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Novel active and passive immunotherapy regimens against nosocomial infections caused by multidrug resistant enterococci

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To Patroklos

and to my parents, Elsa and Tasos

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Abbreviations

AdcAfm	Zinc ABC transporter substrate-binding lipoprotein
BSA	Bovine serum albumin
CDAP	1-cyano-4-dimethylaminopyridinium tetrafluoroborate
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CIES	Carrier-induced-epitopic suppression
CPS	Capsular polysaccharide serotypes
Cyl	Cytolysin
Da	Dalton
DHG	Diheteroglycan
DHG.01	Monoclonal antibody against DHG
DdcP	D-alanyl-D-alanine carboxypeptidase
ELISA	Enzyme-linked immunosorbent assay
epa	Enterococcal polysaccharide antigen
FBS	Fetal Bovine Serum
Galf	Galactofuranose
GelE	Gelatinase
Glcp	Glucopyranose
IgG	Immunoglobulin G
Ka	Association constant
K_D	Affinity constant
Kd	Dissociation constant
LTA	Lipoteichoic acid
LysM	Peptidoglycan-binding protein LysM
mAb	Monoclonal antibody
min	Minute
OD	Optical density
OPA	Opsonophagocytic assay
OPIA	Opsonophagocytic inhibition assay
PAMPs	Pathogen-associated molecular patterns
PBPs	Penicillin binding proteins
PBP5	Low affinity penicillin-binding protein 5

PMNs	Polymorphonuclear nuclear neutrophils
PpiC	Peptidyl-prolyl cis-trans isomerase
PsaAfm	Manganese ABC transporter substrate-binding lipoprotein
rmAbs	Recombinant monoclonal antibodies
rSagA	Recombinant SagA
RT	Room temperature
SagA	Secreted antigen A
SagA.01	Monoclonal antibody against SagA
SPR	Surface Plasmon Resonance
SDS	Sodium dodecyl sulfate
TLRs	Toll-like receptors
US	United States
VRE	Vancomycin-resistant enterococci
WBC	White blood cells
WTA	Wall teichoic acid

List of publications

1. Romero-Saavedra F.*, Laverde D.*, Kalfopoulou E., Martini C., Torelli R., Martinez-Matamoros D., Sanguinetti M., Huebner J. 2019. Conjugation of different immunogenic enterococcal vaccine target antigens leads to extended strain coverage, Journal of Infectious Diseases (in press) *These authors have contributed equally to this work.

2. Kalfopoulou E., Laverde D., Miklic K., Romero-Saavedra F., Malic S., Carboni F., Adamo R., Lenac Rovis T., Jonjic S., Huebner J. 2019. Development of opsonic mouse monoclonal antibodies against multidrug resistant enterococci, Infection and immunity (in press)

1. Introductory summary

1.1 Summary

Enterococci are natural inhabitants of the gastrointestinal tract and they are the second most common Gram-positive pathogens responsible for nosocomial infections. Passive and active immunotherapies targeting capsular polysaccharides and surface-associated proteins have emerged as a potential prevention and/or treatment strategy against this opportunistically pathogenic bacterium.

In the first part of this study we evaluated the potential use of two protein immunogens from *Enterococcus faecium*, the secreted antigen A (SagA) and the peptidyl-prolyl cis-trans isomerase (Ppic), as carrier proteins conjugated with the capsular polysaccharide diheteroglycan (DHG) from *E. faecalis*. These two glycoconjugates (DHG-SagA and DHG-PpiC) could serve as cross-species vaccines against enterococcal infections. For this purpose, rabbits were immunized with the two glycoconjugates, and increasing IgG titers against the immunogens and their components were reported. In addition, the cross-reactivity of the sera was confirmed by evaluation of their *in vitro* opsonophagocytic killing activity and their immunoreactivity in whole cell ELISA against several *E. faecalis* and *E. faecium* strains. The sera, also, conferred protection in mice challenged with the respective enterococcal strains. These results support the potential use of these proteins as carrier proteins and the ability of these glycoconjugate vaccines to provide broader coverage against enterococcal infections. (Publication I)

In the second part of this study we developed two mAbs against two well characterized immunogens, DHG and SagA, from *E. faecalis* and *E. faecium*, respectively. For the development of these mAbs mice were immunized with the glycoconjugate DHG-SagA, also used in the previous study, and the hybridoma technology was used. The implementation of the opsonophagocytic assay for the selection of the hybridomas was investigated. The two mAbs developed through this process were further characterized for their immunoreactivity by *in vitro* immunological assays. Both the generated mAbs, DHG.01 and SagA.01, exhibited high affinity and specificity to their targets. In addition, the *in vitro* opsonophagocytic killing activity of these mAbs against different enterococcal strains was proven, confirming their cross-specificity. These mAbs could serve as a potential immunotherapy against enterococcal infections especially upon humanization. For this purpose, the sequencing of the mAbs and the evaluation of the resulting sequences by the same immunological assays were performed. Finally, the development of these two mAbs upon immunization with DHG-SagA, combined with the previous project, confirm that SagA is a good carrier protein and that the DHG-SagA glycoconjugate is a potential vaccine candidate against prevalent enterococcal species. (Publication II)

1.2 Enterococci

Enterococci are gram-positive, facultative anaerobic oval cocci and lactic acid producers, that form chains of various lengths (1). These bacteria exhibit high tolerance to extreme pH conditions, to a wide range of temperatures and salt concentrations enabling them to colonize a variety of niches. In addition, unlike other bacteria, they are highly tolerant to sodium azide and concentrated bile salts (2). Enterococci are commensal bacteria that colonize the gastrointestinal tract of humans even at the early life stages (3). Some enterococcal strains have also been used as probiotic agents claiming beneficial effects on a number of gastrointestinal and other diseases (4).

Although they are not a threat for healthy individuals, enterococci are opportunistic pathogens associated with hospital-acquired infections making them a serious threat for immunocompromised patients. In particular, enterococci can cause serious diseases including endocarditis, bacteremia, and meningitis, as well as intra-abdominal, wound, and urinary tract infections (5). The increased prevalence of enterococcal infections in humans is mainly attributed to their acquired and intrinsic resistance to antibiotics but also to their ability to acquire virulence factors (6). In addition, the biofilm forming capacity of enterococci contributes to their persistence during infection and increases their ability to endure difficult growth conditions (7).

