar-containing molecule present in the bacterial polar lipids extract might mediate bacterial adhesion to eukaryotic cells. One abundant polar lipid from the enterococcal membrane is a glycolipid, α-diglycosyl-diacylglycerol (DGlcDAG) (8-37% of all membrane polar lipids), and to a lesser extent α-monoglycosyl-diacylglycerol (MGlcDAG) and traces of α-trigylcosyl-DAG (112). To assess whether these structures bind receptors on Caco2 cells, polar lipids from *E. faecalis* (see 3.2.7.1) were degraded with sodium meta-periodate. While native polar lipids inhibited 43% of bacterial binding to Caco2 cells, oxidation of glycolipids by periodate completely abolished their potential to inhibit binding (Fig. 4.14).

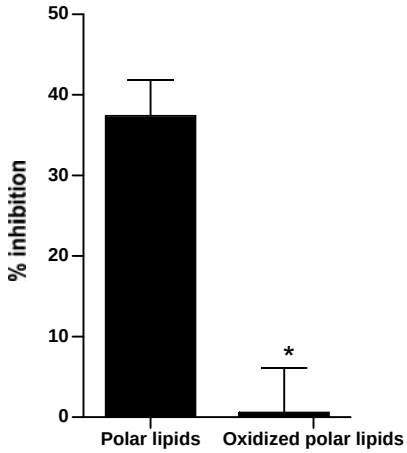


Fig. 4.14 Inactivation of unfractionated polar lipids with sodium meta-periodate eliminates the inhibitory activity. Treatment of polar lipids with 1 mM sodium meta-periodate resulted in a complete loss of the inhibitory activity of enterococcal polar lipids on adherence to Caco2 cells (*p<0.0001 using unpaired t test). Bars represent average plus SEM.

4.2.6. Purification of enterococcal membrane glycolipids

DGlcDAG and MGlcDAG, the two major enterococcal membrane glycolipids were extracted according to the procedure described by Bligh and Dyer (followed by TLC), visualized by α-naphtol staining, and further purified by PLC, as described in 3.2.7. Staining with α-naphtol showed one major band [Retention factor (Rf) 0.47] and 4 minor bands (Rf 0.65; 0.39; 0.25; 0.11) (Fig. 4.15). Purification of the two bands showing the highest mobilitiy in the TLC and analysis by one- and two-dimensional NMR revealed the structure of the two glycolipids: 1,2-diacyl-3-*O*-[α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl]-*sn*-glycerol (DGlcDAG) and 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol (MGlcDAG). Structural details of DGlcDAG and MGlcDAG were specified by Theilacker et al. (91). Glycolipids with a lower mobility in TLC were not purified for structural analysis by us, but according to Fischer, these glycolipids correspond to phosphatidyl-glycolipids, glycerophospho-glycolipids and glycerophospho-phosphatidyl-glycolipids (113).

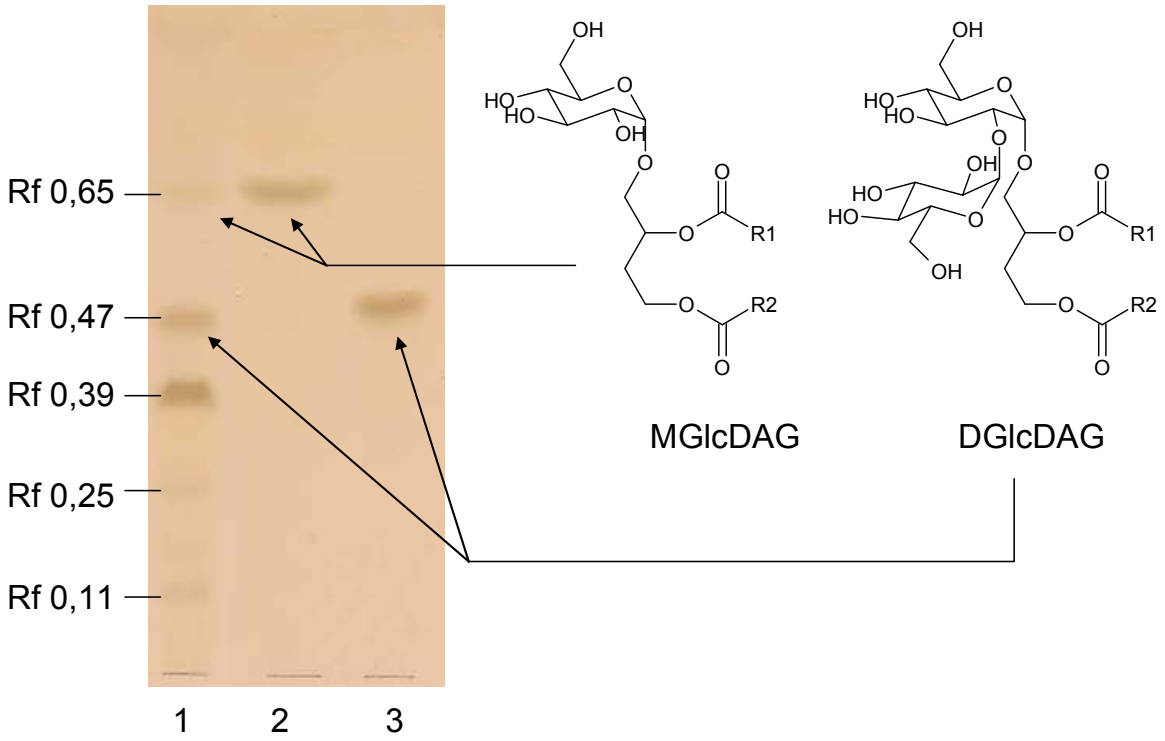


Fig. 4.15 TLC of cell membrane total lipid extracts of *E. faecalis* 12030 (lane 1), purified MGlcDAG (lane 2) and DGlcDAG (lane 3). Glycolipids were separated using a mixture of $CHCl_3$/MeOH/H_20 (65:25:4, v/v/v) and visualized with α-naphtol/sulfuric acid. The structure of MGlcDAG and DGlcDAG as assigned by NMR spectroscopy (91) is shown next to the corresponding TLC band.

4.2.7. Treatment of Caco2 cells with purified bacterial glycolipids

To corroborate the role of membrane glycolipids in bacterial binding to cells and exclude non-specific effects mediated by other polar lipids present in the Bligh-Dyer extract, the two major glycolipids of *E. faecalis* (MGlcDAG and DGlcDAG, purified by PLC as described) were used to inhibit bacterial binding to eukaryotic Caco2 cells. While DGlcDAG inhibited adhesion up to 47% compared with the untreated control, preincubation with MGlcGAG did not influence bacterial binding to eukaryotic cells (Fig. 4.16).

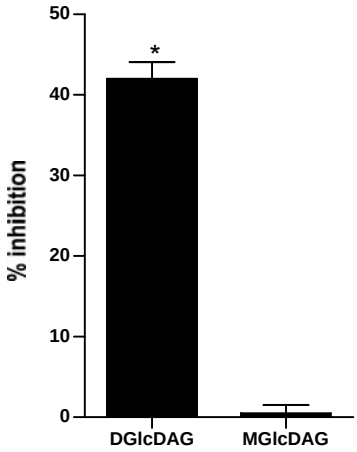


Fig. 4.16 DGlcDAG but not MGlcDAG inhibits enterococcal binding to Caco2 cells. Monolayers of Caco2 cells were incubated with MGlcDAG or DGlcDAG (250 µg/mL) for 30 min before the adherence assay. Cells incubated only with DMEM were used as a negative control. Statistical analysis was done using ANOVA and Tukey´s Multiple Comparison Test (*$p<0.001$ compared with no glycolipids). Bars represents average plus SEM.

4.2.8. The effect of sodium chlorate and/or bacterial polar lipids on bacterial attachment to Caco2 cells

Caco2 cells depleted of sulfuric groups on their GAG chains using sodium chlorate (50 mM, for 48 h) or treated with bacterial polar lipids (250 µg/mL, for 30 min) or with both sodium chlorate and bacterial polar lipids were used in bacterial binding assays. Cells cultivated in the absence of sodium chlorate and polar lipids were used as controls. For all three types of treatments, about 50% inhibition was observed compared to the negative control (Fig. 4.17), suggesting that GAGs and glycolipids together may act as co-receptors in the adhesion, since the same amount of bacterial binding was observed in the presence of both glycolipids and sodium chlorate compared to cells which underwent only one type of treatment.

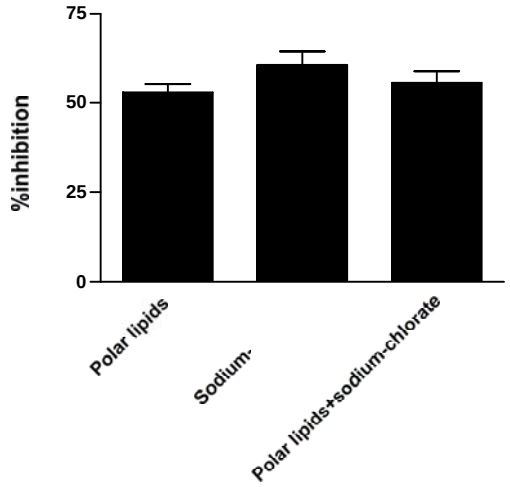


Fig. 4.17 Bacterial adhesion to Caco2 cells treated with polar lipids, sodium chlorate or with both compounds. Differentiated Caco2 monolayers were treated with sodium chlorate for 48 h, with bacterial polar lipids for 30 min or with a combination of both before performing the adhesion assay. Cells unterated with any of these agents were used as negative control. All three types of treatments reduced bacterial adhesion with about 50% compared to the untreated control and no significant difference was observed among the different treatments ($p>0.05$, using ANOVA with Bonferroni's Multiple Comparison Test).

4.2.9. SPR measurement of glycolipid-GAG interactions

To confirm the hypothesis that glycolipids on the bacteria recognize the heparan sulfate proteoglycans on Caco2 cells, the affinity of different GAGs to the two major enterococcal glycolipids was measured by SPR (work done in collaboration with the group of Prof. R. Linhardt, Rensselaer Polytechnic Institute, Troy, New York, United States). Biotinylated GAGs were immobilized on a streptavidin sensor chip as described (see Methods section 3.2.8), and the glycolipids dissolved in running buffer were injected at different concentrations. The SPR measurements were performed with two different chips: one chip with immobilized heparin, the other with several different GAGs (i.e.CSA, CSB, CSC and HS). The density of the immobilized ligand was different between these two chips (~ 70 RU for heparin and ~ 300 RU for other GAGs).

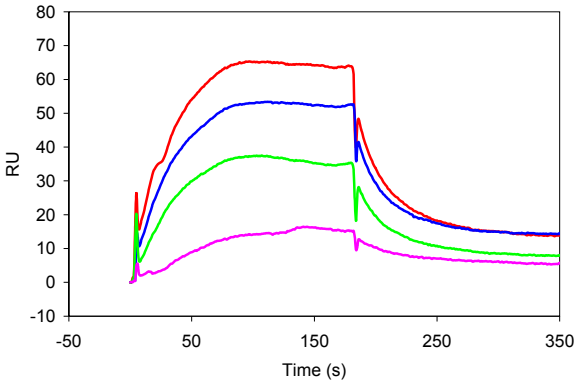


Fig. 4.18 SPR sensorgrams of the interaction between DGlcDAG and heparin. Concentrations of DGlcDAG (from top to bottom) are: 100 (red), 75 (blue), 50 (green), and 25 (pink) μM, respectively.

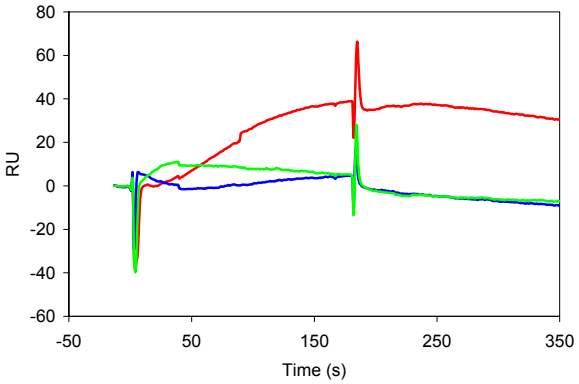


Fig. 4.19 SPR sensorgrams of interaction between DGlcDAG and GAGs. Interaction between HS (red), CSB (green), CSC (blue) and DGlcDAG (100 μM) was measured.

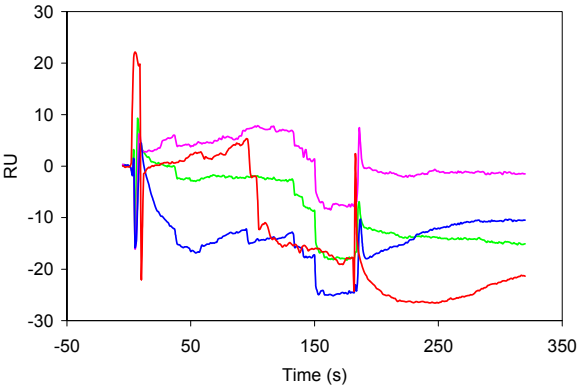


Fig. 4.20 Sensorgrams of interactions between MGlcDAG and GAGs. Interactions between heparin (red), HS (blue), CSB (green), CSC (pink) and MGlcDAG (100 μM) were measured.

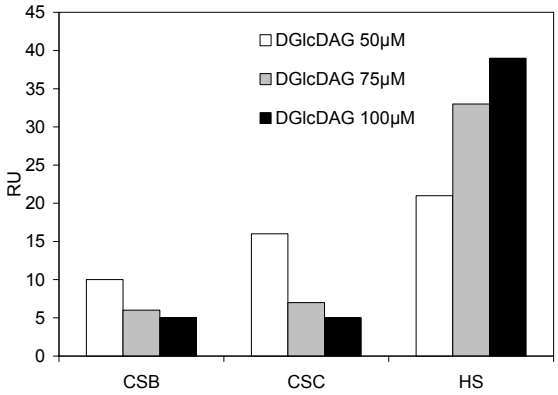


Fig. 4.21 Binding response of DGlcDAG to GAGs. DGlcDAG in different concentrations (50 µM, 75 µM and 100 µM) were used.

The sensorgrams of the DGlcDAG/heparin, DGlcDAG/HS, DGlcDAG/CSB, and DGlcDAG/CSC interactions are shown in Fig. 4.18 - Fig. 4.21. A strong binding signal (RU) could be demonstrated especially for heparin and DGlcDAG (Fig. 4.18). The K_D value for DGlcDAG binding to heparin can be estimated to be ~ 60 µM. A strong interaction was also observed for HS, although the RU for heparin and for HS cannot be compared directly since two different chips with different density of immobilized ligands were used. SPR demonstrated a stronger binding signal (RU) of HS to DGlcDAG, in comparison with CSB or CSC (Fig. 4.19 and Fig. 4.21). The binding of heparin and HS to DGlcDAG was concentration dependent, suggesting a specific interaction. In contrast, binding of CSB or CSC to DGlcDAG was independent of the concentration, and the affinity was generally low. MGlcDAG did not bind to GAGs (negative signal by SPR) (

Fig. 4.20).

