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“My stubbornness came into play, because I’m also not a quitter”

2020 Chemistry Nobel Laureate Jennifer Doudna

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Content

Abstract

Enterococcus faecium and *Staphylococcus aureus,* the Gram-positive pathogens of the ESKAPE group, are known to represent a great threat to human health, especially due to their presence in the healthcare setting where they often present with high virulence and multiple resistances to antibiotics. Together, *S. aureus* and *E. faecium* account for 45% of annual antimicrobial resistance related-deaths in the US.

In this study, we combined several techniques for antigen discovery in an effort to reduce the number of identified proteins to only promising candidates. We used a subtractive proteome analysis that we combined with a false positive analysis of peptides obtained by trypsin shaving. Briefly, the subtractive proteome analysis was conducted on proteins extractions obtained by lysostaphin digestion, SDS boiling or sonication that were run through SDS-PAGE in triplicates. Two of the gels were blotted onto a membrane and detected with either pooled human sera previously tested for the presence of opsonic antibodies, or with the same sera previously depleted of *S. aureus*-specific antibodies. Bands identified with the pooled sera but not with the depleted one were reported to the remaining gel and matching bands were excised for protein identification by mass spectrometry. The false positive analysis was performed using trypsin shaving: bacteria were incubated with or without trypsin in a hypotonic solution and the resulting supernatant was run through mass spectrometry, leading to a list of proteins enriched by the treatment with the enzyme. Combination of the results obtained with both techniques formed a list of 40 potential antigens which was narrowed down to 10 candidates after removal of already described antigens, cytoplasmic and non-immunogenic proteins. We picked five of them: the DUF5011 domain-containing Fe/B12 transporter, and Fe(3+) dicitrate ABC transporter; and two having an amino acid sequence really close to two already discovered antigens in enterococci: PrsA and $AdcA_{au}$. Cross-opsonic effect was investigated and showed that antibodies raised against the two enterococcal antigens can mediate the killing of *S. aureus*. We showed that our selected proteins are able to elicit specific and opsonic antibodies against the *S. aureus* strain MW2. We also demonstrate that PrsA and $AdcA_{au}$ can cross-bind to their homologs and cross-opsonize several *S. aureus, E. faecium* and *E. faecalis* strains.

These findings show that the novel experimental approach for antigen discovery can lead to the identification of promising candidates that can induce the production of opsonic antibodies. We showed that three of the investigated candidates could mediate the killing of *S. aureus*. Also, two of the identified proteins are antigens that could be considered for vaccine formulation against both Gram-positive ESKAPE pathogens.

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List of abbreviations

AB	Ammonium bicarbonate
ABC	ATP-binding cassette
ACN	Acetonitrile
AMR	Antimicrobial resistance
APS	Ammonium Persulfate
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
CA	Community-acquired
CAUTI	Catheter-associated urinary tract infections
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming units
CLABSI	Central line-associated bloodstream infections
CP	Capsular polysaccharide
Cyt	Cytosol
DA-HAI	Device-associated hospital-acquired infections
dHS	Depleted-human sera
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assays
Ext	Extracellular
Fw	Forward
gDNA	Genomic DNA
GI	Gastrointestina
HA	Hospital-acquired
HAI	Hospital-acquired infections
HS	Human sera
I	Immunogenicity
ICU	Intensive-care unit
IgG	Immunoglobulin G
IPTG	Isopropyl-ß-D-thiogalactopyranoside
L	Lysostaphin
LB	Luria Bertani
LFQ	Label-free quantification
LTACH	Long-term acute-care hospitals

MCS	Multiple cloning sites
MDR	Multi-drug resistant
Mem	Membrane
MIP	Macrophage infectivity potentiator
MM	Molecular marker
MRSA	Methicillin-resistant *S. aureus*
MS	Mass spectrometry
NCBI	National center for biotechnology information
Ns	Non significant
OD	Optical density
OM	Outer membrane
OMPBS	Outer membrane bounded periplasmic space
ON	Overnight
ONPG	Ortho-Nitrophenyl-β-galactoside
OPA	Opsonophagocytic assay
OPiA	Opsonophagocytic inhibition assay
P	P-value
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
Per	Periplasmic
PMNs	Polymorphonuclear neutrophils
PPIase	Parvulin-like peptidyl-prolyl cis-trans isomerase protein
PVDF	Polyvinylidene fluoride
RBS	Ribosome binding site
RPMI	Roswell Park Memorial Institute
Rv	Reverse
S	SDS-boiling
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sufalte-PolyAcrylamide Gel Electrophoresis
SEM	Standard error of the mean
SSI	Surgical site infections
SUPRA	Subtractive proteome analysis
TAE	Tris Acetate EDTA
TEMED	Tetramethylethylenediamine
TSA	Tryptic Soy Agar

TSB	Tryptic Soy Broth
US	United States
VAP	Ventilator-associated pneumonia
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *S. aureus*
WHO	World Health Organization
Zn	Zinc

I. Introduction

I.1 Generalities on ESKAPE pathogens

The ESKAPE pathogens group, comprising of *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp*, first described in 2008 (1), clusters bacterial organisms able to "escape" the lethal effect of antibiotics (Figure 1A). They can cause a variety of infections, such as lung, blood, urinary tract or cutaneous infections (2,3). ESKAPE bacteria share many common characteristics. For example, they are all now found in the environment, and they are opportunistic pathogens that are part of the normal microflora (4). All of them present a great concern in the clinical setting, not only because that is where pathogens can spread easily among vulnerable patients, but also because it is where most antibiotic-resistant strains can be found (Figure 1B).

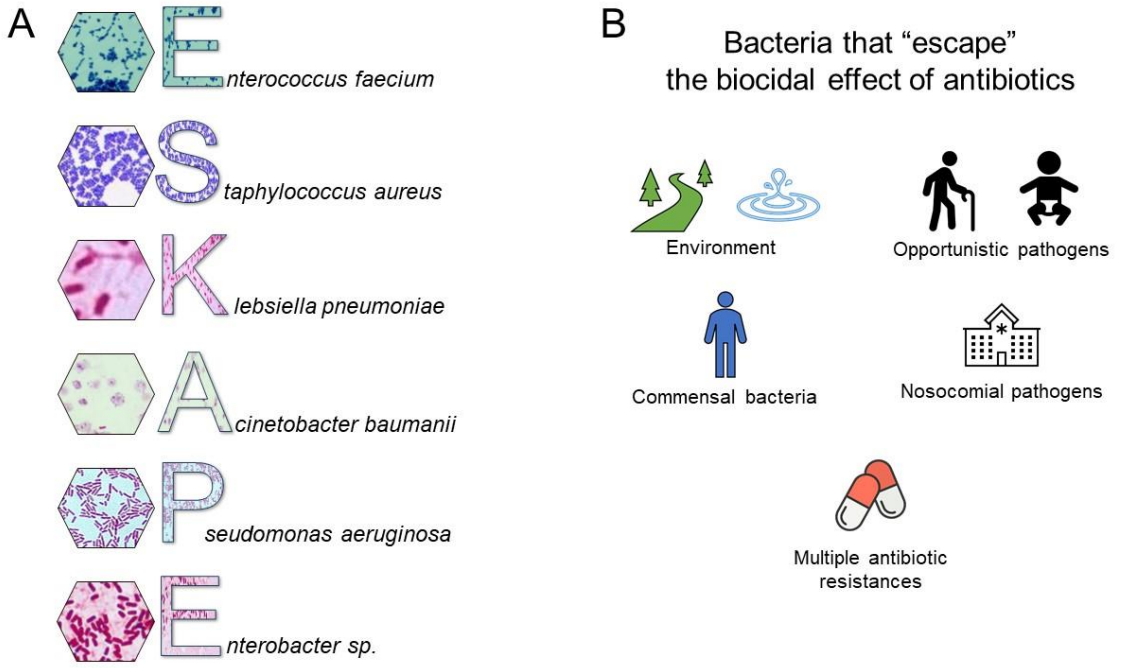


Figure 1. ESKAPE pathogens. (A) Organisms grouped under the ESKAPE acronym. (B) Common characteristics of bacteria pertaining to the ESKAPE group.

I.1.1 Presence in the environment

Bacteria belonging to the ESKAPE group can usually be found in environmental niches and cause community-acquired infections. These bacteria have been commonly isolated from soil, food, water sources, plants or sewage (5). For example, enterococci are easily found in the environment, whether it is in the beach sand, aquatic vegetation, soil or sediments. Their presence in recreational water is even used as an indicator of fetal contamination (6). *S. aureus* can be isolated from a wide variety of places in the community, especially where people gather.

Most of the time, the *S. aureus* found are methicillin-resistant *S. aureus* (MRSA), even if they are isolated from non-hospital related environments (7). Also, multi-drug resistant (MDR) *S. aureus* were isolated from beach water and sand in South Africa (8). The study shows that over 50% of the tested *S. aureus* presented a phenotypic resistance to methicillin and 100% of the isolates showed multiple antibiotic-resistances. MDR *K. pneumoniae* were isolated from several environmental sources, such as rivers and farms (9), MDR *A. baumannii* were found in the soil of damp sites in Croatia (10). As for *P. aeruginosa*, it has been found in multiple environmental habitat such as soil (11), water (12), oil-contaminated environments (13), sinks and drains (14). It can be defined as ubiquitous, or a common soil and water bacterium (15). *Enterobacter spp* are also ubiquitous, found in plants but also in the intestinal tracts of animals, leading to their dissemination to soil, water and sewage (16).

I.1.2 Commensal bacteria

In the early 20th century, the notion of “microbiota” was first explained (17). It was found that microorganisms, especially bacteria, yeasts and viruses were coexisting within the human body (18). Since then, multiple studies have investigated its composition, its implication in our health and the diseases we might face during our lives, as well as how to use it to treat and prevent such diseases (19). It was found that the living bacteria, yeast or viruses were present all over our bodies and the microbiota was even defined as “the hidden organ”, with over 100 trillion microorganisms per person (20). It is now acknowledged that microorganisms profile found in an individual’s microbiota, especially the gut microbiota, can be associated with his health. Different patterns have been associated with healthy people, as well as correlated with diseases including obesity, inflammatory bowel disease, hypertension, autism, cancer and rheumatoid arthritis (21–26).

One characteristic that ESKAPE bacteria share is that they are all found in the human microbiota where they are non-pathogenic but often present with antibiotic resistances (4). Enterococci are mainly found in the gastrointestinal (GI) tract (27,28), but they also colonize the oral cavity and genital tract (29). *S. aureus* is present on the skin and mucous membranes. In fact, humans are the main reservoir for this bacteria (30). It is acknowledged that the bacteria have colonized the nasal cavity of 20-80% of humans (31). These asymptomatic carriers can be divided into two categories: the persistent carriers, which accounts for up to 20% of the world’s population and are colonized with a high bacterial load throughout their entire life; and the non-persistent, who represent the people that were either never colonized (about 20% of the population) or only intermittently with a lower number of bacteria (60%) (32,33). *S. aureus* can also be found in the throat (34), vagina (35) or GI tract (36), though its presence in the anterior nares is the main source of dissemination to other sites via hand transfer (37). *K. pneumonia* colonize the nasopharynx, as well as the mucosal epithelium of the gut (38,39).

The prevalence of gut colonization by *K. pneumonia* varies greatly in between the different world's region: from 4-6% in the US, 40-65% in Senegal and Madagascar and up to 75-87% in Taiwan and Malaysia (40–43). *A. baumannii* is commonly colonizing the GI tract and it can also be found on the skin or in the respiratory tract (44). *P. aeruginosa* could be considered a commensal bacterium, though it generally is not. It is rarely dangerous and often do not cause disease in people with a healthy immune system (45). Oral ingestion of the bacteria do not cause any symptoms in healthy individuals and could even be used as a probiotic to prevent/delay infections in the respiratory tract (46). *Enterobacter spp* also rarely cause diseases in healthy people. It can be found on human skin and in the GI tract of animals and humans (16).

I.1.3 Opportunistic pathogens

ESKAPE bacteria are part of the normal microflora in human and they can be found in a variety of places, from the skin to the vagina or respiratory tract. They can often be found in the GI tract, consistent with the fact that the gut microbiota is the colonization site presenting with the highest numbers of microorganisms, as well with the highest difference in the variety of microorganisms (28). Also, to this date, the gut microbiota is the most studied among scientists, mainly because of its many implications in our health (47–49). Additionally, every bacteria pertaining to the ESKAPE group is either a common environmental bacterium, or has been encountered in the environment. Therefore, our exposition to this pathogenic group is high and constant throughout our lives. Nevertheless, most of the time, contact with these bacteria doesn't lead to the development of infections. This is because the ESKAPE bacteria are opportunistic pathogens (50). They are microorganisms that can cause serious infectious diseases in weaken individuals such as the elderly, newborns or patients presenting with a compromised immune system, or in individuals where a perturbation occurred, whether it is a disease, wound, ageing or surgery (51–53). Opportunists usually arise from the normal microflora or the environment, two places in which the ESKAPE bacteria are typically found. They can be separated into two categories, (i) the commensal opportunists, such as enterococci and *S. aureus*; (ii) the environmental opportunists, like *P. aeruginosa* (54). Studies have shown that the ESKAPE bacteria found in our microbiota or in the environment often contain antibiotic resistance genes or even express a phenotype consistent with antibiotic resistances (55). The combination of those characteristics explains the danger of the ESKAPE group.

I.1.4 Responsible for hospital-acquired infections

Thanks to their opportunistic profile, the ESKAPE bacteria are responsible for many hospital-acquired infections (HAI). They are considered nosocomial pathogens and thrive in clinical

settings, which comprise vulnerable individuals (56,57). HAI are defined as infections acquired during a stay in a hospital or health-care facility. They are often associated with clinical procedures, such as surgeries and invasive procedures, and materials such as prosthetic devices and catheters (58). The different types of HAI are usually categorized into several groups: (i) central line-associated bloodstream infections (CLABSI); (ii) catheter-associated urinary tract infections (CAUTI); (iii) surgical site infections (SSI); (iv) ventilator-associated pneumonia (VAP) (59). A study showed that in the United States (US), between 2011 and 2014, the most common nosocomial infection was CAUTI, with 37.8% of the reported HAI, followed closely by SSI (36.4%), especially abdominal surgeries. CLABSI represented 23.5% and VAP 2.2% (3). A similar study conducted between 2015 and 2017 showed slightly different results with HAI associated with SSI mostly (43.2%), followed by CAUTI and CLABSI, respectively accounting for 29.7% and 25% of the HAI. VAP still being far behind with 2.8% (60). Nosocomial infections represent a great challenge in the clinical setting and are affecting patients considerably, as they have been associated with a higher morbidity and mortality. They also increase greatly the duration of hospital stay and treatments, becoming a financial burden on patients, families, and healthcare systems (61,62). The most common causative agent are bacteria, especially opportunistic organisms such as the ESKAPE bugs. The six microorganisms are among the most frequent bacteria responsible for HAI worldwide (59,63). Five of them are among the top 10 most commonly isolated pathogens associated with HAI in Europe, as reported by a study conducted between 2016 and 2017 (see Figure 2). They detailed the percentage of each bacteria responsible for HAI: *S. aureus* (12.3%), *Klebsiella spp*. (11.4%), *P. aeruginosa* (7.1%), *Enterococcus spp.* (4.8%), *Enterobacter spp.* (2.1%) (64).

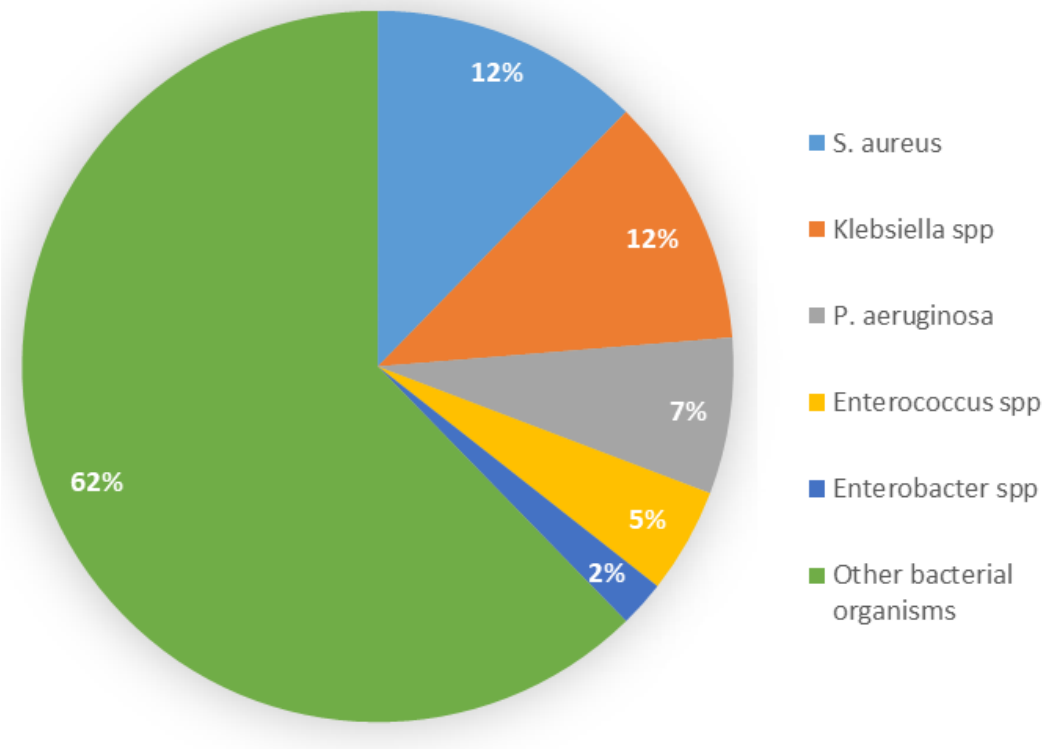


Figure 2. ESKAPE pathogens in HAI. The pie chart shows the percentage of five of the ESKAPE pathogens that were associated with HAI in Europe between 2016 and 2017. Adapted from Suetens et al. 2018 (64).

As previously explained, the main criteria for grouping the ESKAPE pathogens together is their ability to develop antibiotic resistances. This specific characteristic is the one making the ESKAPE bugs so dangerous, especially in the context of nosocomial infections. It has been reported that pathogens responsible for HAI are generally resistant to antibiotics (65).

I.1.1 Antibiotic resistances and global challenge

The discovery of antibiotics was one of the greatest achievement in modern medicine (66). Before humans began to add those molecules to their treatment options, over 50% of the deaths were related to infections (67). This percentage dropped considerably after the introduction of antibiotics in our lives. It is considered that since 1950, the use of antibiotics has saved millions of lives (68). Not only are they used to treat and cure infectious diseases, but they also provide means of infection control, enabling the practice of life-saving medical procedure such as surgeries or organ transplantation (69). In consequence, thanks to the plural influence of antibiotics on global well-being, the health and life expectancy of humans improved dramatically over the 20th century. Between 1900 and 1999, the average human life span grew by 30 years, 25 of which are attributable to advance in public health (70).

However, our extensive use of antibiotics led to the emergence of a worldwide acknowledged threat: the antimicrobial resistance (AMR). Antibiotic-resistant pathogens can be found all over

the world and are responsible for a rising number of infections (71). In 2019, death across the world related to AMR accounted for 1,27 million. It was also reported that by 2025, AMR-related deaths are estimated to rise up to 10 million (72). In 2022, The Lancet published the first comprehensive assessment of the global burden of AMR and report six leading pathogens responsible for 1 million deaths attributable to AMR. Among those six leading pathogens, four are part of the ESKAPE group (*S. aureus, K. pneumoniae, A. baumannii and P. aeruginosa*) (73).

To tackle this rising issue, the World Health Organization (WHO) published in 2017 three lists classifying pathogens that are to be prioritized in the research and development of new antimicrobial treatments (74–76). The Gram-negative ESKAPE pathogens *A. baumannii, P. aeruginosa, and Enterobacter spp* are listed in the first priority list, named “Critical priority”, while the two Gram-positive ESKAPE pathogens *E. faecium* and *S. aureus* are part of the second list annotated as “High priority”. The classification into such lists was performed while taking into account several criteria, including mortality, treatability, transmissibility as well as the ability to prevent infections in the clinical setting and within the community (77).

The risk of AMR infections, as well as the severity of the symptoms induced, are greater in health-care environment where vulnerable patients stay and undergo medical procedures with high infection risks (e.g. surgeries), but the transmission rate is higher than within the community (78). Also, the use of antibiotics is frequent, leading to the thriving of antibiotic resistance development (79). AMR accounts for a rising number of deaths worldwide and especially in the clinical setting, but it is also associated with increases of the hospital stay and treatment costs (80). In the study published in 2020 by Weiner-Lastinger et al, they described the percentage of non-susceptible bugs identified in device-associated HAI (Figure 3). The study shows that 48% of tested *S. aureus* are MRSA, 7% of *E. faecalis* and 82% of *E. faecium* are VRE (vancomycin-resistant *E. faecium*), 21% of *Klebsiella spp* were non-susceptible to ESC, 7% to carbapenes and 13% were MDR, just like 6% of *Enterobacter spp*, 14% of *P. aeruginosa* and 43% of *Acetinobacter spp*.

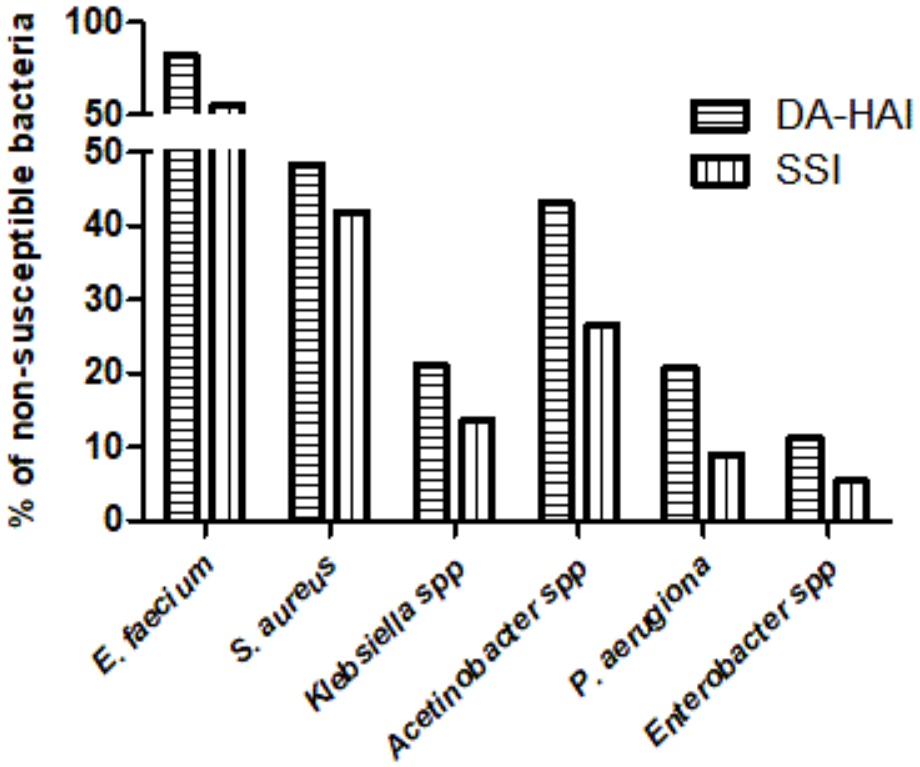


Figure 3. Non-susceptible ESKAPE isolates involved in HAI. The graph shows the percentage of non-susceptible isolates tested in device-associated hospital-acquired infections (DA-HAI) and surgical site infections (SSI) between 2015 and 2017 in the US. The values indicate the percentage of VRE, MRSA, Klebsiella spp resistant to cephalosporins, multi-drug resistant Acetinobacter spp, P. aeruginosa resistant to carbapenems and Enterobacter spp resistant to cefepime. Adapted from Weiner et al. 2020 (60).

Bacterial pathogens, including the ones pertaining to the ESKAPE group, employ different mechanisms to evade the effect of antibiotics. Such mechanisms can include, (i) the alteration of the drug target, for example the modification of topoisomerase enzymes or ribosomal subunits; (ii) the production of biofilms; (iii) production of enzymes degrading/inactivating the antibiotics like the beta-lactamases especially for Gram-positive bacteria; (iv) the expulsion of the drug through efflux pumps that are overexpressed, particularly in the case of Gram-negative pathogens; (v) the decrease of drug uptake by modification of the outer membrane transporters (porins) (81). Given the plurality of mechanisms of resistance that bacteria possess, it is known that ESKAPE pathogens are able to escape lethal effects of several major classes of antibiotics, such as macrolides, tetracyclines, oxazolidinones, β-lactams, lipopeptides, β-lactam-β-lactamase inhibitor combinations, as well as last-line antibiotics such as glycopeptides, carbapenems and polymyxins (79).

I.2 Staphylococcus aureus

I.2.1 Generalities

The word *Staphylococcus* comes from the Greek "*staphyle*", which means grapes, and "*kokkos*" for berry. It was first described in 1880 by the Scottish physician Sir Alexander Ogston who found in pus from a surgical abscess "masses that looked like bunches of grapes" (82). Later, in 1884, a German physician named Friedrich Julius Rosenbach described the *Staphylococcus aureus* (gold in Latin), thanks to its golden colonies (83). First believed to be part of the family *Micrococcaceae*, molecular and phylogenetic analysis revealed many differences and called for a separation of the two families, creating the *Staphylococcaceae* (84). The genus *Staphylococcus* comprises catalase-positive, facultative anaerobic, Gram-positive bacteria, shaped as cocci of 1 µm in diameter, which can be observed in grape-like shape under a microscope. This bacteria does not possess a flagella and do not form spores (85,86). It has a polysaccharide capsule that can help evading the immune system (87). Nowadays, 36 species are recognized within the genus *Staphylococcus*, though the main species encountered are *S. aureus* and *S. epidermis* (88). *S. aureus* can grow with harsh growth conditions, from temperature ranging between 15°C to 45°C, to environments with a high osmolarity, or containing detergents or even alcohol (89). Though its ideal growth conditions are a temperature of 37°C and pH 7.4 (90). Most *S. aureus* strains are hemolytic and can form a transparent ring around the colony on blood agar plates (91). Its identification relies on those specific characteristics and also include deoxyribonuclease tests and the assessment of their ability to ferment mannitol (92).

I.2.2 Pathogenicity

I.2.2.1 Harmless bacteria to dangerous nosocomial pathogen

As explained before, *S. aureus* can be found in the environment or in the normal human microflora. The bacteria can be isolated from a various number of environmental places, such as marine waters, beach waters and sand (93,94). It is also widely distributed in sewage, untreated water or even raw milk (95–97). Its presence in the community can also be linked to dissemination from asymptomatic human carriers that can spread the bacteria, especially in crowded living spaces with poor hygiene and/or lack of sufficient healthcare (98). It has been shown that *S. aureus* can be found in a variety of normal human microflora such as mucous membranes, skin and skin gland, and is even permanently carried in the nasal cavity of about 20% of the population (99).

Dissemination of this opportunistic pathogen can lead to community-acquired (CA) infections that can be particularly menacing when the bacteria present antibiotic resistances like many *S. aureus* do. Indeed, this opportunistic pathogen that can become dangerous and infect susceptible individuals, making it an even bigger threat when infections are hospital-acquired (HA) (100). This pathogen can cause a wide range of infections such as bacteremia, endocarditis, osteoarticular, skin and soft tissue, pleuropulmonary and device-associated infections (101,102). It was reported to be the second most isolated nosocomial pathogen in HA infections in the US between 2015 and 2017 with an identification in 11.8% of adult HAI (60). The same study shows the proportion of each pathogen in the different type of HAI. For CLABSI, *S. aureus* ranked 1st, as the most isolated pathogen associated in hospital wards (15.5%), 3rd in hospital intensive-care units (ICU) and long-term acute-care hospitals (LTACH) with 9.1% and 11.2% respectively. In VAP, *S. aureus* was the most isolated pathogen in hospital ICU (28.8%) and 2nd in hospital wards (20.1%) and LTACH (21.2%). *S. aureus* was also the 1st cause of SSI with 17.5%. The pathogen was also associated with CAUTI for about 2% of infections occurring in hospital wards, ICU or LTACH. Importantly, 48.4% of tested *S. aureus* that were responsible for device-associated HAI, as well as 41.9% of tested pathogens isolated in SSI, were found to be resistant to oxacillin, cefoxitin, or methicillin.

I.2.2.2 Antibiotic resistances

Not only is *S. aureus* responsible for many CA and HA infections, but when it does spread and create a life-threatening infectious disease, it is often difficult to treat as it presents many antibiotic resistances. Even back in 1978, a study already reported that only 7% of tested *S. aureus* strains were still sensitive to penicillin and 40% to cloxacillin (103). These pathogenic bacteria can acquire antibiotic resistances via the transfer of antibiotic-resistance genes but it also possesses intrinsic resistance mechanisms. Some strains, like the aminoglycoside-resistant strains, can reduce the drug uptake by decreasing the outer membrane permeability (104). Others can degrade the antibiotics such as the penicillin, thanks to a large β-lactamase production that can hydrolyze the β-lactam bond present in the molecule, resulting in its inactivation (105–107). Efflux systems are also known as resistance mechanisms naturally present in certain *S. aureus* strains (108,109). The pathogens can also acquire resistance mechanisms via gene transfer, mutation that induce resistance or by forming biofilms. The presence of persistent cells also contributes to the antibiotic resistance dissemination. Acquisition of those resistances by *S. aureus* strains led to the emergence of MRSA, VRSA (vancomycin-resistant *S. aureus*) and other resistant strain that cannot be treated with the newer antimicrobial agents daptomycin and linezolid anymore (110,111).