The first incidence of endocarditis caused by *E. faecalis* was reported in 1899, and since then numerous studies have tried to shed light on this pathogenic bacterium (8). The first outbreak of enterococcal infections was reported in the late 1970s in the United States (US), and currently a new wave, attributed to vancomycin-resistant enterococci (VRE), is affecting not only the US but also Europe (1). In a recent study, Cassini et al. reported 16146 cases of VRE infections and 1081 attributable deaths in the European Economic Area and European Union in 2015 (9). Southern European countries, e.g. Greece and Portugal, have reported the highest rates of VRE associated with nosocomial infections in Europe (10). The two most clinically relevant enterococcal species responsible for infections are *Enterococcus faecalis* and *Enterococcus faecium,* with the highest incidences being initially attributable to *E. faecalis* (11). However, during the last two decades, *E. faecium* has become the leading species responsible for enterococcal infections both in US and European hospitals, probably due to its high incidence of antibiotic resistance compared to *E. faecalis* (12). In 2017, the World Health Organization published a list with 12 antibiotic-resistant pathogens which pose the greatest threat to human health, with *E. faecium* being classified as a high priority for the development of new treatments (13).

1.3 Serotyping of Enterococci

The initial attempt to establish a system to serotype enterococci was conducted by Sharpe M.E. in 1964 (14). In 1992, Maekawa et al. were able to distinguish 21 serovars of *E. faecalis*, and by using sera raised

against these serovars they analyzed a collection of 832 *E. faecalis* strains, from which 77% were typable (14). This classification system was based on sera obtained upon immunization of rabbits with formalin-killed bacteria, providing thus no information regarding defined antigenic structures (e.g. capsules or other cell-wall related antigens) (14).

In 2004, Hufnagel et al. were able to classify 66% of a collection of 29 *E. faecalis* clinical isolates into four capsular polysaccharide serotypes, CPS-A to -D, by immunological selection, using sera raised against the capsular polysaccharides of four representative strains, and genetic methods (15). They further expanded this study by serotyping 157 clinical and laboratory *E. faecalis* isolates from four different countries, being able to categorize only 42% of the isolates into one of the four serotypes (16). Hancock and Gilmore identified a *cps* locus of 11 open reading frames which was responsible for the synthesis of a capsular polysaccharide from *E. faecalis* Type 2 (17). Although all the serotypes CPS-A to -D possess the open reading frames *cpsA* and *cpsB*, only the CPS-C and -D serotypes possess the *cpsC* to *cpsK*, with the *cpsF* existing only in some CPS-C strains (15). Since seven out of the nine genes of the *cps* locus are important for the production of a capsular polysaccharide, the CPS-A and -B serotypes do not express this polysaccharide (18). Theilacker et al. demonstrated that the polysaccharide produced by the *cps* locus is the one that they identified in their study, named diheteroglycan (DHG) (19). A few years later, the study of McBride and co-workers demonstrated that half of the CPS-C strains examined were more virulent compared to the CPS-A and -B strains, due to the presence of multiple virulence and antibiotic resistant traits, as well as to capsular polysaccharides that play critical role in the host-pathogen interaction (20).

1.4 Composition of the enterococcal cell wall

The major cell wall component of Gram-positive bacteria is a peptidoglycan layer, consisting of branches of N-acetylmuramic acid-(β1-4)-N-acetylglucosamine (MurNAc-GlcNAc) repeating units, which are cross-linked through short peptide bridges (21). The peptidoglycan layer in enterococci is decorated with a variety of molecules, which are either covalently linked to the peptidoglycan layer (i.e. polysaccharides, teichoic acids, and surface-anchored proteins) or covalently attached to the plasma membrane (i.e. lipoteichoic acids and lipoproteins) (Figure 1) (22).

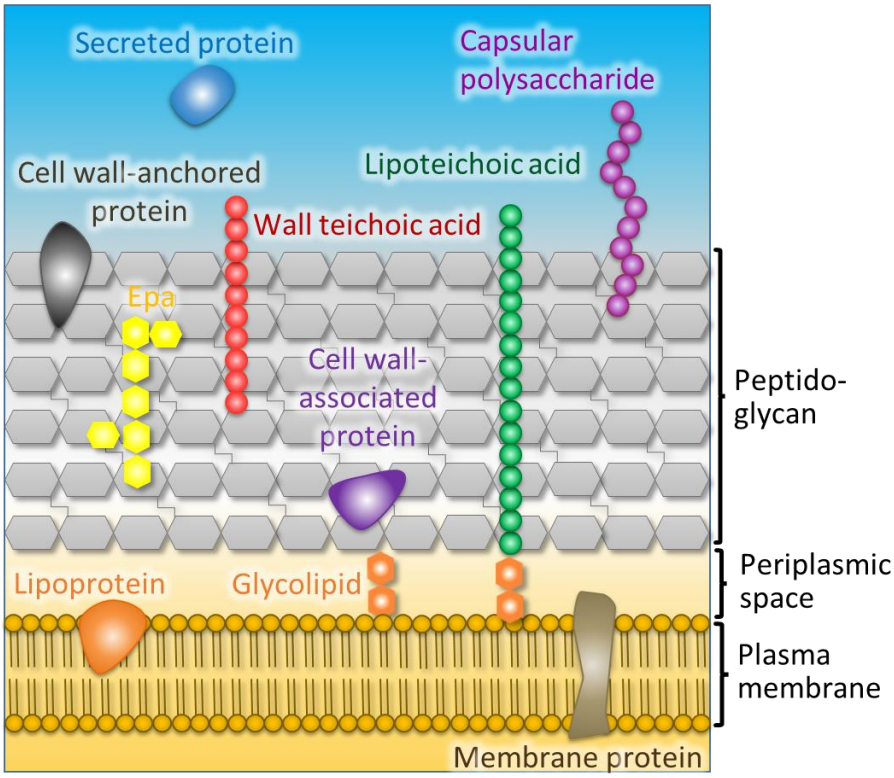

Figure 1. Schematic representation of the enterococcal cell wall. Wall teichoic acids, *epa* (enterococcal polysaccharide antigen), surface-anchored proteins and capsule are bound to the muramyl residues of the peptidoglycan, whereas lipoproteins and lipoteichoic acid are inserted into the plasma membrane (22).

