Staphylococcus aureus biofilm

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***Staphylococcus aureus* biofilm**

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***Staphylococcus aureus* biofilm**

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CHAPTER 1

General introduction and outline of the thesis

Introduction

The biofilm mode of growth

Several characteristics of bacteria contribute to their survival strategy in the human host. They have the ability to persist intracellularly within several types of non-professional phagocytic cells¹, specifically neutrophils². Adhesins like fibronectin-binding protein A and B (*fnbA/B*) have been shown indispensable for adhesion to and internalization by these cells^{1,3}. The intracellular survival of *Staphylococcus aureus* relies on protective antioxidants² such as catalase⁴, staphyloxanthin⁵ and the eponymous golden carotenoid pigment^{6,7}. Especially the small colony variants (SCVs) have been associated with intracellular survival, probably due to their decreased metabolic activity and enhanced expression of adhesins⁸. Other protection mechanisms of micro-organisms to circumvent host defenses and therapeutic intervention involve: (i), the expression of efflux pumps^{9,10}; (ii), the (hyper)production of antibiotic degrading enzymes like beta-lactamases¹¹; (iii), the formation of capsular polysaccharides (CPs)^{12,13} – functioning as important cell wall components that can resist uptake and killing by phagocytes¹⁴ – and the inhibition of antibody- and complement-mediated phagocytosis¹⁵; (iv), the release of virulence factors¹⁵; (v), the modification or genetic diversification of antibiotic target proteins such as penicillin-binding proteins (PBPs)¹⁶; and (vi), the biofilm mode of growth.

During the last decade convincing evidence showed that the capability of bacteria to form biofilm is of unprecedented importance in order to protect themselves against the host immune system and antibiotics. Very likely, bacteria strike back by a mixture of strategies, which could act synergistically. It is believed that conventional resistance mechanisms also contribute to the success of biofilm communities¹⁷. For example, in case of mucosal infections it has been noticed that biofilm aggregates attach to the epithelium to reside in the mucus layer or sequester into intracellular aggregates for persistence¹⁸. Demonstrated intracellular localisation of bacteria inside dysfunctional leucocytes enclosed within a biofilm highlights the relevance of additionally employed strategies by bacteria to limit the efficacy of antibiotics¹⁹. Another example of a mixed protection system is the identification of a biofilm specific efflux pump in *Pseudomonas aeruginosa*^{17,20}. However, the observation that the acquisition of plasmids bearing genes encoding for particular classes of beta-lactamases might lead to a reduced capacity to form biomass²¹, suggests that gaining certain resistance genes could suppress other survival opportunities. Nevertheless, high levels of beta-lactamases entrapped within the biofilm matrix have been observed due to secretion or lysis of biofilm-resident cells^{22,23}.

Biofilm formation represents a protected mode of growth that allows bacterial cells to stay alive in both hostile natural environments as in the human host²⁴, and enables them to disperse and colonize new niches whenever needed. Biofilm embedded bacteria are therefore often causative for recurrent and persistent (or chronic) infections²⁵. Biofilm are mostly adhered to surfaces, including those from natural origin such as oral, respiratory, corneal or urogenital epithelium. These tissues seem to be more prone to bacterial adherence when altered or damaged²⁶. Embedded micro-organisms on any surface constitute

profound implications for the host, because the sessile micro-organisms that are surviving in these matrix-enclosed aggregates are recalcitrant to antibiotic treatment and demonstrate persistence in spite of sustained host defences¹⁸. Another critical issue of biofilm-based infections is that biofilm can contain both multiple bacteria and fungi^{25,27}. It is believed that micro-organisms inside these polymicrobial or multispecies communities gain a fitness advantage²⁸, possibly via the facilitation of microevolutionary processes and mutual horizontal gene transfer²⁹. However, model simulations suggest that within-species cooperation could enhance or disfavour the ability to compete with other species³⁰. A known negative interaction involves a subset of *Staphylococcus epidermidis* which can inhibit *S. aureus* nasal colonization and consequently biofilm formation by secreting the serine protease Esp³¹. Nevertheless, both species cooperated in biofilm infected arthroplasties³². Biofilm formation has not only been demonstrated for *S. aureus*, but also for numerous other pathogens. This thesis focuses on monospecies biofilm formation by *S. aureus*.

The development of biofilm is thought to consist of four consecutive stages: (i), *initial attachment* to a foreign body or tissue; (ii), *accumulation* of extracellular polymeric substances and aggregation; (iii), biofilm *maturation* including expansion and channel formation; and (iv), *dispersal*. Dispersal involves the release of microbes from the biofilm, which might start all over again the vicious cycle³³.

Initial interactions with a surface are believed to occur via non-specific physiochemical forces such as charge, van der Waals forces and hydrophobic interactions³⁴. *Initial adherence* has also been attributed to bacterial surface proteins^{35,36}. Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), surface-anchored adhesins like, among others, fibronectin binding protein A and B (*fnbPA/B*)^{37,38}, fibrinogen binding protein (*fbpA*)³⁹, clumping factor A and B (*clfA/B*)⁴⁰, bone sialoprotein-binding protein (*bbp*)⁴¹, serine-aspartate repeat-containing proteins C, D and E (*sdrC/D/E*)⁴², elastin-binding protein (*ebpS*)⁴³, collagen-binding adhesin (*cna*)⁴⁴, biofilm-associated protein (*bap*)^{45,46}, laminin binding protein (*eno*)⁴⁷ and other adhesins like staphylococcal protein A (*spa*)⁴⁸, autolysin adhesion protein (*aaa*)⁴⁹, extracellular matrix binding homologue (*ebh*)⁵⁰, extracellular fibrinogen-binding protein (*efb*)⁵¹, extracellular matrix binding protein (*emp*)⁵¹ and extracellular adherence protein (Eap)⁵² are utilized to initiate initial adherence or aggregation and strongly adhere to surfaces with a conditioning film of environmental proteins like fibrinogen, fibronectin, collagen and platelets^{48,52-54}. Also non-proteinaceous adhesions, comprising PIA (polysaccharide intercellular adhesin) and wall teichoic and lipoteichoic acids, are able to participate in adherence⁵⁵. Continued growth of bacterial cells on a surface leads to the development of mature biofilm colonies containing tightly packed cells gathered into pillar- and mushroom-shaped masses and surrounded by an extracellular polymeric substance (EPS) matrix^{18,56}. In case of *S. aureus*, the **accumulated extracellular material** could consist of a combination of (i) selfproduced Poly-β(1,6)-N-acetyl-D-glucosamine (PNAG), also referred as slime or PIA⁵⁷; (ii), extracellular DNA (eDNA)⁵⁸ originated from a "sacrificiation" procedure called autolysis⁵⁹⁻⁶¹; (iii), proteins^{34,62} (e.g. surface protein G, SasG)^{63,64}; (iv), cell wall teichoic acids³⁴; (v), lipids⁶⁵, and (vi), other substances taken from the environment⁶⁵. There is also some evidence that *S. aureus* can alternate the

composition of the matrix, depending upon the extracellular niche⁶⁶. Enzymes necessary to synthesize PIA are normally not abundantly present⁶⁷ due to generally low expression levels of *S. aureus* encoded *icaADBC* within a biofilm⁶⁶. Consequently, polysaccharide independent *S. aureus* biofilm do also exist^{33,68}. The embedded bacteria in matrix structures are interspersed with fluid-filled channels which act as a primitive circulatory system, allowing for the exchange of nutrients, oxygen and waste products with their environment³⁴. As a consequence of continuous adaptation to: (i), gradients in acidity; (ii), oxygen deficiency; (iii), concentrations of bacterial extruded signalling molecules and waste materials; and (iv), gradients in nutrient availability from the outer to the inner clusters of the interior of the biofilm^{18,69}, numerous micro-environments exist within the biofilm with bacterial cells in fluctuating physiological states⁷⁰. As a result of carbohydrate consumption during the **maturation phase**, a stationary phase-like dormancy can be created⁷¹ due to nutrient depleted zones and/or enhanced metabolic metabolites in the depths of the biofilm. Consequently this may encourage the emergence of persister cells^{29,72}, which mediates antibiotic resistance in biofilm embedded bacteria. Therefore, limited penetration of nutrients^{73,74} and starvation²⁹, rather than restricted antibiotic diffusion^{75–78}, may contribute to generalized resistance or tolerance to antibiotics¹⁸. The metabolically inactive cells are especially located in the deeper interior of the biofilm. Although the matrix may not inhibit the penetration of antibiotics into the biofilm altogether, it may retard the rate of penetration enough to induce the expression of a variety of genes within the biofilm that mediate resistance. This would lead to species or antibiotic resistance specific related processes such as the accumulation of antibiotic-degrading enzymes or the upregulation of efflux pumps¹⁷. Whereas planktonic cells are considered rather as a homogeneous population, biofilm cells are complex communities displaying structural, chemical and biological heterogeneity⁶⁹.

Bacteria in biofilm also coordinate behaviour by cell-to-cell communication using secreted chemical signals, often referred as quorum sensing^{18,79,80}. Although most of the known quorum sensing signals are species specific, inter-species signalling has also been discovered⁸¹. The opposite, quorum quenching, a process in which bacteria secrete enzymes that degrade the quorum-sensing signals of heterologous species has been described as well^{30,81}. The *S. aureus* quorum-sensing system is encoded by the accessory gene regulator (*agr*) locus⁸² and the communication molecules that it produces and senses are autoinducing peptides (AIPs)⁸³, also referred as autoinducers or pheromones^{14,34}. AIPs are encoded by *agrD* and processed by *agrB*^{34,84}. Upon reaching a critical concentration, AIPs activate the two-component *agrA-agrC* system. The response regulator of this system, *agrA~P* activates promoters P2 and P3 to transcribe RNAII encoding *agrBDCA* and the effector molecule RNAIII, respectively⁸⁵. RNAIII inhibits the expression of genes encoding MSCRAMMs^{84,86}, and stimulates the expression of genes encoding extracellular enzymes and toxins⁸², and as a consequence the activated *agr* system downregulates biofilm formation in general³⁴. It is clear that RNAIII is an important determinant of virulence. Activation of the *agr* quorum sensing system is thought to be dependent on cell density⁸⁴. Quorum sensing might be essential for aggregation and biofilm maturation⁸⁷, involving processes as proliferation, accumulation and intercellular adhesion³⁴. It definitely plays a crucial role during the final stage of the biofilm lifecycle. This phase involves the detachment of cells

via their own quorum sensing molecules initiated active dispersal⁵⁶, which allows them to colonize new locations and to reinitiate the biofilm developmental process⁸⁸. **Dispersal** is principally mediated via a functional and activated *agr* system³⁴. Three distinct modes of biofilm dispersal have been discerned: eroding, sloughing or clumping and swarming or seeding dispersal⁵⁶. Another form of shifting to a new habitat is 'surface dispersal', in which biofilm structures move across surfaces⁸⁹. Furthermore, cell-division-mediated dispersal has been described⁵⁶. Detachment can be caused by external perturbations, such as increased fluid shear or by internal biofilm processes⁹⁰. These processes occur in response to the release of AIPs and the transcription of RNAIII^{83,85} and include the release of detachment factors such as glucosidases, (extracellular) proteases (ECP)^{91–93} and deoxyribonucleases⁵⁶, which are endogenous enzymatic degradation factors. Independently of *agr*, the staphylococcal accessory regulator (*sarA*) counteracts the production of ECPs (e.g. a metalloprotease aureolysin (aur), the two cysteine proteases staphopain A and B (sspB and scpA) and the V8 serine protease (sspA))⁸³ and nucleases^{94,95}. Since ECPs also seem to play a role in autolysis inhibition^{68,96}, besides the suggested enhancement of MSCRAMM downregulation^{92,96}, the interplay between *agr* and *sarA* and its homologues is decisive for the switch between biofilm formation (the adhesive phenotype) and dispersal⁹⁷. During activation of *agr* (the more invasive phenotype), toxin release is upregulated by RNAIII⁸² in conjunction with the influence of *sarA*⁹⁸. Another dispersal approach which also depends on the AIPs is the release of surfactant-like molecules, the phenol soluble modulins (PSMs)³⁴. The RNAIII encoded δ -hemolysin (PSM γ , hld)⁸⁴ and the *hla*-encoded α -toxin (α -hemolysin)⁹⁹ belong both to the PSMs and have strong surfactant properties^{100–102}. Apart from that, like other secreted exotoxins such as Panton-Valentine leukocidin (PVL), the alpha-type PSMs are considered virulence determinants in the host-pathogen interrelationship. They induce pore formation-mediated cytolysis of neutrophils and cause an inflammatory mediator release^{103,104}. Furthermore, it has been suggested that PSM α 1–4 have a role in the interference with competing colonizing pathogen¹⁰⁵, contributing to the proposed competitive ecological advantage of community-associated MRSA (CA-MRSA) lineages like USA300^{106,107}.

Elucidating the physiologies of biofilm-associated bacteria is necessary for our understanding of pathogenesis and to clarify the recalcitrance of these bacteria compared with the resistance exhibited by their planktonic counterparts.

Clinical manifestation and implications of bacterial biofilm

Staphylococci are ubiquitous commensals of the skin and mucous membranes of humans. *S. aureus* and the coagulase-negative staphylococci (CoNSs), in particular *S. epidermidis*, are among the most leading causes of nosocomial infections¹⁰⁸. Infections due to *S. epidermidis* typically are more subacute or even chronic and emerge particularly among predisposed or immunocompromised patients¹⁰⁹. In contrast, the virulent pathogen *S. aureus* causes more acute infections associated with the colonization of tissue or bone (marrow), such as endocarditis or osteomyelitis, which may lead to sepsis. However, *S. aureus* is also a common cause of foreign-body-associated infections and, occasionally, *S. epidermidis* may cause native valve endocarditis¹¹⁰. Nevertheless, biofilm formation is now recognized as causing or exacerbating numerous chronic infections. It is estimated that approximately

80% of all bacterial infections are biofilm mediated^{66,75}, with periodontitis, colitis, vaginitis, urethritis, conjunctivitis, chronic otitis media, chronic wound infections, recurrent urinary tract infections (UTI), recurrent tonsillitis, dental caries and cavities, chronic rhinosinusitis, cystic fibrosis associated pneumonia and device-related infections being just a summarized list of common examples of biofilm-associated infections^{25,111}. Difficulty in identifying *in vivo* biofilm infections has led to the formulation of diagnostic criteria, which are displayed in Box 1.