In 1959, clinicians started to use a modified penicillin, the methicillin, to treat infections by penicillin-resistant bacteria. This new molecule was resistant to the hydrolysis of the β-

lactamase (112,113). But not later than two years after, in 1961, methicillin resistance in *S. aureus* was reported for the first time. While HA-MRSA incidence is decreasing, with 52% of tested isolates related to HAI in the US between 2011 and 2014 and 48% between 2015 and 2017, CA-MRSA infections are rising significantly (3,60,114,115). CA-MRSA are typically acquired via a breach in the skin and are therefore associated with skin and soft tissue infections, while HA-MRSA are generally associated with dangerous conditions such as pneumonia and bloodstream infections (116). However, it is becoming difficult to differentiate CA- and HA-MRSA as some typical CA-MRSA strains are now found in HA bloodstream infections (117,118). Whether the strain is supposedly HA or CA, all MRSA add health care burdens to normal hospital stay with an association with higher morbidity, mortality, length of hospital stay, healthcare costs and quality of life (119). Last-resort treatments started to be used, like vancomycin which became one of the last options to treat MRSA. However, no later than 2002, VRSA appeared, leaving us with very few possible treatments. Additionally, VRSA do not only resist the biocidal effect of methicillin and vancomycin, but can also be resistant to a broad range of antibiotics (120). In 2019, MRSA killed over 121,000 people because of its plurality of antibiotic resistance (121).

I.2.2.3 Immune system evasion

S. aureus also possess a large variety of mechanisms to escape the immune system. In order to undergo the phagocytosis process, performed mostly by phagocytes such as neutrophils or macrophages, the bacteria must be highlighted as a particle to engulf by binding of opsonins to its surface. Opsonins comprises mainly immunoglobulin G (IgG) and the protein iC3b, formed after activation of the complement system. Each of those protein can then be recognized by the Fcγ receptor (for IgG) or integrin CR3 (for iC3b) that are present at the surface of phagocytes. The recognition will enhance the internalization process (122). *S. aureus* owns several immune-modulatory mechanisms that help to prevent the phagocytosis (123). The pathogen produces proteins that can either inhibit the recognition of IgG by the Fcγ receptors, the FLIPr; or even modulate the binding of IgG to the surface of the bacteria, protein A and Sbi. *S. aureus* also secretes proteins that can block the complement's activation, preventing the formation of iC3b. To avoid recognition by molecules from the immune system, the pathogenic bacteria also developed strategies to shield itself, thanks to a capsular polysaccharide structure that surrounds the bacteria; or by secreting the extracellular fibrinogen binding protein, a protein that can bind to both the iC3b that covers the bacterial surface and to fibrinogen, therefore creating a layer of this plasma protein and covering the bacteria (87,124). *S. aureus* typically expresses two capsular polysaccharides (CP), creating the associated serotypes: CP5 and CP8. Both serotypes are constituted of trisaccharide repeating units of N-acetyl mannosaminuronic acid, N-acetyl L-fucosamine and N-acetyl D-

fucosamine but differ because of the linkage type in between the molecules and the sites of O-acetylation (125). Expression of a polysaccharide capsule has been linked to increased virulence and persistence *in vivo* (126). However, its expression and size highly depends on the environment in which the bacteria evolves, and therefore depends on its location within the body (127).

I.3 Enterococcus

I.3.1 Generalities

The word *Enterococcus*, from the Greek "*enteron*", which means gut, was first employed by Thiercelin in 1899 who described a new Gram-positive diplococcus of intestinal origin and capable of causing infection (128). Still in 1899, another organism was identified from a lethal case of endocarditis: the bacteria now known as *E. faecalis* (129). However, several years later, in 1906, its name changed to *Streptococcus faecalis*, thanks to Andrewes and Horder who saw its ability to form short or long chains (130). Enterococci kept being classified as group D streptococci until 1984, creating a long-lasting confusion between the two genus. Thanks to molecular analysis, the two most common enterococci recovered their original names that we still use nowadays: *E. faecalis* and *E. faecium* (131). The genus *Enterococcus*, part of the *Enterococcaceae* family, comprises catalase-negative, facultative anaerobic, lactic acid, Gram-positive bacteria, shaped as non-motile cocci of 0.5-1 µm in diameter, which can be observed under a microscope as single cocci or associated in pairs or short chains (132,133). The bacteria can grow in difficult conditions, surviving a wide pH range (4.6-9.9) and temperatures (5-50°C). Currently, there are 44 species comprised in the *Enterococcus* genus but the most common are *E. faecalis* and *E. faecium* (134).

I.3.2 Pathogenicity

I.3.2.1 Harmless bacteria to dangerous nosocomial pathogen

Enterococcal species are present in the environment, in our microbiota and also in food. They can be isolated from natural sources such as soil, water, sewage or arable land. We can also find them in the digestive systems of humans, animals or insects, as well as in the oral cavity and genital tract (29,135–139). Their presence in fermented products such as cheese, sausages and olives has also been proven and can even be used to enhance flavor taste (140–143). We can find approximately 10^6 to 10^7 Enterococcus in the human intestine, most of them being *E. faecalis* (10^5–10^7 colony-forming units (CFU)/gr feces) or *E. faecium* (10^4–10^5

CFU/gr feces). They help to maintain the pH, participate in the nutrient metabolism, stimulate the immune system and participate in controlling the growth of potential pathogens (144–146).

However, when the fine equilibrium in the microbiota is disturbed, enterococci can become dangerous pathogens. Those commensal opportunists are responsible for a huge variety of infections, from endocarditis, bacteremia, urinary tract and wound infections (133,147,148). They represent a particularly important threat in the healthcare environment. Indeed, a study conducted in the US between 2015 and 2017 reports that *E. faecalis* and *E. faecium* ranked 5th and 8th as the most isolated pathogens in HAI with the two enterococci found in 7.9% and 3.8% of the infections respectively (60). For CLABSI, *E. faecalis* was the most common pathogen in LTACH with 12.1%, it was also associated with 7.6% of infections occurring in the hospital ward and 7.7% in ICU, making it the 4th and 5th most common pathogen for CLABSI in those environments. *E. faecium* was found in 4.8%, 7.2% and 6.4% of CLABSI in hospital wards, ICU and LTACH respectively, making it rank between 6th and 8th. Importantly, it was found to be the 3rd most common pathogen associated with CLABSI in oncology units. For CAUTI, *E. faecalis* was found in about 10% of infections in hospital wards, ICU and oncology services, making it the 4th most observed pathogen. *E. faecium* was isolated less frequently in CAUTI, though it still accounts for 6.7% in LTACH, ranking as the 5th most found pathogen for this category. Both enterococci were found in SSI, *E. faecalis* ranked 3rd (8%) and *E. faecium* 11th (2.9%). Though *E. faecium* was less frequently than *E. faecalis*, it is important to note that 82.1% of tested *E. faecium* in device-associated infections, and 55.6% in SSI, were resistant to vancomycin, while vancomycin-resistant *E. faecalis* were seen in only 7.2% in device-associated HAI and 3.4% in SSI.

I.3.2.2 Antibiotic resistances

Just like the other Gram-positive ESKAPE pathogen, enterococci, and especially *E. faecium*, often present with antibiotic resistances. They possess intrinsic resistance mechanisms such as resistance to penicillin, cephalosporin, low levels of aminoglycosides, and low levels of clindamycin. They can also acquire resistances mechanisms, including resistance to chloramphenicol, erythromycin, high levels of clindamycin, tetracycline, high levels of aminoglycosides, penicillin by means of penicillinase, fluoroquinolones, and/or vancomycin (149,150). VRE represent a huge concern in healthcare environment and have been identified as the most common MDR enterococci in the clinical setting (151). New therapeutics are now used to combat VRE, for example daptomycin, which is the antibiotic of choice against vancomycin-resistant enterococci (152). However, daptomycin-resistant enterococci have already been observed in hospital and in the environment (153,154). While CA-VRE infections are rarely reported, it has already been observed via a burn wound in Malaysia (155).

I.4 Treatment options against *S. aureus* and enterococci

I.4.1 Antibiotics

As previously explained, *S. aureus* and enterococci possess intrinsic resistance mechanisms and are also masters at acquiring new antimicrobial resistances. *S. aureus* was rapidly resistant to penicillin so the methicillin was introduced, but its effectiveness lasted all but two small years before MRSA emerged. Methicillin-susceptible *S. aureus* can still be treated with drugs in the β-lactam class, such as cephalosporins, oxacillin or mafcillin. However, for MRSA, treatments include the use of vancomycin, which also rapidly became ineffective. Since then, several new classes of antibiotics have been studied and are either already commercialized or under study in clinical trials (79). VRE emerged in the 1980s in the US and were associated with the introduction of third-generation cephalosporins. Since then, the antibiotic-resistant pathogen has spread all over the world. VRE can still be treated, however the handling of infected patients calls for specific and expensive measures such as isolation rooms, contact precautions and dedicated room cleaning. Second-line antibiotics can still be used against VRE (tigecycline and daptomycin), although their costs and risk of toxicity are higher than routinely used antibiotics.

The pharmaceutical industry invests less and less in the discovery of new drugs as finding new potent molecules is still possible, but much less frequent than before the 1960s. However, new hope can be placed in the repurposing of already existing drugs. Indeed, this strategy helps reducing the time, cost and risk associated with the investigation of otherwise newly discovered molecules (79). However, development of AMR will always be a lingering threat (156).

I.4.2 Non-drug therapies

To overcome the rising antibiotic resistances, new antimicrobial therapies were studied in the pre-clinical and clinical research and development. Among other, the use of bacteriophages, fecal microbiota transplantation and vaccines are the most promising alternatives to antibiotics.

I.4.2.1 Bacteriophages

Bacteriophage discovery happened in 1915 by William Twort and their first use of lytic bacteriophages was first applied in 1917 by Felix d'Herelle. Primarily disregarded in profit of the newly discovered antibiotics, their potential use is now investigated (157). The idea is to use those biological agents to selectively destroy the targeted bacteria. The phage acts as a "virus for bacteria"; it can infect specifically a host, replicate inside this same host and, induce

its lysis while releasing virion progenies that can restart the infection-lysis cycle all over again (158). Several phages have already been successfully used as therapies against *S. aureus* infections and are described in case reports, especially in bone and joint infections (159), skin and soft tissue infections (160) or in heart and pulmonary infections (161). Mainly because of the lack of laws framing the research on phages, very few clinical trials using phages against infections by *S. aureus* were and will be performed in the near future. For enterococci, different case reports have shown the effects of bacteriophage therapy against the pathogen but very few clinical trials are being carried out (162). So far, no serious adverse effects were observed, confirming the use of bacteriophages as a good option against infectious diseases. However, the emergence of resistance to bacteriophage therapies is a possibility that must be considered. Several strategies can be used to prevent this outcome, like the combination of phages and antibiotics that would kill the pathogens faster than they can replicate (and therefore develop and pass on to daughter cells a resistance mechanism). Another aspect to consider is the potential immune response developed against phage antigens. Phage immunogenicity depends greatly on the phage type, dosage, mode of administration and especially the immunological condition of the host. Little information is available, making the subject a source of conflicting opinions (163).

I.4.2.2 Fecal microbiota transplantation

When treating a patient with broad-spectrum antibiotics, most susceptible bacteria in the microbiota are eradicated. This is particularly true for patients in LTACH that end up with an altered microbiota. Eradication of antibiotic-susceptible bacteria leaves the space for antibiotic-resistant bacteria to thrive. The idea behind fecal microbiota transplantation is to protect against this colonization and a study showed that patients which received this treatment present with a decrease in GI tract colonization by VRE (164).

I.4.2.3 Vaccine development

Vaccination was used before we even knew the biological mechanisms it relies on. In the 1100s, the variolation technique was used in Turkey, Africa, China and Europe. After observing the smallpox survivors never got infected again, physicians started to inoculated children with dried scab material from infected patients. Much later, in 1798, Edward Jenner continued the quest for immunity against smallpox and postulate that protection could be obtained by inoculation with a related and much less dangerous virus: cowpox. He showed that inoculation of an 8-year old boy with liquid recovered from a pustule of a milkmaid offered protection against smallpox. Over 80 years later, Louis Pasteur created the first live attenuated bacterial vaccine. Ever since, vaccine formulations have evolved dramatically, and the source of the

pathogen that we want to be immune from can now be as small as mRNA or proteins and polysaccharides (sub-unit vaccines) (165).

The principle of vaccination rests on the activation of the immune system by an antigen and the creation of an immunological memory. The purpose is to mimic an infection that elicits an immune response and the consequent immunological memory. This memory will help when our body encounters the pathogen again and shorten the associated response, enhancing the immune system to act fast (166). Vaccination is considered one of the most effective method against infections. Its success to prevent polio, smallpox, measles, diphtheria, tetanus, rabies, etc., testify to its potential at reducing the global burden of infectious diseases (167). The scientific and medical community places great hope in vaccine development to combat AMR. However, no effective vaccine formulations are available against ESKAPE pathogens. Many candidates have been studied in clinical trials, but all failed to elicit a protective effect (168).

I.4.2.3.1 Vaccine formulations enrolled in clinical trials

Recent advances in the landscape of *S. aureus* vaccine development comprise the current assessment of four vaccine formulations in clinical trials (see Table 1). However, an important event was the failure of Pfizer's formulation SA4Ag in phase IIb. The formulation contained four staphylococcal antigens, the proteins MntC and ClfA and the capsular polysaccharide CP5 and CP8, all conjugated to a detoxified form of diphtheria toxin (169,170). In the phase I, a single dose could elicit high titers of antibodies whose functionality was successfully proved by opsonophagocytic assays. Yet unfortunately, the formulation did not prove any reduction in BSI, SSI or mortality (171). Other vaccines failed to pass phase II of the clinical trials, like V710 or StaphVax (172,173). Despite the identification of several antigens in enterococci, no clinical trials are currently on-going.

Table 1. Vaccine formulation against S. aureus currently tested in clinical trials.

Clinical trial number	Vaccine name	Composition	Phase	Company
NCT04420221	SA-5Ag	ClfA, Hla, SpA, CP	I: Recruiting	GSK
NCT00974935	1. Stebvax 2. IBT-V02	1. SEB + alum 2. SEB, SEA, TSST-1, LukS, LukF, LukAB, Hla + alum	I: Completed I: Scheduled	Integrated Biotherapeutics
NCT03455309	NDV-3A	Als-3 (C. albicans cross reactive cell wall protein) + Alum	II: Ongoing	Novadigm Therapeutics
NCT03966040	rFSAV	Hla, SpA, SEB, IsdB, MntC + Alum	II: Ongoing	Olymvax

I.4.2.3.2 Antigens identified in *S. aureus* and enterococci

Antigens are molecules that can trigger an immune response and be recognized by specific antibodies. They can be of various chemical background such as proteins, lipids or glycoconjugates. Table 2 presents a non-exhaustive list of vaccine candidates of protein origin against *S. aureus* and enterococci (174,175).

Table 2. Vaccine antigens of protein origin against S. aureus and enterococci.

Name	Description	Reference
***S. aureus* antigens**		
ClfA	clumping factor A	(176)
ClfB	clumping factor B	(176)
CNA	collagen adhesin	(177)
EsxA	Ess extracellular A	(178)
EsxB	Ess extracellular B	(178)
FnBPA	fibronectin binding protein A	(179)
FnBPB	fibronectin binding protein B	(179)
Hla	alpha-hemolysin	(180)
IsdA	iron-regulated surface determinant protein A	(181)
IsdB	iron-regulated surface determinant protein B	(181)
LukF	leukocidin-F	(182)
LukS	leukocidin-S	(182)
MntC	manganese transport protein C	(183)
SdrC	serine-aspartate repeat protein C	(184)
SdrD	serine-aspartate repeat protein D	(184)
Enterococcal antigens		
Ace	collagen adhesin	(185)
Acm	collagen adhesin	(186)
$AdcA_{fm}$	zinc ABC transporter substrate-binding lipoprotein	(187)
DdcP	D-alanyl-D-alanine carboxypeptidase	(188)
EbpA	endocarditis- and biofilm-associated pili A	(189)
GelE	gelatinase	(190)
LysM	peptidoglycan-binding protein	(188)
PBP5	penicillin-binding protein 5	(188)
PpiC	peptidyl-prolyl cis-trans isomerase	(188)
$PsaA_{fm}$	manganese ABC transporter substrate-binding lipoprotein	(187)
SagA	secreted antigen a, bacterial growth and biofilm formation	(191)

II. Context and objectives

II.1 Context

ESKAPE pathogens are a great concern for the global health of our society. Because of their presence in the environment and the normal human microbiota, they can easily disseminate and infect weakened individuals. Those opportunistic pathogens threaten the integrity of our health and their presence in our hospitals can disrupt our safety while receiving care. Adding to those characteristics, they have the ability to escape the biocidal effect of antibodies, which enhance their dangerousness (79).

Since their introduction in our treatment procedures, antibiotics have saved millions of lives. Their discovery is without doubts one of the greatest achievements in modern medicine. However, AMR emerged really quickly and is now widely spread. Clinically available therapies can often be ineffective, especially when treating infections caused by ESKAPE pathogens. The use of broad-spectrum antibiotics is often the only choice as we still lack rapid diagnostic methods to identify infective agents and the presence of AMR genes. Unfortunately, in the case of infections with AMR bacteria, such treatment usually selects resistant bacteria over sensitive ones, contributing to the rise of resistances (73).

While it is clear that urgent solutions must be provided to win back our fight against bacteria, we are still unsure on how to proceed for our next steps. Many hopes can be placed in the discovery of novel antimicrobial molecules, but while we kept discovering more and more drugs during the golden era of antibiotic discovery, between 1945 and 1980, we have entered a “discovery void” and are now struggling to identify new candidates. Bacteriophage therapy, which use phages that can selectively destroy bacteria, has shown some promising results. It presents many advantages such as pathogen selectivity, few side effects and self-multiplication at the infection site. However, the production processes are long, as well as safety control for commercialization. Also, the potential development of AMR will always be a pending threat for these two strategies (153).

We believe that another great achievement in modern medicine can be the answer to this rising problem: vaccines. We strongly think that prevention of bacterial infections by vaccination will not only create a safer environment for every individual in the world, but also prevent the use of antibiotics and the ensuing development of AMR. Vaccination has been used routinely in the last decades and has proven its effectiveness in combatting infectious diseases (168).

Identification of potent vaccine antigens is the key to develop effective vaccine therapies. Not only can they be placed in a vaccine formulation to induce an active immunity but they could also be the target of monoclonal antibodies that can provide immediate protection to high-risk patients (168).

II.2 Objectives

This study aimed to work on novel techniques to identify potential vaccine antigens against *S. aureus* and investigate the potential of selected candidates to prevent infections.

In order to narrow down the list of identified proteins to only those that show promise, we combined a number of antigen discovery approaches in this work. We integrated a false positive analysis of peptides generated by trypsin shaving with a subtractive proteome analysis. To summarize, proteins extracted using lysostaphin digestion, SDS boiling, or sonication were processed in triplicate through SDS-PAGE (Sodium Dodecyl Sufalte-PolyAcrylamide Gel Electrophoresis) and underwent a subtractive proteome analysis. Two of the gels were blotted onto a PVDF (polyvinylidene fluoride) membrane and detected using either pooled human sera that had previously been examined for the presence of opsonic antibodies or the same sera previously depleted of *S. aureus*-specific antibodies. Bands that were detected by the pooled serum but not by the depleted one were reported on the remaining gel, and bands that matched were excised in order to perform protein identification by mass spectrometry (§ IV.1.1.1). Trypsin shaving was used to do the false positive analysis. Bacteria were placed in a hypotonic solution with or without trypsin, and the resulting supernatant was subjected to mass spectrometry (§ IV.1.1.2). A comparison between the proteins identified in the treated and non-treated samples led to a list of proteins that were enriched by the enzymatic treatment. A list of 40 putative antigens was obtained by combining the results of the two approaches and narrowed down to 10 candidates after removal of proteins that were cytoplasmic, non-immunogenic, and previously identified antigens (IV.1.1.3). Five candidates were picked according to the criteria explained in § IV.1.3. The proteins were recombinantly produced and purified to perform the immunization of rabbits (§ III.6.2). A study of the effectiveness of the selected candidates was conducted by an immunological study of polyclonal sera: antibodies were assessed for binding and specific opsonic functions (§ IV.3).

III. Materials and Methods

III.1 Material

Table 3. Chemicals and reagents.

Item	Company
Bacterial media: Brain Heart Infusion (BHI), Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB), Luria Bertani (LB)	Carl Roth
Kanamycin	Sigma
Ampicillin	Sigma
Tris Base	Carl Roth
Acetate	Carl Roth
Ethylenediamine tetraacetic acid (EDTA)	Sigma
Midori Green	Nippon
Generuler 1kb DNA Ladder	Thermo Fisher Scientific
Glycerol	Sigma
Phosphate Buffer Saline (PBS)	Merck Millipore
Sucrose	Carl Roth
Sodium Dodecyl Sulfate (SDS)	Sigma
Loading buffer 5X	Sigma
Coomassie blue	Sigma
Acrylamide	Sigma
Ammonium Persulfate (APS)	Sigma
Tetramethylethylenediamine (TEMED)	Sigma
Glycine	Carl Roth
Methanol	Sigma
Bovine Serum Albumin (BSA)	Carl Roth
Milk powder	Carl Roth
Tween 20	Sigma
Ammonium bicarbonate (AB)	Sigma
Acetonitrile (ACN)	Sigma
Formic acid	Sigma
Trifluoroacetic acid	Sigma
Isopropyl-ß-D-thiogalactopyranoside (IPTG)	Carl Roth
NaH_2PO_4	Merck Millipore
NaCl	Carl Roth
Imidazole	Merck Millipore
Ortho-Nitrophenyl-β-galactoside (ONPG)	Sigma

Table 4. Kits, enzymes antibodies. PCR, Polymerase Chain Reaction; ECL, Enhanced chemiluminescence.

Item	Company
MasterPure™ Gram Positive DNA Purification Kit	Biozym
Q5 High-Fidelity DNA Polymerase kit	New England Biolabs, NEB
Wizard SV Gel and PCR Clean-Up System	Promega
Restriction enzymes: BamHI, KpnI	NEB
Monarch PCR and DNA Cleanup kit	NEB
ElectroLigase	NEB
GoTaq G2 Hot Start Master Mix kit	Promega
PureYieldTM Plasmid Miniprep System	Promega
Lysostaphin	Sigma
Trypsin	Promega
PierceTM ECL Western kit	Thermo Fisher Scientific
Goat anti-human IgG coupled with horseradish peroxidase (HRP)	Southernbiotech
Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G	Sigma

III.2 Bacterial strains and cultures

The project used a variety of strains from *S. aureus*, *E. faecalis*, *E. faecium* and *Escherichia coli* species. Each strain and a brief description are detailed in Table 5.

We used BHI plates and broth to grow *S. aureus* at 37°C. Liquid cultures were agitated to enhance the growth speed. Enterococci were grown in TSB and TSA at 37°C without agitation. *E. coli* M15 containing pREP4 were cultivated in LB and grown at 37°C under agitation. To keep the pressure on pREP4, kanamycin was added to the media at 25 µg/ml. After transformation with pQE30, ampicillin was added at 100 µg/ml to the culture for selection of positive transformants.

Table 5. Bacterial strains used in this study. CA, Community-acquired; MRSA, Methicillin-resistant S. aureus; VRE, Vancomycin-resistant enterococci.

Strains	Description	Reference
Staphylococcus aureus		
S. aureus MW2	CA-MRSA isolate from the USA (North Dakota)	(192)
S. aureus LAC	CA-MRSA isolate from the USA (Los Angeles)	(193)
S. aureus Reynolds	Clinical isolate	(194)
S. aureus MN8	Clinical isolate	(195)
S. aureus 194	Isolate from food environment	(196)
S. aureus SF8300	CA-MRSA isolate from the USA (San Francisco)	(193)
Enterococci		
E. faecium VRE11236/1	VRE isolated from a patient in Germany (Munich)	(197)
E. faecalis 12030	Isolated from a patient in the USA (Cleaveland)	(198)
E. faecalis Type 2	Isolated from a patient in Japan (Sapporo)	(199)
Escherichia coli		
E. coli M15 pREP4	M15 harboring pRep4 plasmid	Invitrogen
E. coli M15/pQE30PpiC	M15 harboring pRep4 and pQE30PpiC plasmids	(188)
E. coli M15/pQE30AdcA$_{fm}$	M15 harboring pRep4 and pQE30AdcA$_{fm}$ plasmids	(187)
E. coli M15/pQE30PrsA	M15 harboring pRep4 and pQE30PrsA plasmids	This study
E. coli M15/pQE30AdcA$_{au}$	M15 harboring pRep4 and pQE30AdcA$_{au}$ plasmids	This study

III.3 Molecular biology

III.3.1 Extraction of genomic DNA from *S. aureus* MW2

Genomic DNA (gDNA) was extracted for *S. aureus* MW2 using the commercial kit MasterPure™ Gram Positive DNA Purification Kit and following the manufacturer's instructions.

III.3.2 Cloning

III.3.2.1 Genes of interest

The genes sequences encoding for potential antigen candidates in *S. aureus* MW2 were retrieved from the National Center for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov/gene/).

III.3.2.2 Plasmid pQE30

The plasmid pQE30 from Qiagen (Figure 4) was used because of its ability to overexpress 6xHis-tagged proteins. The vector presents ideal features for production of recombinant proteins. It contains an optimized promoter–operator element which consists of phage T5 promoter that can be recognized by the RNA polymerase from *E. coli* and two lac operator elements which increase lac repressor binding and ensure efficient repression of the powerful T5 promoter in absence of IPTG. It also has a ribosome-binding site (RBS) placed ahead of the start codon, the his-tag and the multiple cloning site (MCS) which allows the insertion of the gene of interest. The presence of the ampicillin resistance gene also permits the successful selection of bacteria effectively transformed. The plasmid was used to clone the genes encoding for the proteins PrsA (UniProt number: P60749), $AdcA_{au}$ (UniProt number: A0A0H3K1H9), the DUF5011 domain-containing protein (Uniprot number Q2FZJ7), Fe/B12 transporter (Uniprot number Q2FVW9) and Fe(3+) dicitrate ATP-binding cassette (ABC) transporter (Uniprot number Q2FW75).

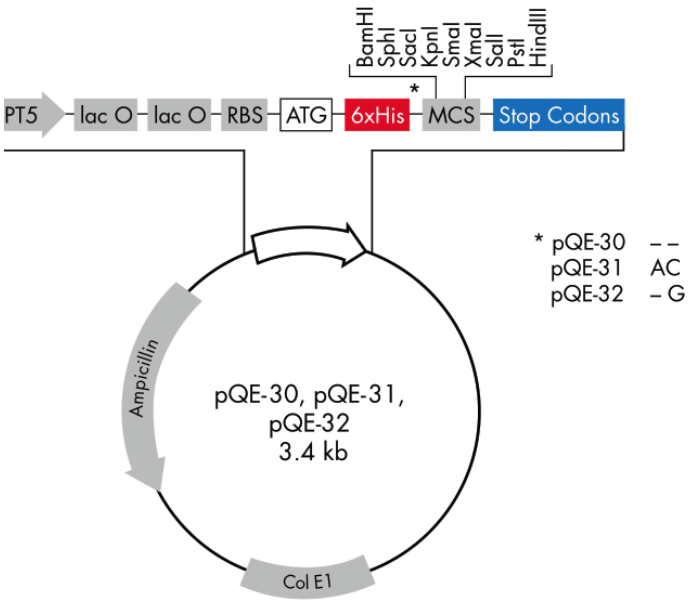


Figure 4. Plasmid pQE30 from Qiagen. PT5, T5 promoter; lac O, lac operator element; RBS, ribosome binding site; ATG, start codon; 6xHis, tag composed of six histidines; MCS, multiple cloning sites.

III.3.2.3 Primers

The Table 6 presents the primers purchased in Eurofins. Some were used to get amplicons from the gDNA of MW2 and effectively create an expression vector containing the genes of interest; and others were used to check the integrity of the insert.

Table 6. Primers used in this study. Fw, forward; Rv, reverse. Bases in orange lowercase letters correspond to the restriction site. Underlined green bases are not complementary to the target sequence.

Name	Sequence 5'-3'	RS	Use
PrsA_ BamHI_Fw	GACTCAggatccGCTTGTGGCGCTAGTGCC	BamHI	Production of the recombinant protein PrsA
PrsA_ KpnI_Rv	GACTCAggtaccTTATTGGCTCATGCCGGATTG	KpnI	Production of the recombinant protein PrsA
$AdcA_{au}$_ BamHI_Fw	GACTCAggatccTGTGGGAATGATGATGGAAAAG	BamHI	Production of the recombinant protein $AdcA_{au}$
$AdcA_{au}$_ KpnI_Rv	GACTCAggtaccTTAATGCGCTAACATTTCTTCTTTG	KpnI	Production of the recombinant protein $AdcA_{au}$
pQE30_Fw	GTGAGCGGATAACAATTTCAC	-	Verification of the insert's integrity
pQE30_Rv	GAGTTCTGAGGTCATTACTG	-	Verification of the insert's integrity

III.3.2.4 Amplification of the genes by polymerase chain reaction

The genes encoding for our candidate antigens were amplified by polymerase chain reaction using the Q5 High-Fidelity DNA Polymerase kit and following the manufacturer's instructions. 10 ng of gDNA were used as template DNA primers were added at a final concentration of 0.5 µM. The PCR products were run by electrophoresis in a 1% agarose gel in Tris Acetate EDTA buffer (TAE: 40 mM Tris Base, 0.1% Acetate, 1 mM EDTA) containing 1 µl of Midori Green at 120 V and 400 mA during 35 min (200). The size of amplicons was verified using Generuler 1 kb DNA Ladder. Successfully amplified PCR products were purified using Wizard SV Gel and PCR Clean-Up System following the manufacturer's instructions.