1.4.1 Enterococcal polysaccharides

In 1999, Wang et al. identified a novel polysaccharide that was present in *E. faecalis* and a vancomycin resistant *E. faecium* strain. (23). Antisera raised to this polysaccharide were able to mediate opsonic killing *in vitro* and protect against *E. faecalis* and *E. faecium* bacteremia (24, 25). The correct structural characterization of this polysaccharide, a lipoteichoic acid (LTA), was performed by Theilacker et al., revealing a 1,3-polyglycerolphosphate backbone substituted at position C-2 with alanine or kojibiose (Figure 2A) (26). While LTA has a glycolipid anchor in the membrane, wall teichoic acids (WTA) have a polyglycerolphosphate or polyribitolphosphate backbone covalently attached to the peptidoglycan layer by a phosphodiester bond (27).

The same polyglycerolphosphate backbone of LTA, is also present in many other clinically important Gram-positive pathogens, such as staphylococci, and some streptococci. Theilacker et al. proved that antibodies targeting the backbone of LTA are opsonic and protective against *E. faecalis* and *S. epidermidis* bacteremia, also conferring protection against *S. aureus* infection (28). In an attempt to

develop a vaccine candidate targeting LTA by synthetic approaches, Laverde et al. identified a synthetic teichoic acid, WH7, able to absorb out the opsonic activity of antibodies raised against enterococcal LTA (Figure 2B). This synthetic oligomer is a promising vaccine candidate against *E. faecalis* and other Gram-positive bacteria (29, 30). Another polysaccharide anchored to the peptidoglycan layer is the enterococcal polysaccharide antigen (Epa), a rhamnopolysaccharide, and its synthesis is attributed to the *epa* locus (31). This polysaccharide has been suggested to play a role in biofilm formation, resistance to neutrophil-mediated phagocytosis, and virulence in a mouse peritonitis model (31–33).

Although LTA is present in all enterococcal serotypes, it is only surface-exposed in the CPS-A and CPS-B serotypes, resulting in the susceptibility of these serotypes to opsonization by the sera raised against LTA (34). Serotypes CPS-C and CPS-D possess a capsular polysaccharide, which masks LTA, characterizing their surface composition and resulting in a different serological recognition compared to serotypes CPS-A and CPS-B (34). This immunogenic capsular polysaccharide DHG was initially identified by Pazur et al. (35). The structural elucidation of DHG was accomplished by Theilacker et al. and Krylov et al. revealing a repeating unit of →6)-β-Gal*f*-(1→3)-β-D-Glc*p*-(1→ with *O*-acetylation in position 5 and lactic acid substitution at position 3 of the Gal*f* residue (Figure 2C) (34–36). In the former study, it was also shown that rabbit serum raised against DHG mediates opsonophagocytic killing of the encapsulated strains *in vitro* and also reduces the bacterial load in livers and kidneys of mice challenged with these strains (34). It was also suggested that passive or active immunotherapy targeting DHG could provide protection against enterococcal infections caused by the encapsulated *E. faecalis* strains (34).

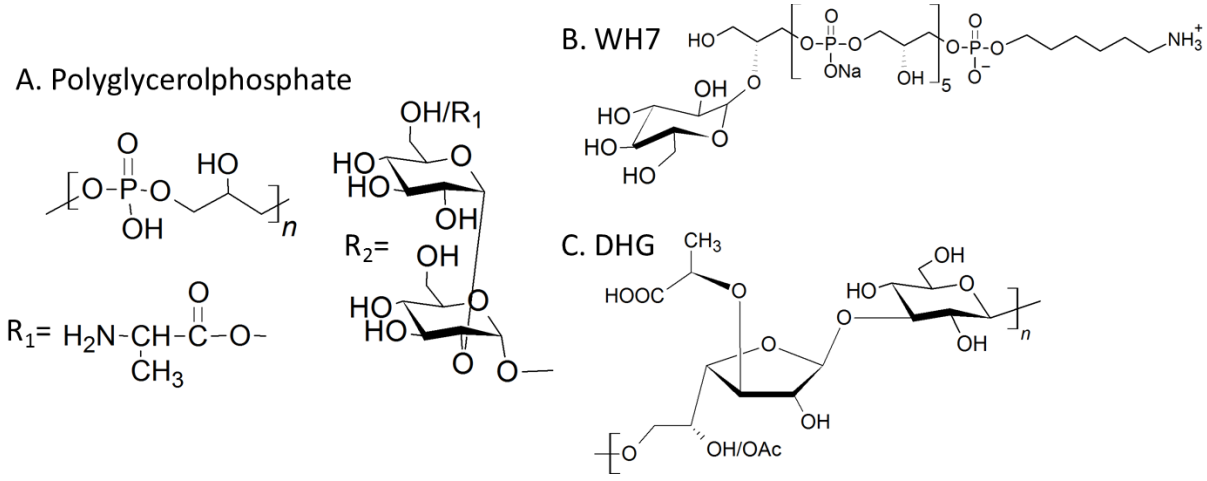

Figure 2. Chemical structure of A. LTA isolated from *E. faecalis* 12030 (26), B. synthetic teichoic acid WH7 (29), and DHG isolated from *E. faecalis* Type 2 (36). The 1,3-polyglycerolphosphate backbone of LTA can be substituted at the position C-2 of the glycerol residues by (R_1) d-Alanine, (R_2) kojibiose or with alanylated kojibiose.

1.4.2 Enterococcal proteins

Bacterial cell-wall-related and secreted proteins play pivotal roles in host-pathogen interactions, including adherence, internalization, toxicity, adaptation to environmental changes, and evasion of the host defense system (37).

