Antibiotics and Host Responses in the Pathogenesis of *Staphylococcus Aureus* Infection

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Antibiotics and Host Responses in the Pathogenesis of *Staphylococcus Aureus* Infection

Antibiotica en gastheerreacties in de pathogenese van *Staphylococcus aureus* infecties

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TABLE OF CONTENTS

Chapter 1

Introduction

Bacterial infections have historically been a major burden in human health (11). Infections remain prevalent today and are involved in approximately 25% of all deaths, and are responsible for 28% of years lost due to disability worldwide (13). Infections occur in the general community, but are also prevalent and dangerous in acute care hospitals (14) and other health care settings. Patients in hospitals and other health care settings often have their skin barriers breached by catheters or surgical interventions, yielding wounds that provide gateways for infection. Hospitalized and health care patients also often have reduced immunity: transplantations, hematological malignancies, chemotherapies and radiation all reduce immune competence. The elderly (15), a growing demographic group, are also more susceptible to infection than the young. Hand hygiene, aseptic surgical techniques (16) and more recently, antimicrobial prophylaxis, screening and targeted decolonization strategies (17) have all reduced, but not eliminated healthcare associated infection. Technologies such as sanitation, vaccination and antibiotics have played major roles in significantly reducing infectious diseases mortality in the community, in the USA from approximately 800 per 100,000 persons per year in the 1900s to around 60 per 100,000 persons per year in the late 1990s (12).

Health care associated infections remain problematic, and may constitute a greater threat today than ever before. The Infectious Disease Society of America (IDSA) warns especially against a collection of healthcare associated pathogens abbreviated in the acronym ESKAPE (2, 18): ***E****nterococcus faecalis* (19, 20), ***S****taphylococcus aureus* (*S. aureus*) (21-23), ***K****lebsiella spp.* (24, 25), ***A****cinetobacter baumannii* (26, 27), ***P****seudomonas aeruginosa* (28, 29) and ***E****nterobacter spp*. (30, 31). These bacteria, also known as superbugs, currently have a significant impact on human health for several reasons: they produce many virulence factors by which they can cause life threatening infections (7, 32) and they are either intrinsically resistant to many antibiotics (33) or easily acquire resistance to many classes of antibiotics. Antimicrobial resistance is an area of special concern, as there are few new antimicrobials being developed, while the currently available compounds are losing effectiveness (2). Since the 1980s only few compounds with a truly novel mode of action have been introduced into clinical practice (13), giving rise to the possibility of a post antibiotic era, in which infections can be diagnosed but no longer treated effectively.

By studying the pathogenesis of infectious diseases we gain more insight in how patients become sick and how we can best treat them. These studies are usually performed without regards of the effects of intervening antimicrobial treatments. However, bacterial infections are commonly treated with antibiotics, which either kill bacteria or halt bacterial growth. After exposure to bacteria (or their products) the human immune system responds. Due to its specificity, the humoral immune response of the host can be used as indication which microbial factors have been expressed and are immunogenic *in vivo*. Differences in the humoral immune responses between patients undergoing differ-

ent types of infections may shed light on which virulence factors are actually involved in pathogenesis (3-6). Examples of bacterial responses to antibiotics or to immune-induced stresses are the production by the bacteria of compounds that modulate the host's immune responses (7), the formation of biofilms (8, 9) (Figure 1), modifying their surface charge and generally altering their behavior, such as their metabolism, in response to antimicrobial peptides.

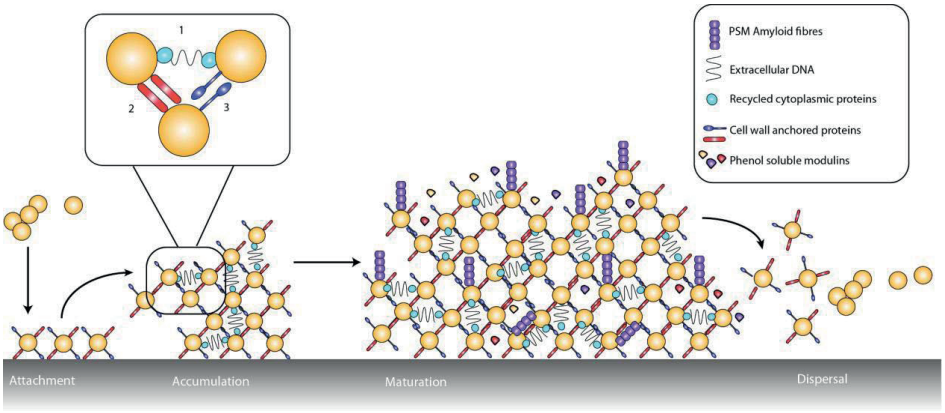

Figure 1: Staphylococcus aureus biofilm formation.
Attachment of *S. aureus* to a surface is mediated by CWA proteins. Cell-to-cell interactions occur during accumulation phase and can be mediated by several factors. The magnified region shows this in more detail: (1) extracellular DNA linking recycled cytoplasmic proteins; (2) CWA proteins binding adjacent cell surfaces; (3) Homophilic interactions between CWA proteins. PSMs form amyloid-like fibers visible at the surface of the biofilm. They also act in the formation of channels within the biofilm to allow nutrient access, while their surfactant properties aid the dispersal phase. The different stages of biofilm formation are detailed from left to right across the diagram. From: Hobley *et al.,* FEMS Microbiol Rev (2015) 39 (5): 649-669, doi: https://doi.org/10.1093/femsre/fuv015

Studying (changes in) the behavior of microbes faced with either antibiotics or challenges from the host's immune system could help explain why antimicrobial therapy and the immune system sometimes fail to clear an infection, as is often the case with invasive *Staphylococcus aureus* infections: 10-20% of all patients suffering from *Staphylococcus aureus* bacteremia (SAB) today still die, even with appropriate antimicrobial therapy. Better understanding of the pathogenesis of infection, accounting for microbe-derived disease-inducing factors (virulence factors), the host's defense response to these microbial challenges and the effects of applying antibiotic treatments, both individually and in concert should lead to a better understanding of infection and ultimately, better strategies to improve the outcomes of patients suffering from serious infectious diseases.

STAPHYLOCOCCUS AUREUS AND OUR IMMUNE SYSTEM

The pathogen studied in detail in this thesis is *Staphylococcus aureus*. This pathogen is present in the anterior nares of approximately 30-50% of all humans, approximately half of whom persistently carry these bacteria whereas others are intermittent carriers. *S. aureus* carriage is usually harmless, but carriage is not without risk: nasal carriers, especially persistent ones, have higher rates of infections by *S. aureus* than non- or intermittent carriers (6, 34-36), although some reports suggest that, once infected, carriers have a decreased risk of dying from *S. aureus* bacteremia compared to non-carriers (37). *S. aureus* can cause many different types of infection (34): from acute bacteremia (10) to chronic osteomyelitis (38), from superficial skin infections (39) to deep seated endocarditis (40) and from relatively mild abscesses (41) to life threatening pneumonia (42). *S. aureus* causes more deaths in the USA annually than HIV and tuberculosis combined, and, worryingly, it is proving increasingly resistant to antibiotic treatment (18, 43, 44).

To successfully cause serious infection *S. aureus* must invade the host and escape the human immune system, which is composed of innate and adaptive responses. Innate immunity is a first line of defense against infection comprising of cells and mechanisms which rapidly repel or clear infectious agents without the generation of immunological memory, although recently it has been proposed that especially monocytes and macrophages, which are important in innate immunity, may develop some level of memory called "trained immunity" or "innate immune memory", most likely via epigenetic changes (111). One of the first innate defenses *S. aureus* must evade are host defense peptides and the complement system (Figure 2). Host defense peptides, such as human beta defensins and cathelicidin can efficiently kill bacteria at low concentrations (56); these peptides target the cellular membrane, but also affect several intracellular targets (56). They further function as signal molecules of infection, attracting other cells to the site of infection (56). Complement is a system of molecules present in blood in inactive form and has several functions (Figure 2) (57): firstly, it functions as an opsonin enhancing phagocytosis of particulate matter (57). Secondly, complement is capable of clumping of agents, targeting them for removal (57). A third function of complement is lysis of cells by forming membrane attack complexes and so rupturing cellular membranes (57). Finally, complement catalyzes its own activation and causes a gradients of metabolites that functions as chemo-attractants to immune cells, including neutrophils (57). Complement can be activated in three different ways: the classical pathway, the alternative pathway and a lectin-mediated pathway. In the classical pathway, complement forms a complex with antibodies after binding to their specific target, generally of class IgM or IgG. The alternative pathway is continuously active at a low level, leading to constant hydrolysis of C3. Finally, in the lectin binding pathway, bacterial sugar moieties containing mannose or glucose bind mannose binding lectin, which in turn activates the complement cascade. All

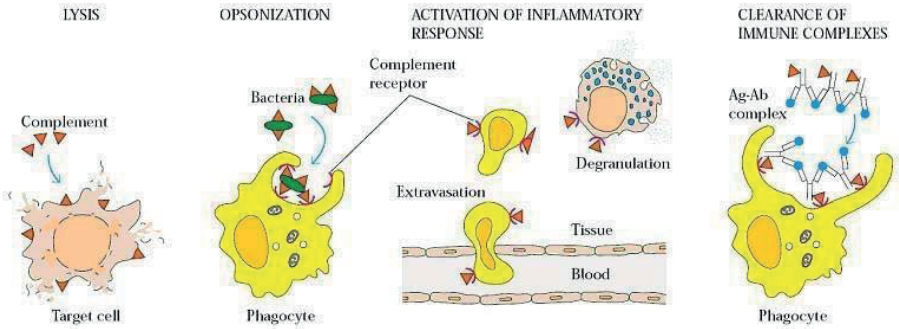

Figure 2: Major functions of the complement system
Complement is a system of molecules present in blood in inactive form and has several functions: firstly, complement lyses cells by forming membrane attack complexes and so rupturing cellular membranes. Secondly, it functions as an opsonin enhancing phagocytosis of antigens. A third function of complement is because complement catalyzes its own activation and causes a gradient it functions as a chemo-attractant to immune cells, such as neutrophils, and further primes them for degranulation. Finally, complement is capable of clumping of antigen-bearing agents, targeting them for removal. (From Kuby *et al.*, Immunology 2003)

complement activation pathways converge at the formation of a C3-convertase complex, activating cleavage of additional inactive complement components present in serum to complete the complement cascade. Neutrophils in the bloodstream sense chemokines such as complement 5a (C5a), interleukin 8 (IL8), macrophage chemo-attractant protein 1 (MCP-1) and leukotriene B4. In response to these chemokines, neutrophils circulating in the bloodstream slow down in a rolling motion and adhere to the endothelial inner surface of blood vessels in the vicinity of the focus of infection, and ultimately leave blood vessels near the site of infection (57, 58). These chemokines also activate neutrophil metabolism and stimulate neutrophil release of granules containing various antibacterial proteins such as the previously mentioned defensins, cathelicidin, and lysozyme (59). Activated neutrophils ingest invading pathogens either tagged with complement C3b using complement receptors CR1, CR3 and CR4 (60) or pathogens tagged with antibodies using Fc receptors (61), in a process called phagocytosis. Phagocytosis is facilitated in the presence of complement or antibodies, which function as opsonins. Ingested bacteria are killed by neutrophils: during respiratory burst hydrogen peroxide (H_2O_2), superoxide anions (O_2^-) and nitric oxide (NO) are generated from molecular oxygen by lysosomal NADPH and other enzymes, causing oxidative stress and ultimately bacterial death (58, 62). Finally, neutrophils can form neutrophil extracellular traps (NETs) (63), which trap invading bacteria in a matrix of DNA and protein, including histones (64), which have antibacterial activity (63). When *S. aureus* invades the human host, it is thus quickly confronted by various innate immune defenses and escape from all these systems is essential for the bacteria to survive and cause an infection.

When innate immune system fails to clear the invading pathogens, approximately 96 hours after primary infection, adaptive immunity responds (65). The adaptive immune response is composed of highly specialized systemic cells and processes that selectively target pathogens for neutralization and destruction, and results in immunological memory. Antigen presenting cells, mainly dendritic cells (DC), ingest invading pathogens and process them to antigens (66). These DC's presenting antigens are transported to lymph nodes, where a response is initiated against the antigens presented via major histocompatibility complex (MHC) molecules (66). B-cells, the main players in humoral immunity of the adaptive immune system, produce antibodies against these DC presented antigens (66). Antibodies consists of the hyper-variable antigen recognition part of the antibody called Fab and the constant part, called the Fc domain which is important for signaling and further immune activation (67). Antibodies have several functions (Figure 3): firstly, they tag microbes or infected cells for destruction by specifically recognizing them (67). Secondly, they neutralize, agglutinate and precipitate foreign antigens. Finally, antibodies function together with complement in opsonization as mentioned earlier, with neutrophils using Fc receptors FcγRI, RIIA, RIIB2 and RIIIB to recognize and ingest pathogens (Figure 2 and 3) (67). Antibodies or immunoglobulins (Ig's) exist in 5

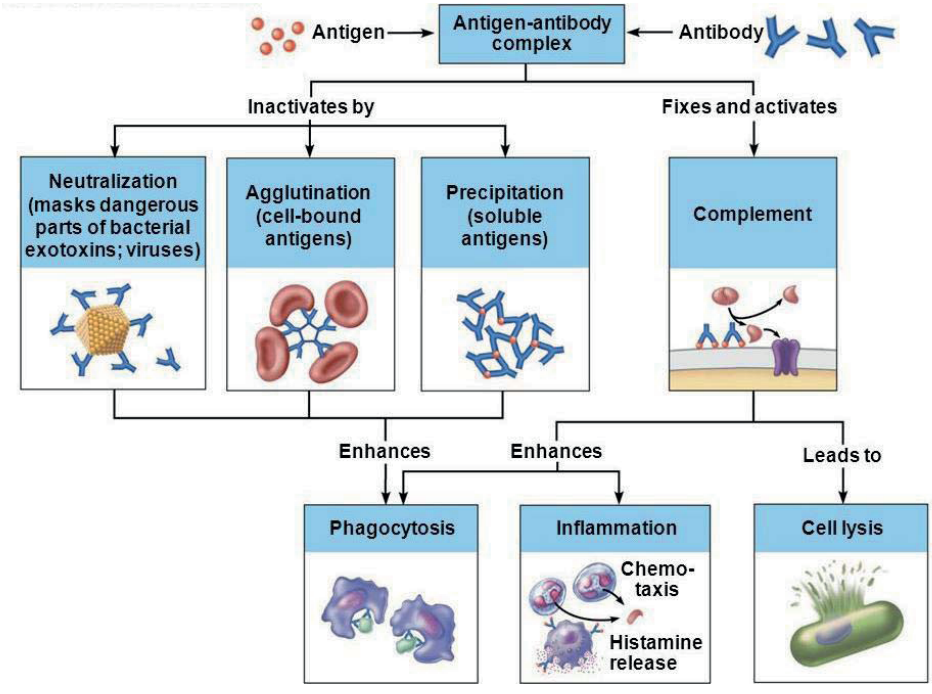

Figure 3: Essentials of Human Anatomy & Physiology (11th Edition) 11th Edition by Elaine N. Marieb

isotypes, with IgG providing the majority of antibody related immunity in the blood to invading pathogens, as 75% of all antibodies in serum are IgG's. IgG is divided into four subclasses each with distinct characteristics and function, which are numbered according to serum abundance. IgG1 is primarily directed against proteins and is the predominant IgG subclass (68). IgG2 is primarily directed to polysaccharides and the 2nd most abundant serum IgG (68). IgG3 is predominantly directed against viral antigens and is less abundant is serum than IgG1 or IgG2 (67, 68). Serum levels of IgG1, IgG2 and IgG3 increase within a week of infection, peak between one and two weeks and resolve after two to three weeks (67, 68). IgG1, 2 and 3 function as pro-inflammatory signals inducing Fc receptor-mediated and complement-mediated phagocytosis (67). IgG4 antibodies, the least abundant type of IgG, are only produced after prolonged exposure, typically taking months or years (68). IgG4 antibodies have less immune- and complement-activating capability than the other IgG antibodies, and IgG4 antibodies are correlated with tolerance after someone has overcome allergy and/or repeated and chronic exposure (67-69).

To actually be able to cause infection, *S. aureus* needs to evade all these previously described innate and adaptive immune responses. The first contact between *S. aureus* and the innate immune system occurs when the pathogen is confronted with host defense peptides and complement. Host defense peptides beta-defensins and cathelicidin also function as signal molecules for infection, attracting other cells to the site of infection (56). *S. aureus* reacts to these membrane stresses by altering the charge of the membrane surface: by adding D-alanines to teichoic acid via the *dlt* system and adding D-lysines to phosphatidylglycerol in the membrane via *mprF*, the net charge of their surface becomes more positive and host defense peptides are repulsed via electrostatic interactions (56). This could further have pathological effects when positively charged antibiotics are reflected, thereby conferring some level of resistance to these antibiotics. Complement activity can be modulated by the staphylococcal complement inhibitor (SCIN), the extracellular complement binding protein (Ecb) and extracellular fibrinogen binding protein (Efb), which all block the activity of the C3 convertase and, thus, block the classical, the alternative and the lectin pathway of complement activation (99, 106). Other molecules also interfere with the complement system and phagocytosis, such as staphylokinase (SAK), clumping factor A (ClfA), *S. aureus* Serine-Aspartate dipeptide repeat protein (SdrE) (100) and Staphylococcal super antigen-like proteins (SSL) 7 and 10, all acting on different targets. Staphylokinase induced plasmin formation actively degrades complement C3b (106). ClfA binds complement regulator I protein (107). SdrE inhibits the alternative pathway of complement by recruiting the complement regulatory protein factor H (108). SSL7 binds and inactivates C3 and C5 (106). SSL7 and SSL10 also sequester antibodies, thus blocking the classical activation of complement (106). Several staphylococcal proteins such as SSL5, SSL11 and FLIPr can bind to the FcR on

phagocytic cells preventing opsonophagocytosis. *S. aureus* is furthermore known to inhibit neutrophil extravasation and chemotaxis (106). Chemotaxis of neutrophils is inhibited by *S. aureus* by molecules such as the chemotaxis inhibitory protein of *S. aureus* (CHIPS), which interferes with the complement receptor C5aR, the formylated peptide receptor (FPR) on neutrophils (101). This FPR is further inhibited by formyl peptide receptor-like 1 inhibitor (FLIPr) and FLIPr-like (FLIPr-L) (106). In addition, SSL5 inhibits the PSGL1 signaling pathway, which allows neutrophils to slowly roll over endothelium (106). Neutrophils and other immune cells, such as monocytes, macrophages, T-cells, dendritic cells and NK cells are further targeted by molecules from the γ-hemolysin family consisting of gamma hemolysin HlgABC and leukocidin (Luk) A, B, D, E, F and S which form pores in the membranes of these cells by binding to the CCR5, CXCR1, CXCR2, CCR2, CD11b, C5aR and C5L2 (58, 109, 110). Finally, *S. aureus* is equipped with several mechanisms to prevent neutrophil-mediated killing (106). When live neutrophils home to the site of infection to form NETS, *S. aureus* can escape these by producing nuclease. This may help explain why MRSA strains excreting nuclease were associated with increased mortality when compared to a nuclease deficient strain (102). After altering innate immune responses to escape clearance, *S. aureus* can evade the adaptive immune response as well. Bacterial super-antigens (Sag), such as staphylococcal enterotoxins (SE) and toxic shock syndrome toxin (TSST-1), modulate adaptive responses by cross-linking MHCII with T-cell receptors, resulting in polyclonal T-cell activation and disruption of adaptive immune response (103). Staphylococcal protein A (Spa) binds antibodies not by their specific antigen recognizing Fab domain, but by their Fc domain (104), hampering downstream signaling of antibodies. Furthermore, Spa is capable of inducing apoptosis in B-cells by binding to the VH3 region of the B-cell receptor (105). Second Immunoglobulin Binding protein (SBI) functions in a similar manner to Spa (106). For an excellent review on all players in staphylococcal manipulation of the host immune responses see (106). Taken together, all these systems show that *S. aureus* is well equipped to respond to pressure caused by both the innate and adaptive immune responses, and that *S. aureus* interacts with several targets via several mechanisms.

Bacteria causing invasive disease *in vivo* are under selective pressure by the host's system and modulate their behavior. Bacterial (pathogenic) behavior is altered by a multitude of factors: partial oxygen pressure, lack of iron ions, elevated temperatures, and exposure to the host's immune responses and by exposure to antibiotics administered to the host during his/her therapy. Two component systems monitor the bacterial environment and inside of the bacteria and alter bacterial behavior or metabolism to maintain homeostasis (80). *S. aureus* actively responds to immune pressure (7), as described previously, and to antibiotic pressure (76, 81).

ANTIBIOTICS AND BACTERIAL BEHAVIOR

Once inside a host, bacteria must escape the innate and adaptive immune system. Generally, patients are treated with antibiotics when the immune system fails to clear the bacteria and a clinical infection has developed. When antimicrobial therapy is prescribed *S. aureus* must also avoid clearance by these antibiotics. Antibiotics, which have only been in clinical use since the 1940s, have various bacterial targets by which interaction they interfere with the life of bacteria (Figure 4). The first antibiotic discovered by Alexander Flemming was penicillin, and was discovered by accident when he observed the fungus *Penicillium* inhibiting the growth of *S. aureus*. Shortly after the introduction of penicillin in clinical practice, *S. aureus* strains resistant to penicillin were detected. By confronting bacteria with antibiotics, selection pressure is applied. Fully susceptible bacteria are killed or halted in their growth, less susceptible or fully resistant variants in the bacterial population tend to remain viable and may even proliferate. As antibiotics are a mainstay in current clinical practice the emergence of resistance is a cause for alarm. Resistance to antibiotics can occur in several ways (Figure 5). *S. aureus* has proven notorious in that it can acquire resistance to many classes of antibiotics, which causes extensive problems in health care settings. This bacterium can show resistance to almost all antibiotics used to treat it, and resistance spreads quickly, not only vertically to progeny, but also through horizontal gene transfer to close relatives, mainly via plasmids and phages. The SSC*mec* cassette genes provide resistance to methicillin, and their presence classify a strain as Methicillin Resistant *Staphylococcus aureus* (MRSA), which is currently causing major problems in health care (21-23). Treatment options are diminishing, leading to fears of a post antibiotic era, in which infections can be diagnosed but no longer treated with antibiotics. Exacerbating this situation is the fact that drugs that have recently been introduced do not have novel modes of action as they are often derivatives of antibiotics currently used in the clinic. Either existing products are modified, or known antimicrobial producing sources are mined for novel compounds, leading to no new treatment options. It is therefore clear that novel antibiotics, with novel modes of action are essential to maintain treatment options.

In addition to evading clearance from either antibiotics or the immune system, *S. aureus* can alter its mode of growth, from planktonic life to a sessile state attached to a substrate (Figure 1); sessile life may facilitate infection and persistence (9). This growth mode is called a biofilm and is reportedly present in over 80% of all infections (95). They consist of an extracellular matrix of DNA, polysaccharide and/or proteins (51), which hinders the diffusion antibiotics prescribed to the diseased host (52) and excludes the immune system from clearing the infection. Much is known on the regulation and generation of biofilms (53), and bacterial virulence factors are typically reported to be produced only in the later phases of the formation of biofilm. In this multicellular sessile

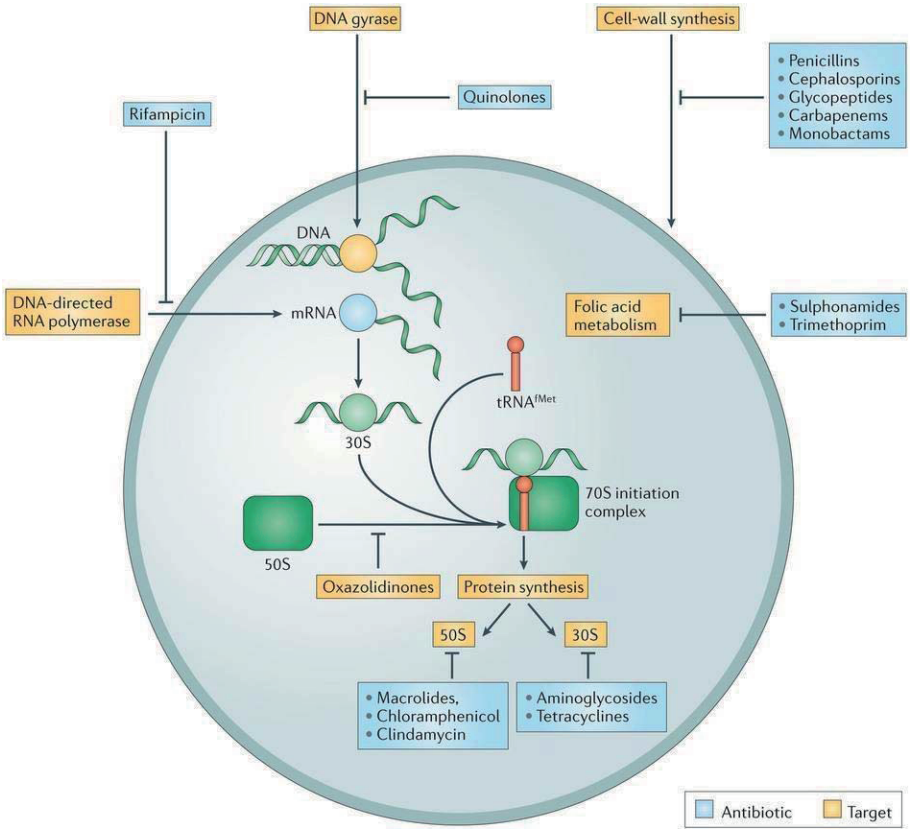

Figure 4: Bacterial targets of antibiotics
The bacterial cell wall is targeted by beta-lactam containing drugs such as penicillins (cephalosporins and carbapenems) and glycopeptides (vancomycin and teichoplanin). These drugs inhibit the correct formation of a complete cell wall, inducing bacterial cell death. Quinolones specifically target bacterial gyrases halting bacterial DNA unwinding and thereby replication, causing bacterial death. Bacterial DNA-dependent RNA-polymerases are targeted by rifampicin, which halts bacterial translation and functions as bacteriostatic agent. Translation of RNA to protein is targeted by several drugs: aminoglycosides (gentamicin and streptomycin), lincosamide (clindamycin), macrolides (clarithromycin and erythromycin) and tetracyclines (tetracycline, tigecycline and doxycycline). The cytosolic bacterial membrane is targeted by colistin (polymyxin E), polymixin B and the previously mentioned host defense peptides, which all act bactericidally. Finally, folic acid synthesis is inhibited by trimethroprim and sulfonamides, inhibiting the formation of bacterial DNA. (From Lewis et al *Nature Reviews Drug Discovery* **12**, 371–387 (2013)

mode of growth, *S. aureus* is much more recalcitrant to clearance for several reasons: bacteria are in different metabolic states, being either active or metabolically dormant, the so-called persister cells, reducing the efficacy of several classes of antibiotics (50). Furthermore, biofilm hinders antibiotics and the immune system to actually reach the bacteria. There are reports showing that some antibiotics influence the formation of biofilm by *S.aureus*, e.g gentamicin induces biofilm formation (101) whereas rifam-

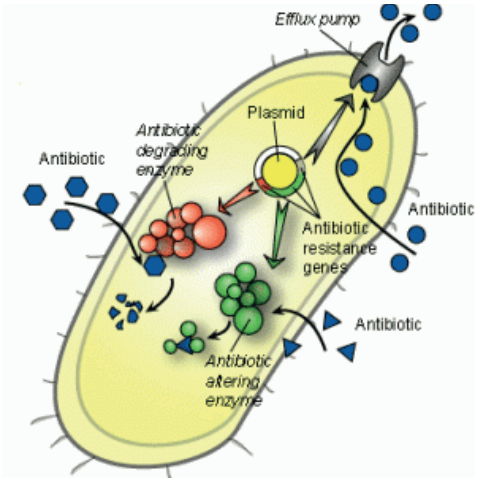

Figure 5: Modes of Antibiotic resistance
Alteration or modification of the antibiotic itself. β-Lactamases break open the β-lactam ring of penicillins, causing these types of antibiotics lose their function. Secondly, under pressure of the antibiotics, bacteria alter the target of these antibiotics, making bacteria no longer susceptible. For example, resistance to rifampicin occurs when the *rpoB* gene is mutated, which encodes the bacterial RNA-polymerase beta subunit, resulting in decreased binding affinity for rifampicin. Antibiotics targeting bacterial metabolic processes, such as sulfanomide, induce bacteria to acquire para-aminobenzoic acid, an alternative for the synthesis of folic acid, and thereby become resistant. Finally, by reducing the accumulation of antibiotics and increasing efflux or decreasing influx of antibiotics, bacteria can become more resistant. For these determinations, a standardized broth is used, which may not mimic the situation encountered *in vivo*. Recent examples of bacterial antibiotic resistance are New Delhi Metallo-Betalactamase (NDM) (104) and Extended Spectrum Beta-lactamases (ESBL) (105) in Gram-negative bacteria. Methicillin resistance in *Staphylococcus aureus* (MRSA) (75) and vancomycin resistance in Enterococci (VRE) (106) are examples of potentially epidemic resistance among Gram-positive bacteria (from: http://textbookofbacteriology.net/resantimicrobial_3.html, Todar's Online Textbook of Bacteriology)

picin inhibits biofilm formation (102). Several stages have been identified in *S. aureus* biofilm formation: attachment, adhesion, maturation and dispersion (Figure 1). During the maturation stage the production of exotoxins, such as alpha-toxin is enhanced. Immune-modulators such as CHIPS and SCIN are also up regulated at this stage. Dispersion of (clusters of) cells from such matrices is the final stage of biofilm formation, by which metastatic infections may occur (49, 100). However, the exact kinetics of the production of virulence factors during biofilm formation remain unknown, especially in the early stages of biofilm formation, and more insight into the dynamics of these processes could influence our therapeutic options. These evasive behaviors of stressed *S. aureus* pose significant challenges, as relatively little is known of the exact status of *S. aureus* (during various phases of infections) *in vivo*.

AIM OF THIS THESIS:

The primary aim of the research described in this thesis was to gain more insight into host pathogen interaction between *Staphylococcus aureus* and the human host by specifically studying the IgG (subclass specific) humoral response against staphylococcal virulence factors in humans with different interactions with *S. aureus*, the kinetics of the production of several of these immune modulators during biofilm formation, and finally, by studying the production of biofilm in staphylococcal bacteremia to gain better understanding of persistence of bacteremia. When the immune system fails to clear an infection, clinicians use antibiotics to treat infections. The secondary aim of this thesis was, therefore, to find antibiotics with truly novel modes of action directed against infections with multi drug resistant ESKAPE pathogens.