Box 1. Diagnostic criteria for biofilm infections based on the modified Parsek–Singh criteria^{18,194}

-
- (1) Pathogenic bacteria are associated* with a surface.
 - (2) Direct examination of infected tissue demonstrates aggregated cells in cell clusters encased in a matrix, which may be of bacterial and host origin.
 - (3) Infection is confined to a particular site in the host.
 - (4) Recalcitrance to antibiotic treatment despite demonstrated susceptibility of planktonic bacteria**
 - (5) Culture-negative result in spite of clinically documented high suspicion of infection (since localized bacteria in a biofilm may be missed in a conventional blood sample or aspirate).
 - (6) Ineffective host clearance evidenced by the location of bacterial cell clusters (macrocolonies) in discrete areas in the host tissue associated with host inflammatory cells.

* The term 'associated' allows the inclusion of aggregates not necessarily firmly attached.

** Since antibiotic susceptibility of planktonic cultures can only be tested upon positive culture from a clinical specimen, it is suggested that in the absence of culture, recalcitrance to antibiotic therapy may be inferred from the presence of live bacterial cells in the biofilm from *in situ* viability staining or reverse transcription polymerase chain reaction (RT-PCR).

From a mature biofilm, individual cells or aggregates can detach. Upon dispersal, bacteria disseminate via the blood stream, which is thought to lead to an extensive dissemination of bacteria. For this reason bacteraemia is frequently associated with metastatic spread of bacteria and particularly hard to treat circumstances. One of the measurements taken is often the removal of the offending device/ foreign body, especially in case of intravascular indwelling catheters. However, in case of implantable devices like hip or knee prostheses, pacemakers or heart valves, removal is complicated and therefore appropriate treatment options are greatly warranted. Various compounds ranging from chemical compounds like efflux pump inhibitors^{112,113}, N-acetylcysteine¹¹⁴, biocides¹¹⁵ including triclosan¹¹⁶ and chitosan^{117,118} to more natural substances such as traditional Chinese medicine¹¹⁹, honey (propolis)^{120,121}, 3'-5'-cyclic diguanylic acid or abbreviated c-di-GMP (a signalling molecule obtained from other biofilm producing pathogens)^{90,122}, vaccines directed against PNAG^{33,123} or MSCRAMMs¹²⁴, antibodies to quorum sensing molecules¹²⁵, and maggot excretions^{126,127} have been tested successfully *in vitro* against *S. aureus* biofilm. Yet, there is a lack of randomized clinical trials to demonstrate the efficacy of these agents for biomaterial-related infections *in vivo*. In contrast to the absence of clinical trials for agents against biofilm-contaminated devices, there is a wide variety of investigations going-on to search for the

most appropriate way to modify biomaterials¹²⁸ in such a manner that biofilm formation could be prevented.

Prevention of biofilm formation

Biofilm formation can occur on natural tissue and on indwelling devices. Since biofilm starts with attachment to a (a)biotic surface, prevention of initial adherence is the most logical approach to prohibit biofilm formation. The ultimate goal is to obtain biomaterials with anti-biofouling features which are fully refractory to microbial adhesion¹²⁹. Modification of biomaterials involves, in most circumstances, impregnation, surface antifouling conditioning (by coating, e.g. through dipping, sputtering, spraying or solvent casting procedures¹³⁰), physico-chemical surface modification, polymer-brushes^{131,132} or the utilization of micro-nanotopologies¹³³. Ideally, drug-eluting carriers, drug-delivery or leaching matrices with preferably controlled release should be manufactured¹³⁴. Incorporation or attachment of antiseptics^{132,135,136}, antibiotics^{135,137,138}, precious pure metals^{132,139–142}, heparin^{132,135,143,144} and/or antimicrobial peptides^{145,146} has been applied. For instance, a commonly used impregnation for central venous catheters (CVCs) in clinical practice with the intention to reduce bacterial colonization is the combination of chlorhexidine and silver-sulfadiazine^{136,147,148}. Prolonged application of CVCs and tip malpositioning are among risk factors associated with the occurrence of catheter-related bloodstream infections (CRBSIs) as a consequence of catheter colonization and/or exit-site contamination^{149,150}. It is estimated that almost 30–60% of all nosocomial bacteraemia originate from some sort of (intra)vascular access device^{2,151}. The incidence of CRBSIs can be as high as two to seven episodes/cases per 1000 central line/catheter days in Western countries¹⁵², while the occurrence in developing countries is much higher (up to 40–60 episodes)¹⁵³. In attempts to reduce the incidence of CRBSIs, antiseptic or antibiotic impregnated or external and/or lumen coated CVCs were introduced. Incorporation of antibiotics or antiseptics like chlorhexidine could be criticized since these might encourage the emergence of resistant micro-organisms^{137,154} or could induce anaphylaxis¹⁵⁵. CRBSIs are related with (asymptomatic) thrombogenic occlusion¹⁵⁰, which might result in serious complications such as embolisation. Thrombus formation starts by enclosure of the CVC with a fibrin sheath^{149,156}, which promotes adherence of bacteria, concurrently¹⁵⁰. Finally, these adhered bacteria might enhance the thrombogenic cascade and may become embedded by a partially self produced exopolysaccharide matrix (biofilm formation), and consequently become exceedingly difficult to eradicate afterwards.

Prevention of thrombotic complications by prescribing anticoagulant prophylaxis is currently not recommended^{157,158}, since it is often contraindicated due to the risk of major bleeding in thrombocytopenic and renal impaired patients. Furthermore, there is absence of consensus due to limited evidence whether it exerts a beneficial effect at all. Strict following hygiene protocols, adherence to aseptic techniques and using sterile barrier precautions during insertion of CVCs are among the best available preventive measurements to reduce the number of CRBSIs¹⁵⁰, but inadequate for full prevention. Also the application of lock (flush) solutions with (antibiotics, taurolidine^{159–161}, chelators like citrate¹⁶² or EDTA^{163,164}) or without (saline, heparin)^{165,166} antimicrobial substances has been advocated, although the latter one is surrounded by concerns about increased risk of bleeding, induc-

tion of thrombocytopenia^{166,167} and promotion of biofilm generation¹⁶⁸. It is evident that improvement in the biomaterial itself leads to better biocompatibility. In order to reduce the necessity for early removal of CVCs, the surfaces/interfaces should be practically non-thrombogenic and must exhibit strong anti-adhesive or biocidal capacities simultaneously.

Disintegration of the biofilm matrix

Under circumstances where biofilm formation could not be prevented, biofilm have to be destructed. For this reason, other treatment modalities beside the prevention strategy of modification of biomaterials are greatly warranted to combat biofilm associated infections. It is believed that disintegrating agents to obtain passive dispersal should be combined with antimicrobial compounds to overcome the risk of colonization of new niches after chemically stimulated dispersal. Since no single mechanism of dispersal utilized by all bacteria exists, the development of a successful disintegrating agent is rather complex. Some classes of agents that may have clinical utility are: (i), biofilm-matrix-degrading or dispersing enzymes^{91,169–172}; (ii), quorum-sensing interferants^{81,90,94,173–175} such as quorum quenching antibodies¹⁷⁶; (iii), (bio)surfactants^{177,178}; and (iv), small molecule inhibitors^{90,125,179} of, for instance, bacterial diguanylate cyclases⁵⁶. It remains to be seen whether any of these agents will have clinical applications. Probably, combinations are necessary to ensure significant reductions in biomass, since many agents act very specifically against only one matrix component, e.g. DNase I disrupts established biofilm due to digestion of eDNA^{57,180}, while Dispersin B hydrolyzes PIA^{57,90}. Intraleural administered DNase is under clinical investigation in pleural biofilm-associated infections¹⁸¹. Aerosolized recombinant human DNase (dornase alfa) has already proven its value in cystic fibrosis (CF) patients¹⁸² due to improvement of airway mucociliary clearance by reducing the viscosity and adhesiveness of infected bronchial secretions^{183,184}. The only substances actually intravenously used in the clinical setting to date are antibiotics, in particular rifampicin¹⁸⁵ combined with another agent. In relation to monotherapy, somehow paradoxically, adjuvant induced hyperthermia has been shown *in vitro* to enhance the antimicrobial effect of various antibiotics on staphylococcal biofilms¹⁸⁶. Rifampicin could not be used as a single agent due to rapid resistance development as a consequence of commonly occurring point mutations in *rpoB*¹⁸⁷. Although efficacy of rifampicin in combination therapy against clinically presumed biofilm infections has been reported^{188,189}, the clinical outcome remains contradictory^{190,191} and rifampicin resistant mutants have emerged during treatment as adjunctive agent¹⁹². Consequently, the biofilm was incompletely sterilized. So far, it is unclear whether these rifampicin induced resistant subpopulations would be responsible for persistent or on the long term reoccurring infections. It appeared that low-level rifampicin resistant mutants have a fitness advantage over their parental rifampicin susceptible strains *in vivo*¹⁹³, which illustrates the risk of rifampicin exposure even more.

The development of appropriate anti-biofilm approaches must take into account at least the following⁶⁶: (i), unique accumulation molecules (e.g. proteinaceous vs. polysaccharide); (ii), the development of persister cells, and (iii), the metabolic heterogeneity that is inherent to growth in a biofilm.

Outline of the thesis

Research in the field of biofilm physiology, development and function has just started to begin. Extended knowledge of *S. aureus* resistance mechanisms and virulence factors involved in the capability to form biofilm, may contribute to the design and implementation of preventive strategies that could minimize the prevalence of biofilm-associated infections or could lead to novel ways to disassembly existing mature biofilm.

Chapter 2 focuses on the genetic background of *S. aureus* in relation to the capability to produce biomass at different glucose concentrations on polystyrene surfaces. Recently, links were made to match strong biofilm producers with *agr* subtype^{195,196}, methicillin susceptibility¹⁹⁷, type of infection¹⁹⁸, isolation site¹⁹⁹ or pathogenic invasive potential of colonizing or commensal strains²⁰⁰. However, the genetic background was not taken into consideration.

Currently, in daily clinical practice, there are no definitive methods for the diagnosis of a biofilm infection, except clinical suspicion and clinical presentation. When immediate removal (and replacement) of the infected indwelling device or implant is unwanted, rifampicin is simply added to standard (empirically) initiated antibiotic treatment in concordance with therapeutical guidelines and recommendations^{189,191,201,202}. However, the rationale behind the supposed working mechanism remains obscure and rifampicin-resistant subpopulations emerge during treatment¹⁹². Possibly, the use of rifampin as an adjunct in biofilm-associated infections is based on the ability to penetrate into biofilms and a presumed activity against dormant bacteria.

It could be speculated that rifampicin-resistant isolates emerge inside the biofilm when the companion agent does not penetrate as quickly as rifampicin is expected to do. Therefore, it is essential to know whether rifampicin is able to destruct the matrix of biofilm-embedded rifampicin-resistant strains, e.g. through pore-formation. **Chapter 3** deals with the efficacy of rifampicin against rifampin-susceptible and isogenic rifampin-resistant methicillin-susceptible *S. aureus* (MSSA) strains. The effects on the biofilm matrix and on viability were assessed with an *in vitro* static biofilm assay.

Since there are still no compounds suitable for intravenous application on the market that effectively destruct preformed mature biofilm it is essential to prevent biofilm formation. This demands substantial improvements in biomaterials properties used for foreign bodies to hinder initial adherence of micro-organisms and/or substances from the environment. It is known that bacterial adhesion and thrombus formation are interrelated¹⁵⁰. For this reason we aimed to develop coatings for central venous catheters that are both anti-microbial, in the sense that they prohibit adhesion of bacteria, and anti-thrombogenic as well. The anti-thrombogenic features of the engineered new hydrophilic coating containing sodium heparin and silver particles are essentially examined in **chapter 4**. The antimicrobial characteristics are further explored in **chapter 5**. In this chapter the silver content and the hydrophilicity of the coating were also taken into account.

It is evident that multiple resistance mechanisms of micro-organisms are involved in their protection against environmental stresses and threats and thus their general survival

strategy. Bacterial cells embedded in biofilm are far more resistant to antimicrobial agents than their planktonic counterparts. It is questioned whether this could be solely attributed to the penetration barrier formed by the matrix. It is likely that the observed dormancy of bacteria in the deeper parts of the biofilm hinders the killing by antibiotics that require an active life-cycle turnover. Still, it is believed that both principles do not fully clarify the recalcitrance to eradication via the host immune system in combination with antibiotic therapy. It has been suggested that other conventional resistance mechanisms ultimately contribute to this phenomenon. A few of these mechanisms are scrutinized in the last experimental chapters. Enhanced extrusion of toxic substances from the cytosol via upregulation of efflux pumps has been suggested. **Chapter 6** focuses on mutations in the (promoter region of the) *norA* gene, associated with the hyperexpression of the NorA efflux pump, in relation to the genetic background of *S. aureus*. Our findings that certain genetic backgrounds were strongly correlated with mutations associated with the capability to augment the expression of *norA* moved us to perform biofilm experiments with efflux pumps inhibitors directed against NorA transmembrane transporters among several distinct clonal lineages. **Chapter 7** comprises this study that was undertaken to unravel the recent observation that efflux pump inhibitors like reserpine or thioridazine could prevent biomass formation by a single tested strain of *S. aureus*. According to recent suggestions, enhanced extrusion of wastes mediated via upregulation of efflux pumps could offer a survival advantage to the biofilm embedded bacterium^{17,112,203}. It also had to be seen whether efflux pump inhibitors could have any influence on mature biofilm.