4.3. The *bgsA* (biofilm-associated glycolipis synthesis A) gene encodes a glycosyltransferase required for synthesis of DGlcDAG from MGlcDAG

4.3.1. TLC and Western blot of *E. faecalis* strain 12030 and its mutant *E. faecalis* 12030Δ*bgsA*

A non-polar deletion mutant *E. faecalis* 12030Δ*bgsA* was provided by Dr. C. Theilacker (University Hospital Freiburg) and the description techniques used in its creation is described in detail in Theilacker et al. (91). *E. faecalis* gene EF_2891 (designated as *bgsA*) encodes a DGlcDAG synthetase which shares high identity and similarity to AL*dgs*, a gene that encodes a diglycosyl-DAG synthetase in *Acheloplasma laidlawii* (114) and with *iagA*,

a gene required for anchoring of LTA to the cell membrane of *Streptococcus agalactiae* (115). The putative function of BgsA is glycosylation of MGlcDAG to yield DGlcDAG.

Polar lipids were isolated from *E. faecalis* strain 12030 and from *E. faecalis* 12030Δ*bgsA* (see 3.2.7**)** and separation by TLC followed by staining with α-naphtol revealed only a single glycolipid band (Fig. 4.22) in the mutant strain, identified as MGlcDAG (91).

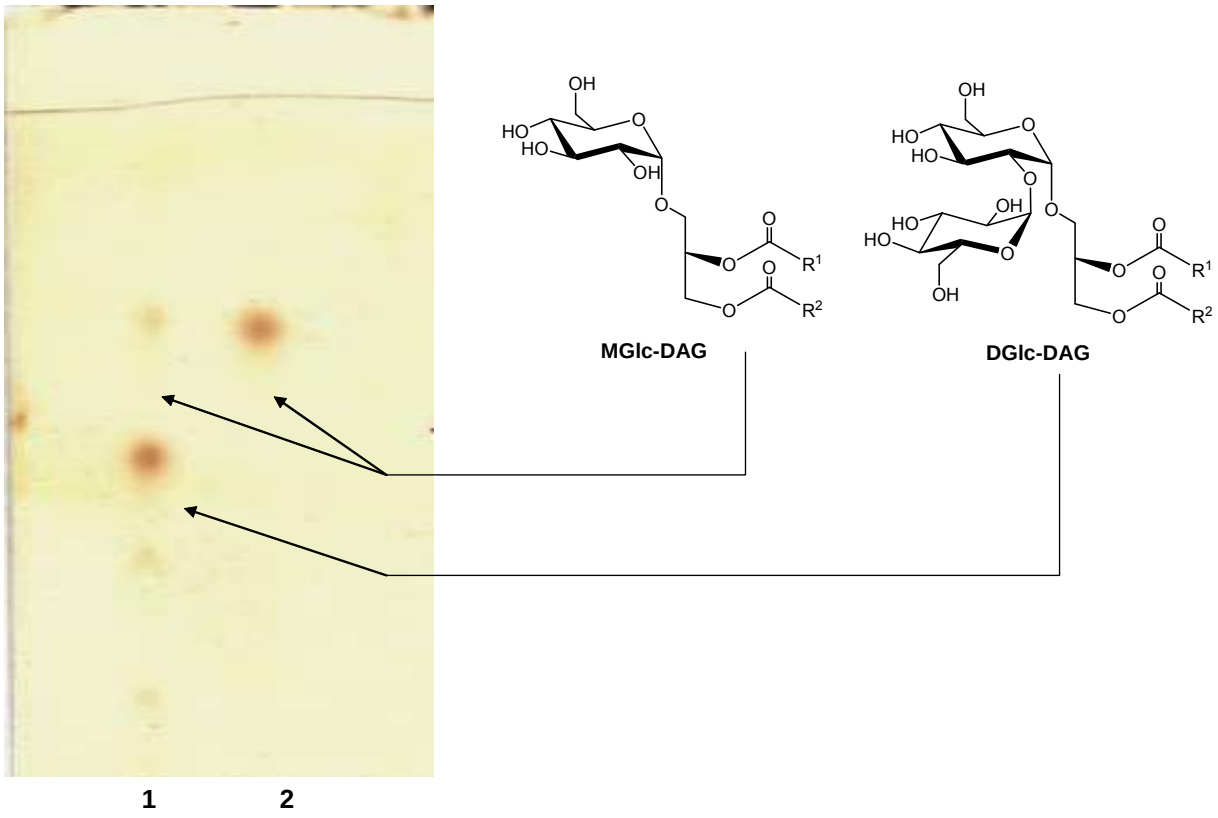


Fig. 4.22 TLC of cell membrane total lipid extracts of *E. faecalis* 12030 (lane 1) and *E. faecalis* 12030*ΔbgsA* (lane 2). Glycolipids were separated using a mixture of $CHCl_3$/MeOH/H_20 (65:25:4, v/v/v) and visualized with α-naphtol/sulfuric acid. The wild type strain produces both MGlcDAG and DGlcDAG, while the mutant produces only MGlcDAG.

Next, we analyzed cell wall-associated antigens of the *E. faecalis* 12030 and 12030*ΔbgsA* by SDS PAGE, followed by Western blot (see 3.2.5.3) revealing two distinct differences of the mutant compared to the wild type strain (Fig. 4.23).

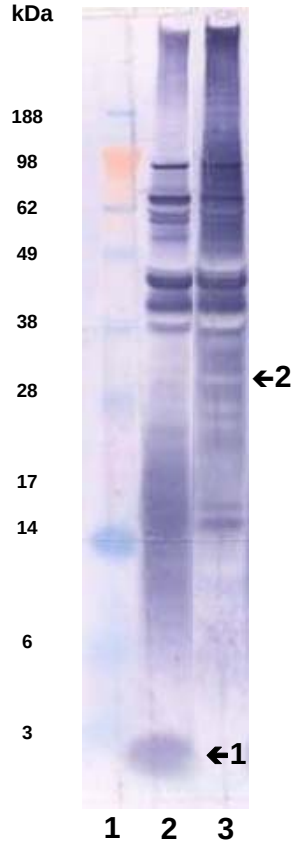


Fig. 4.23 Western blot analysis of cell wall associated antigens of 12030*ΔbgsA* and of the wild type strain. The surface-associated molecules of the 12030 strain (lane 2) and *ΔbgsA* mutant (lane 3) were separated using a 5-12% SDS PAGE and visualized using rabbit serum raised against whole wild type bacteria and anti-rabbit IgG alkaline-phosphatase conjugated. Arrow 1 indicates the low molecular mass band and arrow 2 indicates the LTA from the mutant with lower mobility.

1) a low molecular mass band was missing in *E. faecalis* 12030*ΔbgsA* strain (< 3 kDa). This band represents probably a glycolipid containing α-kojibiosylglycerol, which is recognized by the anti-whole *E. faecalis* strain 12030 serum used in the experiment.

2) electrophoretic mobility of LTA from the mutant strain was retarded compared to the LTA from the wild type strain, (broad band of 6 - 28 kDa in the *E. faecalis* 12030 shifted to 14 - 30 kDa in the 12030*ΔbgsA* mutant). This phenomenon has also been observed by Gründling and Schneewind (116) in mutants defective in the glycosylation of diacylglycerol in *Staphylococcus aureus*. Analysis of cell wall associated proteins showed the same pattern for the two strains, when analyzed by Western blotting.

4.4. Phenotypical traits *not* changed in *E. faecalis* 12030Δ*bgsA*

4.4.1.1. Morphological analysis of bacteria using scanning electron microscopy

The wild type and mutant bacteria were examined by SEM in collaboration with Dr. L. Baldassarri (National Health Institute, Rome, Italy) as described in section 3.2.1.7.

Both *E. faecalis* strain 12030 and the mutant strain exhibited extracellular polysaccharide material. No differences in cell wall morphology of *E. faecalis* 12030 and 12030*ΔbgsA* were observed. SEM of the mutant strain, however, revealed bacterial cells with a minor increase in size and overall increase in pleomorphic cells. The wild type strain had an average size of 0.8±0.01 x 0.74±0.02 µm (lenght x width), while the mutant was 1.4±0.56 x 0.96±0.20 µm.

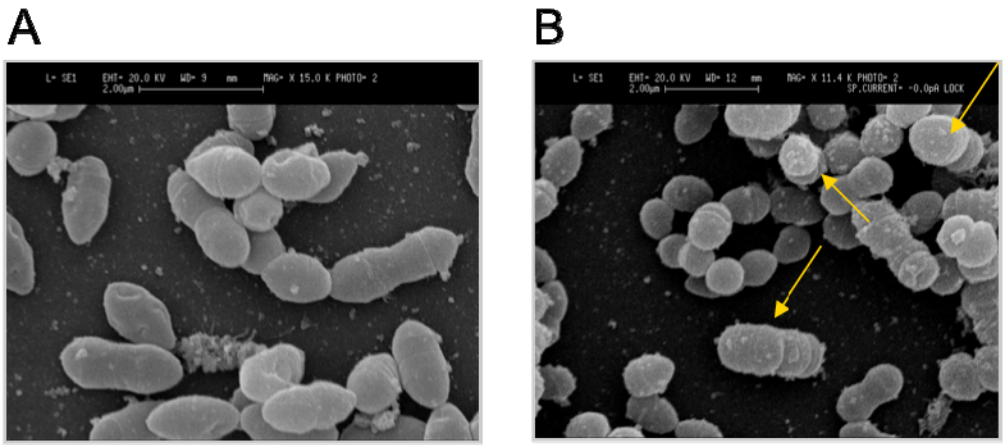


Fig. 4.24 Scanning electron micrographs of exponential-phase cells of *E. faecalis* 12030 WT (A) and the 12030*ΔbgsA* mutant (B). Arrowheads in panel B show aberrant pleomorphs.

4.4.1.2. Opsonophagocytic killing and complement sensitivity

An opsonophagocytic assay (3.2.5.2) was used to investigate the *in vitro* immune response to the two enterococcal strains. Rabbit polyclonal antiserum raised against whole wild-type bacteria (diluted 1:100) was used a source of antibodies. After 90 min incubation with PMNs, complement and antibodies, no significant differences in bacterial killing was observed (96% *E. faecalis* wild type versus 87% Δ*bgsaA* mutant) (Fig. 4.25). When the two bacterial strains were incubated in presence of complement but no antibodies for 90 min, both of them were resistant to complement-mediated killing at a complement concentration of 1.66% (final dilution).

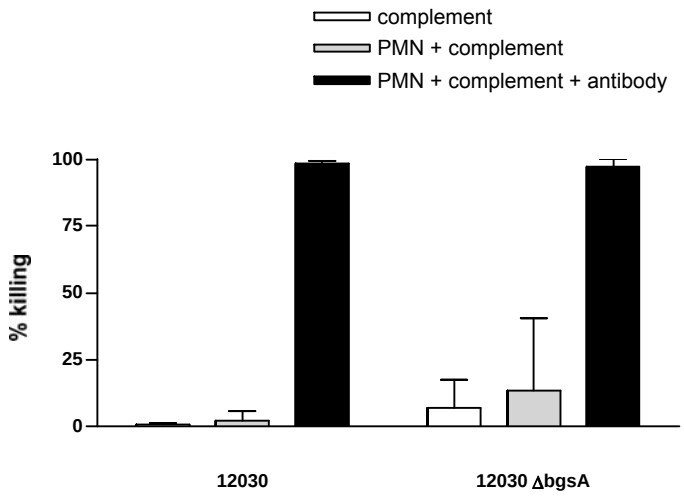


Fig. 4.25 Opsonophagocytic killing and complement susceptibility. The two bacterial strains were compared for their susceptibility to opsonic killing and complement-mediated killing. Only a very small difference in opsonic killing was observed (96% killing of the *ΔbgsA* strain and 87% killing of the wild type strain) and none of the strains was complement-sensitive ($p>0.05$ with ANOVA and Kruskal-Wallis test). Bars represent average plus SEM.

4.4.1.3. Bacterial killing by RAW 264.7 mouse macrophages

The capacity of mouse marcophages to phagocytose and kill *E. faecalis* stains was measured *in vitro* using the mouse macrophage cell line RAW 264.7 (see section 3.2.3.5).

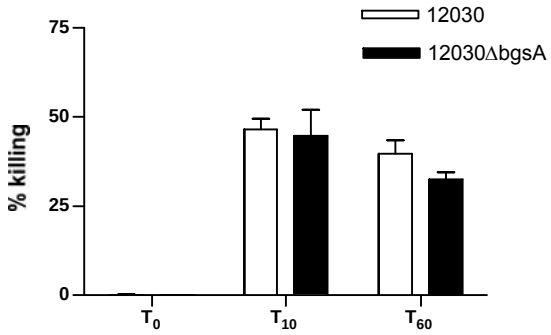


Fig. 4.26 Bacterial killing by RAW 264.7 cells. Mouse macrophages (cell line RAW 264.7) were incubated with bacteria (MOI 100:1) and lysed after 10 or 60 min. Killing was expressed as percentage of killing compared with T0. No significant difference in killing of the two enterococcal strains by mouse macrophages was observed ($p>0.05$ using two-tailed t test). Bars represent average plus SEM.

As observed for human PMNs, no significant difference in killing between the two strains was observed after 10 or 60 min. After 60 min, the percentage of killing was slightly lower for both tested strains, suggesting that bacteria continue to multiply inside the macrophages.

4.4.1.4. Infection of *Drosophila melanogaster* with enterococci

The infection model in *Drosophila* was performed together with Dr. M. Baron from the Chaning Laboratory, Harvard Medical School, Boston, United States. Infection of *Drosophila* as an alternative to mammalian model systems has been used extensively to identify bacterial virulence factors (109). It has been shown that enterococci contain virulence factors required for pathogenesis in hosts such as *Drosophila melanogaster* (30). Here, for each experiment, 25-30 Oregon Red male flies/group were used, as previously described (3.2.9.4). *Drosophila* were infected by pricking the bacterial inoculum into the fly's torsos. Flies inoculated only with TSB were used as controls. No relevant mortality was observed in the control group (97.5% survival). In contrast, significant mortality was seen in flies infected with *E. faecalis* wild type bacteria. The percentage of survival correlated with the bacterial dose (higher bacterial dose lead to higher mortality, data not shown). There was no significant difference in the mortality of flies infected with the wild type or with the mutant strain, respectively (Fig. 4.27).

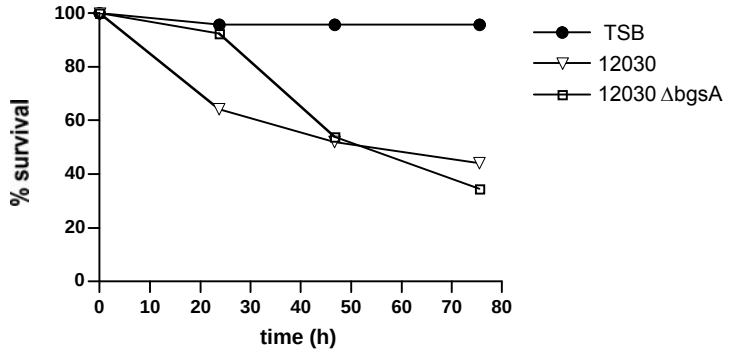


Fig. 4.27 Survival of *Drosophila melanogaster* infected with *E. faecalis* strain 12030 and its *ΔbgsA* mutant. Wild-type Oregon Red flies were infected with $5x10^9$cfu/mL and observed for survival. No significant differences in survival of groups of flies infected with the same bacterial inoculum were observed.

4.5. Biofilm formation and affinity to organic solvents are affected in *E. faecalis* 12030*ΔbgsA*

4.5.1. Biofilm formation on polystyrene surfaces

YpfP encodes a glycosyltransferase that synthetizes the glycosylation of DAG in the membrane of *Staphylococcus aureus* and its deletion leads to an impaired biofilm formation (106). To examine the role of glycolipids in biofilm formation of *E. faecalis*, we

used a polystyrene microtiter assay. Deletion of *bgsA* in *E. faecalis* strain 12030 lead to a greatly reduced ability to form biofilm on plastic surfaces (Fig. 4.28).