HOST PATHOGEN INTERACTIONS

The primary focus of research of this thesis was the pathogenesis of *S. aureus* infections. The humoral immune response can be used as a readout system indicating to which bacterial virulence factors the patient has been exposed and, thus, which antigens were expressed by the bacteria in vivo (3-6). By comparing these responses between different groups of individuals, including carriers versus non-carriers, healthy controls versus infected patients, either acutely so or patients repeatedly or chronically infected ones, *S. aureus* virulence factors may be linked to certain carriage or disease states. This thesis aimed to increase our insight into the antibodies produced against various virulence factors in persons with varying levels of *S. aureus* exposure. New insights generated here will increase our understanding of the sequence of events during bacterial pathogenesis, and possibly facilitate the development of a vaccine, a preventive target that has remained elusive so far. Another important bacterial virulence factor is the switch of the growth mode from planktonic life to biofilm-associated life (8,9) (Figure 1). *In vitro* studies on pathogenic behavior of bacteria are generally performed in nutrient rich broths, facilitating rapid and exponential growth; such conditions are devoid of the stresses posed by the host's innate and adaptive immune responses and of the stress induced by antibiotics. We present an *in vitro* model system which we posit to better mimic *in vivo* growth conditions of these pathogens. (47, 48). Indeed, *S. aureus* grows differently and expresses different virulence factors when their growth conditions are changed (47, 48). I here studied the relationship between biofilm production and virulence factor production. Immune modulator production has so far only been described in mature biofilms, when the biofilm is already formed and viable, and recalcitrant to (immune or antibiotic) clearance. I showed that virulence factors, of which several were identified

in the previous research, are already produced by the bacteria early in the formation of biofilm. By better understanding the formation of biofilm and the virulence factors involved, I aimed to develop new strategies for treatment and biofilm formation may even be prevented. I further studied the role of biofilm formation in *S. aureus* bacteremia, which is defined as the presence of viable bacteria in the bloodstream. Bacteremia can be classified as complicated or uncomplicated, identifiable by clinical markers including the persistence of fever and positive blood cultures for more than 72 hours after the initiation of appropriate treatment (45). In many cases blood cultures remain positive for multiple days despite appropriate parenteral antibiotic therapy (45). Patients with complicated infection have a significantly poorer prognosis compared to patients with uncomplicated infection (46). As complication status can only be definitively determined after 72 hours of directed therapy, I studied what factors, either bacterial or host associated, influence complication status. Early determination of complicated infection based on either bacterial or host factors could lead to improved treatment strategies, and better outcomes for SAB patients.

NOVEL ANTIBIOTICS

If the pathogen is successful in evading the immune system, clinicians use antibiotics to treat infections. As a secondary goal, I aimed to generate novel targets for antimicrobial drug discovery. In this thesis, two possible leads were followed: first, actinomycetes were induced to produce novel compounds that have activity against multi-drug resistant ESKAPE pathogens by using novel culturing techniques. As actinomycetes are responsible for 60% of all antibiotics used in clinical practice today, mining them and inducing them to produce novel compounds is a promising strategy. Secondly, I tested derivatives of gramicidin S for toxicity and antimicrobial activity to facilitate their further development as novel therapies against multi-drug resistant pathogens. Developing novel therapeutics with novel modes of action is essential to be able to continue treatment of patients in hospitals.

OUTLINE OF THIS THESIS:

In **Chapter 2** we analyze several human serum collections for presence of IgG-antibodies directed against *S. aureus* virulence factors. I measure total IgG (IgGt), and subclass IgG1 and IgG4 antibody responses directed against 40 *S. aureus* antigens in serum obtained from patients suffering from four different types of staphylococcal infection from three distinct geographical locations. I also determine IgGt, IgG1 and IgG4 responses against

the same 40 virulence factors in sera obtained from Dutch healthy carriers, Dutch non-carriers and Dutch patients suffering from epidermolysis bullosa, a chronic skin condition associated with *S. aureus* colonization and infection. IgG4 responses, indicative of repeated and prolonged exposure, are found in all human sera, but these responses are only directed against a restricted panel of 17 virulence factors: alpha toxin, CHIPS, ETA and B, HlgB, IsdA, LukD, E, F and S, SCIN, SEC, SSL1, 3, 5 and 9 and TSST-1. I subsequently studied the production of virulence factors during biofilm formation of a selection of these immune modulators: alpha toxin, CHIPS, SCIN and TSST-1 from this cohort, but we also measured FlipR and Nuc. These virulence factors are measured in developing biofilms and described in **Chapter 3**. Bacteria grow in biofilms to evade the host immune system, but immune modulator production has only been described by late phase exponentially growing cells or mature biofilms, leading to the following question: how does *S. aureus* evade the immune system long enough to attain maturity, if it only produces immune modulators when they are already safe in a mature biofilm? I further studied biofilm in the context of bacteremia: biofilm could be a key player in (persistence of) bacteremia, as discussed in **Chapter 4**. *S. aureus* bacteremia can have a complicated (over 72 hours) or uncomplicated (under 72 hours) course. This chapter focuses on host factors and bacterial factors, such as biofilm formation (under antibiotic pressure), that may play a role in determining the complication status of this bacteremia.

Actinomycetes are already a valuable source of antibiotics: approximately 60% of all clinically used antibiotics originate from this group of bacteria. **Chapter 5** focuses on the use of different growth media with actinomycetes strains from remote locations to induce the production of novel antimicrobials, with potent activity and broad spectrum against multi-drug resistant ESKAPE pathogens. **Chapter 6** discusses small antimicrobial peptide antibiotics gramicidin S, which was already discovered in the 1940, but was found too toxic for systematic usage. Here I design new derivatives suitable for systemic use by changing its peptide structure, with the aim of retaining antimicrobial activity but reducing toxic side effects. In **Chapter 7**, I summarize and discuss the results of our studies.

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Chapter 2

IgG4 subclass-specific responses to *Staphylococcus aureus* antigens shed new light on host-pathogen interaction

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ABSTRACT

IgG4 responses are considered indicative for long-term or repeated exposure to particular antigens. Therefore, studying IgG4-specific antibody responses against *Staphylococcus aureus* might generate new insights into the respective host-pathogen interactions and the microbial virulence factors involved. Using a bead-based flow-cytometry assay, we determined total IgG (IgGt), IgG1 and IgG4 antibody responses to 40 different *S. aureus* virulence factors in sera from healthy persistent (non) nasal carriers and patients with various staphylococcal infections from three distinct countries. IgGt responses were detected against all tested antigens. These were mostly IgG1 responses. In contrast, IgG4 antibodies were detected to alpha toxin, CHIPS, ETA and B, HlgB, IsdA, LukD, E, F, and S, SCIN, SEC, SSL1, 3, 5 and 9 and TSST-1 only. Large inter-patient variability was observed, and the type of infection or geographical location did not reveal conserved patterns of response. As persistent *S. aureus* carriers trended towards IgG4 responses to a larger number of antigens than persistent non-carriers, we also investigated sera from patients with epidermolysis bullosa (EB), a genetic blistering disease associated with high *S. aureus* carriage rates. EB patients responded immunologically to significantly more antigens than non-carriers and trended towards even more responses than carriers. Altogether, we conclude that the IgG4 responses against a restricted panel of staphylococcal antigens consisting primarily of immune modulators and particular toxins indicate important roles for these virulence factors in staphylococcal pathogen-host interactions, such as chronicity of colonization and/or (subclinical) infections.

INTRODUCTION

Staphylococcus aureus is responsible for more deaths annually in the USA than HIV/AIDS and tuberculosis combined (1), (2). *S. aureus* infections can range from mild skin and soft tissue infections (3) to more severe bacteremia (4) and osteomylitis (5), and they can be resolving or chronic. *S. aureus* is able to persistently adhere to the anterior nares of 30% of all humans, while in the remaining population this opportunistic pathogen is never or only incidentally detectable (6), (7), (8), (9). It has long been established that nasal colonization is associated with an increased chance of infection (10), (11), (12). In epidermolysis bullosa (EB) patients, a genetic blistering disease that leaves patients highly susceptible to *S. aureus* colonization, nasal carriage rates of 50-80% have been reported, and 75-100% of their skin wounds are culture positive for *S. aureus* (13), (14), (15), (16), (17). Interestingly, although EB patients interact frequently with *S. aureus,* bacteremia is seldom reported in these patients (18).

Numerous *S. aureus* virulence factors have been identified (19), (20), (21), (22), (23), (24). However, the precise roles of most of these virulence factors during colonization and pathogenesis in humans have remained largely unclear, as determination of *in vivo* expression of bacterial virulence factors is technically challenging. Burian *et al.* showed by a qPCR analysis on samples from 4 persistent nasal carriers that the adhesin genes *clf*B, *fnb*A and *isd*A, and the immune modulator gene *chp* are expressed *in vivo* (25). In an artificial inoculation study, *clf*B proved essential for colonization in humans (26).

Instead of direct *in vivo* detection of virulence factors, the human antibody response can be used as an indicator for the *in vivo* expression of *S. aureus* virulence factors (27), (28), (9), and (29). Our group has published several reports on human immune responses to *S. aureus*. Persistent carriers have higher IgG titers directed to TSST-1 than persistent non-carriers (9). During bacteremia, patients developed significantly higher IgG responses than age-matched uninfected controls. These responses were directed to the immune modulators SSL1 and 5, and SCIN, and the toxins gamma-hemolysin B and leukocidin F (28), indicating that these virulence factors are produced *in vivo* during infection. Furthermore, Algerian patients suffering from various *S. aureus* infections showed higher IgG responses directed to ETA, ETB, HlgB, LukD, E and S, SEA, SEE, SEH and SEM compared to controls (30).

When IgG responses are studied usually only the total IgG (IgGt) levels are measured. However IgGt is composed of 4 different subclasses, each with distinct biological functions and induction patterns (31). IgG1 responses, which represent approximately 60% of IgGt, are primarily directed against proteins. They are induced within a week of infection, peak between one and two weeks and resolve after two to three weeks (32). IgG1 functions as a pro-inflammatory signal inducing Fc receptor-mediated and complement-mediated phagocytosis. IgG4 antibodies, which represent approximately

5% of IgGt, are produced after prolonged exposure, typically taking months or years (33), (32). IgG4 antibodies are reported to not or weakly activate complement via the classical pathway in contrast to IgG1 antibodies. Furthermore IgG4 antibodies are correlated with tolerance after allergy (31), (34), (33), they have a low binding affinity to Fc receptors on phagocytes, and IgG4-mediated opsonophagocytosis is reportedly less efficient than IgG-mediated opsonophagocytosis (35).

The aim of our present study was to determine whether analysis of IgGt, IgG1 and IgG4 directed against virulence factors of *S. aureus* could help elucidate the location and duration of exposure to these bacterial factors during infection. Using a previously developed bead-based flow-cytometry assay (xMap, Luminex) (9, 28) we measured total IgGt, IgG1 and IgG4 antibody responses directed to 40 different *S. aureus* virulence factors in sera from patients suffering from 4 different *S. aureus* infections originating from 3 geographical locations. In addition, we studied the humoral responses in sera from healthy carriers, non-carriers and EB patients with well documented *S. aureus* colonization status, all from the Netherlands. We looked for infection-specific responses to the virulence factors of *S. aureus* and the IgG subclasses involved. Furthermore, we determined IgG subclass/IgG total ratios to determine the contribution of the different subclasses to the IgGt response to *S. aureus*. By studying these responses, we obtained new insights on the (chronicity of) human exposure to *S. aureus* and the virulence factors involved, which have implications for anti-staphylococcal vaccine development.

MATERIALS AND METHODS

Serum from healthy volunteers and patients

We included serum from 19 Dutch persistent carriers and 26 Dutch persistent non-carriers (9). An individual was defined as a persistent nasal carrier when 3 out of 3 nasal swabs taken 2 weeks apart were positive and as a persistent non-carrier when all swabs were negative for *S. aureus* (9). All swabs were processed as described by Nouwen *et al. (36)*. In addition, we included sera isolated from 10 Dutch patients with bacteremia at diagnosis and 1, 2 and 3 weeks after diagnosis (37), and sera from 13 EB patients (18). Furthermore, serum samples were included from 10 patients without *S. aureus*-related infections admitted to the Mustapha Pacha Hospital, Algiers, Algeria and patients with either *S. aureus* skin infections (n=10), joint infections (n=10), or respiratory infections (n=10) 14 days (range 7-34) after strain identification (30). Serum samples were collected from 60 healthy Sudanese volunteers at the University of Khartoum and 25 Sudanese citizens with *S. aureus* skin infections. The Medical Ethics Committee in the Erasmus Medical Centre approved the study (MEC-2007-106) for work performed in Rotterdam, the Netherlands. The Medical Ethics Committee of the University Medical Center Gron-

ingen approved the collection of sera from EB patients (approval no. NL27471,042,09). Local ethical committees reviewed and approved both Algerian (30) and Sudanese studies. All serum donors provided written informed consent.

Antigens

The antigens used were isolated upon over-expression and comprised 10 cell wall-associated and 30 secreted *S. aureus* antigens: Alpha toxin (A-Tox); chemotaxis inhibitory protein of *S. aureus* (CHIPS); clumping factors A and B (ClfA and ClfB); extracellular fibrinogen-binding protein (Efb); exfoliative toxins A and B (ETA and B); fibronectin binding proteins A and B (FnbpA and B); γ hemolysin B (HlgB); iron-responsive surface determinants A and H (IsdA and H); leukocidin (Luk) S-PV, LukF-PV, LukD-PV, and LukE-PV; *S. aureus* surface protein G (SasG); staphylococcal complement inhibitor (SCIN); serine-aspartate dipeptide repeat protein D and E (SdrD and SdrE); staphylococcal enterotoxins A-E, G-J, M-O, Q, and R (SEA - SEE, SEG - SEJ, SEM - SEO, SEQ, SER); staphylococcal superantigen-like proteins 1, 3, 5, 9, and 11 (SSL1, SSL3, SSL5, SSL9, and SSL11) and toxic shock syndrome toxin 1 (TSST-1) (30, 38-50) (Table S1). Besides *S. aureus* antigens, IgGt (16-16-090707, Athens Research & Technology, Athens, Georgia, USA), IgG1 (16-16-090707-1M, Athens Research), and IgG4 (16-16-090707-4M, Athens Research) were used to assess whether cross-reactivity existed between subclass-specific detection antibodies. Beads without antigens were used as a negative control.

Measurement of anti-staphylococcal antibodies

IgGt antibodies in serum directed against the different *S. aureus* antigens were simultaneously quantified in a multiplex assay using a bead-based flow cytometry technique (xMap; Luminex Corporation), following previously described protocols (9), (51), (28). The Median Fluorescence Intensity (MFI) was determined as the median fluorescence of 100 beads and is used as measure of immunoglobulins bound to the antigens coupled the beads. IgGt was detected using Goat-Anti-Human IgG-PE from Jackson Immuno Research (Newmarket, Suffolk, United Kingdom). Subclass-specific responses were determined using monoclonal mouse anti-human-IgG1 of IgG1 subclass (05-3300, Zymed, Paisley, United Kingdom), or monoclonal mouse-anti-human-IgG4 of IgG1-k subclass (05-3800, Invitrogen, Paisley, United Kingdom). All subclass-specific antibodies were detected using IgG Goat Anti Mouse-PE (Abcam, Cambridge, United Kingdom). To assess cross reactivity between subclass-specific detection antibodies, IgG1 and IgG4 were coupled to microspheres and incubated with the anti-IgGt, anti-IgG1 or anti-IgG4 detection antibodies. As a positive control, pooled serum from 36 healthy volunteers was used.

Data analysis

Coefficient of variation (CV) values were determined by dividing the standard deviation of the measurements by the mean of the measurements. Values with a CV exceeding 25% were excluded from further analysis. Control beads without protein coupled were included in each experiment to determine non-specific binding. The non-specific MFI values were subtracted from the antigen-specific results. Groups were compared using a Mann Whitney U test in IBM SPSS Statistics 20. Bonferroni correction was applied to P values to correct for multiple testing. The ratios were calculated by dividing the IgG (subclass) signal by the IgGt signal. The median of these ratios is shown in the (supplemental) figures, to determine subclass-specific contributions to the IgGt signal and to facilitate inter-group comparisons. An increase of signal in the bacteremic patients was defined as a ratio >1 for all time points and this ratio was calculated by dividing the signal of the later time points by the first time point.

RESULTS

Validation of IgG subclass-specific Luminex assay

To compare *S. aureus* antigen-specific IgG levels in serum samples, the bead-based multiplex Luminex assay was applied as described previously (28), (30), (52), (51), and the level of cross-reactivity between our detection antibodies for IgG1 and IgG4 and coupled antibodies was assessed by incubating a bead mixture containing IgGt, IgG1 and IgG4 with either the anti-IgG1 antibody or the anti-IgG4 antibody. IgG1 antibodies showed (291/10759)*100=2.7% cross-reactivity to IgG4-coupled antibodies. IgG4 antibodies gave (65/16096)*100 = 0.4% cross reactivity to the coupled IgG1 antibodies. Thus, less than 5% cross reactivity of the detection antibodies was observed. The CV values ranged between 5% and 37% and, on average 20% of all measurements had to be excluded for exceeding the CV value cut-off of 25%, comparable to previous reports (9, 28, 51-55). High CV's were measured mainly for low MFI signals in the IgG4 Luminex assays (<1000 MFI). In all determinations done here, human pooled serum gave median MFI values of 2883 (range 49 – 15655) for IgGt, of 794 (range 6 – 16700) for IgG1 and 73 (range 0 – 9379) for IgG4. Taken together, we conclude that our Luminex assay is suitable to determine both total and subclass-specific responses against *S. aureus* antigens.

IgGt, IgG1 and IgG4 anti-staphylococcal antibodies in an Algerian discovery cohort

To expand our previous IgGt data (30), the IgGt, IgG1 and IgG4 responses directed against 40 *S. aureus* antigens were determined in a discovery cohort of Algerian patients with either *S. aureus* joint (10 patients), respiratory (10 patients) or skin infections (10

patients) and 10 Algerian control patients. Significant differences in IgGt, IgG1 and IgG4 between groups are shown in Table S2A.

IgG subclass / IgG total ratios were calculated to determine the contribution of the different subclasses to the IgGt response to 40 different *S. aureus* antigens. IgG1, which constitutes approximately 60% of IgGt (31), (32), showed responses directed to almost all tested *S. aureus* antigens, similar to results obtained for IgGt (Fig. 1A). Strikingly, in all groups we observed IgG4 responses against a restricted panel of antigens consisting of alpha toxin, CHIPS, ETA and B, HlgB, IsdA, LukD, E, F, and S, SCIN, SEC, SSL1, 3, 5 and 9, and TSST-1 (Fig.1B), with only few individuals showing high responses against alpha toxin, ETB, IsdA, SEC, SLL3 and 5 (Fig. 1, A and B). These *S. aureus* virulence factors are almost all secreted immune-modulators. No defined patterns of IgG1 or IgG4 responses were observed for the different types of *S. aureus* infection. Skewed ratios for IgG4/IgGt for SER and SSL11 in joint infections and SEB in respiratory infections were caused by single patients with high responses. The interquartile ranges of all ratios are given in Fig. S1, A and B.

Anti-staphylococcal IgGt, IgG1 and IgG4 antibodies in an expanded cohort of Sudanese patients with *S. aureus* skin infections and healthy controls

To study the antibody responses in skin infections in a larger cohort from a different geographical setting, we measured the relative IgGt, IgG1 and IgG4 levels in sera of 25 Sudanese patients with *S. aureus* skin infections and 60 healthy Sudanese volunteers. For this purpose, the same antigens were used as in the above analysis of the Algerian serum sets (except SasG and SEB). Significant differences in the IgGt, IgG1 and IgG4 levels between groups are shown in Table S2B. IgG1 responses against all tested antigens were detected both in sera from patients and healthy controls, similar to the Algerian discovery cohort (Fig. 2A). Intriguingly, also for the Sudanese serum sets, IgG4 antibody responses were detected against alpha toxin, CHIPS, ETA, ETB, HlgB, IsdA, LukD, E, F and S, SCIN, SEC, SSL1, 3, 5, 9 and 11 and TSST-1 (Fig. 2B). Interquartile ranges of all ratios are given in Fig. S2, A and B. Altogether, the sera from Algerian and Sudanese patients and the respective control sera revealed IgG4 responses to a similar sub-set of the tested antigens.

Induction of IgGt, IgG1 and IgG4 anti-staphylococcal antibodies during progression of bacteremia

As previous reports have shown that anti-staphylococcal IgGt responses reach peak values after a median of 21 days after diagnosis of bacteremia (range 5-50) (37), (28), we studied the contribution of IgG1 and IgG4 to the increase of IgGt. We determined the IgGt, IgG1 and IgG4 levels to 40 *S. aureus* antigens in serum samples taken at acute phase, 1, 2 and 3 weeks after diagnosis of bacteremia in 10 Dutch patients. For the last

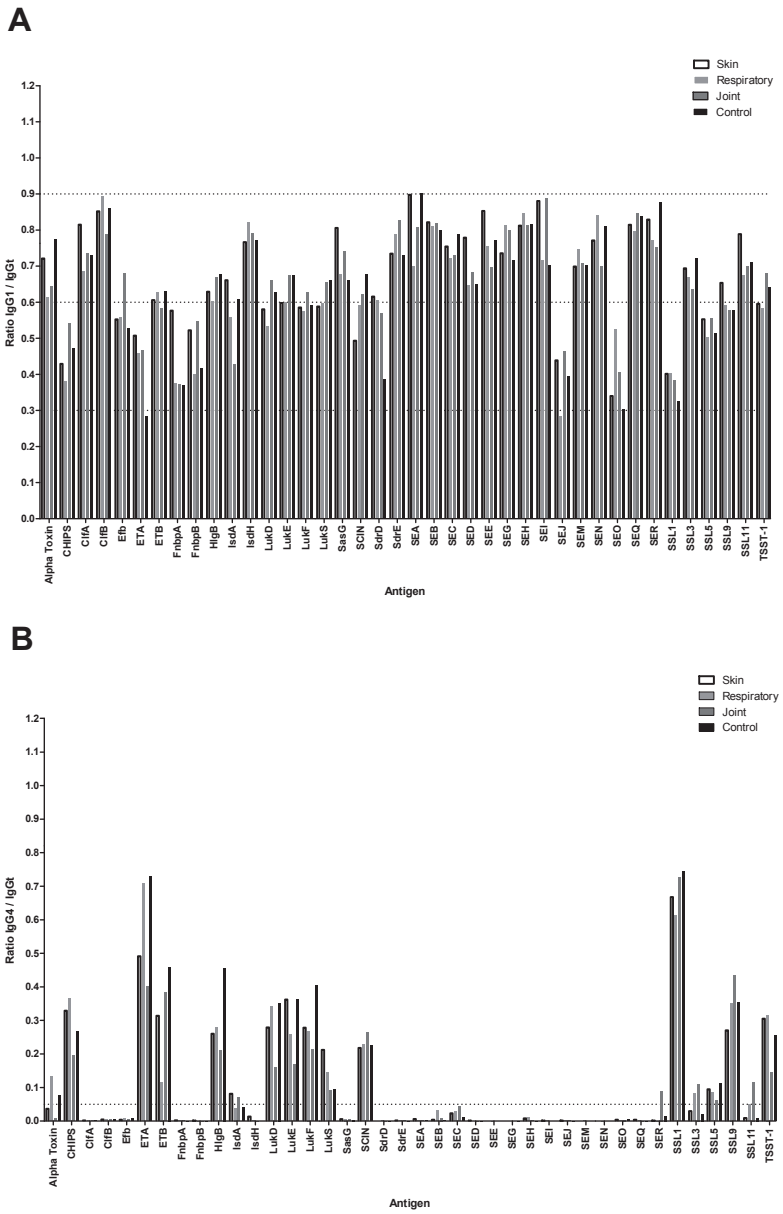

Figure 1: Ratios of IgG1/IgGt and IgG4/IgGt in sera from 40 Algerian volunteers.
A: Median of the ratios of IgG1/IgGt in sera from 10 Algerian patients with either joint (dark grey), respiratory (light grey bars) or skin (white bars) *S. aureus* infections, and sera from 10 Algerian control patients without *S. aureus* infection (black bars). On the x-axis the 40 tested *S. aureus* antigens are listed. The y-axis shows the median of the ratios of the IgG1/IgGt signal for each particular antigen. Dotted lines mark 60% (the reported ratio of IgG1/IgGt), 30% (50% of this reported value) and 90% (150% of this reported value)
B: Same as for Figure 1A, but showing the IgG4/IgGt ratios. The dotted line marks 5%, which is the reported ratio of IgG4/IgGt.

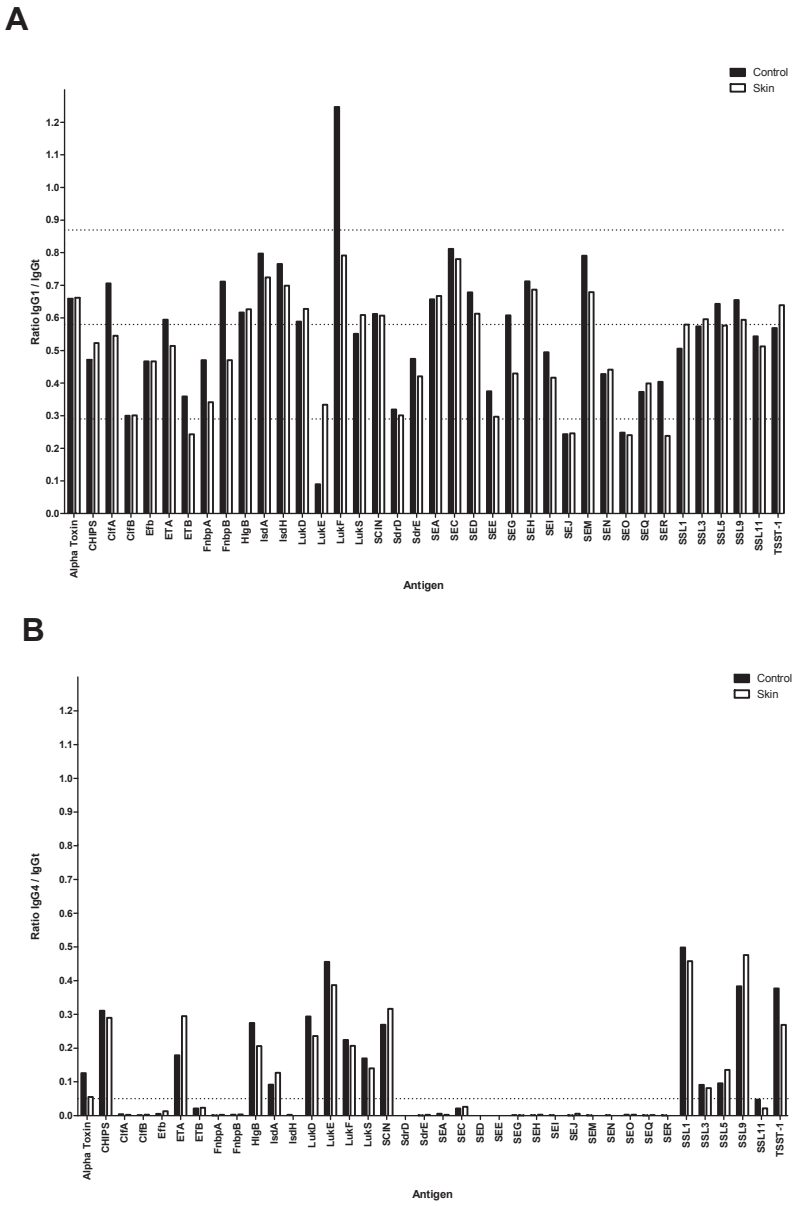

Figure 2: Ratios of IgG1/IgGt and IgG4/IgGt in sera from 25 Sudanese patients with *S. aureus* skin infection and 60 healthy Sudanese volunteers.

A: Median of the ratios of IgG1/IgGt in sera of 25 Sudanese patients with *S. aureus* skin infection (white bars) and 60 Sudanese volunteers (black bars). On the x-axis the 38 tested *S. aureus* antigens are listed (note that SasG and SEB were not included in this particular analysis). The y-axis shows the median of the ratios of the IgG1/IgGt signal for each particular antigen. Dotted lines mark 60% (the reported ratio of IgG1/IgGt), 30% (50% of this reported value) and 90% (150% of this reported value).

B: Same as for Figure 2A, but showing the IgG4/IgGt ratios. The dotted line marks 5%, the reported ratio of IgG4/IgGt.

time point, 3 samples were not available. Antigens against which increased responses could be determined were counted. Indeed, the IgGt level was increased during the 3 week observation period with bacteremic patients, specifically showing increased responses to 6-23 antigens (median 16.5) (Table S3, A and B). Patients showed increased IgG1 responses during the 3 week observation period to 1-21 antigens (median 11.5) (Table S3,A and B). Ratios of IgG1/IgGt were calculated for antigens showing an increase in IgGt, IgG1 and IgG4 signal (Fig. 3A). Notably, the IgG1/IgGt ratios did not vary over time, showing that an increase in IgGt was mainly caused by an increase in IgG1. The IgG4 responses were poorly conserved between patients and, also in these serum sets, these responses were only detectable in a restricted panel of antigens, namely: alpha toxin, CHIPS, ETA and B, HlgB, IsdA, LukD, LukE, LukF, LukS, SCIN, SEC, SSL1, 3, 5 and 9, and TSST-1 (Fig. 3B). During a period of 3 weeks after the onset of bacteremia, patients showed increased IgG4 responses to 0 – 13 antigens (median 6.5) (Table S3,A and B). Ratios of IgG4/IgGt were calculated for antigens showing an increase in IgGt, IgG1 and IgG4 signal. This showed that the IgG4/IgGt ratios did not change over time. Thus, the IgG4 signals increased together with IgGt signals. Interquartile ranges of all ratios are given in Fig. S3, A and B.

Anti-staphylococcal IgGt, IgG1 and IgG4 antibodies in a cohort of Dutch volunteers with long-term *S. aureus* exposure

To determine whether carriers and non-carriers differed in their immune responses against *S. aureus*, serum was collected from 19 persistent nasal carriers and 26 persistent nasal non-carriers. Significantly higher IgGt levels directed to TSST-1 were measured for carriers than non-carriers. Significantly higher IgG1 levels directed to TSST-1 were measured for carriers than non-carriers. Finally, carriers showed significantly higher IgG4 levels directed to TSST-1 than non-carriers (Table S3C). Also, the numbers of antigens to which responses were detectable were determined. Non-carriers showed IgGt and IgG1 responses to similar numbers of antigens as carriers, but trended towards fewer IgG4 responses (mean of 2.68, range 0-11) than carriers (mean 2.95, range 0-12) (Fig. 4). Based on these findings, we studied sera of EB patients to determine whether long-term exposure to different *S. aureus* types influenced the numbers of antigens to which IgG4 responses are elicited. Previous studies had shown that up to six different types of *S. aureus* could be cultured from individual patients with EB (18) (16), (17). IgGt showed similar levels compared to a previous report (18). EB patients differed significantly from non-carriers in IgGt responses to ClfA and in IgG4 responses SCIN. EB patients differed significantly from carriers in IgGt responses to ClfA and IgG4 responses to SCIN.