III.3.2.5 DNA digestion by endonucleases

Inserts and vectors were digested by the corresponding restriction enzymes for one hour at 37°C following the manufacturer's instruction. After the enzymatic digestion, products were purified with the Monarch PCR and DNA Cleanup kit following the manufacturer's instruction.

III.3.2.6 Ligation

Digested and purified vector and inserts were mixed at a ratio 1:3 and ligated using the ElectroLigase following the manufacturer's instruction. The ligation was conducted for an hour at room temperature and the reaction was stopped by inactivation for 15 minutes at 65°C. To assess the yield of the ligation, a 1:10 dilution of the ligated products was run by PCR with GoTaq G2 Hot Start Master Mix kit and primers at a final concentration of 0.5 µM.

III.3.3 Transformation

III.3.3.1 Preparation of electrocompetent *E. coli* M15

The electrocompetent *E. coli* cells were prepared following a previously described protocol (201). Briefly, 500 ml of liquid LB media were inoculated with 10 ml of an overnight culture, placed at 37°C under agitation and let to grow until the culture reached an optical density at 600nm (OD_{600nm}) of 0.8. The bacterial suspension was centrifuged for 30 minutes at 5,000 x g. The pellet was washed twice with 50 ml and then 250 ml of cold sterile ultra-pure water, followed by two washes with 100 ml of cold sterile glycerol 10%. Finally, cells were resuspended in 1 ml of cold sterile glycerol 10% and aliquoted in 100 µl portions before storage at -80°C.

III.3.3.2 Electroporation of competent *E. coli* M15

E. coli M15 were transformed with pQE-30 previously modified to contain the gene of interest. 100µl of electrocompetent cells and 2 µl of the ligated plasmid were mixed. Bacteria were transformed by electroporation with BTX Harvard Apparatus using a resistance of 200 Ω, a capacitance of 25 F, and a voltage of 2.5 kV in an ice-cold cuvette (2 mm, ROTH). Immediately after the pulse, bacteria were incubated for 45 min at 37°C in LB with agitation at 220 rpm.

III.3.3.3 Selection of positive clones

Transformants were plated on LB agar containing ampicillin and kanamycin. After an incubation overnight at 37°C, grown CFU were screened to test the presence of the modified plasmid. Each CFU were resuspended in 10 µl of LB containing antibiotics and used as a DNA source for PCR. The GoTaq G2 Hot Start Master Mix kit was used with the same parameters and primers described before. PCR products were analyzed by electrophoresis with a 1% agarose. Liquid cultures of positive colonies were incubated overnight (ON) at 37°C with shaking.

III.3.3.4 Verification of the insert's quality

To verify that the inserts cloned into the plasmid did not undergo any mutation during the cloning process, plasmid preparation were performed from the selected positive transformants using the PureYieldTM Plasmid Miniprep System following the manufacturer's instructions. Minipreps were sent to Eurofins Genomics who performed the sequencing based on the Sanger method (202). After assessment of the correct insertion of the insert and integrity of the sequence, the clones were stored at -80°C until further use.

III.4 Biochemical methods

III.4.1 Protein extraction methods

III.4.1.1 Protein extraction by lysostaphin

A protocol already described was adapted and followed (203): *S. aureus* MW2 and MN8 were grown in 200 ml of BHI, inoculated with an overnight liquid culture diluted at OD_{600nm} of 0.05. Once OD_{600nm} reached 0.6, the bacterial culture was harvested by centrifugation at 5,000 x g for 10 minutes at 4°C. The pellet was washed twice in cold PBS, followed by one wash with cold digestion buffer (PBS, 30% sucrose). Bacteria were resuspended in 2 ml of cold digestion buffer containing 200 µg of lysostaphin and incubated for one hour at 37°C under light agitation. Supernatant-containing protein was obtained by centrifugation at 2,500 x g for 15 minutes at 4°C and clarified of potential residues by centrifugation at top speed for two minutes and filtration with 0.22 µM filters (Carl Roth).

III.4.1.2 Protein extraction SDS boiling

Preparation of protein extracts by SDS boiling was performed following a protocol already described by Nandakumar et al in 2005 (204). Briefly, bacteria were grown in 100 ml of BHI until mid-exponential phase (OD_{600nm} 0.6). Cultures were harvested and washed twice with cold PBS by centrifugation at 5,000 x g for 10 minutes at 4°C. The pellet was resuspended in extraction buffer (PBS pH 8.0 + 2% SDS) at 10 µl per milligram of wet pellet. The suspension was heated at 95°C for three minutes and then placed back on ice. The proteins were isolated from the cells by centrifugation at 5,000 x g for 15 minutes at 4°C. The supernatant was centrifuged at top speed and run through a 0.22 µM filter to be clarified of potential residues.

III.4.1.3 Total protein extraction by sonication

A procotol already published in PLoS One was adapted to perform the total protein extractions by sonication (205). 100 ml of bacterial culture to mid-exponential phase were harvested by centrifugation at 5,000 x g for 10 minutes at 4°C. The pellet obtained was washed twice in cold PBS and resuspended in 4 ml of cold PBS. The suspension was sonicated at 30% amplitude (750-Watt Ultrasonic processor) for a duration of 20 minutes including on/off pulses of 9 seconds. Centrifugation at 10,000 x g for 20 minutes at 4°C was used to separate cell residues to extracted proteins. Further clarification was performed by filtration through a 0.22 µM filter.

III.4.1.4 Trypsin shaving

Preparation of peptide extracts by trypsin shaving was performed as previously described (206). Briefly, 40 ml of bacterial culture was cultivated from OD_{600nm} 0.05 to 0.6. Cells were harvested, washed twice with cold PBS and once with cold digestion buffer (PBS, 30% sucrose) thanks to centrifugation at 5,000 x g for 15 minutes at 4°C. The pellet was resuspended in 2 ml of cold digestion buffer and the subsequent bacterial suspension was separated in two equal volumes. One volume was kept as it was and the other was used to resuspend 20 µg of trypsin. Both tubes were incubated for 15 minutes at 4°C with light agitation. Supernatants were collected by pelleting bacteria by centrifugation at 1,000 x g for 15 minutes at 4°C. Potential residues were removed by centrifugation of the supernatants at top speed and by filtration through a 0.22 µM filter.

III.4.2 Subtractive proteome analysis (SUPRA)

III.4.2.1 Separation of proteins by SDS-PAGE

SDS-PAGE to separate the proteins present in protein extractions were performed according to an already described protocol (207). Gels were prepared as explained in Table 7, the stacking gel was poured on top of the resolving gel once it was polymerized. Protein samples were mixed with 5X loading buffer, heated at 95°C for 5 minutes and 10 µl of each were loaded similarly on three identical gels following a similar pattern presented in Table 8. Gels were run at 100 V until the protein samples reached the resolving gel and then at 120 V for about 1,5 h. One gel was kept for staining with Coomassie blue and the two others were further analyzed by western immunoblotting.

Table 7. Composition of acrylamide gels.

Components	12% acrylamide resolving gel	Stacking gel
1.5 M Tris base (ml)	4.8	1
10% SDS (µl)	300	75
30% acrylamide (ml)	12	1
10% APS (µl)	300	75
1:1 TEMED (µl)	30	7.5
Water (ml)	9.6	5.4

Table 8. Loading layout. MM, molecular maker; S, sonication; L, lysostaphin

Well	1	2	3	4	5	6	7	8
Sample	MM	S	L	SDS	MM	S	L	SDS
Strain		←	*S. aureus* MW2	→		←	*S. aureus* MN8	→

III.4.2.2 Immunoblotting

The two gels kept for immunoblotting were transferred onto Immobilon-P® PVDF membranes (Merck Millipore). The membranes were activated by incubation in methanol for 30 seconds and 5 seconds in dH_2O. After equilibration in transfer buffer (0.2 M Glycine, 25 mM Tris base, 20% methanol), the blotting was performed for 16-18h at 4°C and 30 mA (Bio-rad wet/tank blotting system) in wet conditions.

III.4.2.3 Depletion of *S. aureus*-specific antibodies from pooled sera from healthy human donors

Sera isolated from eight healthy human donors was pooled and is thereafter mentioned as “HS” for human sera. The volume of pooled antibodies was separated in two and half of it was used to create sera depleted of *S. aureus*-specific antibodies “dHS” (depleted-HS). Firstly, bacteria were grown overnight in 50 ml of media, pelleted by centrifugation and washed twice with PBS. The washed pellet was resuspended in 5 ml of serum previously diluted to 1:10 in PBS and incubated overnight at 4°C under a light agitation with either *S. aureus* MW2 or MN8. The following day, the suspension was centrifuged and the collected supernatant was filtered to remove any potential remaining bacteria.

III.4.2.4 Immunodetection

After the transfer of the proteins from the gels, the membranes were incubated with blocking buffer (5% BSA, 3% milk powder in PBS) overnight at 4°C to avoid unspecific binding. After three successive one-minute incubation with washing buffer (0.02% Tween 20), the membranes were incubated for four hours at 4°C with either HS or dHS for attachment to the recognized proteins. Membranes were then washed again and incubated for one hour at 4°C with the secondary antibody: goat anti-human IgG coupled with HRP. After washing in the abovementioned conditions, membranes were detected by chemiluminescence on a Vilber Fusion Fx (Vilber, France) imaging system using the PierceTM ECL Western kit, according to manufacturer’s instructions. The bands revealed by chemiluminescence were compared in between the two membranes and bands identified with HS, but not with dHS were matched with the corresponding protein bands observed after destaining the associated gel.

III.4.2.5 Gel excision

The interesting bands were then excised to identify the proteins present in the bands of interest by liquid chromatography-mass spectrometry. Briefly, bands were excised using a clean scalpel, incubated in 100 mM AB/ACN (1:1) for 30 min-incubation and occasionally vortexed for destining. The resulting distained gels pieces were incubated in neat ACN until they shrank and then incubated in trypsin buffer (13 ng/µL trypsin in 10 mM AB containing 10% ACN) for two hours on ice. 100 mM AB were added and the mixture was incubated overnight at 37ºC to digest the proteins within the gel into peptides. The remaining pieces were then incubated with extraction buffer (5% formic acid/ACN (1:2)) at 37°C for 15 minutes to extract the digested peptides from the gel pieces. To exchange the buffer containing the digested peptides, supernatants were transferred to new tubes, dried in a vacuum and redissolved in 0.1% trifluoroacetic acid. To properly get a solution and no remaining pieces of dried peptides, the solution was sonicated for five minutes and centrifuged for 10 min at 10,000rpm, then finally dried in a vacuum Eppendorf Concentrator 5301 (208).

III.4.3 Mass spectrometry analyses

Protein extracts obtained by trypsin shaving and peptides obtained by band excision and in-gel digestion were analyzed by mass spectrometry (MS) to determine the identity of proteins in each sample.

III.4.3.1 MS analysis of trypsin shaving extracts

Results for protein identities in control and treated samples obtained by trypsin shaving were compared by calculation in order to see which proteins were enriched by treatment with the trypsin. Four different types of calculation were performed from the data obtained for each identity found: difference in number of peptides, in percentage of peptides, in intensity and in label-free quantification (LFQ) intensity. Each type of calculation led to a list that was then ranked from highest to lowest difference and only the five top percent of each four lists were kept for further analysis and considered as surface-exposed.

III.4.3.2 MS analysis of protein identified in excised bands from SUPRA technique

A table for each sample run through MS was given by the platform where the analysis was conducted. Out of this table, all potential contaminants or proteins identified with a sequence overage under 15% were removed. Then, by comparison to where the band was excised, proteins identified with a molecular weight variation over 20% were removed from the list of

potential candidates. The remaining candidates were considered as proteins recognized by opsonic antibodies.

III.4.3.3 Combination of results obtained by both techniques and short-listing

The frequency of identification for each candidate was analyzed. Proteins found in both false-positive analysis and SUPRA, as well as in both bacterial strains, were kept for further analysis. All the candidates identified were studied to short-list according to specific criteria. First, proteins that were already studied as antigens in *S. aureus* were removed from the list as their potential was already known. Then, bibliographic research and bioinformatics techniques were used to gather information on each remaining candidate. Information on the sub-cellular location and the immunogenicity were characteristics collected bioinformatically for each candidate to short-list the proteins identified by MS.

III.4.4 Production of recombinant proteins

III.4.4.1 Information on proteins cloned

Table 9. Information on proteins cloned

Name	Uniprot number	Accession number	Sequence
Fe/B12 transporter	Q2FVW9	WP_182257875.1	MKKLLLPLIIMLLVLAACGNQGEKNNKAETKSYKMDDGKTVDIPKDPKRIAVVAPTY AGGLKKLGANIVAVNQQVDQSKVLKDKFKGVTKIGDGDVEKVAKEKPDLIIVYSTD KDIKKYQKVAPTVVVDYNKHKYLEQQEMLGKIVGKEDKVKAWKKDWEETTAKDG KEIKKAIGQDATVSLFDEFDKKLYTYGDNWGRGGEVLYQAFGLKMQPEQQKLTAK AGWAEVKQEEIEKYAGDYIVSTSEGKPTPGYESTNMWKNLKATKEGHIVKVDAGT YWYNDPYTLDFMRKDLKEKLIKAAK
Fe(3+) dicitrate ABC transporter	Q2FW75	WP_001214661	MRGLKTFSILGLIVALLLVAACGNTDNSSKKESSTKDTISVKDENGTVKVPKDAKRI VVLEYSFADALAALDVKPVGIADDGKKKRIIKPVREKIGDYTSVGTRKQPNLEEISKL KPDLIIADSSRHKGINKELNKIAPTLSLKSFDGDYKQNINSFKTIAKALNKEKEGEKR LAEHDKLINKYKDEIKFDRNQKVLPAVVAKAGLLAHPNYSYVGQFLNELGFKNALS DDVTKGLSKYLKGPYLQLDTEHLADLNPERMIIMTDHAKKDSAEFKKLQEDATWK KLNAVKNNRVDIVDRDVWARSRGLISSEEMAKELVELSKKEQK
DUF5011 domain-containing protein	Q2FZJ7	MBG1315796	MNKLLQSLSALGVSATLVTPNLNADATTNTTPQIKGANDIVIKKGQDYNLLNGISAF DKEDGDLTDKIKVDGQIDTSKSGKYQIKYHVTDSDGAIKISTRYIEVK
Foldase protein PrsA	Q2G2S6	WP_000782123	MKMINKLIVPVTASALLLGACGASATDSKENTLISSKAGDVTVADTMKKIGKDQIAN ASFTEMLNKILADKYKNKVNDKKIDEQIEKMQKQYGGKDKFEKALQQQGLTADKY KENLRTAAYHKELLSDKIKISDSEIKEDSKKASHILIKVKSKKSDKEGLDDKEAKQKA EEIQKEVSKDPSKFGEIAKKESMDTGSAKKDGELGYVLKGQTDKDFEKALFKLKD GEVSEVVKSSFGYHIIKADKPTDFNSEKQSLKEKLVDQKVQKNPKLLTDAYKDLLK EYDVDFKDRDIKSVVEDKILNPEKLKQGGAQGGQSGMSQ
Zinc ABC transporter AdcA	Q2G1U8	WP_000727762	MKKKLGMLLLVPAVTLSLAACGNDDGKDKDGKVTIKTTVYPLQSFAEQIGGKHVK VSSIYPAGTDLHSYEPTQKDILSASKSDLFMYTGDNLDPVAKKVASTIKDKDKKLSL EDKLDKAKLLTDQHEHGEEHEHEGHDHEKEEHHHHHGGYDPHVWLDPKINQTFA KEIKDELVKKDPKHKDDYEKNYKKLNDDLKKIDNDMKQVTKDKQGNAVFISHESIG YLADCYGFVQKGIQNMNAEDPSQKELTKIVKEIRDSNAKYILYEDNVANKVTETIRK ETDAKPLKFYNMESLNKEQQKKDNITYQSLMKSNIENIGKALDSGVKVKDDKAESK HDKAISDGYFKDEQVKDRELSDYAGEWQSVYPYLKDGTLDEVMEHKAENDPKKS AKDLKAYYDKGYKTDITNIDIKGNEITFTKDGKKHTGKYEYNGKKTLKYPKGNRGV RFMFKLVDGNDKDLPKFIQFSDHNIAPKKAEHFHIFMGNDNDALLKEMDNWPTYY PSKLNKDQIKEEMLAH

III.4.4.2 Bacterial culture and cell lysis

The bacteria *E. coli* M15 containing pREP4 and the modified pQE30/protein-gene was cultivated in 1 L of LB supplemented with antibiotics at 37°C with agitation. At early exponential phase (OD_{600nm} of 0.5), IPTG was added to the culture at 0.5 mM and the induction took place for two hours at 37°C. Bacteria were harvested by centrifugation at 5,000 x g for 15 minutes at 4°C and subsequently washed with PBS. The bacteria were resuspended in 5ml of PBS per gram of pellet. Lysozyme at 1 mg/ml was added to the bacterial suspension and the lysis was performed for 30 minutes at 4°C. To further improve the yield, a mechanical lysis was carried out by adding glass beads (Biospec products) to the sample and following a vortexing cycle (6 times 0.5 min vortex/4 min rest). The lysate was recovered by centrifugation at 20,000 x g for 15 minutes at 4°C and clarified by filtration through a 0.45 µM filter (Carl Roth).

III.4.4.3 Affinity purification through a nickel resin

The His-tagged proteins were purified through a nickel resin, Protino® Ni-NTA Agarose resin (Macherey-Nagel). Equilibration of the resin was done by three consequent washes with 4 bed volumes of equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl). Binding of His-tagged proteins was achieved by incubation of the lysate with the resin for one hour at 4°C with light shaking. After incubation the mix was poured into a Pierce column (Thermo Fisher Scientific) and allowed to drain by gravity. The flowthrough was re-circulated once more, followed by two washes with 10 bed volumes of equilibration buffer and two similar washes with equilibration buffer containing 20 mM of imidazole. The elution was performed by circulating 10 x 2 ml of elution buffer (equilibration buffer, 250 mM imidazole) and the purity of the fraction was assessed by SDS-PAGE. Protein-containing fractions were pooled and concentrated using a 10,000 MWCO Amicon® Ultra 15 mL (Merck Millipore) following the manufacturer's instructions and the proteins were stored at -20 °C.

III.5 Bioinformatics

III.5.1 Pairwise alignments

The sequence alignments of PpiC with PrsA and $AdcA_{fm}$ with $AdcA_{au}$ was conducted with EMBOSS Needle (209).

III.5.2 Prediction of protein subcellular localization

The subcellular localization was obtained on the UniProt website (https://www.uniprot.org/) by accessing information for each candidate through their UniProt number. Further bioinformatical

analysis were conducted using prediction tools: CELLO v.2.5 (http://cello.life.nctu.edu.tw/) (210) and Gpos-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/Gpos-multi/) (211).

III.5.3 Prediction of immunogenicity

The Vaxijen tool was used for antigenicity prediction, which uses algorithms based on principal amino acid properties of a protein sequence (212).

III.6 Immunological assays

III.6.1 Immunodotblot

Protein samples obtained by the different protein extractions were diluted using a factor 2 until 1:256 in PBS. 2 µl of each dilution were applied to a PVDF membrane and left to dry for 15 minutes. The membrane was then blocked overnight at 4°C with blocking buffer (PBS containing 3% BSA), washed thrice and incubated with 10 ml of HS or dHS at 4°C for one hour. After washing, the membrane was incubated at 4°C for one hour with a secondary antibody, a goat anti-human IgG coupled with HRP and diluted at 1:1000. After washing, membranes were detected by chemiluminescence on a Vilber Fusion Fx (Vilber, France) imaging system using the PierceTM ECL Western kit (Thermo Fisher), according to manufacturer's instructions.

III.6.2 Production of polyclonal antibodies by rabbit immunization

Recombinant proteins were used to immunize New Zealand rabbits and produce polyclonal antibodies. The animals were previously selected by assessment of their level of pre-existing opsonic antibodies by opsonophagocytic assay (OPA). Later, as described in Table 9. Briefly, they were immunized by subcutaneous injections containing the recombinantly produced protein and a proprietary adjuvant from Biogenes. At day 1, pre-immune serum was collected, and the animals received 200 µg of pure protein. At day 7, day 14 and day 42, they received three additional injections containing 100 µg of protein. The anti-protein sera were collected on day 49 and heat-inactivated for 30 minutes at 56°C. The different rabbit sera used in this study are listed in Table 10.

Table 10. Immunization schedule.

Day	Manipulation	Product
- 14	Selection	1,5ml of pre-immune serum
1	Pre-bleeding Immunization: 200µg of protein	10ml of pre-immune serum
7	Boost: 100µg of protein	
14	Boost: 100µg of protein	20ml of antiserum
42	Boost: 100µg of protein	
49	Terminal bleeding	50ml of terminal bleed

Table 11. Sera used in this project.

Name	Description	Reference
Pre-PpiC	Pre-immune serum collected from rabbits immunized with PpiC	(188)
Anti-PpiC	Anti-protein serum collected from rabbits immunized with PpiC	(188)
Pre- $AdcA_{fm}$	Pre-immune serum collected from rabbits immunized with $AdcA_{fm}$	(187)
Anti- $AdcA_{fm}$	Anti-protein serum collected from rabbits immunized with $AdcA_{fm}$	(187)
Pre-PrsA	Pre-immune serum collected from rabbits immunized with PrsA	This study
Anti-PrsA	Anti-protein serum collected from rabbits immunized with PrsA	This study
Pre- $AdcA_{au}$	Pre-immune serum collected from rabbits immunized with $AdcA_{au}$	This study
Anti- $AdcA_{au}$	Anti-protein serum collected from rabbits immunized with $AdcA_{au}$	This study

III.6.3 Immunodetection by enzyme linked immunosorbent assays (ELISA)

ELISA was carried out as previously described by Franco et al (213). To coat Nunc-immuno MaxiSorp 96-well plates (Haus), recombinant proteins were diluted at 1 µg/ml in coating buffer (0,2 M sodium carbonate/bicarbonate, pH 9.4) and incubated overnight in the wells. After washing, plates were blocked for one hour with PBS-BSA (phosphate buffer saline containing 3% bovine serum albumin). Sera were diluted in PBS-BSA, then added to the plate and incubated for one hour. Following washing, incubation for one hour was performed by adding alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G diluted to 1:1000. Finally, the plates were washed five times and 1mg/ml of ONPG was added to each well. After two hours, the absorbance was measured at 405 nm.

III.6.4 Opsonophagocytic assays

Table 12. Chemicals and reagents used in OPA.

Item	Company
Roswell Park Memorial Institute (RPMI) 1640 Medium	Life Technologies
FBS	Gibco
Lyophilized baby rabbit serum	Cedarlane Laboratories
Dextran	Sigma
Heparin-sufate sodium salt	Sigma
Ammonium chloride	Carl Roth
Intravenous immunoglobulin (IVIG)	Biotest

The *in vitro* OPAs were performed as described elsewhere (191,198). This experiment comprises four components: (i) a source of antibodies, either human sera or polyclonal antibodies raised in rabbits; (ii) baby rabbit complement; (iii) polymorphonuclear neutrophils (PMNs) freshly isolated from healthy human donors; (iv) bacteria. (i) Antibodies were diluted in sterile RPMIF (RPMI 1640 Medium, 15% FBS previously heat inactivated for 30 min at 56°C) to a chosen dilution depending on the experiment. (ii) Lyophilized baby rabbit serum was resuspended in sterile RPMIF and diluted to 1:15 or 1:30, in the same medium, for use in OPA against enterococci and *S. aureus* respectively. Potentially present IgG were adsorbed by incubation with bacteria freshly taken from an agar plated grown overnight and incubation for one hour at 4°C under light agitation. The suspension was centrifuged and filtered sterilized before use in the assay. (iii) PMNs were freshly isolated form blood of healthy human donors as follow: 25 ml of blood was incubated for 45 minutes at 37°C with 25 ml of heparin-dextran buffer (4.5 g NaCl, 10 g dextran, 32.8 mg heparin-sulfate sodium salt in 500 ml injectable water). The upper phase was kept for further isolation, transferred into a new tube and centrifuged (2,700 rpm, 10 min, 10°C). The supernatant was discarded, the pellet of leukocytes was washed with 10 ml of RPMIF and incubated for 20 minutes at RT in 10 ml of lysis buffer (1% ammonium chloride solution). After centrifugation, the remaining cells were washed once more with RPMIF and resuspended to get a concentration of 18.10^6 cells/ml. (iv) A 7 ml-culture of bacteria was set at OD_{600} 0.1 from isolated colonies on plates and grown until OD_{600} reached 0.4. 1ml of the culture was harvested by centrifugation, washed and resuspended with an equal volume of RPMIF. The bacterial suspension was then diluted to a chosen dilution dependent on the organism studied and the particular strain.

100 µl of each four prepared components were incubated to get a final incubation volume of 400 µl. For each antibodies' dilution tested, the mixture was made in duplicated, one with the PMNs, and one replacing the PMNs by RPMIF. The samples were incubated for 90 minutes

at 37°C with gentle shaking and diluted to 1:100 before plating on agar plates. The next day, the percentage of killing mediated by each antibodies' dilution was calculated as follow:

$$\% \text{ Killing} = 100 - \left[100 \times \frac{(\text{mean CFU sample } PMN's^{+} \text{ at t90})}{(\text{mean CFU } sample\ PMN's^{-} \text{ at t90})}\right]$$

Four controls were always applied to the assay to verify the behavior of each biological material in the said experiment. Number of colonies counted for each control was compared to a number of colonies obtained from a sample containing bacteria incubated with no other component. Three negative controls assessed the complement-associated lysis, the sensitivity to PMNs and the complement-mediated phagocytosis. One positive control tested the capacity of each other component to play their role in the phagocytosis by incubation with either IVIG or with anti-protein sera already described to mediate killing of the studied strain.

III.6.5 Opsonophagocytic inhibition assays (OPiA)

To evaluate the specificity of the mediated killing observed when incubating the anti-protein sera in OPA, we performed a similar assay that aims to test the inhibition capacities of the antigenic determinants. The recombinant proteins used in the immunization schedule were diluted in RPMIF at different concentrations ranging from 200 to 8 µg/ml and incubated overnight at 4°C under gentle shaking with the anti-protein sera diluted at 1:25 in RPMIF. The following day, the inhibited sera were used as a source of antibodies in the classical OPA protocol. The inhibition of each sample was assessed by counting the CFU on the nextday and applying a slightly different formula:

$$\% \text{ Inhibition} = 100 - \left[100 \times \frac{(\%\text{Killing}_{with\ inhibitor} \text{ at t90})}{(\%\text{Killing}_{without\ inhibitor} \text{ at t90})}\right]$$

III.6.6 Statistical analysis

The statistical analyses were conducted using the GraphPad PRISM software version 5.00. Bars in the bar graphs are expressed as the mean values and whiskers represent the standard error of the mean (SEM). Statistical differences between biological conditions to compare; for example, between pre- and anti-protein sera, or with or without inhibitors in OPiA; were calculated using an unpaired two-tailed T-test with a 95% confidence interval. A p-value (P) superior to 0.05 was considered non-significant and is annotated "ns" in graphs. A p-value inferior or equal to 0.05 was considered significant and significance degree were attributed as follow: * P ≤ 0.05, ** P ≤0.01, *** P≤0,001.

IV. Results

IV.1 Antigen identification in *S. aureus*

The commonly used approaches for antigen identification frequently result in an extensive number of possible candidates whose testing for antigenic potential is time-consuming, labor-intensive, and costly. In order to reduce the number of identified protein antigen candidates in *S. aureus* as much as possible, we opted to combine multiple strategies that had previously been shown to be beneficial, either with the organism we tested or in other bacterial species.

IV.1.1 Subtractive proteome analysis combined with false-positive analysis of trypsin shaving protein extractions

IV.1.1.1 Subtractive proteome analysis

Our approach to the well-known subtractive proteome analysis was novel. Three distinct methods of extracting proteins were used: boiling in a buffer containing SDS, lysostaphin digestion of the peptidoglycan in hypotonic solution, or conventional sonication. We opted to perform subtractive immunoblotting using antibodies from healthy individuals that we pooled together and split in half to use as it is or after incubation with the whole bacterium to deplete of *S. aureus*-specific antibodies.

IV.1.1.1.1 Depletion of *S. aureus*-specific antibodies

Human sera from healthy donors were depleted of *S. aureus*-specific antibodies by incubation with the whole bacteria. Bacteria and human sera were mixed in a tube and incubated overnight at 4°C to allow the antibodies that recognize surface-exposed proteins to bind. Centrifugation was used to separate antibody-bacterium complexes from the unbound antibodies, leaving only unspecific antibodies in the supernatant.

Immunodot blot was used to assess the effectiveness of the depletion. Briefly, a 2 µl drop of each protein extractions (paragraph (§) III.4.1.1/2/3) previously diluted from 1:1 to 1:256 was placed on two separate membranes. Each membrane was blocked with BSA, incubated with either HS or dHS, then with anti-human IgG coupled with HRP and finally revealed using ECL chemiluminescence kit. The figure 5 presents the results obtained.