Another observation is the entrapment and accumulation of beta-lactamases inside the biofilm matrix^{22,23}. A subset of *S. aureus* strains that could be potentially more prone to beta-lactamase induction during penetration of a beta-lactam into the biofilm are the borderline oxacillin-resistant *S. aureus* (BORSA). These strains could not be differentiated from their fully susceptible counterparts by routine automatic susceptibility testing and will also not be isolated whenever screened for the *mecA* gene. Consequently they will be initially exposed to a beta-lactam antibiotic.

In an attempt to unravel whether planktonic BORSA could be killed with a standard beta-lactam, such as oxacillin, various BORSA isolates were tested in a time-kill experiment. Alternatives like vancomycin, linezolid and daptomycin were also taken into account. **Chapter 8** describes the pharmacodynamic analysis of all time-kill experiments comparing the EC_{50} and E_{max} values of the different antibiotics. Upon oxacillin exposure, the survival behaviour of pBORa53-like plasmid-containing BORSA²⁰⁴ was intentionally compared with non-methicillinase producing BORSA with oxacillin MICs ≥ 4 mg/L.

Finally, a general discussion (**chapter 9**) is presented in which the findings of all studies are put into perspective and in which recommendations for further investigations are discussed. A brief overview of our findings regarding *S. aureus* biofilm formation, prevention and disassembly can be found in the summary (**chapter 10**).

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CHAPTER 2

***Staphylococcus aureus* biofilm formation at the physiologic glucose concentration depends on the *S. aureus* lineage**

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Abstract

Background

Since bacteria embedded in biofilms are far more difficult to eradicate than planktonic infections, it would be useful to know whether certain *Staphylococcus aureus* lineages are especially involved in strong biofilm formation. For this reason, *in vitro* biofilm formation of 228 clinical *S. aureus* isolates of distinct clonal lineages was investigated.

Results

At 0.1% glucose, more than 60% of the *S. aureus* strains associated with multilocus sequence typing (MLST) clonal complex (CC)8 produced large amounts of biomass, compared to 0–7% for various other clonal lineages. Additionally, *S. aureus* bloodstream isolates associated with MLST CC8 and CC7 had similar biofilm forming capacities as their commensal counterparts. Furthermore, strong biofilm formation could not be attributed to a specific accessory gene regulator (*agr*) genotype, as suggested previously. The *agr* genotypes were strictly associated with the clonal lineages. Moreover, strong biofilm formation was not related to slime formation. Congo red agar (CRA) screening is therefore not useful as a qualitative screening method for biofilm formation.

Conclusion

The adherence to polystyrene surfaces under physiologic glucose concentration (0.1%) was dependent on the clonal lineage. Strains associated with MLST CC8 were markedly more often classified as strong biofilm former at glucose concentrations of 0%, 0.1% and 0.25%.

The present study reveals that the MLST CC8 associated genetic background was a predisposing factor for strong biofilm formation *in vitro*, under all tested glucose concentrations.

Background

One of the defense mechanisms of *Staphylococcus aureus* is the capacity to form biofilms. Bacteria embedded in biofilms are often difficult to eradicate with standard antibiotic regimens and inherently resistant to host immune responses^{1, 2}. As a result, treatment of many chronic *S. aureus* biofilm related infections, including endocarditis, osteomyelitis and indwelling medical device infections is hindered³. Biofilm formation is a multistep process, starting with transient adherence to a surface. Subsequently, specific bacterial adhesins, referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMS) promote the actual attachment⁴. Next, during the accumulation phase, bacteria stick to each other and production of extracellular polymeric substances (EPS) and/or incorporation of host derived components, such as platelets, takes place, resulting in a mature biofilm. In circumstances of nutrient deprivation, or under heavy shear forces, detachment of bacteria appears through autonomous formation of autoinducing peptides (AIP)⁵, with release and dispersal of bacteria as a consequence. It has been shown that expression of the accessory gene regulator (*agr*) locus, encoding a quorum-sensing system, results in expression of surfactant-like molecules, such as δ -toxin⁶, contributing to the detachment.

Essential for biofilm development in *S. aureus* is the regulatory genetic locus staphylococcal accessory regulator (*sarA*), which controls the intracellular adhesin (*ica*) operon and *agr* regulated pathways⁷. It has been suggested that biofilm formation in methicillin-resistant *S. aureus* (MRSA) is predominantly regulated by surface adhesins, which are repressed under *agr* expression, while biofilm formation in methicillin-susceptible *S. aureus* (MSSA) is more dependent on cell to cell adhesion by the production of *icaADBC*-encoded polysaccharide intercellular adhesin (PIA), also referred as poly-*N*-acetylglucosamine (PNAG) or slime⁸. However a clear role for the *ica* locus of *S. aureus* is not as evident as that of *Staphylococcus epidermidis*⁹.

In general, the presence of glucose represses the *agr* system through the generation of a low pH^{10, 11}. So far, biofilm development in physiologic glucose-supplemented medium (1 g/L), corresponding to normal blood glucose levels¹², has not been investigated. Biofilm formation often occurs on medical devices, like catheters and heart valves, which are in direct contact with normal (floating) blood. Furthermore, since it has been shown that the regulatory pathways for biofilm formation vary between strains⁸, the question arose whether these strain-to-strain differences could be attributed to different clonal lineages.

The aim of the present study was to examine the contribution of the genetic background of both MRSA and MSSA to biofilm formation under physiologic glucose concentration. MRSA associated with the five major multilocus sequence typing (MLST) clonal complexes (CCs), i.e. CC5, CC8, CC22, CC30 and CC45¹³ and MSSA with the same MLST CCs, and also CC1, were included in this study, since it has been suggested that these lineages possess the ability to become MRSA¹⁴. The results were compared with those obtained with lineages normally not related to MRSA, i.e. CC7, CC12, CC15, CC25 and CC121¹⁵. Fur-

thermore, the aim was to evaluate whether slime production is indicative for strong biofilm formation in *S. aureus*.

Methods

Bacterial strains

S. aureus strains (72 MRSA and 156 MSSA) investigated were isolated during 2005 to 2008 in the Maastricht University Medical Center, a tertiary 715-bed hospital, and originate from surveillance cultures (commensal flora) from individual patients, recovered from nasal swabs. MRSA and/or MSSA strains associated with MLST CC1, CC5, CC8, CC22, CC30, CC45, CC7, CC12, CC15, CC25 and CC121, were randomly selected from our institutional collection (Table 1). All MRSA strains were tested positive for the MRSA-specific *mecA* gene, by real-time PCR³⁴. Additionally, 26 MSSA blood stream isolates from individual patients and associated with either MLST CC8 or CC7 were tested. These isolates were considered invasive.

Characterization of the genetic background

Typing of the *spa* locus was carried out as described previously¹⁹. The *spa* types were assigned through the Ridom SpaServer (<http://spaserver.ridom.de>) and clustered into *spa*-CCs using the algorithm based upon repeat pattern (BURP) with Ridom StaphType 1.4 using the default settings^{35, 36}. Although, *spa* typing alone sometimes lacks discriminatory power, due to related *spa* repeat patterns within different clonal lineages and the emergence of homoplasies among *spa* sequences³⁷, it has been shown that *spa* typing/BURP results are often in agreement with results obtained by MLST^{36, 38}. Therefore, the associated MLST CCs were allocated through the SpaServer. To confirm the association between MLST and *spa* typing, in combination with BURP, MLST was performed on a representative set of 16 strains of each major *spa* type and associated MLST CC^{39, 40}.

Phenotypic detection of slime producing ability onto Congo red agar

MRSA ($n=72$), MSSA with MRSA associated MLST CCs ($n=75$), i.e. CC1, CC5, CC8, CC22, CC30 and CC45, and MSSA with MSSA associated MLST CCs ($n=81$), i.e. CC7, CC12, CC15, CC25 and CC121, were cultured on Congo red agar (CRA) plates, either consisting of trypticase soy or brain heart infusion agar (both from Becton Dickinson) with 0.8 g/L Congo red (Pro-labo, Leuven, Belgium) and without or with 5% sucrose (Merck, Darmstadt, Germany). Colony morphology and color were evaluated after incubation at 37°C for 24 h. Colonies with a dry crystalline (rough) morphology were considered deviant and slime producing positive¹⁶, smooth round colonies were classified as low-slime producers.

Table 1. Distribution of *spa* types and associated MLST CCs among *S. aureus* strains included in this study

associated MLST CC	ST	No. of MRSA strains	No. of MSSA strains	<i>agr</i> geno-type	<i>spa</i> types MRSA strains (No.)	<i>spa</i> types MSSA strains (No.)
1	ST1	NA ^a	16	III	NA ^a	t127 (15), t1787
5	ST5/ST5	15	15	II	t002 (4), t003, t041, t045, t447 (8)	t002 (12), t179, t311, t2212
8	ST8/ST1411 ^a	26	15	I	t008 (12), t052 (6), t064, t068 (5), t303, t622	t008^a (10), t190, t648, t701 (2), t2041
22	ST22/ST22	10	15	I	t223 (10)	t005 (9), t223, t474, t790, t1433, t1629, t2681
30	ST36/ST714 ^b	10	15	III	t012 (7), t253 (2), t1820	t012 (2), t021^b (4), t238, t300, t318 (2), t438, t1130, t1504, t2572, t2854
45	ST45/ST45	11	15	I	t038 (8), t445 (2), t740	t015 (2), t026, t050, t065, t102, t230 (3), t583, t589, t620 (2), t772 (2)
7	ST7	-	15	I	-	t091 (15)
12	ST12	-	10	II	-	t060, t156 (2), t160 (5), t213, t771
15	ST15	-	15	II	-	t084 (11), t085, t491 (2), t1716
25	ST25	-	10	I	-	t078 (4), t081, t087, t258, t353, t1671, t1898
121	ST720 ^c	-	15	IV	-	t159 (2), t171^c (4), t284, t408 (4), t645 (2), t659, t2213
Total		72	156			

not available

Boldface indicates *spa* types on which multilocus sequence typing analysis was performed (ST, sequence type).

^a The strain *spa* typed as t008 had ST1411, a double locus variant of ST8 at the *gmk* and *tpi* locus.

^b The strain *spa* typed as t021 had ST714, a single locus variant of ST30 at the *arcc* locus.

^c The strain *spa* typed as t171 had ST720, a single locus variant of ST121 at the *yqil* locus.

Detection of biofilm biomass with crystal violet staining

The polystyrene crystal violet adherence assay was carried out as described previously⁴¹, with some modifications. Briefly, overnight cultures in Trypticase Soy Broth (TSB) without dextrose (Becton Dickinson, Le pont de Claix, France) were diluted until 10⁸ CFU/mL in TSB containing 0%, 0.1%, 0.25% and 0.5% glucose. Individual wells of polystyrene, flat-bottomed 96-well plates (Greiner Bio-One, Frickenhausen, Germany) were filled with 100-μL aliquots of the cultures. As a negative control, uninoculated medium was used. *S. aureus* ATCC 25923 and one clinical *S. aureus* isolate from our collection, known to form fully established biofilms (*A*₅₉₀ values within the highest range and stable) as observed during a

pilot experiment, were added to each plate as reference standard¹⁷ and positive control, respectively. After 4 h of adhesion at 37°C on a rocking platform at 25 oscillations min⁻¹, the medium containing non-adhered cells, was replaced by 100 µL fresh broth and the plates were further incubated for 24 h. Next, the wells were washed three times with 200 µL 0.9% NaCl. Biofilms were fixed at 60°C during 1 h. Subsequently, 100 µL crystal violet solution (0.3% wt/vol) was added to all wells. After 15 min., the excess crystal violet was rinsed off by placing the plates under running tap water. Finally, after drying the plates, bound crystal violet was released by adding 100 µL 70% (vol/vol) ethanol with 10% isopropyl alcohol (vol/vol). Absorbance was measured spectrophotometrically at 590 nm (A_{590}) and was proportional to biofilm biomass. All assays were performed in triplicate, and repeated on three occasions. The intra- and interday coefficients of variation for the assay were 14% and 23%, respectively. To obtain a threshold A_{590} value for which strong biofilm formation commences, the A_{590} values of all strains at the different glucose concentrations were sorted in ascending order and divided into quartiles. The distribution of A_{590} values in the lower three quartiles was similar at glucose concentrations of 0%, 0.1% and 0.25% and therefore used to determine the cut-off value (two standard deviations above the mean A_{590} value). The threshold A_{590} value was 0.374. Bacteria with A_{590} values above this value were considered strong biofilm formers.

Figure 1. Congo red agar screening of *S. aureus* isolates. CRA screening for *S. aureus* with a dry crystalline colony morphology, which was considered indicative for slime formation. In the left panel (A), the black bar (not visible, 0%) represents MRSA ($n=72$), the dark grey bar represents MSSA with MRSA associated MLST CCs ($n=75$) and the light grey bar represents MSSA with MSSA associated MLST CCs ($n=81$). Asterisks denote statistically significant difference $P < 0.01$ (A) and statistically significant difference of individual CCs versus all other associated MLST CCs (B) $P < 0.01$.

Determination of the *agr* type

The *agr* types of all *S. aureus* isolates were determined by a real-time multiplex PCR assay, as described previously⁴².

Statistical analysis

SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Chi-square analysis was used for comparison of the prevalence of strong biofilm formation or slime formation between the specified groups. Mann-Whitney U analysis was used to compare the A_{590} values between groups of strong biofilm formers. A P value of < 0.05 was considered to be statistically significant.