Three secreted virulence factors have been identified so far in enterococci, named cytolysin (Cyl), gelatinase (GelE), and secreted antigen A (SagA) (38). The secreted antigen A (SagA) was initially identified in *E. faecium* by Teng et al., and has been shown to be essential for the bacterial growth as well as to bind a number of extracellular matrix proteins, including fibrinogen, collagen type I, collagen type IV, fibronectin, and laminin (37). In a recent study, Paganelli et al. identified SagA as the major secreted protein during biofilm formation and studied its susceptibility to degradation, its localization in the biofilm matrix, and its contribution in biofilm formation of *E. faecium* (39). Kropec et al. demonstrated that immunization with recombinant SagA induces opsonic antibodies against *E. faecium* VRE strains and promotes bacterial clearance in mice challenged with the same bacterial strains. These results suggest that active immunotherapy using only SagA or SagA conjugated with polysaccharides could serve as a promising vaccine candidate against enterococcal infections (40, 41). Another class of virulence factors are the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), such as Ace(42) and Acm (43), and the pilus proteins that promote biofilm formation (44, 45).

Although virulence factors are present in many vaccine formulations, any bacterial antigen exposed to the immune system can serve as a potential vaccine candidate (46). In this context, Romero-Saavedra et al. identified 6 enterococcal proteins that could serve as potential vaccine candidates against enterococcal infections by the implementation of transcriptomic (AdcAfm and PsaAfm) and proteomic (LysM, DdcP, PpiC, and PBP5) approaches (41, 47). In both studies, rabbits were immunized with the recombinant proteins and the resulting sera were evaluated in opsonophagocytic assay (41, 47). The sera raised against the proteins were opsonic against the homologous strain (*E. faecium* E155) but also against a collection of *E. faecalis* and *E. faecium* strains (41, 47). Moreover, the sera were found to be protective in a mouse bacteremia model (41, 47). Both results, indicate the potential use of these proteins as vaccine candidates with a broad cross-reactivity and serotype-independent coverage against enterococcal infections (41, 47).

Two of these proteins, AdcAfm and PsaAfm, are a zinc and a manganese ABC transporter substrate-binding lipoprotein. Lipoproteins are substrate-binding proteins which deliver the substrate to the corresponding ABC transporters (48). Interestingly, a homologue of PsaAfm, PsaA, has been studied as a promising vaccine candidate with broad coverage against *Streptococcus pneumoniae*, but also as a carrier protein with a synthetic oligosaccharide from *S. pneumoniae* serotype 14 (49). On the other side, a peptidoglycan-binding protein LysM, a D-alanyl-D-alanine carboxypeptidase (DdcP), a peptidyl-

prolyl cis-trans isomerase (PpiC) and a low affinity penicillin-binding protein 5 (PBP5) are surface-exposed proteins, which are associated with peptidoglycan (50). Although the function of these proteins has not been completely elucidated, these four proteins have been associated with resistance to ampicillin and high salt concentrations, and they seem to be involved in bacterial virulence and infection (47, 51–54). Finally, apart from targets being localized in the bacterial cell wall, several of these proteins were identified in membrane vesicles (MVs) of *E. faecium* correlating MVs, making enterococcal MVs interesting vaccine candidates (50).

1.5 Enterococcal infection and immunity

Enterococci are natural colonizers of the gastrointestinal tract, usually comprising only a small portion of the healthy gut microbiota (55). Exposure of hospitalized patients to antibiotics against Gram-negative bacteria distorts the gut microbiota, increasing the prevalence of mostly VRE in the gastrointestinal tract (1). Under healthy conditions, lipopolysaccharide and flagellin from Gram-negative bacteria induce the production of REGIIIγ. REGIIIγ suppresses the overgrowth of the Gram-positive bacteria, including *E. faecium*. Elimination of the Gram-negative bacteria population decreases REGIIIγ leading to the overgrowth of VRE in the gastrointestinal tract (56, 57). Similar shifts in the gut microbiota have also been reported in patients undergoing allogeneic hematopoietic stem cell transplantation, where the VRE prevalence in the gut was followed by bloodstream enterococcal infections (58).

The innate immune system constitutes the first line of defense against pathogen invasion. This type of defense depends on the recognition of the pathogen-associated molecular patterns (PAMPs), which are solely present in the pathogens (59). PAMPs are recognized through pattern recognition receptors, e.g. the components of the complement system and the Toll-like receptors (TLRs) (59). There is evidence that TLR2 interacts with CD14, playing an important role in the innate immune response against Gram-positive bacteria by recognizing peptidoglycan and LTA (60). Using an *E. faecium* peritonitis mouse model, Leendertse et al. showed that *E. faecium* is recognized through TLR2 mediating neutrophil influx to the site of infection and bacterial clearance (61). In the same model it was also found that peritoneal macrophages (62), neutrophils (61) and the complement system (63) are essential for the rapid eradication of this bacterium in the early stages of the infection.

Apart from the direct interaction of the pathogen with the phagocyte through the PAMPs-TLR interaction, there is also an indirect pathway mediated through a class of molecules called opsonins, comprised of immunoglobulins and complement components (64). Activation of the alternative complement pathway elicits deposition of the complement component C3b on the bacterial surface, which is subsequently recognized by complement receptors on the phagocytes (65). On the other side, IgGs trigger the FcγRs and activate the classical complement pathway resulting in the uptake of the

bacteria by the neutrophils (65). In encapsulated Gram-positive bacteria, like enterococci, the combination of these two mechanisms is crucial for efficient phagocytosis of the bacteria (65–67). Immediately upon formation of the phagosome its maturation starts, and the phagosome subsequently is fused with the lysosome for the formation of the phagolysosome, which is a microbicidal organelle (64).

In a study of Arduino et al. it was found that 50% of the *E. faecium* strains tested were resistant to phagocytosis, evading internalization by PMNs, which may be attributable to carbohydrate structures in their cell surface (68). In addition, there have also been reported cases where enterococci were able to resist killing despite their internalization by the phagocytes (69–71). The incompetence of the immune system to kill the intracellular enterococci may lead to their systemic spread (5).