The intensity of the spots in the membranes presenting dilutions of *S. aureus* MW2 protein extractions differs greatly between the one incubated with HS and the one incubated with dHS (Figure 5A). As we can see that the intensity is higher when the immunodetection is done with HS when compared to dHS, it means that the recognition of protein is reduced with dHS, especially for protein extracted by the sonication technique. Indeed, with HS, spots are detected up to a dilution of the protein extract of 1:128, while no spots are detected when

incubating with dHS. For, SDS boiling, with dHS, spots are no longer detected when diluting the protein extracts more than 1:32 but can be detected up to 1:256 when incubating with HS. For protein extracts obtained by peptidoglycan digestion with lysostaphin, detection with HS goes up to 1:128 and up to 1:8 with dHS. The results indicate that the *S. aureus*-specific antibodies depletion is not complete with the strain MW2, but it is still efficient as the incubation with the whole bacteria did reduce greatly the number of antibodies able to bind to the protein extracts. However, for the strain MN8 (Figure 5B), the depletion was even more effective. Not only did the incubation with the whole bacteria reduced the quantity of binding antibodies but it completely removed them. Indeed, no detection was possible using dHS, while each protein extract can be detected by HS, up to 1:64 for the lysostaphin-mediated extraction, 1:16 for SDS boiling and 1:32 for sonication.

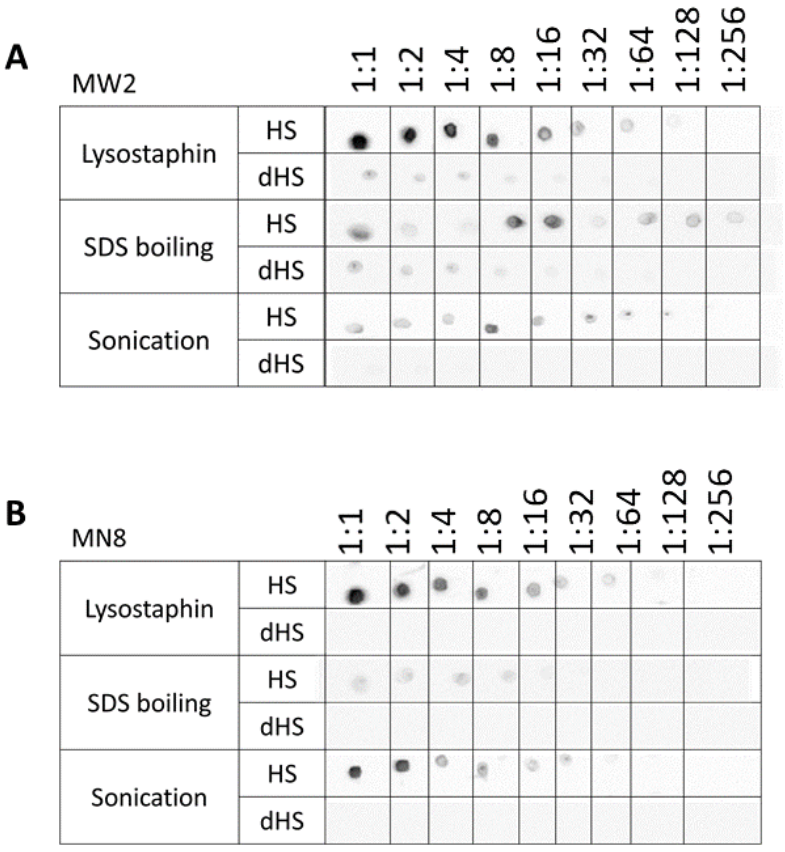


Figure 5. Human sera depletion of specific antibodies – Immunodotblot. A drop of each diluted protein extractions was placed on the PVDF membranes and left to dry. After blocking, membranes were incubated with either HS or dHS, then with a conjugated anti-human IgG and revealed. (A) Proteins extracted from S. aureus MW2. (B) Proteins extracted from S. aureus MN8.

To investigate the *in vitro* efficiency of the antibodies present in human sera and also verify the depletion's success, opsonophagocytic assays were performed against *S. aureus* MW2 and MN8 (Figure 6A and B). The killing mediated by antibodies in HS ranged from 60 to 17% for MW2 and between 47 and 4% against *S. aureus* MN8. When using dHS as a source of

antibodies in the assay, the killing dropped to a 42-7% range against MW2 and 32-3% range against MN8. The results show a dose-dependent mediated-killing while using either of the sera and against both strains. At 1:100 dilution, the depletion reduced the mediated killing by approximately 16.5% for both strains. Statistics show a killing significantly lower with dHS when compared to the same dilution of HS.

Overall, the results show that the depletion protocol was successful in reducing the binding antibodies and opsonic killing mediated by the antibodies present in the human sera.

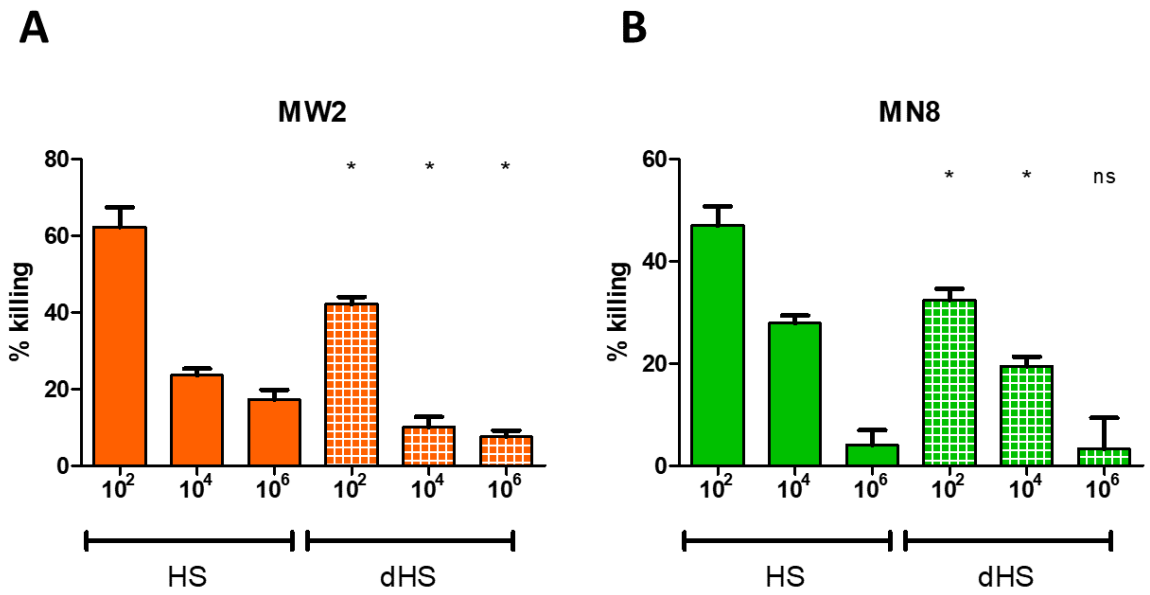


*Figure 6. Human sera depletion of specific antibodies – OPA. OPA against (A) S. aureus MW2 and (B) S. aureus MN8 using HS (plain bars) or dHS (white squares). Values from the same dilution of HS and dHS were compared statistically using an unpaired two-tailed T-test with a 95% confidence interval. Bars and whiskers represent mean values ± standard error of the mean. NS, not significant (P > 0.05), *P ≤ 0.05.*

IV.1.1.1.2 Identification of protein targets

In order to perform the identification of interesting bands for potential protein targets, three SDS-PAGE were run simultaneously using the same protein extractions obtained by either sonication, lysostaphin digestion or SDS boiling. After being blotted onto a PVDF membrane, two of the triplicates were incubated using either the HS or dHS while the third was stained using Coomassie blue (Figure 7). Much less bands were detected by incubation with dHS when compared to HS, which was expected given the previous results obtained by immunodotblot. Comparison between the two membranes obtained was carried out to highlight bands identified with the non-depleted serum but not with the depleted serum. In the case of *S. aureus* MW2, comparison of both membranes showed two positive hits while a similar comparison for MN8 led to the detection of seven bands showing a signal with HS but not dHS. The proteins found using this matching technique were reported to the gel and the respective

bands were excised (orange rectangles). After in-gel digestion with trypsin and extraction, the samples were analyzed by MS to identify the proteins present in the excised bands, supposedly proteins that are the targets of opsonic antibodies, and therefore good vaccine candidates.

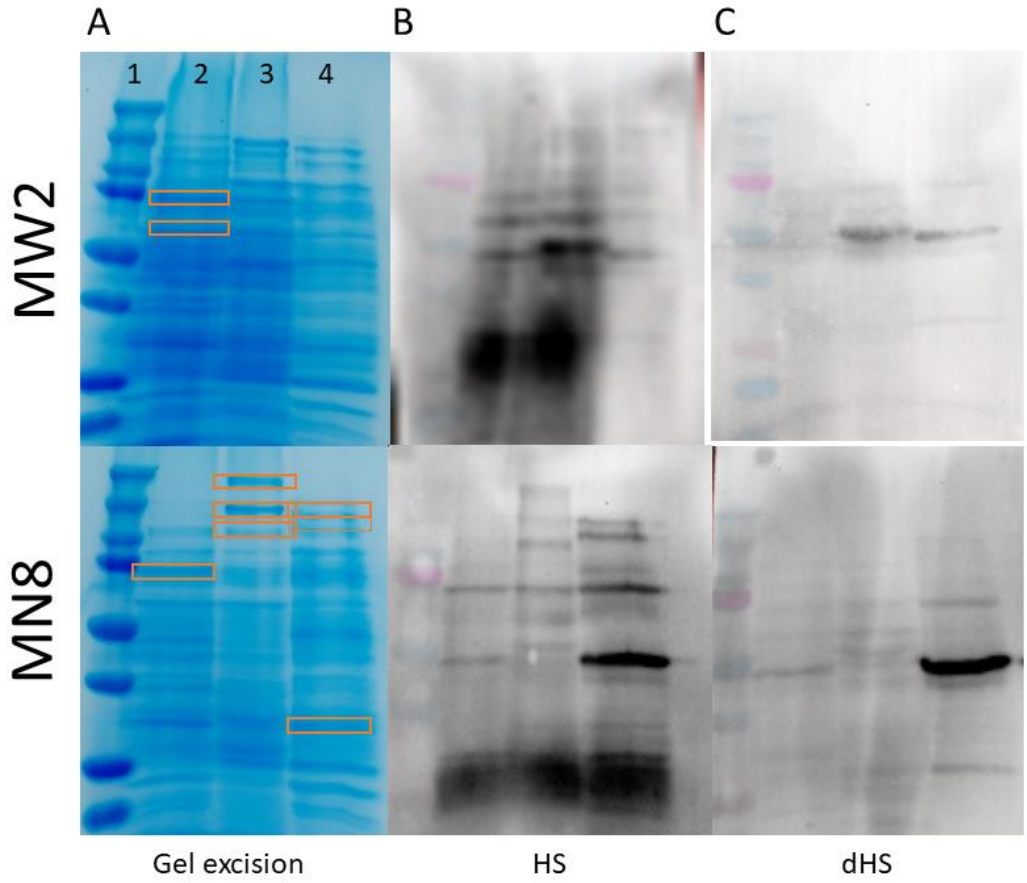


Figure 7. Identification of potential protein targets – SUPRA. (A) SDS-PAGE and the associated blotted PVDF membranes incubated with (B) human sera or (C) depleted-human sera. Line 1, molecular marker. Line 2, sonication. Line 3, lysostaphin. Line 4, SDS boiling.

IV.1.1.1.3 Mass spectrometry data analysis

The list of proteins identified by MS from samples obtained by gel excision of interesting bands were analysed as explained in § III.4.3.2. The resulting list of proteins was considered to summarize proteins potentially recognized by opsonic antibodies.

IV.1.1.2 False-positive analysis

Using the trypsin shaving procedure, we were able to better expose surface-exposed proteins for the enzyme to break them down by swelling up the bacteria in a hypotonic solution. After performing a false-positive analysis, which compares the treated sample with the control to reveal protein enrichment in the supernatant due to trypsin digestion, the peptides released

were analyzed by mass spectrometry. Aiming to show the enrichment of proteins due to the trypsin activity, we performed a set of four calculations to highlight the differences as explained in § III.4.3.1. The goal of this technique was to find surface-exposed proteins, which are a feature of possible vaccine candidates.

IV.1.1.3 Short-listing of potential candidates

The results obtained by SUPRA showing proteins potentially recognized by opsonic antibodies were combined to the results obtained with the false-positive analysis that identified surface-exposed proteins. Short-listing of the candidates was performed as described in the Materials and Methods section in § III.4.3.3. Table 13 shows the characteristics of all candidates remaining after removing cytosolic proteins and proteins already described as possible vaccine antigens. It is important to note that many already identified antigens were uncovered using this novel approach, proving its effectiveness in identifying vaccine antigens. This was the case for IsdA, the iron-regulated surface determinant protein A or the protein MntC, manganese transport protein C, for example.

Table 13. Summary of the identified candidates and their associated characteristics. Subcellular location given by Uniprot website (https://www.uniprot.org/) or predicted using CELLO v.2.5 (http://cello.life.nctu.edu.tw/) and Gpos-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/Gpos -multi/). Ext, extracellular; Mem, membrane; Cyt, cytosol; OMBPS, outer membrane bounded periplasmic space; OM, outer membrane; Per, periplasmic. I, Immunogenicity is given as Vaxijen score with a threshold of 0.40.

Protein name	Uniprot number	Accession number NCBI	Subcellular location			I
			Uniprot	CELLO v.2.5	Gpos-mPLoc	
Neutral metalloproteinase	Q2FUX4	WP_142352938	Ext	Ext	Ext	0.61
Lipoteichoic acid (LTA) synthase	Q2G093	WP_000098285	Mem	OM-Per	Ext	0.51
Fe/B12 transporter	Q2FVW9	WP_182257875.1	OMBPS	Mem-Ext	Mem	0.61
Thermonuclease	Q2G010	AIU85438	Mem	Per	Ext	0.68
Fe(3+) dicitrate ABC transporter	Q2FW75	WP_001214661	OMBPS	Cyt-Per	Mem	0.48
Quinol oxidase	Q2FZJ9	WP_000032835	Mem	Per	Mem	0.69
DUF5011 domain-containing protein	Q2FZJ7	MBG1315796	Unknown	Ext-Per	Ext	0.83
Molybdenum ABC transporter	Q2FVX4	WP_000782818.1	OMBPS	Per	Cyt-Ext	0.58
Foldase protein PrsA	Q2G2S6	WP_000782123	Mem	Per	Cyt	0.77
Zinc ABC transporter AdcA	Q2G1U8	WP_000727762	Mem	Per	Mem	0.70

IV.1.2 Investigation of cross-opsonic activity

IV.1.2.1 Sequence homology between two candidates and already described antigens in enterococci

A bibliographic research showed that two of the identified proteins were playing the same role and having the same function as two other proteins in enterococci that were already studied and described as effective antigens against enterococcal infections. Indeed, the foldase protein PrsA is a parvulin-like peptidyl-prolyl *cis-trans* isomerase protein (PPIase), which plays an essential role in protein folding and catalyzing the isomerization of peptide bonds in *S. aureus*. In enterococci, the protein PpiC also has the same function and was described by Romero-Saavedra and colleagues as an antigen present in both *E. faecium* and *E. faecalis* (188). Additionally, the zinc ABC transporter AdcA identified by our combination of antigen discovery techniques is a ZinT domain-containing protein that takes part of zinc intake. A protein with similar function and bearing the same name ($AdcA_{fm}$) was also already described as an efficient antigen for both *E. faecium* and *E. faecalis* by Romero-Saavedra and colleagues (187).

Because of the similarities in the effector function of our candidates with already studied and characterized antigens in another Gram-positive ESKAPE pathogen, we decided to compare their amino acid sequences (Figure 8). The overall pairwise sequence identity of the PrsA and PpiC, computed using EMBOSS Needle is 24.9% (Figure 8A). However, a strongly conserved regions is observed between residues 219-232 (210-223 in PpiC, sequence identity – 71.4%, similarity 92.9%), followed by the region 71-82 (73-84 in PpiC, sequence identity 58.3%, similarity 83.3%). These regions are located on the parvulin domain and in the NC domains, respectively. For $AdcA_{au}$ and $AdcA_{fm}$, the total sequence alignment using EMBOSS Needle has shown sequence identity of 41,2% (Figure 8B). Highly conserved regions are located on the ZinT and ZnuA domains of those proteins and are observed between the residues 342-376 of $AdcA_{au}$ (334-368 in $AdcA_{fm}$, sequence identity – 85.7%, similarity 85.7%) and the region 131-147 (125-141 in $AdcA_{fm}$, sequence identity – 52.9%, similarity 64.7%).

Antigen prediction was performed on the whole protein sequences and the specific conserved sequences using Vaxijen (Table 14). Consistent with our experimental results, all proteins are predicted to be strong antigens, with high antigenicity scores according to Vaxijen. Significantly stronger antigenicity scores characterize the identified sequence regions in the homologs.

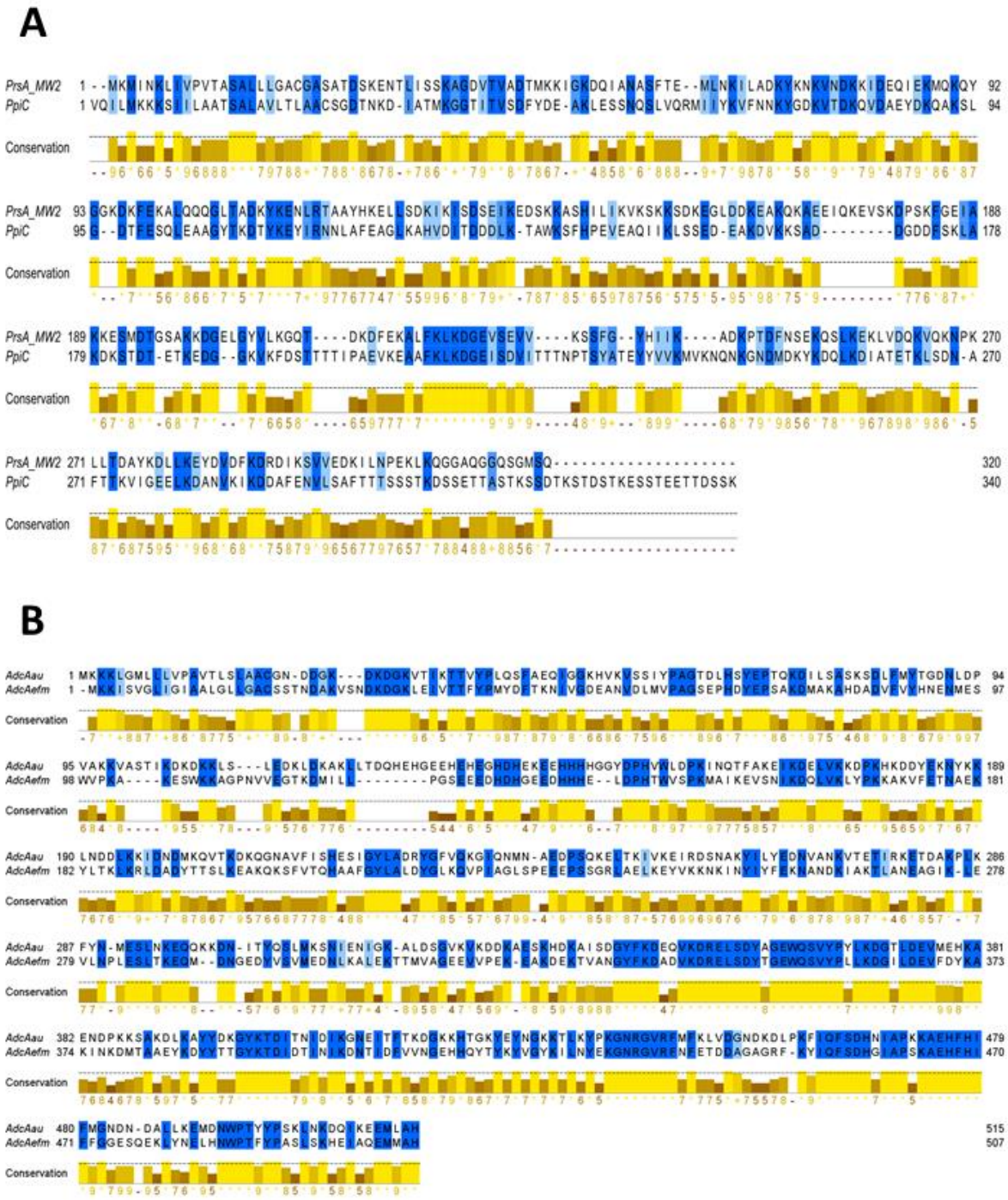


Figure 8. Pairwise alignments between protein sequences. EMBOSS Needle was used to perform pairwise alignment between (A) PrsA and PpiC and between (B) the zinc ABC transporter AdcA from S. aureus and $AdcA_{fm}$.

Table 14. Antigenicity prediction of full length proteins and conserved regions. Immunogenicity is given as Vaxijen score with a threshold of 0.40).

Sequence	Immunogenicity
Full length PrsA	0.77
Full length PpiC	0.86
PrsA 219-232 (ALFKLKDGEVSEVV)	1.06
PpiC 210-223 (AAFKLKDGEISDVI)	1.21
PrsA 71-82 (KYKNKVNDKKID)	1.92
PpiC 73-84 (KYGDKVTDKQVD)	1.67
Full length $AdcA_{au}$	0.70
Full length $AdcA_{fm}$	0.56
$AdcA_{au}$ 342-376 (GYFKDEQVKDRELSDYAGEWQSVYPYLKDGTLDEV)	0.43
$AdcA_{fm}$ 334-368 (GYFKDADVKDRELSDYTGEWQSVYPLLKDGILDEV)	0.42
$AdcA_{au}$ 131-147(EHEHEGHDHEKEEHHHH)	1.7
$AdcA_{fm}$ 125-141 (GSEEEDHDHGEEDHHHE)	1.57

IV.1.2.2 Cross-opsonic activity

Given the closeness of PpiC's and $AdcA_{fm}$'s sequence to proteins in *S. aureus* identified by our antigen identification technique and the presence of highly conserved antigenic regions, we decided to test whether a cross-opsonic effect could be seen. As the two enterococcal homologs had been previously studied in our lab, we decided to use the polyclonal sera from those studies (187,188). Using opsonophagocytic assays, we tested whether rabbit sera raised against PpiC (anti-PpiC) or against $AdcA_{fm}$ (anti-$AdcA_{fm}$) could mediate opsonic killing of *S. aureus* (Figure 9). Three staphylococcal strains were used in OPA with the anti-protein sera. Whether against MW2 (Figure 9A,D), LAC (Figure 9B,E) or Reynolds (Figure 9C,F), the killing mediated by anti-protein sera was dose-dependent and always significantly higher when comparing with the killing seen by incubation with pre-immune sera (pre-PpiC and pre-$AdcA_{fm}$). The observed killing was up to approximately 65% with the lowest tested dilution of 1:12.5. Those data show that anti-PpiC and anti-$AdcA_{fm}$ are able to cross-opsonize staphylococcal strains and strengthen our belief that both pairs of proteins share relevant epitopes.

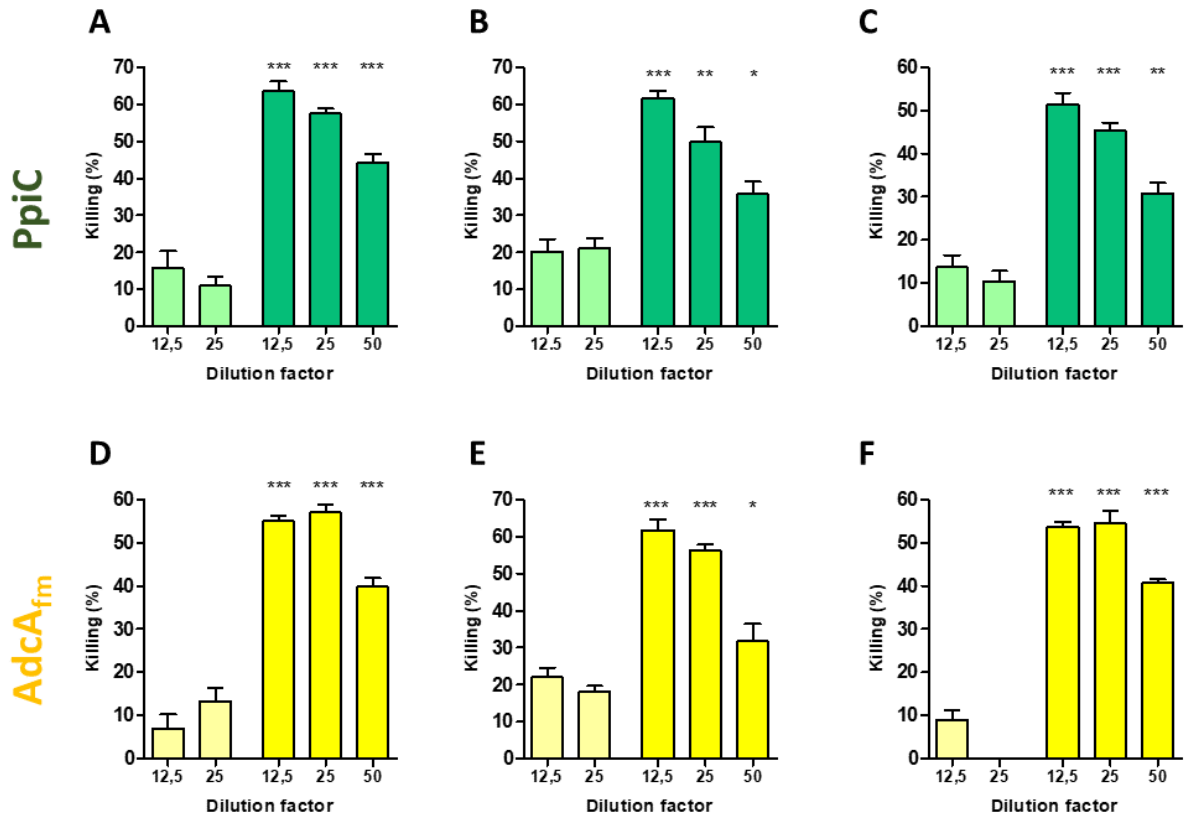


*Figure 9. Cross-opsonic activity of anti-PpiC and anti-AdcA$_{fm}$ – OPA. Various S. aureus strains were tested for cross-opsonic activity: MW2 (A,D), LAC (B,E) and Reynolds (C, F). The graphs show the opsonic killing observed using anti-PpiC (green) and anti-AdcA$_{fm}$ (yellow), previously obtained by immunization of rabbits with the associated recombinant protein. The graphs also present results obtained by incubation with pre-PpiC (light green) and pre-AdcA$_{fm}$ (light yellow), pre-immune sera isolated from rabbit blood before any immunization. Statistical differences between pre-immune sera and anti-protein sera at similar concentrations were tested by unpaired two-tailed T-test with a 95% confidence interval. Bars and whiskers represent mean values ± standard error of the mean (SEM). *P ≤ 0.05, ** P ≤0.01, *** P≤0,001.*

Opsonophagocytic inhibition assays were used to verify that the killing observed was due specifically to the antibodies raised against the enterococcal proteins. Anti-PpiC and anti-AdcA$_{fm}$ were incubated overnight with several dilutions ranging from 200 to 8 µg/ml of the associated recombinantly produced proteins, allowing the antibodies to bind to their target. Those inhibited sera were used as a source of antibodies in a classical OPA. In figure 10A and B, the graphs show that the killing mediated by anti-PpiC and anti-AdcA$_{fm}$ is significantly reduced and therefore inhibited due to the previous incubation with their associated antigens. These data indicate that the antibodies specifically raised against both enterococcal proteins can mediate the killing of *S. aureus*.

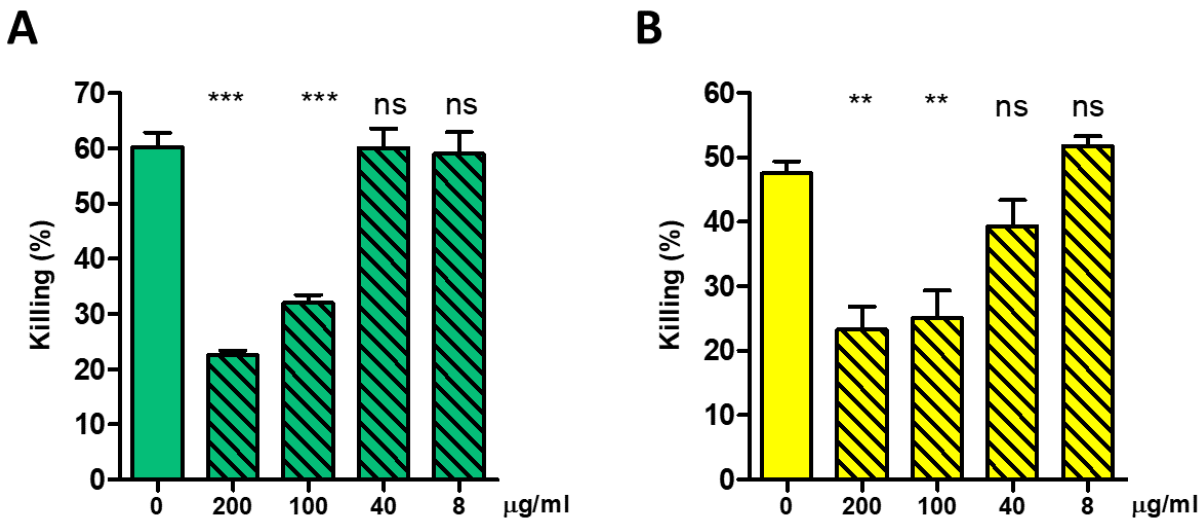


*Figure 10. Inhibition of the cross-opsonization mediated by anti-PpiC and anti-AdcA$_{fm}$ – OPiA. OPiA were performed against S. aureus MW2 using (A) anti-PpiC (green) and (B) anti- AdcA$_{fm}$ (yellow) diluted at 1:25 and previously incubated overnight with their associated recombinantly produced proteins at concentration ranging from 200 to 8 µg/ml (vertical stripes). The mediated killings observed for each antigen concentration were statistically compared to the positive controls, the anti-protein sera (empty bars). Statistical differences were calculated by unpaired two-tailed T-test with a 95% confidence interval. Bars and whiskers represent mean values ± SEM. NS, not significant (P > 0.05), ** P ≤0.01, *** P≤0,001.*

IV.1.3 Selection of worthy candidates to investigate

The previous results identified 10 proteins as potential vaccine antigen candidates. Further bioinformatics analysis showed that two of them had an amino acid sequence close to already described protein antigens in *E. faecium* and *E. faecalis*. Therefore, both the foldase protein PrsA and the zinc ABC transporter AdcA (later referred as AdcA$_{au}$) were selected for further analysis to determine their potential as a vaccine antigen against *S. aureus* infections. The two proteins were also investigated for their possible role as a vaccine antigen against enterococcal infections.