Figure 2. Quantification of strong biofilm formation in MSSA and MRSA. Quantification of strains of the specified group defined as strong biofilm former at different glucose concentrations. Black bars represent MRSA, dark grey bars represent MSSA with MRSA associated MLST CCs and light grey bars represent MSSA with MSSA associated MLST CCs. Asterisks denote statistically significant difference, (*) $P < 0.05$ and (**) $P < 0.01$.

Results

Characterization of the genetic background

The *spa* types and associated MLST CCs of all tested strains are shown in Table 1. The results of *spa* typing/BURP and MLST were in accordance for a representative set of 16 strains of each major *spa* type and associated MLST CC.

Phenotypic detection of slime producing ability onto Congo red agar

The different Congo red agar (CRA) screening methods described in the literature were evaluated^{16–18}. The choice of the agar medium, either brain heart infusion or trypticase soy, did not influence the morphology. The majority of *S. aureus* strains (91%) displayed colonies with a normal morphology (smooth round colonies), indicating that most strains were low-slime producers. Without sucrose, all colonies were colored (bright) red to bordeaux

red, irrespective of the agar medium used. Addition of sucrose to both agar media resulted in more dark colonies and made the dry crystalline morphology harder to recognize. With sucrose, all colonies on brain heart infusion agar with Congo red were colored red to bordeaux red, while strains on trypticase soy agar with Congo red displayed mostly purple to black colonies. Nuances in color were not corresponding to differences in morphology.

Figure 3. Biomass quantification of MRSA and MSSA. Absorbance (A_{590}) of the crystal violet stained biofilm matrix for strong biofilm formers (with A_{590} above the threshold value of 0.374, represented by the horizontal dashed line) at different glucose concentrations. Boxplots at the left show MRSA, in the middle MSSA with MRSA associated MLST CCs and at the right MSSA with MSSA associated MLST CCs. The lower and higher boundary of the box indicates the 25th and 75th percentile, respectively. The line within the box marks the median. Whiskers above and below the box indicate the 90th and 10th percentiles. Open circles indicate the 95th and 5th percentiles. Asterisks denote statistically significant difference, (*) $P < 0.05$ and (**) $P < 0.01$.

MSSA strains showed more often a deviant, dry crystalline (rough) morphology (slime producing positive) than MRSA isolates, 14% (22 of 156) and 0%, respectively. A significant distinction in slime formation was observed between MRSA and MSSA with MSSA associated MLST CCs, i.e. CC7, CC12, CC15, CC25 and CC121, and with MRSA associated MLST CCs, i.e. CC1, CC5, CC8, CC22, CC30 and CC45 ($P < 0.01$), as shown in Figure 1A. MSSA associated with MLST CC121 had the highest prevalence of a deviant morphology, 67% (10 of 15) (Figure 1B).

Detection of biofilm biomass with crystal violet staining

Under physiologic glucose (0.1%) concentration, 13% ($n=30$) of all strains formed a strong biofilm and all these strains were MRSA or had a MRSA associated MLST CC. MRSA and MSSA with MRSA associated MLST CCs, i.e. CC1, CC5, CC8, CC22, CC30 and CC45, were significantly more capable than MSSA with MSSA associated MLST CCs, i.e. CC7, CC12, CC15, CC25 and CC121, to form strong biofilms in the presence of 0.1% glucose ($P < 0.01$),

but not at glucose concentrations of 0.25% and 0.5% (Figure 2). The higher the glucose concentration, the more strains produced biofilm above the A_{590} threshold value and were consequently classified as strong biofilm former. At glucose concentrations of 0.25% and 0.5%, the amount of biomass of the biofilms of strong biofilm forming strains was still significantly more for MRSA compared to MSSA irrespective of the MLST CCs ($P < 0.01$) (Figure 3). Of all strains classified as strong biofilm producers, MRSA and MSSA associated with MLST CC8 produced the most biomass under all tested glucose concentrations (Figure 4A and 4B). Strains defined as strong biofilm formers and associated with MLST CC5, CC25 and CC30 approached approximately the same level of biomass at the following glucose concentrations, i.e. CC5 at 0.25%, CC25 at 0.5% and CC30 at 0.5% glucose, respectively.

Figure 4. Biomass formation related to the genetic background of *S. aureus*. Absorbance (A_{590}) of the crystal violet stained biofilm matrix of strong biofilm forming *S. aureus* strains in relation to different associated MLST CCs (A) and of strong biofilm forming strains associated with MLST CC1, CC5, CC8, CC22, CC30 and CC45 (B). R in the legend represents MRSA and S represents MSSA. Quantification of strains of the specified genetic background defined as strong biofilm former at different glucose concentrations, (C) and (D). Asterisks denote statistically significant difference, (B) and (D), and statistical significant difference of individual CCs versus all other associated MLST CCs, (A) and (C), except #, (*) $P < 0.05$ and (**) $P < 0.01$.

The main contributors to the higher prevalence of MRSA and MSSA with MRSA associated MLST CCs to produce strong biofilms at 0.1% glucose were MLST CC8 isolates, approximately 60% (26 of 41), (Figure 4C), especially with a tendency towards MRSA (Figure 4D).

Additionally, blood stream isolates of MSSA associated with MLST CC8 and MLST CC7 were included in the study, to address the question whether the isolation site is an (additional) predisposing factor for strong biofilm formation. MSSA associated with MLST CC7 are one of the main clonal lineages among blood stream isolates in our hospital¹⁹. No differences in the ability to produce strong biofilms were observed between bloodstream isolates and isolates of commensal origin among MSSA associated with MLST CC8 and CC7 (Figure 5A and 5B). Furthermore, no significant differences in slime-forming ability were observed (Figure 5C).

Figure 5. Biofilm formation in *Staphylococcus aureus* isolates of bloodstream infections and commensal origin. Biofilm formation between *S. aureus* isolates of the same clonal lineage from blood stream infections (CC8 $n=15$, CC7 $n=11$) and of commensal origin (CC8 $n=15$, CC7 $n=15$), no significant differences were found (A). S in the legend represents MSSA, BSI represents bloodstream isolates and C represents commensal isolates. Number on each bar refers to number of isolates. Absorbance (A_{590}) of the crystal violet stained biofilm matrix of strong biofilm formers at different glucose concentrations (B). CRA screening for colonies with a dry crystalline morphology (C).

Correlation between slime formation and development of biofilm biomass

In order to investigate whether slime production is indicative for strong biofilm formation, the correlation between these two characteristics was addressed. Phenotypic detection of slime production on CRA was not related to the quantitative detection of strong biofilms, measured by crystal violet staining, which was used as a gold standard. The sensitivity and specificity of the CRA method for *S. aureus* was approximately 9% and 90%, respectively (Table 2). Only a part of the slime producing strains surpassed the A_{590} threshold value for strong biofilm formation, namely 5%, 15%, 45% and 90% at 0%, 0.1%, 0.25 and 0.5% glucose, respectively.

Table 2. Correlation between slime formation (Congo red agar screening) and development of biofilm biomass (crystal violet staining).

Glucose (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	CRA+ / CV+	CRA- / CV+	CRA+ / CV-	CRA- / CV-
Number of <i>S. aureus</i> strains								
0	6.3	91.0	5.0	92.8	1	15	19	193
0.1	9.7	91.3	15.0	86.5	3	28	17	180
0.25	11.6	93.0	45.0	63.5	11	76	9	132
0.5	8.3	80.0	90.0	3.9	18	200	2	8

(PPV) positive predictive value, (NPV) negative predictive value, (CRA) Congo red agar screening, (CV) crystal violet staining

Distribution of *agr* types

Clonal lineages MLST CC7, CC8, CC22, CC25 and CC45 harbored *agr*-I, all CC5, CC12 and CC15 were characterized by *agr*-II, while all CC1 and CC30 were detected as *agr*-III. Furthermore, CC121 isolates carried *agr*-IV (Table 1). No consistent relationship was found between *agr* genotype and the ability to produce biofilm.

Discussion

In vitro quantification of biofilm formation in distinct clonal lineages of *S. aureus* was performed to investigate whether there were differences in the capacity to form fully established biofilms. This study revealed that at 0.1% glucose, enhanced biofilm formation of *S. aureus* was strongly associated with MLST CC8 and observed in 60% of these isolates, while it varied between 0–7% for the other clonal lineages tested.

A higher percentage of MSSA (14%) than MRSA (0%) was found positive for slime producing ability, in concordance to the more important role of PIA/PNAG in MSSA than in MRSA biofilm development⁸. Addition of sucrose to CRA did not influence slime formation, suggesting that slime formation was carbohydrates independent. The results were consistent with previous findings in MRSA and MSSA isolates of O'Neill *et al.* In MSSA isolates increased *ica* expression and PIA/PNAG production (as determined with PIA/PNAG immunoblot) was correlated with 4% NaCl-induced biofilm formation, but not with glucose-induced biofilm production⁸. In addition, in MRSA, *ica* operon transcription was more potentially activated by NaCl than by glucose, but did not result in PIA/PNAG formation⁸. Since it has recently been suggested that, in general, PIA/PNAG is a minor matrix component of *S. aureus* biofilms^{5, 9}, and thus possibly hardly detectable by CRA screening, a low prevalence of slime producing strains was expected. Knobloch *et al.* and Mathur *et al.* re-

ported a positive CRA assay result in only 4–5% of the *S. aureus* strains tested, in relative accordance with the results of this study, while Grinholm *et al.* mentioned 47% and 69% for MRSA and MSSA, respectively^{16–18}. Jain *et al.* reported differences between blood stream isolates and commensal *S. aureus* isolates with regard to positive CRA screening, 75% and 20%, respectively²⁰. The variations could be due to differences in genetic backgrounds of the strains used, or to differences in interpretation of the colonies. The definition of slime-forming strains used by Grinholm *et al.* and Jain *et al.* was based on the color of the colonies and not on the morphology. Furthermore, they both found a high consistency (96% and 91%, respectively) between CRA screening and biofilm biomass crystal violet staining^{17, 20}. In contrast, both in this study, as well in the studies by Knobloch *et al.*, Rode *et al.*, and Mathur *et al.*^{16, 18, 21}, no correlation was found between slime producing MRSA and MSSA isolates and an enhanced tendency to form large amounts of biomass. These studies strongly suggest that CRA screening forms no alternative for crystal violet staining to detect biofilm formation. Probably, the cell to cell adhesion, stimulated by the formation of PIA/PNAG, is less efficient than the expression of surface adhesins, in their contribution to produce more biomass.

As described before, the *agr* genotypes were strictly associated with the clonal lineages^{22, 23}. However, exceptions have been observed^{24–27} which might be due to interstrain recombination and intrastrain rearrangements²⁸. The association between *agr* genotypes and the genetic background explains the absence of a relationship between the enhanced ability to form biofilm and specific *agr* genotype(s). Although, there was a tendency that the *agr*-I genotype was associated with an enhanced capacity to form strong biofilms (data not shown), this was a reflection of the biofilm-forming capacity of strains associated with MLST CC8. In contrast to our results, Cafiso *et al.* described a link between *agr*-II genotype and the capacity to form strong biofilms, since all strains with *agr*-II genotype were associated with strong biofilm formation at 0.25% glucose. However, the genetic background was not taken into consideration²⁹. Our findings revealed that strains associated with MLST CC5, CC12 and CC15 (all harboring *agr*-II) were classified as strong biofilm formers in only 21%, 9% and 53% of the cases at 0.25% glucose, respectively. Furthermore, the *agr*-II genotype encompass diverse strains, not including strains associated with MLST CC8^{22, 23}.

Biofilm formation was induced by increasing glucose concentrations up to 0.5% in both MRSA and MSSA isolates, which is entirely consistent with previously reported data^{8, 21}. However, MRSA produced significantly more biomass than MSSA with MSSA associated MLST CCs, under all tested glucose conditions. Especially strains associated with MLST CC8 contributed to this phenomenon. Furthermore, MSSA with MRSA associated MLST CCs were also capable to produce more biomass than MSSA with MSSA associated MLST CCs at 0.1% glucose. Variations in biofilm forming capacities in clonal lineages of *S. aureus* could be explained by unique combinations of surface-associated and regulatory genes²³ or by different expression levels of genes that regulate the different phases of biofilm formation. Since this study showed that the biofilm formation on polystyrene surfaces was the strongest for the MLST CC8 associated genetic background, further studies with other material or tissue are warranted. Recently, differences in adhesion to human airway epithelial cells have been observed between strains belonging to MLST CC8 and CC5, the latter demonstrating a generally lower adherence in both representatives of MRSA and MSSA³⁰.

An enhanced ability to adhere and invade these cells has also been shown for MRSA associated with the Brazilian/Hungarian clone, which belongs to MLST CC8¹⁵, compared to a population of MSSA with an unknown genetic background³¹. Furthermore, strains associated with the same clone were not included among our MLST CC8 isolates, but were previously classified as strong biofilm producers and designated superior in their ability to produce biofilm compared to isolates associated with the Pediatric clone (MLST CC5)³².

To analyse possible other predisposing factors besides the MLST CC8 associated genetic background, bloodstream and commensal isolates of the same clonal lineage were compared. The biofilm forming capacity between MSSA bloodstream and commensal isolates, associated with MLST CC8 and CC7, was similar and consistent with the findings of Smith *et al.*, who compared the biofilm forming capacity of Scottish clinical *S. aureus* strains collected from different isolation sites³³. In contrast, Jain *et al.* described more frequently strong biofilm formers among *S. aureus* bloodstream isolates than commensal²⁰. A possible explanation might be that all bloodstream isolates came from patients with peripheral intravenous devices, while this was not an inclusion criterion in the study by Smith *et al.* . Peripheral or central line intraluminal colonization might be associated with strains that easily attach to (catheter) surfaces and as a consequence these strains could be dominant in leading to bloodstream infections.