Notably, while IgG4 responses to the same restricted panel of antigens were observed in EB patients as for the other groups described above, the EB patients showed IgG4 responses to significantly more antigens than non-carriers (mean 5.38 , range 1-11

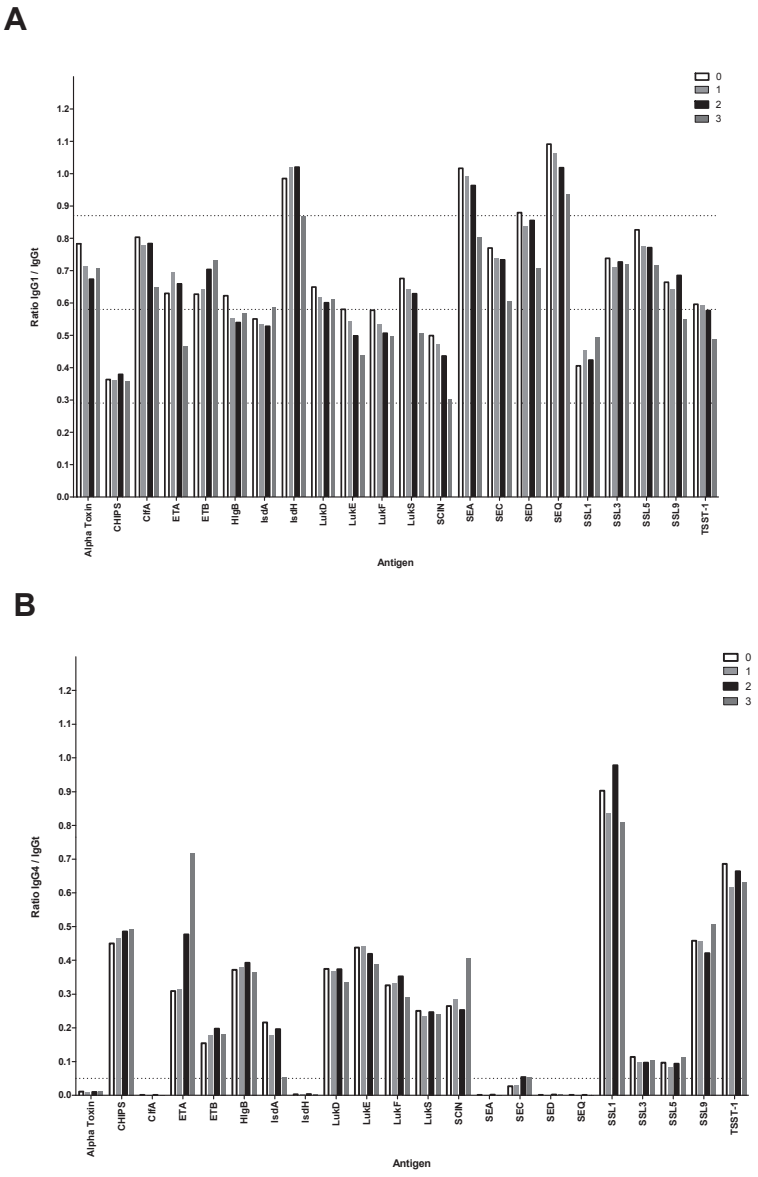

Figure 3: Ratios of IgG1/IgGt and IgG4/IgGt in sera from 10 Dutch bacteremic patients during disease progression

A: Median of the ratios of IgG1/IgGt in 10 Dutch bacteramic patients during disease progression. White bars, IgG1/IgGt ratio at diagnosis. Light grey bars, IgG1/IgGt ratio 1 week after diagnosis. Dark grey bars, IgG1/IgGt ratio at 2 weeks after diagnosis. Black bars, IgG1/IgGt ratio 3 weeks after diagnosis. On the x-axis the 17 antigens with increase in either IgGt, IgG1 and IgG4 signal are depicted. The y-axis shows the median of the ratios of the IgG1/IgGt signal for each particular antigen. The dotted lines mark 60% (the reported ratio of IgG1/IgGt), 30% (50% of this reported value) and 90% (150% of this reported value).

B: Same as for Figure 3A, but showing the IgG4/IgGt ratios. The dotted line at 5% marks the reported ratio of IgG4/IgGt.

versus mean 2.68, range 0-11, p=0.0013), and they showed a trend towards responding to more antigens than *S. aureus* carriers (mean 5.38, range 1-11 versus mean 2.68, range 0-12, p=0.1275) (Fig. 4). This implies that the intense exposure of EB patients to different *S. aureus* types results in increased numbers of staphylococcal antigens to which IgG4 responses will develop.

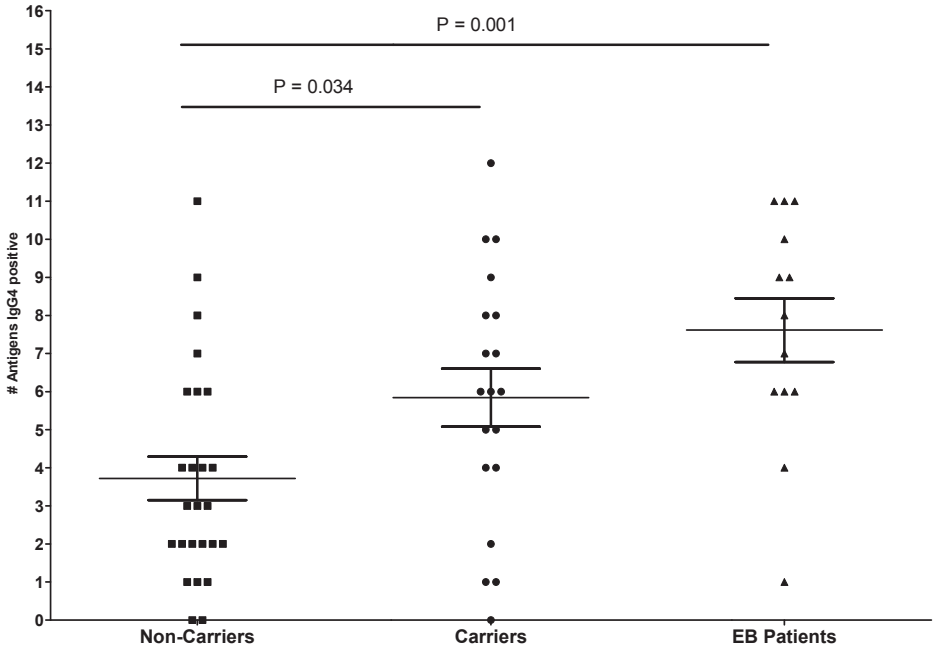

Figure 4: Number of *S. aureus* antigens to which IgG4 responses were detectable in sera from 19 Dutch carriers (circles), 26 Dutch non-carriers (squares) and 13 Dutch EB patients (triangles)
Dutch carriers showed a trend to IgG4 serum responses to more antigens than non-carriers (mean 2.95 , range 0-12 versus mean 2.68, range 0-11, p=0.0339). EB patients showed IgG4 responses to significantly more antigens than non-carriers (mean 5.38, range 1-11 versus mean 2.68, range 0-11, p=0.0013), and trended towards more IgG4 responses than carriers (mean 5.38 , range 1-11 versus mean 2.98, range 0-12, p=0.1275). Mean and SEM are plotted.

DISCUSSION

In the present study, we investigated the IgG subclass-specific responses directed against 40 different *S. aureus* virulence factors. These responses were measured in the sera from patients from 3 geographical locations suffering from 4 different types of *S. aureus* infections. In addition, we studied the humoral response in sera from healthy human carriers, non-carriers and patients suffering from epidermolysis bullosa, with well documented *S. aureus* colonization status, to gain more insights into the bacterial fac-

tors involved in pathogen-host interaction. Total IgG responses were detected against almost all antigens in our panel, in agreement with our previous analyses (9, 30, 37), (28). IgGt responses consisted mostly of IgG1 responses, consistent with the previously reported finding that IgG1 composes of 60% of IgGt (31), (32). In contrast, in all serum sets analyzed here we observed that IgG4 antibodies, which represent approximately 5% of the IgGt response, were detected to a core panel of *S. aureus* antigens consisting almost exclusively of secreted immune modulators, irrespective of the type of human-pathogen interaction.

IgG4 responses were observed against alpha toxin, CHIPS, ETA and B, HlgB, IsdA, LukD, E, F and S, SCIN, SEC, SSL1, 3, 5 and 9, and TSST-1. These immune modulators interact with both the human innate and acquired immune systems on many levels. Innate responses affected are chemotaxis, which is modulated by CHIPS (56), extravasation, modulated by SSL3 and SSL5 (57), complement activity, which is modulated by SCIN (58), and TLR2 signaling, which is affected by SSL3 (59). SEC and TSST-1 modulate adaptive responses by non-antigen directed binding of MHC II with T cell receptors, resulting in polyclonal T cell activation (60). Neutrophils are targeted by the γ-hemolysin family (HlgB, LukD, E, F and S) (20), desmosomes are targeted by exfoliative toxins (ETA and B) (61), and alpha toxin lyses mononuclear immune cells and platelets (62). SSL9 binds to monocytes and dendritic cells, and it blocks the complement system (63, 64), and no clear function has thus far been described for SSL1. Interestingly, patterns of IgG4 response varied extensively between volunteers, indicating that each person is exposed to different virulence factors and/or reacts differently. The different exposure to virulence factors could be explained by the fact that various genetic backgrounds of *S. aureus* contain different sets of virulence factors and variation may also be due to differences in regulators or gene expression in various strains (65), (29), (66), (67), (68), (69), (24).

IgG4 responses were found to be directed against more different antigens in EB patients than in healthy non-carriers. EB patients are highly susceptible to blistering upon minor trauma due to mutations in structural proteins of the epidermis and the epidermal-dermal junction. Most likely as a consequence of their fragile skin, 62% to 75% of these patients are nasal *S. aureus* carriers. EB patients with chronic wounds show higher carriage rates than patients without chronic wounds (16), (18). Importantly, *S. aureus* wound colonization was detected in 92% of the EB patients with chronic wounds and 69% of the patients without chronic wounds (13), (16). Serial sampling of three wounds, the left and right anterior nares, and the throat revealed that 58.3% of the EB patients with chronic wounds and 43.5% of the EB patients without chronic wounds carried alternating *S. aureus* types over a period of ~2 years and, during this period, the same *S. aureus* type was only encountered in 42.5% of all sampled patients (17, 18) (16),(70). This suggests that these patients were exposed to diverse staphylococcal virulence factors over a prolonged period of time. Accordingly, our present IgG4 data

indicate that repeated exposure to *S. aureus* in EB patients has led to IgG4 responses directed against more different staphylococcal antigens, although we cannot exclude that other forms of (previous) exposure might result in the development of IgG4. Intriguingly, our IgG4 data indicate a chronic and repeated exposure for all humans to *S. aureus*, and indicated that repeated exposure as in EB patients leads to higher levels of IgG4 responses directed against more antigens than is the case in healthy volunteers. Not all staphylococcal isolates produce all of the virulence factors tested in the present study, and it therefore seems likely that some of them have a higher potential to elicit an IgG4 response than others. Importantly, the presence of IgG4 levels against *S. aureus* antigens in human individuals may be an indication of past (chronic) or repeated exposure, possibly in the form of a-symptomatic, self-limiting infections or colonization (33), (32).

IgG4 is important in neutralizing antibody responses during tolerance after allergy (34), (71), vaccine development (72) and immune therapy (73). The increased interest in IgG4 is predominantly caused by the fact that IgG4 antibodies activate the immune system to a lesser degree by Fc receptor-mediated and complement-mediated phagocytosis than other IgG subclasses, making IgG4 ideal for passive immunization therapies. Our finding that EB patients have the widest spectrum of IgG4 responses, while being fairly resistant to bacteremia may prove interesting clues for further vaccination research: possibly new vaccination strategies should induce neutralizing IgG4 antibodies by repeated exposure, although a protective role of other adaptive immune responses cannot be excluded.

The findings we report here were generated in several cohorts, each from distinct geographical locations. As with many clinical studies, acquiring sufficient samples and finding appropriately matched controls is challenging, laborious and time-consuming. Therefore, we performed an explorative analysis with descriptive statistics based on study cohorts that were relatively small. Accordingly, no power analysis could be done prior to measurement. In this respect, it has to be noted also that previous reports have shown large inter patient variability (9, 19, 28, 30, 37). Nevertheless, we still observed the restricted panel of antigenic immune modulators to which IgG4 responses were mounted in all cohorts analyzed. This observation that IgG4 responses are mounted to a restricted panel of secreted immune modulators of *S. aureus* is novel and therefore of value to report. Differences between non-carriers and carriers with respect to the number of antigens responded to, as shown in Figure 4, reach significance without correction ($p < 0.05$), but do not remain significant after the Bonferroni correction ($p < 0.0167$). We observe a clear trend in the number of antigens showing IgG4 responses and exposure to *S. aureus*, but larger longitudinal follow-up studies based on power analyses and initial results will be needed to further substantiate these findings.

To the best of our knowledge, this is the first report on IgG4 responses directed to *S. aureus* antigens. As we found little cross-reactivity between the different subclass-

specific detection antibodies, we conclude that our Luminex assay is robust, and has potential for application with other clinically relevant pathogens. Lastly, our study demonstrates a remarkable variation in the composition of the human subclass-specific antibody responses to various antigens of *S. aureus*, predominantly secreted immune modulators. This has been consistently observed since the start of measuring such antibodies and is fully in line with the outcomes of our previously published analyses (9, 19, 28, 30, 37). Our present data suggests that there is widespread (asymptomatic) exposure to this *S. aureus* in the community and this applies to all groups studied here, from infected patients to persistent nasal non-carriers. We therefore hypothesize that interactions between humans and *S. aureus* occur extensively and repeatedly and are even more diverse than currently appreciated. This might have major implications for research on the respective host-pathogen responses *in vivo* and for the development of immunotherapeutic strategies such as active and passive vaccination.

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Chapter 3

Staphylococcus aureus immune modulators Chemotaxis Inhibitor Staphylococcal Protein (CHIPS) and Staphylococcal Complement Inhibitor Protein (SCIN) are produced during the early stages of biofilm formation *in vitro*

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ABSTRACT

Immune modulators have been reported to be produced by mature biofilms and during different stages of planktonic growth of *Staphylococcus aureus* (*S. aureus*). Little is known about their production during the early stages of biofilm formation, raising the following question: how does *S. aureus* protect itself at these stages from the innate immune responses? Therefore we determined the expression of the immune modulators Chemotaxis inhibitor Staphylococcal protein (CHIPS), Staphylococcal complement inhibitor protein (SCIN), Formyl peptide receptor-like 1 inhibitor (FLIPr), Gamma-hemolysin component B (HlgB), Leukocidin D, E, and S (LukD, LukE and LukS) and Staphylococcal Enterotoxin A (SEA) during *in vitro* biofilm formation in Iscove`s Modified Dulbecco`s Medium (IMDM) at different time points using a competitive Luminex assay and Mass Spectrometry. With both methods we demonstrated the presence of immune modulators SCIN and CHIPS already during the early stages of biofilm formation. Using green fluorescence protein (GFP) promoter fusion technology, we also confirmed *scn* and to lesser extent of *chp* gene transcription during the early stages of biofilm formation. Further, we were able to demonstrate that SCIN could inhibit human complement activation by early biofilm. Our *in vitro* data indicate that *S. aureus* is able to modulate the innate immune system already during the early stages of biofilm formation.

INTRODUCTION

Biofilms are reported in 80% of all bacterial infections (1) and they are composed of clusters containing bacteria with heterogeneous metabolic activity (2-5). These bacterial clusters are embedded in a matrix composed of extracellular DNA (eDNA), proteins and/or polysaccharides from endogenous and exogenous origin (6). *Staphylococcus aureus* (*S. aureus*) is well-known for its ability to form biofilms causing a broad range of biofilm related infections, including chronic osteomyelitis (7, 8), endocarditis (9), chronic wound infection (10, 11) and infections of indwelling medical devices (11). *S. aureus* biofilm-related infections are of concern as they are associated with treatment failure and can act as persistent foci of infections, even after administration of adequate antibiotic therapy (1, 12). The fact that host immune responses and antibiotics penetrate poorly into the biofilms (13) and the presence of bacteria with low metabolic activity within the biofilm (3) lead to reduced treat-ability of these staphylococcal biofilm-associated infections.

During the early phase of the maturation phase of biofilm formation the production of immune modulators like SCIN and CHIPS has been ascertained (2). CHIPS and SCIN are known to be produced by planktonically growing *S. aureus*. These molecules can inhibit chemotaxis and subsequently phagocytosis by host immune cells (20). In this study we focused on the kinetics of immune modulator production and expression during early biofilm formation. Both in mature planktonic growth and in mature biofilms, *S. aureus* are known to produce immune modulators (2, 5). However, little is known about the early stages of biofilm formation, raising the question how sessile *S. aureus* in the first stage of biofilm formation escapes from the rapid response of the complement system and avoids opsonophagocytosis by neutrophils? To study this, we determined the presence of immune modulators CHIPS, SCIN, FLIPr, HlgB, LukE, LukD, LukS and SEA during the development of *S. aureus* biofilms and ascertained the effect of SCIN during the early stages of biofilm formation on complement activation.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Clinical and laboratory *S. aureus* isolates were used in this study (Table 1). *S. aureus* confirmation was performed by Bactec (Becton Dickinson, Breda, the Netherlands) and Slidex Staph Plus (bioMerieux, Zaltbommel, the Netherlands) agglutination assays. All wild type *S. aureus* strains were grown on Trypticase™ Soy Agar with 5% sheep blood (TSA) overnight at 37°C. The GFP containing strains were plated on TSA supplemented with 10 µg/mL chloramphenicol.

Table 1: *S. aureus* strains used in this study.

***S.aureus* strain**	**Genetic background**	**Description**	**Source**
M82	ST20	Isolated from a patient with an osteomyelitis infection in Indonesia	this study
M116	ST239	Isolated from a patient with an osteomyelitis infection in Indonesia	this study
RWW50	ST8	Clinical isolate from thecollection of MMIZ Erasmus MC, The Netherlands	W. van Leeuwen
Newman	ST8	Laboratory strain	
8325-4	ST8	Wild type, GFP negative control	A. Cheung
8325-4 GFP	ST8	GFP positive control, Page repressor promoter cloned into EcoR1- Xbal site of pACL1484	S. Rooijakkers (30)
8325-4 *scn*-GFP	ST8	*scn* Promoter cloned into EcoR1-Xba1 site of pACL1484	S. Rooijakkers (29)
8325-4 *chp*-GFP	ST8	*chp* Promoter cloned into EcoR1-Xba1 site of pACL1484	S. Rooijakkers (29)

Immune modulators' genes determination

All strains were tested for the presence of immune modulator genes (*scn, chp, flir, hlgB, lukE/D, luks* and *sea*), according to a PCR protocol as described previously (23).

Biofilm mass assessment

S. aureus cells grown overnight on TSA were suspended in 4 mL of 0.9% NaCl until OD_{660} of 2.0 (± 0.2). One microliter of this suspension was added to 199 µL Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Bleiswijk, The Netherlands) in separate wells of a sterile flat-bottom 96-well polystyrene tissue culture plate (CELLSTAR® Greiner Bio-One, Oberosterreich, Germany) (24). Plates were incubated for 1, 2, 4 and 24 hours with 150 rpm orbital shaking at 37°C. At these times points, formed biofilms in the wells were washed twice with 200 µL sterile water, air dried for 30 minutes and stained for 2 minutes with 30 µL of 1% crystal violet (Sigma Aldrich, Zwijndrecht, The Netherlands). Excess crystal violet was removed by washing the plates 5 times with 200 µL of sterile water. Biofilms were dissolved in extraction solution containing 50% dH_2O, 40% ethanol (Sigma Aldrich, Zwijndrecht, The Netherlands) and 10% acetic acid (Sigma Aldrich, Zwijndrecht, The Netherlands). Finally, to determine the biofilm mass, the amount of crystal violet was measured spectrofotometrically at 490 nm with a micro-plate reader (Epoch 2 Microplate reader, BioTek instrument, Inc., Winooski, VT, USA)

Production of immune modulators during *S. aureus* biofilm formation

To determine the production of immune modulators during biofilm formation of *S. aureus*, we cultured *S. aureus* M82 (ST20), M116 (ST239), RWW50 (ST8) and Newman as described above. We cultured the biofilm for either 1, 3 or 23 hours. At these time points

we discarded the supernatant to remove all the non-attached or planktonic bacteria, washed the biofilm with 200 µL IMDM once and added 200 µL of fresh IMDM medium to wells and proceeded the incubation for 1 hour at the conditions described above. Subsequently, we collected the supernatants, and labelled them as the 2nd, 4th, and the 24th hour biofilm supernatants. All individual supernatants from all time points produced by all different strains were filtered with 0.2 µm sterile filter (Whatman® GE Healthcare Life Sciences, Buckinghamshire, UK) and stored at -20^{0} C until further determination. The supernatant collected after the 1st hour of incubation served as reference control.

Quantitation of biofilm derived immune modulators by competitive Luminex assay

Collected supernatants, as described in the previous section, were three-fold diluted in PBS-BN buffer (PBS containing 1% bovine serum albumin [Sigma] and 0.05% sodium azide [pH 7.4]) to create a 1, 3, 9, 27 and 81 times dilution. The competitive Luminex assay was performed, as described previously (22, 25). Briefly, 30 µL of specified dilutions of biofilm derived supernatant were added to 30 µlL of 1/200 diluted HPS (human pooled serum) in U shape 96-well micro-plates (Greiner Bio-One, Oberosterreich, Germany) and incubated with continuous shaking (800 rpm) at 22 ^{0}C during 35 minutes on a Thermostat plus (Eppendorf, Hamburg, Germany). Subsequently, 50 µL of these mixtures were directly used for the semi-quantitative determination of *S. aureus* biofilm derived immune modulators by Luminex bead based flow cytometry (Luminex Corporation). Essentially, this assay measures the level of unbound immune modulator specific IgG antibody remaining in the mixture after the 35 minutes of its incubation. For each immune modulator the fraction of specific IgG bound during the incubation can thus be calculated and taken to represent the amount of each immune modulator that was present in the supernatant. Antigens used in this assay were: Chemotaxis Inhibitor Staphylococcal Protein (CHIPS), Staphylococcal Complement Inhibitor Protein (SCIN), Formyl Peptide receptor-like 1 inhibitor (FLIPr), Gamma-hemolysin component B (HlgB), Leukocidin D, E, and S (LukD, LukE and LukS) and Staphylococcal Enterotoxin A (SEA). All antigens were coupled to MagPlex beads (Luminex Corporation, Austin, TX, USA) according to the protocol as provided by the manufacture. The antigen concentration used for coupling was 2.5 µg per antigen per 10^{6} MagPlex beads, as optimized previously (26). The beads were diluted to a final concentration of 1,500 beads/µL. Negative control beads (beads to which no antigen was added during coupling) were included in all assays. The Luminex assay was performed as described previously (27) with one modification, we measured 50 beads per region, as optimized previously (26). All samples were analyzed on the Luminex BioPlex 200 System using Bio-Plex® Manager software version 6.1 (Bio-Rad Laboratories, Inc., USA). All data are based on three independent experiments. The Median Fluorescence Intensity (MFI) values of these triplicates were averaged if the CV value was lower than 25% and the Standard Error of

the Mean (SEM) was calculated. Using the results of the competitive Luminex assay, log dose-response curves of assessed antigens were generated for all biofilm time-points and all *S. aureus* strains tested. The relative signal for the antigens tested in the test samples was calculated by comparing the Luminex signals of the test samples with the signal of the supernatant collected from the 1st hour of biofilm formation as our reference control. The reference control is defined as 1 unit per biofilm.

Identification of the immune modulators by mass spectrometry

One hundred microliters of supernatants derived from the 4th and the 24th hour biofilms formation of strains RWW50 (ST8) and Newman were precipitated at 1:10 v/v with ice-cold acetone (Sigma Aldrich, Zwijndrecht, The Netherlands) before further sample preparation. These mixtures were centrifuged for 10 minutes at 18407 x g at 4°C and pellets were washed twice with 50 µL of cold acetone (Sigma Aldrich, Zwijndrecht, The Netherlands). The final pellets were left to air dried for 30 minutes and subsequently dissolved in 10 µL of 50 mM NH_4HCO_3 (Sigma Aldrich, Zwijndrecht, The Netherlands). Solution digestion was performed according to an adjusted protocol as described previously by Dekker, *et al* (28). Ten micro-liters of 0.2% Rapigest (Waters Corporation, Milford, MA) which was previously diluted in 50 mM NH_4HCO_3 (Sigma Aldrich, Zwijndrecht, The Netherlands) was added into 10 µL sample. This mixture then was reduced with 5 mM dithiothreitol (DTT) (Sigma Aldrich, Zwijndrecht, The Netherlands) at 60 °C for 30 minutes. After it cooling to room temperature, this mixture was alkylated in the dark with 15 mM iodoacetamide (Sigma Aldrich, Zwijndrecht, The Netherlands) at room temperature for 30 min, and digested overnight with 1:100 (w/w) trypsin (Promega, Madison, WI). Five percent trifluoroacetic acid (Sigma Aldrich, Zwijndrecht, The Netherlands) was added, to obtain a final concentration of 0.5% trifluoroacetic acid (pH<2). After 45 min of incubation at 37 °C the samples were centrifuged at 13,000 g for 10 minutes. Mass spectrometry and data analyses was performed as described elsewhere (28). Database search was performed against the uniprot_sprot_v151112 database (selected for Bacteria, 332280 entries).

Measurement of CHIPS and SCIN immune modulators transcription

To determine the transcription kinetics of immune modulators CHIPS and SCIN during early biofilm formation, we selected GFP containing *S. aureus* strains (Table 1). *In vitro* biofilms were generated from all GFP-containing strains (Table 1) according to the method as described above. After 1 hour of incubation at 37^0C, the growth medium was replaced with 200 µL fresh IMDM medium. The biofilms were then incubated in FLUOstar Optima micro-plate fluorescence reader (BMG lab technologies) at 37^0C with 150 rpm periodic rotational shaking. The accumulation of fluorescence, as a measure of gene transcription, was determined automatically every 30 min over 20 hours of incubation with an excitation wave length of 485 nm, and emissions tracked at 520 nm with a gain

setting at 800. Median Fluorescence Intensity (MFI) of the *chp* promoter-GFP (CHIPS) and *scn* promoter-GFP (SCIN) were compared to the MFI of the GFP positive control and were depicted as relative percentage of the last. For visualization of fluorescence emission, biofilms were mounted onto a fluorescence microscope after 4 hours of biofilm formation. Before imaging, planktonic cells were removed by refreshing the medium.

Measurement of complement activation

We performed an ELISA inhibition assay for Human Complement 3a (C3a) and Complement 5a (C5a) to determine the ability of SCIN to modulate the activity of complement during the early stages of biofilm formation. Two hundred micro-liters of diluted human sera in IMDM (1:5000 for C3a assay and 1:50 for C5a assay) was added to 3 hours old biofilms of the SCIN and CHIPS negative strain 8325-4 (Table 1) in the presence or absence of either 3 µg/mL recombinant SCIN (rSCIN) or recombinant CHIPS (rCHIPS). After 1 hour incubation at 37^0C, the supernatants were harvested and filtered with 0.2 µm sterile filter (Whatman® GE Healthcare Life Sciences, Buckinghamshire, UK) and processed according to the protocol of Human C3a and C5a ELISA KIT II (BD OptEIATM, San Jose, USA). C3a and C5a activation were measured at 450 nm (λ correction at 570 nm) using a Micro-plate reader (Epoch 2 Micro-plate reader, BioTek instrument, Inc., Winooski, VT, USA)

Statistical analysis

Statistical analysis was performed by using the Prism 5.0 package (Graph Pad Software, San Diego, CA, USA) and Microsoft Excel 2010. We used unpaired t-test for data analysis with ρ value ≤ 0.05 considered statistically significant. All experiments were independently repeated at least three times and the mean with Standard Error of the Mean (SEM) or the median with range were depicted.

RESULTS

Biofilm formation

Biofilm formation has been described as one of the important virulence factors of *S. aureus*. The ability to form biofilm was assayed by incubating our clinical isolates M82 (ST20), M116 (ST239) and RWW50 (ST8) and the laboratory strain Newman and 8325-4 in a polystyrene micro-plate containing IMDM at 37^0C. All tested strains were able to produce detectable biofilms after 2 hours, and biofilms increased in a time dependent manner (Fig. 1). However, despite being microscopically visible, we were not able to detect the 1^{st} hour biofilms with the crystal violet staining procedure. Apparently, the biofilm formed during

the first hour contained either too few cells or were still loosely attached to the polystyrene surface and largely washed away during the staining procedure.

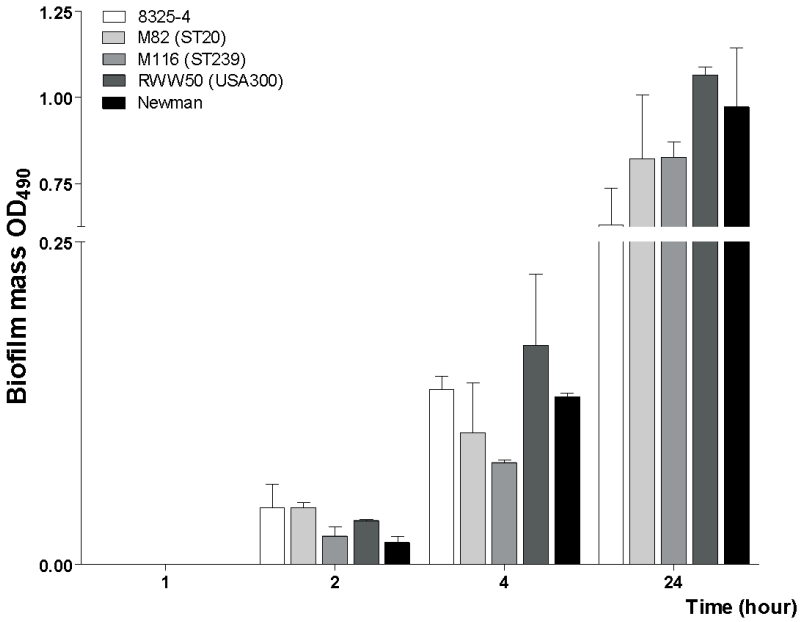

Figure 1: Biofilm formation of various *S. aureus* strains after 1, 2, 4 and 24 hours of incubation at 37°C in IMDM. Incubation time points are plotted on the x-axis and the biofilm masses at 490 nm are plotted on the y-axis. Error bars represent mean with range SE (n = 3).

Detection of Immune modulator production of *S. aureus* biofilms by competitive Luminex

Using the competitive Luminex assay (8, 22), we monitored the production of immune modulators CHIPS, SCIN, FLIPr, HlgB, LukD, LukE, LukS and SEA during the 2nd, 4th and the 24th hours of biofilm formation. The presence of the immune modulator was calculated by determining the signal shift between Luminex signal of the 2nd, 4th or the 24th hour biofilm supernatant and the signal of the 1st hour supernatant (reference control) (Fig. 2A). Based on these calculations, the average immune modulator production per biofilm was generated as depicted in the Figure 2B-D. Four hours old biofilm of M82 (ST20), RWW50 (ST8) and Newman produced on average 1.3, 3.7 and 6.5 fold ($\rho \leq 0.05$) (Fig. 2B) respectively, more CHIPS than the reference control. During the 4th hour of biofilm formation by strains M82(ST20), M116 (ST239), RWW50 (ST8) and Newman produced 1.3, 2.7, 2.3 and 12.7 units of SCIN per biofilm, respectively (Fig 2C). FLIPr was produced with averages of 1.3, 7.3 and 5.3 units by strains M116 (ST239), RWW50 (ST8) and Newman, respectively, during the 4th hour of biofilm (Fig 2D). Other immune modulators such as HlgB, LukE, LukD, LukS and SEA were not produced by any of the tested strains (data not shown). Our ST239 strain lacks

the CHIPS gene, and M82 (ST20) does not have the FLIPr gene, therefore, these strains can be taken as negative controls for CHIPS and FLIPr respectively (Table 2).

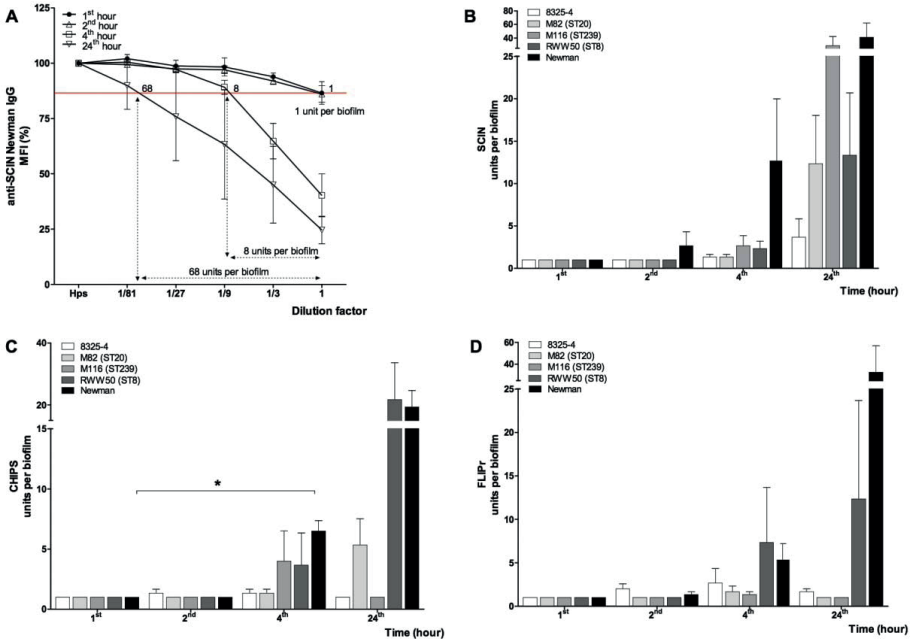

Figure 2: Production of the immune modulators CHIPS, SCIN and FLIPR during the 2nd, 4th and 24th hour of biofilm formation of *S. aureus* M82 (ST20), M116 (ST236), RWW50 (ST8) and Newman. Using the competitive Luminex assay we indirectly monitored the presence of several immune modulator by determining the shift of the Luminex signal between the supernatant of the 2nd , 4th and the 24th hour of biofilm compared to the 1st hour biofilm supernatants as our reference control (A). From this calculation the production was derived and expressed as mean ± SEM units/biofilm of immune modulators CHIPS (B), SCIN (C) and FLIPr (D) by *S. aureus* M82 (ST20), M116 (ST236), RWW50 (ST8) and Newman strain. *S. aureus* 8325-4 strain are used as negative control for SCIN and CHIPS. Data are based on at least three separate experiments Note: the more Immune modulator present in the supernatant, the lower the signal observed, as the presence of un-captured specific IgG is inversely related to the presence of the immune modulators in the supernatants (A).