1.6 Antibiotic resistance and options for treatment

Enterococci not only possess intrinsic resistance to several antibiotics, but also may develop acquired resistance through sporadic mutations or by the acquisition of exogenous resistance genes (i.e. by pheromone-sensitive plasmids, broad host range plasmids, or through transposon movement) (6). Approximately 90% of the clinical isolates from *E. faecium* are ampicillin resistant (1). In most cases resistance to β-lactam antibiotics in enterococci is attributed to the expression of low affinity penicillin binding proteins (PBPs) (72). Since enterococci are intrinsically resistant to aminoglycosides (e.g. gentamycin and streptomycin) due to their low bacterial uptake, *in vitro* studies have supported the synergistic effect of penicillin co-administration (1, 73). Still there are instances where this scheme is inadequate, especially when high-level resistance to aminoglycosides is encountered (74).

The first VRE strain was isolated in 1986 (75). Vancomycin belongs to the glycopeptides, a class of antibiotics that inhibits the peptidoglycan synthesis (76). VRE modify the D-alanyl-D-alanine terminus of the N-acetylmuramic acid and N-acetylglucosamine peptides to D-Ala-D-lactate or D-Ala-D-serine, reducing the affinity of the glycopeptides to the peptides but not affecting the biosynthesis of the cell wall (76). The van gene clusters are responsible for this type of resistance, with VanA and VanB being the most prevalent in Europe, providing a series of enzymes that facilitate the signal transduction, the synthesis of the D-lactate and D-serine, and their subsequent ligation to the precursor molecules, as well the elimination of the alternative biosynthetic pathway (10, 76). Each of these clusters provides different susceptibility levels to the enterococcal species, for instance the VanA cluster provides high-level resistance to vancomycin and teicoplanin, whereas strains possessing the VanB cluster retain their susceptibility to teicoplanin (77).

Alternative antibiotic agents, such as quinupristin-dalfopristin, daptomycin, tigecycline, and linezolid, have entered the clinical practice to fight VRE infections. However, resistance to these agents has

already been reported (10). Resistance to streptogramins and quinupristin/dalfopristin includes enzymatic acetylation of the drugs (78, 79), methylation of the 23S rRNA (80), and efflux pumps (80, 81). Clinical data suggest that quinupristin/dalfopristin could be beneficial in the treatment of *E. faecium* endocarditis especially as part of a combination therapy (82, 83). The two cell membrane proteins GdpD and LiaF are associated with resistance to daptomycin (84). Daptomycin is approved for skin and soft tissue VRE infections, and its efficacy against infective endocarditis, either as a monotherapy or in combination with aminoglycosides, ampicillin, or tigecycline, should be further investigated (85, 86). Resistance to linezolid has been attributed to mutations in the domain V of the 23S rRNA or alterations in the methylation of the 23S rRNA (87, 88). Generally, it is recommended linezolid as an alternative treatment for VRE endocarditis when other therapeutic options are not available (85, 86).

1.7 Alternative treatment methods against Enterococci

A current challenge in the treatment of enterococcal infections in the clinical setting is their resistances to most conventional antibiotics (89). This underscores the necessity for the development of new types of treatment or prevention, such as passive or active immunotherapies. As mentioned above, capsular and cells wall polysaccharides, but also cell-surface associated protein antigens can serve as targets for the development of immunotherapies.

An enterococcal vaccine could be beneficial in high risk patients for enterococcal infection, reducing the mortality rates and the hospital stay of this population (90). For this purpose studies that establish risk factors in well-defined patient populations are of major importance (91, 92). All the polysaccharides mentioned above could serve as good antigens in vaccine formulations against enterococcus. However, polysaccharides are poorly immunogenic, triggering T cell-independent immune response and in most cases are unable to elicit memory B cells (93). Chemical conjugation of polysaccharides with a carrier protein can overcome these obstacles. In particular, the carrier protein directs the processing of the glycoconjugate by polysaccharide-specific B cells. As a consequence the processed antigen is presented through the MHC class II molecule to the carrier-peptide-specific T cells, provoking thus, T cell-dependent immune responses, affinity maturation and B cell memory (Figure 3) (93). Currently, several licensed glycoconjugate vaccines have been proven to be safe and successful in the prevention of infectious diseases against *Haemophilus influenza*, meningococcus serogroups A, C, and ACWY, 10 to 13 serotypes of pneumococcus, and *Salmonella typhi* (94). Apart from their implementations in the vaccine industry, glycoconjugates have also served as immunogens for the production of polysaccharide specific mAbs in mice (95–97).