Conclusions

In summary, the present study reveals that the MLST CC8 associated genetic background was a predisposing factor for strong biofilm formation *in vitro*, under all tested glucose concentrations, i.e. 0%, 0.1%, 0.25% and 0.5%. At physiologic glucose concentration (0.1%), 0–7% of *S. aureus* from various clonal lineages were defined as strong biofilm former, compared to 60% for the *S. aureus* associated MLST CC8.

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CHAPTER 3

Unpredictable effects of rifampicin as adjunctive agent in elimination of rifampicin-susceptible and -resistant *Staphylococcus aureus* grown in biofilm

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Abstract

The use of rifampicin as adjunct in biofilm-associated infections is based on the ability to penetrate into biofilms and a presumed activity against dormant bacteria. Yet, its efficacy remains contradictory and rifampicin-resistant strains frequently emerge during therapy. Therefore, the efficacy against rifampicin-susceptible and isogenic rifampicin-resistant methicillin-susceptible *Staphylococcus aureus* (MSSA) strains was evaluated.

Biofilms were generated under static conditions using MSSA with various genetic backgrounds. Oxacillin alone or with rifampicin at various concentrations were subsequently added and after 24h biomass and viable cell counts were determined.

Upon rifampicin addition, inter-strain variations in viable count change, ranging from a tendency towards antagonism to synergy, were observed among all strains tested, irrespective of the genetic backgrounds of the strains. Similar variations were observed in changes in biomass. The decrease in viable count upon rifampicin addition was negatively correlated to formation of large amounts of biomass, since strains embedded by more biomass showed a diminished reduction in viable count.

Rifampicin (1 $\mu\text{g/mL}$) as adjunct to oxacillin achieved greater reductions in biomass produced by most rifampicin-susceptible isolates, ranging from 17 to 54% compared to 4% for oxacillin alone. In contrast, rifampicin had no additional value in reduction of biomass of isogenic rifampicin-resistant mutants. At subinhibitory concentrations of rifampicin (0.008 $\mu\text{g/mL}$), none of the strains tested yielded an extra reduction in biomass $\geq 40\%$.

In conclusion, the effect of rifampicin as adjunct on biomass and viable count was unpredictable and the use of rifampicin against biofilm containing rifampicin-resistant strains seems unwarranted.

Introduction

Bacteria embedded in biofilm are often hard to eradicate, which will often result in failure of antibiotic therapy. Biofilm can appear and persist on implanted or indwelling devices. Adherence to bone or natural tissue can also occur. As a consequence of biofilm formation, foreign body infections are a risk factor for persistent bacteremia¹.

Staphylococcus aureus biofilm infected devices often require removal in combination with antimicrobial therapy². Even if an infection seems to be cured, a subset of bacteria can survive within the partly unremoved biofilm and re-manifest after several years. These are the so-called low-grade infections³. The ideal antibiotic should be able to diffuse properly into biofilms, eradicate dormant bacteria and kill phagocytized *S. aureus*, since foreign-body infections were associated with intracellular *S. aureus*^{4,5}. Rifampicin meets these criteria partially⁶. Rifampicin penetrates biofilms adequately⁷ and improves the diffusion of a companion agent through the biofilm⁸. It also reduces the adherence of bacteria to surfaces⁶. Furthermore, rifampicin addition eliminates intrinsic rifampicin-resistant *Pseudomonas aeruginosa* (MIC 32 µg/mL) from biofilms⁹. Therefore, it could be hypothesized that the presence of rifampicin leads to biofilm matrix disintegration. However, rifampicin can not be used alone due to the rapid emergence of resistance¹⁰ and it is not as effective as presumed against slow growing bacteria^{7,11}. An alternative approach for *S. aureus* biofilm-associated infections is the addition of rifampicin to a companion drug. In clinical practice, rifampicin addition has occasionally demonstrated to be beneficial in terms of clinical or bacteriological cure rates¹², especially for prosthetic device-related infections^{5,13,14}. However, its effect remains poorly defined since the number of supporting human studies is limited and most of them are underpowered^{5,13–15}. Moreover, recently performed *in vitro* studies have demonstrated antagonistic effects after addition of rifampicin in experimental foreign-body infection and endocarditis models^{16,17}.

Exposure to combination therapy including rifampicin frequently results in the emergence of rifampicin-resistant subpopulations, which might hinder clearance of the infection¹⁸. This is probably due to the fact that the pharmacokinetics of the companion drug were different from that of rifampicin. Preferable, the companion drug needs to penetrate easily through diverse layers of a biofilm to prevent the development of rifampicin resistance. But, although some antibiotics penetrate adequately through biofilms, bacteria may be protected from killing by thickening of the cell wall⁷. A high bacterial load is another causative reason for rapid resistance development¹⁹. To overcome the emergence of resistance it is recommended that rifampicin should be initiated after the companion drug has been administered for 2 to 5 days, to reduce the high density of the initial burden²⁰, or at least until a substantial reduction in bacterial load has been achieved^{18,21}.

Since inter-strain variability in response to rifampicin addition might be responsible for contradictory results obtained by various authors, the aim of the present study was to elucidate whether this variability does exist for strains between and within the *S. aureus* genetic backgrounds associated with multilocus sequence type (MLST) CC5, CC8 and CC30. Furthermore, since rifampicin-resistant mutants emerge during therapy and rifam-

picin is used for a hypothesized destruction of the biofilm matrix, we evaluated whether continuation of exposure to rifampicin is useful against biofilms containing rifampicin-resistant methicillin-susceptible *S. aureus* (MSSA). In addition, we examined whether a potential effect was also present at a subinhibitory concentration.

Materials and methods

Bacterial strains

As part of an ongoing surveillance of *S. aureus* carriers, seventeen MSSA isolates from individual patients, recovered from nasal swabs in the Maastricht University Medical Center, a tertiary 715-bed hospital, were evaluated. The isolates were randomly selected from a group of MSSA associated with MLST CC5 ($n=6$), CC8 ($n=5$) and CC30 ($n=6$) and a rifampicin MIC of 0.016 $\mu\text{g/mL}$ (Table 1). All three lineages possess the ability to become methicillin-resistant *S. aureus* (MRSA) by acquisition of a Staphylococcal Cassette Chromosome *mec* (SCC*mec*) element and belong to pandemic lineages, CC30 in Europe and CC8 in the USA²². This study was restricted to MSSA isolates, since the prevalence of MRSA is still low in the Netherlands²³ and the Scandinavian countries²⁴.

Table 1. Characteristics of *S. aureus* strains and rifampicin-resistant mutants included in this study.

associated MLST CC	ST	No. of isolates	No. of mutants	Oxacillin MIC ($\mu\text{g/mL}$)		Rifampicin MIC ($\mu\text{g/mL}$) / Disk diffusion		<i>spa</i> types (No.)
				RSS	RRM	RSS	RRM	
5	ST5	6	6	0.125 – 0.5	0.125 – 0.5	0.016 / S	> 4 / R	t002 (6)
8	ST1411 ^a	5	5	0.125 – 0.5	0.125 – 0.5	0.016 / S	> 4 / R	t008^a (5)
30	ST714 ^b	6	6	0.125 – 0.5	0.125 – 0.5	0.016 / S	> 4 / R	t021^b (2), t012 (2), t238 (1), t1130 (1)

RSS, rifampicin susceptible strain ; RRM, rifampicin resistant mutant

Boldface indicates *spa* types on which multilocus sequence typing analysis was performed (ST, sequence type).

^a The strain *spa* typed as t008 had ST1411, a double locus variant of ST8 at the *gmk* and *tpi* locus.

^b The strain *spa* typed as t021 had ST714, a single locus variant of ST30 at the *arcC* locus.

Characterization of the genetic background

Real-time amplification of the *spa* locus, followed by sequencing, was performed as described before²⁵. The *spa* types were assigned through the Ridom SpaServer (<http://spaserver.ridom.de>) and clustered into *spa*-CCs using the algorithm based upon repeat pattern (BURP) with Ridom StaphType 1.5 using the default settings²⁶. Since it has been shown that *spa* typing/BURP, yields results consistent with typing results obtained by MLST^{26,27}, the associated CCs, as determined with MLST, were allocated through the Ridom SpaServer. To confirm the association between MLST and *spa* typing, in combination with

BURP, MLST was performed on one isolate of each major *spa* type and associated MLST CC, as described previously^{25,28}.

Isolation of rifampicin-resistant mutants

Selective rifampicin containing (16 µg/mL = 1000 × MIC) 5% sheep blood agar plates (Oxoid CM0331, Wesel, Germany) were seeded with 100 µL of a suspension of approximately 10⁹ CFU/mL of MSSA and incubated overnight at 37°C. The colonies that appeared on these plates were picked, purified and cultured overnight on plates containing rifampicin (16 µg/mL). Afterwards, rifampicin-resistant mutants were kept frozen at -20°C, until further examination. Prior to each test, mutants were plated on non-antibiotic containing blood agar plates (Oxoid PB5039A, Basingstoke, UK).

Susceptibility testing

Susceptibility testing of the parent strains was performed using a micro-dilution technique according to the CLSI guidelines²⁹. Prior and after each experiment, rifampicin resistance of the rifampicin-resistant mutants was confirmed using a disk diffusion method (Neo-Sensitabs 30 µg, Rosco Diagnostica A/S, Taastrup, Denmark) on Mueller-Hinton II agar plates (Becton Dickinson Ref:221177, USA), according to the manufacturer's recommendations and the CLSI breakpoints³⁰. Rifampicin susceptibility of the rifampicin-susceptible isolates was likewise determined.

Biofilm formation in microtiter plates and detection of biomass and viable count

MSSA were allowed to establish biofilms during 24h in 96-well (biomass) and 24-well (viable count) flat-bottomed non-tissue culture treated polystyrene microtiter plates (Greiner Bio-One, Frickenhausen, Germany and Becton Dickinson, Le Pont de Claix, France, respectively) at 37°C on a rocking platform at 25 oscillations min⁻¹. Individual wells were filled with 100 µL (biomass) and 800 µL (viable count) of an overnight culture which was diluted until 10⁸ CFU/mL in Trypticase Soy Broth (TSB) without dextrose (Becton Dickinson, Le Pont de Claix, France), respectively, and supplemented with 0.1% glucose. After 24h biofilm formation, growth medium with planktonic cells was discarded and replaced by fresh broth containing antibiotics, except for controls, where medium was added only. Oxacillin (Sigma-Aldrich, Zwijndrecht, the Netherlands) was added at 2 and 4 × MIC (six replicates per concentration). Furthermore, rifampicin (Sigma-Aldrich) at ½ (0.008 µg/mL) and 62½ × MIC (1 µg/mL) was added to oxacillin. Oxacillin was also tested alone. The plates were incubated for an additional 24h. Subsequently, the wells were washed three times with 200 µL (biomass) and 1.6 mL NaCl 0.9% (viable count), respectively.

Similar concentrations of rifampicin were used when the rifampicin-resistant mutants were tested: a rifampicin concentration of 1 µg/mL was considered clinically achievable in serum. The subinhibitory concentration (½ × MIC) was used to reveal whether a low concentration affects the ability to form biomass and influences bacterial replication and/or killing.

Biomass formation in 96-well plates was quantified using the crystal violet adherence assay, carried out as described previously²⁵ and performed in six fold. Biofilms grown in 24-wells plates were dispersed with a disposable cell scraper, adjusted to 1 mL, diluted and plated on blood agar plates to determine the number of CFU per well. Viable count experiments were repeated once. The intra- and interday coefficients of variation for the biomass assay were 10% and 15%, respectively.

Synergy for the viable count measurements was defined as a decrease of $\geq 2\text{-log}_{10}$ CFU per well with a combination of two antimicrobials versus the most active single agent. Antagonism was defined as an increase of $\geq 1\text{-log}_{10}$ CFU per well with the combination compared with the least active single agent.

Figure 1. Categorization of the effect of rifampicin as adjunctive agent on *S. aureus* biofilm. Categorization of rifampicin-susceptible (RIF-S) isolates and rifampicin-resistant (RIF-R) mutants for viable count of biofilm-embedded bacteria (A) and biomass (B) measurements. The effect was expressed as the difference between oxacillin alone and oxacillin in combination with rifampicin and subsequently categorized. The adjunctive use of rifampicin was evaluated at 0.008 µg/mL and 1 µg/mL, respectively. The number of strains tested are specified per category in each bar. An extra reduction of $\geq 1\text{-log}_{10}$ CFU per well (including synergism) upon rifampicin and oxacillin exposure was considered a positive outcome. Similarly, an extra decrease in biomass of $> 20\%$ in the presence of rifampicin, compared to oxacillin alone, was considered positive. All positive outcomes were clustered and are shown with slope striped patterns. Grey or white filled bars display a negative outcome and were clustered as well.

Statistical analysis

SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. ANOVA with Bonferroni correction was used to compare individual runs of the biomass experiments. Chi-square analysis was used for comparison of the prevalence of defined categories between the specified groups. Mann-Whitney U analysis was used to compare biomass values and changes in viable count. A *P* value of ≤ 0.05 was considered to be statistically significant.

Figure 2. The effect of rifampicin as adjunctive agent on *S. aureus* biofilm per clonal lineage. Change in viable count (**A**) or biomass of biofilm-embedded bacteria (**B**) caused by oxacillin (OXA) combined with rifampicin (RIF) at 1 µg/mL, compared with control (dark grey filled bars). The effects of oxacillin alone at 2 × MIC are also shown (light grey filled bars). The results of the biomass experiments with BF35 were excluded, since BF35 showed a totally different outcome than the other isolates associated with MLST CC8. Error bars indicate standard error of the mean. The abbreviations RIF-S and RIF-R represent rifampicin-susceptible isolates and rifampicin-resistant mutants, respectively.