Also, three other proteins were selected for further analysis, and studied to bring a stronger proof of concept to our novel antigen discovery method: the DUF5011 domain-containing protein, as it has the highest predicted immunogenicity score; as well as two transporters, Fe/B12 transporter and Fe(3+) dicitrate ABC transporter.

IV.2 Production of polyclonal sera

IV.2.1 Rabbit selection

As previously explained in § I.2.2.1 and § I.3.2.1, *S. aureus* and enterococci are ubiquitous in the environment, as well as present in the microbiota of humans and animals. Given those characteristics, it is easy for the two opportunistic pathogens to spread among animal facilities, either by being part of the animal's microbiota or brought by animal handler or food. As the bacteria are mostly harmless to non-weakened organisms, it can be difficult to determine whether the animals carry the bacteria. When colonized, the animals may develop specific antibodies that could interfere with downstream experiments we may wish to carry out. For this reason, prior to immunization, rabbits were bled and pre-immune sera were collected to assess the presence of pre-existing antibodies. OPA were performed using those pre-immune sera as a possible source of antibodies. A first screening diluting the antibodies at 1:50 was performed (Figure 11). The killing mediated by antibodies present in the pre-immune sera of the twenty tested rabbits ranged from 0 to 45%. Seven rabbits were selected for further analysis by OPA.

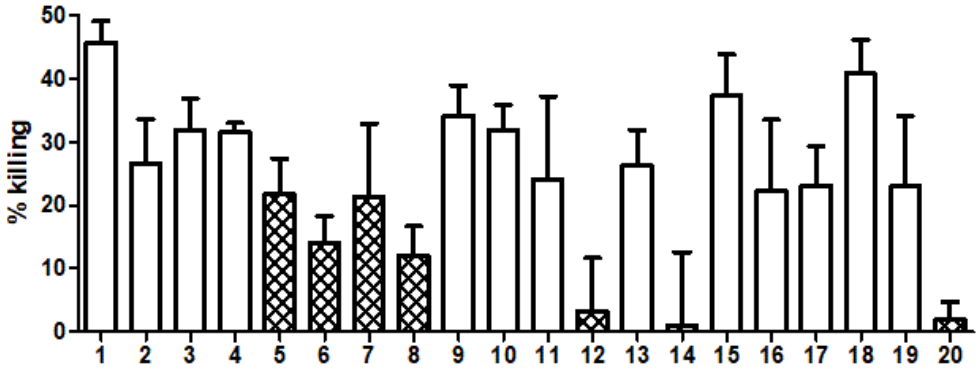


Figure 11. Initial screening for rabbit selection - OPA. Twenty pre-immune rabbit sera were diluted at 1:50 and screened for the presence of pre-existing antibodies mediating the killing of S. aureus MW2. White bars represent rabbits not selected for the next steps of selection while bars containing diamonds represent rabbits whose sera were further analyzed.

The sera from rabbits 5, 6, 7, 8, 12, 14 and 20 were analyzed again to confirm the results obtained in the first screening and to assess the killing they can mediate at a lower dilution (Figure 12). As expected, and previously seen in figure 11, pre-immune sera from rabbits 5 and 7 showed the highest killing obtained in the experiment. Other rabbits showed a killing at 1:50 always lower to about 5% and most of the time less to 5% as well for pre-immune sera diluted at 1:25, except for the rabbit 6 who still showed a really low killing of 15%. The rabbits 6, 8, 12, 14 and 20 were deemed suitable for use in further experiments as they showed a low level of opsonic pre-existing antibodies.

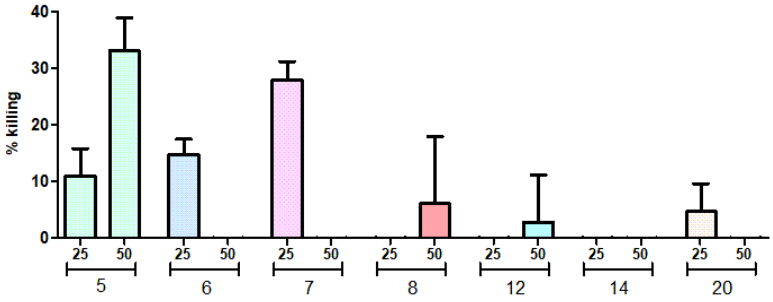


Figure 12. Final screening for rabbit selection - OPA. Pre-immune sera from rabbits 5, 6, 7, 8, 12, 14 and 20 were tested in OPA against S. aureus MW2 at 1:25 and 1:50.

IV.2.2 Rabbit immunization

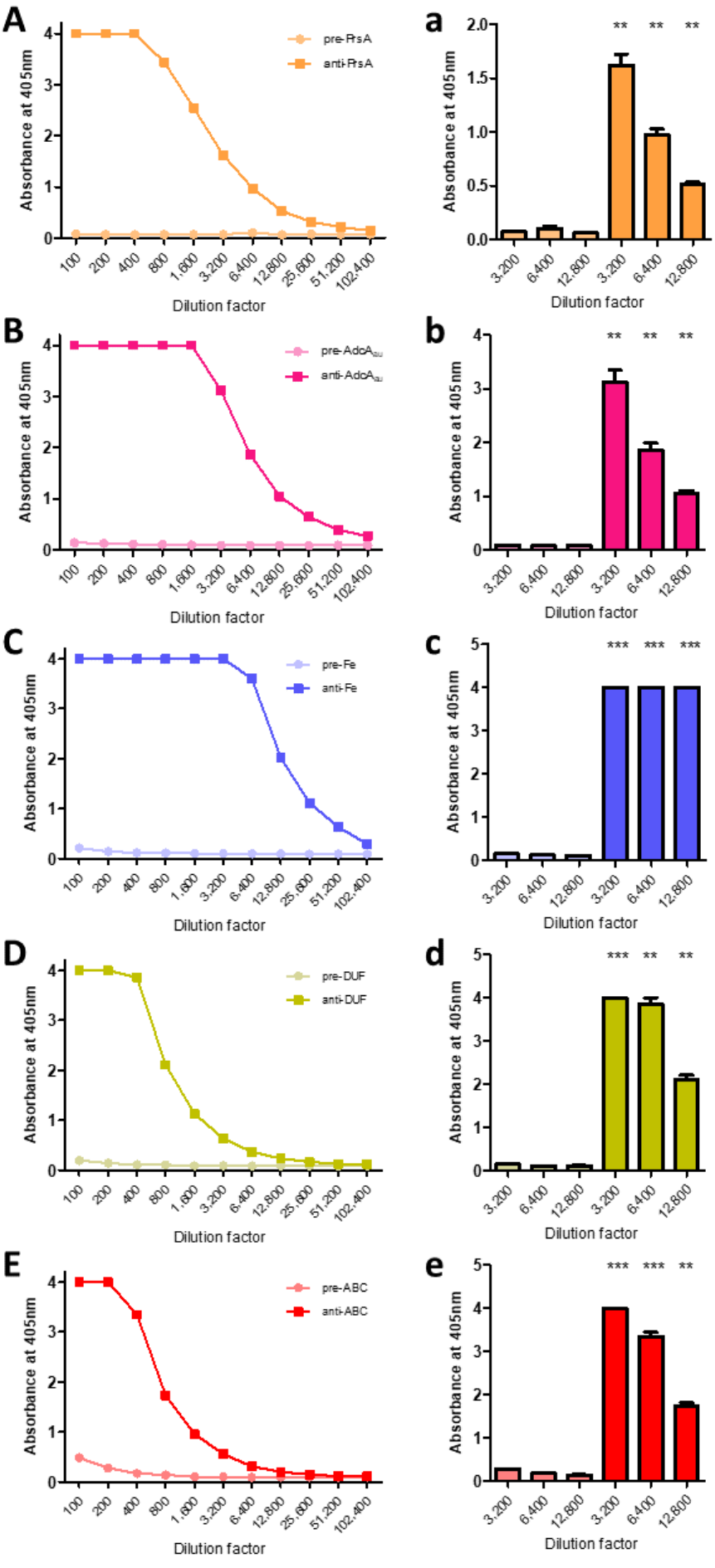
The selected rabbits were immunized as explained in § III.6.2, following the immunization schedule presented in table 9. The following antigens were produced for injection in the rabbit as defined in § III.4.4: PrsA, $AdcA_{au}$, the DUF5011 domain-containing protein (later referred as "DUF"), the Fe/B12 transporter (later mentioned as "Fe") and Fe(3+) dicitrate ABC transporter (referred as "ABC").

IV.3 Efficacy assessment of polyclonal sera *in vitro*

IV.3.1.1 Immunoreactivity towards recombinantly produced proteins

IV.3.1.1.1 Binding studies

After immunization and collection of the anti-protein sera, the antibodies raised in each rabbit were tittered to verify the immunization efficacy and the presence of antigen-binding antibodies. Enzyme-linked immunosorbent assays were performed using the five different proteins, PrsA, $AdcA_{au}$, Fe, DUF and ABC. The figure 13A-E shows the results obtained by incubation with a wide range of antibody dilutions. The S-like shape indicates that the antibodies are specifically binding to their associated proteins, as the dose-dependent profile means that the effect is due to the diluted compound. For each profile, we showed saturations of the binding and we also completely titered out the effect. The data for three dilutions, 1:3200, 1:6400 and 1:12800, were plotted into bar graphs to perform statistical analyses (Figure 13a-e). The results show that the binding observed with every anti-protein serum is significantly higher when compared to the binding obtained with pre-immune sera. This indicates that the immunization did succeed in raising protein-binding specific antibodies. The results obtained demonstrate that the candidates we chose are immunogenic, capable of raising specific antibodies that bind to their target when applying the immunization schedule followed in this study.


A
pre-PrsA
anti-PrsA
Absorbance at 405nm
Dilution factor
a
Absorbance at 405nm
Dilution factor
B
pre-AdcA
anti-AdcA
Absorbance at 405nm
Dilution factor
b
Absorbance at 405nm
Dilution factor
C
pre-Fe
anti-Fe
Absorbance at 405nm
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Absorbance at 405nm
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pre-ABC
anti-ABC
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Dilution factor

*Figure 13. Immunoreactivity towards the selected candidates – ELISA. ELISAs were performed to verify that rabbit polyclonal sera raised against our antigens could bind to their target. Briefly, Nunc-Immuno MaxiSorp 96-well plates were coated with the recombinantly produced PrsA (Aa – orange), $AdcA_{au}$ (Bb – pink), Fe (Cc – blue), DUF (Dd – yellow/green) and ABC (Ee – red) and different dilutions of the polyclonal antibodies were added to the plates. Results show the absorbance after detection with anti-rabbit IgG and incubation with ONPG. A wide range of dilutions was tested to show the specificity (A-E) and three dilutions were picked to calculate statistical difference between pre-immune and anti-protein sera (a-e). An unpaired two-tailed T-test with a 95% confidence interval was used to statistically compared the absorbance values obtained for the same dilutions. Bars and whiskers represent mean values ± standard error of the mean (SEM). ** P ≤0.01, *** P≤0,001.*

IV.3.1.1.2 Cross-binding studies

To investigate whether the sequence homology between the two pairs of homologs leads to the production of antibodies capable of recognizing both proteins, and to biologically assess the involvement of PrsA and $AdcA_{au}$ in the cross-reactive opsonic activity previously shown, we decided to perform binding studies using ELISA. The recombinantly produced proteins were used to coat Nunc-Immuno MaxiSorp 96-well plates and the associated anti-homolog sera was added at different dilutions: plates coated with PrsA were incubated with anti-PpiC (Figure 14A), and in reverse, anti-PrsA was incubated in plates previously coated with PpiC (Figure 14B); for the second pair of homologs, the immunoreactivity towards $AdcA_{au}$ (Figure 14C) and $AdcA_{fm}$ (Figure 14D) was assessed by the anti-homologous protein sera anti-$AdcA_{fm}$ and anti-$AdcA_{au}$ respectively. The results show that each anti-homologous serum can react with its associated protein. Also, a dose-dependent effect is seen in graphs B, C and D, demonstrating that the antibodies present in the sera are responsible for the binding seen. The same diluting effect can be seen with anti-PpiC but the anti-protein sera needs to be diluted further (data not shown). Importantly, the comparison between the absorbance relating to the binding of the pre-immune sera and the absorbance obtained by adding the anti-protein sera to the plates is statistically significant. These data show that it is indeed the immunization with the candidate antigens that raised antibodies capable of binding to their homologs in other organisms. Altogether, these results proved that immunization with either protein led to the production of antibodies that can bind to both their intended target and other proteins from other organism that share a high sequence identity. The data also strengthen our hypothesis that both PrsA and $AdcA_{au}$ are the homologous protein in *S. aureus* responsible for the cross-reactivity of anti-PpiC and anti-$AdcA_{fm}$ against both Gram-positive strains. It confirms that PrsA and PpiC share epitopes, as well as $AdcA_{au}$ does with $AdcA_{fm}$, and that those epitopes are targets of antibodies binding to both proteins.

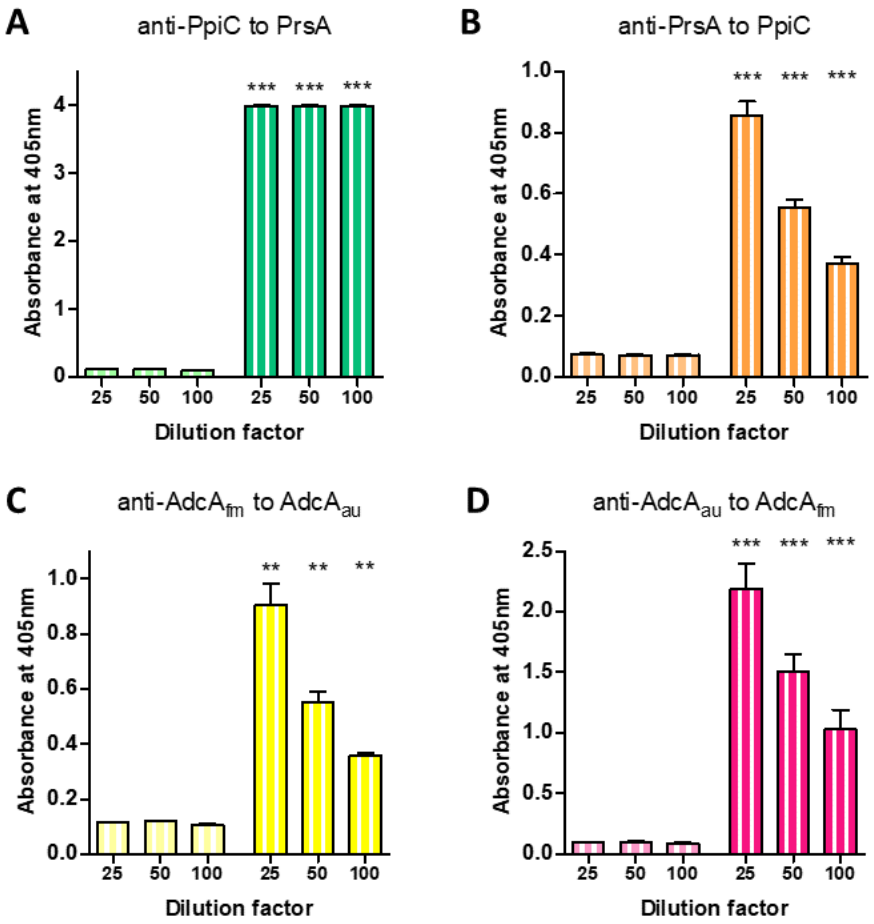

*Figure 14. Immunoreactivity towards the homologs – ELISA. The binding capacities of antibodies to their homologs were assessed by ELISA. The recombinant proteins (A) PrsA, (B) PpiC, (C) AdcA$_{au}$ and (D) AdcA$_{fm}$ were used to coat Nunc-Immuno MaxiSorp 96-well plates by incubation overnight. After washing and blocking, the plates were placed with different dilutions of the anti-homolog sera. Values for the same dilution were statistically compared using an unpaired two-tailed T-test with a 95% confidence interval. Bars and whiskers represent mean values ± SEM. ** P ≤0.01, *** P≤0,001*

IV.3.1.2 Inhibition of the cross-opsonic activity of anti-PpiC and anti-AdcA$_{fm}$

As sera raised against enterococcal proteins can bind to their homologs, we decided to test whether the recombinantly produced staphylococcal proteins could inhibit the killing of *S. aureus* by anti-PpiC and anti-AdcA$_{fm}$ observed in figure 9. Opsonophagocytic inhibition assays were performed using the anti-protein sera previously incubated overnight with several dilutions of their homologous proteins (from 200 to 8 µg/ml). Those potentially inhibited sera were used as a source of antibodies in a classical OPA (Figure 15). The results show that the killing mediated by anti-PpiC and anti-AdcA$_{fm}$ is significantly reduced with a previous incubation with PrsA and AdcA$_{au}$, respectively. These data suggested that both staphylococcal proteins could bind to the antibodies and inhibit the killing they mediate. This shows that the antibodies

specifically raised against both enterococcal proteins can mediate the killing of *S. aureus* via opsonization through PrsA and $AdcA_{au}$.

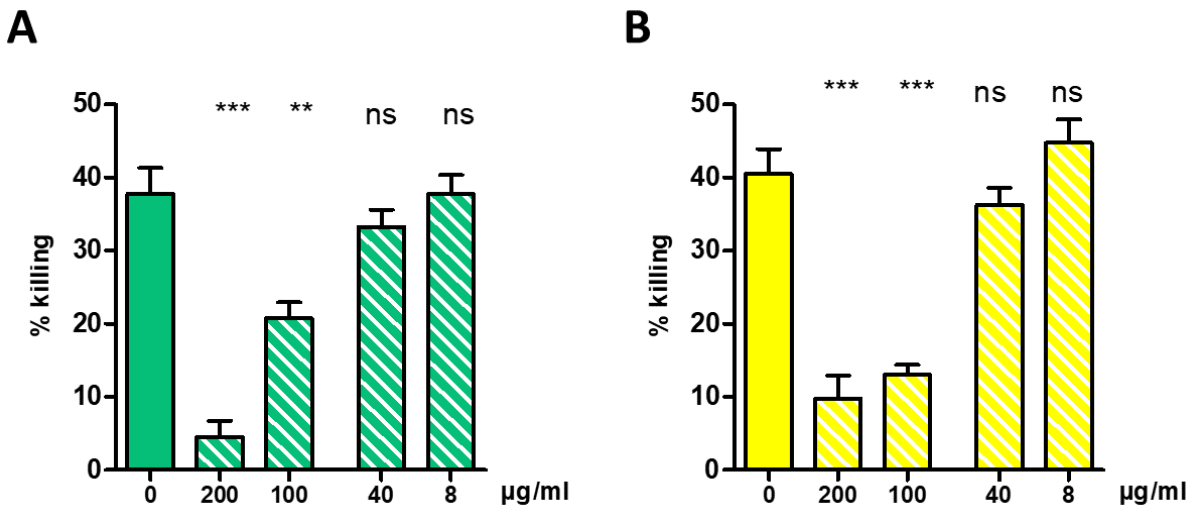

*Figure 15. Inhibition of the cross-reactive activity of anti-PpiC and anti- anti-$AdcA_{fm}$ with their homologs – OPiA. OPiA against S. aureus MW2 were performed to assess the inhibition of (A) anti-PpiC (green) diluted at 1:20 and previously incubated with the recombinantly produced protein PrsA diluted to final concentrations ranging from 200 to 8 µg/ml (vertical stripes) or (B), the inhibition of anti-AdcAfm (yellow) at 1:40 by incubation with $AdcA_{au}$ at similar concentrations (vertical stripes). Statistics were used to compare the killing seen without inhibition to each killing observed with previous incubation with homolog proteins. Statistical differences were calculated by unpaired two-tailed T-test with a 95% confidence interval. Bars and whiskers represent mean values ± SEM. NS, not significant (P > 0.05), ** P ≤0.01, *** P≤0,001.*

IV.3.1.3 Opsonic activity against *S. aureus* MW2

To assess the efficacy of the polyclonal antibodies obtained by immunization with our candidates, OPA were performed against *S. aureus* MW2. For the two most promising candidates we identified as potential vaccine antigens, a large range of dilutions was tested (Figure 16AB). The results obtained by incubation with anti-PrsA ranged from 35 to 10% and 35 to 20% with anti-$AdcA_{au}$. The wide range of dilutions allowed to titer out the mediated killing and confirmed the antibodies in the sera were responsible for the effect seen. Also, the killing observed was significantly higher when comparing the anti-protein sera directed against PrsA or $AdcA_{au}$ to their corresponding pre-immune sera. These results show that the immunization of rabbits with our candidates led to the production of opsonic antibodies capable of mediating the killing of *S. aureus* MW2 in *in vitro* assays.

As the study of the other three candidates was aiming to prove that the novel experimental approach to identify potential antigens was effective, we decided to perform a preliminary investigation on the opsonic function of polyclonal antibodies raised against these proteins. All sera were diluted at 1:25 and showed killing up to approximately 55% with anti-Fe and anti-

ABC. The antibodies raised against DUF could mediate a killing up to 40% when diluted at 1:25. Importantly, the values obtained with anti-protein sera were always significantly higher when compared to the values obtained with the pre-immune sera. Altogether, these data show that our three candidates used in a formulation to immunize rabbits leads to the production of polyclonal antibodies capable of opsonizing *S. aureus* MW2 and mediating its killing. Although these results are preliminary and need a deeper investigation to draw final conclusions, they strongly suggest that the three proteins, the two transporters, Fe/B12 transporter and the Fe(3+) dicitrate ABC transporter, as well as the DUF5011 domain-containing protein, seem like promising candidates for vaccine formulations against *S. aureus*. The data also show that the novel technique we used for antigen discovery can be effective, leading to a successful identification of potential antigens and therefore should be further used in the future.

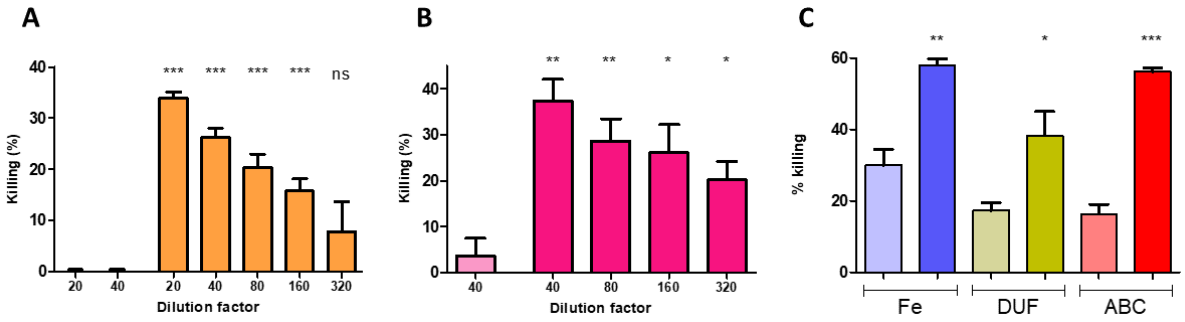

*Figure 16. Opsonic functions of rabbit sera against S. aureus MW2 – OPA. The opsonic killing antibodies present in the polyclonal sera was assessed by OPA against MW2. The killings obtained by incubation with anti-protein sera (darker colors) were compared to the killings observed at the same dilution of pre-immune sera (lighter color). The figure represents the opsonic killing mediated by (A) anti-PrsA, (B) anti-AdcA$_{au}$ and (C) anti-Fe (blue), anti-DUF (yellow) and anti-ABC (red) diluted at 1:25. Statistical differences between pre-immune sera and anti-protein sera at the same dilution were determined using unpaired two-tailed T-test with a 95% confidence interval. Bars and whiskers represent mean values ± SEM. NS, not significant (P > 0.05). *P ≤ 0.05, ** P ≤0.01, *** P≤0,001.*

The specificity of the killing observed was tested by OPiA against *S. aureus* MW2 (Figure 17). Polyclonal sera raised against PrsA and AdcA$_{au}$ were incubated overnight with their associated proteins at concentrations ranging from 200 to 8 µg/ml. The next day, those potentially inhibited sera were used as a source of antibodies in OPA. The results show a decreased killing by incubation of the sera with the recombinant proteins that is dose-dependent. This demonstrates that the mediated killing can be inhibited by incubation with PrsA and AdcA$_{au}$ and is therefore specifically due to antibodies raised against these two proteins.

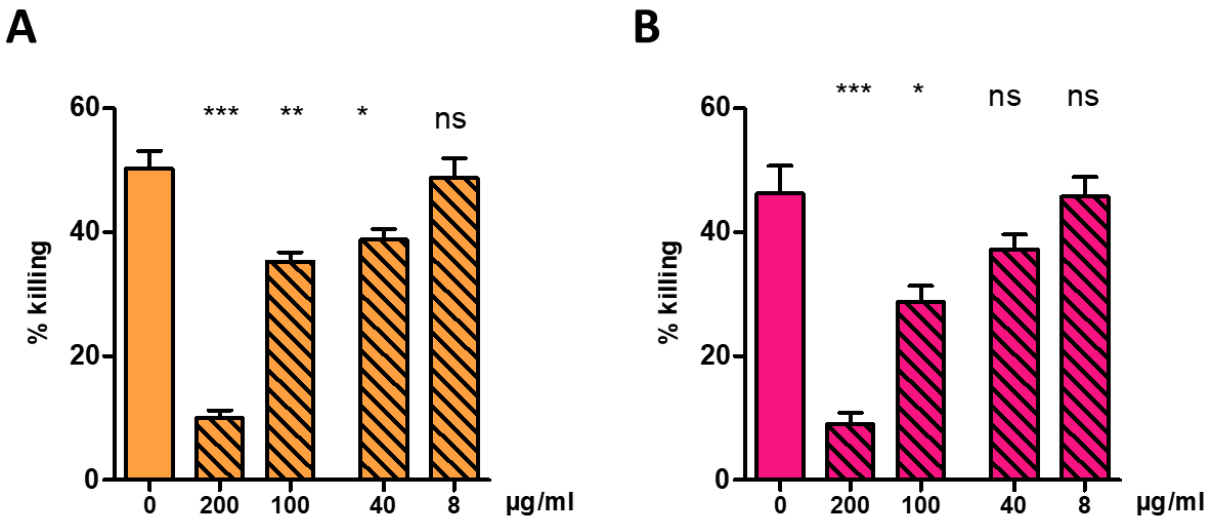

*Figure 17. Specificity of anti-PrsA and anti-AdcA$_{au}$ – OPiA. OPiA were performed against S. aureus MW2 to check the specificity of (A) anti-PrsA and (B) anti-AdcA$_{au}$. Anti-protein sera were used at 1:20 and 1:40 respectively and incubated with their associated proteins at concentrations ranging from 200 to 8 µg/ml (vertical stripes) or without any inhibitors (empty bars). The mediated killings observed for each antigen concentration were statistically compared to the mediated killing observed without protein. Statistical differences were calculated by unpaired two-tailed T-test with a 95% confidence interval. Bars and whiskers represent mean values ± SEM. NS, not significant (P > 0.05), *P ≤ 0.05, ** P ≤0.01, *** P≤0,001.*

IV.3.1.4 Cross-opsonic activity

The capacity of polyclonal antibodies raised against PrsA (Figure 18A-D) and AdcA$_{au}$ (Figure 18E) to mediate opsonic killing of other *S. aureus* strains was assessed by OPA. Anti-PpiC and anti-AdcA$_{fm}$ were used as well in the assay to see if the cross-activity previously shown was also true for a larger strain coverage. Rabbit sera raised by immunization with our candidates mediated a killing significantly higher when comparing to the antibodies present in the pre-immune sera. Also, in every strain tested, the opsonic killings mediated by antibodies contained in anti-PpiC or anti-AdcA$_{fm}$ were always slightly lower when compared to the percentage of killing observed with antibodies present in anti-PrsA or anti-AdcA$_{au}$. Those results show that antibodies raised against both PrsA and AdcA$_{au}$ can mediate the opsonic killing of several *S. aureus* strains.