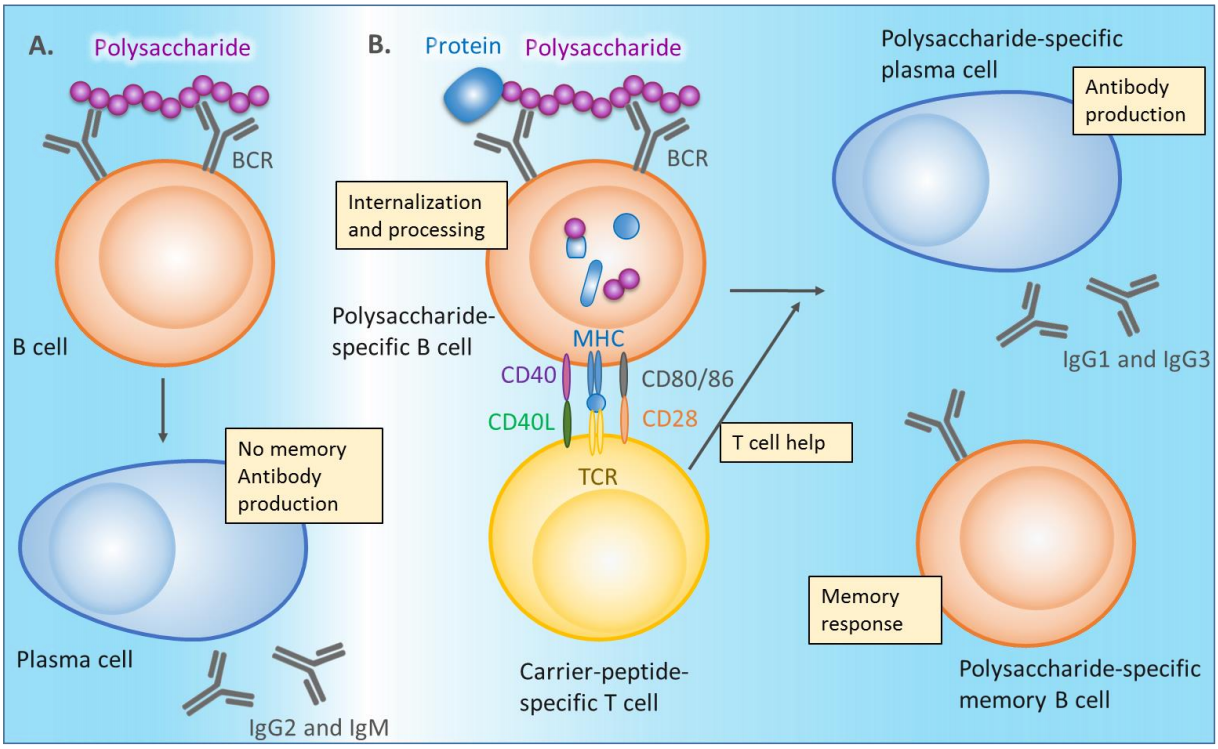

Figure 3. Immune response to polysaccharide and glycoconjugate vaccines. A. Immunization with polysaccharides stimulates B cells, and promotes the production of antibodies, without eliciting B cell memory. B. Glycoconjugate vaccines are processed by polysaccharide-specific B cells, and the resulting peptides are presented to carrier-peptide-specific T cells, inducing T cell help for the production of memory B cells and plasma cells. CD40L, CD40 ligand; TCR, T-cell receptor; B-cell receptor; modified from (93).

As it was discussed above, carrier proteins in glycoconjugate vaccines facilitate the T cell-dependent immune response to the conjugated polysaccharide, which is a T cell-independent antigen. The currently licensed carrier proteins for glycoconjugated vaccines are diphtheria toxoid, tetanus toxoid, CRM_{197}, *Haemophilus* protein D, and the outer membrane protein complex of serogroup B meningococcus (98). In an attempt to simplify vaccine formulations, to broaden the vaccine coverage, and to overcome carrier-induced-epitopic suppression (CIES), there is an emerging interest in the identification of new carrier proteins (98, 99). In this context, conjugates of polysaccharide and protein virulence factors from the same bacterium, where proteins play a dual role not only as a carrier protein but also as an immunogen, have been proposed (98).

Passive immunotherapy using mAbs is an emerging field with many promising candidates to fight these health threats (100). Despite their short-lasting effect, mAbs possess several advantages over vaccines. In particular, they can have a faster effect, with lower number of doses, could be easily produced at industrial level, and most importantly even immunosuppressed individuals can profit from this type of

treatment (101). Up to date a limited number of mAbs against enterococci exists in the literature. In a recent study from Rossmann et al., two opsonic mAbs targeting enterococci were developed, exhibiting promising results *in vivo* and *in vitro* (102). In this study, it was also proposed that these mAbs are directed against LTA, providing a limited coverage to the *E. faecalis* serotypes, since anti-LTA antibodies fail to opsonize the CPS-C and -D *E. faecalis* serotypes (34, 102). Two other mAbs targeting the enterococcal proteins Adhesin to collagen (Ace) and the major component of pili (EbpC) are also in preclinical phase (103, 104). The effectiveness of these mAbs against non-encapsulated *E. faecalis* strains provides further support for the development of mAbs against enterococcal infections. In addition, further research has to be performed in the selection of the immunogenic targets for the development of mAbs since the major barrier of mAb development is the antigenic heterogeneity of clinically relevant pathogens (105). The potential of this type of treatment has also been proven by the evaluation of human hyperimmune globulin preparations, which contain opsonic and protective antibodies against multidrug resistant Gram-positive and -negative bacteria (106).

1.8 Aim of the thesis

The intrinsic and acquired resistance of enterococcal species to most commonly used antibiotics has contributed to a high frequency of isolates in European and US hospitals. The overall aim of this PhD was to develop alternative treatments against multidrug resistant enterococci. For this purpose, active (Publication I) and passive (Publication II) immunotherapy regiments were developed.

In the first publication, we evaluated the potential use of two enterococcal proteins, SagA and Ppic, as antigens and carriers for enterococcal carbohydrate immunogens in order to develop cross-species vaccines against enterococci. As it has been discussed above, both of these antigens have been proven to be good immunogens for the development of vaccines against enterococci (Section: *Enterococcal proteins*). The increasing demand for multivalent vaccines and vaccine co-administration can reduce the efficacy of the glycoconjugate vaccines by affecting the immune response, e.g. by CIES or bystander interferences (107). This obstacle can be overcome by the use of alternative carrier proteins that could furthermore contribute in the development of vaccines targeting several virulence factors of pathogens (107). The incorporation of multiple bacterial components, e.g. toxoids, capsular polysaccharides, and cell-wall associated proteins, could contribute in the development of an effective vaccine (108). Addressing this issue in the first publication we semi-synthesized the glycoconjugates of SagA and PpiC with the enterococcal capsular polysaccharide DHG and we evaluated them in rabbits. Since a protective immune response against enterococci is mediated through PMNs and requires both antibodies and complement, the opsonophagocytic assay is a reliable *in vitro* method to be used as a surrogate of protective immune response in order to evaluate the efficacy of enterococcal vaccines (25, 47, 109).

For this purpose, the sera raised in rabbits upon immunization with the two glycoconjugates were evaluated by opsonophagocytic assay against several enterococcal strains. In addition, the cross-reactivity of the sera was evaluated in whole cell ELISA for enterococcal strains that could not be evaluated in the opsonophagocytic assay. Animal studies were performed to assess whether the elicited antibodies were able to confer protection in mice challenged with the respective bacteria. These results confirmed that the antigens DHG-PpiC and DHG-SagA were able to elicit opsonic and protective antibodies targeting both their components. Moreover, the glycoconjugates from this project could not only serve as vaccine candidates, but could also be used in the second part of this PhD as immunogens for the production of antigen specific mAbs in mice (95–97).