Results

Rifampicin as adjunct against rifampicin-susceptible isolates

Following biofilm formation under static conditions, we examined the inter-strain variability of rifampicin addition to oxacillin on biomass and viable count among genetically related biofilm-embedded MSSA strains. Rifampicin as an adjunctive agent at 1 µg/mL ($62\frac{1}{2} \times$ MIC) reduced the viable count of these isolates and the amount of biomass formed generally more than oxacillin alone at $2 \times$ MIC. Increased bacterial killing of $\geq 1 \log_{10}$ CFU per well compared to oxacillin was observed in 10 out of 17 isolates, of which four demonstrated synergism (Figure 1A). However, inter-strain variability in response to rifampicin was found since an increase in viable count was observed with four other isolates. Beside that, an additional decrease in biomass of 20% or more was noticed in 15 out of 17 isolates (Figure 1B), whereas one strain showed an increase in biomass. This isolate (BF35), associated with MLST CC8, demonstrated an increase of approximately 300% in biomass formation upon addition of rifampicin at $62\frac{1}{2} \times$ MIC to oxacillin (Figure 2B). This increase was accompanied by less effective killing of bacterial cells: $-1.04 \log_{10}$ versus an average of $-2.69 \log_{10}$ for the other rifampicin-susceptible isolates associated with MLST CC8 (Figure 2A). Contrary to this observation, the relative change in biomass of the rifampicin-susceptible isolates was independent on the genetic background (Figure 2B) when rifampicin was used as adjunctive agent at $62\frac{1}{2} \times$ MIC. However, rifampicin addition led to a smaller decline in CFU per well ($-1.52 \log_{10}$) against a few isolates associated with MLST

CC5 compared to CC8 ($-2.69 \log_{10}$) (except isolate BF35) and CC30 ($-2.43 \log_{10}$) (Figure 2A), but there was a tendency that the former were embedded by more biomass (Figure 3 and 4C).

Figure 3. The additional change in viable count ($\log_{10}$ CFU per well) of biofilm-embedded bacteria in the presence of rifampicin ($1 \mu\text{g/mL}$) as an adjunct to oxacillin ($2 \times \text{MIC}$), compared to the change with oxacillin alone. The dashed horizontal line indicates the distinction between increase (upper panel) and decrease (lower panel) in viable count. The dotted line represents the threshold values for a synergistic effect of rifampicin. The amount of biomass (absorbance at 590 nm) of the controls is represented on the x-axis. The dashed vertical line indicates the previously defined threshold value for strong biomass formation. The symbol fillings of all tested strains associated with MLST CC8 (circles), CC5 (triangles downwards) and CC30 (squares) represent the change in biomass upon addition of oxacillin in combination with rifampicin. Unfilled and filled symbols indicate an increase and decrease in biomass compared to control, respectively. The abbreviations S and R represent rifampicin-susceptible isolates and rifampicin-resistant mutants, respectively.

To evaluate the concentration of the companion agent, we evaluated the effect on biomass of $2 \times \text{MIC}$ oxacillin compared with $4 \times \text{MIC}$ oxacillin, both combined with rifampicin at 0.008 and $1 \mu\text{g/mL}$. No differences in relative change in biomass were observed between these two oxacillin concentrations (data not shown).

A subinhibitory concentration of rifampicin was tested to assess whether rifampicin augments biofilm formation, as described for various antibiotics against different bacterial species^{31,32}. In general, compared to oxacillin alone, addition of rifampicin at $0.008 \mu\text{g/mL}$ ($\frac{1}{2} \times \text{MIC}$) to oxacillin neither triggered biomass formation, nor enhanced the reduction in biomass (Figure 1B). Moreover, compared to rifampicin at $1 \mu\text{g/mL}$, the number of isolates without any effect on viable count increased from 3 to 8 out of 17 isolates (Figure 1A).

Rifampicin as adjunct against rifampicin-resistant mutants

Besides a direct antimicrobial effect on RNA polymerase¹⁰, we hypothesized that rifampicin exercises an anti-biofilm effect by causing matrix degradation, thereby improving the diffusion of the companion agent. Therefore, we evaluated whether rifampicin was also able to destruct biofilms of rifampicin-resistant strains. Although we observed a synergistic bacterial killing effect for one isolate, when rifampicin was added at 1 µg/mL to oxacillin (Figure 1A), 7 out of 17 rifampicin-resistant mutants showed a slight increase in viable count (Figure 1A). Most rifampicin-resistant mutants (13 out of 17) showed indifference at 0.008 µg/mL rifampicin. Although the viable count of some mutants associated with MLST CC5 and CC8 was reduced slightly more in the presence of rifampicin, compared with oxacillin alone (Figure 2A), the amount of biomass was rather unaltered (Figure 2B and 3). Overall, biomass was not affected by the presence of rifampicin in both concentrations: 15 out of 17 rifampicin-resistant mutants showed an indifferent effect when rifampicin was added to oxacillin (Figure 1B).

Figure 4. The amount of biomass (absorbance at 590 nm) produced during 48h (A), the number of colony forming units (CFU) per biofilm (B) and the number of viable micro-organisms per biomass (C), per genetic background, in the absence of antibiotics. Error bars represent standard error of the mean. The abbreviations RIF-S and RIF-R represent rifampicin-susceptible isolates and rifampicin-resistant mutants, respectively.

Effect of rifampicin in relation to biomass formation

Some strains associated with MLST CC8 or CC5, including both rifampicin-susceptible isolates and rifampicin-resistant mutants, possessed an absorbance value above a previously defined threshold value of strong biomass formation at 0.1% glucose²⁵ (Figure 3). When strong biomass formation was taken into account, the viable counts of both rifampicin-susceptible and rifampicin-resistant strains embedded by large amounts of biomass, were hardly affected by rifampicin addition to oxacillin. Additional changes in viable count were generally less than 1-log₁₀ CFU per well (Figure 3). On the other hand, compared to strains with the highest amounts of biomass, half of all low biomass-producers (irrespective of the clonal lineage) showed a substantially larger additional reduction in viable count (up to 3-log₁₀) upon rifampicin addition. However, mutants associated with MLST

CC30, which were also low biomass-producing strains, showed approximately 20% enhanced biomass formation in the presence of rifampicin (Figure 2B and 3). Consequently, these bacteria became more embedded upon rifampicin addition, resulting in rather no difference in effect on viable count, compared to oxacillin alone (Figure 2A and 3).

Discussion

Medical device associated infections such as orthopedic implant-related infections and prosthetic valve endocarditis or infections due to contaminated intravenous lines have been associated with bacteria embedded in biofilm^{12,13,15}. In clinical practice, rifampicin is used as additional therapy in foreign body-related infections due to *S. aureus*². Since contradictory results of rifampicin as adjunctive agent for the treatment of *S. aureus* biofilm related infections have been reported^{5,13}, we performed *in vitro* studies and demonstrated that eradication with rifampicin in combination with oxacillin displays inter-strain variability, both for rifampicin-susceptible isolates and rifampicin-resistant mutants.

Both the concentration of the adjunctive agent and the companion agent are essential for the outcome of the combination. A dose depending effect of the companion drug have been described for beta-lactams and fluoroquinolones^{16,33}, but was not observed with oxacillin in our experiment. Similar results were obtained with oxacillin at 2 and 4× MIC. Furthermore, the reduction in the amount of biomass at 0.008 µg/mL of rifampicin added to oxacillin at 2× MIC was similar as for oxacillin alone, both for rifampicin-susceptible *S. aureus* isolates and rifampicin-resistant mutants. Apparently, subinhibitory concentrations of rifampicin were not sufficient to improve biofilm disintegration. In general, rifampicin at 0.008 µg/mL did not induce biomass formation in contrast to what has been described for subinhibitory concentrations of certain antibiotics and various (subsets of) bacterial species^{31,32}. However, a possible inducible effect of a subinhibitory concentration of rifampicin could not be ruled out, since it was previously demonstrated that a particular subinhibitory concentration (range) could be responsible for induction³¹. To prevent exposure of bacteria to subinhibitory concentrations of rifampicin, in clinical practice a high-dosage regimen (such as twice daily 600 mg) of rifampicin should be recommended, taking in consideration the potential risks against the assumed benefits, which will be discussed below.

When rifampicin at 1 µg/mL was used as an adjunct to oxacillin, an additional biomass reduction ≥ 20% was observed for 15 out of 17 rifampicin-susceptible *S. aureus* isolates, whereas only 10 out of 17 showed a reduction in viable count of more than 1-log₁₀ CFU per well compared to the most active single agent. It is likely that the outer parts were mainly destructed by the antibiotics, resulting in a decrease in biomass, whereas the majority of bacteria located at the centre of the biofilm was less effected by rifampicin.

Environmental triggers, including subinhibitory antibiotic concentrations, can specifically activate biomass formation of certain bacterial species, for example by influencing the production of exopolysaccharides or by disturbing the expression of signal transduction molecules that regulate cell surface adhesiveness^{31,32,34,35}. Although the multiples of the MIC used for oxacillin and rifampicin were not subinhibitory for planktonic bacteria,

they might be the optimal subinhibitory concentration for bacteria embedded in biofilm to stimulate biomass formation. Due to the relatively strong biomass forming capacity of strain BF35 probably no high antibiotic levels inside the biofilm could be achieved, resulting in stimulation of biomass formation by oxacillin or rifampicin. It needs to be elucidated whether this observation is a rare example, or representative for other strong biomass producing strains.

In contrast to the rifampicin-susceptible parent isolate of BF35, the rifampicin-resistant mutant did not induce biomass upon rifampicin exposure. For unknown reasons, the rifampicin-resistant mutants of strains associated with CC30 showed the opposite, i.e. stimulation of biomass formation by rifampicin, whereas this was not observed for the corresponding rifampicin-susceptible isolates. Killing of embedded *S. aureus* was impaired in most cases in which biomass formation was stimulated by rifampicin or oxacillin. Inter-strain variability of responses to rifampicin addition might be clarified by differences in composition of biofilm matrices. Factors such as differences in expression of certain surface adhesins³⁶ and dissimilarities in release of genomic DNA^{37,38}, are the main causes of variations in composition.

Overall, rifampicin was significantly less successful in achieving additional changes to the effect of oxacillin alone on biomass ($P < 0.01$) and viable count ($P < 0.05$) of biofilms of rifampicin-resistant mutants. After oxacillin and rifampicin exposure, no decrease in biomass of these mutants was observed and only 3 out of 17 displayed (a tendency towards) a synergistic effect on reducing the viable count, while the opposite was found for 7 out of 17 mutants. These results might suggest that continuation of rifampicin use in case of rifampicin-resistant mutants should not be recommended. Long-lasting persistent infections may be attributed to the emergence of resistant subpopulations, which unfortunately could not always be detected by routine susceptibility testing.

Beside the disadvantage of the emergence of rifampicin-resistant isolates and the unpredictable outcome of the adjunctive use of rifampicin, it is necessary to take other disadvantages of the use of rifampicin into consideration, which include hepatotoxicity⁵ and drug-interactions¹⁸. A novel rifampicin derivate was developed to overcome poor clinical tolerance and important drug interactions due to induction of the cytochrome p450 system, especially CYP3A and CYP2C³⁹. However, this compound has not been extensively investigated and is not yet commercially available. Other compounds active against dormant bacteria are under investigation¹¹.

In conclusion, based on current knowledge summarized in reviews^{5,12-14}, adding rifampicin to standard antimicrobials warrants a careful risk-benefit assessment for the individual patient since its efficacy is uncertain, severe side effects and important drug-interactions which can not be waved aside are known and rifampicin-resistant mutants can emerge. The unpredictable outcome of rifampicin tested *in vitro* against *S. aureus* biofilm emphasizes the need for controlled clinical trials of sufficient power to provide firm answers. Furthermore, it seems prudent to omit rifampicin administration at the moment that resistant mutants are observed or suspected, since rifampicin demonstrated to be inefficacious against biofilms of rifampicin-resistant mutants *in vitro*.

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CHAPTER 4

Hydrophilic surface coatings with embedded biocidal silver nanoparticles and sodium heparin for central venous catheters

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Abstract

Central venous catheters (CVCs) have become indispensable in the treatment of neonates and patients undergoing chemotherapy or hemodialysis. A CVC provides easy access to the patient's circulation, thus enabling facile monitoring of hemodynamic parameters, nutritional support, or administration of (cytostatic) medication. However, complications with CVCs, such as bacterial bloodstream infection or thromboembolism, are common. Bloodstream infections, predominantly caused by *Staphylococcus aureus*, are notoriously difficult to prevent and treat. Furthermore, patients receiving infusion therapy through a CVC are at risk for deep-vein thrombosis, especially of the upper limbs. Several recent clinical trials have shown that prophylactic anticoagulation (low-molecular-weight heparin or vitamin K antagonists) is not effective. Here, we report on the systematic development of a new bifunctional coating concept that can –uniquely– be applied to make CVC surfaces antimicrobial and antithrombogenic at the same time. The novel coating consists of a moderately hydrophilic synthetic copolymer of N-vinylpyrrolidinone (NVP) and n-butyl methacrylate (BMA), containing embedded silver nanoparticles (AgNPs) and sodium heparin. The work demonstrates that the AgNPs strongly inhibit adhesion of *S. aureus* (reference strain and clinical isolates). Surprisingly, heparin not only rendered our surfaces practically non-thrombogenic, but also contributed synergistically to their biocidal activity.