Table 2: PCR results for immune modulators gene.

	detected by PCR in strain				
Gene	**8325-4**	**M82 (ST20)**	**M116 (ST239)**	**RWW50 (ST8)**	**Newman**
chp (CHIPS)	Neg	Pos	Neg	Pos	Pos
scn (SCIN)	Neg	Pos	Pos	Pos	Pos
flr (FLIPr)	Pos	Neg	Pos	Pos	Pos
hlgB (HlgB)	Pos	Pos	Pos	Pos	Pos
lukD/E (LukD/E)	Pos	Pos	Pos	Pos	Pos
lukS (LukS)	Neg	Neg	Neg	Pos	Neg

Detection of Immune modulator production of *S. aureus* biofilms by Mass Spectrometry

To confirm the production of immune modulators during the early stages of biofilm formation we performed Mass Spectrometry on supernatants collected at the 4th and

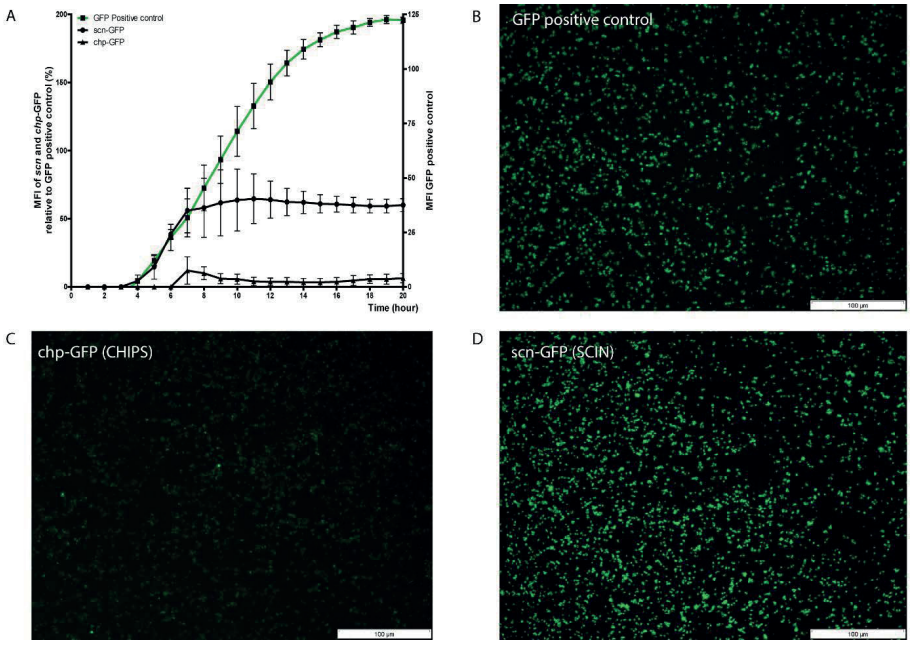

Figure 3: The kinetics of *chp* and *scn* transcription of *S. aureus* during 10 hours of biofilm formation. Transcription of the *chp* and *scn* promotor during the first 10 hours of biofilm formation are presented as percentage (%) of the positive control. Biofilm formation during the first 10 hours is represented by MFI's of the GFP Positive control strain. Bars represent means with SEM (n = 3) (A).
Promotor activity of the positive control (B), *chp* (C) and *scn* (D) after 4 hours of biofilm formation are visualized by fluorescence microscope. These pictures are representative of 3 independent experiments.

the 24th hour of biofilm formation of strains RWW50 (ST8) and Newman. We were able to identify immune modulators CHIPS, SCIN and FLIPr in the 4th hours of biofilms supernatant of RWW50 (ST8) and Newman. This result supports the hypothesis that immune modulators indeed may already be produced during the early stages of *S. aureus* biofilm formation.

Transcription of *scn*- and *chp*-promoters during biofilm formation

Based on the above results, the transcription of both *scn* and *chp* during biofilm formation was studied by applying GFP promoter fusion technology in the guise of *scn*- and *chp*-promoter-GFP fusion constructs cloned in 8325-4 strains in biofilm assays (29). As a positive control we used a constantly green 8325-4 strain (Table 1), previously described by Rooijakkers, *et al.* (30). The transcription of the *chp*- and *scin*-promoters during biofilm formation was detectable after 2 hours of incubation and steadily increased until 3.5 hours for *chp* and until 4 hours for *scn*. Transcription of the *scn* promoter was higher compared to *chp*-promoter throughout 10 hours of biofilm formation. This result corresponds with our finding that during early stages of biofilm formation more SCIN was detected than CHIPS.

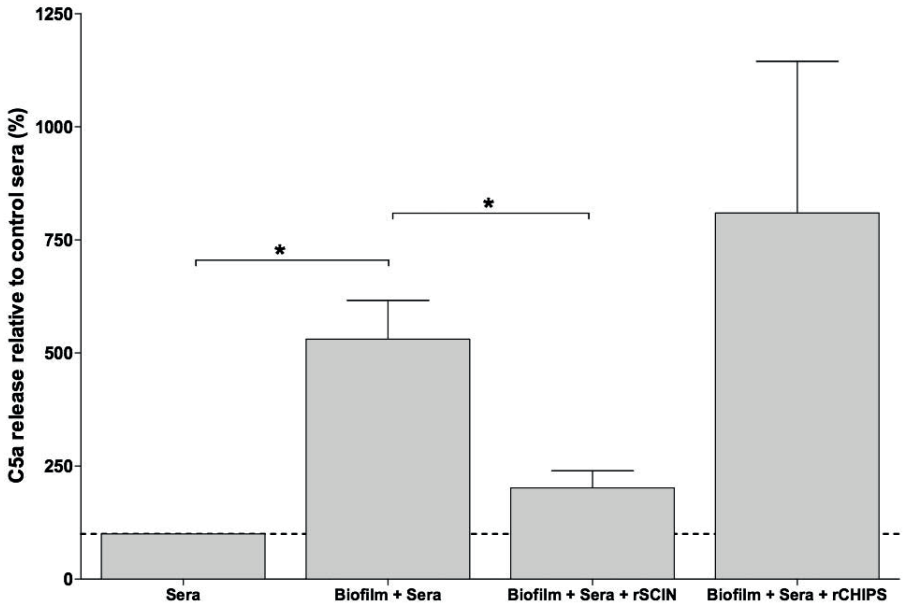

Figure 4: Inhibition of C5a release by SCIN. Human pooled serum was added to a 3 hours old biofilms of *S. aureus* 8325-4 in the presence or absence of rSCIN or rCHIPS. After 1 hour incubation at 37^{0}C, the released C5a was measured using an ELISA . Bars represent mean and SEM from 3 independent experiments.

SCIN inhibits biofilm-induced C5a release

Considering the significant expression of SCIN and CHIPS already during the early stages of biofilm formation, we also studied whether these immune modulators can protect young biofilm from host innate immune responses such as complement activation. Therefore, we added recombinant SCIN or CHIPS protein during the 4th hour of biofilm formation to the SCIN and CHIPS negative strain 8325-4 and measured the formation of human complement component 3a (C3a) and complement 5a (C5a) in the presence of human serum. As can be seen in Fig. 4, we were able to show a significant reduction of C5a release in the biofilms spiked with rSCIN compared to the ones without rSCIN ($p=0.024$). Addition of rSCIN to biofilms did not reduce the biofilm-induced release of C3a (data not shown).

DISCUSSION

Using a competitive Luminex assay and Mass spectrometry, we were able to show that during the early stages of biofilm formation, *S. aureus* produces immune modulator SCIN and to a lesser extent CHIPS and FLIPr. Additionally, during this growth phase transcription of genes coding for both CHIPS and SCIN was observed. These findings provide convincing evidence that SCIN and CHIPS are not only expressed during the exponential growth of planktonic cells (29) but also during the early stages of biofilm formation, and adds FLIPr to this list of early produced immune modulators. Archer *et al.* speculated on the production of immune modulators, including SCIN and CHIPS during the early stages of biofilm formation (2). Our observations confirm the earlier proposed production of immune modulators during the early stages of biofilm formation.

Generally, *S. aureus* biofilm formation can be divided into four stages (14): attachment, adhesion-multiplication, maturation and dispersion. Attachment, occurring after 1-2 hours of growth, involves elevated expression of cell wall-associated adhesins (15, 16). In the adhesion phase bacteria multiply and form small aggregates known as microcolonies embedded in biofilm associated protein (BAP) and secreted glycocalyx (17). An additional stage following the multiplication stage was later described by Moormeier, *et al* (18), and is called the exodus stage. During this stage, the production of DNAse facilitates the release of a subset of adhered bacteria, facilitating the formation of "tower-like" structures of biofilm (18). Next is the maturation phase, in which subsequently multilayered cell clusters form a matrix of eDNA, proteins and polysaccharides (12, 19). The final stage of biofilm formation is dispersion, where the bacterial cells begin to dissociate from the biofilm as the result of protease and DNAse production, promoting bacterial detachment and seeding (5, 21).

Although immune modulator production of biofilms was observed previously, particularly during the maturation stage, there is still little evidence available concerning the consequence of their presence. SCIN is present in 90% of all clinical *S. aureus* strains and it can reduce the complement mediated phagocytosis of planktonic cells as it is a potent C3 convertase inhibitor (20, 31). SCIN stabilizes C3 convertase molecules by causing dimerization of two convertases (32). These dimers prevent opsonophagocytosis (33) on one hand and also the formation of C5 convertases on the other hand. Under normal conditions the generation of C5 convertase will facilitate C5a release, which is an effective chemoattractant for phagocytes including the neutrophils toward the site of infection (33, 34). Therefore, SCIN has a major impact on the ability of the innate immune system to remove viable bacteria. In this study we show that recombinant SCIN was able to inhibit biofilm induced C5a release, however, we were unable to demonstrate that SCIN reduces C3a release. Thus, it seems that in our biofilm setting, the alternative pathway of complement activation occurs, resulting in C3a formation that cannot be blocked by SCIN as its' blocking capacity is downstream in the complement cascade and depends on the generation of C3 convertases. Interestingly, we do see blocking of biofilm induced C5a after addition of SCIN, indicating that SCIN facilitated dimerization of C3 convertases and as such protects biofilms from the impact of the innate immune system as well. Furthermore, our data show that C5a is a good marker for *de novo* biofilm-induced complement activation, allowing us to study the impact of different (bacterial) components on this part of the human innate immune system.

During the early stages of biofilm formation, *S. aureus* is able to manipulate the immune system, which could facilitate the survival of the biofilm-forming bacteria leading to the establishment of a biofilm. We anticipate that in addition to CHIPS, SCIN and FLIPr, other immune modulators are involved in this immune avoidance during the early stages of biofilm formation. Therefore, these results may serve as a steppingstone towards elucidating the role of immune modulators in the establishment of biofilms *in vivo*. The setup used in this study is not only a rapid screening method to determine which factors are produced during the early stages of biofilm formation but it also allows us to determine their role in the establishment of a biofilm, which gives us opportunities to develop strategies to prevent this. For several reasons biofilms of *S. aureus* are an important clinical problem (35), and helping the immune system to neutralize biofilm-forming bacteria will be tremendously beneficial to human healthcare.

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Chapter 4

Bacterial determinants of persistent *Staphylococcus aureus* bacteremia

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ABSTRACT

Introduction: Complicated and uncomplicated *Staphylococcus aureus* bacteraemia (SAB) are distinguished by host factors and persistence of infection (Infectious Disease Society of America (IDSA). Most studies on bacterial factors associated with complicated and uncomplicated bacteremia have been performed in areas with a high prevalence of MRSA. Currently, little is known about *S. aureus* strains causing complicated and uncomplicated bacteraemia in areas with low incidence of MRSA, such as the Netherlands.

Methods: 126 *S. aureus* strains were isolated from 126 consecutive patients with complicated and uncomplicated SAB. Clinical data and host factors were retrieved by chart review. All *S. aureus* strains were typed by Pulsed Field Gel Electrophoresis (PFGE). In a subset of 15 patients with complicated and 15 patients with uncomplicated SAB, antibiotic susceptibility to flucloxacillin, amoxicillin clavulanic acid, vancomycin, and ceftaroline fosfamil, *spa* type and capacity to form biofilm (under antibiotic pressure) in eukaryotic (IMDM) and prokaryotic (TSB^+) growth media were determined.

Results: No differences in genetic background were found between strains isolated from patients with complicated (15) or uncomplicated SAB (15). All strains were susceptible to the antibiotics tested. Biofilm formation was similar for strains causing complicated and uncomplicated SAB, and more biofilm was formed in TSB^+ than IMDM. In IMDM strains causing complicated SAB formed significantly more biofilm in the presence of vancomycin in comparison to uncomplicated SAB strains ($p = 0.0173$).

Conclusion: *S. aureus* strains causing complicated SAB formed significantly more biofilm than strains causing uncomplicated SAB when grown under vancomycin pressure in IMDM. Although only few of our patients were actually treated with vancomycin,we hypothesize that *in vitro* vancomycin-resistant biofilm formation may represent an *in vivo* (stress) response that facilitates persistence of SAB infection.

INTRODUCTION:

S. aureus bacteraemia (SAB) is fatal in 20 – 40% of all cases (24) and it causes more deaths annually in America than HIV, hepatitis and tuberculosis combined (5, 6, 15). The Infectious Disease Society of America uses several (host) factors to distinguish complicated from uncomplicated SAB: blood cultures remaining positive for more than 48 hours after initiation of directed therapy, fever persisting beyond 72 hours, endocarditis, indwelling prosthetic material and metastatic infection all define SAB as a complicated SAB (19). The genetic background of the causative *S. aureus* also has been reported to be associated with mortality, as shown for USA300 (14). One important bacterial factor during SAB may be the ability of *S. aureus* to form biofilm; a collection of bacterial cells within an extracellular matrix composed of DNA, proteins and polysaccharides that is firmly attached to either a host or foreign substrate (4). Strains causing complicated SAB have been shown to produce a significantly higher amount of polysaccharides than strains isolated from cases of uncomplicated SABs in prokaryotic cell culture media (20). These data suggest that formation of biofilm may be a *S. aureus* characteristic that is more frequently present in strains causing a complicated SAB. Antibiotic treatment affects biofilm formation (11, 23), with reports showing vancomycin to induce biofilm formation in vancomycin-non-susceptible strains of *S. aureus* (12). Most of the studies regarding biofilm formation have been done in areas with a high prevalence of Methicillin Resistant *Staphylococcus aureus* (MRSA). Currently, the capacity to form biofilm of *S. aureus* strains causing complicated and uncomplicated bacteremia in areas with low incidence of MRSA is not clear. Therefore, we determined whether *S. aureus* strains causing complicated SAB differ from strains causing uncomplicated SAB in the capacity to form biofilm in presence and absence of antibiotics in different media.

In vitro assays to study biofilm formation currently only use prokaryotic cell culture media, of which the chemical composition is not completely known such as: Brain Heart Infusion (BHI) or Tryptic Soy Broth (TSB) supplemented with glucose and sodium chloride. In well characterized *S. aureus* strains isolated from patients in the Netherlands (a low MRSA country), the association between bacterial factors, such as genotype, antibiotic resistance, biofilm formation (under antibiotic pressure) in either prokaryotic (TSB^+) or eukaryotic and chemically defined culture medium (IMDM) with a complicated course of SAB was determined. More insight in persistence of SAB will bring better understanding of *S. aureus* pathogenesis, ultimately leading to better treatment strategies for SAB.

MATERIALS AND METHODS

Patient characteristics

All patients 18 years and older from whom a *S. aureus* strain was isolated from a blood culture at the microbiology laboratory of the Erasmus University Medical Centre, Rotterdam, were eligible. Inclusion was from July 2009 to June 2011. Only the first positive blood culture of a patient was included in the analysis. Strains obtained from patients without follow up blood cultures and from patients with poly-microbial infections were excluded (Fig. 1). Clinical data were retrospectively obtained by chart review. Hospital acquired infection was defined as an infection starting 48 hours after admission. Community acquired infection was defined as a positive blood culture taken within 48 hours of admission. Healthcare associated SAB was defined as a positive blood culture taken 48 hours after admission in a patient, or from a patient that had been admitted up to 3 months previously, or who lived in a nursing home, or a patient undergoing chronic hemodialysis. Complicated and uncomplicated SAB were defined according to the IDSA guidelines (19). This study was approved by the Medical Ethics Committee of the Erasmus University Medical Centre Rotterdam (MEC-2007-106).

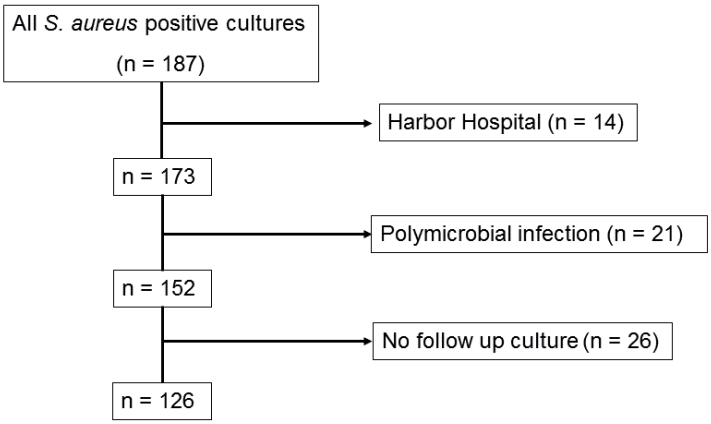

Figure 1: Flow chart of selected patients. In total, 187 patients were eligible for this study. After exclusion criteria were applied, 126 patients remained.

Genetic characterization of *S. aureus*

Pulsed-field gel electrophoresis (PFGE) of *SmaI* (Fermentas, Waltham, USA) digested chromosomal DNA from all *S. aureus* strains was performed as described previously (16). Concentrated bacteriophage lambda DNA (Bio-Rad laboratories, Veenendaal, The Netherlands) was used to provide a molecular size marker. Banding patterns were interpreted according to the guidelines described by Melles *et al* (29). PCR fingerprints were visually

inspected and evaluated with Bionumerics software (version 3.0, Applied Maths, Gent, Belgium)

Spa-typing was performed as described by Aires-de-Sousa *et al*. (28) and *spa*-sequences were analyzed using the Ridom StaphType software (Ridom GmbH, Würzburg, Germany).

In all cases *S. aureus* strains were freshly grown on Columbia blood agar plates (CBA, Becton Dickinson, Breda, The Netherlands) prior to experimental use.

MIC determination

Bacterial cells were cultured for 18-24 hours on Columbia blood agar (CBA) and suspended in 0.9% NaCl to an optical density of 0.5 (±0.05) MacFarland and diluted 1:100 in Mueller Hinton Broth (MHB, Oxoid, Badhoevedorp, The Netherlands). Of this suspension 100 µl was added to wells containing serial twofold dilutions of antibiotics: amoxicillin-clavulanic acid (GlaxoSmithKline, Middlesex, United Kingdom), flucloxacillin (ACS Dobfar Generics, Luxembourg, Luxembourg), vancomycin (Xellia, Oslo, Norway) and ceftaroline fosfamil (Zinforo, Astra Zeneca, Zoetermeer, The Netherlands). Plates were incubated for 18 hours at 37°C and MIC values were determined visually following the CLSI guidelines.

Biofilm formation determination

S. aureus was grown overnight on CBA and suspended in 0.9% NaCl to an OD_{600} 0.5 (± 0.05). Of this suspension, 10µl was dispensed into wells of sterile flat-bottom 96-well polystyrene tissue culture plates (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) containing 190µl Tryptic Soy Broth (TSB, Oxoid, Badhoevedorp, The Netherlands) with 0.5% glucose and 3% NaCl (TSB^+) or Iscove's Modified Dulbecco Medium (IMDM, Gibco, Bleijswijk, the Netherlands). Plates were incubated for 18 - 24 h at 37°C with orbital shaking at 150 rpm. Subsequently, biofilms present in the wells were washed twice with sterile phosphate-buffered saline (PBS), air dried for at least 30 minutes at room temperature and stained for 2 minutes with 1% crystal violet (Sigma Aldrich, Zwijndrecht, The Netherlands). Excess crystal violet was removed by washing the plates with dH_2O 5 times and the crystal violet was extracted in an extraction solution consisting of 50% dH_2O, 40% EtOH (Sigma Aldrich, Zwijndrecht, The Netherlands) and 10% acetyl acid (Sigma Aldrich, Zwijndrecht, The Netherlands). Absorbance was measured using a micro plate reader (Bio-Rad, Hercules, USA) at 490 nm. Biofilm formation was determined at 0 MIC and 1 MIC of each antibiotic. MICs were determined in Mueller Hinton Broth as described previously for each separate experiment for flucloxacillin, amoxicillin-clavulanic acid, vancomycin and ceftaroline fosfamil. No ceftaroline fosfamil was used clinically, as this antimicrobial agent was not available until 2012 in the Netherlands. All experiments were performed in triplicate.

Table 1. Baseline characteristics of 126 patients included in this study, and of a subset of patients with complicated or uncomplicated infection.

	All SAB	Complicated SAB (n = 15)	Uncomplicated SAB (n = 15)
Men	78/126 (62%)	10/15 (66%)	8/15 (53%)
Age	21-88	37-85	22-86
Overall mortality	47/126 (37%)	7/15 (47%)	3/15 (20%)
Diabetes	31/126 (25%)	6/15 (40%)	2/15 (13%)
Prosthetic material present	36/126 (39%)	4/15 (27%)	0
Catheter in situ	61/126 (48%)	4/15 (27%)	0
Catheter removed	41/61 (67%)	4/15 (27%)	6/15 (40%)
Immune modulating suppressive therapy	40/126 (32%)	6/15 (40%)	5/15 (33%)
Origin of infection			
Hospital acquired	68/128 (52%)	3/15 (20%)	6/15 (40%)
Health care associated	40/126 (32%)	5/15 (33%)	5/15 (33%)
Community acquired	18/126 (14%)	7/15 (47%)	4/15 (27%)
Infection site			
Unknown	41/126 (32%)	10/15 (66%)	3/15 (33%)
Intravasculair	39/126 (31%)	2/15 (13%)	4/15 (27%)
Abcess	11/126 (9%)	1/15 (7%)	1/15 (7%)
Skin	9/126 (7%)	0	3/15 (33%)
Surgical Site Infections	7/126 (6%)	0	1/15 (7%)
Urosepsis	5/126 (4%)	0	1/15 (7%)
Osteomyelitis	3/126 (6%)	1/15 (7%)	0
Other†	11/126 (9%)	1/15 (7%)	2/15 (14%)
Endocarditis	9/37 (24%)	5/15(33%)	2/15 (13%)
Empirical therapy	97/126 (77%)	12/15 (80%)	0
Cephalosporins iv	22/97 (23%)	7/15 (47%)	7/15 (47%)
Amoxicillin-clavulanic acid iv	20/97 (21%)	2/15 (13%)	4/15 (27%)
Vancomycin iv	20/97 (21%)	1/15 (7%)	1/15 (7%)
Combination therapy$	20/97 (21%)	3/15 (30%)	0
Duration therapy‴	1 - 12 days (median = 3)	1 - 9 (median = 2.5)	1 - 4 days (median = 2)
Targeted therapy#			
Cephalosporins‡	4/126 (3%)	1/15 (7%)	15/15 (100%)
Flucloxacillin / Cotrimoxazol	1/126 (1%)	0	0
Meropenem / Vancomycine	1/126 (1%)	0	0
Additional Rifampicin	51/126 (41%)	13/15 (87%)	0
Additional Gentamicin	30/126 (24%)	6/15 (40%)	2/15 (13%)
Duration Therapy	1 – 57 (median 13 days)	7 - 57 (median 15,5)	7 - 14 (median 11.5)
Culture Negative After 48 72 hours	34/126 (27%)	0/15 (0%)	15/15 (100%)

* Defined according to IDSA guidelines
# After culture results were known.
$ Consisting of ceftazidime with vancomycin (n = 2). Cefazolin with amoxicillin/clavulanic acid (n =1). Ceftriaxone with doxycycline (n =1), amoxicillin/clavulanic acid (n =1), or amoxicillin (n =1). Cefotaxim with ciprofloxacin (n =4), amoxicillin / clavulanic acid (n =1) or vancomycin (n =1). Cefuroxime with amoxicillin/clavulanic acid (n =1), vancomycin (n =4), gentamicin (n =2) or flucloxacillin (n =1).
†: 1 diabetic foot, 2 wound, 1 spondylodiscitis (1 in restricted cohort for complicated SAB), 3 heamatoma (1 in restricted cohort for uncomplicated SAB), 3 pcn (1 in the restricted for uncomplicated SAB) 1 drain neurosurgery
‡Cephalosporins = cefuroxim, cefotaxime, ceftazolin
‴ Duration of therapy before targeted therapy was initiated

Statistical analysis

Univariate analysis of host factors influencing SAB persistence was performed. Factors were subsequently analyzed by multivariate logistic regression analysis. Groups were compared using a Mann Whitney U test in IBM SPSS Statistics 20. Differences were considered to be significant when *P*-value ≤0.05.

RESULTS

Patient population

From 187 patients with SAB were eligible. Of these, 14 patients (7%) were excluded because they were treated in a hospital elsewhere and clinical data were not available. 21 patients (11%) were excluded because their bacteremia was poly-microbial. Furthermore, 26 patients (14%) were excluded because no follow up cultures were available. Mortality rate in the latter group was 9/26 (35%), not different from our study population. MSSA isolates were available from 126 patients, no MRSA strains were isolated during the study period (Table 1). The age ranged from 21 to 88 year and 78/126 (62%) were male. Empirical therapy was initially given to 97/126 patients (77%) with a median duration of 2-3 days (range 1-12 days) before it was switched to targeted therapy (Table 1). Targeted antibiotic therapy consisted mainly of flucloxacillin with additional rifampicin or gentamicin (Table 1). Of the 126 patients, 34 (27%) were culture negative within 72 hours of treatment, of whom 15 patients with uncomplicated SAB, as defined by the IDSA criteria. The other 19 patients were classified as complicated SAB based on secondary host-factors. Of the 92 patients with positive cultures persisting beyond 72 hours after the start of appropriate antibiotic therapy, 15 patients with complicated SAB were selected. To increase the chance of finding bacterial factors, we chose patients with the least host factors from the IDSA definition for complicated SAB and excluded patients with multi-organ failure, or death (Table 1). Overall mortality was 37% (47/126), mortality was higher in the 15 patients with complicated SAB (47%) than in those with uncomplicated SAB (20%).

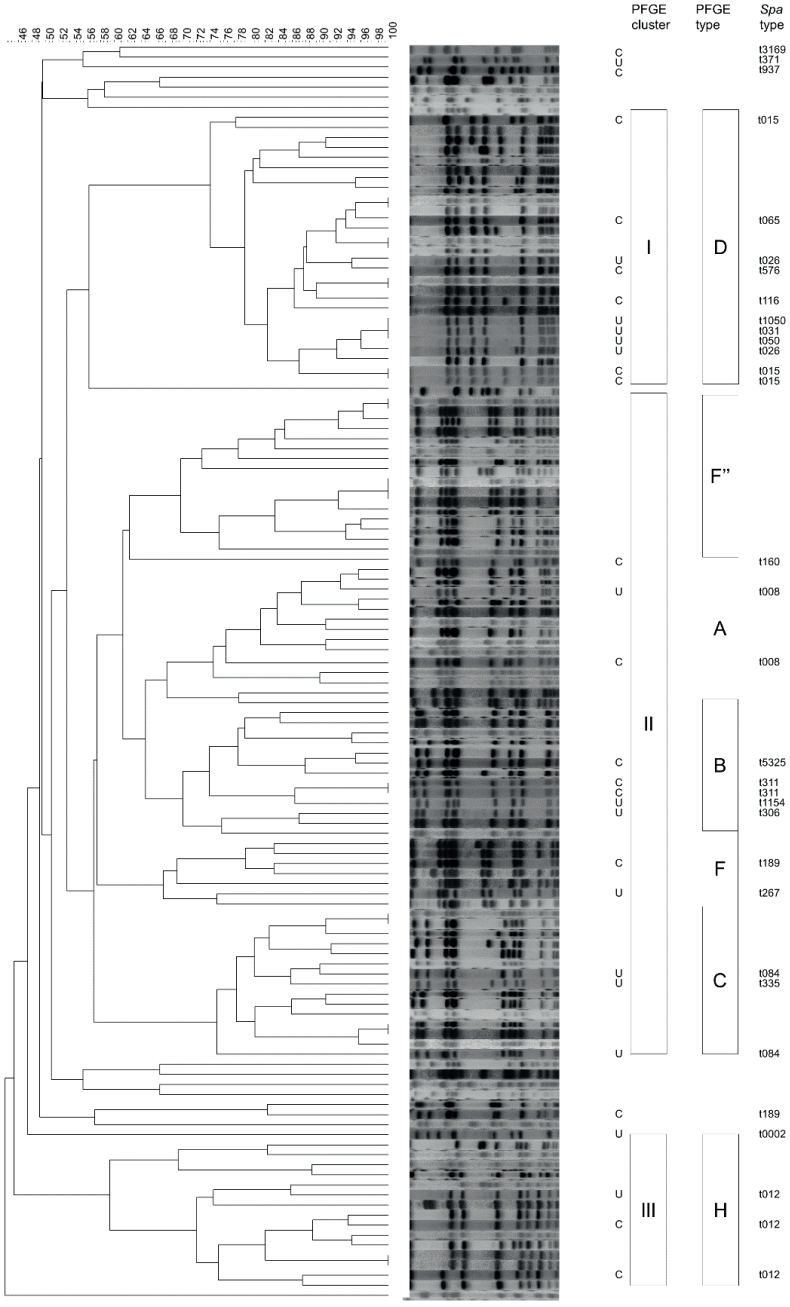

Figure 2: PFGE and *spa* type of 126 strains. From the Ridom server, *spa* type could be correlated to MLST. By combining PFGE type and *spa* typing, several clusters are obvious: Cluster I, Pulsed Field Type D contains primarily ST45 (7/10 strains). Cluster II, Pulsed Field type B contains primarily ST5 (2/5 strains). Cluster III, containing Pulsed Field Type H, contains ST30 (3/3 strains)

Genetic analysis of *S. aureus* strains

All 126 MSSA strains causing SAB were typed using PFGE. Three main clusters emerged (Fig. 2). Cluster I comprised 26/126 (20%) *S. aureus* strains of PFGE type D. Cluster II included 5 different PFGE types: F" (17 strains, 13%), A (14 strains, 11%), B (13 strains, 10%), F (7 strains, 6%) and C (15 strains, 12%), comprising a total of 66/126 of all strains (52%). Cluster III contained 15/127 (12%) of all strains, which all had PFGE type H. Other strains, 19/126 (15%), were not part of these major clusters and formed smaller clusters (Fig. 2).