The aim of the second part of this PhD was to identify and develop opsonic and protective mAbs that target two well-characterized antigens, DHG and SagA, from *E. faecalis* and *E. faecium*, respectively. These mAbs could be used as a prophylactic therapy against enterococci either as monotherapy or as a supplementary therapy with antibiotics having a synergistic effect on the eradication of enterococcal infections. The antigenic heterogeneity of pathogens isolated from patients limits the successful application of therapeutic mAbs (105). This antigenic variation in clinical pathogens necessitates either cross-reacting mAbs, cocktails of mAbs, or rapid accurate diagnosis prior to administration (105). Despite the fact that the high specificity of the mAbs may limit the coverage that they provide compared to polyclonal sera, mAbs can provide many advantages since they are well-defined reagents with high consistency in production (110). Enterococci have been classified to a limited number of serotypes (Section: *Serotyping of enterococci*) (90). Taking this into consideration, these two newly developed mAbs targeting the CPS-C and -D serotypes of *E. faecalis* (mAb against DHG) and *E. faecium* (mAb against SagA) combined with the previously developed mAb targeting the CPS-A and -B serotypes (mAb against LTA) (102), could provide an extended coverage to enterococcal strains. Other monoclonal antibodies against enterococci in the literature have been tested only against one (103, 104) maximum two enterococcal strains (102), and despite the great importance of these studies, this issue is substantially critical and is addressed in our study. For this project we decided to use hybridoma technology, which is a well-established technique for the generation of mAbs targeting polysaccharide and protein antigens against bacterial pathogens (95, 96, 111–113). For this purpose, mice were immunized with the glycoconjugate DHG-SagA, which was reported in the previous study, in order to elicit opsonic and protective antibodies against both antigens. The opsonophagocytic activity of the mAbs was validated. This test is a good indicator of the mAb activity, since it has been observed that *in vitro* opsonophagocytic activity of mAbs is usually correlated with a protective immune response against bacterial pathogens *in vivo* (114). The affinity and specificity of the two mAbs was evaluated by *in vitro* immunological assays and kinetic studies. Future studies should validate the *in vivo* protective efficacy of these mAbs and attempt to humanize them in order to increase their therapeutic potential. In an

effort to pursue this latter goal we performed sequencing of the variable regions of the mAbs and we validated the synthetic constructs of the heavy and light chain with ELISA and opsonophagocytic assay. Moreover, the elucidation of the protective epitope and the minimal binding requirements of the anti-polysaccharide mAb could contribute to the rational design of structurally defined vaccines that are easily manufactured through synthetic approaches (115). Furthermore, the anti-protein mAb could be used in the epitope mapping of the protein for elucidation of the immunogenic epitopes that should be preserved upon conjugation (98).

Finally, both studies describe the feasible development of therapeutic tools that could be implemented in the eradication of enterococcal infections in the clinical setting. In addition, both studies pursue to combat the antigenic variability of the pathogen by targeting multiple antigens, which according to serotyping, the elucidation of the carbohydrate composition of enterococci, and to our current knowledge regarding enterococcal antigens, could provide a broad protection against these pathogenic strains. Studies in serotyping as well as the examination of a bigger collection of clinically isolated enterococcal strains would provide further insights in the therapeutic potential of these two strategies.

1.9 Contribution to publications

In the first article the main research work was conducted by Dr Diana Laverde and Dr Felipe Romero-Saavedra (first co-authors). I participated in the immunogen preparation (protein purification), the preparation of the sera (sera purification), in the analysis of the results and in the writing of this manuscript by reviewing, correcting and editing the manuscript in all the submissions as well as in all the revision steps. The analysis of the results of this work lead to the use of the same glycoconjugate, DHG-SagA, for the development of the mAbs of the second project. The second article was the main focus of my work and thus lead to my first-author publication. I started this project under the supervision of Prof Dr med Johannes Hübner and Dr Diana Laverde, and I was responsible for the planning, management and implementation of this project. I participated in all the experiments described and in their analysis. The hybridomas were developed during my secondment in the Center of Proteomics, Rijeka, Croatia, under the guidance and assistance of Karmela Miklic and Prof Dr Tihana Lenac Rovis, and the SPR experiments were performed during my secondment in GSK, Siena, Italy, under the guidance of Dr Filippo Carboni and Dr Roberto Adamo. I performed the ELISAs, Western blots and the majority of the opsonophagocytic assays presented in the figures. In addition, I performed the sequencing of the mAbs and I reconstructed them under the guidance of Dr Felipe Romero-Saavedra, and I performed the expression of the recombinant mAbs in the eykaryotic cells and their final evaluation by ELISA and opsonophagocytic assays. Finally, I performed the analysis of all these data as well as writing and submitting the final manuscript presented in this PhD.

2. Publication I

Romero-Saavedra F.*, Laverde D.*, Kalfopoulou E., Martini C., Torelli R., Martinez-Matamoros D., Sanguinetti M., Huebner J.

Conjugation of different immunogenic enterococcal vaccine target antigens leads to extended strain coverage.

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*These authors have contributed equally to this work.

3. Publication II

Kalfopoulou E., Laverde D., Miklic K., Romero-Saavedra F., Malic S., Carboni F., Adamo R., Lenac Rovis T., Jonjic S., Huebner J.

Development of Opsonic Mouse Monoclonal Antibodies against Multidrug-Resistant Enterococci.