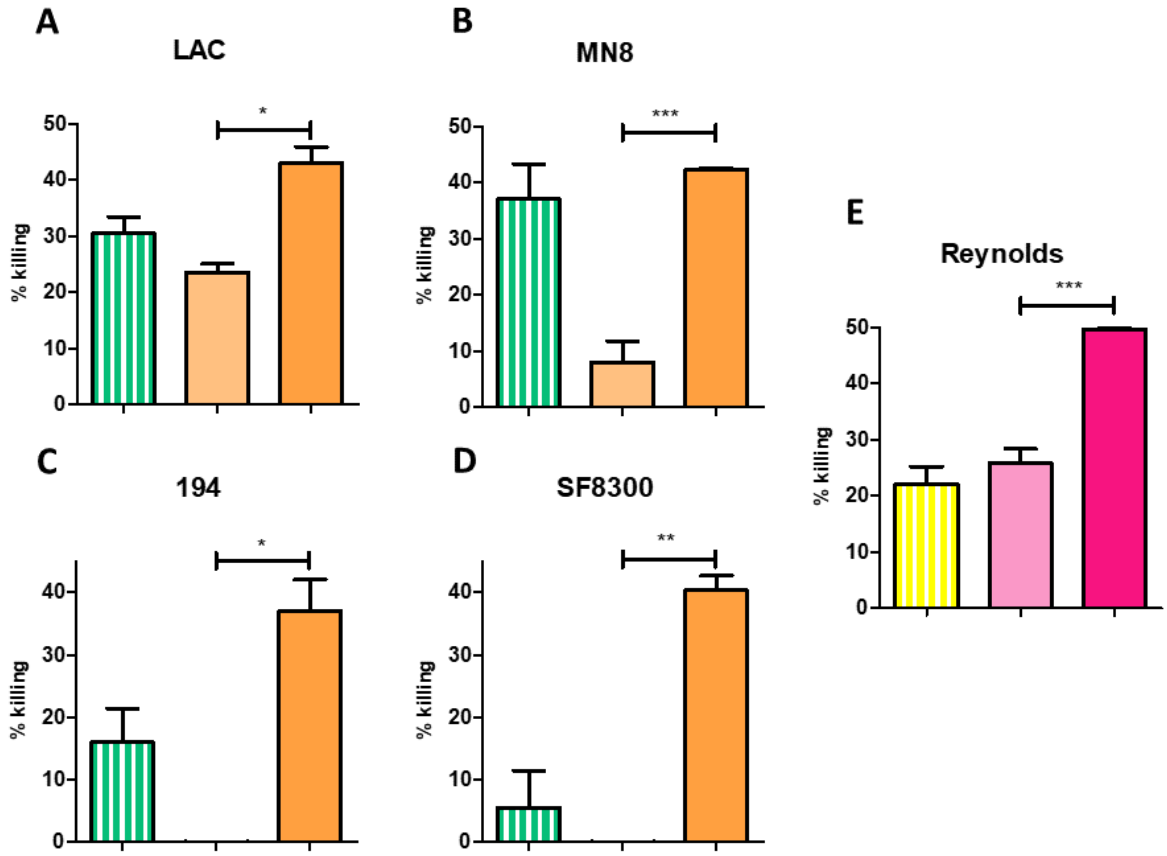

*Figure 18. Cross-opsonization of other S. aureus strains – OPA. Several strains were tested to see if the raised polyclonal antibodies could mediate the opsonic killing of additional staphylococcal strains. The sera were diluted at 1:20 for use in OPA against all five strains: (A) LAC, (B) MN8, (C) 194, (D) SF8300 and (E) Reynolds. In A-D, the killings mediated by anti-PpiC (green, white bars) and pre- (light orange) and anti-PrsA (orange) were assessed and statistically compared. The figure E shows the killings measured by incubation with anti-AdcA$_{fm}$ (yellow, white bars) or pre- (light pink) and anti-AdcA$_{au}$ (pink) which were also compared using statistics. An unpaired two-tailed T-test with a 95% confidence interval was used to calculate the statistical differences. *P ≤ 0.05, ** P ≤0.01, *** P≤0,001.*

As previously shown, the rabbit sera raised against the enterococcal proteins PpiC and AdcA$_{fm}$ were able to mediate the opsonic killing of bacteria pertaining to another family: *S. aureus*. Also, cross-binding studies have shown that each of the four sera; anti-PpiC, anti-PrsA, anti-AdcA$_{fm}$ and anti-AdcA$_{au}$; could bind in a dose-dependent manner to their associated homolog. Considering those results, we decided to test whether the polyclonal sera produced during this study, and directed towards staphylococcal proteins could also mediate the killing of enterococcal strains. OPA were performed against one vancomycin-resistant *E. faecium* 11236/1 (Figure 19A,D) and two *E. faecalis* strains: Type 2 (Figure 19B,E) and 12030 (Figure 19C,F). The polyclonal antibodies directed against PpiC and PrsA (Figure 19A-C) as well as against the iron transporters (Figure 19D-F) were diluted in RPMIF and used in the assay. The killings observed when incubating with anti-protein sera, raised by immunization, were always significantly higher when compared to the pre-existing antibodies collected in rabbits before

the injection of our candidates. Interestingly, anti-PrsA and anti-$AdcA_{au}$ mediate a killing that is either equal or slightly higher when compared to anti-PpiC and anti-$AdcA_{fm}$. The data strongly suggests that both identified proteins, PrsA and AdcAau, are good antigens and could even be slightly better than the previously discovered enterococcal proteins. The results demonstrate that our candidates should be considered for vaccine formulation against the two Gram-positive pathogens of the ESKAPE group.

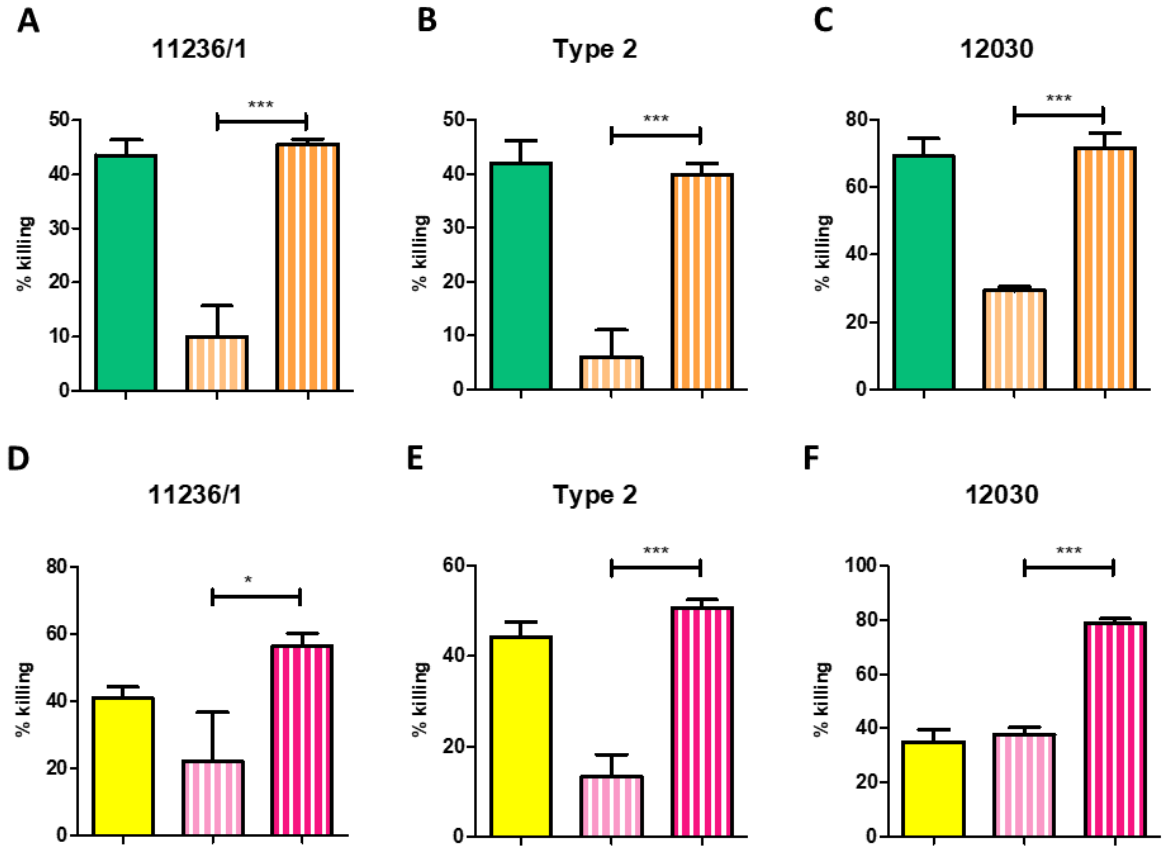

*Figure 19. Cross-opsonic activity against enterococcal strains – OPA. Three enterococcal strains were also tested for cross-opsonic function of polyclonal antibodies. Sera were diluted at 1:20 for use in OPA against (A,D) Enterococcus faecium 11236/1 and (B,E) Enterococcus faecalis Type 2, and at 1:50 while tested against (C,F) Enterococcus faecalis 12030. Each strain was probed for killing mediated by antibodies present in anti-PpiC (green) and anti-$AdcA_{fm}$ (yellow). Also, the killing mediated by pre-PrsA (light orange, white bars), anti-PrsA (orange, white bars), pre-$AdcA_{au}$ (light pink, white bars) and anti-$AdcA_{au}$ (pink, white bars). Values for each pre-immune serum and its associated anti-protein serum were statistically compared using an unpaired two-tailed T-test with a 95% confidence interval. Bars and whiskers represent mean values ± SEM. *P ≤ 0.05, *** P≤0,001.*

V. Discussion

ESKAPE bacteria represent a great threat to the human health. They are opportunistic pathogens found in the environment, and in the microbiota of humans and animals (1). The two Gram-positive ESKAPE pathogens *S. aureus* and enterococci have intrinsic antimicrobial resistance but also acquired new mechanisms over time due to the overuse of antibiotics (79). Combined together, those characteristics explain why these pathogens are serious threat in the clinical setting, where vulnerable patients can be found. The ESKAPE pathogens are all around and over us, so they can infect people while procedures with a high risk of infection are performed, for example surgeries. As most of the time they also present with antimicrobial resistance, preventive measures are often ineffective and treatments difficult (5). These led the two Gram-positive pathogens of the ESKAPE group to represent 23.5% of HAI in the US between 2015 and 2017, *S. aureus* was the second most isolated pathogen with 11.8%, *E. faecalis* was associated with 7.9% of the HAI. While *E. faecium* represent only 3.8% of HAI, over 85 % of those isolated bacteria were resistant to vancomycin (60). Those results only highlight how big of a concern the two organisms represent to global health. Indeed, the "2019 Antibiotic Resistance Threats Report," released in 2019 by the Centers for Disease Control and Prevention (CDC), includes the most recent information on deaths and infections caused by a list of 18 antimicrobial-resistant bacteria in the United States. The classification of these bacteria based on their level of risk for human health is indicated in the study. MRSA and VRE are both categorized as "Serious Threats" (214). According to the CDC, antimicrobial-resistant organisms cause around 2,8 million infections and 35,000 fatal infections annually in the United States. In 2017, MRSA was accountable for 10,600 deaths (30%) and VRE for 5,400 deaths (15%) (215,216).

Given the ability of the pathogens to develop antibiotic resistances, new measures must be taken to tackle this issue. Effective vaccines can prevent dangerous diseases but have also been associated with reduced antibiotic use, cheaper treatment costs, and shorter hospital stays, all of which are enormous benefits to our society (80,217,218). Unfortunately, despite several trials and a huge number of discovered antigens, no vaccine has been successfully developed to prevent infections by enterococci or *S. aureus* (219–222).

This study aims to discover new antigens in *S. aureus* and to assess the effectiveness of polyclonal sera raised against the identified proteins to determine their potential as antigenic determinants. We used a combination of already described methods in an effort to narrow down the list of candidates to only a small number of promising proteins to experimentally investigate. A false positive analysis of peptide digested from surface-exposed proteins was combined to a subtractive proteome analysis using protein extractions obtained by lysostaphin digestion of the peptidoglycan, SDS boiling and sonication which were detected using human sera from healthy donors and the same sera but previously depleted for *S. aureus*-specific antibodies.

The combination of both techniques led to a short list of 10 potential candidates. Out of those proteins, three were selected for further analysis, the DUF5011 domain-containing protein, Fe/B12 transporter and Fe(3+) dicitrate ABC transporter. Two other proteins in the list presented with a similar function as already described antigens in enterococci. We demonstrated that anti-protein sera raised against the enterococcal proteins was able to mediate the killing of *S. aureus* strains. We decided to further study this cross-opsonic effect and kept the two staphylococcal proteins for a deeper investigation.

The candidates were produced in *E. coli* and purified by affinity purification. The recombinant proteins were used to immunize pre-selected rabbits and produce polyclonal sera. To show the potential of our candidates as antigens, we studied the antibodies raised in rabbits. We first checked the binding function of the raised antibodies by performing ELISA. We showed that the produced antibodies contained in the polyclonal sera could bind to their target, confirming the effectiveness of the immunization schedule, and mostly importantly, the immunogenicity of the candidates we selected. Then we decided to assess the effectiveness of the IgGs by OPA to check their capacity to mediate the opsonic killing of *S. aureus* MW2. We showed that the antibodies raised by immunization with our candidates can mediate specific opsonic killing of this staphylococcal strain.

We were also curious and keen to investigate the cross-reactive effect seen of antibodies raised against the enterococcal proteins. We performed pairwise alignment which highlighted the sequence similarity between PrsA and PpiC, a previously described antigen in *E. faecium*, also interesting in formulations against *E. faecalis* (188), as well as between $AdcA_{au}$ and $AdcA_{fm}$, also considered a great antigen against infections caused by *E. faecium* and *E. faecalis* (187). Especially some highly conserved regions showed a high immunogenicity score (obtained via analysis through Vaxijen). Taking into account that information, we decided to use anti-PpiC and anti-$AdcA_{fm}$ polyclonal sera in OPA against staphylococcal strains and the results showed that those antibodies could mediate a cross-opsonic killing of *S. aureus*. Later, after immunization of rabbits with PrsA and $AdcA_{au}$, we showed that each four sera could bind to their homologous target. These data biologically confirmed that each pair share one epitope. Inhibition assays showed that both PrsA and $AdcA_{au}$ could inhibit the killing observed by anti-PpiC and anti-$AdcA_{au}$ respectively and confirmed that the homologs pairs are responsible for the cross reactivity observed with anti-PpiC and anti-$AdcA_{fm}$. Cross-opsonic studies were conducted using OPA and the results show that anti-protein sera raised against staphylococcal proteins not only mediate the opsonic killing of a large variety of *S. aureus* strains, but can also mediate the killing of both *E. faecalis* and *E. faecium*.

These results are consistent with the function of each protein. Indeed, PpiC is a parvulin-like peptidyl-prolyl *cis-trans* isomerase protein, which plays an essential role in protein folding and

catalyzing the isomerization of peptide bonds. PPIase are proteins ubiquitous in all types of cells. In bacteria, they are known to be surface-exposed, involved in penicillin-binding protein folding, essential to bacterial growth, and involved in β-lactam resistance which makes them important virulence factors (223–226). They are usually composed of three domains: (i) the N-terminal domain, which is covalently linked to a di-acyl glycerol and provides anchorage to the membrane (227); (ii) the parvulin PPIase domain, which catalyzes isomerization of peptide bonds preceding proline residues and limits the folding of proteins; and (iii) the C-terminal domain (228). Given those characteristics, it is not surprising that a protein from another organism could present with a sequence similarity with PpiC and that antibodies raised against PPIase could mediate the killing of several bacterial strains from different species.

In addition to catalyzing the post-translocational folding of exported proteins, the membrane-anchored foldase protein PrsA is involved in the physiology and pathogenicity of numerous bacteria, including *Helicobacter pylori* and *Listeria monocytogenes* (229–231). The prolyl isomerase activity of the PPIase proteins from *S. aureus*, *B. subtilis*, and *L. monocytogenes* was verified using protease-coupled PPIase tests (232–234). The PPIase domains of *S. aureus* and *B. subtilis* share a common parvulin fold, which is made up of four α-helices around a four-stranded antiparallel β-sheet (233). It has also been demonstrated that PrsA contributes to streptococci's pathogenicity (235). The discovery that penicillin-binding proteins are unstable in PrsA null mutants also indicates that PrsA is necessary for penicillin-binding protein folding and, consequently, for bacterial growth (224).

In line with our findings, subtractive genomics and reverse vaccinology methods also suggested PrsA as a possible vaccine target in MRSA (236). In numerous organisms, it has been discovered that PPIase make intriguing targets for vaccines. By using reverse vaccination on *Streptococcus sanguinis*, a bacteria that can cause infective endocarditis, PrsA was found to be a good antigen candidate (237). Furthermore, it is known that bacterial Macrophage Infectivity Potentiator (MIP)-like PPIases may be potential candidates for vaccines that protect humans and animals. *Neisseria gonorrhoeae* and *Neisseria meningitidis*, which cause meningitis and sepsis, and the sexually transmitted disease gonorrhea, both express MIP-PPIase, which may be involved in the intracellular survival and aid in their immune system evasion. Moreover, MIP-PPIase-specific antibodies were found in the blood of *N. gonorrhoeae*-infected individuals, indicating the antigenicity of the pathogen and its possible application as a vaccine target. Several bacterial strains whose MIP-like PPIase has been successfully researched as a vaccine antigen are listed in the review by Humbert et al (238).

The second pair of proteins in this study that were related to cross-reactive behavior, are ATP-binding cassette transporter substrate-binding lipoproteins. In *E. faecium*, $AdcA_{fm}$ is a substrate binding protein in the ABC transport system and belong to the metal binding lipoproteins family

who regulates zinc (Zn) homeostasis (239). All life forms require zinc, a d-block transitional metal ion that is involved in numerous biological processes like carbon metabolism and DNA transcription control (240). It has a structural and catalytic role in about 5% of bacterial proteins. Zn is absorbed by bacteria through Zn-binding proteins, including AdcA*fm*, which helps them to overcome the host-derived restriction of the ion and live in the harsh environment (241,242). In a range of bacterial species, mutations in Zn-regulating genes consistently decrease virulence and increase vulnerability to host defensive mechanisms (243–247). Significant issues with growth and survival as well as reduced resistance to ampicillin, bacitracin, and daptomycin are also brought on by inactivating the gene encoding the protein (239). Zn is also linked to biofilm formation where SasG protein promotes adhesion between cells in a Zn-dependent manner (248). It's interesting to note that the protein's expression has been demonstrated to rise during infection, and proteins with related functions have previously been studied in other Gram-positive pathogens as possible vaccine antigens or virulence factors (249,250).

To summarize, our dual approach for antigen identification led to the discovery of several candidates. Five of them were investigated to biologically assess their antigenic potential. The study successfully showed that those five proteins should be further investigated for use in vaccine formulation against *S. aureus*. This approach proved to be fast and effective, two important qualities for the development of new medicines. Additionally, two of the antigenic determinants identified and studied showed a sequence homology with other dangerous opportunistic Gram-positive pathogens: *E. faecalis* and *E. faecium*. Our results show that both proteins can be considered for vaccine formulation against the two nosocomial pathogens.

Further investigation should be undertaken to validate the potential of the antigens discovered for vaccine formulation. For example, the polyclonal sera used in this study could be used to perform passive immunization in animal experiments, which consist in the injection of the antibodies shortly before and after a challenge with bacteria. Additionally, active immunization could also be used to prove the effectiveness of the antigens. It is usually performed following an immunization schedule of three injections with the antigen and a challenge with bacteria. Depending on the bacterial species, different ways of challenging the animals after immunization can be employed. In the past, skin or muscle infection models were performed with *S. aureus* (251). Systemic infection models were also used with *S. aureus* and enterococci (221).

In the case of PrsA and $AdcA_{au}$, several highly conserved regions were identified in comparison with PpiC and $AdcA_{fm}$ respectively. These regions were also predicted to be highly immunogenic. One future direction for this investigation could be to produce these regions and investigate their potential for vaccine development.

The study could also be extended to several other pathogenic bacterial species as our candidates present with essential functions within the cells. As proven in this study, such characteristics can mean the presence of similar proteins in other organisms and lead to cross-reactive effects. Targeting essential proteins could help to develop multivalent vaccines, especially if investigation only conserved and immunogenic regions. These potential epitopes were situated in the effective regions of the proteins in our study, so it would be interesting to study their potential against other pathogens which have proteins with similar functions.

In 2021, Ismail and colleagues proposed an interesting strategy to better control WHO priority pathogens and address global antibiotic resistances; they talked about pan-vaccinomics whose aim is to create a universal vaccine targeting multiple pathogens (252). We believe this approach to be clever and strongly feel that study like ours could lead someday to the achievement of this goal. Indeed, we think that investigation of already-discovered antigens who are known to have essential function within bacterial cells and therefore to be ubiquitous is a great strategy to implement in the frame of pan-vaccinomics.

References

1. Rice LB. Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE. J INFECT DIS. 2008 Apr 15;197(8):1079–81.

2. Vale de Macedo GHR, Costa GDE, Oliveira ER, Damasceno GV, Mendonça JSP, Silva L dos S, et al. Interplay between ESKAPE Pathogens and Immunity in Skin Infections: An Overview of the Major Determinants of Virulence and Antibiotic Resistance. Pathogens. 2021 Feb 2;10(2):148.

3. Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, et al. Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011–2014. Infect Control Hosp Epidemiol. 2016 Nov;37(11):1288–301.

4. Lamaudière MTF, Arasaradnam R, Weedall GD, Morozov IY. The Colorectal Cancer Gut Environment Regulates Activity of the Microbiome and Promotes the Multidrug Resistant Phenotype of ESKAPE and Other Pathogens. mSphere. 2023 Feb 27;8(2):e00626-22.

5. Denissen J, Reyneke B, Waso-Reyneke M, Havenga B, Barnard T, Khan S, et al. Prevalence of ESKAPE pathogens in the environment: Antibiotic resistance status, community-acquired infection and risk to human health. International Journal of Hygiene and Environmental Health. 2022 Jul;244:114006.

6. Byappanahalli MN, Nevers MB, Korajkic A, Staley ZR, Harwood VJ. Enterococci in the Environment. Microbiol Mol Biol Rev. 2012 Dec;76(4):685–706.

7. Sexton JD, Reynolds KA. Exposure of emergency medical responders to methicillin-resistant Staphylococcus aureus. Am J Infect Control. 2010 Jun;38(5):368–73.

8. Akanbi OE, Njom HA, Fri J, Otigbu AC, Clarke AM. Antimicrobial Susceptibility of Staphylococcus aureus Isolated from Recreational Waters and Beach Sand in Eastern Cape Province of South Africa. IJERPH. 2017 Sep 1;14(9):1001.

9. Ebomah KE, Okoh AI. Detection of Carbapenem-Resistance Genes in Klebsiella Species Recovered from Selected Environmental Niches in the Eastern Cape Province, South Africa. Antibiotics. 2020 Jul 21;9(7):425.

10. Hrenovic J, Durn G, Music MS, Dekic S, Troskot-Corbic T, Skoric D. Extensively and multi drug-resistant Acinetobacter baumannii recovered from technosol at a dump site in Croatia. Science of The Total Environment. 2017 Dec;607–608:1049–55.

11. Ferguson MW, Maxwell JA, Vincent TS, da Silva J, Olson JC. Comparison of the exoS gene and protein expression in soil and clinical isolates of Pseudomonas aeruginosa. Infect Immun. 2001 Apr;69(4):2198–210.

12. Mena KD, Gerba CP. Risk assessment of Pseudomonas aeruginosa in water. Rev Environ Contam Toxicol. 2009;201:71–115.

13. Haritash AK, Kaushik CP. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. J Hazard Mater. 2009 Sep 30;169(1–3):1–15.

14. Purdy-Gibson ME, France M, Hundley TC, Eid N, Remold SK. Pseudomonas aeruginosa in CF and non-CF homes is found predominantly in drains. J Cyst Fibros. 2015 May;14(3):341–6.

15. Crone S, Vives-Flórez M, Kvich L, Saunders AM, Malone M, Nicolaisen MH, et al. The environmental occurrence of Pseudomonas aeruginosa. APMIS. 2020;128(3):220–31.

16. Rogers L, Power K, Gaora PÓ, Fanning S. Escherichia coli and Other Enterobacteriaceae: Occurrence and Detection. In: Caballero B, Finglas PM, Toldrá F, editors. Encyclopedia of Food and Health [Internet]. Oxford: Academic Press; 2016 [cited 2024 Feb 7]. p. 545–51. Available from: https://www.sciencedirect.com/science/article/pii/B9780123849472002592

17. Farré-Maduell E, Casals-Pascual C. The origins of gut microbiome research in Europe: From Escherich to Nissle. Human Microbiome Journal. 2019 Dec 1;14:100065.

18. Ursell LK, Haiser HJ, Van Treuren W, Garg N, Reddivari L, Vanamala J, et al. The Intestinal Metabolome—an Intersection Between Microbiota and Host. Gastroenterology. 2014 May;146(6):1470–6.

19. Hou K, Wu ZX, Chen XY, Wang JQ, Zhang D, Xiao C, et al. Microbiota in health and diseases. Sig Transduct Target Ther. 2022 Apr 23;7(1):1–28.

20. Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J. 2017 May 16;474(11):1823–36.

21. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. Elife. 2013 Nov 5;2:e01202.

22. Sharon G, Sampson TR, Geschwind DH, Mazmanian SK. The Central Nervous System and the Gut Microbiome. Cell. 2016 Nov 3;167(4):915–32.

23. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature. 2009 Jan;457(7228):480–4.

24. Wilck N, Matus MG, Kearney SM, Olesen SW, Forslund K, Bartolomaeus H, et al. Salt-responsive gut commensal modulates TH17 axis and disease. Nature. 2017 Nov;551(7682):585–9.

25. Wlodarska M, Kostic AD, Xavier RJ. An integrative view of microbiome-host interactions in inflammatory bowel diseases. Cell Host Microbe. 2015 May 13;17(5):577–91.

26. Zitvogel L, Daillère R, Roberti MP, Routy B, Kroemer G. Anticancer effects of the microbiome and its products. Nat Rev Microbiol. 2017 Aug;15(8):465–78.

27. Lebreton F, Manson AL, Saavedra JT, Straub TJ, Earl AM, Gilmore MS. Tracing the Enterococci from Paleozoic Origins to the Hospital. Cell. 2017 May 18;169(5):849-861.e13.

28. Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, et al. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microorganisms. 2019 Jan 10;7(1):14.

29. Murray BE. The life and times of the Enterococcus. Clin Microbiol Rev. 1990 Jan;3(1):46–65.

30. Taylor TA, Unakal CG. Staphylococcus aureus Infection. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 [cited 2024 Jan 15]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK441868/

31. Brown AF, Leech JM, Rogers TR, McLoughlin RM. Staphylococcus aureus Colonization: Modulation of Host Immune Response and Impact on Human Vaccine Design. Frontiers in Immunology [Internet]. 2014 [cited 2024 Feb 8];4. Available from: https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2013.00507

32. Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev. 1997 Jul;10(3):505–20.

33. van Belkum A, Verkaik NJ, de Vogel CP, Boelens HA, Verveer J, Nouwen JL, et al. Reclassification of Staphylococcus aureus nasal carriage types. J Infect Dis. 2009 Jun 15;199(12):1820–6.

34. Mertz D, Frei R, Jaussi B, Tietz A, Stebler C, Flückiger U, et al. Throat swabs are necessary to reliably detect carriers of Staphylococcus aureus. Clin Infect Dis. 2007 Aug 15;45(4):475–7.

35. Bourgeois-Nicolaos N, Lucet JC, Daubié C, Benchaba F, Rajguru M, Ruimy R, et al. Maternal vaginal colonisation by Staphylococcus aureus and newborn acquisition at delivery. Paediatr Perinat Epidemiol. 2010 Sep;24(5):488–91.

36. Acton DS, Plat-Sinnige MJT, van Wamel W, de Groot N, van Belkum A. Intestinal carriage of Staphylococcus aureus: how does its frequency compare with that of nasal carriage and what is its clinical impact? Eur J Clin Microbiol Infect Dis. 2009 Feb;28(2):115–27.

37. Sivaraman K, Venkataraman N, Cole AM. Staphylococcus aureus nasal carriage and its contributing factors. Future Microbiol. 2009 Oct;4(8):999–1008.

38. Joseph L, Merciecca T, Forestier C, Balestrino D, Miquel S. From Klebsiella pneumoniae Colonization to Dissemination: An Overview of Studies Implementing Murine Models. Microorganisms. 2021 Jun 12;9(6):1282.

39. Martin RM, Bachman MA. Colonization, Infection, and the Accessory Genome of Klebsiella pneumoniae. Front Cell Infect Microbiol. 2018 Jan 22;8:4.

40. Conlan S, Kong HH, Segre JA. Species-level analysis of DNA sequence data from the NIH Human Microbiome Project. PLoS One. 2012;7(10):e47075.

41. Gorrie CL, Mirceta M, Wick RR, Edwards DJ, Thomson NR, Strugnell RA, et al. Gastrointestinal Carriage Is a Major Reservoir of Klebsiella pneumoniae Infection in Intensive Care Patients. Clin Infect Dis. 2017 Jul 15;65(2):208–15.

42. Huynh BT, Passet V, Rakotondrasoa A, Diallo T, Kerleguer A, Hennart M, et al. Klebsiella pneumoniae carriage in low-income countries: antimicrobial resistance, genomic diversity and risk factors. Gut Microbes. 2020 Sep 2;11(5):1287–99.

43. Lin YT, Siu LK, Lin JC, Chen TL, Tseng CP, Yeh KM, et al. Seroepidemiology of Klebsiella pneumoniae colonizing the intestinal tract of healthy Chinese and overseas Chinese adults in Asian countries. BMC Microbiol. 2012 Jan 19;12:13.

44. Bartal C, Rolston KVI, Nesher L. Carbapenem-resistant Acinetobacter baumannii: Colonization, Infection and Current Treatment Options. Infect Dis Ther. 2022 Apr;11(2):683–94.

45. Bashir A, Brown JS. Pseudomonas aeruginosa. In: Janes SM, editor. Encyclopedia of Respiratory Medicine (Second Edition) [Internet]. Oxford: Academic Press; 2022 [cited 2024 Feb 8]. p. 14–22. Available from: https://www.sciencedirect.com/science/article/pii/B9780081027233002584

46. Forestier C, Guelon D, Cluytens V, Gillart T, Sirot J, De Champs C. Oral probiotic and prevention of Pseudomonas aeruginosa infections: a randomized, double-blind, placebo-controlled pilot study in intensive care unit patients. Crit Care. 2008;12(3):R69.