The 15 strains of patients with uncomplicated SAB and the 15 strains from patients with complicated SAB were *spa*-typed. Among the strains causing complicated SAB *spa*-types t015 (3 times), t012 (2 times), t189 (2 times) and t311 (2 times), and, t008, t065, t116, t160, t576, t937, t3169 and t5325 were found (Figure 2). Uncomplicated SAB strains varied in *spa* type t084 (2 times), t026 (2 times), t002, t008, t012, t031, t050, t267, t306, t335, t371, t1050 and t1154 (Figure 2). Complicated and uncomplicated SAB strains were present in similar PFGE and *spa*-types, indicating that no genetic lineage was uniquely associated with complicated or uncomplicated SAB.

Antibiotic Susceptibility

The susceptibility for amoxicillin with clavulanic acid, flucloxacillin, vancomycin and ceftaroline fosfamil was determined for the selected 30 SAB strains. All 15 strains from complicated and 15 strains from uncomplicated SAB were susceptible to the 4 tested antibiotics with MIC's varying from 0.06 – 1 µg/ml for flucloxacillin (median 0.25 µg/ml), 0.125 – 2 µg/ml for vancomycin (median 0.75 µg/ml), <0.06 – 4.0 µg/ml for amoxicillin-clavulanic acid (median 1 µg/ml) and 0.125 – 1 µg/ml for ceftaroline fosfamil (median 0.25 µg/ml). No significant differences in MIC values were found between strains from patients with complicated and uncomplicated SAB.

Biofilm Formation and Biofilm Formation Inhibition by Antibiotics

We determined the capacity to form biofilm in TSB^+ and IMDM for strains causing complicated and uncomplicated SAB. Growth in TSB^+ resulted in significantly more biofilm formation than in IMDM (p <0.05) (Fig. 3). This difference was evident for most, but not all strains tested: a few strains showed the reverse, i.e. more biofilm in IMDM than in TSB^+ (Fig. 3). No significant difference in biofilm formation was found between strains obtained from patients with uncomplicated (n=15) and complicated (n=15) SAB in either TSB^+ or IMDM. We subsequently tested influence of beta-lactam antibiotics flucloxacillin, amoxicillin-clavulanic acid and ceftaroline fosfamil in TSB^+ and IMDM on biofilm formation. Biofilm formation was reduced in the presence of beta-lactam antibiotics. Addition of either flucloxacillin or ceftaroline fosfamil at 1 MIC in TSB^+ significantly reduced biofilm formation compared to untreated biofilms for strains from both uncomplicated and complicated SAB ($p < 0.0001$, Figure 4A). No significant effect on biofilm formation was

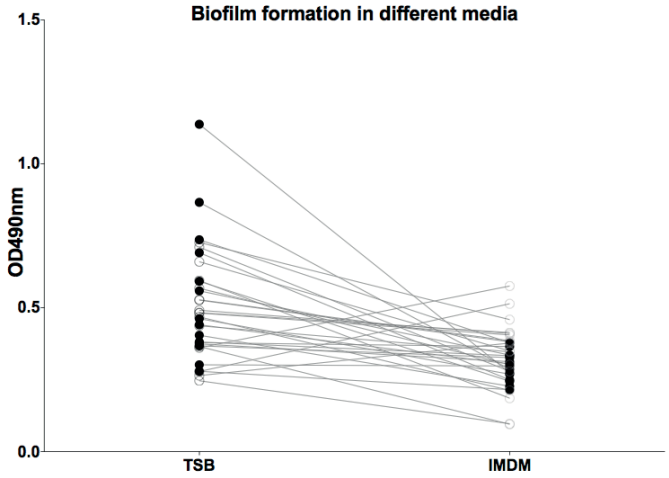

Figure 3: Biofilm formed in TSB$^+$ and IMDM by 15 strains causing complicated SAB (open circles) and 15 causing uncomplicated SAB strains (circles symbols). Biofilm readings of individual strains in TSB$^+$ are linked to readings in IMDM via lines

observed by addition of beta-lactams when the bacteria were grown in IMDM (Figure 4B). No differences between strains from uncomplicated and complicated SAB were measured in biofilm formation after 18-24 hours in TSB$^+$ or IMDM under beta-lactam antibiotic pressure. We finally tested biofilm inhibition by the glycopeptide vancomycin. There was no significant difference in biofilm formation in TSB$^+$ between strains causing complicated or uncomplicated SAB in the presence of 1 MIC of vancomycin. Strikingly, in the eukaryotic cell culture medium IMDM, strains causing complicated SAB formed significantly more biofilm in the presence of 1 MIC vancomycin than strains causing uncomplicated SAB ($p = 0.0173$) (Figure 5).

DISCUSSION

In our study, differences in bacterial factors between *S. aureus* strains causing complicated SAB and complicated SAB were studied. Antibiotic resistance, PGFE and *spa*-typing were not different in *S. aureus* strains causing complicated and uncomplicated SAB. Biofilm formation without antibiotics was similar, but biofilm formation in the presence of vancomycin in the eukaryotic cell culture medium IMDM was inhibited in *S. aureus* strains causing uncomplicated SAB.

Under vancomycin stress conditions in IMDM, SAB strains causing uncomplicated SAB were significantly less able to form biofilm compared to strains isolated from complicated SAB. In nutrient-rich bacterial growth medium TSB$^+$, however, no such distinction

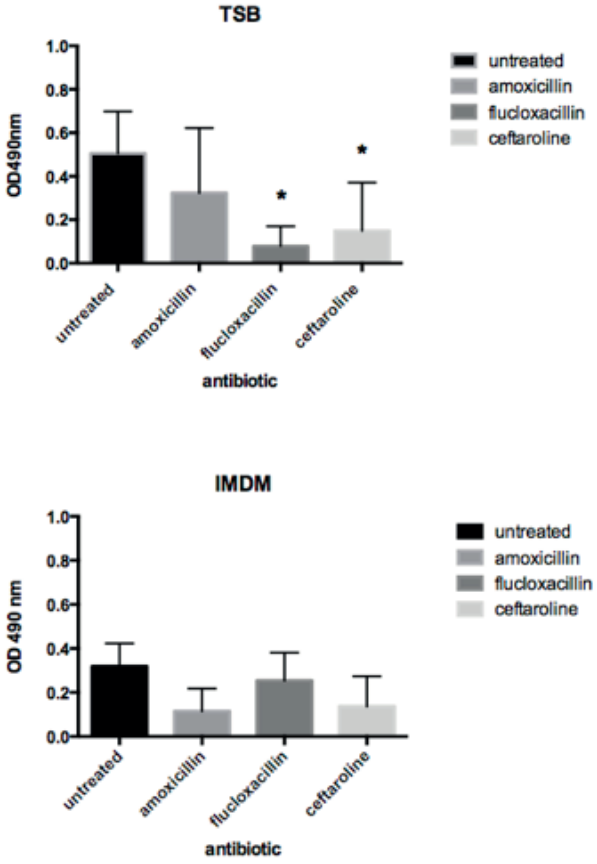

Figure 4: Biofilm formed by 30 selected strains in TSB^{+} (A) or IMDM (B). Antibiotics used are shown on the x-axis.

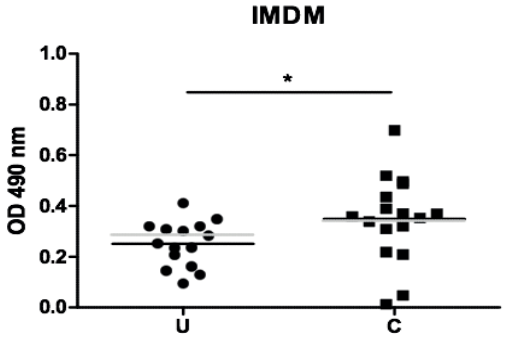

Figure 5: Biofilm formation in IMDM medium by 15 strains causing complicated SAB (C) and 15 causing uncomplicated SAB (U) in the presence of 1x MIC of vancomycin.

in the amounts of biofilm formed was observed between the two groups of strains: both were similarly affected by the presence of vancomycin in TSB^+. Bacteria generally show different behavior in different growth media (23, 26): in eukaryotic growth media, which are relatively devoid of iron, *S. aureus* grows slower and produces much larger amounts of iron-regulated surface proteins such as IsdA, where they produce little IsdA in TSB (26). We studied biofilm formation in the chemically defined eukaryotic cell culture medium IMDM, as opposed to the standard TSB^+ medium usually applied for biofilm experiments. Generally, *S. aureus* biofilm formation was less pronounced when performed in IMDM instead of TSB^+, indicating that conditions to form biofilm *in vivo* may well differ from those in laboratory media. We suggest that using eukaryotic cell culture medium better mimics the *in vivo* situation and indicates a good direction for future research.

Antibiotics do not only have direct effects on bacterial survival, as antibiotics have been reported to have an effect on *S. aureus* biofilm formation, (1, 12), with rifampicin inhibiting biofilm formation and, in contrast, gentamicin showing an induction of biofilm. We studied biofilm formation in the presence of antibiotics, using antibiotics routinely used in clinical practice to treat SAB. Generally, the strains produced less biofilm when grown under antibiotic pressure induced by adding 1 MIC of several agents to the experiments. In contrast, *S. aureus* strains causing complicated disease were significantly less reduced in biofilm formation when grown under pressure of the glycopeptide vancomycin in IMDM compared to uncomplicated SAB strains. The other beta-lactam antibiotics tested - amoxicillin-clavulanate, flucloxacillin and ceftaroline fosfamil - did not have such differential effects on biofilm formation in either of the growth media tested. It is already known that vancomycin resistance might be related to, but is not a causative factor of complication and mortality (11). In this study it was demonstrated that SAB with a higher vancomycin MIC is associated with an increase in 30-day mortality, irrespective of the antibiotic actually was used in treatment. All the strains in our study had MIC values for vancomycin of less than 2.0 ug/ml, similar to those reported in the study of Holmes (11), but we could not find significant differences in vancomycin resistance between uncomplicated and complicated SAB strains. Another explanation for the biofilm formation during vancomycin exposure may be that vancomycin induces transcriptional responses, especially the up-regulation of the cell wall stress stimulon VraSR, that are similar to the transcriptional responses to stresses induced by human derived antimicrobial peptides (27). So it could very well be that treatment with vancomycin in our *in vitro* experiments actually reflects the natural exposure to antimicrobial peptides. Taken together, the ability of certain strains of *S. aureus* to maintain biofilm production under stress conditions encountered *in vivo*, including that exerted by antimicrobial therapy, might explain, the persistence of SAB in our cohort of patients. This observed persistence does seem related to a biofilm mode of growth and locations that are hard to treat, such as endocarditis and osteomyelitis, which are observed more

in the complicated group compared to the uncomplicated group of SAB. As biofilms are thought to mediate persistence of infection, we hypothesise that our finding could become relevant in distinguishing between complicated and uncomplicated episodes of SAB in an early stage of the infection.

We further attempted to discriminate strains causing complicated from those causing uncomplicated SAB by studying their genetic background. SAB genetics is related to mortality, with USA 300 showing an increased mortality for SAB compared to non-USA300 strains. In our study, neither PFGE nor *spa* typing were discriminatory for complicated SAB, and complicated and uncomplicated SAB strains occurred in the same PFGE and *spa* types. To our knowledge, this is the first study on the influence on persistence of SAB by different genetic backgrounds.

We compared two, relatively small, subsets of patients selected from the total cohort of SAB cases. Therefore, our results cannot be simply extrapolated to all strains causing complicated SAB and further studies are needed to confirm or refute our hypothesis that biofilm formation under appropriate conditions may discriminate complicated from uncomplicated episodes of SAB at an early stage of the diseases.

We finally studied differences in antibiotic susceptibility between complicated and uncomplicated SAB's. No differences were found when comparing the antibiotic susceptibility to flucloxacillin, amoxicillin-clavulanic acid or ceftaroline fosfamil profiles of strains causing complicated versus those causing uncomplicated SAB. The IDSA advises 6-8 weeks of intravenous antibiotic therapy for patients with complicated SAB, and 2-4 weeks of therapy for patients with uncomplicated SAB. Rapid detection of strains prone to cause complicated SAB is an advantage over the current diagnostic methods which currently can take several days to establish. Testing the infecting strains ability to form biofilm under pressure of the glycopeptide antibiotic vancomycin in IMDM may facilitate more rapid determination of SAB complication status and help guide clinical management of SAB. Although our finding needs confirmation in other studies, use of bacterial factors may be a new approach to determine the risk of complicated SAB.

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Chapter 5

Eliciting antibiotics active against the ESKAPE pathogens in a collection of actinomycetes isolated from mountain soils

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SUMMARY

The rapid emergence of multiply drug resistant (MDR) bacterial pathogens poses a major threat for human health. In recent years, genome sequencing unveiled many poorly expressed antibiotic clusters in actinomycetes. Here we report a well-defined ecological collection of over 800 actinomycetes obtained from sites in the Himalaya and Qinling Mountains, and use these in a concept study to see how efficiently antibiotics can be elicited against MDR pathogens isolated recently from the clinic. Using 40 different growth conditions, 96 actinomycetes were identified - predominantly *Streptomyces* – that produced antibiotics with efficacy against the MDR clinical isolates referred to as ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and/or *Enterobacter cloacae*. Antimicrobial activities that fluctuated strongly with growth conditions were correlated to specific antibiotics, including borrelidin, resistomycin, carbomethoxy-phenazine and 6,7,8- and 5,6,8-trimethoxy-3-methylisocoumarin, of which the latter was not previously described. Our work provides insight into the potential of actinomycetes as producers of drugs with efficacy against recently emerged clinical isolates and also underlines the importance of targeting a specific pathogen.

INTRODUCTION

Infections caused by multiple drug-resistant bacteria continue to be a world-wide problem. Society currently faces rapidly growing resistance among Gram-positive and Gram-negative pathogens that cause infection in the nosocomial environment and the general community (Giske *et al.*, 2008; Rice, 2008; Spellberg *et al.*, 2008). Several multiple drug resistant (MDR) pathogens, especially *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp.* - collectively referred to by the acronym ESKAPE - cause the majority infections within the nosocomial environment (Rice, 2008). Current antimicrobial therapies have reduced efficiency or are inactive against these isolates, which leads to increased mortality among patients (Rice, 2008), and more people die of these increasingly antibiotic resistant pathogens than of HIV/AIDS and tuberculosis combined (Boucher & Corey, 2008; Klevens *et al.*, 2006). This underlines the urgent need for new antimicrobials.

The actinomycetes are a diverse family of filamentous bacteria that produce a wealth of secondary metabolites, including two-thirds of the antibiotics in clinical use (Miyadoh, 1993; Okami, 1988). Of the actinomycetes, streptomycetes are particularly prolific antibiotic producers, which grow as a branched multicellular network of hyphae, called the mycelium; on solid surfaces they reproduce through spores formed by a specialized aerial mycelium, which coincides with antibiotic production. Many studies have been conducted in search of novel actinomycetes (Lazzarini *et al.*, 2000; Ng & Amsaveni, 2012; Singh *et al.*, 2006; Takahashi & Omura, 2003; Thakur *et al.*, 2007). However, the intensive screening programs in the pharmaceutical industry failed to yield significant numbers of novel drugs (Payne *et al.*, 2007). New antibiotics have been estimated to occur at frequencies of less than one per million in randomly chosen actinomycetes grown under routine conditions (Baltz, 2007; Baltz, 2008). Sequencing of *Streptomyces* genomes established the presence of silent antibiotic biosynthetic gene clusters, suggesting that the potential of these organisms for novel drug production is much larger than originally anticipated (Challis & Hopwood, 2003; Hopwood, 2007).

A major challenge is therefore to find ways to elicit the production of yet unknown antibiotics. Such strategies have been in use in industry for many decades, but the discovery of cryptic antibiotics has renewed the interest in identifying specific conditions or agents that elicit the expression of antibiotics (Craney *et al.*, 2012; van Wezel *et al.*, 2009; Zhu *et al.*, 2013). An example is *N*-acetylglucosamine (GlcNAc), which in streptomycetes is imported by the phosphoenolpruvate (PEP)-dependent phosphotransferase system PTS and converted into glucosamine-6-phosphate (Nothaft *et al.*, 2010; Swiatek *et al.*, 2012); once imported, it forms a signaling cascade with GlcN-6P acting as a ligand for the antibiotic repressor DasR, thereby rendering it inactive and triggering antibiotic production in *Streptomyces coelicolor* (Rigali *et al.*, 2006; Rigali *et al.*, 2008). Recently,

screening of a chemical library for new elicitor molecules resulted in the identification of chemical probes that activated antibiotic production by interfering with fatty acid biosynthesis (Ahmed *et al.*, 2013; Craney *et al.*, 2012). These approaches show the power of elicitors for rational drug discovery approaches.

Media composition has a major impact on antimicrobial agent production, with glucose and phosphate as well-known suppressors of antibiotic production (reviewed in (Sanchez *et al.*, 2010; van Wezel & McDowall, 2011)). Clearly, screening under different growth conditions is a strategy that has been used by the pharmaceutical industry for many decades, although with less success in recent years (Payne *et al.*, 2007). In this study we present a novel collection of actinomycetes from soil samples obtained from remote mountains and analyzed their antibiotic-producing potential. This provides new insights towards the efficacy of the antibiotics produced, against MDR pathogens that currently threaten the health of hospitalized patients. We identified a number of antibiotics whose expression varied greatly with the growth conditions, including a previously unidentified isocoumarin-type antibiotic.

METHODS

Selective isolation of actinomycetes from soil

Soil samples were collected from the Qinling Mountains and the Himalaya Mountains (China). The Qinling samples were obtained from different places in Xi'an, Shaanxi Province, China, namely Shandi Village at longitude 109° 22′ 39″ and latitude 34° 3′ 28″, height 660 meters; Gepai Village at longitude 109° 30′ 8″ and latitude 33° 54′ 54″, height 1088 meters; Muzhai Village at longitude 109° 22′ 03″ and latitude 34° 21′ 02″, height 985 meters; Huafeng Village at longitude 108° 39′ 4″ and latitude 34° 00′ 58″, height 533 meters; Daohe Village at longitude 108° 41′ 10″ and latitude 34° 00′ 58″, height 692 meters; Langshan Village at longitude 109° 0′ 53″ and latitude 34° 1′ 5″, height 720 meters; Xinlian Village at longitude 108° 48′ 16″ and latitude 34° 1′ 43″, height 692 meters. The Himalayan sample was obtained at 5000 m height, collected near a hot water spring. Every soil sample was collected at a depth of 10 to 20 cm and put in sterilized plastic bags and stored at 4 °C before processing.

The soil was enriched with 6% yeast extract (Hayakawa & Nonomura, 1989), and dilutions plated onto selective media, which were modified starch casein agar (MSCA) (Küster & Williams, 1964), humic acid agar (HA) (Hayakawa & Nonomura, 1987), glucose casein agar (GCA) (soluble starch 15.0g, KNO_3 0.5g, K_2HPO_4 0.5g, $MgSO_4 \cdot 7H_2O$ 0.5g, NaCl 5.0g,KCl 5.0g, $FeSO_4 \cdot 7H_2O$ 0.01g ,vitamins 0.5mg, Agar 18.0g,add water to 1000ml, pH 7.2~7.4), soy flour mannitol medium (SFM) or minimal medium (MM) (Kieser *et al.*, 2000). Nystatin (50 µg/mL) and nalidixic acid (10 µg/mL) were added for the inhibition of fungi

and bacteria respectively during initial selection. Single actinomycete colonies were streaked onto SFM agar plates until pure cultures were obtained. Imaging of actinomycetes by phase contrast, stereo microscopy and cryo-scanning electron microscopy was conducted as previously described (Colson *et al.*, 2008).

Indicator microorganisms, growth conditions and antimicrobial assays

Bacillus subtilis 168 (no resistance markers) and *E. coli* ET8 (Amp^R $Apra^R$ Cam^R Kan^R Neo^R Str^R Tet^R) were used as indicator strains. ET8 is a derivative of *Escherichia coli* ET12567 (contains the *cmr, aadA* and *tet* genes that confer resistance to chloramphenicol, streptomycin and tetracyclin, respectively; (MacNeil *et al.*, 1992)) harboring cosmid supercos 1 (Agilent technologies; contains the *bla* gene for ampicillin resistance and *aph* for resistance to kanamycin and neomycin), and in addition the apramycin resistance cassette *aacC4*. MDR clinical isolates *E. faecium, S. aureus, K. pneumoniae, A. baumannii*, *P. aeruginosa* and *E. cloacae* were obtained from Erasmus Medical Centre (Rotterdam, Netherlands). Indicator bacteria were cultured in LB media at 37 °C. Antimicrobial assays were conducted using the double-layer agar method. Briefly, MM agar plates, containing supplements as detailed in Table 1, were inoculated with actinomycetes. Plates were incubated for 4 days at 30 °C, or longer if lower temperatures were used, overlaid with LB top agar (0.6% w/v agar) containing one of the indicator strains pre-grown in liquid LB to exponential phase (OD_{500} 0.4 - 0.6), and then incubated overnight at 37 °C. The following day, antibacterial activity was determined as zones of inhibition (in mm) surrounding the actinomycete colonies.

16S ribosomal gene sequencing and phylogenetic analysis

The 16S rRNA genes of the actinomycetes were amplified by PCR from liquid-grown mycelia using primers F1 (5'-GCGTGCTTAACACATGCAAG-3') and R1 (5'-CGTATTACCGCGGCTGCTG-3'), corresponding to nt positions 15-34 and 465-483 of the 16S rRNA locus of *S. coelicolor* A3(2), respectively. PCRs were performed as described (Kieser et al., 2000) and sequenced using oligonucleotide Seq F1 (5'-TGCTTAACACATGCAAGTCG-3'). Sequencing was done by BaseClear (Leiden, the Netherlands). 16S sequences were deposited in the GenBank database as a batch under the accession number BankIT 1698416. 16S rRNA sequences were used as query for BLASTN analysis. Subsequently, a phylogenetic tree based on these partial 16S rRNA sequences was constructed, essentially as described previously (Girard et al 2013). To that aim, sequences of known actinomycetes were retrieved from GenBank. Sequences were aligned with Mafft (Katoh *et al.*, 2009). After visual inspection, alignments were trimmed for gaps where more than 5% of the sequences were missing, using the Extractalign tool of the eBioX package (http://www.ebioinformatics.org/ebiox/). The phylogenetic tree was generated using maximum-likelihood algorithms with default parameters as implemented in MEGA

Table 1. Media supplements used to elicit antibiotic production. For details see Materials and Methods section.

No.	Supplement	No.	Supplement	No.	Supplement
1	25mM GlcNAc (Fluka)	14	0.1% Vitamins (Vitamins With Minerals Tablets, Hangzhou, China) (W/V)	27	1% Cellobiose (W/V) (Sigma)
2	0.3% $NaNO_2$ (W/V) (Fluka)	15	1% Xylose (W/V) (Sigma)	28	1% Galactose (W/V) (Merck)
3	0.7% KNO_3 (W/V) (Merck)	16	0.6% Yeast extract (W/V) (Bacto)	29	1% Lactose (W/V) (Sigma)
4	pH 10 (adjusted using 5M NaOH)	17	0.5% Malt extract (W/V) (Bacto)	30	1% Mannose (W/V) (JANSSEN, Belgium)
5	pH 8 (adjusted using 5M NaOH)	18	0.8% Peptone (W/V) (Difco)	31	1% Saccharose (W/V) (BDH)
6	pH 6 (adjusted using 2M HCl)	19	0.001% Jasmonic acid (W/V) (Sigma)	32	0.5% Trehalose (W/V) (Brunschwig)
7	pH 4 (adjusted using 2M HCl)	20	2.5% Fungal extract (W/V) (In house extraction)	33	0.8% Dextrin (W/V) (Merck)
8	1% Starch (W/V) (Difco)	21	1.2% Milk powder (W/V) (Friso)	34	0.5% Glycine (W/V) (Merck)
9	1% $MgCl_2$ (W/V) (Merck)	22	2% Oatmeal (W/V)	35	0.5% Cellulose (W/V) (Sigma)
10	0.1% $CaCl_2$ (W/V) (Merck)	23	1% Maltose (W/V) (Sigma)	36	0.1% Glucosamine (W/V) (Sigma)
11	0.6% NaCl (W/V) (Merck)	24	0.8% Glucose (W/V) (Sigma)	37	1% Chitin (W/V) (Sigma)
12	1% Casein (W/V) (Difco)	25	1% Fructose (W/V) (Sigma)	38	High temperature (45 °C) for overnight
13	25 mM Na-K-Phosphate buffer	26	1% Arabinose (W/V) (Sigma)	39	Low temperature (4 °C) for overnight

version 5 (Tamura *et al.*, 2011). The tree reliability was estimated by bootstrapping with 1,000 replicates. Since groupings supported by poor bootstrap values are not reliable, internal branches with a bootstrap value of less than 50% were collapsed so as to emphasize the reliable branching patterns.

Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC)

Cultures were extracted with ethyl acetate (EtOAc), which was then removed under vacuum at 40^0C, after which the residue was dissolved in methanol (MeOH). Chemical analysis was conducted by TLC and HPLC. Specifically, TLC silica gel 60 F_{254} (Merck, Darmstadt, Germany) plates were developed using chloroform ($CHCl_3$) and MeOH as the solvent system and visualized under UV light 254 nm and 365 nm; HPLC analysis was performed using an Agilent Technologies 1200 series chromatographic system with a photodiode array detector (DAD) and separated on a Phenomenex Luna C18 column

(4.6 mm x 250 mm, 5 µm) with MeOH-water as the mobile phase, applying a gradient of 20% - 100% MeOH over 40 min at a flow rate of 1 mL/min. The TLC-bioautography assay was done by placing the developed TLC plate onto the bioassay petri dish overlaid with soft LB agar (Hispanagar) (0.6%) containing *Bacillus subtilis* as an indicator. Following two hour incubation, the TLC plate was removed and incubated overnight at 37 °C. A control plate was also processed using the same solvents excluding test material to ensure the TLC and solvents themselves do not affect the growth of the indicator strain. The activity assessment was based on inhibition zones of the indicator strain.

For chromatographic methods we used normal phase silica gel (Merck, Darmstadt, Germany; pore size 60 Å, 230-400 mesh), Sephadex LH-20 (Pharmacia, The Netherlands), reversed-phase C18 semi-preparative HPLC and preparative TLC (PLC silica gel 60 F_{254} TLC (Merck, Darmstadt, Germany)).

NMR and mass spectrometry

Structure elucidation of pure active compounds was done by nuclear magnetic resonance (NMR) measurement and mass spectrometry (MS) analysis. The spectra of ^{1}H NMR, correlation spectroscopy (COSY), *J*-resolved, heteronuclear single quantum coherence (HSQC), heteronuclear multi-bond correlation spectroscopy (HMBC), and ^{13}C attached proton test (APT) of purified compounds were recorded on a 600 MHz DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz (1H) and 150.13 MHz (^{13}C). The specific 1H NMR experiment measurement parameters were set as described (Kim *et al.*, 2010).

Fourier Transform Mass Spectrometry (FTMS) (Bruker) was used to determine the exact mass of the compounds. The analyses were performed by DI-nanoESI-MS in the positive ion mode using the automated Advion NanoMate Triversa system (type 'A' chip) coupled to a LTQ-FT Ultra (Thermo Fisher Scientific).

RESULTS

A collection of actinomycetes and growth conditions to elicit antibiotic production

A new collection of actinomycetes was created from soil samples obtained from unspoiled areas in the Qinling and Himalaya Mountains (China). In total 816 different actinomycetes were recovered from the soil samples (see Materials and Methods section for details). The strains were initially tested for their ability to inhibit the growth of indicator strains *B. subtilis* 168 and of *E. coli* ET8, which carries several resistance cassettes. The vast majority of the actinomycetes inhibited growth of *B. subtilis* even under routine growth conditions, and production of antibiotics with efficacy against Gram-positive bacteria

was therefore only pursued with MDR pathogens (see below). When analyzing inhibition of *E. coli* ET8, 108 actinomycetes inhibited its growth under control growth conditions, while an additional 207 could also be induced by at least one of the media supplements to inhibit growth of ET8; in other words, a total of 315 out of 816 strains (39% of the strain collection) produced inhibitory activity against this multi-resistant Gram-negative strain. Of the 40 different growth conditions, pH 10, starch (1% w/v) and peptone (0.8% w/v) had the strongest stimulatory effect on antibiotic production. The strongly stimulatory effect of high pH values is particularly noteworthy as it by no means a common condition for screening of antibiotics. All growth conditions also repressed antibiotic

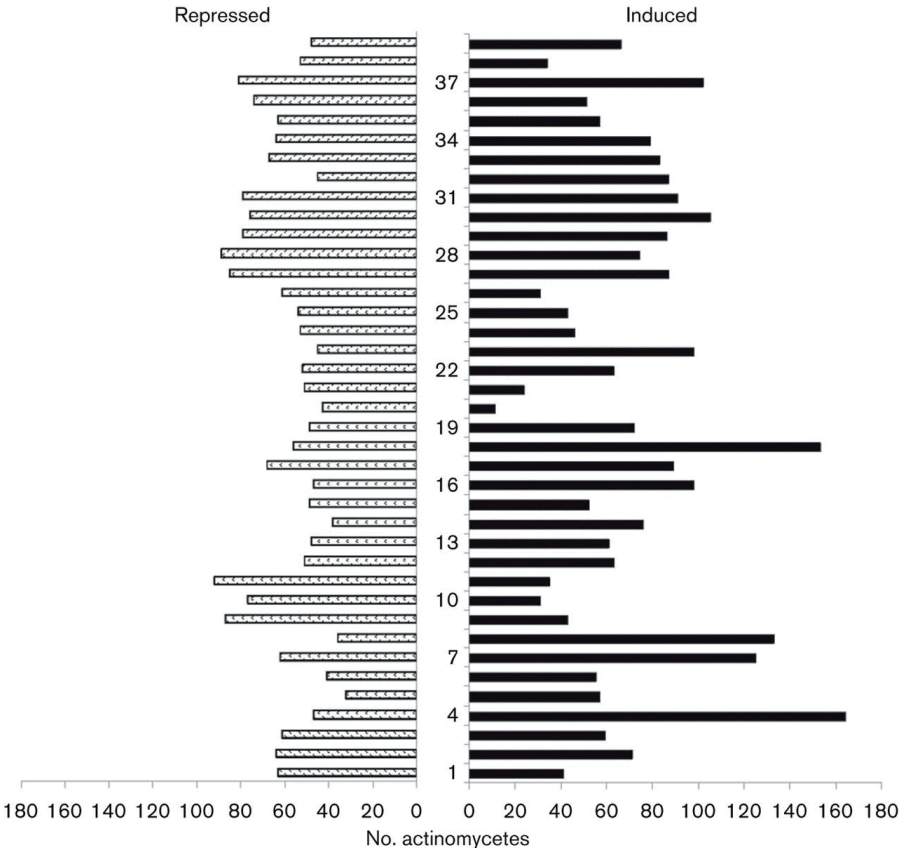

Figure 1. Quantification of induction or repression of antimicrobial activity in actinomycetes against *E. coli* ET8 depending on the growth conditions. Numbers on vertical axis represent growth conditions as listed in Table 1 and those on the horizontal axis represent the total number of actinomycetes whose antibiotic production was either induced or repressed under each of the 40 growth conditions. Strains were grown on MM agar plates with 0.5% mannitol and 1% glycerol as carbon sources, with or without additives, and then overlaid with top agar containing indicator strain *E. coli* ET8. Inhibition zones were then measured as zones of clearing. Only significant changes (changes of more than 1 mm and 10% in diameter) in the size of the zones in all replicates of the triplicate experiment were considered.

production by a number of the species in the collection (Figure 1). As an example, we compared the producing capacity of eight *Streptomyces* species from the collection, namely MBT1, MBT7, MBT11, MBT12, MBT22, MBT27, MBT28 and MBT33, which were all grown on MM agar plates or MM with added GlcNAc, starch or peptone, or MM pH 10. Again using *E. coli* ET8 as the indicator strain, the inhibition zones varied strongly per species and by growth conditions (Figure 2). MBT1 and MBT12 inhibited growth of *E. coli* ET8 most strongly when grown on routine MM agar, while production of antibiotics by these species was blocked by growth at pH 10; conversely, MBT7 and MBT11 were induced by growth at pH 10. Antibiotic production by MBT22 and MBT33 was induced by GlcNAc, while the same compound repressed antibiotic production by MBT27 and MBT28 (Figure 2).