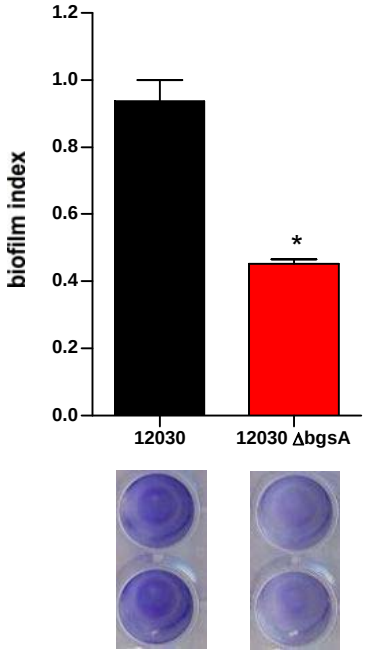


Fig. 4.28 Biofilm formation. *E. faecalis* strain 12030 and the *ΔbgsA* mutant were tested for biofilm production in medium with 1% glucose (TSBG). The amount of biofilm is expressed as the biofilm index. The mutant strain forms significantly less biofilm on plastic surfaces compared with the wild type strain (*p=0.22 with t test). Error bars represent average and SEM. The pictures below the graph are photographs taken from representative wells.

4.5.2. Time course of biofilm accumulation

To investigate whether the *E. faecalis* 12030*ΔbgsA* is impaired in initial adherence to plastic surfaces or in biofilm accumulation, the density of biofilm was monitored for both strains over time (2 to 24 h) as described in 3.2.6.2. The initial adherence in the first 2 h of incubation was comparable for the two enterococcal strains. In later stages, the wild type strain continued to form biofilm, while the *bgsA* mutant did not increase its biofilm beyond the initial level (Fig. 4.29).

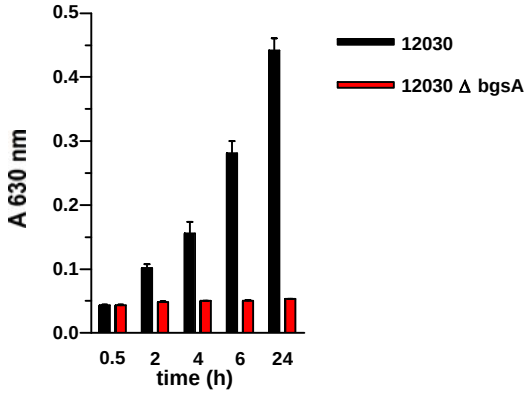


Fig. 4.29 Time course of biofilm accumulation. Development of biofilm on polystyrene of *E. faecalis* 12030 and 12030*ΔbgsA* was measured over time. After incubation periods of 4 h or more *E. faecalis* 12030 elaborated significantly more biofilm than the respective deletion mutant ($p < 0.001$, using unpaired, two-tailed t test). Bars represent means of 4 replicates and error bars the SEM.

4.5.3. Affinity to organic solvents

Affinity of bacteria to organic solvents is related to their physiochemical surface properties. Enterococcal strains were compared for their ability to associate with aqueous buffers or organic solvents after vigorous shaking as described (3.2.6.3).

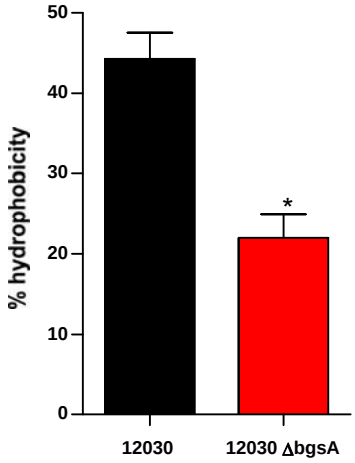


Fig. 4.30 Hydrophobicity of bacterial cells. Bars represent the percentage of bacteria remaining in the organic phase after partitioning of an aqueous buffer/dodecane solvent system. Significantly less mutant bacteria diffused in the organic phase (*p=0.0024 with two-tailed t test). Data represent the means of at least 4 determinations and error bars depict the SEM.

Compared to the wild type strain, significantly less *E. faecalis* 12030*ΔbgsA* could be detected in the organic phase (dodecane). These results correlate with the reduced ability to form biofilm on plastic surfaces suggesting a role of hydrophobic interaction in biofilm formation by enterococci (Fig. 4.30).

4.6. Adherence of the *bgsA* mutant to different cell lines

4.6.1. Adherence to colonic carcinoma cell line Caco2

Since adherence to plastic surfaces was impaired in *E. faecalis* 12030Δ*bgsA* we wondered, if interaction with eukaryotic cells may also be affected by the deletion of *bgsA* gene. Adherence to human colonic cells is thought to be the first step of enterococci to translocate across the gastrointestinal mucosa. We therefore measured adhesion of *E. faecalis* strains to Caco2 cells. In this cell culture model using a MOI of 100:1, the attachment of the 12030*ΔbgsA* mutant was 50.3% lower compared to the wild type (Fig. 4.31). Our results confirm the role of DGlcDAG in adhesion to colonic epithelial cells. Both strains were unable to invade the Caco2 cells in significant numbers at MOI 100:1.

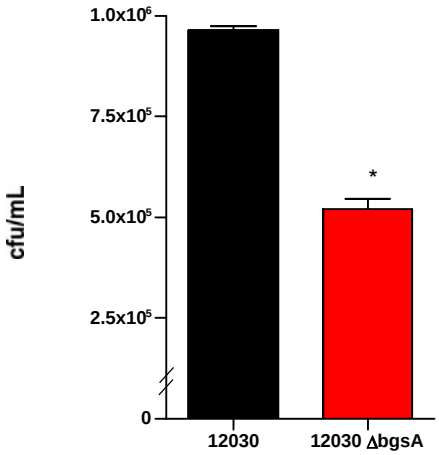


Fig. 4.31 Adherence to Caco2 cells of *E. faecalis* 12030 (black bars) and *ΔbgsA* (red bars). Caco2 cells were seeded in 24-well plates to a density of $1x10^5$ cells/well, cultivated to differentiate, and incubated for 2 h with bacteria grown to mid-log phase at a bacterium to cell ratio of 100:1. Significantly less mutant bacteria adhered to the cells monolayers (*$p<0.0001$ using two-tailed t test). Total cell-associated bacteria include surface-adherent and intracellular bacteria. Data represent the means and error bars the SEM.

4.6.2. Translocation across Caco2 monolayers

Enterococcal translocation across Caco2 monolayers was evaluated using a transwell system. In this model, neither strain was able to cross the epithelial barrier formed on the filters. None of the two strains were detected in the lower chamber after 2, 4 or 6 h of incubation with bacteria (Fig. 3.32) and TEER value did not change during the experiment. The enteroinvasive *Salmonella enterica* serovar Oranienburg was used as positive control and was recovered in large numbers from the basal chamber after 2 h of incubation; the number of bacteria translocating increased after longer incubation periods (360 min). Also,

a drop in the TEER value was observed during incubation of monolayers with *Salmonella enterica*, reflecting a disruption of the tight junctions (data not shown).

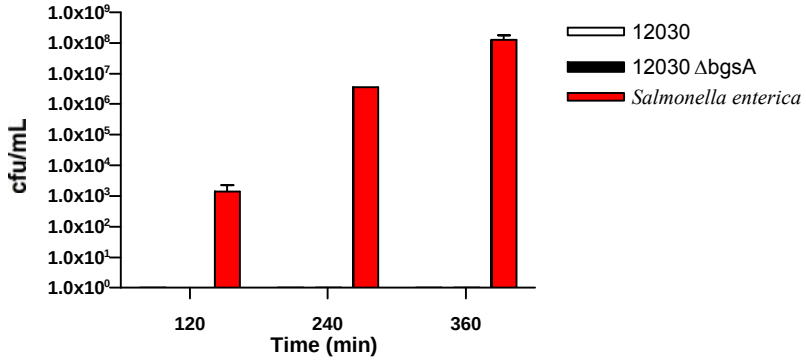


Fig. 4.32 Translocation of bacteria through Caco2 monolayers. *E. faecalis* strain 12030 and its *ΔbgsA* mutant were tested for their ability to translocate Caco2 monolayers cultivated in a transwell system. None of the two strains was able to translocate the epithelial cells, compared to *Salmonella enterica*, which was used as positive control.

4.6.3. Adherence to larynx carcinoma cell line Hep2

In a previous study of a mutant *E. faecalis* deficient in Alanination of the teichoic acids, the ability to form biofilm on inanimate surfaces correlated with adhesion to larynx carcinoma cells, (73).

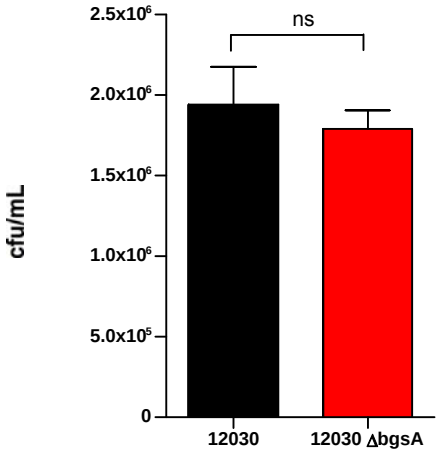


Fig. 4.33 Adherence to Hep2 cells of *E. faecalis* 12030 (black bar) and 12030*ΔbgsA* (red bar). Hep2 cells were cultivated in 24 well plates to a density of $1x10^5$ cells/well for 24 h and incubated for 2 h with bacteria grown to mid-log phase at a bacterium to cell ratio of 100:1. No significant difference ($p=0.58$ with two-tailed t test) in adhesion of the two enterococcal strains to Hep2 cells was observed. Data represent the means ± SEM.

In our case, no significant differences were found between the wild type strain and the mutant regarding their ability to adhere to Hep2 cells after 2 h of incubation at MOI 100:1,

suggesting that adhesion of *E. faecalis* to Hep2 cells is not mediated by interactions with glycolipids or LTA (Fig. 4.33).

4.7. Virulence in a mouse sepsis model

To study the persistence of strain 12030 and its Δ*bgsA* mutant in the bloodstream, mice were injected via the tail vein with an inoculum of $2x10^9$ cfu/animal as described (3.2.9.1) and sacrificed 72 h after the bacterial challenge. The high inoculum was chosen since lower infectious doses are cleared rapidly from the blood stream. A high enterococcal inoculum, on the other hand, causes bacteremia, which lasts several days, without being lethal to the animals. The bacterial counts in the blood of mice sacrificed at 72 h after infection were significantly lower for *E. faecalis* 12030*ΔbgsA* compared to wild type infection (Fig. 4.34).

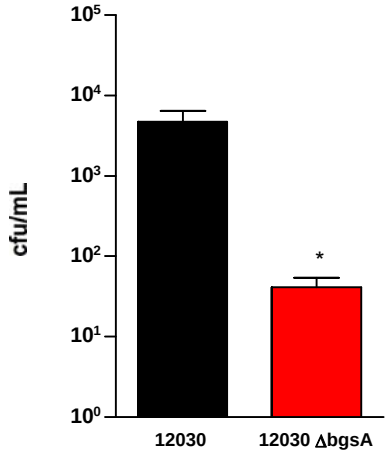


Fig. 4.34 Bacterial virulence in a mouse sepsis model. Bacterial counts were performed in the blood 72 h after i.v. challenge of 6 - 8 week old female BALB/c mice with *E. faecalis* strains (2 x 10^9 cfu/mouse) via the tail vein. Data represent the median bacterial counts and error bars the SEM. Significantly less bacteria were recovered from the blood of mice infected with the mutant strain (*$p < 0.001$ using ANOVA with Dunn's multiple comparison test).

4.8. Virulence in a mouse peritonitis model

4.8.1. Survival of Balb/c mice infected with enterococci

In vivo virulence was also evaluated in mouse peritonitis model. In a first approach, mice were injected i.p. with 5% mucin, as described by Knudsen et al. (117) followed after 2 h by a high bacterial inoculum. In contrast to the attenuation in virulence of *E. faecalis* 12030*ΔbgsA* shown in various *in vitro* (4.5.1 and 4.6.1) and *in vivo* models (4.7), we observed an *increased* mortality in mice infected with mutant *E. faecalis* 12030*ΔbgsA* in

the peritonitis model. In more detail, a highly significant mortality rate was observed in the group of mice infected with the *ΔbgsA* strain after 24 h (7 out of 8 mice in the Δ*bgsA* group compared to 1 out of 8 in the 12030 group). No animal died in the following 4 d. (Fig. 4.35A)

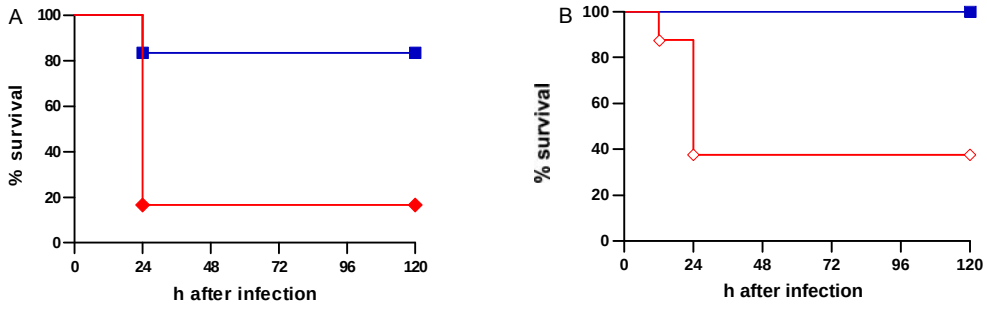


Fig. 4.35 Mouse peritonitis model. The virulence of *E. faecalis* 12030 (blue) and *ΔbgsA* (red) was compared in a mucin-enhanced peritonitis model (A) or in a peritonitis model where mucin was omitted (B). Female Balb/c mice were injected i.p. with 5% mucin (A) followed by $1.3x10^9$ cfu/mouse bacterial inoculum (A) or $1.4x10^9$ cfu/mouse (B). Mice were observed for survival for 5 d. A highly significant difference in survival between the two groups of mice was observed (significantly more mice survived in the group infected with the wild type bacteria; A: $p<0.027$ and B: $p<0.001$ using Logrank test).

In a next set of experiments, mucin was eliminated from the model and mice were injected i.p. only with the bacterial inoculum. Using approximately the same bacterial inoculum, mortality in the group infected with the mutant strain was lower, but still significantly higher than in the group infected with the wild type bacteria (five mice out of eight died in the *ΔbgsA* group and none in the 12030 group) (Fig. 4.35B).

4.8.2. Bacterial load in blood, kidneys and PLF of mice with *E. faecalis* peritonitis

To unravel the mechanism that underly the increased virulence of the *ΔbgsA* mutant in a mouse peritonitis model, animals infected i.p. were sacrificed after 3 h or 12 h and bacterial counts in blood, kidneys and PLF were determined (for methods see 3.2.9.3).

After 3 h, elevated bacterial counts were observed in blood, kidneys and PLF, but no significant differences between mice infected with the wild type and the mutant bacteria were detected.

After 12 h, the bacterial counts were in general slightly lower compared to 3 h, indicating that the host immune system started clearence of bacteria. Still, no difference between the bacterial counts of the two groups of mice could be detected. Hence, mortality is not likely

to be a result of an increased replication within the host organism or evasion of phagocytic killing by *E. faecalis* 12030*ΔbgsA* (Fig. 4.36-Fig. 4.38).