Introduction

Central venous catheters (CVCs) are used ubiquitously during treatment of critically ill cancer patients. According to recent estimates, more than 5 million cancer patients in the US require central venous access each year¹. A similar estimate was made for Europe. CVCs offer important advantages, such as facile sustained administration of cytostatic or pain-killing medication, infusion of stem cells, continuous measurement of hemodynamic parameters, or sustained nutritional support. However, application of CVCs is associated with a significant risk for adverse effects, particularly bloodstream infection²⁻⁵ and thromboembolism⁶⁻¹⁰. In the US, approximately 80,000 CVC-related nosocomial bloodstream infections occur annually. The associated extra cost is in the range of \$300 million to \$2.3 billion per year, and the attributable mortality is around 20%. On an average, survivors usually remain one extra week in the intensive care unit, or 2–3 additional weeks in the hospital.

Hence, prevention of CVC-related complications is of paramount importance. Regarding infection, preventive strategies include the use of (i), a maximum sterile barrier during CVC insertion; (ii), innovative catheter hubs, and (iii), chlorhexidine-containing cutaneous antiseptics^{2-5,11}. Moreover, strict adherence to evidence based protocols for hygiene and sterility proved highly successful¹¹. Prevention of thrombotic complications is mostly attempted through administration of anticoagulants during treatment⁶⁻¹⁰. Despite all efforts, it is evident that there is a need for improved biomaterials for the manufacture of safer catheters. Engineering into this direction must focus on the catheter's surface, which must have broad-spectrum antimicrobial activity as well as excellent blood compatibility. We describe the systematic development of new bifunctional surface coatings that –uniquely– meet these requirements.

Materials and methods

Formulation

Six different coating solutions were prepared as follows: (i), 600 mL of 10 % solution of the hydrophilic copolymer (SS) in NMP was equally divided over six 500-mL glass bottles. (ii), Sodium heparin (3 × 1.5 g, purchased from Celsus Laboratories, Cincinnati, OH, USA) was dissolved (mechanical stirring) in formamide (75 mL). The solution was split into three equal parts, and these were mixed with three of the SS solutions as indicated in Table 1. (iii), Silver nanoparticles, AgNPs, (Ag6V or Ag4E; 3.0 g, purchased from Metalor SA, Neuchâtel, Switzerland; Ag6V and Ag4E differed with respect to the hydrophilic surface coating used for their stabilization) were dispersed in NMP and mixed with two of the SS solutions as follows: Coating #1 (SS): SS solution (100 mL) + NMP (50 mL). Coating #2 (SS-Hep): SS solution (100 mL) + NMP (25 mL) + 1.5 g heparin dissolved in formamide (25 mL). Coating #3 (SS-Ag6V): SS solution (100 mL) + NMP (50 mL) + nanosilver (1.5 g). Coating #4 (SS-Ag6V-Hep): SS solution (100 mL) + NMP (25 mL) + nanosilver (1.5 g) + 1.5 g heparin dissolved in formamide (25 mL). Coating #5 (SS-Ag4E): SS solution (100 mL) + NMP (50 mL) +

nanosilver (1.5 g). Coating #6 (SS-Ag4E-Hep): SS solution (100 mL) + NMP (25 mL) + nanosilver (1.5 g) + 1.5 g heparin dissolved in formamide (25 mL). Coatings had a thickness of 3.0 μm , and differed only with respect to the embedded species. Fabrication of the specimens involved three consecutive steps: (i) formulation of coating suspensions through mixing of the NVP/BMA copolymer (designated SS) + AgNPs suspended in N-methylpyrrolidone and/or sodium heparin dissolved in formamide; (ii) application of the coating onto a long (approximately 300 m) and thin (178 μm diameter) stainless steel wire in a continuous process; (iii), coiling of the coated wire around a rotating mandril of 600 μm diameter^{12–14}.

Table 1. Atomic concentrations (percentages) measured by XPS. All data represent averages of two independent measurements

Number	Coating	SS solution (mL)	NMP (mL)	Nanosilver (g)	Formamide (mL)	Sodium heparin (g)
1	SS	100	50	-	-	-
2	SS-Hep	100	25	-	25	1.5
3	SS-Ag6V	100	50	1.5	-	-
4	SS-Ag6V-Hep	100	25	1.5	25	1.5
5	SS-Ag4E	100	50	1.5	-	-
6	SS-Ag4E-Hep	100	25	1.5	25	1.5

Methods and equipment

The platelet-stabilizing anticoagulant mixture, citrate-theophylline-adenosine-dipyridamole (CTAD) was purchased from Becton-Dickinson (Alphen a/d Rijn), the Netherlands. Brain Heart infusion broth and Mueller-Hinton agar were from Oxoid BV (Badhoevedorp, the Netherlands). The lactate dehydrogenase (LDH) assay was performed using the Cyto-Tox 96 Non-Radioactive Cytotoxicity Assay from Promega Benelux BV (Leiden, the Netherlands). Platelet activation was quantified with the Assachrom β -TG linked immunosorbent assay (ELISA), obtained from Roche Diagnostics Nederland BV (Almere, the Netherlands). Fluorescence tracings were recorded on a SpectraMax M2 spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA). Absorbances were measured on an ELx808 Absorbance Microplate Reader (BioTek Instruments Inc., VT, USA). Sputter coating for electron microscopy was performed with a Sputter Coater 108/SE (Cressington Scientific Instruments Ltd., Watford, UK). Scanning electron microscopy was performed with a Philips XL30 instrument (Philips, Eindhoven, the Netherlands). X-ray Photoelectron Spectroscopy measurements were carried out in a Quantera SXM instrument from Ulvac-PHI (Q2). During the measurements, the angle between the axis of the analyzer and the sample surface was 45°. The information depth is then appr. 6 nm. The measurements have been performed using monochromatic AlK α radiation in High Power mode (100 Watt, measuring spot 100 μm , scanned over 1400 μm x 500 μm).

Experiments with bacteria

Overnight cultures were prepared by inoculation of a bacterial colony into 10 mL growth medium (37 g/L heart infusion broth). From this culture, a bacterial suspension of 10⁷

CFU/mL was prepared in 0.9 % NaCl. Coated coils (length 30 mm) were incubated at 37 °C for 60 min in 1.5 mL bacterial suspension, in a shaking incubator. The samples were subsequently transferred to a fresh volume of 1.5 mL 0.9 % NaCl, and vortexed for 1 s. Vortexing resulted in detachment of loosely adhered bacteria. Then, the samples were transferred again to fresh volumes of 1.5 mL of 0.9 % NaCl, and left for 4 h under continuous shaking. Subsequently, the catheter samples were carefully removed, washed in 0.9 % NaCl, and rolled over a Mueller-Hinton blood agar plate (38g/L Mueller-Hinton agar, 5 % defibrinated sheep blood). Plates were incubated overnight and photographed and evaluated on the next day. The six sample groups were subjected to these tests in three-fold.

Thrombin generation experiments

Freshly prepared human platelet-rich blood plasma (PRP) was used in the thrombin generation assays. There were two donor groups: a first group consisting of five healthy male volunteers (ages 22, 23, 24, 25, and 51 years), who were non-smokers and non-users of any drugs that could possibly influence hemostasis. The second group consisted of a cohort of nine patients who were under treatment in the Maastricht University Medical Center. These patients all received high-dose cytostatic medication through a central venous catheter; patient data are summarized in Table 2.

Table 2. Data on patients and/or blood donors

Patient	Age (gender)	Length (m) / mass (kg)	Specified malignancy	Thrombocyte counts (10 ⁹ /mL) [†]	Leukocyte Counts (10 ⁹ /mL) [‡]
1	50 (f)	1.64 / 47	AML*	134	1.4
2	61 (m)	1.72 / 64	Histiocytic sarcoma	52	0.2
3	61 (f)	1.58 / 53	AML	145	3.0
4	60 (m)	1.85 / 104	AML	51	1
5	58 (f)	1.63 / 69	Amyloidosis	107	10.5
6	66 (m)	1.83 / 65	MGUS + C1-esterase deficiency	281	4.5
7	76 (m)	1.75 / 60	Mantle cell NHL**	120	0.8
8	44 (m)	1.80 / 77	AML	252	6.2
9	55 (m)	1.72 / 109	Multiple myeloma	27	1.5

*AML = acute myeloid leukemia

**NHL = non-Hodgkin's lymphoma

[†]Normal thrombocyte concentration: 150 – 450 × 10⁹ / L

[‡]Normal leukocyte concentration: 4.5 – 10 × 10⁹ / L

Patient #5 was the only patient receiving anticoagulant medication (low-molecular-weight heparin) at the time of blood sampling.

Informed consent was obtained in accordance with the Declaration of Helsinki, and the study was approved by the Maastricht University Medical Center ethical committee. The volunteers each donated approximately 40 mL blood through venipuncture. The collection tubes contained citrate for anticoagulation (end-concentration 0.013 M citrate). The patients donated 20 mL blood each. In these cases, Vacutainers containing citrate for anticoagulation were used. PRP was isolated through centrifugation (200g, 15 min, room temperature). PRP was carefully transferred into new tubes and kept at 37 °C until further use.

Thrombin generation experiments were done according to a one-donor-on-one-day scheme. On such an experimental day, 30 thrombin generation curves were measured (6 experimental coatings, each experiment in five-fold). The experiments were done in four consecutive steps: (i), Five pieces of 25 mm were cut out of each of the six different coils. Then, each piece was cut further into five approximately equal pieces, and these were transferred into one single well of a 96 well plate, (ii), The fluorogenic substrate for thrombin, Z-Gly-Gly-Arg-AMC (a product of Bachem Holding AG (Bubendorf, Switzerland; ref. I-1140) was added to the citrated PRP to a final concentration of 400 μM . Then, the PRP was “recalcified” through adding CaCl_2 stock solution (0.5 M) up to a final concentration of 20 mM Ca^{2+} . Consequently the mechanism for intrinsic (i.e. biomaterial-surface-induced) coagulation is no longer inhibited, (iii), The PRP was then rapidly distributed over the wells; 200 μL was transferred into each well. All pieces of the coiled wire were submersed in PRP. Wells without coil-samples served as controls. (iv), Fluorescence tracings were recorded at 37 °C. Wavelengths of excitation and measuring were 368 and 460 nm, respectively. Data were collected every 30 s; the plate was gently shaken for 2 s prior to each measurement. The fluorescence intensity was converted into nanomolar concentrations of thrombin. This technique resulted in a thrombin generation curve for each well.

Statistical analysis

Data were analyzed with a one-way analysis followed by a Bonferroni correction for determining differences between groups. Differences were considered as statistically significant with $P < 0.05$.

Table 3. Atomic concentrations (percentages) measured by XPS. All data represent averages of two independent measurements.

Entry	Surface	C1s	N1s	O1s	Ag3d	S2p (-SO ₃ H)
1	SS	79.7	4.6	14.4	-	0.21
2	SS-Hep	78.6	4.3	14.5	-	0.43
3	SS-Ag6V	80.4	4.2	12.6	0.22	0.21
4	SS-Ag6V-Hep	79.2	5.0	14.2	0.11	0.42
5	SS-Ag4E	79.3	6.6	13.0	0.15	0.24
6	SS-Ag4E-Hep	78.9	5.3	14.6	0.19	0.35

Results

Samples and sample characterization

The continuous coating process afforded a huge inventory of virtually identical specimens for every formulation. This approach distinguishes this study from other investigations on biomaterial surface coatings obtained through dipping or spraying, which are discontinuous methods that generally introduce substantial noise in subsequent experimental data. Specimens were first studied with X-ray photoelectron spectroscopy (XPS). Figure 1B shows a representative wide-scan spectrum. Data were collected with a spot size of only 100 μm at two positions per specimen, i.e., on the top of two individual windings. The

information depth was approximately 6 nm. Figure 1C shows narrow-scan XPS spectra of SS-Ag4E-Hep (sulfur and silver lines). The apparent atomic concentrations are compiled in Table 3.

Figure 1. XPS spectra of surface coating SS-Ag4E-Hep, containing silver nanoparticles and heparin. (A) Photograph of a typical specimen. Note the coiled structure. (B) Wide-scan XPS spectrum of the typical specimen SS-Hep-Ag4E. A small spot size (100 μm) was used, which enabled scanning on the top of one of the specimen's windings. The signal intensity is reported in counts per second (CPS) (C) Narrow-scan X-ray photoelectron spectra of the outermost layer of the SS-Ag4E-Hep surface; left: Ag3d doublet due to surface-exposed AgNPs, right: S2p peak due to the SO_3H groups of heparin.

All duplicate measurements were in good agreement. As expected, only specimens #3–#6 expose silver at their surface. The specimens containing heparin (#2, #4 and #6) show clearly elevated concentrations of sulfur ($-\text{SO}_3\text{H}$; 167.9 ± 0.1 eV) at their surfaces (0.43, 0.42 and 0.35% respectively, vs. 0.21, 0.21 and 0.24% for specimens #1, #3, and #5). Hence, XPS confirms the presence of heparin at the surface of #2, #4 and #6. To the best of our knowledge, this is the first example of the use of XPS to verify the presence of heparin in the outermost regions of biomaterial surface coatings. The presence of sulfur traces in speci-

mens #1, #3, and #5 can be attributed to the binding layer of poly(ethersulfone), which is routinely used in the continuous coating procedure.

Samples and sample characterization

The system used for systematic comparison of the effects of embedded AgNPs and/or heparin is shown in Figure 1A. Fabrication of the specimens involved three consecutive steps: (i) formulation of coating suspensions through mixing of the NVP/BMA copolymer (designated SS) + AgNPs suspended in N-methylpyrrolidone and/or sodium heparin dissolved in formamide; (ii) application of the coating onto a long (approximately 300 m) and thin (178 μm diameter) stainless steel wire in a continuous process; (iii), coiling of the coated wire around a rotating mandril^{12–14}. Note that the continuous coating process afforded a huge inventory of virtually identical specimens for every formulation. This approach distinguishes this study from other investigations on biomaterial surface coatings obtained through dipping or spraying, which are discontinuous methods that generally introduce substantial noise in subsequent experimental data. Note furthermore that two different types of AgNPs were used (designated Ag6V and Ag4E); these AgNPs differed with respect to the hydrophilic surface coating used for their stabilization.