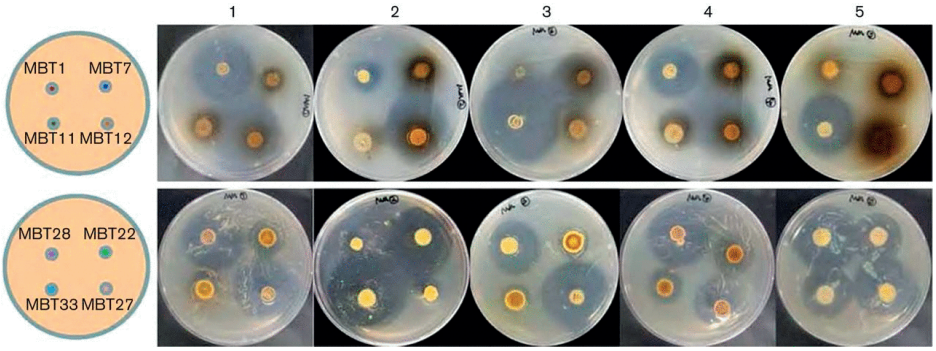

Figure 2. Antimicrobial activity of eight actinomycetes against *E. coli* ET8 under five different growth conditions. Strains were grown on MM agar plates with 0.5% mannitol and 1% glycerol as carbon sources, with or without additives. **1,** no additives; **2,** 25 mM GlcNAc; **3,** pH adjusted to 10; **4,** starch (1% w/v); **5,** peptone (0.8% w/v). The experiment serves as a representative example of the inducibility of antibiotic production in actinomycetees, under the chosen conditions. For details on the effect of elicitors on the 96 strains we refer to Supplemental Tables S1 and S4.

To see if the specific changes to the growth media - and in particular growth at pH 10 - affected the susceptibility of the indicator strains against antibiotics, we determined the inhibition zones for reference antibiotics against *E. coli* ET8 and *B. subtilis*. This showed that the zone of inhibition caused by ampicillin, apramycin, chloramphenicol or kanamycin did not increase when the indicator strains were grown at pH 10 (Supplemental Figure S1). These data corroborate that the growth conditions did not affect the susceptibility of *B. subtilis* or *E. coli*, but instead elicited antibiotic production in the actinomycetes.

Analysis of 96 promising strains for antibiotic production against MDR clinical isolates

Based on the screening of the strain collection, the 96 actinomycetes most prolific antibiotic-producing actinomycetes were identified, with their antibiotic production typically induced under specific growth conditions. Of these 96, in total 89 strains were identified as *Streptomyces* (Zhu, 2014; Supplemental Table S2 and Supplemental Figure S2). Other genera were *Kitasatospora* (MBT63, MBT64, MBT66 and MBT69), *Nocardia* (MBT52), *Micromonospora* (MBT87) and *Amycolatopsis* (MBT80). All of the 96 actinomycetes inhibited *B. subtilis* when grown on MM agar plates (not shown). To obtain detailed insight into their producing capacity, antibiotic production was further assessed with the multi-resistant *E. coli* ET8 as the indicator strain. For this, the actinomycetes were grown for four days on MM agar, MM agar with starch (1% w/v), MM agar with peptone (0.8% w/v) or MM agar at pH 10, and inhibition zones determined (Supplemental Table S1). This revealed that all strains were able to inhibit growth of *E. coli* ET8 under at least one of the growth conditions.

The antibiotic-producing potential of the 96 strains that all showed strong antimicrobial activity against the MDR *E. coli* strain ET8 was then assessed for possible clinical relevance, by analyzing their ability to inhibit the growth of six MDR clinical isolates. These clinical isolates were all isolated recently at the Erasmus Medical Center (Rotterdam, The Netherlands). The resistance profile of these isolates (Supplemental Table S3) was determined as MIC values using the VITEK II (BioMerieux) system (AST-P586 for *E. faecium*, AST-P608 for *S. aureus* and AST-N140 for *K. pneumoniae, A. baumannii*, *P.* aeruginosa and *E. cloacae*).

In accordance with previous reports on antimicrobial susceptibility (Burt, 2004; Nair & Chanda, 2006), Gram-positive pathogens (*S. aureus, E. faecium*) were more sensitive to the antibiotics produced by the actinomycetes than Gram-negative pathogens (*A. baumanii, E. cloacae, K. pneumoniae, P. aeruginosa*) (Supplemental Table S4). An example of the differential antimicrobial activity produced by 24 of the actinomycetes of the collection against the six pathogens is shown in Supplemental Figure S3. Growth of the Gram-positive MRSA (methicillin-resistant *S. aureus*) was inhibited by 82 of the actinomycetes grown on routine MM agar plates, and by another 10 strains when they were grown under specific conditions. Under the same growth conditions, 53 actinomycetes inhibited growth of the Gram-positive *E. faecium* and an additional 36 actinomycetes inhibited *E. faecium* under other growth conditions. Most Gram-negative bacteria were inhibited by some 40% of the actinomycetes growth on MM agar plates, namely *K. pneumoniae* by 41 strains, *A. baumannii* by 37 strains and *E. cloacae* by 34 strains. Changing the growth media activated antibiotic production against the latter three pathogens in another 16, 27 and 15 strains, respectively. Notably, the opportunistic pathogen *P. aeruginosa* was extremely resilient, with only seven of the 96 actinomycetes able to

exert some degree of growth inhibition to this strain when grown on standard MM agar plates. However, antibiotic production against *P. aeruginosa* could be elicited in 33 of the 89 non-producing actinomycetes by growth on MM with added starch or peptone, or at pH 10 (Figure 3A).

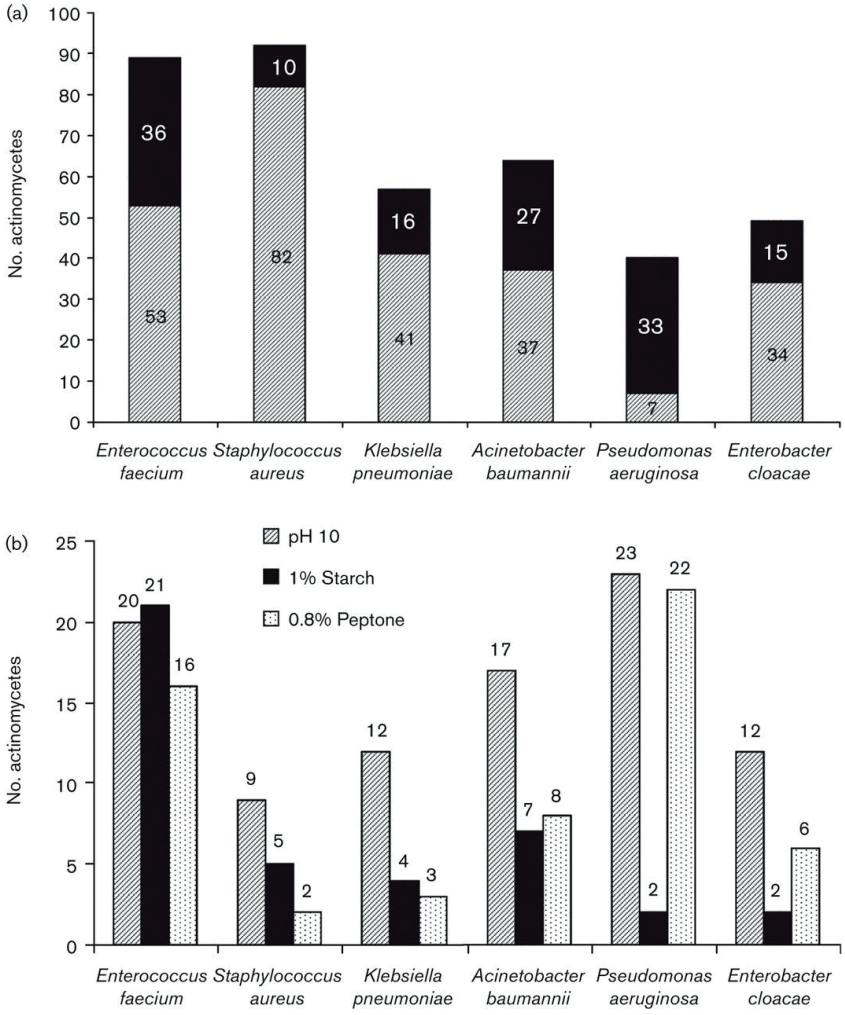

Figure 3. Inducibility of antimicrobial activity of 96 selected actinomycetes against MDR pathogens and effect of media conditions.

(A) Bar diagram of total number of actinomycetes that could inhibit the indicated MDR pathogens when grown under non-induced (light grey) or induced (black) conditions. **(B)** Bar diagram of the number of actinomycetes that were induced to produce antibiotics with efficacy against the MDR strains by growth on MM at pH10 (hashed), MM with starch (black) or MM with peptone (small dots). Antimicrobial assays were conducted using the double-layer agar method (see Materials and Methods section). The tested pathogens were *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter cloacae*.

It is important to note that actinomycetes that inhibited one Gram-negative pathogen often did not inhibit any of the other Gram-negative pathogens, which strongly suggests that different antibiotics were produced (Supplemental Table S4). As an example, strains MBT32, MBT44, MBT94 and MBT96 only inhibited growth of *A. baumannii* while they had no effect on growth of the other MDR Gram-negative clinical isolates of *K. pneumoniae*, *E. cloacae* and *P. aeruginosa*, while, conversely, MBT82 only inhibited the growth of *E. cloacae*. Intriguingly, MBT10 (when grown on MM with added peptone) exclusively inhibited the growth of MDR *P. aeruginosa* and none of the other Gram-negative pathogens, even though *P. aeruginosa* was by far the most resilient of all pathogens in our analyses.

Expectedly, antimicrobial activity varied significantly with the growth conditions (Figure 3B). When grown on MM with starch, antibiotics were produced with activity against the Gram-positive *E. faecium* and *S. aureus* in 21 and five actinomycetes, respectively, for MM at pH 10 this was the case for 20 and nine actinomycetes, and for MM with peptone for 16 and two actinomycetes, respectively. For the induction of antibiotics active against *K. pneumoniae* and *A. baumannii*, increasing the pH to 10 was most effective, activating the production of antibiotics by 17 actinomycetes against *A. baumannii* and by 12 against *K. pneumoniae*. When grown on MM with starch or peptone, seven and eight strains inhibited growth of *A. baumannii*, respectively, and for *K. pneumoniae* these numbers were four and three, respectively.

Venn diagrams (Figure 4) visualize the specificity of the antibiotics produced by the 96 actinomycetes. Despite the multiple resistance cassettes present in *E.coli* ET8 for dereplication purposes, all of the 96 strains inhibited growth of this indicator strain under at least one of the growth conditions. Furthermore, 30 out of 96 actinomycetes could be activated by one or more growth conditions to produce antibiotics against all of the tested MDR pathogens (Supplemental Table S4). Considering the broad resistance spectra of these pathogens, this underlines the potential of these actinomycetes in terms of antibiotic production. Of these, 34 actinomycetes had the potential to produce antibiotics active against *P. aeruginosa* and *K. pneumonia*, six specifically inhibited growth of *P. aeruginosa* and not of *K. pneumonia*, while the reverse was true for 23 actinomycetes. This strongly suggests that many of the actinomycetes produced different antibiotics. In a similar comparison but now with the Gram-negative *E. cloacae* and *A. baumannii*, a total of 44 actinomycetes were active against both MDR pathogens, with 20 only against *A. baumannii*, and five only against *E. cloacae*. Comparing the Gram-positive MDR pathogens *E. faecium* and *S. aureus*, 86 out of 96 tested actinomycetes were active against both indicator strains, six of which were specific against *S. aureus* and three specific against *E. faecium* (Figure 4).

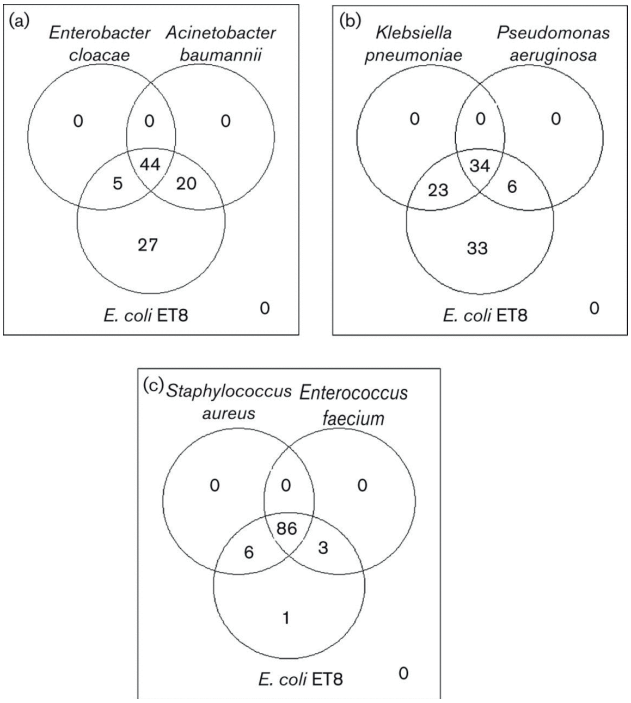

Figure 4. Venn diagrams of antimicrobial activities of 96 selected actinomycetes against different groups of pathogens. Numbers refer to the number of actinomycetes that produced antibiotics that inhibited the indicated MDR pathogens, under any of the four conditions listed in Figure 4. The groups of pathogens are **(A)** *Enterobacter cloacae, Acinetobacter baumannii* and *E. coli* ET8, **(B)** *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *E. coli* ET8, and **(C)** *Staphylococcus aureus*, *Enterococcus faecium* and *E. coli* ET8.

Identification of antibiotics

The activity-guided assay demonstrated that all actinomycetes produced varying levels of growth inhibiting bio-activities. However, a question that needed to be answered was as to what extent the growth inhibition was indeed caused by antibiotics with possible application as clinical drugs, rather than representing general disruptive activities. Examples of the latter include surfactants, acids or lytic agents and agents with a mode of action that can also have a detrimental effect on eukaryotic cells, including agents that target DNA, induce cell lysis or damage the cell membrane. Therefore, bio-activities that fluctuated strongly depending on the growth condition, were selected for further characterization. For this validation study, *Streptomyces* species MBT28, MBT70, MBT73 and MBT76 (all part of the sub-collection of 96 actinomycetes) were selected as they showed strongly inducible activity and therefore served as reference strains for this study. Importantly, as discussed below, all of the antimicrobial bio-activities that were characterized were indeed identified as antibiotics. However, it should be noted that all of the strains produce multiple antibiotics (not shown), and the entire antibiotic-pro-

ducing capacity of the strains is currently under investigation. A summary of the data is presented below. For details on the compound isolation procedure the reader is referred to Supplemental data file 1, and for NMR data the reader is referred to Supplemental data file 2 and Supplemental Figures S4 and S5.

A bioactivity that was specifically produced by *Streptomyces* sp. MBT73 on MM with starch (see HPLC chromatograms in Figure 5A) was isolated as 4.6 mg of a pure orange compound with a molecular formula of $C_{22}H_{16}O_6$ based on an ESI-MS $[M + Na]^+$ accurate mass of *m/z* 375.08926. This compound was identified by NMR analysis on the isolated compound as the antimicrobial and anticancer compound resistomycin ((3, 5, 7, 10-tetrahydroxy-1, 1, 9-trimethyl-2H-benzo [cd] pyrene-2, 6(1H)-dione), which was described previously and produced by *Streptomyces resistomycificus*, *Streptomyces griseoincarnatus* and *Streptomyces aurantiacus* (Sajid *et al.*, 2011; Vijayabharathi *et al.*, 2011). The proton NMR spectrum is presented in Supplemental Figure S4. Further confirmation was provided by genome sequencing, which revealed a gene cluster with high similarity (95% nucleotide level; not shown) to that of the previously published resistomycin gene clusters from *S. griseoincarnatus* and *S. aurantiacus*.

An antibiotic induced during growth at high pH from *Streptomyces* sp. MBT28 (Figure 5B) was isolated as 8 mg of a pure and colorless compound with a molecular formula of $C_{28}H_{43}NO_6$ based on an ESI-MS $[M + Na]^+$ accurate mass of *m/z* 488.30438. The compound was identified by NMR analysis as the antimicrobial, antiviral and antimalarial agent borrelidin (2-(7-cyano-8,16-dihydroxy-9,11,13,15-tetramethyl-18-oxooxacyclooctadeca-4,6-dien-2-yl)-cyclo-pentane-carboxylic acid; (supplemental Fig. S4)). Borrelidin is a natural product first identified in *Streptomyces rochei* (Wakabayashi *et al*., 1997). The gene cluster was identified by genome sequencing in MBT28 (not shown), and shared 89%, 97% and 93% nt identity to the borrelidin biosynthetic gene clusters of *S. rochei, S. parvulus* and *S. griseus*, respectively.

Growth on MM with peptone elicited the production of at least three distinct antibacterial agents in *Streptomyces* sp. MBT70, as shown by thin layer chromatography (Figure 5C). For one of these, 1.9 mg of a pure and brown compound was obtained. The compound was readily characterized by NMR on the purified compound (Supplemental Figure S4) as 1-carbomethoxy-phenazine, with a molecular formula of $C_{14}H_{10}N_2O_2$ (Römer, 1982). Phenazines are produced by pseudomonads and actinomycetes, and the biosynthetic gene clusters from *Streptomyces* MBT70 and of *Streptomyces cinnamonensis* (Karnetová *et al.*, 1983) share 95% nt identity (not shown).

Growth of *Streptomyces* sp. MBT76 on MM with peptone resulted in the isolation of two active compounds (see HPLC chromatograms in Fig. 5D). For the first compound 2.3 mg of a colourless compound was obtained with a molecular formula of $C_{13}H_{15}O_7$ based on the ESI-MS $[M + Na]^+$ accurate mass of m/z 273.0905. For the second compound, 2.8 mg of a yellowish solid with the same predicted molecular formula of $C_{13}H_{15}O_7$

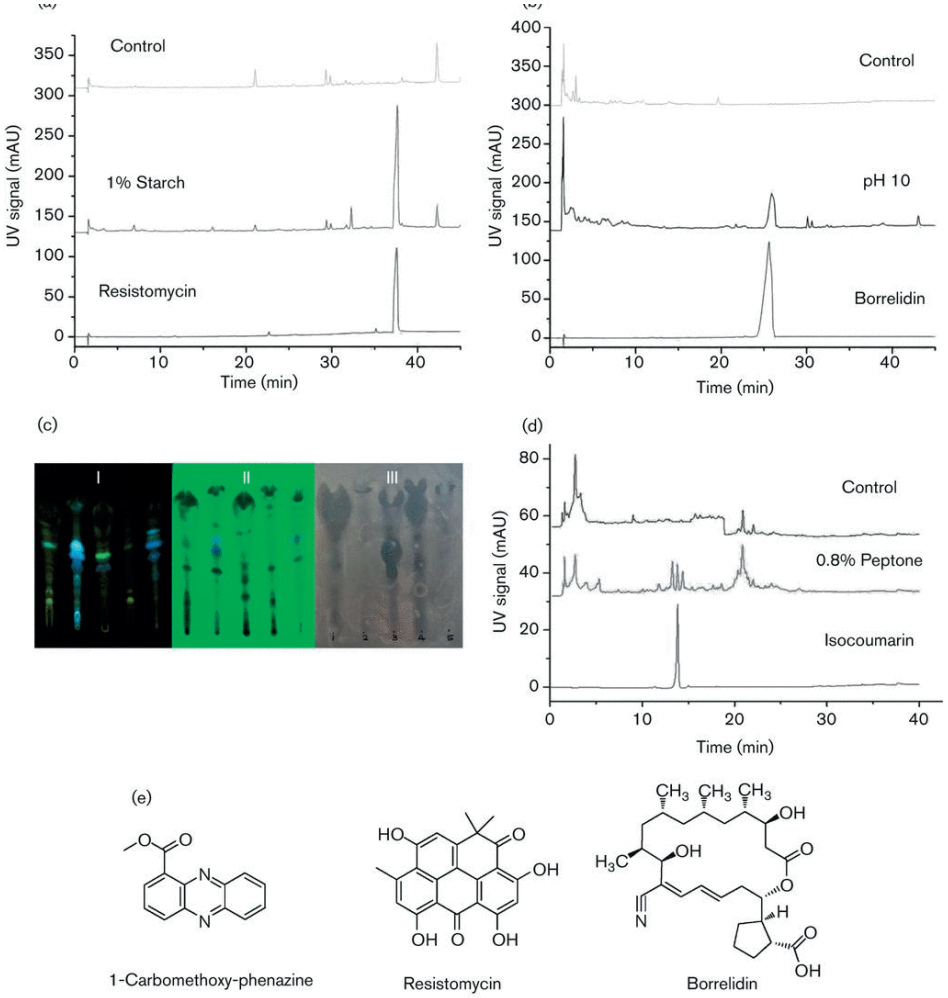

Figure 5. Induction of antibiotic production. Resistomycin, borrelidin, and 1-carbomethoxy-phenazine, and the isocoumarins 5,6,8-trimethoxy-3-methylisocoumarin and 6,7,8-trimethoxy-3-methylisocoumarin were produced by *Streptomyces* species MBT73, MBT28, MBT70 and MBT76, respectively, under specific growth conditions. **(A)** HPLC chromatograms (366 nm) of EtOAc extracts of MBT73 grown under control conditions (MM; top) and starch-supplemented cultures (middle); purified resistomycin (bottom) is presented as control. (**B)** HPLC chromatograms (245 nm) of EtOAc extracts of MBT28 grown under control conditions (MM; top) and at pH 10 (middle); purified borrelidin (bottom) is presented as control. **(C)** Thin layer chromatography of extracts of MBT70 grown in (from left to right) **1**, MM; **2**, MM with 25mM NAG; **3**, MM with peptone; **4**, MM pH10; **5**, MM with starch. TLC solvent was a 10:1 mixture of chloroform and methanol. The TLC plates show: **i,** separated compounds under UV 366nm; **ii,** separated compounds under UV 254 nm; **iii,** antimicrobial activity against *B.subtilis* via an agar overlay assay. **(D)** HPLC chromatograms (366 nm) of EtOAc extracts of MBT76 grown under control conditions (MM; top) and peptone-supplemented cultures (middle); purified 5,6,8-trimethoxy-3-methylisocoumarin (bottom) is presented as control. **(E)** Chemical structures of the identified antibiotics.

(ESI-MS $[M + Na]^+$ accurate mass of m/z 273.0932). NMR analysis showed that the two compounds were similar but distinct isocoumarin-type compounds. The first isocoumarin was identified as the previously described 6,7,8-trimethoxy-3-methylisocoumarin (Hegde *et al.*, 1989), while the second isocoumarin was identified as 5,6,8-trimethoxy-3-methylisocoumarin, which has not yet been described in the literature. For details on the ^{1}H and ^{13}C NMR data of the isocoumarins see Supplemental Figure S5, Table S5 and Supplemental Data File 1. These data show that the observed antimicrobial activities were caused by pharmaceutically relevant natural products.

All compounds were tested for antimicrobial activity against the ESKAPE pathogens, using MIC assays as described in the Methods section. 1-carbomethoxy-phenazine, borrelidin, resistomycin and the novel compound 5,6,8-trimethoxy-3-methylisocoumarin, showed activity against the Gram-positive *E. faecium* (Table 2). However, the known isocoumarin 6,7,8-trimethoxy-3-methylisocoumarin did not display noticeable antimicrobial activity. Borrelidin and resistomycin inhibited growth of the Gram-negative strains *A. baumannii* and *K. pneumoniae*, with resistomycin showing very strong activity (MIC <0.3 µg.ml) against both of the Gram-negative MDR pathogens, while borreldin showed similarly strong activity against *K. pneumoniae*, but only mild activity against *A. baumannii*. No bioactivity was found for any of the compounds against MRSA, nor against the Gram-negative pathogens *E. cloacae* and *P. aeruginosa*.

Table 2. MIC values (µg/ml) for the isolated antibiotics against the ESKAPE pathogens.

	E. faecium	*S. aureus*	*A. baumannii*	*K. pneumoniae*	*P. aeruginosa*	*E. cloacae*
1-carbomethoxy-phenazine	37,5	>37,5	>37,5	>37,5	>37,5	>37,5
Borrelidin	9,375	>37,5	18,75	0,29	>37,5	>37,5
Resistomycin	0,59	>37,5	0,15	0,29	>37,5	>37,5
5,6,8-trimethoxy-3-methylisocoumarin	18,75	>37,5	18,75	>37,5	>37,5	>37,5
6,7,8-trimethoxy-3-methylisocoumarin	>37,5	>37,5	>37,5	>37,5	>37,5	>37,5

DISCUSSION

In this work, we have isolated over 800 actinomycetes from remote mountain areas in the Qinling and Himalaya Mountains and tested their capacity to produce antimicrobial agents with efficacy against Gram-positive and Gram-negative bacteria, and in particular antibiotics that inhibit growth of one or more of the six MDR 'ESKAPE' pathogens isolated recently from a nosocomial environment in The Netherlands. The observed bioactivity

profiles of the actinomycetes underlined the importance of screening for antibiotics against specific pathogens, in addition to the search for a broadly applicable antibiotic. Gram-negative pathogens are a major problem in the nosocomial environment (Rice, 2008) and therefore many screening efforts focus on finding novel antibiotics that target these pathogens. In our study, 82 of the 96 isolates that were identified as the best antibiotic producers of the strain collection, inhibited the Gram-positive MRSA even when grown on control MM agar plates and 53 inhibited *E. faecium*, while a significantly lower number of strains inhibited growth of Gram-negative MDR pathogens when grown on these standard media. Many of the actinomycetes that produced antibiotics against the Gram-negative MDR *K. pneumoniae* or *E. coli* under a certain growth condition did not inhibit *P. aeruginosa* and *vice versa*. In fact, *P. aeruginosa*, which is the major pathogen found in cystic fibrosis lungs (Davies, 2002), was by far the most resilient of the six clinical isolates tested, with only seven of the 96 best antibiotic producing strains of our collection inhibiting growth of this bacterium under routine growth conditions. For comparison, 34 of the 96 strains inhibited growth of *K. pneumoniae*, which is also a very dangerous MDR pathogen recently associated with major outbreaks in hospitals. It is therefore particularly interesting to note that *Streptomyces* strain MBT10 exclusively inhibited the growth of MDR *P. aeruginosa* and none of the other Gram-negative pathogens, and we are therefore currently investigating the possible application of this strain for the development of drugs against this dangerous opportunistic pathogen. These experiments underline that specific antimicrobial agents that act against a certain pathogen may have advantages over traditional broad spectrum agents, whereby resistance development and side effects could potentially be reduced (Fischbach & Walsh, 2009). An example of the success of antibiotics acting against a specific clinical pathogen is the recent development of the lipopeptide antibiotic surotomycin for treatment of diseases caused by *Clostridium difficile*, an anaerobic spore-forming Gram-positive bacterium of the lower gastrointestinal tract (Tran *et al.*, 2013). With the rapidly increasing occurrence of MDR pathogens in the clinic, the development of drugs with efficacy against specific infectious diseases appears to be a logical strategy.

Screening under different growth conditions is a strategy that has been used by the pharmaceutical industry for many decades, but the low success rates in the more recent years led to a steep decline in drug discovery efforts (Payne *et al.*, 2007). We analyzed the efficacy of the strains of our collection against MDR pathogens that currently threaten the health of patients in the nosocomial environment, whereby the strains were grown under 40 different growth conditions. This included more traditional growth conditions as well as novel ones, such as the addition of GlcNAc, which inhibits the activity of the global antibiotic repressor DasR and was previously shown to activate antibiotic production, including the cryptic polyketide Cpk in *S. coelicolor* (Rigali *et al.*, 2008). However, of the conditions tested in this study, in particular high pH values (pH 10) appeared to be

a promising new condition for screening, as it efficiently elicited antibiotic production. Previous studies on productivity by the fungus *Sclerotinia sclerotiorum* using a pH range between 5-9 showed optimal production at pH 7 (Song *et al.*, 2012). We here show that the effect of pH varies from strain to strain, and that in fact there is strong potential of using in particular high pH values for screening purposes, while low pH (acidic conditions) was less effective in our assays. Perhaps counter-intuitively, growth at high pH did not affect the susceptibility of the indicator strains *B. subtilis* or *E. coli*, nor did it noticeably influence growth and development of the actinomycetes. As an example of application of pH 10 during screening, strain MBT68 inhibited growth of *E. faecium*, *P. aeruginosa*, *K. pneumoniae*, *E. cloacae* and *A. baumannii* only when grown on MM with pH 10. With the observed strong effects on antibiotic production in many of the strains in our collection, very high pH appears to be a very promising growth condition for antimicrobial screening efforts.

Detailed analysis of the antimicrobial compounds produced by a number of the isolates identified both new and previously identified compounds. This is exemplified by the metabolic profiles of four *Streptomyces* species grown under various conditions, which revealed inducible borrelidin production at high pH by MBT28, peptone-inducible production of 1-carbomethoxy-phenazine by MBT70 and of both 5,6,8- and 6,7,8-trimethoxy-3-methylisocoumarin by MBT76, and starch-inducible resistomycin production by MBT73. It should be noted that - as is often the case for streptomycetes - all of the four strains can produce multiple antibiotics, so that not all antimicrobial activity can be explained by the identified compounds (not shown). Borrelidin and resistomycin are commercially highly relevant natural products. Borrelidin is a nitrile-containing macrolide that acts as a potent inhibitor of both bacterial and eukaryotic threonyl-tRNA synthetases (Habibi *et al.*, 2012; Nass & Hasenbank, 1970), while resistomycin is a pentacyclic polyketide metabolite that inhibits RNA polymerase (Haupt *et al.*, 1975), HIV-1 protease (Roggo *et al.*, 1994) and apoptosis (Shiono *et al.*, 2002) and was first applied as drug for the treatment of tuberculosis. 1-carbomethoxy-phenazine is known to have weaker antimicrobial activity. Of the isocoumarins, 6,7,8-trimethoxy-3-methylisocoumarin acts on calmodulin-sensitive cAMP- and cGMP-phosphodiesterases (Hegde *et al.*, 1989), while its antimicrobial activity has not yet been described. Applicability of isocoumarins as an antibiotic, and in particular also that of the novel antibiotic 5,6,8-trimethoxy-3-methylisocoumarin discovered in this work, should be analysed in more detail.

In conclusion, thorough analysis of a novel collection of actinomycetes from soil samples obtained from remote mountains shows their antibiotic-producing potential when grown under a range of different growth conditions, of which high pH proved to be particularly efficient. The study provides new insights in the efficacy of antibiotics produced against recently isolated MDR pathogens, and identified a number of antibiotics whose expression varied greatly with the growth conditions, including a previously

unidentified isocoumarin-type antibiotic. In particular, the highly differential sensitivity of the various MDR 'ESKAPE' pathogens to the antibiotics produced by the actinomycetes, underlines that an important strategy may be to search for drugs that target a specific pathogen.

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Chapter 6

Structure, toxicity and antibiotic activity of gramicidin S and derivatives

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ABSTRACT:

Development of new antibiotics is declining whereas antibiotic resistance is rising, heralding a post-antibiotic era. Antimicrobial peptides such as gramicidin S (GS), exclusively topically used due to its hemolytic side-effect, could still be interesting as therapeutic compounds.