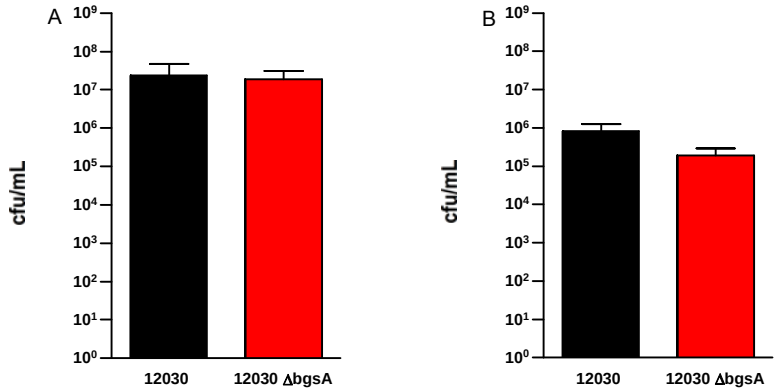


Fig. 4.36 Mouse peritonitis - bacterial loads in mouse blood. Animals infected i.p. with the wild type *E. faecalis* 12030 (10^9 cfu/animal) (black bars) or with the *ΔbgsA* mutant (10^9 cfu/animal) (red bars), without mucin, were sacrificed 3 h (A) or 12 h (B) after inoculation. Blood was obtained by cardiac puncture, plated and after 18 h the cfu were enumerated. No significant difference in the bacterial loads was observed in blood of the animals both after 3 or 12 h ($p>0.05$ using ANOVA with Kruskal-Wallis Test).

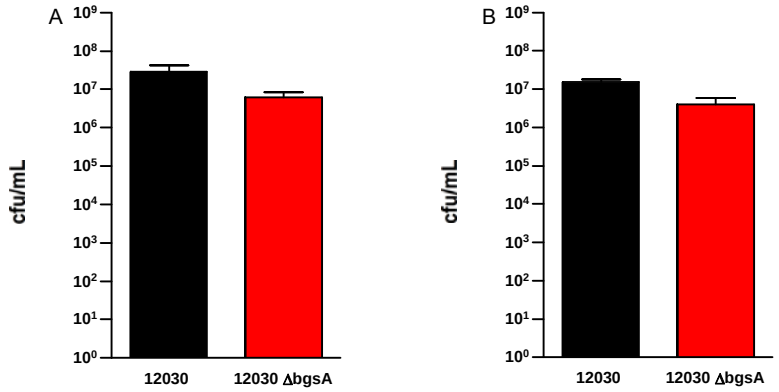


Fig. 4.37 Mouse peritonitis - bacterial loads in mouse kidneys. After infection i.p. with wild type *E. faecalis* 12030 (10^9 cfu/animal) (black bars) and the *ΔbgsA* (10^9 cfu/animal) (red bars) mutant, without mucin, animals were sacrificed after 3 h (A) or 12 h (B). Kidneys were harvested, homogenized, plated and after 18 h the cfu were enumerated. No significant difference in the bacterial loads was observed in kidneys of the animals both after 3 or 12 h ($p>0.05$ using ANOVA with Kruskal-Wallis Test).

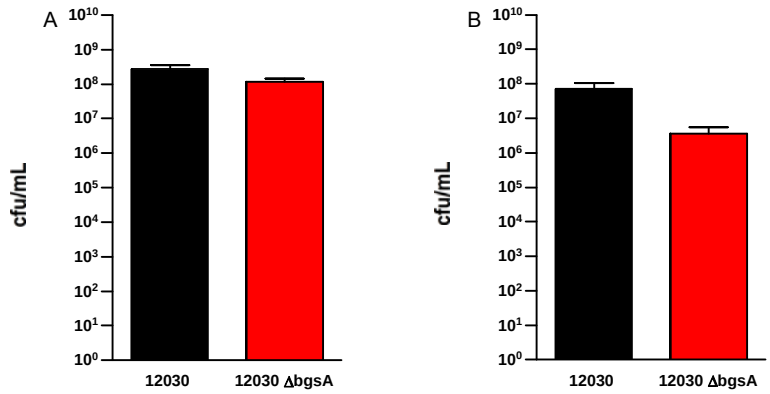


Fig. 4.38 Mouse peritonitis - bacterial loads in PLF. Mice infected i.p. with the wild type *E. faecalis* 12030 (10^9 cfu/animal) (black bars) and the *ΔbgsA* (10^9 cfu/animal) (red bars) mutant, without mucin, were sacrificed 3 h (A) or 12 h (B) after the infection. Peritoneal lavage was performed with sterile PBS, plated, and cfu were enumerated after 18 h. No significant difference in the bacterial loads was observed in PLF both after 3 or 12 h ($p>0.05$ using ANOVA with Kruskal-Wallis Test).

4.8.3. Total cell counts in PLF of mice with peritonitis

As previously demonstrated by Leendertse et al. (90), peritoneal macrophages play a crucial role in the first steps of enterococcal peritonitis in mice. To evaluate if increased mortality of mice infected with *E. faecalis* 12030*ΔbgsA* was mediated by increased peritoneal inflammation we determined the number of leukocytes in PLF 3 h or 12 h after infection

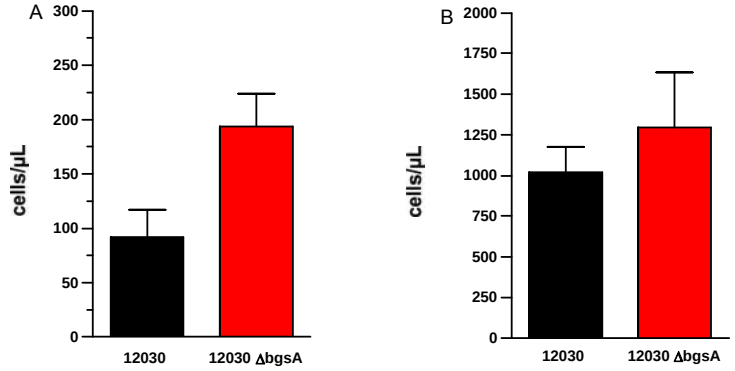


Fig. 4.39 Total cell counts in PLF. Cell count was performed from the PLF of Balb/c mice infected i.p. with strain 12030 (10^9 cfu/animal) (black bars) or with the *ΔbgsA* mutant (10^9 cfu/animal) (red bars) after 3 h (A) or after 12 h (B). Significantly more cells were present in PLF of mice infected with the mutant bacteria after 3 h (A, *$p=0.025$), while the difference in cell counts observed 12 h after the infection was not significant (B, $p>0.05$, using two-tailed t test).

4.8.4. Cytokine measurements in PLF

Due to differences in lethality of mice infected with the wild type and the mutant strain in the peritonitis model, several cytokines relevant for the inflammation-related immune response were measured by ELISA, as described in 3.2.5.1.

Previous studies (108) indicated that cytokine expression levels in bacterial peritonitis due to enterococci are reaching a peak in the first 2-6 h after infection. Therefore levels of TNF-α, IFN-γ and IL-4 in the PLF of mice were measured 3 and 12 h after the induction of peritonitis with enterococci.

Three h after induction of peritonitis, both TNF-α and IL-4 levels are significantly elevated in the mice infected with the mutant strain (Fig. 4.39A and C and 4.40A and C), while IFN-γ level was low both 3 and 12 h after infection in both groups of animals (Fig. 4.39 B and Fig. 4.40 B).

The early production of TNF-α was followed by a decreased level of this cytokine after 12 h in the group of mice infected with the *ΔbgsA* mutant and a delayed production of TNF-α by the mice infected with the wild type strain (Fig. 4.39A.and Fig. 4.40A). After 12 h, both groups of mice displayed low levels of IL-4 (Fig. 4.39 C and Fig. 4.40 C).

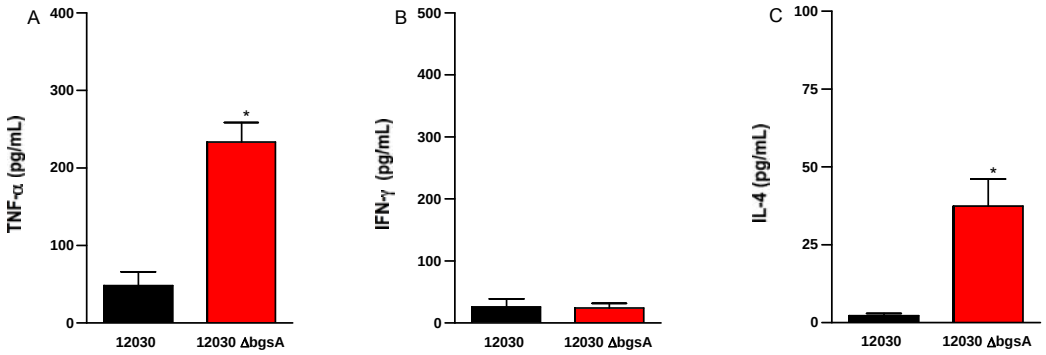


Fig. 4.40 Cytokine levels in PLF after 3 h. Groups of 6-8 Balb/c mice infected with strain 12030 (black bars) or *ΔbgsA* (red bars) were sacrificed after 3 h and TNF-α (A), IFN-γ (B) or IL-4 (C) levels were measured by ELISA. High levels of TNF-α (*p=0.022) and IL-4 (*p=0.0023) were detected in PLF of mice infected with mutant bacteria while the levels of IFN-γ measured were low and not different between groups.

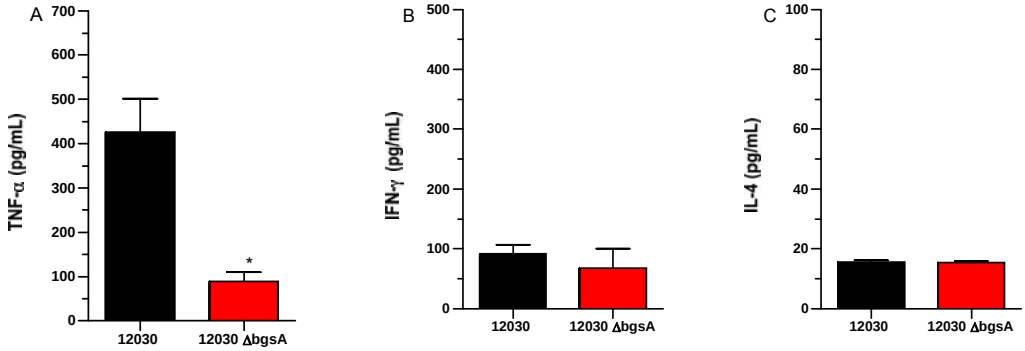


Fig. 4.41 Cytokine levels in PLF after 12 h. Groups of 6-8 Balb/c mice infected with strain 12030 (black bars) or *ΔbgsA* (red bars) were sacrificed after 12 h and TNF-α (A), IFN-γ (B) or IL-4 (C) levels were measured by ELISA. High levels of TNF-α (*p=0.022) were detected in PLF of mice infected with wild type bacteria, while the levels of IFN-γ and IL-4 measured were low and not different between different groups of animals.

4.8.5. Survival of Balb/c and CD1d-/- mice infected with *E. faecalis* strain 12030 and the *ΔbgsA* mutant

Inactivation of the glycosyltransferase responsible for the synthesis of DGlcDAG leads to accumulation of large amounts of MGlcDAG in the membrane of the *ΔbgsA* mutant. The structure of this glycolipid resembles the strucure of α-galactosyl diacylglycerol isolated from *Borrelia burgdorferi*, described as being a strong stimulator of the host invariant natural killer T (*i*NKT) cells (118). *i*NKT cells bear an antigen receptor - T cell antigen receptor (TCR) which is able to recognize glycolipids presented by cluster of differentiation (CD) 1 molecules on antigen-presenting cells. The presence of higher amounts of MGlcDAG in the membrane of the *ΔbgsA* mutant might explain the high mortality, the early cytokines response, and the cell influx into the peritoneum.

CD1d-/- mutant mice lack *i*NKT cells as well as some other CD1-reactive T cells (119), (120). They were used to investigate whether MGlcDAG recognition by the *i*NKT cells might be responsible for some or all the effects previously observed (4.8.1-4.8.4). Therefore, wild type Balb/c mice and CD1d-/- mice created in a Balb/c background (a gift from Dr. F. Loewenich, Institut für Medizinische Mikrobiologie, Uniklinik Freiburg) were infected i.p. with either the wild type strain or the *ΔbgsA* mutant and observed for lethality for 7 d. Mice infected with the wild type bacteria did not show any significant difference in lethality (5 out of 8 Balb/c mice and 5 out of 7 CD1d-/- mice died after 48 h) (Fig. 4.42).

A significant difference in lethality of the 2 groups of mice infected with the mutant bacteria was observed (all 8 Balb/c mice and 4 out of 7 CD1d-/- mice died after 96 h), indicating that mice lacking the *i*NKT cells are probably partially protected against hyper-inflammation produced via the MGlcDAG molecule (Fig. 4.43).

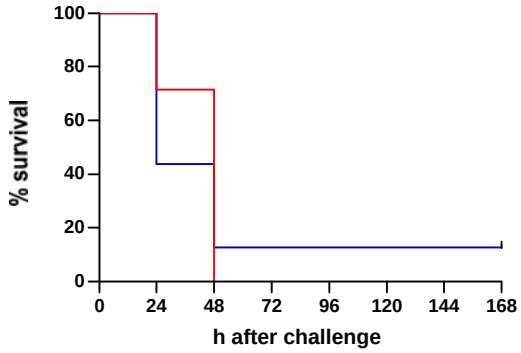


Fig. 4.42 Survival of Balb/c and CD1d-/- mice. Balb/c (blue line) and CD1d-/- (red line) mice generated in a Balb/c background were infected i.p. with 1.9×10^9 wild-type bacteria/animal and observed for 7 d. No statistical difference in mortality between the two groups of mice was observed (p=0.69 using Logrank test).

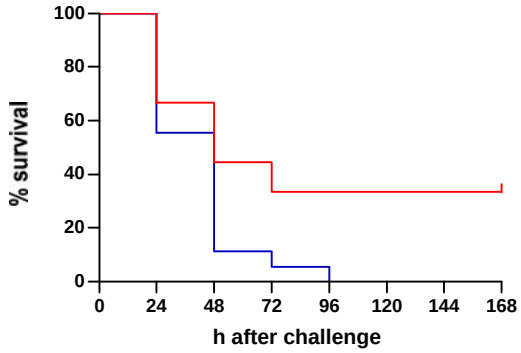


Fig. 4.43 Survival of Balb/c and CD1d-/- mice. Balb/c (blue line) and CD1d-/- (red line) mice generated in a Balb/c background were infected i.p. with 1.2×10^9 *ΔbgsA* bacteria/animal and observed for 7 d. CD1d-/- mice were partially protected against hypervirulence of the mutant strain (p=0.017 using Logrank test).

5. Discussion

The focus of this thesis was to analyze the role of amphiphilic cell wall glycoconjugates in *E. faecalis* virulence using different *in vitro* and *in vivo* models. In clinically relevant Gram-positive organisms, cell wall carbohydrates have been previously shown to be involved in different aspects of pathogenicity, such as attachment to plastic surfaces (73), adhesion to eukaryotic cells (121), (122), sensitivity to antimicrobial agents, [reviewed by Neuhaus et al.(81)] or modulation of the immune system (123). In the present study, these aspects have been investigated with the main focus on enterococcal cell wall glycolipids and LTA. We were able to show that these molecules participate in complex interactions with the host, involving adherence to animate and inanimate surfaces as well as recognition by the immune system of the host.