Infect Immun. 2019;87

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PERSONAL INFORMATION

Ermioni Kalfopoulou

Sex Female | Date of birth 15/02/1991| Nationality Greece

EDUCATION AND TRAINING

2016- Present
Expected finishing date: December 2019

PHD candidate

Dr. von Haunersches Kinderspital, Ludwig-Maximilians-Universität München

- ESR in Marie-Sklodowska Curie ITN- GLYCOVAX-Rational design of glycoconjugated vaccines
- Thesis: *Production of monoclonal antibodies against gram positive bacteria.*

Training activities:

- Analysis of antibodies in a glycan microarray. Leiden University, Leiden, Netherlands. (October 2017)
- Production of monoclonal antibodies through the hybridoma technology. Center for Proteomics, Rijeka, Croatia (University of Rijeka Medical Faculty). (January-March 2018)

Training in Glaxosmithkline (GSK)

- Kinetic analysis of antibody-antigen interactions using Surface plasmon resonance (March 2019)

2013- 2015

Master's Degree in "Pharmaceutical Biotechnology - Molecular Diagnostics"

School of Pharmacy, Aristotle University of Thessaloniki, Greece

- Grade Point Average 9.25/10 (Excellent)
- Master thesis: *Studies on the folding of recombinant proteins expressed as triple hybrids with the molecular chaperone DnaK.*

2008- 2013

Diploma in Pharmacy

School of Pharmacy, Aristotle University of Thessaloniki, Greece

- Grade Point Average 8.18/10 (4th in a class of 116 students)
- Undergraduate research thesis: *Replacement of the Herpes Simplex Virus type I glycoprotein D gene with the beta-galactosidase gene in the virus genome.*

2012-2013

Pharmacist trainee (Practical training)

- Private Pharmacy (Dimitrios Gourgiotis, Averof 2, Kavala, GR-65302, (9 months)
- Hospital Pharmacy (Theageneio - Anticancer Hospital of Thessaloniki, Al. Simeonidi 2, Thessaloniki , GR- 54007, (3 months)

PERSONAL SKILLS

Mother tongue(s): Greek

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C2	C2	C2	C2	C2
	Certificate of Proficiency in English (University of Michigan) C2 level				
German	B2	B2	B2	B2	B2
	Staatszertifikat über Sprachkenntnisse B2 level				

Communication skills/ scientific writing

- Knowledge transfer and motivational skills acquired by tutoring undergraduate students in lab rotations
- Experience in verbal presentation and negotiation skills gained in the context of undergraduate and postgraduate courses, undergraduate and Master's thesis, conferences, GLYCOVAX meetings, journal club and lab meetings
- Team work and collaboration skills as well as working independently gained as a member of the ITN network GLYCOVAX, LMU Course: "Working in International Teams - Strategies for Intercultural Collaboration" and during my PhD project
- Scientific article writing, Scientific reporting, LMU Course: Grant writing

Organisational / managerial skills / leadership skills

- Ability to plan, prioritize and provide frequent reports in order to achieve deliverables obtained during my PhD which was funded by a grant consisting of work packages and deliverables.
- Scientific conference organization and management. Served as a member of the organizing committee for the 2nd Young Scientists' Forum of the Hellenic Society for Biochemistry and Molecular Biology, Thessaloniki, Greece (2014). Organization of the GLYCOVAX meeting 8-12 October (2018).
- Course: Project management and intellectual property (GLYCOVAX)
- Supervision of undergraduate students performing their undergraduate research thesis.

Research-related skills

- Basic lab skills (working is S2 conditions, solution preparation etc.)
- Nucleic acid technologies- Molecular biology (DNA and cDNA isolation, cloning, PCR, etc.)
- Biochemistry techniques and bacterial protein expression technologies, protein purification methods, SDS-PAGE, western blot analysis, Luciferase assays, etc , as well as protein refolding methods.
- Cell culture techniques (cell line maintenance, monoclonal antibody expression in mammalian cells)
- Migration assay, PBMCs isolation, Opsonophagocytic assay, ELISA
- Production of glycoconjugates
- Development of monoclonal antibodies in mice through the Hybridoma technology
- Antibody sequencing, vector construction, expression in eykaryotic cells, CDR grafting (humanization)
- Antibody-antigen kinetic analysis by Surface plasmon resonance
- Animal handling (TierSchVersV)

Awards and scholarships

2016-19: Marie Sklodowska-Curie fellowship

2014: 2nd best Poster award in the 2nd congress of Pharmaceutical sciences, Patras, Greece.

2013: Greek State's Fellowship Foundation (IKY) Fellowship of excellence for postgraduate students in Greece (IKY-SIEMENS fellowship program).

2011: Scholarship offered by the Department of Studies of Aristotle University of Thessaloniki.

2008: - Scholarship from: the pharmaceutical association of Thessaloniki, the Chamber of Commerce and Industry of Kavala, and Eurobank. Award «ΑΙΕΝ ΑΡΙΣΤΕΥΕΙΝ» (Strive for Excellence) from the IKY offered to students having achieved the best scores in the Entry Exams to Higher education Institutions

Posters

E. Kalfopoulou, K. Miklic, S. Malic, D. Laverde, F. Romero-Saavedra, V. Abramova, T. Lenac Rovis, S. Jonjic, J. Huebner. (2018) Production of mouse monoclonal antibodies against enterococcal polysaccharides. Summer School on Infection Research, 27-31 May 2018,Wernigerode, Germany.

E. Kalfopoulou, D. Laverde, F. Romero-Saavedra, F. Berni, E. Tringou, A. Walther, J. D. C. Codée, J. Huebner. (2018) Analysis of monoclonal antibodies that target polysaccharides. 28th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), 21–24 April 2018, Madrid, Spain.

Kalfopoulou, E.A., Chatsisvili, A., Panagiotidis, C.H. and Panagiotidis, C.A. (2014) A study on the effects of chaperone fusions on protein folding and activity using firefly luciferase fusions with DnaK and GroEL. *FEBS – EMBO 2014 Conference*, August 28-Setember 4, 2014, Paris, France, Abstract 3684.

Publications

Kalfopoulou E., Laverde D., Miklic K., Romero-Saavedra F., Malic S., Carboni F., Adamo R., Lenac Rovis T., Jonjic S., Huebner J. (2019) Development of opsonic mouse monoclonal antibodies against multidrug resistant enterococci, Infection and immunity (Accepted)

Romero-Saavedra F.*, Laverde D.*, Kalfopoulou E., Martini C., Torelli R., Martinez-Matamoros D., Sanguinetti M., Huebner J. (2019) Conjugation of different immunogenic enterococcal vaccine target antigens leads to extended strain coverage, Journal of Infectious Diseases (Accepted) (*These authors equally contributed to this work)

Computer skills

- Good command of Microsoft Office™ tools (Word, Excel, Powerpoint, Access) [ECDL certification], Adobe Photoshop and Illustrator, etc.
- Good command of Bioinformatic tools such as BLAST analysis, Expasy tools, ImageJ, prism etc.