By modifying the amino-acid composition of GS, we synthesized GS analogues. We now show that derivative VK7 has a lower MIC (7.8–31.2 μg/ml, median 15.6 μg/ml) against strains of multi-drug resistant (MDR) *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* than GS has (3.9–62.5 μg/ml, median 31.3 μg/ml). Low MICs for both VK7 and GS were observed for *Staphylococcus aureus* and *Enterococcus faecium*. VK7 showed reduced hemolysis and less lactate dehydrogenase release. All compounds were fully bactericidal at MIC values. Modification of GS enables production of novel derivatives potentially useful for systemic treatment of human infections.

INTRODUCTION

Six bacterial pathogens with a propensity for developing multi-drug resistance (MDR) are specifically warned for by the Infectious Disease Society of America (IDSA) (ESKAPE: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*). These species are causing the majority of human infections and efficiently acquire additional resistance traits [1], which implies that new antibiotics have to be effective against these actively evolving MDR pathogens. The incidence of vancomycin-resistant *Enterococci* (VREs) has increased dramatically over recent years [2]. *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA), currently causes more deaths in the USA annually than HIV and tuberculosis combined [2]. ESBLs continue to be on the rise and limit treatment options [3]. *P. aeruginosa* is becoming increasingly resistant to multiple classes of drugs [4], whereas *Acinetobacters* are naturally resistant to many classes of antibiotics [5]. Increasing antibiotic resistance leads to extended hospitalization, rising treatment costs, and increased morbidity and mortality.

The rise of antibiotic-resistant pathogens has sparked research into currently disregarded antimicrobial peptides including gramicidin S (GS). GS is naturally produced by *Aneurinibacillus migulanus* [6] and was first discovered in 1941 [7]. GS shows antimicrobial activity against both Gram-positives and Gram-negatives in a MIC range from 4–64 μg/ml [8]. The lowest MICs are seen for Gram-positive bacterial species [9]. Despite its good antimicrobial activity, GS cannot be used systemically due to its hemolytic side-effect [10] and is therefore only applied topically to treat superficial infections [11]. GS is a cyclic, C2-symmetrical deca-peptide with the sequence cyclo(Pro-DPhe-Leu-Orn-Val)2. The two Pro-DPhe dipeptides form two type II β-turns, and the two Leu-Orn-Val stretches form an antiparallel β-sheet. GS has been reported to kill bacteria by forming pores in the outer membranes [8]. Native GS is a natural scaffold for amino acid alteration in such a way that antimicrobial activity is retained but toxicity is reduced. In previous attempts to modify GS, several strategies have been followed: nonnatural amino-acids were included [12], the size of the ring has been modified [8], and the β-turn region [13] and β-strand region have been changed [14]. Still, few new derivatives of GS have been identified that show retention of antimicrobial activity with reduced toxicity [8, 15–18]. We here study the β-strand-modified GS analogue VK7 [14] and the β-turn modified derivative 20 [13]. Studying naturally occurring antimicrobial peptides such as GS could help with the design and development of novel derivative drugs to combat multi-drug resistance.

MATERIAL AND METHODS

ESKAPE panel collection and characterization

Except for the *S. aureus* USA 300 and MRSA 252 strains [19], ESKAPE strains were collected at the Department of Medical Microbiology and Infectious Disease in the Erasmus Medical Centre, Rotterdam, The Netherlands. Thirty clinical isolates of *E. faecium* (5), *S. aureus* (5), *K. pneumonia* (5), *A. baumannii* (5), *P. aeruginosa* (5) and *E. cloacae* (5) were isolated from January 2010 to October 2011 from different wards (Table 1). All strains were cultured on Columbia agar plates with 5 % sheep blood (Becton Dickinson, Breda, The Netherlands) overnight at 37 °C before antibiotic susceptibility testing. Antibiotic resistance was determined using disk diffusion following Clinical and Laboratory Standards Institute (CLSI) guidelines and VITEK2 (bioMérieux, Zaltbommel, The Netherlands) following manufacturer's protocol; susceptibility was determined using EUCAST breakpoints [20].

MIC determination

Antimicrobial activity of GS and derivatives (Fig. 1) was determined following the CLSI guidelines and in triplicate [21]. Briefly, bacterial cells were cultured overnight on Columbia agar plates with 5 % sheep blood (Becton Dickinson, Breda, The Netherlands). Colonies were suspended in 0.9 % NaCl to a density of 0.5 McFarland, then diluted 1:100 in Müller–Hinton Broth (MHB, Oxoid, Badhoevedorp, The Netherlands); 100 µl of this suspension was added to wells containing GS, derivatives 3, 20, or VK7 ranging from a concentration of 0.95 µg/ml to 62.5 µg/ml in MHB. Ninety-six well plates (Greiner Bio One, Alphen aan den Rijn, The Netherlands) were incubated for 18–24 hours at 37 °C, and MIC values were determined visually. To determine whether antimicrobial activity was bactericidal, 200 µl of the suspension was plated onto new Columbia agar plates with 5 % sheep blood, and colonies were counted.

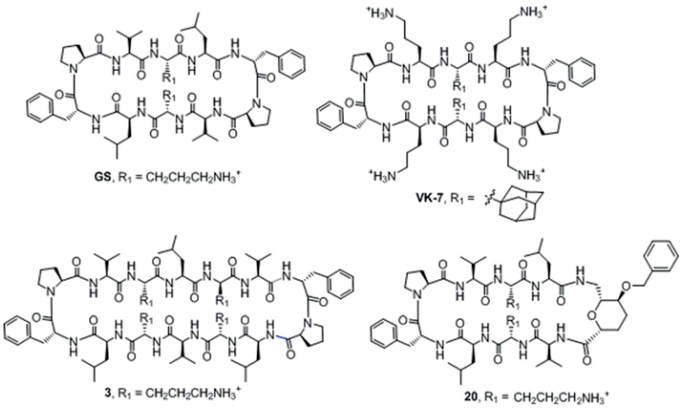


Table 1:

	Strain #	3	20	GS	VK-7
Enterococcus faecium	1	7,8	15,6	3,9	3,9
	2	7,8	15,6	3,9	3,9
	3	7,8	7,8	3,9	3,9
	4	7,8	7,8	3,9	3,9
	5	7,8	3,9	3,9	3,9
Staphylococcus aureus	1	31,3	7,8	3,9	7,8
	2	7,8	3,9	3,9	3,9
	3	62,5	62,5	7,8	15,6
	4	31,3	7,8	3,9	7,8
	5	31,2	7,8	3,9	7,8
Klebsiella pneumoniae	1	62,5	62,5	31,3	15,6
	2	31,3	62,5	62,5	15,6
	3	62,5	62,5	62,5	15,6
	4	31,3	62,5	31,3	15,6
	5	15,6	15,6	7,8	15,6
Acinetobacter baumannii	1	31,3	62,5	31,3	15,6
	2	31,3	62,5	62,5	7,8
	3	62,5	62,5	31,3	15,6
	4	62,5	62,5	15,6	15,6
	5	31,3	62,5	62,5	31,2
Pseudomonas aeruginosa	1	31,3	62,5	31,3	7,8
	2	31,3	31,3	31,3	7,8
	3	31,3	31,3	31,3	7,8
	4	62,5	62,5	62,5	7,8
	5	62,5	62,5	62,5	7,8
Enterobacter cloacae	1	3,9	7,8	3,9	7,8
	2	3,9	7,8	7,8	15,6
	3	7,8	7,8	3,9	7,8
	4	3,9	1,95	1,95	7,8
	5	62,5	62,5	62,5	15,6
toxic dose 50% (hemolyse)		41,6	nd	35,2	nd
toxic dose 50% (LDH)		49,8	62,5	18,7	nd
MIC lowest values		3,9	1,95	1,95	3,9
MIC highest values		62,5	62,5	62,5	31,2
TI following hemolysis range		0,66 - 10,67	nd	0,56 - 18,05	nd
TI following LDH release range		0,80 - 12,77	1 - 32,05	0,30 - 9,59	nd

Hemolysis assay

Hemolysis assays were performed as described before [13]. Freshly drawn heparinized blood from healthy volunteers was centrifuged for 10 min at 1000 g at 10 °C. The pellet was washed three times with 0.9 % saline and diluted with saline to a 1/25 packed volume of red blood cells. Triton-X100 (1 %) was used as a positive control. GS or derivative 3, 20, and VK7 were diluted in 100 µl PBS in U bottom 96-well plates (Greiner Bio One). Serial dilution resulted in a concentrations ranging from 62.5 µg/ml to 0.95 µg/ml. DMSO (Sigma–Aldrich, Zwijndrecht, The Netherlands) was used as a solvent control. Subsequently, 50 µl of the red blood cell suspension was added to the wells, and the plates were incubated at 37 °C for 4 h. After incubation, the plates were centrifuged at 1, 000 g at 10 °C for 4 min, and 50 µl of the supernatant of each well was dispensed into new flat-bottom 96-well plates (Greiner Bio One), and absorbance was measured at 415 nm in a Bio-Rad 680 spectrophotometer (Bio-Rad, Veenendaal, The Netherlands). OD values were plotted as a percentage of the positive control. Experiments were performed in triplicate.

Cytotoxicity testing

Human colorectal adenocarcinoma cells (HT-29, ATCC number HTB-38, Wesel, Germany) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Bleiswijk, The Netherlands) with 10 % FCS (Gibco) and penicillin–streptomycin (Gibco). Colourless DMEM (Gibco) with 1 % FCS (Gibco) was used as assay medium. HT-29 cells were seeded at a density of 2.0 × 10^4 cells/well in a Costar flat-bottom 96-well plate (Corning, Amsterdam, The Netherlands) and incubated overnight. Serial dilutions of GS and derivatives were added and incubated for 4 h at 37 °C. Plates were centrifuged for 10 min at 1,000 g, and the amount of LDH in the supernatants was determined (LDH release kit, Roche, Woerden, The Netherlands) following the protocol. Cytotoxicity testing was independently repeated three times.

Therapeutic indices

The therapeutic index was defined as a measure of toxicity, either the 50 % hemolysis or the 50 % LDH release, divided by the lowest and highest MIC values seen for each strain tested. Therapeutic indices are given as a range to be compared between GS and its GS derivatives to determine improved performance.

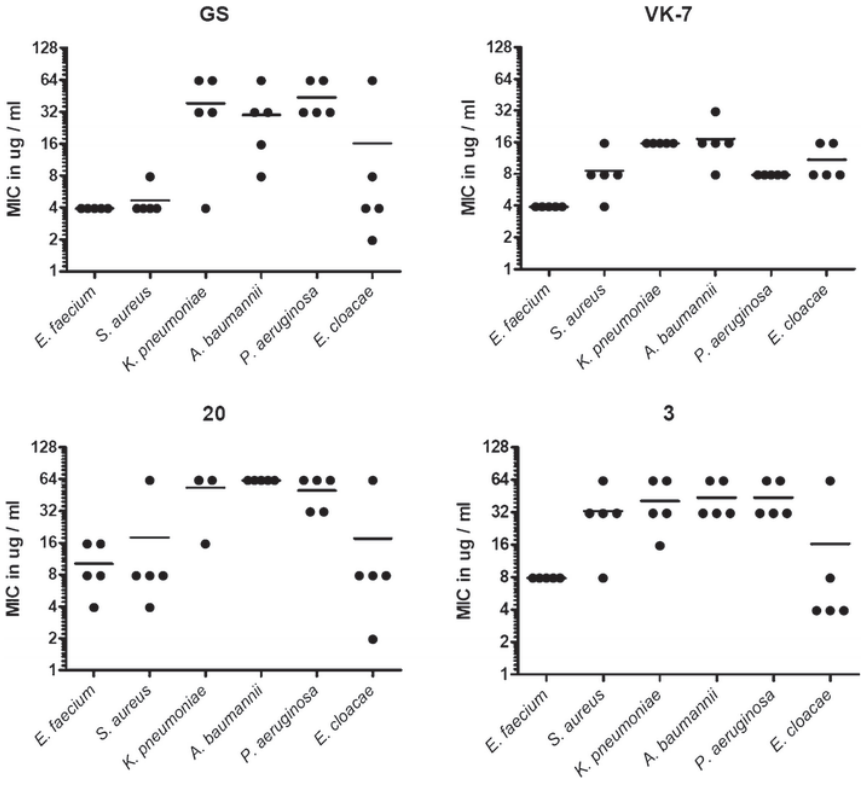
RESULTS

Cohort collection and characterization

Five strains each of *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa*, and *E. cloacae* were collected from various wards of the Erasmus MC in 2010 and 2011 (Table 1). Extensive antibiotic resistance was observed. *S. aureus* and *E. faecium* showed resistances to penicillins, cephalosporins, lincosamides, tetracyclines, macrolides, fusidic acid, aminoglycosides, carbapenems, (fluoro)quinolones, oxazolidononones, monoxycarbolic acid, nitrofuran derivatives, rifamycins, sulfanomides, and glycopeptides. Extensive drug resistance was also observed among *K. pneumoniae, A. baumannii, P. aeruginosa*, and *E. cloacae* including aminoglycosides, (ureido)penicillins (in combination with beta lactamase inhibitors), fluoroquinolones, polymyxins, carbapenems, nitrofuran derivatives, and trimethroprim with sulfamethoxazole. Each strain showed a unique profile with resistance to several clinically used antibiotics.

MIC determination

GS was active against *S. aureus* and *E. faecium* at 3.9–7.8 µg/ml (median 3.9 µg/ml) (Fig. 2).



MIC values against *E. cloacae, P. aeruginosa, K. pneumoniae* and *A. baumannii* for GS ranged between 3.9–62.5 µg/ml (median 31.3 µg/ml).

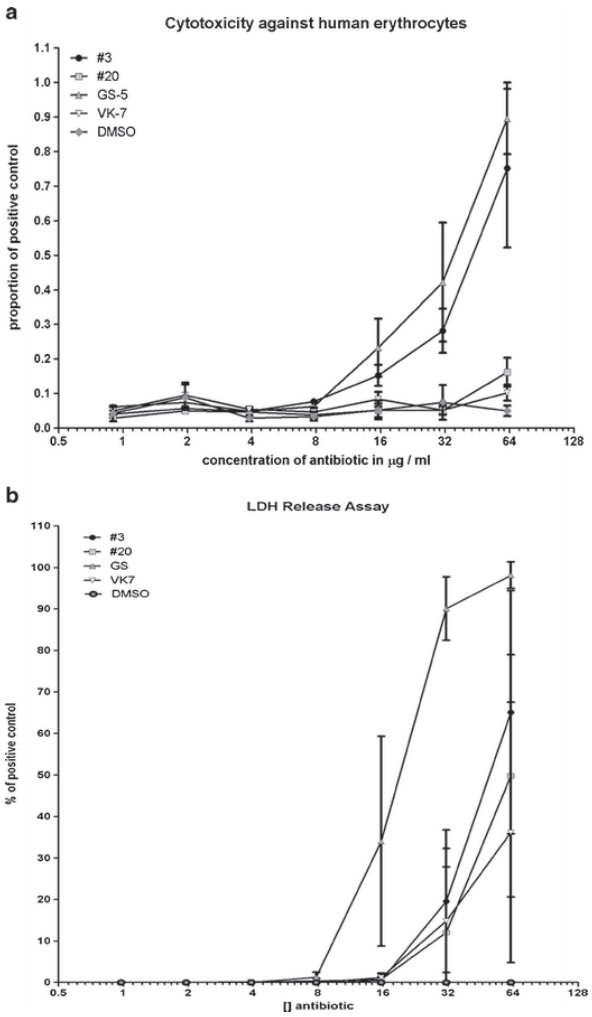
The beta-strand variant VK7 showed activity against *S. aureus* and *E. faecium* in the range of 3.9–15.6 µg/ml (median 3.9 µg/ml), comparable to parental GS. VK7 showed activity in the range of 7.8–31.2 µg/ml (median 15.6 µg/ml) against *E. cloacae, P. aeruginosa, K. pneumoniae*, and *A. baumanii*. The MIC values against all *P. aeruginosa* and *A. baumannii* strains and most *K. pneumoniae* strains are 2- to 8-fold lower for VK7 than the GS MIC values.

The β-turn variant 20 showed slightly reduced activity against *S. aureus* and *E. faecium* as compared to GS. MIC values for compound 20 were in the range of 7.8–62.5 µg/ml (median 7.8 µg/ml). Derivative 20 showed activity against *E. cloacae, P. aeruginosa, K. pneumoniae,* and *A. baumannii* in the range of 1.95–62.5 µg/ml (median 62.5 µg/ml), which is slightly less than measured for the parental compound.

Derivative 3 showed activity against *S. aureus* and *E. faecium* in the range from 7.8–62.5 µg/ml (median 7.8 µg/ml), which is slightly less than parental GS. Derivative 3 shows activity against the MDR Gram-negative strains *E. cloacae, P. aeruginosa, K. pneumoniae*, and *A. baumannii* in the range from 3.9–62.5 µg/ml (median 31.3 µg/ml), which is comparable to the parental compound. All compounds tested were bactericidal, as sub-culturing of medium from wells without visible growth on agar media did not result in detectable growth.

Hemolysis assay and therapeutic indices

Hemolysis is clearly concentration-dependant. Canonical GS showed 50 % hemolysis at 35.2 µg/ml (Fig. 3a). As the MIC values of GS varied from 3.9 to 62.5 µg/ml, the TIhem for GS was calculated to be between 0.56 and 18.5. Derivative 3 showed 50 % hemolysis at 41.6 µg/ml (Fig. 3a), which is in a similar range as for the parental compound. As derivative 3 had MIC values varying from 3.9 and 62.5 µg/ml, the TIhem of derivative 3 was calculated to be 0.6 to 10.6, also comparable to the parental compound. This shows that derivative 3 is not an improved antibiotic in comparison with GS. VK7 and compound 20 did not show hemolysis at 62.5 µg/ml, which was the highest concentration tested. Hence, the exact TIhem for compound 20 and VK7 could not be determined, but still, these derivatives are clearly less hemolytic than GS (Fig. 2).



LDH release assay and therapeutic indices

The values of 50 % LDH release were 18.7 µg/ml for GS (Fig. 3b). As the MIC values were between 3.9 and 62.5 µg/ml, TILDH was calculated to be between 0.3 and 9.6. Derivative 3 showed 50 % LDH release at 49.8 µg/ml. As the MIC values were between 3.9 and 62.5 µg/ml, TILDH was calculated to be between 0.8 and 12.8, in the same range as documented for GS. Derivate 20 showed 50 % LDH release at 62.5 µg/ml. As MIC values were between 1.95 and 62.5 µg/ml, TILDH was calculated to be between 1 and 32.1 µg/ml, which shows slight improvement compared to GS. VK7 did not reach 50 % LDH release at concentrations tested here. Therefore, no TILDH could be calculated, indicating again that VK7 is less cytotoxic than GS (Table 1).

DISCUSSION

Beta-strand modification of GS seemed to be promising for the development of new systemically applicable antibiotics. Derivative VK7 showed activity against *E. cloacae* which was equal to that of GS. Against all *P. aeruginosa* and *K. pneumoniae* and most *A. baumannii* there was a 2–8-fold increase in activity. The antimicrobial activity of VK7 against Gram-positive MDR pathogens such as *S. aureus* and *E. faecium* was similar to that of GS. In addition, we observed reduced toxicity for VK7 towards human erythrocytes and the human colorectal adenocarcinoma cell-line HT-29. The β- strand-modified VK7 has the same overall secondary structure as GS, but probably displays an elevated cationic character counterbalanced by two robust hydrophobic adamantane groups. The data indicate that β-strand modification of GS can generate interesting new antibiotics combining antimicrobial activity and lowered toxicity.

The β-turn-modified derivative 20 showed reduced toxicity compared to the parental compound, with a slight decrease in antibiotic activity compared to GS, especially when used against Gram-negatives. This β-turn-modified derivative encompasses a substituted sugar amino acid (SAA) dipeptide isoster as turn mimetic. The six-ring SAA in our lead was found to have better conformational and hydrophobic characteristics than a 4-ring (oxetane) and 5-ring (furanoid) SAA [9]. Beta-turn modification could still be promising, as at least some reduction of toxic potential is observed. Not all modifications of GS have a positive effect on antimicrobial activity and toxicity: derivative 3 shows comparable antimicrobial activity to the parental compound but a similar toxicity profile.

The synthesis of modified GS derivatives has been reported by other groups [8, 12, 15, 17]. One of the key factors important in the balance between cytotoxicity and antimicrobial activity is overall hydrophobicity–hydrophilicity. Derivatives that are slightly less hydrophobic than the parental GS generally show good antimicrobial activity, while showing reduced hemolysis. Using solid- and liquid-phase organic synthesis, derivatives of GS can be obtained containing non-naturally occurring amino acids, which show reduced cytotoxicity and reduced hemolysis, while retaining antimicrobial activity.

Antimicrobial peptides may have a bright future in combating infection, as they generally do not have a single conserved target, but affect multiple bacterial processes. Modifications of the β-strand of GS in which the hydrophobic side chains have been varied are promising leads for the development of novel compounds. New derivatives of GS can possibly address the growing problem of multi-drug resistant bacteria and lead to new therapeutic compounds for systemic use, as is suggested on the basis of our current data.

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Chapter 7

Discussion and Summary

Infectious diseases remain a major burden on human health [53], despite many previous breakthroughs in various fields such as treatment, prevention and vaccination [54, 55]. This thesis focuses on the pathogen *Staphylococcus aureus*, which causes a broad range of infectious diseases: from acute bacteremia [56] to chronic osteomyelitis [57], from superficial skin infections [58] to deep-seated endocarditis [59] and from relatively mild abscesses [60] to life threatening pneumonia [61]. *S. aureus*, especially the methicillin resistant *S. aureus* variant MRSA and the emerging vancomycin resistant mutants [1-4], have complicated treatment of *S. aureus* infection. Infection is thus an increasing burden on human health [10-12]. It is, therefore, essential to develop new interventions against this pathogen. By gaining new insights in bacterial pathogenesis, novel interventions may be devised, including anti-staphylococcal vaccination and immunotherapy. Also, these insights might lead to the technology whereby bacterial behavior *in vivo* can be altered into a less pathogenic mode. Furthermore, antibiotics with new modes of action must be found to expand our options for applying conventional treatment approaches.

More insight into host pathogen interactions between *S. aureus* and humans, the primary aim of this thesis, was gained by studying: (I) how, in humans with different types of interactions with *S. aureus,* their humoral immune system responds to known staphylococcal virulence factors, (II) the (kinetics of) production of virulence factors during biofilm formation, and (III) the link between (the formation of) biofilm and persistence of infection in patients suffering from staphylococcal bacteremia. The second aim of this thesis was to find new leads for antibiotics with novel modes of action directed against infections with multi-drug resistant ESKAPE pathogens. Actinomycetes, the origin of up to 60% of all clinically available antibiotics, were induced to produce novel compounds by culturing strains from remote locations in various laboratory conditions. Furthermore, gramicidin S was used as a scaffold to design new derivatives by adding non-naturally occurring amino-acids to attain reduced toxicity profiles, while retaining antimicrobial activity.

MAIN FINDINGS

In this thesis, we studied humoral immune responses directed against *S. aureus* virulence factors produced *in vivo*. In **Chapter 2** we studied IgG, as it is the most common isotype of antibody present in blood. IgG occurs in different subclasses, each with their own unique function *in vivo* [17, 18]. Importantly, we have found IgG4 responses against a broad panel of *S. aureus* derived immune modulators. IgG4 responses, which are indicative of repeated and prolonged exposure [18], were found in all human sera against multiple *S. aureus* components. However, when we tested antibody responses against 40 *S. aureus* virulence factors, we found these IgG4 responses only against a restricted

panel of 17 virulence factors: alpha toxin, CHIPS, ETA and B, HlgB, IsdA, LukD, -E, -F and -S, SCIN, SEC, SSL1, 3, 5 and 9 and TSST-1. These staphylococcal products are known to interact with and even counteract the human immune system on many levels. Among the innate immune responses that are affected are chemotaxis (by CHIPS), extravasation (by SSL3 and SSL5), complement activity (by SCIN) and TLR2 signaling (by SSL3). Neutrophils themselves are targeted by the γ-hemolysin family (HlgB, LukD, E, F and S) and alpha toxin lyses mononuclear immune cells and platelets. SSL9 binds to monocytes and dendritic cells [19], but its exact function remains unknown. SSL1 is a potent inhibitor of neutrophil matrix metalloproteinases (MMP's), which are endopeptidases that degrade components of the extracellular matrix of human tissues, but also modulate inflammation [5]. SEC and TSST-1 modulate adaptive responses by non-antigen directed binding of MHC II with T cell receptors, resulting in polyclonal T cell activation [20]. Interestingly, IgG4 responses to these antigens were found to be dependent on the type of host pathogen interaction and/or on the carrier status of the individuals at the time they were sampled. We found IgG4 responses to be most commonly directed against immune modulators secreted by *S. aureus*, indicating that we as humans are repeatedly and perhaps chronically exposed to these virulence factors; most IgG4 responses were measured in sera from patients suffering from Epidermolysis Bullosa (EB). IgG4 class antibodies are correlated with the induction of tolerance in the framework of allergy therapies and with repeated or chronic exposure for other reasons [17, 23]. We, therefore, hypothesize that humans interact more extensively and repeatedly with *S. aureus*, than currently appreciated. This might mean that we need to adapt our view of interactions between *S. aureus* and humans, in either disease or carriage states. This concept may have implications for the development of effective immunotherapy, such as (active and passive) vaccination and immune modulation interventions in the treatment of *S. aureus* infections.

S. aureus does not only escape clearance by the immune system by producing immune modulators, but it also produces biofilm. Reportedly, 80% of all infections are biofilm related [24]. Late in the maturation phase of biofilms, staphylococcal cells disperse from the biofilm and seeding of distant sites may occur, together with the production of the immune modulators stated above [25, 26]. This leads to the question: how does *S. aureus* protect itself during the early stages of biofilm formation, if immune modulators are reportedly only produced late in biofilm formation? In **Chapter 3** we show that some immune modulators are produced and secreted earlier than currently reported, and that they are produced for much longer periods of time. Our experiments with biofilm formation and virulence factor production were performed in the eukaryotic cell culture medium IMDM. Growing bacteria in eukaryotic cell culture media is a strategy followed by several other research groups [6, 7], as *S. aureus* has previously been reported to behave differently in eukaryotic medium compared to prokaryotic cell culture media.

We hypothesized that using eukaryotic cell culture medium mimics the situation bacteria will encounter in a human body better compared to using prokaryotic cell culture medium. We have used IMDM for experiments in **Chapter 3 and 4**. Most model systems use less well-defined bacterial growth media, that do not resemble the internal milieu of the human host at all. Bacterial cell culture media are also not constant in composition, they can vary from batch to batch, as their composition is not completely chemically defined. Better model systems are required to mimic the *in vivo* situation. The immune modulators we found in early biofilms are mainly directed against the early innate immune system: CHIPS, SCIN and FLIPr. SCIN modulates C3b complement deposition and thereby C3b mediated opsonophagocytosis [27]. CHIPS binds to the C5a receptor and formylated peptide receptor inhibiting neutrophil chemotaxis [28]. FLIPr is a a potent FcγR antagonist. Using our model system we provided evidence for the production of these immune modulators in the beginning stages of biofilm formation, an area not previously researched. Further study in this direction may lead to better understanding of the process of virulence factor production during early versus late biofilm formation, facilitating a better understanding of the first phase of biofilm formation and possibly new (or better timed) treatment options.

Biofilm is seen in many different types of infection, and it plays an important role in resisting clearance. *S. aureus* in a biofilm is much less sensitive to clearance by the immune system and to antibiotics compared to when it is growing in a planktonic form [24]. Biofilm hinders clearance by facilitating the trapping and physical exclusion of antibiotics and immune cells and proteins, in addition to secreting immune modulators (**Chapter 3**). Furthermore, a biofilm consists of bacterial cells in various metabolic states, reportedly making them heterogeneously sensitive to certain classes of antibiotics [30]. Persistence is seen in many infections, and causes significant concern in patients with *S. aureus* bacteremia; even when patients do receive adequate antimicrobial therapy, symptoms of invasive infection may last for prolonged periods of time and 10-20% of all patients die. The Infectious Diseases Society of America (IDSA) has produced a guideline that advises 6-8 weeks of intravenous antibiotic therapy for patients with complicated SAB (defined as patients that have positive blood cultures for more than 72 hours under appropriate therapy), and 2-4 weeks of therapy for patients with uncomplicated SAB (when the patient's blood becomes culture negative within 72 hours of appropriate therapy) [31]. Rapid detection of the type of SAB is thus essential for treating patients correctly, but by definition, this takes up to 72 hours after treatment initiation to establish or refute. In **Chapter 4,** we searched for bacterial and host factors that could predict complications at an earlier stage. Strains causing complicated SAB, when cultured in the eukaryotic cell culture medium IMDM and in the presence of 1 x MIC of vancomycin, produced more biofilm than strains causing uncomplicated SAB. It was described by Pietiäinen et al. [8], that vancomycin induces transcriptional responses, especially the

up-regulation of the cell wall stress stimulon VraSR, that are similar to the transcriptional responses to stresses induced by human derived antimicrobial peptides [32]. Thus, the effect of treatment with antibiotics in our *in vitro* experiments may actually resemble the effects of natural exposure to host-derived antimicrobial peptides. Taken together, we hypothesize that the ability of certain strains of *S. aureus* to maintain biofilm production under stress conditions encountered *in vivo*, including stresses exerted by antimicrobial therapy, could explain, at least in part, the persistence of bacteremia in *S. aureus* infected patients. This direction of a more systems-based mode of research by combining media and antibiotics to better mimic the *in vivo* situation deserves more attention since it could possibly yield a better *ex vivo* model for staphylococcal infection. Such a model could also give more insight into the pathogenesis of *S. aureus* infections and possibly explain why biofilm is seen in so many infections: irrespective of the antibiotics actually used in clinical practice, the driving force behind biofilm formation could actually be our own immune system. Testing the infecting strains ability to form biofilm under vancomycin pressure in IMDM may be a rapid ascertainment of the probability that a SAB will be complicated or not, and so help guide clinical management of SAB.

For the treatment of infectious diseases, antibiotics are still the mainstay and will remain so in the foreseeable future. A worrying trend seen in the treatment of infectious disease is the ever-increasing bacterial resistance to antibiotics. Not only is there currently extensive antibiotic resistance in our clinics, the IDSA is concerned that some pathogens are well on their way to become multi- or totally drug-resistant. These are ***E****nterococcus spp. [33, 34]*, ***S****taphylococcus aureus [10-12]*, ***K****lebsiella spp. [35, 36]*, ***A****cinetobacter baumannii* [37, 38]*,* ***P****seudomonas aeruginosa [39, 40]* and ***E****nterobacter spp*. [41, 42]. They have coined the acronym ESKAPE-pathogens. New therapeutic options are thus of the greatest importance. In **Chapter 5** we have induced actinomycetes, already known to produce up to 60% of all antibiotics used in clinical practice, to produce novel compounds. By growing these actinomycetes in non-standard culture conditions, they started producing interesting molecules that can potentially be developed into medicines. We are currently only at the beginning of this approach, and estimates of microorganisms that can be cultured vary widely: in our work, only culturable actinomycetes are used for research and induction purposes. Here, we show that the vast majority of tested actinomycetes can be induced to inhibit the growth of *S. aureus*. Many of the actinomycetes that produced antibiotics against one pathogen under a certain growth condition did not inhibit another, and *vice versa*, showing that choosing the correct culture condition is key. Of the various conditions tested, alkalinity (pH 10) appears to be promising for eliciting antibiotic production. The highly differential sensitivity of the various MDR ESKAPE pathogens to the antibiotics produced by the actinomycetes tested here underlines that an important strategy may be to search for drugs that target a specific pathogen.