5.1. Mechanisms of adhesion of *E. faecalis* to the colonic epithelium

Adhesion to eukaryotic cells is considered to be the first step for many bacterial pathogens in establishing infections. As shown in the RESULTS section, proteoglycans were investigated for their role as receptors for enterococcal adhesion to cells and we could demonstrate that heparin and HS mediate binding of *E. faecalis* strain 12030 to colonic epithelial cells. Also, we investigated DGlcDAG either itself or as a partial structure of LTA, for its role in mediating bacterial binding to enterocytes by recognizing heparin and/or HS on the surface of the cells.

Proteoglycans are abundant components of the extracellular space and cell surface of eukaryotic cells (124), (125). They occur as heteroproteins to which GAG chains are linked covalently. Chains of GAGs consist of 50 to 1000 disaccharide units formed of uronic acids (glucuronic or iduronic) and aminosugars (*N*-acetyl-glucose or galactosamine). The proportions of uronic acids to aminosugars represent distinct traits of specific tissue and species (126). During coevolution of pathogens and their respective hosts, bacteria learned to exploit cell surface GAGs as receptors for attachment. A large body of literature supports the role of host GAGs as receptor molecules for bacteria, parasites and viruses [reviewed by Rostand et al. (85)]. The relatively low affinity of GAGs, their prominent extracellular location and ubiquitous presence in all mammalian organisms make them an ideal structure used by pathogens to adhere to host tissues. Many pathogenic bacteria establish infections after adhering to host surfaces by means of GAGs.

It has been shown in previous studies that enterococci, like other bacteria, use host GAGs to adhere to macrophages or epithelial colonic cells (88), (87).

An important goal of our study was to elucidate the role of GAGs as specific receptors for *E. faecalis* on cultured enterocytes. We used Caco2 cells as a useful tool to study bacterial adhesion. However, it is important to point out that only this cell line and six *E. faecalis* strains were evaluated. Therefore, additional studies are needed before the results presented here may be generalized to define the properties of the whole species. In this study we showed that the strength of interaction between enterococcal cells and GAGs is highly dependent on their molecular structure with heparin and HS having the highest affinity for *E. faecalis*. The inhibitors used and their concentrations were selected based on previously published work (88). When we used exogenous heparin or HS purified from porcine intestinal mucosa as inhibitors of the bacterial binding, both were able to partially block adhesion of *E. faecalis* strain 12030 to Caco2 cells. Several studies indicate that these two GAGs are widely expressed on epithelia of mammalian cells and are structurally similar, but HS has a far more variable composition with fewer *N*- and *O*-sulfate groups and more *N*-acetyl groups (126), which may explain the small differences observed when we used these two molecules as inhibitors.

Although we obtained only a partial inhibition of the bacterial adhesion using heparin and HS, this was in accordance with results obtained by other groups investigating the role of GAGs in binding of *Staphylococcus* spp. (127) (highest inhibition about 60%) or *Listeria monocytogenes* (128) (46-57% inhibition). We believe that this amount of inhibition suggests that additional mechanisms and other receptors are involved in bacterial binding to mammalian tissues. Rostand and coworkers postulated that GAGs mediate an initial interaction between pathogens and host cells, and that this interaction seems to be relatively weak (85). In contrast to heparin and HS, by using CSB we were not able to inhibit enterococcal adhesion, indicating that bacterial ligands recognize specifically a particular class of GAGs structures. Baldassarri and coworkers showed that heparin, HS and CS act as the host receptors for enterococci on phagocytes (88). Our data corroborate to some extent the findings of Baldassarri et al. (88). In contrast to their results, however, we did not see any inhibition by CS in our experiments. These differences may be explained by the fact that we used a different cell line derived from colon carcinoma while Baldassarri and coworkers used professional and nonprofessional phagocytes. These

results may point towards a difference in target structures that may explain the tropism observed with different bacterial species.

To further confirm these findings, we preincubated Caco2 cells with Heparinase I from *Flavobacteruim heparinum* (which selectively cleaves heparin and HS chains at the linkage between hexosamines and *O*-sulfated iduronic acids) and then infected them with *E. faecalis* strain 12030. Enzymatic degradation of heparin and HS chains reduced bacterial adhesion in a concentration dependant manner by 25 to 52.5%. When chondroitinase ABC from *Proteus vulgaris* (which removes CS and dermatan sulfate side-chains) was used to remove CS residues, this treatment had no or very little effect on bacterial adhesion, confirming that CS species do not mediate the interaction between enterococci and Caco2 cells.

In recent years, numerous HS biosynthetic enzymes, which are involved in the modification of the HS chain, have been identified. These modifications include 2-*O*-sulfation of iduronic and glucuronic acid, *N*-sulfation of glucosamine, as well as 6-*O*-sulfation and 3-*O*-sulfation of glucosamine (129). Here we provide evidence that *N*-sulfation and 2-*O*- but not 6-*O*-sulfation are required for recognition of enterococci by heparin. This conclusion is supported by the observations that 6-*O*-desulfated heparin was able to impair bacterial binding to the same amount as unmodified heparin, while 2-*O*-desulfated heparin as well as *N*-desulfated heparin partially lost their inhibitory activity (21% and 23% inhibition compared to 58% inhibition of native heparin). These findings support the conclusion that specific sulfate groups on cellular heparin sulfate rather than the total level of sulfation may be important for mediating interactions between enterococci and host cells. Interestingly, the sulfation pattern required for inhibition of enterococcal binding was different from the sulfation pattern mediating hepatitis C virus envelope glycoprotein - HS interaction (130) or *Chlamydia trachomatis* - HeLa 229 cells interaction (131). These findings further confirmed us the specificity of interaction between eukaryotic GAGs chains and enterococal cells.

We also investigated the role of GAGs sulfation in bacteria-host cells interaction by treatment of Caco2 monolayers with sodium chlorate, which depletes the GAGs of their sulfuric groups. Our results support the role of sulfate groups in bacterial recognition of the GAGs. It is noteworthy to mention in this context that heparan sulfate proteoglycans present on differentiated Caco2 cells are among the highest sulfated species (132). The same approach was used to investigate whether utilization of GAGs as receptors for

enterococcal binding is strain-specific. Since adhesion of three out of six *E. faecalis* strains tested could be inhibited by sodium chlorate treatment of the enterocytes, we conclude that, although proteoglycans may not act as receptor for all the enterococcal strains, this mechanism is not a unique feature of only a single *E. faecalis* strain (*E. faecalis* 12030). It is tempting to speculate that the property of a given strain to adhere to the GI epithelium may differentiate colonizing strains from pathogenic strains involved in other infections (i.e. UTI, endocarditis, etc.). Further studies comparing strains isolated from different infection sites are necessary to confirm such a hypothesis.

Our hypothesis of the involvement of GAGs in end-organ recognition by Gram-positive bacteria is supported by a recent publication (133). In the study of Shi et al. GAGs extracted from various rat organs differed significantly regarding chains length and the degree of sulfation. It has been postulated that specific recognition of GAG patterns present in different organs plays an important role in tropism of bacteria. The specific expression of GAGs at different sites within a host could explain why enterococci are able to infect some organs and not others. For example, *E. faecalis* is a well-established pathogen in UTI and endocarditis while infections of the respiratory tract are exceedingly rare. As demonstrated for other pathogen-GAGs interactions, the carbohydrate chains do not serve as the sole receptor for host-pathogen interaction, but rather as primary receptor to facilitate access to a specific organ (134).

The binding partners for GAGs on the bacterial surface have been characterized for some bacterial species, while others are still controversial. All the known binding ligands of proteoglycans described to date are surface proteins. For example, Baron and colleagues found that alpha-C protein of *Streptococcus agalactiae* binds to proteoglycans on the ME180 cervical epithelial cell line (135). In *Streptococcus pyogenes,* Frick and colleagues showed that there is a strong correlation between M protein expression and the ability of the bacteria to bind to GAGs, especially dermatan sulfate (136). A different protein, ActA, has been shown to be involved in attachment of *Listeria monocytogenes* to host cell heparin and HS (128).

To our knowledge, no molecular structures involved in adhesion to GAGs have been identified so far in enterococci. Since all the receptors described in other Gram-positive bacteria are proteins, our first approach was to determine whether digestion of cell wall associated proteins would affect adhesion to eukaryotic cells. Surprisingly, adhesion of proteinase-treated bacteria was significantly higher compared to native bacteria. This

observation confirms several previous studies showing that enterococcal adherence was enhanced by proteolytic digestion of whole bacterial cells. Zareba and coworkers showed that proteinase treatment of enterococci increased adhesion to extracellular matrix proteins such as lactoferrin and vitronectin (137). In a more recent study, the adhesive properties of a clinical *E. faecalis* isolate to Int-407 cells (derived from human jejunum and ileum) and Girardi heart cell line were investigated (138). In this study, enterococcal clinical isolates also bound more avidly to Int-407 cells after treatment with trypsin (1.4 to 11-fold more compared to untreated bacteria). In both reports, the exact nature of enterococcal adhesins was not determined, but the authors speculated that bacterial components might become surface-exposed after treatment with proteinases. Our data confirmed also a study that investigated the role of heparan sulfate proteoglycans in bacterial translocation across the gut epithelial barrier (87). In this publication, enterococci - in contrast to *Listeria* spp., streptococci, and staphylococci - were the only bacterial species unable to express heparin-binding proteins.

To further investigate host-pathogen interactions, we preincubated *E. faecalis* 12030 with sodium meta-periodate, which oxidizes and thereby denatures carbohydrates. These bacteria were used in adhesion assays, and untreated enterococci served as negative control. In this experimental setting, carbohydrate oxidation decreased enterococcal binding to cells in a dose-dependent manner without affecting bacterial viability. From these results we concluded that sugar-containing molecules rather than proteins seem to mediate adhesion of enterococci to Caco2 cells.

There are only few examples of glycoconjugates mediating adhesion of low GC Gram-positive bacteria to eukaryotic cells. Guzman and colleagues (139) treated *E. faecalis* isolates with sodium meta-periodate and showed that adherence to human cells is mediated by carbohydrate residues on the bacterial surface. They also showed that the adhesins involved in adherence to urinary tract epithelial cells contain D-mannose and/or D-glucose, while those involved in adherence to Girardi heart cells also contain D-galactose and L-fucose (139). Beachey and colleagues studied more than three decades ago the adhesion of group A streptococci to different host cells and found that LTA takes part in a two step process of adhesion (122). The role of LTA in invasion of brain microvascular endothelial cells by group B streptococci was studied by Doran et al. using a deletion mutant in the *iagA* gene. IagA is a glycosyltransferase responsible for the synthesis of DGlcDAG, the cell surface anchor of LTA. According to the study of Doran, the anchoring of LTA is

critical for bacterial invasion in the brain microvascular endothelial cells (115). WTA, another major component on the surface of Gram-positive bacteria, was shown to play an important role in *Staphylococcus aureus* binding to rat nasal epithelial cells and human endothelial cells, although the structures on host cells involved in adhesion remain unidentified (140), (121). In an other study it was suggested that WTA enhances staphylococcal adhesion to extracellular matrix proteins indirectly by a bridging mechanism (141).

Based on the above-mentioned previous findings, in our study we used highly purified LTA and WTA isolated from *E. faecalis* strain 12030 at various concentrations to block adhesion of the homologous strain to Caco2 cells. However, none of the two polymers was able to inhibit enterococcal binding to colonic epithelial cells even at high concentrations.

LTA is inserted into the cell membrane by a glycolipid anchor, DGlcDAG. Non-substitued DGlcDAG can also be found as a component of the cell membrane and represents the most abundant polar lipid (37%) in *Enterococcus* spp. (112). To examine the role of glycolipids in bacterial adhesion, we extracted the total polar lipids from *E. faecalis* strain 12030 using the Bligh/Dyer method and applied them as inhibitors of attachment to Caco2 cells. Caco2 cells preincubation with enterococcal polar lipids inhibited bacterial attachment to the eukaryotic cells in a dose-dependent manner. Also, when we treated this lipid mixture with sodium meta-periodate, which oxidizes the sugar residues, it lost the inhibitory activity, suggesting that a carbohydrate-containing molecule mediates adhesion of enterococci to colonic cells. To gain a better understanding of this interaction, we purified the two most important glycolipids from the enterococcal membrane, MGlcDAG and DGlcDAG, by preparative TLC and used them as inhibitors of bacterial binding to colonic cells. Our experiments show that only DGlcDAG, but not MGlcDAG (which differs only by the lack of a terminal α-D-glucose) inhibited bacterial binding.

Using DGlcDAG, we could decrease bacterial binding to colonic epithelial cells and we also demonstrated that enterococci use highly sulfated heparan sulfate proteoglycans as receptors on the host cells. Next, we wanted to assess whether these molecules act as co-receptors and we were able to show that cells treated with sodium chlorate, with bacterial polar lipids or with a combination of both compounds showed the same amount of inhibition (about 50%) as cells treated with only one of the components. From these results we conclude bacterial adhesion to host cells might be mediated by interaction between heparan sulfate proteoglycans and DGlcDAG.

So far, however, our evidence to support this hypothesis was only indirect. To measure direct interaction between the two molecules we employed SPR. The SPR technique uses an optical method to measure the change of the refractive index. The change of the refractive index is a result of the binding between the two analyzed molecules (see MATERIALS AND METHODS). Using this experimental approach, a high affinity interaction of heparin was measured for DGlcDAG while for MGlcDAG no detectable binding was observed. As for the binding of DGlcDAG to different GAGs, high affinity binding was observed to heparin and HS, which represent the most sulfated GAGs present on eukaryotic cells and show the highest inhibitory effect. No specific binding was observed between various types of CS and DGlcDAG, confirming our findings from the inhibition experiments using Caco2 cells. The K_D value of the binding between heparin and DGlcDAG was estimated to be around 60 μM. Compared to other biological relevant protein-protein interactions, this K_D value is relatively small. Similar K_D values in the micromolar range have been also measured, however, for other microbial carbohydrate molecules, e.g. for *Candida* spp. glycolipids binding to immunoglobulins G and M (142).

Because of the spatial arrangement of the cell wall, a direct interaction of membrane-bound enterococcal glycolipids with host GAGs is difficult to envision. Both glycolipids and LTA, however, are shed permanently from the cells during growth and turnover of the bacterial cell wall (143). It has been demonstrated that the LTA molecule becomes reoriented on the bacterial surface, exposing its glycolipid anchor towards the outside of the bacterial cell and enabling binding to host cells via its lipid moiety (144). The work of Beachey et al. confirms the reorientation of LTA suggesting that LTA molecule inserts its glycolipid anchor into eukaryotic membranes (145, 146). We therefore hypothesize that LTA shed from planktonic bacteria is inserted into the eukaryotic membrane and interacts with GAGs through the kojibiose residue of the DGlcDAG anchor. The polyglycerolphosphate moiety, which now extends towards the interface between host cells and pathogens, probably binds to a bacterial cell surface receptor, which yet needs to be identified. A similar model was suggested for *Streptococcus pyogenes*: in this case the molecule responsible for stabilizing the polyglycerolphosphate chain at the bacterial surface was shown to be the M protein (143). The model is supported by our experimental findings because i) saturating the LTA-binding sites on the eukaryotic cell by preincubation with DGlcDAG, a bridging by reoriented LTA would be blocked; ii) using the LTA molecule for preincubation no such inhibition would be achieved due to the

additional presence polyglycerolphosphate in the inhibitor, the putative binding moiety to the bacterial cell surface receptor.