47. Ogunrinola GA, Oyewale JO, Oshamika OO, Olasehinde GI. The Human Microbiome and Its Impacts on Health. Int J Microbiol. 2020 Jun 12;2020:8045646.

48. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett C, Knight R, Gordon JI. The human microbiome project: exploring the microbial part of ourselves in a changing world. Nature. 2007 Oct 18;449(7164):804–10.

49. Ursell LK, Metcalf JL, Parfrey LW, Knight R. Defining the Human Microbiome. Nutr Rev. 2012 Aug;70(Suppl 1):S38–44.

50. Ma YX, Wang CY, Li YY, Li J, Wan QQ, Chen JH, et al. Considerations and Caveats in Combating ESKAPE Pathogens against Nosocomial Infections. Adv Sci (Weinh). 2020 Jan;7(1):1901872.

51. De Rosa FG, Corcione S, Raviolo S, Montrucchio C, Aldieri C, Pagani N, et al. Candidemia, and infections by Clostridium difficile and carbapenemase-producing Enterobacteriaceae: new enteropathogenetic opportunistic syndromes? Infez Med. 2015 Jun;23(2):105–16.

52. CDC. Guidelines for Prevention and Treatment of Opportunistic Infections in HIV-Infected Adults and Adolescents. Recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America [Internet]. 2009 [cited 2024 Feb 9]. Available from: https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5804a1.htm

53. Riccardi N, Rotulo GA, Castagnola E. Definition of Opportunistic Infections in Immunocompromised Children on the Basis of Etiologies and Clinical Features: A Summary for Practical Purposes. Curr Pediatr Rev. 2019 Nov;15(4):197–206.

54. Brown SP, Cornforth DM, Mideo N. Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. Trends Microbiol. 2012 Jul;20(7):336–42.

55. Aguilar-Salazar A, Martínez-Vázquez AV, Aguilera-Arreola G, de Jesus de Luna-Santillana E, Cruz-Hernández MA, Escobedo-Bonilla CM, et al. Prevalence of ESKAPE Bacteria in Surface Water and Wastewater Sources: Multidrug Resistance and Molecular Characterization, an Updated Review. Water. 2023 Jan;15(18):3200.

56. Ayobami O, Brinkwirth S, Eckmanns T, Markwart R. Antibiotic resistance in hospital-acquired ESKAPE-E infections in low- and lower-middle-income countries: a systematic review and meta-analysis. Emerging Microbes & Infections. 2022 Dec 31;11(1):443–51.

57. Tabah A, Koulenti D, Laupland K, Misset B, Valles J, Bruzzi de Carvalho F, et al. Characteristics and determinants of outcome of hospital-acquired bloodstream infections in intensive care units: the EUROBACT International Cohort Study. Intensive Care Med. 2012 Dec;38(12):1930–45.

58. Monegro AF, Muppidi V, Regunath H. Hospital-Acquired Infections. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 [cited 2024 Feb 9]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK441857/

59. Sikora A, Zahra F. Nosocomial Infections. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 [cited 2024 Feb 9]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK559312/

60. Weiner-Lastinger LM, Abner S, Edwards JR, Kallen AJ, Karlsson M, Magill SS, et al. Antimicrobial-resistant pathogens associated with adult healthcare-associated infections: Summary of data reported to the National Healthcare Safety Network, 2015–2017. Infect Control Hosp Epidemiol. 2020 Jan;41(1):1–18.

61. Kollef MH, Sharpless L, Vlasnik J, Pasque C, Murphy D, Fraser VJ. The Impact of Nosocomial Infections on Patient Outcomes Following Cardiac Surgery. CHEST. 1997 Sep 1;112(3):666–75.

62. Sheng WH, Chie WC, Chen YC, Hung CC, Wang JT, Chang SC. Impact of nosocomial infections on medical costs, hospital stay, and outcome in hospitalized patients. J Formos Med Assoc. 2005 May;104(5):318–26.

63. Liu JY, Dickter JK. Nosocomial Infections: A History of Hospital-Acquired Infections. Gastrointest Endosc Clin N Am. 2020 Oct;30(4):637–52.

64. Suetens C, Latour K, Kärki T, Ricchizzi E, Kinross P, Moro ML, et al. Prevalence of healthcare-associated infections, estimated incidence and composite antimicrobial resistance index in acute care hospitals and long-term care facilities: results from two European point prevalence surveys, 2016 to 2017. Euro Surveill. 2018 Nov 15;23(46):1800516.

65. D'Accolti M, Soffritti I, Mazzacane S, Caselli E. Fighting AMR in the Healthcare Environment: Microbiome-Based Sanitation Approaches and Monitoring Tools. Int J Mol Sci. 2019 Mar 27;20(7):1535.

66. Katz L, Baltz RH. Natural product discovery: past, present, and future. Journal of Industrial Microbiology and Biotechnology. 2016 Mar 1;43(2–3):155–76.

67. Armstrong GL. Trends in Infectious Disease Mortality in the United States During the 20th Century. JAMA. 1999 Jan 6;281(1):61.

68. Erdem H, Tetik A, Arun O, Besirbellioglu BA, Coskun O, Eyigun CP. War and infection in the pre-antibiotic era: the Third Ottoman Army in 1915. Scand J Infect Dis. 2011 Sep;43(9):690–5.

69. Cook MA, Wright GD. The past, present, and future of antibiotics. Sci Transl Med. 2022 Aug 10;14(657):eabo7793.

70. CDC. Ten Great Public Health Achievements -- United States, 1900-1999 [Internet]. 1999 [cited 2024 Feb 6]. Available from: https://www.cdc.gov/mmwr/preview/mmwrhtml/00056796.htm

71. WHO. GLASS Report: Early Implementation 2020 [Internet]. 2020 [cited 2024 Feb 6]. Available from: https://www.who.int/publications-detail-redirect/9789240005587

72. U.N. Environment Programme. UNEP - UN Environment Programme. 2020 [cited 2024 Jan 15]. Antimicrobial resistance: a global threat. Available from:

http://www.unep.org/explore-topics/chemicals-waste/what-we-do/emerging-issues/antimicrobial-resistance-global-threat

73. Murray CJL, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet. 2022 Feb;399(10325):629–55.

74. O'Neill J. Antimicrobial resistance : tackling a crisis for the health and wealth of nations [Internet]. Government of the United Kingdom; 2014 [cited 2024 Feb 6]. Available from: https://wellcomecollection.org/works/rdpck35v

75. O'Neill J. Tackling drug-resistant infections globally: final report and recommendations [Internet]. Government of the United Kingdom; 2016 May [cited 2024 Feb 6]. Available from: https://apo.org.au/node/63983

76. WHO. WHO publishes list of bacteria for which new antibiotics are urgently needed [Internet]. 2017 [cited 2024 Feb 6]. Available from: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

77. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. The Lancet Infectious Diseases. 2018 Mar;18(3):318–27.

78. Cassini A, Plachouras D, Eckmanns T, Abu Sin M, Blank HP, Ducomble T, et al. Burden of Six Healthcare-Associated Infections on European Population Health: Estimating Incidence-Based Disability-Adjusted Life Years through a Population Prevalence-Based Modelling Study. Harbarth S, editor. PLoS Med. 2016 Oct 18;13(10):e1002150.

79. De Oliveira DMP, Forde BM, Kidd TJ, Harris PNA, Schembri MA, Beatson SA, et al. Antimicrobial Resistance in ESKAPE Pathogens. Clin Microbiol Rev. 2020 Jun 17;33(3):e00181-19.

80. Vekemans J, Hasso-Agopsowicz M, Kang G, Hausdorff WP, Fiore A, Tayler E, et al. Leveraging Vaccines to Reduce Antibiotic Use and Prevent Antimicrobial Resistance: A World Health Organization Action Framework. Clinical Infectious Diseases. 2021 Aug 16;73(4):e1011–7.

81. Darby EM, Trampari E, Siasat P, Gaya MS, Alav I, Webber MA, et al. Molecular mechanisms of antibiotic resistance revisited. Nat Rev Microbiol. 2023 May;21(5):280–95.

82. Ogston A. Micrococcus Poisoning. J Anat Physiol. 1882 Oct;17(Pt 1):24–58.

83. Rosenbach FJ. Mikro-organismen bei den Wund-Infections-Krankheiten des Menschen [Internet]. Wiesbaden: J.F. Bergmann; 1884. Available from: https://www.biodiversitylibrary.org/bibliography/22955

84. Gherardi G, Di Bonaventura G, Savini V. Chapter 1 - Staphylococcal Taxonomy. In: Savini V, editor. Pet-To-Man Travelling Staphylococci [Internet]. Academic Press; 2018 [cited 2024 Feb 13]. p. 1–10. Available from: https://www.sciencedirect.com/science/article/pii/B9780128135471000017

85. Kadariya J, Smith TC, Thapaliya D. Staphylococcus aureus and Staphylococcal Food-Borne Disease: An Ongoing Challenge in Public Health. Biomed Res Int. 2014;2014:827965.

86. Pollitt EJG, Diggle SP. Defining motility in the Staphylococci. Cell Mol Life Sci. 2017;74(16):2943–58.

87. O'Riordan K, Lee JC. Staphylococcus aureus Capsular Polysaccharides. Clin Microbiol Rev. 2004 Jan;17(1):218–34.

88. Götz F, Bannerman T, Schleifer KH. The Genera Staphylococcus and Macrococcus. The Prokaryotes. 2006;5–75.

89. Missiakas DM, Schneewind O. Growth and Laboratory Maintenance of Staphylococcus aureus. Curr Protoc Microbiol. 2013 Feb;CHAPTER 9:Unit-9C.1.

90. Gardete S, Tomasz A. Mechanisms of vancomycin resistance in *Staphylococcus aureus*. J Clin Invest. 2014 Jul 1;124(7):2836–40.

91. Sato A, Yamaguchi T, Hamada M, Ono D, Sonoda S, Oshiro T, et al. Morphological and Biological Characteristics of Staphylococcus aureus Biofilm Formed in the Presence of Plasma. Microbial Drug Resistance. 2019 Jun;25(5):668–76.

92. Lowy FD. Staphylococcus aureus infections. N Engl J Med. 1998 Aug 20;339(8):520–32.

93. Goodwin KD, McNay M, Cao Y, Ebentier D, Madison M, Griffith JF. A multi-beach study of Staphylococcus aureus, MRSA, and enterococci in seawater and beach sand. Water Res. 2012 Sep 1;46(13):4195–207.

94. Thapaliya D, Hellwig EJ, Kadariya J, Grenier D, Jefferson AJ, Dalman M, et al. Prevalence and Characterization of Staphylococcus aureus and Methicillin-Resistant Staphylococcus aureus on Public Recreational Beaches in Northeast Ohio. Geohealth. 2017 Dec;1(10):320–32.

95. Deddefo A, Mamo G, Leta S, Amenu K. Prevalence and molecular characteristics of Staphylococcus aureus in raw milk and milk products in Ethiopia: a systematic review and meta-analysis. International Journal of Food Contamination. 2022 Sep 10;9(1):8.

96. Kozajda A, Jeżak K. Occupational exposure to Staphylococcus aureus in the wastewater treatment plants environment. Med Pr. 2020 May 15;71(3):265–78.

97. Nyenje ME, Odjadjare CE, Tanih NF, Green E, Ndip RN. Foodborne pathogens recovered from ready-to-eat foods from roadside cafeterias and retail outlets in Alice, Eastern Cape Province, South Africa: public health implications. Int J Environ Res Public Health. 2012 Aug;9(8):2608–19.

98. Loewen K, Schreiber Y, Kirlew M, Bocking N, Kelly L. Community-associated methicillin-resistant Staphylococcus aureus infection. Can Fam Physician. 2017 Jul;63(7):512–20.

99. Lakhundi S, Zhang K. Methicillin-Resistant Staphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology. Clin Microbiol Rev. 2018 Oct;31(4):e00020-18.

100. Guo Y, Song G, Sun M, Wang J, Wang Y. Prevalence and Therapies of Antibiotic-Resistance in Staphylococcus aureus. Front Cell Infect Microbiol. 2020;10:107.

101. Humphreys H. Staphylococcus aureus: the enduring pathogen in surgery. Surgeon. 2012 Dec;10(6):357–60.

102. Tong SYC, Davis JS, Eichenberger E, Holland TL, Fowler VG. Staphylococcus aureus Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. Clinical Microbiology Reviews. 2015 May 27;28(3):603–61.

103. Kimberlin C, Khalid F, Hariri A. Resistance of staphylococci to penicillin-G and cloxacillin. Pahlavi Med J. 1978 Apr;9(2):182–92.

104. Andrade JC, Morais-Braga MFB, Guedes GMM, Tintino SR, Freitas MA, Menezes IRA, et al. Enhancement of the antibiotic activity of aminoglycosides by alpha-tocopherol and other cholesterol derivates. Biomedicine & Pharmacotherapy. 2014 Oct 1;68(8):1065–9.

105. Harada Y, Chong Y, Shimono N, Miyake N, Uchida Y, Kadowaki M, et al. Nosocomial spread of meticillin-resistant Staphylococcus aureus with β-lactam-inducible arbekacin resistance. Journal of Medical Microbiology. 2014;63(5):710–4.

106. Hashizume H, Takahashi Y, Masuda T, Ohba S ichi, Ohishi T, Kawada M, et al. In vivo efficacy of β-lactam/tripropeptin C in a mouse septicemia model and the mechanism of reverse β-lactam resistance in methicillin-resistant Staphylococcus aureus mediated by tripropeptin C. J Antibiot. 2018 Jan;71(1):79–85.

107. Khan S, Sallum UW, Zheng X, Nau GJ, Hasan T. Rapid optical determination of β-lactamase and antibiotic activity. BMC Microbiology. 2014 Apr 4;14(1):84.

108. Foster TJ. The remarkably multifunctional fibronectin binding proteins of Staphylococcus aureus. Eur J Clin Microbiol Infect Dis. 2016 Dec 1;35(12):1923–31.

109. Jang S. Multidrug efflux pumps in Staphylococcus aureus and their clinical implications. J Microbiol. 2016 Jan 1;54(1):1–8.

110. Gould IM, David MZ, Esposito S, Garau J, Lina G, Mazzei T, et al. New insights into meticillin-resistant Staphylococcus aureus (MRSA) pathogenesis, treatment and resistance. Int J Antimicrob Agents. 2012 Feb;39(2):96–104.

111. Rodvold KA, McConeghy KW. Methicillin-resistant Staphylococcus aureus therapy: past, present, and future. Clin Infect Dis. 2014 Jan;58 Suppl 1:S20-27.

112. Khoshnood S, Heidary M, Asadi A, Soleimani S, Motahar M, Savari M, et al. A review on mechanism of action, resistance, synergism, and clinical implications of mupirocin against *Staphylococcus aureus*. Biomedicine & Pharmacotherapy. 2019 Jan 1;109:1809–18.

113. Rayner C, Munckhof WJ. Antibiotics currently used in the treatment of infections caused by Staphylococcus aureus. Internal Medicine Journal. 2005;35(s2):S3–16.

114. CDC. Antibiotic resistance threats in the United States, 2019 [Internet]. Centers for Disease Control and Prevention (U.S.); 2019 Nov [cited 2024 Feb 14]. Available from: https://stacks.cdc.gov/view/cdc/82532

115. Kourtis AP. Vital Signs: Epidemiology and Recent Trends in Methicillin-Resistant and in Methicillin-Susceptible Staphylococcus aureus Bloodstream Infections — United States. MMWR Morb Mortal Wkly Rep [Internet]. 2019 [cited 2024 Feb 14];68. Available from: https://www.cdc.gov/mmwr/volumes/68/wr/mm6809e1.htm

116. DeLeo FR, Otto M, Kreiswirth BN, Chambers HF. Community-associated meticillin-resistant Staphylococcus aureus. The Lancet. 2010 May 1;375(9725):1557–68.

117. Dermota U, Jurca T, Harlander T, Košir M, Zajc U, Golob M, et al. Infections Caused By Community-Associated Methicillin-Resistant European Clone (ST80) In Slovenia Between 2006 And 2013. Slovenian Journal of Public Health. 2016 Jun 1;55(2):131–5.

118. Drougka E, Foka A, Liakopoulos A, Doudoulakakis A, Jelastopulu E, Chini V, et al. A 12-year survey of methicillin-resistant Staphylococcus aureus infections in Greece: ST80-IV epidemic? Clinical Microbiology and Infection. 2014 Nov 1;20(11):O796–803.

119. Pada SK, Ding Y, Ling ML, Hsu LY, Earnest A, Lee TE, et al. Economic and clinical impact of nosocomial meticillin-resistant Staphylococcus aureus infections in Singapore: a matched case–control study. Journal of Hospital Infection. 2011 May 1;78(1):36–40.

120. Cong Y, Yang S, Rao X. Vancomycin resistant Staphylococcus aureus infections: A review of case updating and clinical features. J Adv Res. 2020 Jan;21:169–76.

121. Chan A. Among superbugs, MRSA is at the forefront of antimicrobial resistance | Institute for Health Metrics and Evaluation [Internet]. 2022 [cited 2024 Feb 14]. Available from: https://www.healthdata.org/news-events/insights-blog/acting-data/among-superbugs-mrsa-forefront-antimicrobial-resistance

122. Uribe-Querol E, Rosales C. Phagocytosis: Our Current Understanding of a Universal Biological Process. Frontiers in Immunology [Internet]. 2020 [cited 2024 Feb 14];11. Available from: https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2020.01066

123. de Jong NWM, van Kessel KPM, van Strijp JAG. Immune Evasion by Staphylococcus aureus. Microbiol Spectr. 2019 Mar;7(2).

124. Ko YP, Kuipers A, Freitag CM, Jongerius I, Medina E, van Rooijen WJ, et al. Phagocytosis Escape by a Staphylococcus aureus Protein That Connects Complement and Coagulation Proteins at the Bacterial Surface. PLoS Pathog. 2013 Dec 12;9(12):e1003816.

125. Jones C. Revised structures for the capsular polysaccharides from Staphylococcus aureus Types 5 and 8, components of novel glycoconjugate vaccines. Carbohydr Res. 2005 May 2;340(6):1097–106.

126. Watts A, Ke D, Wang Q, Pillay A, Nicholson-Weller A, Lee JC. Staphylococcus aureus Strains That Express Serotype 5 or Serotype 8 Capsular Polysaccharides Differ in Virulence. Infect Immun. 2005 Jun;73(6):3502–11.

127. Herbert S, Newell SW, Lee C, Wieland KP, Dassy B, Fournier JM, et al. Regulation of Staphylococcus aureus Type 5 and Type 8 Capsular Polysaccharides by CO2. J Bacteriol. 2001 Aug;183(15):4609–13.

128. Thiercelin. Morphologie et modes de reproduction de l'enterocoque. In 1899. p. 551–3. (Comptes Rendus des Seances de la Societe de Biologie et des ses Filiales.; vol. 11).

129. Maccallum WG, Hastings TW. A CASE OF ACUTE ENDOCARDITIS CAUSED BY MICROCOCCUS ZYMOGENES (NOV. SPEC.), WITH A DESCRIPTION OF THE MICROORGANISM. J Exp Med. 1899 Sep 1;4(5–6):521–34.

130. Andrewes F, Horder T. A study of streptococci pathogenic for man. In: Lancet. 1906. p. 852–5. (168).

131. Schleifer KH, Kilpper-Bälz R. Transfer of Streptococcus faecalis and Streptococcus faecium to the Genus Enterococcus nom. rev. as Enterococcus faecalis comb. nov. and

Enterococcus faecium comb. nov. International Journal of Systematic and Evolutionary Microbiology. 1984;34(1):31–4.

132. Fisher K, Phillips C. The ecology, epidemiology and virulence of Enterococcus. Microbiology (Reading). 2009 Jun;155(Pt 6):1749–57.

133. Tendolkar PM, Baghdayan AS, Shankar N. Pathogenic enterococci: new developments in the 21st century. Cell Mol Life Sci. 2003 Dec;60(12):2622–36.

134. Giraffa G. ENTEROCOCCUS. In: Robinson RK, editor. Encyclopedia of Food Microbiology [Internet]. Oxford: Elsevier; 1999 [cited 2024 Feb 14]. p. 617–24. Available from: https://www.sciencedirect.com/science/article/pii/B0122270703005201

135. Abriouel H, Omar NB, Molinos AC, López RL, Grande MJ, Martínez-Viedma P, et al. Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical samples. International Journal of Food Microbiology. 2008 Mar 31;123(1):38–49.

136. Martin JD, Mundt JO. Enterococci in insects. Appl Microbiol. 1972 Oct;24(4):575–80.

137. Micallef SA, Rosenberg Goldstein RE, George A, Ewing L, Tall BD, Boyer MS, et al. Diversity, distribution and antibiotic resistance of *Enterococcus* spp. recovered from tomatoes, leaves, water and soil on U.S. Mid-Atlantic farms. Food Microbiology. 2013 Dec 1;36(2):465–74.

138. Nowakiewicz A, Ziółkowska G, Trościańczyk A, Zięba P, Gnat S. Determination of resistance and virulence genes in *Enterococcus faecalis* and *E. faecium* strains isolated from poultry and their genotypic characterization by ADSRRS-fingerprinting. Poultry Science. 2017 Apr 1;96(4):986–96.

139. Sadowy E, Luczkiewicz A. Drug-resistant and hospital-associated Enterococcus faecium from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. BMC Microbiology. 2014 Mar 14;14(1):66.

140. Ben Omar N, Castro A, Lucas R, Abriouel H, Yousif NMK, Franz CMAP, et al. Functional and safety aspects of Enterococci isolated from different Spanish foods. Syst Appl Microbiol. 2004 Feb;27(1):118–30.

141. Fontana C, Gazzola S, Cocconcelli PS, Vignolo G. Population structure and safety aspects of Enterococcus strains isolated from artisanal dry fermented sausages produced in Argentina. Lett Appl Microbiol. 2009 Sep;49(3):411–4.

142. Franz C, Holzapfel WH, Stiles ME. Enterococci at the crossroads of food safety? Int J Food Microbiol. 1999 Mar 1;47(1–2):1–24.

143. Franz C, Stiles ME, Schleifer KH, Holzapfel WH. Enterococci in foods--a conundrum for food safety. Int J Food Microbiol. 2003 Dec 1;88(2–3):105–22.

144. Krawczyk B, Wityk P, Gałęcka M, Michalik M. The Many Faces of Enterococcus spp.—Commensal, Probiotic and Opportunistic Pathogen. Microorganisms. 2021 Sep 7;9(9):1900.

145. Lebreton F, Willems RJL, Gilmore MS. Enterococcus Diversity, Origins in Nature, and Gut Colonization. In: Gilmore MS, Clewell DB, Ike Y, Shankar N, editors. Enterococci: From Commensals to Leading Causes of Drug Resistant Infection [Internet]. Boston:

Massachusetts Eye and Ear Infirmary; 2014 [cited 2024 Feb 8]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK190427/

146. Wan LYM, Chen ZJ, Shah NP, El-Nezami H. Modulation of Intestinal Epithelial Defense Responses by Probiotic Bacteria. Crit Rev Food Sci Nutr. 2016 Dec 9;56(16):2628–41.

147. Kao PHN, Kline KA. Dr. Jekyll and Mr. Hide: How *Enterococcus faecalis* Subverts the Host Immune Response to Cause Infection. Journal of Molecular Biology. 2019 Jul 26;431(16):2932–45.

148. Krawczyk B, Wysocka M, Kotłowski R, Bronk M, Michalik M, Samet A. Linezolid-resistant Enterococcus faecium strains isolated from one hospital in Poland -commensals or hospital-adapted pathogens? PLoS One. 2020;15(5):e0233504.

149. Hollenbeck BL, Rice LB. Intrinsic and acquired resistance mechanisms in enterococcus. Virulence. 2012 Aug 15;3(5):421–569.

150. Murray BE. Vancomycin-resistant enterococcal infections. N Engl J Med. 2000 Mar 9;342(10):710–21.

151. Lee T, Pang S, Abraham S, Coombs GW. Antimicrobial-resistant CC17 *Enterococcus faecium*: The past, the present and the future. Journal of Global Antimicrobial Resistance. 2019 Mar 1;16:36–47.

152. Kamboj M, Cohen N, Gilhuley K, Babady NE, Seo SK, Sepkowitz KA. Emergence of Daptomycin-Resistant VRE: Experience of a Single Institution. Infection Control & Hospital Epidemiology. 2011 Apr;32(4):391–4.

153. Furtula V, Jackson CR, Farrell EG, Barrett JB, Hiott LM, Chambers PA. Antimicrobial Resistance in Enterococcus spp. Isolated from Environmental Samples in an Area of Intensive Poultry Production. International Journal of Environmental Research and Public Health. 2013 Mar;10(3):1020–36.

154. Lellek H, Franke GC, Ruckert C, Wolters M, Wolschke C, Christner M, et al. Emergence of daptomycin non-susceptibility in colonizing vancomycin-resistant *Enterococcus faecium* isolates during daptomycin therapy. International Journal of Medical Microbiology. 2015 Dec 1;305(8):902–9.

155. Raja NS, Karunakaran R, Ngeow YF, Awang R. Community-acquired vancomycin-resistant Enterococcus faecium: a case report from Malaysia. Journal of Medical Microbiology. 2005;54(9):901–3.

156. David MZ, Daum RS. Treatment of Staphylococcus aureus Infections. In: Bagnoli F, Rappuoli R, Grandi G, editors. Staphylococcus aureus: Microbiology, Pathology, Immunology, Therapy and Prophylaxis [Internet]. Cham: Springer International Publishing; 2017 [cited 2024 Feb 14]. p. 325–83. (Current Topics in Microbiology and Immunology). Available from: https://doi.org/10.1007/82_2017_42

157. Clokie MR, Millard AD, Letarov AV, Heaphy S. Phages in nature. Bacteriophage. 2011;1(1):31–45.

158. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. Bacteriophage. 2011;1(2):66–85.

159. Fish R, Kutter E, Wheat G, Blasdel B, Kutateladze M, Kuhl S. Bacteriophage treatment of intransigent diabetic toe ulcers: a case series. J Wound Care. 2016 Jul;25(Sup7):S27–33.

160. Zhvania P, Hoyle NS, Nadareishvili L, Nizharadze D, Kutateladze M. Phage Therapy in a 16-Year-Old Boy with Netherton Syndrome. Front Med (Lausanne). 2017 Jul 3;4:94.

161. Kvachadze L, Balarjishvili N, Meskhi T, Tevdoradze E, Skhirtladze N, Pataridze T, et al. Evaluation of lytic activity of staphylococcal bacteriophage Sb-1 against freshly isolated clinical pathogens. Microb Biotechnol. 2011 Sep;4(5):643–50.

162. Rodríguez-Lucas C, Ladero V. Enterococcal Phages: Food and Health Applications. Antibiotics. 2023 May;12(5):842.

163. Plumet L, Ahmad-Mansour N, Dunyach-Remy C, Kissa K, Sotto A, Lavigne JP, et al. Bacteriophage Therapy for Staphylococcus Aureus Infections: A Review of Animal Models, Treatments, and Clinical Trials. Front Cell Infect Microbiol. 2022 Jun 17;12:907314.

164. Bilinski J, Grzesiowski P, Sorensen N, Madry K, Muszynski J, Robak K, et al. Fecal Microbiota Transplantation in Patients With Blood Disorders Inhibits Gut Colonization With Antibiotic-Resistant Bacteria: Results of a Prospective, Single-Center Study. Clinical Infectious Diseases. 2017 Aug 1;65(3):364–70.

165. Riedel S. Edward Jenner and the history of smallpox and vaccination. Proc (Bayl Univ Med Cent). 2005 Jan;18(1):21–5.

166. Ahmed R, Gray D. Immunological memory and protective immunity: understanding their relation. Science. 1996 Apr 5;272(5258):54–60.

167. Sallusto F, Lanzavecchia A, Araki K, Ahmed R. From Vaccines to Memory and Back. Immunity. 2010 Oct 29;33(4):451–63.

168. Jansen KU, Knirsch C, Anderson AS. The role of vaccines in preventing bacterial antimicrobial resistance. Nat Med. 2018 Jan;24(1):10–9.

169. Begier E, Seiden DJ, Patton M, Zito E, Severs J, Cooper D, et al. SA4Ag, a 4-antigen *Staphylococcus aureus* vaccine, rapidly induces high levels of bacteria-killing antibodies. Vaccine. 2017 Feb 22;35(8):1132–9.

170. Hassanzadeh H, Baber J, Begier E, Noriega DC, Konishi H, Yato Y, et al. Efficacy of a 4-Antigen Staphylococcus aureus Vaccine in Spinal Surgery: The STaphylococcus aureus suRgical Inpatient Vaccine Efficacy (STRIVE) Randomized Clinical Trial. Clin Infect Dis. 2023 Jul 26;77(2):312–20.

171. Pfizer. A PHASE 2B, RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED STUDY TO EVALUATE THE SAFETY AND EFFICACY OF STAPHYLOCOCCUS AUREUS 4 ANTIGEN VACCINE (SA4AG) IN ADULTS UNDERGOING ELECTIVE OPEN POSTERIOR SPINAL FUSION PROCEDURES WITH MULTILEVEL INSTRUMENTATION [Internet]. clinicaltrials.gov; 2020 Dec [cited 2024 Jan 1]. Report No.: NCT02388165. Available from: https://clinicaltrials.gov/study/NCT02388165

172. Fowler VG, Allen KB, Moreira ED, Moustafa M, Isgro F, Boucher HW, et al. Effect of an investigational vaccine for preventing Staphylococcus aureus infections after cardiothoracic surgery: a randomized trial. JAMA. 2013 Apr 3;309(13):1368–78.