In **Chapter 6** we focus on gramicidin S, a well-known and old antibiotic, that has not reached clinical practice for systemic usage due to its toxicity profile in humans [43-45]. By using gramicidin S as a scaffold, new derivatives were designed that have a different mode of action than classically used antibiotics [43-45]. Not all modifications of GS have a positive effect on antimicrobial activity and toxicity: one derivative showed comparable antimicrobial activity to the parental compound but had decreased toxicity towards eukaryotic cells. New derivatives of existing antimicrobial peptides may also have a future in combating infectious disease, as they generally do not have a single conserved target, but affect multiple bacterial processes and can possibly address the growing problem of multi-drug resistant bacteria and lead to new therapeutic compounds for systemic use.

MAIN CONCLUSIONS:

1: The finding of IgG4 responses against a range of *S. aureus* virulence factors indicate repeated and prolonged exposure to *Staphylococcus aureus*, much more extensively so than previously appreciated. These 17 virulence factors are alpha toxin, CHIPS, ETA and B, HlgB, IsdA, LukD, -E, -F and -S, SCIN, SEC, SSL1, 3, 5 and 9 and TSST-1.

2: In patients suffering from Epidermolysis Bullosa IgG4 antibodies were found directed against a wider range of virulence factors than carriers, and in carriers IgG4 antibodies were found directed against a wider range of virulence factors than non-carriers, showing a descending order of the frequency of (immunological) interaction between *S. aureus* and its human host.

3: *Staphylococcus aureus* often forms biofilms. SCIN, CHIPS and FLIPr are important in the immune evasion of *S. aureus* in the early phase of biofilm formation. We humans are exposed to SCIN, CHIPS and FLIPr repeatedly and for prolonged periods of time.

4: Strains causing complicated SAB produce more biofilm than strains causing uncomplicated SAB when grown in eukaryotic cell culture medium in the presence of vancomycin.

5: Actinomycetes can be induced to produce novel antibiotics against MDR-ESKAPE pathogens, especially when grown in alkaline environments.

6: Modifications of the β-strand of GS, in which the hydrophobic side chains are modified provide promising leads for the development of compounds that are safer when compared to the parental compound, while retaining antimicrobial activity comparable to the parental compound.

PERSPECTIVES ON *S. AUREUS* RESEARCH

No clear antibody response profile could be linked to the site or type of exposure to *S. aureus* (**Chapter 2**). This indicates that each host *S. aureus* interaction may have a unique virulence profile, which does not bode well for the prospect of vaccination and other immune based therapies. Patterns of IgG4 response varied extensively between volunteers, indicating that each person is exposed to different virulence factors. The different exposure to virulence factors could be explained by the fact that various genetic backgrounds of *S. aureus* contain different sets of virulence factors. Variation may also be due to differences in regulators and/or gene expression in various *S. aureus* strains [46-49]. Further complicating this host pathogen interaction study is the fact that hosts may show different humoral responses. Host IgG4 responses were predominantly directed against secreted immune modulators of *S. aureus*, a constant finding since the start of measuring such antibodies [13-16, 50]. We hypothesize that host *S. aureus* interactions occur extensively and repeatedly and are even more varied than currently appreciated, regardless of disease state or carriage status. As IgG4 is indicative of repeated and prolonged exposure, we may be currently underestimating the interaction that occurs between the human host and the opportunistic pathogen *S. aureus* (**Chapter 2**). This might have major implications for research on host-pathogen responses *in vivo* and for the development of immuno-therapeutic strategies such as active vaccination. Patients suffering from EB are often chronically colonized with high densities of *S. aureus* while not frequently suffering from invasive *S. aureus* infections such as bacteremia, leading to the most responses against *S. aureus* virulence factors. This observation could be interesting for further vaccination research: as IgG4 antibodies are produced mainly after repeated exposure, inducing them might require prolonged and repeated exposure.

Our experiments with virulence factor production (**Chapter 3**) and biofilm formation (**Chapter 4**) were performed in an eukaryotic cell culture medium (IMDM). Growing bacteria in eukaryotic cell culture media is a strategy followed by several other groups, as *S. aureus* behaves differently in prokaryotic medium compared to eukaryotic cell culture media [51, 52]. In eukaryotic growth media, which are relatively devoid of iron, *S. aureus* grows slower and produces much larger amounts of iron-regulated surface proteins such as IsdB, in comparison to TSB grown bacteria [52]. Conditions *in vivo* may well differ from those in laboratory media and we suggest that using eukaryotic cell culture medium better mimics the *in vivo* situation and indicates a good direction for future research. Although much is known about the production of virulence factors of mature biofilm, our knowledge is far from complete concerning early biofilms; we are still far from a complete insight into the kinetics of immune modulator production during the formation of biofilms. Other secreted virulence factors may also play a role in the escape from immune clearance, and the previously identified virulence factors warrant further

research. The results generated here are a stepping stone towards elucidating the role of virulence factors during biofilm formation, and gives a method to screen and determine which factors are important in pathogenesis *in vivo*. This test system was further used for studying the production of biofilm in the context of staphylococcal bacteremia (**Chapter 4**). Complicated infections persist much longer than uncomplicated infections, and we researched if this difference could be caused by biofilm formation. We found that strains causing complicated SAB produce more biofilm than strains causing uncomplicated SAB when they were grown in IMDM with vancomycin. As vancomycin induces similar responses to host defense peptides [9], our finding may indicate that human immune system is actually the driving force in the production of biofilm. This cell wall stress could be used by bacteria as a signal that they are "inside the host" and in response to these signals they might alter their behavior, either by producing virulence factors and/or by forming biofilm. Taken together, we hypothesize that the ability of certain strains of *S. aureus* to maintain biofilm production under stress conditions encountered *in vivo*, including that exerted by antimicrobial therapy, could explain, at least in part, the observed persistence of bacteremic infection in our cohort of patients. This observed persistence does seem related to an aggregated form of life and sites of infection that are difficult to treat, such as endocarditis and osteomyelitis, which are associated with complicated form of SAB. These findings also highlight the importance of taking into account not only the host, but also the bacterium and finally the antibiotic therapy used when researching infectious diseases. Several avenues of research in this direction could be: the addition of components of the immune system, such as complement, serum, plasma and / or neutrophils (or other cells of the immune system) to growing bacteria or biofilms at time points when the bacteria would encounter these components *in vivo* during infection. Also, growing biofilms in flow conditions is an interesting route of new research that warrants future research, especially in the context of *S. aureus* bacteremia.

After primarily focusing on the bacterium and the host in the first half of this thesis, antibiotics take center stage in the second half of this work. Here we focus mainly on generating new leads for antimicrobials. New strategies in the treatment of infectious disease should follow a more targeted approach and find treatment options that are highly selective. Instead of broad spectrum antibiotics, more focused antibiotics could be useful, which only act on a sample of bacterial species. This would further necessitate the rapid identification of the pathogen responsible for disease. This route was followed in **Chapter 5**, where we researched actinomycetes that were culturable in laboratory setting, and found several likely leads, which were highly specific. Future study should be directed towards cultivating actinomycetes more in their natural habitat, which could lead to further new compounds. The culturable micro-organisms form the tip of the iceberg, with many strains not being researched as they cannot be cultivated yet.

By better culture methods [10] and better mimic systems, more biologically relevant findings will be generated.

Instead of targeted treatment, more broad-spectrum antibiotics remain useful, especially in empirical therapies. **Chapter 6** focuses on one of these: gramicidin S has a very broad spectrum and targets the cell membrane of bacteria. Furthermore, gramicidin S works well against a wide spectrum of gram positive bacteria, and is slightly less effective against gram negative bacteria. As antimicrobial peptides generally do not have a single conserved target, but affect multiple bacterial processes, they could be very useful: resistance against such antimicrobials is slow and difficult to evolve, possibly allowing long usage of such therapeutics. We show here that β-strand modification of GS could generate interesting new antibiotics combining high antimicrobial activity and lowered toxicity and are thus promising leads for the development of novel compounds.

In conclusion, to remain able to treat *S. aureus* infections in the future we require a two-pronged approach:

1: We need to know more about how *Staphylococcus aureus* actually causes different types of infection, so that we may be able to modulate and interfere with pathogenic behavior, either by vaccination or by alternative therapies.
2: We need to continue searching for novel antibiotics: as soon as a new compound is approved for usage in humans, resistance against that compound occurs, necessitating even newer antimicrobials.

Combining these two avenues of research remains essential to help future health care patients; especially the elderly, the hospitalized patient population that routinely requires antibiotic prophylaxis to remain free of infections, the ever increasing group of immunocompromised patients (HIV /AIDS, diabetics, transplanted persons) and other patients in the hospital. If we keep studying these 2 subjects, and research them in tandem, we may be able to stave off a post antibiotic era, in which infections can be rapidly and effectively diagnosed but no longer treated.

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Chapter 8

Nederlandse samenvatting

Infectieziekten blijven een groot effect op menselijke gezondheid hebben, ondanks doorbraken in meerdere gebieden zoals behandeling, preventie en vaccinatie. Dit proefschrift heeft als hoofdonderwerp de opportunistische pathogeen *Staphylococcus aureus*, die een breed scala aan ziektes veroorzaakt zowel in de algemene bevolking als in ziekenhuizen: van acute bacteriemie, tot chronische osteomyelitis, van oppervlakkig huidinfecties tot diepgelegen endocarditis. Het opduiken van meticilline-resistente varianten van *S. aureus* (MRSA) hebben de behandeling van deze infecties verder bemoeilijkt. MRSA-infecties worden veelal met vancomycine behandeld, maar ook tegen vancomycine blijken dergelijke stammen resistent te kunnen worden. MRSA infecties geven daarmee een toenemende belasting van de gezondheidszorg. Het is daarom essentieel om nieuwe therapieën tegen deze verwekker te ontwikkelen. Door nieuwe inzichten in de bacteriële pathogenese kunnen nieuwe interventies ontwikkeld worden, zoals vaccinatie en immunotherapie. Mogelijk leiden deze inzichten ook tot interventies, waarbij het gedrag van *S. aureus in vivo* aangepast kan worden zodat ze minder of geen schade aan de gastheer, en daardoor ziekte, veroorzaken. Daarnaast moeten antibiotica met nieuwe werkingsmechanismen gevonden worden, om het arsenaal aan behandelingsmogelijkheden uit te breiden.

Er is met het onderzoek beschreven in dit proefschrift meer inzicht verkregen in de interactie tussen *S. aureus* en de menselijke gastheer: op de eerste plaats is onderzocht hoe, in mensen met verschillende *S. aureus* interacties, het humorale immuunsysteem reageert op reeds bekende *S. aureus* virulentie factoren. Daarnaast is onderzocht hoe de productie van virulentie factoren tijdens de vorming van biofilm verloopt, en tenslotte is onderzocht of er een verband bestaat tussen de vorming van biofilms en aanhouden van *S. aureus* bacteriemie. Het 2^{e} doel van het onderzoek beschreven in dit proefschrift was het vinden van antibiotica met nieuwe werkingsmechanismen, die werkzaam zijn tegen multiresistente varianten van bacteriën uit de zogenoemde ESKAPE groep micro-organismen; die ESKAPE groep bestaat uit de bacteriegeslachten en -soorten *Enterococcus spp., Staphylococcus aureus, Klebsiella spp., Acinetobacter spp., Pseudomonas spp.* en *Enterobacter* spp., die veel infecties veroorzaken en vaak antibioticaresistent zijn. Hiertoe werden Actinomyceten, de bron van ongeveer 60% van al onze klinisch gebruikte antibiotica, geïsoleerd uit exotische locaties en vervolgens gestimuleerd om nieuwe metabolieten met antibiotische werking te produceren. Daarnaast is het gramicidine S molecuul als uitgangspunt gebruikt om nieuwe derivaten te ontwikkelen met niet natuurlijk voorkomende aminozuren met als doel het behouden en vergroten van antibiotische activiteit en het verkleinen van de toxiciteit.

VOORNAAMSTE BEVINDINGEN

In dit promotieonderzoek heb ik de humorale immuunrespons bestudeerd gericht tegen *S. aureus* virulentie factoren die geproduceerd werden *in vivo*. In **hoofdstuk 2** hebben we IgG bestudeerd, omdat dat het meest voorkomende isotype immuunglobuline in bloed is. IgG heeft verschillende subtypen, elk met een eigen functie *in vivo*. Ik heb IgG4 responsen gevonden tegen een breed scala aan *S. aureus* virulentie factoren. IgG4 responsen wijzen in het algemeen op herhaalde en langdurige blootstelling aan een immunogeen. IgG4 gericht tegen *S. aureus* componenten werden in alle menselijke sera gevonden, en zij waren gericht tegen meerdere, maar niet alle onderdelen van *S. aureus*. Wij vonden IgG4 responsen gericht tegen 17 van 40 verschillende virulentie factoren die werden onderzocht; dat waren alfa-toxine, CHIPS, ETA en B, HlgB, IsdA, LukD, -E, -F en -S, SCIN, SEC, SSL1, -3, -5 en -9 en TSST-1. Van deze virulentie factoren is bekend dat ze op meerdere niveaus reageren met het immuunsysteem, c.q. de afweer van de mens tegen infecties tegenwerken. Belangrijke afweermechanismen die door deze virulentiefactoren beïnvloed worden zijn de chemotaxis van witte bloedcellen (CHIPS), de extravasatie van witte bloedcellen (SSL3 en 5), complement activatie (SCIN), en TL2 signalering (SSL3). Neutrofiele granulocyten zijn gevoelig voor de toxische werking van *S. aureus* gamma hemolysines (HlgB, LukD, E, F en S) en alfa-toxine grijpt aan op mononucleaire cellen en bloedplaatjes. SSL9 bindt aan dendritische cellen en monocyten, maar de exacte functie van dit eiwit is onbekend. SSL1 is een sterke remmer van metallo-proteases en endopeptidases van de granulocyten, die een rol spelen in ontsteking. SEC en TSST-1 grijpen aan op de verkregen afweer door niet-antigen gerelateerde binding van MHC-2 receptoren op T-cellen, wat resulteert in polyclonale T-cel activatie. De gemeten IgG4 responsen bleken afhankelijk van de type interactie tussen *S. aureus* en de menselijke gastheer. Daarnaast speelde de dragerschapsstatus van de individuen ten tijde van serumafname een rol. De gemeten IgG4 responsen waren vooral gericht tegen virulentiefactoren van *S. aureus*, die door de bacterie worden uitgescheiden. Deze waarnemingen geven aan dat mensen langdurig en herhaaldelijk blootgesteld worden aan deze virulentie factoren. De meest uitgebreide IgG4 responsen werden gevonden in het bloed van patiënten die lijden aan de ziekte Epidermolysis bullosa. Omdat IgG4 responses gepaard gaan met de inductie tolerantie in de behandeling van allergie en met herhaalde en langdurige blootstellingen voor andere redenen, veronderstellen wij dat mensen in het algemeen langer en vaker met *S. aureus* in aanraking komen dat tot nu gedacht werd. Dit betekent dat we onze kijk op de interactie tussen *S. aureus* en de mens enigszins bij moeten stellen, zowel met betrekking tot *S. aureus* dragerschap als met betrekking op infecties veroorzaakt door *S. aureus*. Dit concept kan ook gevolgen hebben voor het ontwikkelen van (passieve of actieve) vaccinaties en andere immuun-modulerende behandelingen van *S. aureus*.

S. aureus ontsnapt niet alleen aan het immuunsysteem door het produceren van immuun modulerende virulentiefactoren, maar ook door het produceren van biofilms. In de wetenschappelijke literatuur worden biofilms van belang geacht bij ongeveer 80% van alle infecties. Vast staat dat eenmaal volgroeide biofilms cellen kunnen loslaten die uitzaaien en elders in het lichaam secundaire haarden (zogenaamde strooihaarden) kunnen vormen; daarbij produceren volgroeide biofilms veel van de eerder genoemde immuun-modulerende virulentiefactoren waarmee ze zich beschermen tegen de afweer van de gastheer. Of dergelijke virulentie factoren ook in de vroege fase van biofilmvorming geproduceerd worden was onduidelijk. Dit leidde tot de volgende vraag: hoe beschermt *S. aureus* zich tijdens de vroege fases van biofilm vorming tegen de gastheer afweer, als immuun-modulatoren alleen maar tijdens de late fase van biofilm vorming worden geproduceerd. In **hoofdstuk 3** laten we zien dat bepaalde immuun-modulatoren eerder gemaakt worden dan tot nu toe werd aangenomen, en dat ze daarnaast langer geproduceerd worden dan eerst werd gedacht. Onze experimenten met de vorming van biofilm werden uitgevoerd in het eukaryote celkweek medium IMDM. Het kweken van *S. aureus* in eukaryoot medium is een strategie die gevolgd wordt door meerdere onderzoeksgroepen, omdat er aanwijzingen zijn dat *S. aureus* zich anders gedraagt in eukaryoot dan in prokaryoot celkweek medium. We stelden de hypothese dat de eukaryote kweekcondities een beter *in vitro* equivalent zijn van de situatie die de bacteriën *in vivo* tegen zullen komen dan de prokaryote media die tot nu toe veelal gebruikt worden. Wij hebben dit eukaryoot medium gebruikt in **hoofdstuk 3 en 4**. De meeste andere model systemen gebruiken chemisch ongedefinieerde prokaryote kweekmedia, die niet overeenkomen met de samenstelling van het interne milieu van de mens. Daarnaast zijn deze prokaryote kweekmedia niet constant van samenstelling en kunnen er aanzienlijke verschillen tussen batches zijn, omdat de chemische samenstelling niet vastgelegd is. Er zijn betere *in vitro* modelsystemen nodig om de *in vivo* situatie na te bootsen.

De immuun modulatoren die wij vonden in jonge biofilms (CHIPS, SCIN en FLIPr) zijn vooral gericht tegen het aangeboren immuunsysteem. SCIN moduleert C3b depositie en heeft daardoor een effect op C3b gemedieerde fagocytose. CHIPS remt de chemotaxis van neutrofielen. FLIPr is een sterke antagonist van de receptor voor Fc-gamma. In ons modelsysteem hebben we bewijs gevonden voor de productie van deze immuunmodulatoren in de vroege fase van biofilmvorming, een tot op heden weinig bestudeerde fase. Verder onderzoek in deze richting zal meer inzicht leveren in de productie van virulentie factoren in de vroege versus late fases van biofilmvorming. Mogelijk leidt een beter begrip van de vroege fases van biofilm tot nieuwe (of beter getimede) behandelmogelijkheden.

Biofilms worden gezien in vele soorten infecties, en spelen een belangrijke rol in de overleving van micro-organismen. *S. aureus* cellen zijn veel minder gevoelig voor het immuunsysteem en voor antibiotica als ze in een biofilm groeien dan wanneer ze planktonisch groeien. Biofilms werken de behandeling van infecties tegen door het wegvangen en het uitsluiten van antibiotica en gastheer componenten (**hoofdstuk 3**). Daarnaast bestaat biofilm uit bacteriële cellen in verschillende metabole toestanden, waardoor ze veelal verminderd gevoelig zijn voor bepaalde antibiotica, vooral die antibiotica die aangrijpen in het delingsproces zoals beta-lactam antibiotica. Persistentie van een infectie wordt vaak gezien en is bijzonder zorgwekkend in het geval van bacteriemie, waarbij levende bacteriën dagenlang in de bloedbaan kunnen blijven circuleren, zelfs als patiënten de juiste antimicrobiële therapie ontvangen. In dergelijke gevallen kunnen symptomen van invasieve infectie aanhouden en de sterftekans bij een *S. aureus* bacteriemie is dan ook nog steeds 10-20% De Infectious Diseases Society of America raadt in geval van gecompliceerde bacteriemie (gedefinieerd als onder de juiste therapie toch meer dan 72 uur positief blijvende bloedkweken) tussen de 6 tot 8 weken intraveneuze therapie aan, terwijl 2 tot 4 weken intraveneuze therapie volstaat voor patiënten met een ongecompliceerde bacteriemie. Snelle vaststelling van de complicatie status is dus essentieel voor de juiste behandeling van patiënten, maar per definitie duurt het minstens 5-7 dagen om door middel van dagelijks afgenomen bloedkweken vast te stellen wat die status is. In **hoofdstuk 4** zochten we naar gastheer en bacteriële factoren die bij een bacteriemie een gecompliceerde status konden voorspellen in een vroeger stadium. Stammen die gecompliceerde bacteriemie veroorzaakten, als ze gekweekt worden in het eukaryote celkweek medium IMDM in aanwezigheid van het antibioticum vancomycine bleken significant meer biofilm te produceren dan stammen die een ongecompliceerde bacteriemie veroorzaakten. Vancomycine induceert bepaalde transcriptionele responsen, in het bijzonder een verhoogde afschrijving van het celwand stress operon VraSR, een fenomeen dat overeenkomt met de responses die geïnduceerd worden wanneer *S. aureus* blootgesteld wordt aan antimicrobiële eiwitten van menselijke oorsprong. Wij hypothetiseren daarom dat het effect van behandeling met (bepaalde) antibiotica in onze *in vitro* experimenten zou overeen kunnen komen met de effecten van natuurlijke blootstelling aan gastheer afgeleide antimicrobiële eiwitten.

Uit dit onderzoek concluderen wij dat de mogelijkheid van bepaalde *S. aureus* stammen om biofilm te blijven produceren onder stress condities die ze *in vivo* tegenkomen, zoals bijvoorbeeld door antimicrobiële therapie, een verklaring zou kunnen zijn voor de persistentie van *S. aureus* bacteriemie. Deze vorm van onderzoek, waarbij er rekening gehouden wordt met zowel het effect van antibiotica als effecten van het kweekmedium, verdient naar onze mening meer aandacht: het zou mogelijk leiden tot een beter *ex vivo* model voor stafylokokkeninfecties. Zo'n model geeft meer inzicht in de pathogenese van *S. aureus* infecties, en zou mogelijk verklaren waarom biofilm in zoveel infecties wordt

gezien: ons eigen immuunsysteem is mogelijk de drijfveer voor bacteriën om bioflms te vormen, en niet de antibiotica die gebruikt in de klinische behandeling van de infectie. Het testen van de mogelijkheid van de infecterende stam om biofilm te vormen in IMDM onder vancomycine druk zou, indien bevestigd in grootschaliger nader onderzoek, een snelle manier kunnen zijn om vast te stellen of een *S. aureus* bacteriemie gecompliceerd zal verlopen of niet, en zo helpen met de klinische behandeling van *S. aureus* infecties.

In de behandeling van infectieziekten zijn antibiotica een zeer belangrijke pijler, en dat zullen ze in de nabije toekomst ook blijven. Een zorgwekkende ontwikkeling in de behandeling van infectieziekten is de immer toenemende resistentie tegen antibiotica. Niet alleen is er uitgebreide resistentie nu al aanwezig in de kliniek, mondiaal zijn er grote zorgen dat een aantal pathogenen volledig resistent tegen alle antibiotica zullen worden. Deze pathogene bacteriesoorten staan inmiddels bekend als de ESKAPE pathogenen (zie boven). Het ontwikkelen antimicrobiële middelen met geheel nieuwe werkingsmechanismen is daarom van het hoogste belang. In **hoofdstuk 5** hebben we daartoe Actinomyceten, al bekend als bron van ongeveer 60% van alle klinisch gebruikte antibiotica, gestimuleerd om nieuwe metabolieten te produceren met antimicrobiële eigenschappen. Voor deze inductie zijn de Actinomyceten op niet standaard media gekweekt, maar onder bijzondere condities. Het overgrote merendeel van deze Actinomyceten bleek in staat om stoffen te produceren die de groei van *S. aureus* remmen. Actinomyceten die de groei van een micro-organisme onder een bepaalde omstandigheid remden, remden deze groei niet onder een andere kweekcondities. Van de verscheidene kweekcondities die wij hier hebben onderzocht leek vooral een basisch milieu (pH10) ervoor te zorgen dat de Actinomyceten antibiotica produceerden. De differentiële gevoeligheid van verschillende ESKAPE pathogenen voor de metabolieten geproduceerd door de Actinomyceten geeft aan dat in de zoektocht naar nieuwe antibiotica we niet alleen breed spectrum antibiotica moeten zoeken, maar juist stoffen met een beperkt antimicrobieel spectrum kunnen vinden.

In **hoofdstuk 6** hebben we Gramicidine S onderzocht, een al lang bestaand antimicrobieel middel, dat niet in de kliniek gebruikt kan worden doordat het als bijwerking de rode bloedcellen oplost (hemolyse). We hebben in dit onderzoek het Gramicidine S molecuul als uitgangspunt gebruikt om nieuwe derivaten te maken die een ander aangrijpingspunt hebben dan de meeste antibiotica. Niet alle modificaties hadden een positief effect op de antimicrobiële activiteit en toxiciteit: één derivaat had een antimicrobiële activiteit die vergelijkbaar was met Gramicidine S, maar bleek een verhoogde toxiciteit voor eukaryote cellen te hebben. Nieuwe derivaten van bestaande antimicrobiële peptiden hebben wellicht een toekomst in de bestrijding van infectieziekten, omdat ze niet specifiek één doelwit hebben, maar vaak aangrijpen op meerdere processen, wat het voor bacteriën moeilijker maakt om er resistent tegen te worden.

Appendices

Dankwoord
Curriculum Vitae
List of publications
PhD portfolio

DANKWOORD

Welkom bij het meest gelezen stuk van mijn proefschrift. Of althans, het stuk wat mensen als eerste gaan lezen. Zonder jullie was het niet tot dit eindresultaat gekomen. Tot zover het kortste dankwoord in de geschiedenis van promoties.

Ik wil toch wat woorden tot een aantal mensen richten. Sta je er niet persoonlijk tussen? Dat is geen onwil, dat is de haast geweest om alles op tijd af te krijgen tussen het afronden van het schooljaar aan de Hogeschool, het emigreren naar Hong Kong en de duizend-en-één andere zaken die ik nu aan mijn hoofd heb. Daarnaast wil ik niet een al te klef dankwoord schrijven, en door eerder groepen te benoemen zie je niemand over het hoofd.

Ten eerste, professor doctor doctor Alex van Belkum: jij bent degene die mij deze promotieplek aangeboden hebt in oktober 2009. Daarnaast heb je na je vertrek bij de Medische Microbiologie en Infectieziekten nog op afstand begeleid en geholpen bij de publicatie van hoofdstuk 6. Zonder jou hulp en advies was het boekje er niet gekomen. Hartelijk dank voor je inzet en hulp.

Professor doctor Verbrugh, geachte Henri: na het vertrek van Alex heb jij het stokje overgenomen als promotor en mij begeleid richting de eindstreep. Zeer veel dank voor het doorlezen en managen van het geheel. Daarnaast heb je als moderator en manager van de promotie opgetreden, waarvoor ik je ook zeer veel dank schuldig voor ben. Zonder de maandelijkse meetings met jou erbij was het boekje ook zeker niet afgekomen.

Doctor Willem van Wamel, dank voor je niet aflatende focus op detail. Zonder jouw focus, inspanning en scherpte was het boekje niet tot stand gekomen en zeker niet in deze vorm. Ik heb je door de jaren heen als stevige sparringpartner leren kennen, en waardeer de vele discussies die we over *S. aureus* gehad hebben erg. Dank voor het naar een hoger plan tillen van dit boekje.

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Daarnaast zou ik ook de rest van de commissie willen bedanken voor hun tijd, aandacht en oppositie.

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je Rotterdamse nuchterheid en dank voor je altijd beschikbare hulp om iets last minute te organiseren. Ook na mijn aanstelling bij de Hogeschool heb ik met velen van jullie contact gehouden, en ik hoop dat verder te onderhouden vanaf de andere kant van de wereld.

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Maude, wife, little lover. The dog days are over. In a month I will join you in Hong Kong, and our adventure together can truly start there. Thank you for being patient with me (mostly): making me work through last summer really was the push this thesis needed, and I got it from you. You make me a better person, and I am lucky to be married to you. I love you to Hong Kong and back and can't wait to start our adventure together there. Je t'aime, mon PSdA.

CURRICULUM VITAE

Jasper Wybe Swierstra was born on the 27th of July 1984 in Atlanta, Georgia, the United Stated States of America. He completed his secondary education at the Gemeentelijk Gymnasium in Hilversum in 2002. He continued to study at the University Utrecht, where he attained a Bachelors degree in Biomedical Sciences in 2005 and wrote his bachelor thesis on the lama antibodies (VHH's), under the supervision of Dr. H. Adams and Dr. J. Stam. After a sabbatical of a year in the board of the student-society Biton in Utrecht as a vice-president, he continued his studies and started his Masters degree in Immunity and Infection at the Utrecht University. There he completed a 9 month internship at the department of Virology at the department of Veterinary Medicine researching toro-viruses under the supervision of Dr. R. de Groot. He then performed a 6 month internship at the department of Molecular Immunology at the Wilhelmina's Children Hospital focussing on the role of FoxP3 acetlyation under the supervision of J. van Loosdregt and Prof. dr. P. Coffer. To attain his degree he wrote a thesis on the interaction between TLR-9 and flagellin under the supervision of B. Bardoel and Prof. dr. Jos van Strijp. Following his masters degree, he started his PhD project on finding novel antibiotics with truly novel modes of action, funded by the Dutch technology foundation Stichting Toegepaste Wetenschappen (STW) at the department Medi cal Microbiology and Infectious Disease of the Erasmus Medical Centre in Rotterdam, under the supervision of Dr. W. van Wamel, Prof. dr. dr. A. van Belkum and Prof. dr. Henri Verbrugh. In August 2014 he started teaching at the University of Applied Sciences of Rotter dam, where he currently still teaches microbiology, immunology and specializes in training technicians in microbiological diagnostics.

LIST OF PUBLICATIONS

Author:

Eliciting antibiotics active against the ESKAPE pathogens in a collection of actinomycetes isolated from mountain soils
Microbiology. 2014 Aug;160(Pt 8):1714-25

IgG4 subclass-specific responses to *Staphylococcus aureus* antigens shed new light on host-pathogen interaction
Infect Immun. 2015 Feb;83(2):492-501

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Eur J Clin Microbiol Infect Dis. 2016 May;35(5):763-9

Staphylococcus aureus immune modulators SCIN and CHIPS are produced during the early stages of biofilm formation
Manuscript submitted to Immunity and Infection, under review

Bacterial determinants of persistent Staphylococcus aureus bacteremia
Manuscript submitted

Editing:

Over Drank, Meneer Wateetons, 2016
Over Rot, Meneer Wateetons, 2015
Over Worst, Meneer Wateetons & Sjoerd Mulder 2016

PHD PORTFOLIO

Name PhD student: Jasper W. Swierstra
Erasmus MC department: Medical Microbiology and Infectious Disease
PhD period: 2009-2017
Research school: Postgraduate school Molecular Medicine
Promotor: Prof. Dr. H. Verbrugh
Copromotor: Dr. W.J.B van Wamel

PhD training

Courses

Introductory course Molecular Medicine 2009
English writing for PhD students 2013
Workshop Microbial Pathogenesis 2013
Epidemiology Course 2014

Seminars and workshops

NVMM 2011 (poster presentation)
NVMM 2012 (poster presentation)
NVMM 2013 (poster presentation)
NVMM 2014 (poster presentation)
Department Journal Clubs 2009 - 2014
Department Research Meeting 2009 - 2014
PhD Day Erasmus MC 2012
PhD Day Erasmus MC 2013
Research Day Department MMID 2013 (organizser)
Mol Med Day 2012
Mol Med Day 2013
Mol Med Day 2014

International conferences

"International Symposium on Staphylococci and Stapylococcal Infection" Bath, United Kingdom 2010 (poster presentation)
"International Symposium on Staphylococci and Stapylococcal Infection" Lyon, France, 2012 (oral presentation)
TRR34 Pathophysiology of *Staphylococci*, 2010, Greifswald, Germany (poster presentation)

Teaching

Supervision of medial students "Vaardigheidsonderwijs"
Supervision of bachelor and master students