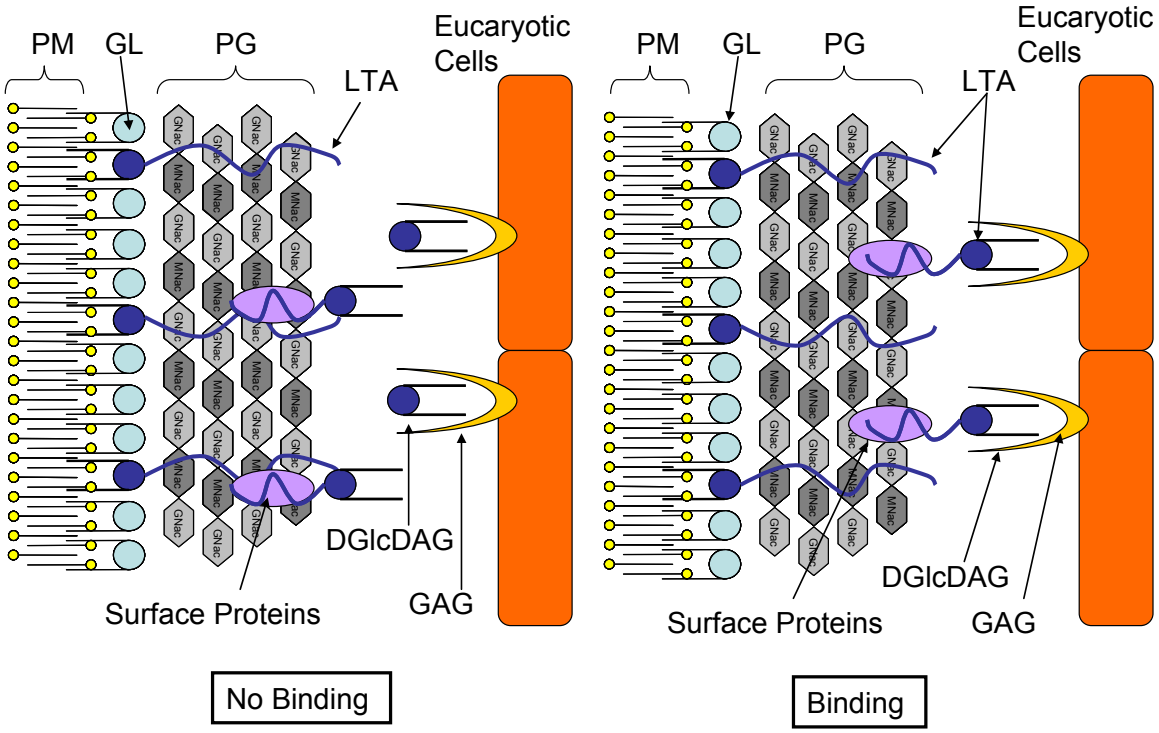


Fig. 5.1 Hypothetical mechanism of *E. faecalis* binding to GAGs on the eukaryotic cells using the DGlcDAG anchor of the LTA (PM - plasma membrane, GL - glycolipids, PG – peptidoglycan, GNa - *N*-acetylglucosamine, MNa - *N*-acetylmuramic acid).

5.2. Role of enterococcal membrane glycolipids in biofilm formation and virulence *in vivo*

Since our experiments suggested that enterococcal glycolipids are involved in bacterial attachment to host tissues, we were interested how defects on bacterial glycolipid synthesis may affect bacterial physiology and virulence. To address these questions, we evaluated a deletion mutant of *bgsA* (biofilm-associated glycolipid synthesis) in *E. faecalis* strain 12030. *bgsA* encodes for a putative glycosyltransferase (TIGR number EF_2891) that shares sequence identity with AL*dgs* gene from *Acholeplasma laidlawii* (48%) and *cpoA* gene from *Streptococcus pneumoniae* (28%) (91). This glycosyltransferase catalyzes the glycoslysation of MGlcDAG with D-glucose (*Acholeplasma laidlawii*) or D-galactose (*Streptococcus pneumoniae*) from UDP-glucose or UDP-galactose to yield DGlcDAG (114) (Fig. 5.2A). *iagA*, the homolog of *bgsA* in *Streptococcus agalactiae* also shares high identity (55%) with the *bgsA* gene from *E. faecalis*. A *ΔiagA* mutant failed to produce DGlcDAG, synthesizing only MGlcDAG (115). A second gene located immediately downstream of *bgsA*, *bgsB* is encoding for an enzyme that catalyzes the transfer of D-

glucose from UDP-glucose to DAG to form MGlcDAG (Theilacker et al., manuscript submitted).

Interestingly, the glycosyltransferases required for the biosynthesis of DGlcDAG in *Bacillus subtilis* and *Staphylococcus aureus* are encoded by a different gene, *ypfP,* which seems to be the only diglycosyldiacylglycerol synthase catalyzing the synthesis of DGlcDAG in these species. YpfP is an enzyme with a lower substrate specificity that utilizes both DAG and MGlcDAG as sugar acceptors for UDP-glucose to produce DGlcDAG (147), (148), (116), (106) (Fig. 5.2B).

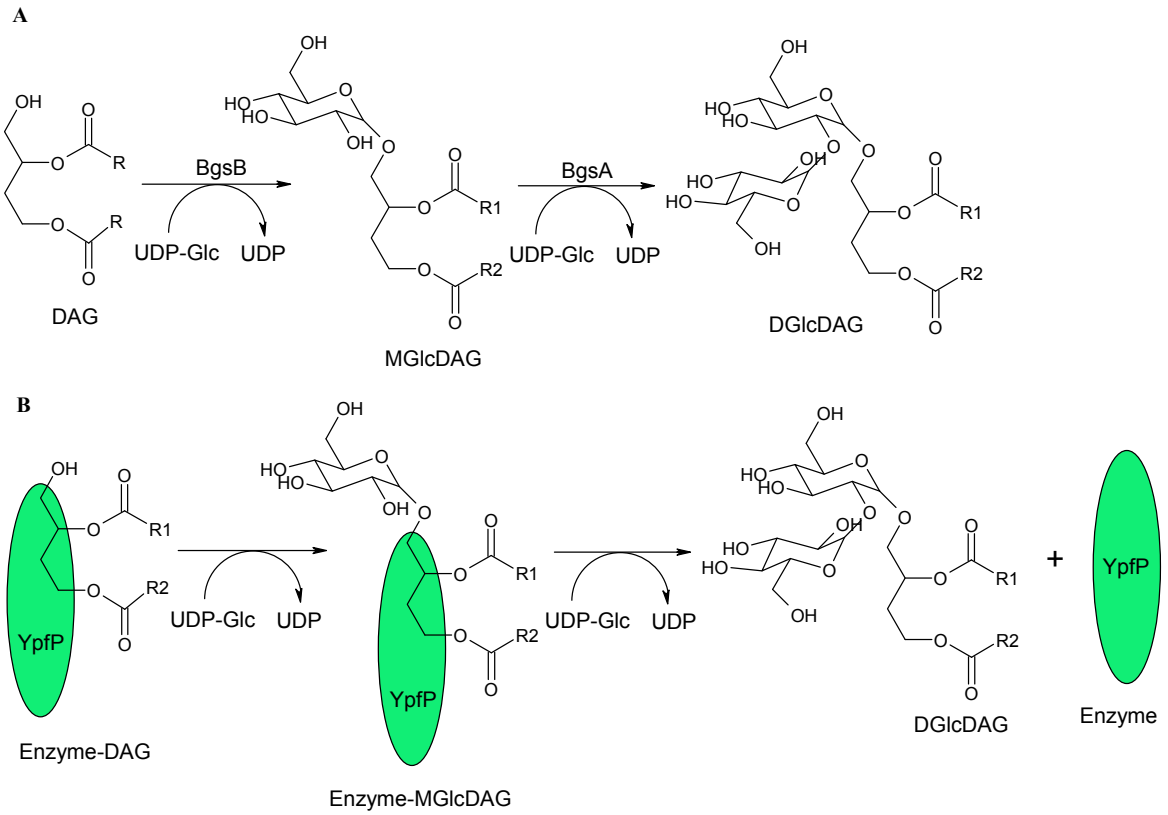


Fig. 5.2 Hypothetical biosynthetic pathways mediated by the glycosyltransferases BgsA, BgsB (in *E. faecalis*, A) and YpfP (in *Staphylococcus aureus*, B)

5.2.1. Structural and morphological modifications due to deletion of the *bgsA* gene

To examine the phenotype of *E. faecalis* strain 12030*ΔbgsA* in regard to its changed glycolipid expression profile, we compared glycolipid contents in the wild type strain and in the mutant after extraction according to Bligh/Dyer, subsequent TLC and staining with α-naphtol, which stains specifically the carbohydrates. The cell wall of strain 12030*ΔbgsA* contained no DGlcDAG, while its putative precursor, MGlcDAG was overexpressed. Our findings suggest that BgsA catalyzes the glycosylation of MGlcDAG to form DGlcDAG, the same function ascribed to its homologue in *Acholeplasma laidlawii* (114). Besides DGlcDAG, the wild type strain contained also minor amounts of MGlcDAG confirming Fischer's findings that a small proportion of the polar lipids of the cell membrane in *E. faecalis* are represented by MGlcDAG (112), while the predominant species is the DGlcDAG.

Western blot analysis of surface associated antigens from the wild type and the mutant strain revealed us two major changes in the phenotype of the mutant 12030*ΔbgsA*. A low molecular mass band representing probably DGlcDAG was recognized by serum raised against whole bacteria. This band was present in the wild type strain but was absent in the mutant strain. Another phenotypic change observed in the immunoblot is the lower mobility of the LTA of the mutant strain, a phenomenon observed also in an *ypfP* mutant created by Gründling and colleagues in *Staphylococcus aureus* (116). Taken together, these results suggest that the inactivation of *bgsA* as well as *ypfP* leads to a change in structure of LTA produced by the mutant bacteria.

To examine the impact of the deletion of *bgsA* on the cell shape of *E. faecalis*, we employed SEM as well as phase contrast microscopy (data not shown). While transmission electron microscopy did not show major alteration of the cell size or shape for the mutant (91), visualisation by SEM revealed a higher variability in cell size compared to the wild type bacterial cells. Aberrant cells have been observed before, when mutants in the *ypfP* gene of *Staphylococcus aureus* and *Bacillus subtilis* were examined for their morphology (147), (148). In the *ypfP* mutants, evidence suggests that defective glycolipid synthesis may affect cell wall integrity. For *E. faecalis* 12030*ΔbgsA,* we did not observe significant alterations in the growth rate or osmotic resistance (data not shown).

As stated above, our results suggest that BgsA in *E. faecalis* has the same function as alDGS from *Acholeplasma laidlawii*. The role of the alDGS was studied in depth by the group of Wislander (149), (114). According to their work, membrane elasticity and porosity is determined by the ratio of the bilayer-forming glycolipid DGlcDAG and the non-bilayer forming glycolipid MGlcDAG. As opposed to DGlcDAG, which is the major bilayer-forming glycolipid in *Acholeplasma laidlawii*, increasing concentration of MGlcDAG makes the two membrane lipid monolayers to curl concavely toward the water phase. Since the ratio of both glycolipids is critical for membrane physiology, bacterial division and growth, the enzyme activity of alDGS is highly regulated in *Acholeplasma* spp. (114). From our knowledge of the function of membrane glycolipids in *Acholeplasma laidlawi*, one would expect that the loss of DGlcDAG in *E. faecalis* strain 12030*ΔbgsA* would have a profound effect on membrane physiology of this deletion mutant. Except for minor changes such as pleomorphic and size-increased bacterial cells, we did not observe a major change in the shape of the *ΔbgsA* deletion mutant.

5.2.2. Biofilm formation and affinity to organic solvents

Formation of multicellular communities of bacteria may be seen as an adaptive mechanism and also considered a virulence factor in both Gram-positive and Gram-negative pathogens. The most important Gram-positive nosocomial pathogens, staphylococci and enterococci, are well known for their ability to produce biofilm. Several enterococcal infections such as endocarditis or foreign body infections are associated with biofilm formation. The formation and maturation of a biofilm involves several steps, including initial adherence to foreign body surfaces, accumulation, and matrix formation and finally, detachment and dissemination to new body compartments (150). Following attachment and biofilm development bacteria might undergo phenotypic shifts such as reduced cell division and increased resistance to antibiotics (151) or to opsonic killing *in vitro* (152). In recent years, a large number of assays and experimental techniques have been developed to examine biofilm formation. Most studies, however, rely on a microtiterplate assay. Hydrophobicity and roughness of the microtiter plate surface are recognized as important determinants for bacterial adhesion and biofilm production in this assay.

Hydrophilic surface-exposed polymers such as LTA influence the physico-chemical properties of the bacterial cell surface and in consequence, bacterial co-aggregation and adherence to foreign bodies. D-Alanine esterification of LTA is a means of bacterial cells to modulate the properties of the cell envelope according to its current needs. Alanine

residues are positively charged and their addition to teichoic acids reduces the negative net surface charge of this polymer. Availability of mutants defective in D-alanylation of teichoic acids have demonstrated a critical role of this chemical substitution on biofilm formation. Mutants lacking the D-Alanine carrier protein ligase gene (*dltA*) impaired in biofilm formation were generated in a number of Gram-positive pathogens including *Staphylococcus aureus* (153) and in *E. faecalis* (73). The *dltA* mutant created by Gross and colleagues in *Staphylococcus aureus* was highly compromised in biofilm formation on glass and polystyrene surfaces. Deletion of the *dltA* gene in *E. faecalis* strain 12030 resulted in a reduced ability to form biofilm on plastic surfaces (50% less biofilm compared to the wild type strain).

To investigate the role of the *bgsA* deletion in bacterial adhesion to plastic surfaces, a microtiter plate assay followed by staining with crystal violet was employed. According to results of our study, deletion of the *bgsA* gene in the same strain background led to an even more drastic decrease in biofilm formation. A more detailed analysis of the process of biofilm formation revealed, that initial adherence to polystyrene microtiter plates of the 12030*ΔbgsA* strain was not affected. Instead, mutant bacteria failed to accumulate in the growing biofilm. This is a phenomenon which requires further investigations, since *bgsA* seems to be the first gene in *E. faecalis* discovered so far that is involved not in adherence, but accumulation of a mature biofilm (91). In *Bacillus subtilis,* mutations in the synthetic pathway of UDP-glucose, the substrate of the glycolipid synthetase YpfP also led to a decrease in biofilm formation (148). Similar to *E. faecalis*, the absence of YpfP in *Staphylococcus aureus* strain SA113 resulted in a highly impaired ability to form biofilm (106). In the *Staphylococcus aureus ypfP* mutant, impairment of biofilm formation was correlated with increased hydrophobicity of the mutant. This is in contrast with our findings, which demonstrate reduced hydrophobicity in the *E. faecalis* 12030*ΔbgsA* mutant. Possibly this phenomenon is related to the difference in LTA-content of the cell wall of mutant *Staphylococcus aureus ypfP* and *E. faecalis bgsA*. According to the findings of Fedkte and coworkers (154), the SA113 *ypfP* mutant has a strongly reduced LTA content, while deletion of *bgsA* in *E. faecalis* resulted in a 3.3 fold increase in cell wall LTA (data not shown). Hydrophobic interactions have been shown to be of major importance in biofilm formation and reduced hydrophobicity, as shown by the lower affinity for organic solvents (dodecane) is a possible mechanism for the impaired biofilm production of *E. faecalis* 12030*ΔbgsA*.