173. Shinefield H, Black S, Fattom A, Horwith G, Rasgon S, Ordonez J, et al. Use of a Staphylococcus aureus Conjugate Vaccine in Patients Receiving Hemodialysis. New England Journal of Medicine. 2002 Feb 14;346(7):491–6.

174. Jahantigh HR, Faezi S, Habibi M, Mahdavi M, Stufano A, Lovreglio P, et al. The Candidate Antigens to Achieving an Effective Vaccine against Staphylococcus aureus. Vaccines. 2022 Jan 27;10(2):199.

175. Kalfopoulou E, Huebner J. Advances and Prospects in Vaccine Development against Enterococci. Cells. 2020 Nov 2;9(11):2397.

176. Colque-Navarro P, Palma M, Söderquist B, Flock JI, Möllby R. Antibody Responses in Patients with Staphylococcal Septicemia against Two Staphylococcus aureus Fibrinogen Binding Proteins: Clumping Factor and an Extracellular Fibrinogen Binding Protein. Clin Diagn Lab Immunol. 2000 Jan;7(1):14–20.

177. Patti JM, Bremell T, Krajewska-Pietrasik D, Abdelnour A, Tarkowski A, Rydén C, et al. The Staphylococcus aureus collagen adhesin is a virulence determinant in experimental septic arthritis. Infect Immun. 1994 Jan;62(1):152–61.

178. Cruciani M, Etna MP, Camilli R, Giacomini E, Percario ZA, Severa M, et al. Staphylococcus aureus Esx Factors Control Human Dendritic Cell Functions Conditioning Th1/Th17 Response. Front Cell Infect Microbiol. 2017 Jul 21;7:330.

179. Shinji H, Yosizawa Y, Tajima A, Iwase T, Sugimoto S, Seki K, et al. Role of Fibronectin-Binding Proteins A and B in In Vitro Cellular Infections and In Vivo Septic Infections by Staphylococcus aureus ▿. Infect Immun. 2011 Jun;79(6):2215–23.

180. Berube BJ, Bubeck Wardenburg J. Staphylococcus aureus α-Toxin: Nearly a Century of Intrigue. Toxins (Basel). 2013 Jun 13;5(6):1140–66.

181. Hammer ND, Skaar EP. Molecular mechanisms of Staphylococcus aureus iron acquisition. Annu Rev Microbiol. 2011;65:10.1146/annurev-micro-090110–102851.

182. Diep BA, Chan L, Tattevin P, Kajikawa O, Martin TR, Basuino L, et al. Polymorphonuclear leukocytes mediate Staphylococcus aureus Panton-Valentine leukocidin-induced lung inflammation and injury. Proc Natl Acad Sci U S A. 2010 Mar 23;107(12):5587–92.

183. Salazar N, Castiblanco-Valencia MM, da Silva LB, de Castro ÍA, Monaris D, Masuda HP, et al. Staphylococcus aureus Manganese Transport Protein C (MntC) Is an Extracellular Matrix- and Plasminogen-Binding Protein. PLoS One. 2014 Nov 19;9(11):e112730.

184. Sabat A, Melles DC, Martirosian G, Grundmann H, van Belkum A, Hryniewicz W. Distribution of the Serine-Aspartate Repeat Protein-Encoding sdr Genes among Nasal-Carriage and Invasive Staphylococcus aureus Strains. J Clin Microbiol. 2006 Mar;44(3):1135–8.

185. Singh KV, Nallapareddy SR, Sillanpää J, Murray BE. Importance of the Collagen Adhesin Ace in Pathogenesis and Protection against Enterococcus faecalis Experimental Endocarditis. PLoS Pathog. 2010 Jan 8;6(1):e1000716.

186. Nallapareddy SR, Singh KV, Murray BE. Contribution of the Collagen Adhesin Acm to Pathogenesis of Enterococcus faecium in Experimental Endocarditis. Infect Immun. 2008 Sep;76(9):4120–8.

187. Romero-Saavedra F, Laverde D, Budin-Verneuil A, Muller C, Bernay B, Benachour A, et al. Characterization of Two Metal Binding Lipoproteins as Vaccine Candidates for Enterococcal Infections. Freitag NE, editor. PLoS ONE. 2015 Aug 31;10(8):e0136625.

188. Romero-Saavedra F, Laverde D, Wobser D, Michaux C, Budin-Verneuil A, Bernay B, et al. Identification of Peptidoglycan-Associated Proteins as Vaccine Candidates for Enterococcal Infections. Woo PCY, editor. PLoS ONE. 2014 Nov 4;9(11):e111880.

189. Flores-Mireles AL, Pinkner JS, Caparon MG, Hultgren SJ. EbpA vaccine antibodies block binding of Enterococcus faecalis to fibrinogen to prevent catheter-associated bladder infection in mice. Sci Transl Med. 2014 Sep 17;6(254):254ra127.

190. Kazemian H, Pourmand MR, Siadat SD, Mahdavi M, Yazdi MH, Avakh Majelan P, et al. Molecular Cloning and Immunogenicity Evaluation of PpiC, GelE, and VS87_01105 Proteins of Enterococcus faecalis as Vaccine Candidates. Iran Biomed J. 2019 Sep;23(5):344–53.

191. Kropec A, Sava IG, Vonend C, Sakinc T, Grohmann E, Huebner J. Identification of SagA as a novel vaccine target for the prevention of Enterococcus faecium infections. Microbiology. 2011 Dec 1;157(12):3429–34.

192. CDC. Four pediatric deaths from community-acquired methicillin-resistant Staphylococcus aureus — Minnesota and North Dakota, 1997-1999. MMWR Morb Mortal Wkly Rep. 1999 Aug 20;48(32):707–10.

193. Diep BA, Palazzolo-Ballance AM, Tattevin P, Basuino L, Braughton KR, Whitney AR, et al. Contribution of Panton-Valentine Leukocidin in Community-Associated Methicillin-Resistant Staphylococcus aureus Pathogenesis. Ratner AJ, editor. PLoS ONE. 2008 Sep 12;3(9):e3198.

194. McKenney D, Hübner J, Muller E, Wang Y, Goldmann DA, Pier GB. The ica locus of Staphylococcus epidermidis encodes production of the capsular polysaccharide/adhesin. Infect Immun. 1998 Oct;66(10):4711–20.

195. Kreiswirth BN, Löfdahl S, Betley MJ, O'Reilly M, Schlievert PM, Bergdoll MS, et al. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature. 1983 Oct 20;305(5936):709–12.

196. Di Ciccio P, Vergara A, Festino AR, Paludi D, Zanardi E, Ghidini S, et al. Biofilm formation by Staphylococcus aureus on food contact surfaces: Relationship with temperature and cell surface hydrophobicity. Food Control. 2015 Apr;50:930–6.

197. Romero-Saavedra F, Laverde D, Kalfopoulou E, Martini C, Torelli R, Martinez-Matamoros D, et al. Conjugation of Different Immunogenic Enterococcal Vaccine Target Antigens Leads to Extended Strain Coverage. The Journal of Infectious Diseases. 2019 Oct 8;220(10):1589–98.

198. Huebner J, Wang Y, Krueger WA, Madoff LC, Martirosian G, Boisot S, et al. Isolation and chemical characterization of a capsular polysaccharide antigen shared by clinical isolates of Enterococcus faecalis and vancomycin-resistant Enterococcus faecium. Infect Immun. 1999 Mar;67(3):1213–9.

199. Maekawa S, Yoshioka M, Kumamoto Y. Proposal of a New Scheme for the Serological Typing of *Enterococcus faecalis* Strains. Microbiology and Immunology. 1992 Jul;36(7):671–81.

200. Green MR, Sambrook J. Molecular cloning: a laboratory manual. 4th ed. Cold Spring Harbor: Cold Spring Harbor laboratory press; 2012.

201. Dower WJ, Miller JF, Ragsdale CW. High efficiency transformation of E. coli by high voltage electroporation. Nucleic Acids Res. 1988 Jul 11;16(13):6127–45.

202. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A. 1977 Dec;74(12):5463–7.

203. Taverna F, Negri A, Piccinini R, Zecconi A, Nonnis S, Ronchi S, et al. Characterization of cell wall associated proteins of a Staphylococcus aureus isolated from bovine mastitis case by a proteomic approach. Vet Microbiol. 2007 Jan 31;119(2–4):240–7.

204. Nandakumar R, Nandakumar MP, Marten MR, Ross JM. Proteome Analysis of Membrane and Cell Wall Associated Proteins from Staphylococcus aureus. J Proteome Res. 2005 Apr 1;4(2):250–7.

205. Sianglum W, Srimanote P, Wonglumsom W, Kittiniyom K, Voravuthikunchai SP. Proteome analyses of cellular proteins in methicillin-resistant Staphylococcus aureus treated with rhodomyrtone, a novel antibiotic candidate. PLoS One. 2011 Feb 4;6(2):e16628.

206. Tjalsma H, Lambooy L, Hermans PW, Swinkels DW. Shedding & shaving: disclosure of proteomic expressions on a bacterial face. Proteomics. 2008 Apr;8(7):1415–28.

207. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(5259):680–5.

208. Badalian AM, Simonov YuA, Shevchenko VI. Method of vacuum correlation functions: Results and prospects. Phys Atom Nuclei. 2006 Oct 1;69(10):1781–802.

209. Madeira F, Pearce M, Tivey ARN, Basutkar P, Lee J, Edbali O, et al. Search and sequence analysis tools services from EMBL-EBI in 2022. Nucleic Acids Res. 2022 Jul 1;50(W1):W276–9.

210. Yu CS, Cheng CW, Su WC, Chang KC, Huang SW, Hwang JK, et al. CELLO2GO: A Web Server for Protein subCELlular LOcalization Prediction with Functional Gene Ontology Annotation. PLoS One. 2014 Jun 9;9(6):e99368.

211. Shen HB, Chou KC. Gpos-mPLoc: a top-down approach to improve the quality of predicting subcellular localization of Gram-positive bacterial proteins. Protein Pept Lett. 2009;16(12):1478–84.

212. Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC Bioinformatics. 2007 Jan 5;8:4.

213. Franco AR, Sadones O, Romerio A, Artusa V, Shaik MM, Pasco ST, et al. Novel TLR4-Activating Vaccine Adjuvant Enhances the Production of *Enterococcus faecium* -binding Antibodies. J Med Chem. 2024 Mar 21;acs.jmedchem.3c02215.

214. CDC. Centers for Disease Control and Prevention. 2022 [cited 2024 Jan 22]. The biggest antibiotic-resistant threats in the U.S. Available from: https://www.cdc.gov/drugresistance/biggest-threats.html

215. CDC. Methicillin-Resistant Staphylococcus Aureus. 2019;

216. CDC. Vancomycin-Resistant EnterococcI. 2019;

217. Bloom DE, Black S, Salisbury D, Rappuoli R. Antimicrobial resistance and the role of vaccines. Proc Natl Acad Sci USA. 2018 Dec 18;115(51):12868–71.

218. Micoli F, Bagnoli F, Rappuoli R, Serruto D. The role of vaccines in combatting antimicrobial resistance. Nat Rev Microbiol. 2021 May;19(5):287–302.

219. Kim HK, Kim H, Schneewind O, Missiakas D. Identifying protective antigens of *Staphylococcus aureus* , a pathogen that suppresses host immune responses. FASEB j. 2011 Oct;25(10):3605–12.

220. Laverde D, Wobser D, Romero-Saavedra F, Hogendorf W, Van Der Marel G, Berthold M, et al. Synthetic Teichoic Acid Conjugate Vaccine against Nosocomial Gram-Positive Bacteria. Miyaji EN, editor. PLoS ONE. 2014 Oct 21;9(10):e110953.

221. Laverde D, Probst I, Romero-Saavedra F, Kropec A, Wobser D, Keller W, et al. Targeting Type IV Secretion System Proteins to Combat Multidrug-Resistant Gram-positive Pathogens. The Journal of Infectious Diseases. 2017 Jun 15;215(12):1836–45.

222. Middleton JR. *Staphylococcus aureus* antigens and challenges in vaccine development. Expert Review of Vaccines. 2008 Aug;7(6):805–15.

223. Galat A. Peptidylprolyl Cis / Trans Isomerases (Immunophilins): Biological Diversity - Targets - Functions. CTMC. 2003 Aug 1;3(12):1315–47.

224. Hyyryläinen H, Marciniak BC, Dahncke K, Pietiäinen M, Courtin P, Vitikainen M, et al. Penicillin-binding protein folding is dependent on the PrsA peptidyl-prolyl *cis - trans* isomerase in *Bacillus subtilis*. Molecular Microbiology. 2010 Jul;77(1):108–27.

225. Kouri ED, Labrou NE, Garbis SD, Kalliampakou KI, Stedel C, Dimou M, et al. Molecular and Biochemical Characterization of the Parvulin-Type PPIases in *Lotus japonicus*. Plant Physiology. 2009 Jul 2;150(3):1160–73.

226. Roch M, Lelong E, Panasenko OO, Sierra R, Renzoni A, Kelley WL. Thermosensitive PBP2a requires extracellular folding factors PrsA and HtrA1 for Staphylococcus aureus MRSA β-lactam resistance. Commun Biol. 2019 Nov 15;2(1):1–11.

227. Stoll H, Dengjel J, Nerz C, Götz F. Staphylococcus aureus Deficient in Lipidation of Prelipoproteins Is Attenuated in Growth and Immune Activation. Infect Immun. 2005 Apr;73(4):2411–23.

228. Jousselin A, Manzano C, Biette A, Reed P, Pinho MG, Rosato AE, et al. The Staphylococcus aureus Chaperone PrsA Is a New Auxiliary Factor of Oxacillin Resistance Affecting Penicillin-Binding Protein 2A. Antimicrob Agents Chemother. 2016 Feb 26;60(3):1656–66.

229. Cahoon LA, Freitag NE. Listeria monocytogenes virulence factor secretion: don't leave the cell without a chaperone. Front Cell Infect Microbiol [Internet]. 2014 [cited 2024 Jan 15];4. Available from: http://journal.frontiersin.org/article/10.3389/fcimb.2014.00013/abstract

230. Jakob RP, Koch JR, Burmann BM, Schmidpeter PAM, Hunkeler M, Hiller S, et al. Dimeric Structure of the Bacterial Extracellular Foldase PrsA. Journal of Biological Chemistry. 2015 Feb;290(6):3278–92.

231. Jiang X, Yang Y, Zhou J, Liu H, Liao X, Luo J, et al. Peptidyl isomerase PrsA is surface-associated on *Streptococcus suis* and offers cross-protection against serotype 9 strain. FEMS Microbiology Letters [Internet]. 2019 Jan 1 [cited 2024 Jan 15];366(2). Available from: https://academic.oup.com/femsle/article/doi/10.1093/femsle/fnz002/5281431

232. Alonzo F, Xayarath B, Whisstock JC, Freitag NE. Functional analysis of the Listeria monocytogenes secretion chaperone PrsA2 and its multiple contributions to bacterial virulence. Mol Microbiol. 2011 Jun;80(6):1530–48.

233. Heikkinen O, Seppala R, Tossavainen H, Heikkinen S, Koskela H, Permi P, et al. Solution structure of the parvulin-type PPIase domain of Staphylococcus aureus PrsA – Implications for the catalytic mechanism of parvulins. BMC Struct Biol. 2009 Mar 24;9:17.

234. Vitikainen M, Lappalainen I, Seppala R, Antelmann H, Boer H, Taira S, et al. Structure-function analysis of PrsA reveals roles for the parvulin-like and flanking N- and C-terminal domains in protein folding and secretion in Bacillus subtilis. J Biol Chem. 2004 Apr 30;279(18):19302–14.

235. Hamilton A, Robinson C, Sutcliffe IC, Slater J, Maskell DJ, Davis-Poynter N, et al. Mutation of the Maturase Lipoprotein Attenuates the Virulence of *Streptococcus equi* to a Greater Extent than Does Loss of General Lipoprotein Lipidation. Infect Immun. 2006 Dec;74(12):6907–19.

236. Naorem RS, Pangabam BD, Bora SS, Goswami G, Barooah M, Hazarika DJ, et al. Identification of Putative Vaccine and Drug Targets against the Methicillin-Resistant Staphylococcus aureus by Reverse Vaccinology and Subtractive Genomics Approaches. Molecules. 2022 Mar 24;27(7):2083.

237. Ge X, Kitten T, Munro CL, Conrad DH, Xu P. Pooled Protein Immunization for Identification of Cell Surface Antigens in Streptococcus sanguinis. Xue C, editor. PLoS ONE. 2010 Jul 26;5(7):e11666.

238. Humbert MV, Almonacid Mendoza HL, Jackson AC, Hung MC, Bielecka MK, Heckels JE, et al. Vaccine potential of bacterial macrophage infectivity potentiator (MIP)-like peptidyl prolyl *cis/trans* isomerase (PPIase) proteins. Expert Review of Vaccines. 2015 Dec 2;14(12):1633–49.

239. Lam LN, Brunson DN, Molina JJ, Flores-Mireles AL, Lemos JA. The AdcACB/AdcAII system is essential for zinc homeostasis and an important contributor of *Enterococcus faecalis* virulence. Virulence. 2022 Dec 31;13(1):592–608.

240. Andreini C, Banci L, Bertini I, Rosato A. Zinc through the Three Domains of Life. J Proteome Res. 2006 Nov 1;5(11):3173–8.

241. Capdevila DA, Wang J, Giedroc DP. Bacterial Strategies to Maintain Zinc Metallostasis at the Host-Pathogen Interface. Journal of Biological Chemistry. 2016 Sep;291(40):20858–68.

242. Xia P, Lian S, Wu Y, Yan L, Quan G, Zhu G. Zinc is an important inter-kingdom signal between the host and microbe. Vet Res. 2021 Dec;52(1):39.

243. Bayle L, Chimalapati S, Schoehn G, Brown J, Vernet T, Durmort C. Zinc uptake by Streptococcus pneumoniae depends on both AdcA and AdcAII and is essential for normal bacterial morphology and virulence. Mol Microbiol. 2011 Nov;82(4):904–16.

244. Burcham LR, Le Breton Y, Radin JN, Spencer BL, Deng L, Hiron A, et al. Identification of Zinc-Dependent Mechanisms Used by Group B *Streptococcus* To Overcome Calprotectin-Mediated Stress. Cook L, McDaniel LS, editors. mBio. 2020 Dec 22;11(6):e02302-20.

245. Gao H, Dai W, Zhao L, Min J, Wang F. The Role of Zinc and Zinc Homeostasis in Macrophage Function. Journal of Immunology Research. 2018 Dec 6;2018:1–11.

246. Grim KP, San Francisco B, Radin JN, Brazel EB, Kelliher JL, Párraga Solórzano PK, et al. The Metallophore Staphylopine Enables *Staphylococcus aureus* To Compete with the

Host for Zinc and Overcome Nutritional Immunity. Torres VJ, editor. mBio. 2017 Nov 8;8(5):e01281-17.

247. Makthal N, Do H, Wendel BM, Olsen RJ, Helmann JD, Musser JM, et al. Group A Streptococcus AdcR Regulon Participates in Bacterial Defense against Host-Mediated Zinc Sequestration and Contributes to Virulence. Freitag NE, editor. Infect Immun. 2020 Jul 21;88(8):e00097-20.

248. Formosa-Dague C, Speziale P, Foster TJ, Geoghegan JA, Dufrêne YF. Zinc-dependent mechanical properties of Staphylococcus aureus biofilm-forming surface protein SasG. Proc Natl Acad Sci U S A. 2016 Jan 12;113(2):410–5.

249. Brown JS, Ogunniyi AD, Woodrow MC, Holden DW, Paton JC. Immunization with components of two iron uptake ABC transporters protects mice against systemic Streptococcus pneumoniae infection. Infect Immun. 2001 Nov;69(11):6702–6.

250. Garmory HS, Titball RW. ATP-binding cassette transporters are targets for the development of antibacterial vaccines and therapies. Infect Immun. 2004 Dec;72(12):6757–63.

251. Som A, Navasa N, Percher A, Scott RW, Tew GN, Anguita J. Identification of Synthetic Host Defense Peptide Mimics That Exert Dual Antimicrobial and Anti-Inflammatory Activities. Clin Vaccine Immunol. 2012 Nov;19(11):1784–91.

252. Ismail S, Shahid F, Khan A, Bhatti S, Ahmad S, Naz A, et al. Pan-vaccinomics approach towards a universal vaccine candidate against WHO priority pathogens to address growing global antibiotic resistance. Computers in Biology and Medicine. 2021 Sep;136:104705.

Affidavit

Affidavit

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I hereby declare, that the submitted thesis entitled:

Vaccine antigen identification against dangerous Gram-positive ESKAPE pathogens

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.
I further declare that the dissertation presented here has not been submitted in the same or similar form to any other institution for the purpose of obtaining an academic degree.

Munich, 05/11/2024 Océane Sadones

place, date Signature doctoral candidate

Confirmation of congruency

Confirmation of congruency between printed and electronic version of

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Curriculum vitae

Océane SADONES

Work experience and associated skills

2020-2024 - MSCA ITN BactiVax - Early Stage Researcher
Klinikum der Ludwig-Maximilian-Universität - MÜNCHEN (GERMANY)
Vaccine antigen identification against systemic infection - handling of S2 bacteria, opsonophagocytic assay, bacterial culture, cell culture, cloning, protein production and purification, Ig quantification and titers determination by ELISA, whole-cell ELISA, immunodot, surface protein extraction, rabbit selection for immunization, western blot, set up of mice protection model, supervision of students

2020 - **4-months** - Technician on COVID test platform
CHU de Poitiers - Diagnostic laboratory - POITIERS (FRANCE)
Samples recording for anonymous tests, handling of COVID-infected samples, use of robots for RNA extractions, RT-qPCR.

2019-2020 - **8-months** internship as part of the Master's degree
Laboratory of the Department of Pharmacological and biomolecular science - MILANO (ITALY)
Study of gene expression in *E.coli* under LPS stress - Vector construction, bacterial growth, β-galactosidase assay

2019 - **3-months** internship as part of the Master's degree
Laboratory of the School of Medicine - Universidad Católica de Valencia - VALENCIA (SPAIN)
RNaseL in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome patient subtyping - Northern Blot, RT-qPCR, cell culture

2017 - **2-months** internship as part of the 12th grade
Laboratoire STIM (Signalisation et Transports Ioniques Membranaires) - POITIERS (FRANCE)
Study of lipointoxication on muscular cells - Western Blot, viability assays, confocal microscopy

2016 - **2-months** internship as part of the 12th grade
Laboratoire EcoLab INRA (Institut National de la Recherche Agronomique) - TOULOUSE (FRANCE)
Towards functional validation of candidate genes for plant resistance to root disease - Genetic transformation methods

Publications

Ovejero T, **Sadones O**, Sánchez-Fito T, Almenar-Pérez E, Espejo JA, Martín-Martínez E, Nathanson L, Oltra E. Activation of Transposable Elements in Immune Cells of Fibromyalgia Patients. International Journal of Molecular Sciences. 2020; 21(4):1366. https://doi.org/10.3390/ijms21041366

Franco AR#, **Sadones O#,** Romerio A, et al. Novel TLR4-Activating Vaccine Adjuvant Enhances the Production of Enterococcus faecium-binding Antibodies. J Med Chem. doi:10.1021/acs.jmedchem.3c02215

Sadones O, Kramarska E, Laverde D, Berisio R, Huebner J, Romero-Saavedra F. Investigation of cross-opsonic effect leads to the discovery of PPIase-domain containing protein vaccine candidate to prevent infections by Gram-positive ESKAPE pathogens. *BMC Microbiol*. 2024;24(1):280. doi:10.1186/s12866-024-03427-w

Kramarska E, Toumi E, Squeglia F, Laverde D, Napolitano V, Frapy E, Autiero I, **Sadones O**, Huebner J, Skurnik D, Romero-Saavedra F, Berisio R. A rationally designed antigen elicits protective antibodies against multiple nosocomial Gram-positive pathogens. *NPJ vaccines*, *9*(1), 151. https://doi.org/10.1038/s41541-024-00940-x

Jurado-Martín, I., Tomás-Cortázar, J., Hou, Y., Sainz-Mejías, M., Mysior, M. M., **Sadonès, O.**, Huebner, J., Romero-Saavedra, F., Simpson, J. C., Baugh, J. A., & McClean, S. (2024). Proteomic approach to identify host cell attachment proteins provides protective Pseudomonas aeruginosa vaccine antigen FtsZ. *NPJ vaccines*, *9*(1), 204. https://doi.org/10.1038/s41541-024-00994-x

Two other manuscripts have been or will be submitted before the end of the year. One as first author and one as co-author.

Education

2020-2024 - **Doctor of Philosophy - PhD in Medical research - Grade: 1.42 (very good)**
- Ludwig Maximilians University - MÜNCHEN (GERMANY)
  2-month secondments in UCD Dublin and CIC bioGUNE Bilbao

2018-2020 - **Master's degree in Health and Biology - Microbiology and Immunology - with honors**
- 1st year - Université de Poitiers - POITIERS (FRANCE) - with honor, 2nd in the class ranking
- 2nd year - Università degli studi di Milano - La statale - MILANO (ITALY) - Classes in English

2017-2018 - **Bachelor of Science in Biochemistry - with honors**
- 1st semester - Université de La Rochelle - LA ROCHELLE (FRANCE)
- 2nd semester - Universidad Católica de Valencia - VALENCIA (SPAIN) - Classes in English

2015-2017 - **12th grade - two-year technical degree in Biotechnology - with honors**
- Lycée la Découverte - DECAZEVILLE (FRANCE)

2014-2015 - **Scientific High School degree - with honors**
- LEGTA Poitiers-Venours - ROUILLÉ (FRANCE)

Technical skills - acquired during the technical degree

- **Microbiology:** cell-counting, culture monitoring, cell typing, antibiogram, phage production and titration, transduction, yeast complementation assay, mold identification, biochemical and molecular bacterial identification (Api galleries, 16S rRNA gene sequencing)
- **Immunology:** flow cytometry, Ouchterlony method (immunoprecipitation, double immunodiffusion), simple immunoelectrophoresis, blood group determination
- **Cell culture:** mammalian cell line maintenance, cryoconservation, lymphocyte purification, animals and plant cells culture, primary culture, treatments applications, transfection
- **Biochemistry of proteins:** protein dosages (BCA, Folin-Lowry, Bradford, Biuret, Kjeldahl), chromatography (FPLC, CCM, BPLC, CPG), SDS-PAGE, IEF, 2D electrophoresis, Western blot, enzyme characterisation, ELISA, purification and yield calculation, β-galactosidase assay
- **Molecular biology:** DNA digestion and cartography, PCR, electrophoresis, DNA purification and quantification, RT-PCR, qPCR, cloning, Sanger sequencing, RNA extraction, Northern Blot, creation of vector
- **Fermentation engineering:** Kla determination by cooper and statistic method, use of fermentation units (small capacities - mantling, dismantling, control, pH/O2... probes), discontinuous fermentation campaign
- **Computer science and bioinformatic:** use of R software, Igor software (linear and hyperbolic regression), Graphpad, Microsoft softwares, BLAST tools, CloneManager, online softwares, primer design, Mega, BioEdit, Galaxy, Benchling and Mendeley

Other interests and skills

Languages:
- French - mother tongue
- English - C1 level: able to understand, speak, write and read reports or scientific articles without any difficulty - fluent
- Spanish - B2 level: speaking fluently
- German - A2 level: basic knowledge, able to communicate
- Italian - A1 level: basic knowledge

Hobbies: Cooking, travelling, dancing, reading

List of publications

https://orcid.org/0000-0002-5042-9724

Ovejero T, **Sadones O**, Sánchez-Fito T, Almenar-Pérez E, Espejo JA, Martín-Martínez E, Nathanson L, Oltra E. Activation of Transposable Elements in Immune Cells of Fibromyalgia Patients. International Journal of Molecular Sciences. 2020; 21(4):1366. https://doi.org/10.3390/ijms21041366

Franco AR#, **Sadones O#**, Romerio A, et al. Novel TLR4-Activating Vaccine Adjuvant Enhances the Production of Enterococcus faecium-binding Antibodies. J Med Chem. doi:10.1021/acs.jmedchem.3c02215

Sadones O, Kramarska E, Laverde D, Berisio R, Huebner J, Romero-Saavedra F. Investigation of cross-opsonic effect leads to the discovery of PPIase-domain containing protein vaccine candidate to prevent infections by Gram-positive ESKAPE pathogens. BMC Microbiol. 2024;24(1):280. doi:10.1186/s12866-024-03427-w

Kramarska E, Toumi E, Squeglia F, Laverde D, Napolitano V, Frapy E, Autiero I, **Sadones O**, Huebner J, Skurnik D, Romero-Saavedra F, Berisio R. A rationally designed antigen elicits protective antibodies against multiple nosocomial Gram-positive pathogens. NPJ vaccines, 9(1), 151. https://doi.org/10.1038/s41541-024-00940-x

Jurado-Martín, I., Tomás-Cortázar, J., Hou, Y., Sainz-Mejías, M., Mysior, M. M., **Sadonès, O.**, Huebner, J., Romero-Saavedra, F., Simpson, J. C., Baugh, J. A., & McClean, S. (2024). Proteomic approach to identify host cell attachment proteins provides protective Pseudomonas aeruginosa vaccine antigen FtsZ. NPJ vaccines, 9(1), 204. https://doi.org/10.1038/s41541-024-00994-x

Two other manuscripts have been or will be submitted before the end of 2024. One as first author and one as co-author.