5.2.3. Impact of the *bgsA* mutation on the interaction with the host: *in vitro* and *in vivo* models

Previous studies have described a number of enterococcal surface proteins involved in adhesion to host cells. Among the proteins studied in binding to eukaryotic cells are the Esp (enterococcal surface protein (37)), Ace (adhesion to collagen from *E. faecalis* (155)) and pili (50).

Our results indicate, that aside from these well studied proteins, bacterial carbohydrates are also involved in the binding to host cell receptors. In our *E. faecalis* 12030□*bgsA* mutant, replacement of DGlcDAG by MGlcDAG as the major cell membrane glycolipid led not only to a decreased ability to bind to plastic surfaces, but also to an impaired bacterial adhesion to Caco2 cells. In our cell culture adhesion assay, the 12030*ΔbgsA* mutant partially lost its ability to bind to colonic epithelial cells. The weak interaction of the altered glycolipid anchor of LTA of the *ΔbgsA* mutant with highly sulfated heparin on the surface of the cell could be a possible mechanism for the reduced adherence. Although we have not been able to prove the altered glycolipid structure of LTA derived from the mutant, there is strong evidence in the literature that defects in the biosynthesis of DGlcDAG lead in consequence to a replacement of this glycolipid in the anchor structure of LTA (106, 116, 156). This important finding confirmed our inhibition experiments regarding the interaction between heparan sulfate proteoglycans on the Caco2 cells and DGlcDAG. In our adherence experiments, binding of *E. faecalis* 12030*ΔbgsA* was reduced by approximately 50%, the same amount of inhibition we observed when DGlcDAG or heparin was used as inhibitors. Deletion of the *bgsA* homologue in group B streptococci (*iagA)* in a recent publication by Doran and coworkes also affected the host-pathogen interaction (115). In this study, deletion of the gene encoding for the DGlcDAG synthetase was associated with reduced invasiveness of the blood-brain barrier, although the bacterial adhesion to the brain microvascular cells was not affected by this mutation. The authors speculate that LTA may act as an invasin and incorrect anchoring of this molecule (due to the replacement of DGlcDAG with MGlcDAG) results in decreased invasion of the blood-brain barrier. It is somehow difficult to compare the effect of the respective mutation in *E. faecalis* and *Streptococcus agalactiae*, because *E. faecalis* is unable to invade eukaryotic cells (such as Caco2) in significant numbers, even at a high MOI.

The cell line Hep2 (derived from larynx carcinoma) was previously used by Fabretti et al. as a model to investigate the role of LTA alanylation in enterococcal adhesion to eukaryotic cells. In that study, significant differences in adhesion of the wild type strain and the *dltA* mutant could be observed after 2 h of incubation (73). In contrast, adherence of *E. faecalis* 12030*ΔbgsA* to Hep2 cells was not impaired. These findings may suggest that glycolipids - rather than being unspecific factors involved in adherence - mediate increased binding to specific cell types only.

In vivo, adherence of *E. faecalis* to host cells is sometimes followed by translocation across the intestinal mucosal barrier. It has been suggested that bacteria indigenous to the GI tract such as enterococci are continuously translocating in small numbers from the GI tract even in the immunocompetent hosts without establishing infections (84). Intestinal bacterial overgrowth, deficiencies in the immune system, or damage of the intestinal barrier promotes bacterial translocation and establishment of systemic infections.

In this work, we used *in vitro* transcytosis assay to study the enterococcal translocation across polarized monolayers of Caco2 cells. Using this cell line, we did not observe translocation of *E. faecalis* strain 12030 and the 12030*ΔbgsA* mutant across the intestinal epithelial cells even at a high MOI. The failure to translocate across colonic epithelium, however, may be strain-related. This is suggested by the observations of Zeng and colleagues (98). In this work, only 8 out of 14 enterococcal human isolates translocated across T84 polarized monolayers, a human colon carcinoma-derived cell line.

Recent studies of Theilacker and colleagues demonstrate the role of LTA as target for opsonic (77) and protective (manuscript submitted) antibodies in the same strain background, *E. faecalis* 12030. We wanted to determine whether modification of the membrane glycolipids affects the strain susceptibility to opsonic killing. In an opsonaphagocytic assay, a slightly higher proportion of the *bgsA* mutant cells were killed by the polyclonal rabbit anti-whole bacteria serum. When using rabbit antiserum specific for enterococcal LTA, however, the opsonic killing of the two strains was identical (data not shown). Since opsonic antibodies to *E. faecalis* strain 120303 target LTA, the biologic significance of the moderately increased sensitity the *bgsA*-deletion mutant remains unclear.

In the recent years the immune system of *Drosophila melanogaster* was used as a model organism in many fields of biology including infectious disease research. Reasons for the

popularity of *Drosophila* as an *in vivo* model are numerous: the fruit fly is genetically well described, has a short generation time and some of the components of its immune system are similar to the human innate immune system. Previously characterized bacterial virulence determinants important for mammalian infection are also required for pathogenicity in *Drosophila* (30, 109), making *Drosophila* a suitable model for studies of human host–pathogen interaction. Also, the GAGs repertoire of this organism is similar to that of human cells, but expressing only three characterized membrane heparan sulfate proteoglycan (110). Baron and coworkers showed in a recent study that cell surface GAGs confer susceptibility to infection with group B streptococcus of the flies by recognizing and binding alpha C protein on the surface of the bacteria.

For comparison of *E. faecalis* strain 12030 and the 12030*ΔbgsA* mutant, we infected *Drosophila* by direct inoculation into the thorax. Using this infection model, we would have expected a lower mortality of the flies infected with bacterial lacking DGlcDAG, due to a lower interaction with the GAGs on the host cells. However, no difference in mortality between the flies infected with the two strains was observed, but survival rate inversely correlated to the inoculum for both wild type and the mutant strain. We can only speculate that, due to the variable GAGs expression in different organs of *Drosophila* (110) the wound colonization model used in this case versus the gastrointestinal colonization model used with Caco2 cells does not allow comparison.

Aside from the *Drosophila* model, we studied the persistence of the wild type strain and of the 12030*ΔbgsA* mutant in the bloodstream using a mouse bacteremia model. Compared to wild type bacteria, the *bgsA* deletion mutant was cleared more rapidly from the bloodstream. Since *E. faecalis* 12030 and the corresponding mutant strain are both complement-resistant, vary very little in their sensitivity to opsonophagcytic killing and do not differ in their susceptibility to antimicrobial peptides (data not shown), our results suggest, that persistence of *E. faecalis* in the blood stream might be related to its ability to form biofilm. These findings are in agreement with previous studies from our group using this model. Deletion mutants of *bopD* (67) and *dltA* (unpublished observations) genes in *E. faecalis,* which are also defective in biofilm formation, were also less virulent in the mouse bacteremia model .

In humans, the role of enterococci as primary pathogens in polymicrobial peritonitis has been demonstrated for severely ill, immunocompromised patients [reviewed by Harbarth et

al. (157)]. However, the knowledge of which virulence factors are playing a role in peritonitis is limited. We investigated here the impact of the *bgsA* deletion on bacterial virulence using a mouse peritonitis model. In this model, bacteria are injected directly into the peritoneal cavity. Compared to other Gram-positive cocci, enterococci are not very pathogenic in the peritonitis model and the LD 50 is high (108). Accordingly, we were able to induce lethal peritonitis only if a high bacterial inoculum was used (> $5x10^8$ cfu/animal). Lower bacterial inocula are cleared by mice without signs of illness (data not shown). Two strategies have been often used to render enterococci more virulent and to lower the bacterial inoculum needed to produce peritonitis: the use of rat sterile faeces (49, 158) or of hog gastric mucin solution (Gilmore M., personal unpublished observation) administrated together with the bacterial inoculum. In a first approach to establish a peritonitis model, we administrated 200 µl mucin 5% i.p., followed by the bacterial inoculum 2 h later. This led to a quite surprising result: peritonitis caused by the 12030*ΔbgsA* mutant, which is was shown to have an attenuated phenotype *in vitro* and mouse bacteremia model *in vivo* led to highly increased mortality compared to the wild type bacteria. These findings contradict our results obtained in the sepsis model, but alternative mechanisms involved in bacterial clearance for different routes of infection might explain these discrepant observations. To get a better understanding of these results, we tried to reproduce the experiment without the use of mucin. The same bacterial inoculum was associated, as expected, with lower overall mortality of the animals, but still significantly more mice infected with the 12030*ΔbgsA* strain compared to mice infected with the wild type strain died. Several mechanisms could account for the observed phenomenon. The first line of defence encountered by bacteria in the peritoneum is represented by the resident peritoneal macrophages - the most prominent leukocytes within the healthy peritoneum. As suggested in a recent study by Leendertse et al. peritoneal macrophages rapidly ingest and eliminate enterococci, starting as early as one h after infection (108). Since infection with the 12030*ΔbgsA* strain led to an increased mortality of the animals, we first hypothesized that maybe the mutant is able to evade the killing by peritoneal macrophages. Therefore, we compared the two enterococcal strains for their susceptibility to killing by mouse macrophages (RAW 264.7 cell line) *in vitro*. According to this assay, however, the two strains were equally susceptible to killing by macrophages, suggesting that the high mortality of the mice infected with the mutant is due to other reasons.

We then hypothesized that the 12030*ΔbgsA* mutant might be more invasive than wild type bacteria, being able to spread to other organs more rapidly and in higher numbers. To test this hypothesis, we infected mice i.p. with a high bacterial inoculum (10^9 cfu/mouse) and sacrificed them after 3 h or after 12 h. Bacterial counts were performed from blood, PLF and homogenized kidneys. However, in both groups of mice, bacterial counts from blood, PLF and kidneys showed no difference between the two strains for both time points. These results differ from the sepsis model, where *E. faecalis* 12030*ΔbgsA* was cleared more rapidly. These findings indicate that the higher and early mortality in the peritonitis model was not caused by an increased bacterial burden in the peritoneum, blood or kidneys.

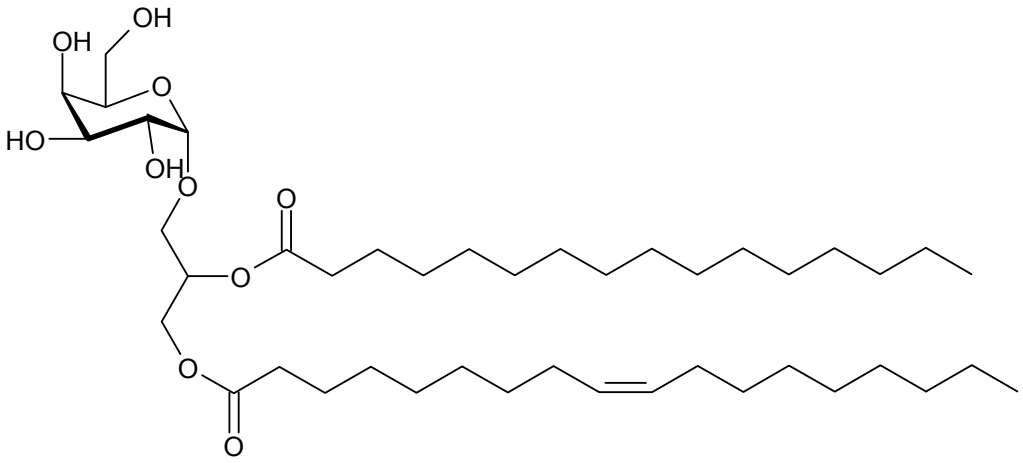
A recent study investigating the role of *E. faecium* in a mouse peritonitis model (159) showed that attraction of large numbers of neutrophils in the PLF is an important and early event involved in the rapid clearance of this bacterium. Using the same experimental setup as mentioned above, leukocyte counts from the PLF of infected mice were performed. The cell counts revealed a higher influx of leukocytes in the peritoneum of the mice infected with the mutant 3 h after infection. At 12 h after infection the numbers of cells were higher for both groups without a significant difference between the two groups. Our data imply an earlier recruitment of leukocytes to the site of infection with the 12030*ΔbgsA* strain, possibly induced by an early hyper-inflammatory reaction produced by this strain.

To further evaluate the inflammatory response induced by the two enterococcal strains in peritonitis, the levels of TNF-α, IL-4 and IFN-γ from the PLF were measured. For mice infected with the mutant strain, early influx of inflammatory cells 3 h after the infection correlated with a higher production of the pro-inflammatory cytokine TNF-α and - to a lesser degree - of the anti-inflammatory cytokine IL-4 in mice infected with 12030*ΔbgsA*. After 12 h, high levels of TNF-α were measured for mice infected with the wild type bacteria only and IL-4 levels were below detection limit of the ELISA assay in both groups. An early proinflammatory response by *E. faecalis* strain 12030*ΔbgsA* may be an explanation of the higher mortality of mice infected with this strain. It is known that production of cytokines at the site of infection can cause serious tissue damage and propagate the inflammatory response, leading to higher and rapid lethality of the animals [reviewed by Hack et al. (160)]. TNF-α is one of the major endogenous mediators of sepsis reaching a peak level few hours after bacterial challenge. In several sepsis models it has been shown that TNF-α is secreted in response to infecting microorganisms or their secreted products leading to lethal complications (160).

In our case, the only major difference between the two bacterial strains used to induce mice peritonitis is the replacement in the mutant of the major membrane glycolipid, DGlcDAG, with its precursor MGlcDAG.

In the last decade, the discovery of molecules capable to recognize and to present lipid antigens to T cells and NKT cells broadened our understanding regarding the immune response to infectious pathogens. The molecular mechanism of the lipid antigens recognition involves presentation of the antigens by CD1 (a MHC class I homologue) present on the antigen presenting cells to a subpopulation of T-lymphocytes known as *i*NKT cells. CD1 molecules contain two or four hydrophobic pockets able to bind lipid tails. Lipid antigen tails must be of acceptable size and shape to fit properly into CD1 pockets [reviewed by Zajonc et al. (161)]. CD1-restricted T cells display a T cell receptor able to recognize microbial glycolipids antigens presented by the CD1 molecules. In response to activation by the lipid antigens, *i*NKT cells produce a number of pro- and anti-inflammatory cytokines. By far the best-characterized agonist of *i*NKT cells is the glycolipid α-galactosylceramide isolated from a marine sponge during a screening for compounds that could prevent tumor metastasis (162). Most mammalian sphingolipids have a β-linkage to the sphinosine base preventing autoreactivity of the T cells with self-glycolipids (163) and leading to the recognition of the α-linkage of the microbial antigens. It seemed unlikely, though, that a conserved T-cell population was selected to recognize a rather atypical antigen from a marine organism only. Subsequent studies demonstrated that *i*NKT cells are able to recognize glycolipids from several bacteria [reviewed by Cohen et al. (164)], suggesting that these cells can contribute to microbe-specific immune response. Recently, an α-linked galactosyl diacylglycerol isolated from *B. burgdorferi* has been shown to bind to CD1 molecules and to activate *i*NKT cells (118). This α-linked compound resembles very closely the major glycolipid of the 12030*ΔbgsA* mutant, MGlcDAG. The only difference between the two glycolipids is the replacement of α-galactose with α-glucose in MGlcDAG, as depicted in Fig. 5.3.

A



B

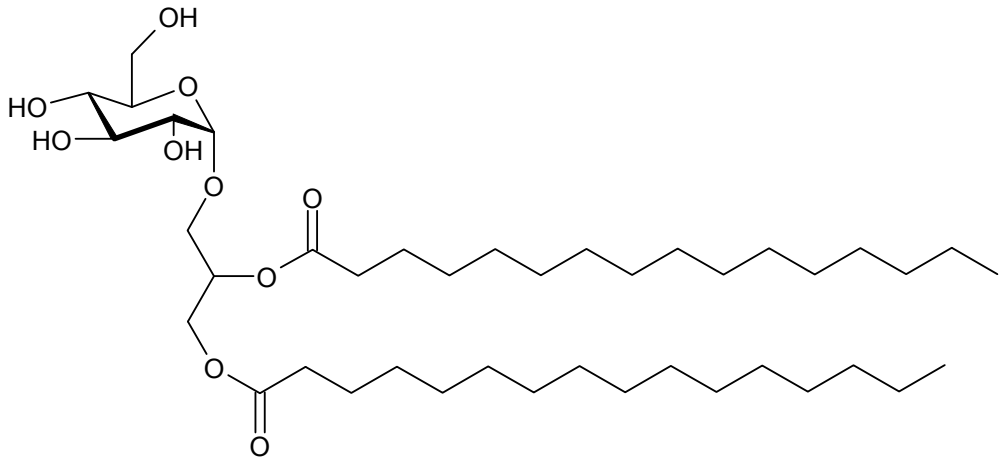


Fig. 5.3 Chemical structures of the α-galactosyl diacylglycerol from *B. burgdorferi* (A) and the α-glucosyl diacylglycerol from *E. faecalis* (B)

The ability to precisely discriminate the carbohydrate structure of the monosaccharide glycolipid antigens is typical for CD1 T-restricted cells (Fig. 5.4) [reviewed by Zajonc et al. (161)].

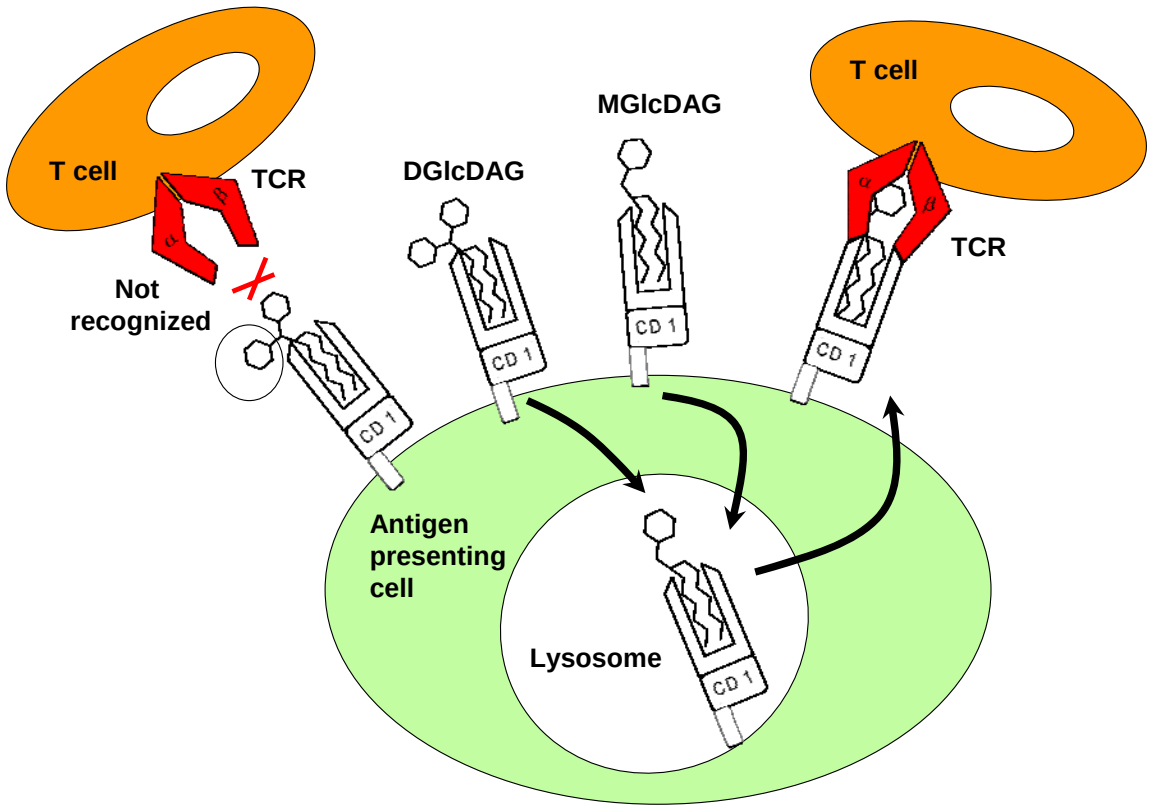


Fig. 5.4 Recognition mechanism of the MGlcDAG by the CD1 T-restricted cells [modified after (165)]

Numerous studies demonstrated the protective role of *i*NKT cells in the immune response to microbes using CD1d deficient mice [reviewed by Cohen et al. (164)]. In infections with *Pseudomonas aeruginosa* (166) or *Streptococcus pneumoniae* (167), mice lacking CD1 reactive T cells were more susceptible to infections compared to wild type mice. Few reports (168) demonstrated a detrimental role of *i*NKT cells in controlling bacterial infections. Mattner and colleagues (168) demonstrated that *i*NKT cells activated by glycolipids are able to promote a cytokines storm associated with high mortality in wild type mice infected with high doses of *Sphingomonas*. In contrast, infection of NKT-deficient mice resulted in lower levels of cytokines and higher survival.

In the experiments presented in our study, higher mortality and early TNF-α and IL-4 productions was observed for the wild type mice infected with the 12030*ΔbgsA* mutant producing MGlcDAG instead of DGlcDAG. In addition, less mortality was observed for NKT-deficient mice (CD1d-/-) infected with the 12030*ΔbgsA* mutant, compared to wild type mice. In contrast, the mortality rate was similar for wild type and CD1d-/- mice infected with *E. faecalis* strain 12030. Our findings suggest that *i*NKT cell activation by MGlcDAG may play a role in hyperinflammation found in the 12030*ΔbgsA* mutant.

5.3. Conclusions and perspectives

The aim of this study was to provide newer insights into enterococcal pathogenesis needed to develop alternative therapeutic strategies for the increased number of antibiotic resistant bacteria.

Since microorganisms adhesion to host cells is the initial stage of the infectious process, the use of agents able to interfere with the adherence to host tissues might be considered an interesting alternative to the classical antimicrobial therapy. We were able to identify molecules involved in enterococcal adhesion to human enterocytes, both on the surface of the eukaryotic cells and on the bacteria. GAGs on the host cells are recognized by the bacterial glycolipids during the adhesion process. Further studies to clarify the exact mechanism of interaction of the two types on molecules are needed.

Furthermore, an *E. faecalis* gene (*bgsA* – encoding a glycosyltransferase) involved in biofilm formation has been identified and its role in other phenotypical traits related to virulence has been studied using a deletion mutant. Several *in vitro* and *in vivo* models have been employed to study the effect of *bgsA* deletion on the bacterial virulence. Aside from biofilm formation, the inactivation of this gene impairs bacterial adhesion to colonic cells and shortens the time of the bacteremia *in vivo.* Surprisingly, we found that in a peritonitis model, the virulence of *E. faecalis* deletion mutant was exacerbated. So far, it seems that bacterial glycolipids produced by the mutant are playing a role in an inflammatory response mediate by *i*NKT cells. Ongoing studies employing antigenic stimulation of the *i*NKT cells using bacterial glycolipids and vaccination of the mice with glycolipids prior enterococcal infection are in progress.

6. SUMMARY

Enterococcus faecalis is regarded nowadays as one of the most important nosocomial pathogens. These bacteria have emerged both because they are intrisically resistant to many antibiotics and because they have acquired resistance genes to antibiotics that were effective in the past. The intestinal mucosa is considered the main entry site for these microorganisms. A better understanding of the factors and mechanisms leading to enterococcal translocation of the intestinal mucosa and dissemination to distant sites might help designing therapeutic strategies against these microorganisms.

Adhesion to human enterocytes, which represents the first step in bacterial colonization, was studied in detail in this thesis and two different aspects – i.e. molecules of the host extracellular matrix and components of the bacterial cell wall were investigated in more detail. Since enterococci are gut commensals, a colonic carcinoma cell line - Caco2 - was used as a model to investigate the interaction between enterococci and the intestinal mucosa. GAGs are important components of the extracellular matrix of virtually all animal cells and there is substantial evidence that they are recognized by a large number of pathogens infecting the host. In this study, their role as receptors for enterococci on the gut epithelial cells has been confirmed. By competitive bindig assays, enzymatic digestion and decrease of the sulfation of the GAGs chains, we could show that heparin and HS, but not CS are acting as receptors for enterococci on human enterocytes. The binding partners on the bacterial surface were also investigated using modifications of the cells surface and purified cell-wall bacterial compounds as inhibitors of the bacterial binding. By using the enterococcal glycolipid DGlcDAG, but not other carbohydrate cell wall components, bacterial adhesion to eukaryotic cells could be partially inhibited. SPR measurements revealed that heparin and HS on the host cells are recognized by bacterial glycolipid DGlcDAG, (either itself or as a part of the LTA molecule) but not its metabolic precursor, MGlcDAG.

Biofilm formation is regarded as an important way for enterococci to cause infections by adhering to inanimate surfaces. A deletion mutant previously constructed lacking DGlcDAG (*ΔbgsA*) was tested for its ability to form and accumulate biofilm on plastic surfaces. Inactivation of the glucosyltransferase catalyzing the synthesis of DGlcGAG leads to an almost complete loss of the ability to develop biofilm. As expected, the lack of DGlcDAG, shown to be responsible for binding to Caco2 cells, leaded to an impaired

adhesion of the *ΔbgsA* mutant to this cell line, compared to the wild type *E. faecalis* strain 12030. In a mouse bacteremia model, the *ΔbgsA* mutant was cleared more rapidly from the bloodstream, compared to the wild type bacteria. However, in a mouse peritonitis model, the infection with the mutant strain led to a high mortality, associated with a higher influx of inflammatory cells in the peritoneum and early TNF-α and IL-4 production. CD1d-/- mice (which lack the NKT cells) are partially protected against bacterial mortality, when infected with the mutant, suggesting a role of *i*NKT cells of the host in recognizing bacterial MGlcDAG, synthesized by the mutant instead of DGlcDAG.

7. ZUSAMMENFASSUNG

Enterococcus faecalis ist heutzutage einer der wichtigsten Verursacher nosokomialer Infektionen. Die Verbreitung dieser Mikroorganismen wird dadurch begünstigt, daß sie eine intrinsische Resistenz gegenüber vielen Antibiotika besitzen und schnell Resistenzen gegenüber weiteren Antibiotika entwickeln oder erwerben, die früher wirksam waren.

Die gastrointestinale Mukosa des Menschen wird als Eintrittspforte dieser Mikroorganismen angesehen. Ein besseres Verständnis der Mechanismen und Faktoren, die zur Translokation von Enterokokken durch die gastrointestinale Schleimhaut und in der Folge zur Verbreitung im menschlichen Körper führen, kann dazu beitragen, neue Therapieoptionen gegen diese Erreger zu entwickeln.

In der vorliegenden Arbeit wurde die Adhäsion von Enterokokken an menschliche Enterozyten, die die erste Stufe bakterieller Kolonisation darstellt, näher untersucht. Dabei wurden sowohl die extrazellulären Moleküle des Wirtes, als auch die Komponenten der bakteriellen Zellwand näher untersucht. Da Enterokokken gastrointestinale Kommensale sind, wurde eine humane Kolon-Karzinomzellinie – Caco2 – als Model zur Untersuchung der Interaktion von Enterokokken und menschlicher Intestinalmukosa gewählt.

Glycosaminoglycane (GAGs) sind wichtige Komponenten der Extrazellulärmatrix von nahezu allen tierischen Zellen. Sie werden von einer großen Anzahl von humanpathogenen Erregern als Adhäsionsmolekül benutzt. In dieser Studie konnte die Rolle der GAGs als Rezeptoren für Enterokokken auf gastrointestinalen Zelloberflächen bestätigt werden. Durch kompetitive Bindungsassays, enzymatischen Verdau und spezifischer Hemmung der Sulfatierung von GAGs konnte gezeigt werden, dass Heparin und Heparansulfat, nicht aber Chondroitinsulfat als Rezeptoren für Enterokoken auf menschlichen Enterocyten fungieren. Die korrespondierenden Bindungspartner auf der bakteriellen Zelloberfläche wurden untersucht, indem Modifikationen der Zelloberfläche vorgenommen, oder gereinigte Komponenten der Bakterienzellwand als Bindungsinhibitoren eingesetzt wurden. Durch den Zusatz von DGlcDAG (Diglucosyldiacylglycerol), einem Glycolipid der Enterokokkenzellwand, konnte die Adhäsion an eukaryotische Zellen teilweise inhibiert werden, wohingegen andere Zellwandbestandteile keine inhibitorische Wirkung zeigten.

Oberflächenplasmonresonanzmessungen ergaben, dass Heparin und Heparansulfat auf den Wirtszellen durch das bakterielle Glycolipid DGlcDAG (sowohl in reiner Form, als auch als Bestandteil von Lipoteichonsäuren), nicht aber durch seinen metabolischen Vorläufer MGlcDAG (Monoglucosyldiacylglycerol) gebunden werden.

Da Enterokokken durch Biofilmbildung an unbelebte Oberflächen adhärieren können, wird diese als wichtiger Pathomechanismus angesehen. Eine kürzlich generierte Deletionsmutante im Gen *bgsA* (einer Glycosyltransferase, die die Synthese von MGlcDAG zu DGlcDAG katalysiert) wurde auf ihre Fähigkeit untersucht, Biofilme auf Plastikoberflächen auszubilden. Dabei zegite sich, dass die Inaktivierung von *bgsA* zu einem nahezu vollständigen Verlust der Biofilmbildung führte. Dabei kam es durch den Verlust von DGlcDAG, das wie bereits gezeigt, verantwortlich für die Bindung an Caco2 Zellen ist, zu einer verringerten Adhäsion der *bgsA* Mutante verglichen mit dem Wildtyp *E. faecalis* 12030. In einem Maus Bakteriämie Modell wurde die *bgsA* Mutante schneller aus der Blutbahn eliminiert als der Wildtypstamm. Demgegenüber führte die Infektion mit der *bgsA* Mutante in einem Maus Peritonitis Modell zu einer höheren Mortalität, verbunden mit einem höheren Einstrom von inflammatorischen Zellen ins Peritoneum und einer früheren TNF und IL-4 Produktion. CD1d -/- Mäuse zeigten eine geringere Mortalität bei Infektion mit der *bgsA* Mutante. Dies deutet auf eine wichtige Rolle der *i*NKT Zellen des Wirtes bei der Erkennung von bakteriellem MGlcDAG hin, das in der Mutante anstatt des DGlcDAG vermehrt synthetisiert wird.

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Weiterhin versichere ich Georgeta Irina Sava, geboren am 29.11.1978 in Comanesti, Rumänien, dass ich meine Dissertation zur Erlangung der Doktorwürde gemäß Promotionsordnung der Technisch-Naturwissenschaftlichen Fakultät der Universität zu Lübeck nach § 8 Abs. 1 - 8 ohne fremde Hilfe durchgeführt, sowie keine anderen als die in der Arbeit genannten Hilfsmittel benutzt habe.

Weiterhin versichere ich gemäß § 8 Abs. 1- 9, dass ich weder vorher oder gleichzeitig andernorts einen Zulassungsantrag gestellt oder die Dissertation vorgelegt habe.

Auch erkläre ich hiermit dass ich mich nach § 8 Abs. 1- 10 noch keinem anderen Promotionsverfahren unterzogen habe.