Biocidal activity

The roll-plate method according to Maki *et al.*¹⁵ was used to assess adherence of the reference strain *Staphylococcus aureus* ATCC 29213 to the different coatings. This technique visualizes bacterial colonies on each roll track, provided that bacterial adhesion occurred during the incubation phase. Our cylindrical specimens were convenient to use in this assay, and data-reproducibility was excellent. Virtually no bacteria could be found on the agar plates for SS-Ag6V, SS-Ag6V-Hep, SS-Ag4E, and SS-Ag4E-Hep, whereas bacteria were abundantly present on the agar plates for SS and SS-Hep (Figure 2 and Figure 3A, B and C). These data confirm the strong antibacterial activity of the AgNPs Ag6V and Ag4E. Furthermore, it appeared that heparin exerts a pronounced antibacterial effect by itself (compare SS and SS-Hep). The antimicrobial effects of AgNPs and heparin are additive: it is clear that much less bacteria adhered to the SS-Ag6V-Hep surface than to SS-Ag6V alone. Analogously, SS-Ag4E-Hep has less adhered bacteria (if any are present at all) than SS-Ag4E. At first sight, we were puzzled by this heparin effect; several recent papers pointed out that heparin *per se* has no antibacterial effects¹⁶, and even that heparin may *promote* biofilm formation, especially for *S. aureus*^{17,18}. Interestingly, recent work of Schmidtchen *et al.* may explain our observations in terms of an indirect effect of heparin: proteins carrying structural characteristics that impart affinity for heparin (positive charges and consensus regions such as the Cardin motif or the Weintraub motif), *also* have antimicrobial properties^{19,20}. For example, heparin-binding plasma proteins such as fibronectin, vitronectin, protein-C inhibitor and von Willebrand factor are biocidal for Gram-positive and Gram-negative bacteria. Thus, if incubation of our coatings in plasma first leads to adsorption of proteins with an affinity for heparin, then these proteins introduce biocidal activity at the coating's surface in turn.

Figure 2. Bacterial colonies that developed on the sheep-blood columbia agar plates (roll-plate method according to Maki *et al.*¹⁵). Experiments were done with six different coatings (one per column), and in four-fold (four rows). The specimens were carefully rolled from the right side to the left side of the dishes, thereby releasing bacteria that generated visible colonies afterwards. Note the presence of many colonies for the SS coating, compared to the SS-Hep coating. Bactericidal effects of the AgNPs are clearly visible.

Next to *S. aureus* ATCC 29213, we studied two clinical isolates (MRSA BF110 and MSSA BF45), i.e., *S. aureus* bacteria that were isolated from patients with a catheter-associated bloodstream infection. The biocidal effects of our materials on the clinical isolates (BF110 and BF145) were, in fact, stronger than those found for the reference strain (Figure 3D).

Figure 3. Roll-plate analysis carried out in agar-covered Petri dishes. In each dish, two identical specimens were rolled horizontally from one end of the Petri dish to the other end. Colonies were allowed to grow for 24 h. Specimens were not removed. (A) Enlarged view of the abundant growth of bacterial colonies that were transferred from the SS specimen to the agar. (B) Same experiment, carried out with SS-Hep. Compared to SS, less colonies are found, showing that heparin exerts a significant biocidal effect by itself. (C) Same experiment with SS-Ag4E-Hep, showing the combined antimicrobial effect of heparin and AgNPs. Note the presence of the specimens at the top of A, B, and C. (D) Quantitative data of the roll-plate analysis. Data are expressed as mean $\pm$ SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ SS-Ag4E-Hep or SS-Ag6V-Hep versus SS-Ag4E or SS-Ag6V without heparin, respectively; # $P < 0.001$ SS-Hep versus SS only.

***In vitro* assessment of blood compatibility: the thrombin generation assay**

The six different surfaces were subjected to a thrombin generation assay^{12,13}. Platelet-rich blood plasma (PRP) was used from five healthy volunteer donors and from nine patients undergoing high-dose chemotherapy against a hematological malignancy in the Maastricht University Medical Center (Table 2). We measured so-called thrombin generation curves ([thrombin] vs. time) in six-fold for every specimen-PRP combination. Thrombin lag times, i.e. time elapsing between the moment of recalcification and the moment at which [thrombin] becomes > 2 nM, were abstracted from all curves^{12,13}. Note that the assay measures intrinsic activation of the coagulation system: the biomaterial's surface is the only trigger for thrombin formation (i.e., the assay uses no tissue factor or any other procoagulant agent). As we advocated previously, the thrombin lag time is a valuable measure of

the thrombogenicity of the biomaterial under investigation^{12,13}. The longer the lag time, the higher the level of blood compatibility *in vitro*. Figure 4A compiles lag times measured for our heparin-free specimens in contact with PRP from volunteer donors and patients.

Figure 4. Thrombin generation in PRP from nine patients (abbreviated as P) on high-dose chemotherapy against a hematological malignancy and from five healthy volunteer donors (abbreviated as D) Thrombin lag times measured for coatings SS (black, lower bars), SS-Ag6V (light grey, middle bars), and SS-Ag4E (dark grey, upper bars) (A). Averaged thrombin lag times for the nine patients (viz. Figure 4A) plotted vs. thrombocyte counts. Thrombin lag times were measured for SS (black dots), SS-Ag6V (white triangles), and SS-Ag4E (black squares) (B).

The volunteer data show a clear donor-dependency, which is in agreement with previous findings¹². All heparin-containing specimens, for volunteer donor PRP and patient-derived PRP, had lag times > 60 min, revealing that the anticoagulant activity of heparin is by no means neutralized or significantly compromised by the AgNPs. The thrombin lag times measure for the patients also show considerable spreading. Data measured for patients #3, #6, #7 and #8 compare well with those of the volunteers, others were somewhat longer with appr. 20 min max. Taken together, all thrombin generation lag times are grossly similar, despite the fact that most patients show clear leukopenia and/or thrombocytopenia (Table 2). This reflects, albeit from an unusual point of view, that the regulation mechanism of the blood coagulation system manages to maintain hemostatic balance, even under conditions of severe illness and high-dose chemotherapy. Closer inspection of the patient data showed that one of the patients (#5) received low-molecular-weight heparin at the time of blood sampling. For the other eight patients, the thrombin lag times appeared to correlate inversely with the thrombocyte counts ($r^2 = 0.76$; Figure 4B). A similar but weaker correlation ($r^2 = 0.45$) was found between the thrombin lag times and the leukocyte counts of these patients. This may point out that patients with normal or close-to-normal platelet (and leukocyte) concentrations are likely to be at the largest risk for catheter-associated thrombus formation. Clinical testing of this hypothesis falls outside the scope of this study

but is certainly worthwhile: if true, then simple thrombocyte counting would provide an indication whether or not to provide prophylactic anticoagulation during the catheter-based treatment period (e.g. through (subcutaneous) administration of low-molecular-weight heparin).

***In vitro* assessment of blood compatibility: thrombocyte adherence**

Adhesion of blood platelets to the six experimental coatings was studied further with PRP from healthy donors #2 and #3. The SS surface had the largest density of adherent platelets (LDH assay, appr. 20,000/cm coil). Inclusion of heparin (SS-Hep) or AgNPs (SS-Ag6V) reduced this number to appr. 6,000/cm coil and 5,000/cm coil, respectively. The combination of heparin and AgNPs (Ag6V) reduced the density of adhered platelets further (down to appr. 2,000/ cm coil). Remarkably, the use of Ag4E nanoparticles *per se* (SS-Ag4E) already reduced the density of adhered platelets to approximately 2,000/cm coil. No further effect due to inclusion of heparin (SS-Hep-Ag4E) could be observed.

Scanning electron microscopy showed that platelets on the SS surface were mostly discrete with partly spread morphology. Small pseudopodia by which they attach to the surface were observed. It was very difficult to discern platelets on the three heparin-containing surfaces. On the other hand, adherent platelets could be found easily on the two coatings that contain merely AgNPs (SS-Ag6V and SS-Ag4E). These platelets were mostly associated with distorted morphologies as was observed previously^{12,21,22}.

Discussion

Hydrophilic surface coatings for medical devices have drawn widespread attention during the last years. Adherent hydrophilic coatings provide catheters and guidewires with a “slippery-when-wet” lubricious surface, and this is extremely important for indwelling catheters, but also for delicate interventional procedures, such as percutaneous transluminal coronary angioplasty (PTCA) and super-precise embolization of solid tumors. New developments are focused on embedding of active components in hydrophilic surface coatings. This principle can lead to controlled release of active agents from the device’s surface, and/or to exposure of active molecules at the device’s surface. The combination of hydrophilic coatings with heparin can lead to anticoagulant and lubricious surfaces.

Research on drug-containing hydrophilic surface coatings requires a highly accurate and reproducible coating protocol. Obviously, variations of the coating quality (thickness, adherence, etc.) would generate substantial experimental noise, which could easily obscure effects of active constituents of the coating. Our extrusion-like coating procedure to apply NVP/BMA copolymers as uniform thin layers on thin metallic wires generates a platform of precisely defined experimental coatings. These can be used in detailed comparative studies. For instance, the hydrophilic nature of the materials can be tuned through the composition: the molar ratio NVP (hydrophilic):BMA (hydrophobic) determines parameters like contact angle, aqueous swelling ratio, etc. NVP/BMA coatings can be engineered such that the bio-active compound is either slowly released upon immersion in an aqueous

medium, or remains immobilized at the coating's surface. This principle has already led to the development of, e.g., new devices for controlled delivery of drugs to the tear film of the eye, and heparin releasing/exposing guidewires for intravascular use featuring an excellent level of blood compatibility^{13,24,25}.

Here, we studied NVP/BMA coating formulations in which silver nanoparticles and sodium heparin were co-embedded, with the aim to develop coatings for medical devices that effectively combine antimicrobial AND antithrombogenic features. Our data reveal that AgNPs and heparin can be combined effectively. Interestingly, synergetic effects of heparin and nanosilver were observed: (i), heparin enhances the biocidal activity of the AgNPs, and (ii), no lysis of contacting thrombocytes occurs.; rupture of platelets was observed previously with NVP/BMA coatings that merely contain embedded AgNPs²³. In addition, it is clear that our methodology can readily be scaled-up and transformed into an industrial procedure. We anticipate that this work can provide the basis for the development of safer central venous catheters. There is a clear unmet need for biomaterials and surfaces that combine excellent biocidal properties with zero or close-to-zero thrombogenicity in vivo. Improved CVCs will, next to other measures to prevent infection and thromboembolism, help to improve treatment of many critically ill patients, and to reduce health care cost, especially when prolonged hospitalization can be prevented indeed.

Conclusion

Copolymers of NVP and BMA generate a platform of biocompatible coatings for medical devices. The hydrophilic nature of these materials can be tuned through the composition; the molar ratio NVP (hydrophilic): BMA (hydrophobic) determines parameters like contact angle, aqueous swelling ratio, etc. Furthermore, it has been established that the hydrophobic constituent is responsible for the adherence of the coating to the underlying substrate (polyethersulfone).

The NVP/BMA coatings offer number of interesting options. Firstly, they can be applied onto long wires or tubes in a precisely controlled extrusion-type procedure. Secondly, biologically active species can be embedded in the coatings. The NVP/BMA coatings can be engineered such that the bio-active compound is either slowly released upon immersion in an aqueous medium, or remains immobilized at the coating's surface. This principle has led to the development of, e.g., new devices for controlled delivery of drugs to the tear film of the eye, and heparin releasing/exposing guidewires for intravascular use featuring an excellent level of blood-compatibility.

Now, we have identified NVP/BMA coating formulations that effectively combine biocidal and anticoagulant features. This combination of bioactivities was obtained after embedding both silver nanoparticles and sodium heparin in the NVP/BMA coatings. Silver nanoparticles are known to have a strong biocidal effect against a wide range of microorganisms. Noteworthy, the presence of heparin in the coating has two very beneficial effects: (i), heparin enhances the biocidal activity of the AgNPs, and (ii), no lysis of contacting thrombocytes occurs (this was observed previously with NVP/BMA coatings that merely

contain embedded AgNPs). To the best of our knowledge, no other biocompatible coatings that feature both biocidal and non-thrombogenic behavior have been described so far. In addition, it is clear that our methodology can readily be scaled-up and transformed into an industrial procedure.

We anticipate that this work can potentially provide the basis for the development of safer central venous catheters. Regarding CVCs, there is a clear unmet need for biomaterials and surfaces that combine excellent biocidal properties with zero or close-to-zero thrombogenicity *in vivo*. Improvement of CVCs is a particularly important objective, since complications due to infection and thrombosis are common and hazardous for many patients. Improved CVCs will, next to other measures to prevent infection and thromboembolism, help to improve treatment of many critically ill patients and to reduce health care cost, especially when prolonged hospitalization can indeed be prevented.

Hydrophilic surface coatings for medical devices featuring both biocidal and non-thrombogenic behavior can be prepared. The methodology as described in this work can be scaled-up and transformed into an industrial procedure. This work may help to realize the goal of manufacturing catheters and other medical devices that combine excellent biocidal properties with zero or close-to-zero thrombogenicity *in vivo*.

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CHAPTER 5

Antimicrobial and anti-thrombogenic features combined in hydrophilic surface coatings for skin-penetrating catheters. Synergy of co-embedded silver particles and heparin

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Abstract

Percutaneous (skin-penetrating) catheters such as central venous catheters (CVCs), are used ubiquitously in the treatment of critically ill patients, although it is known that the risks for serious complications, particularly bloodstream infection and thromboembolism, are high. Materials science and engineering offer important new perspectives regarding further improvement of CVCs. A promising approach is the use of synthetic biocompatible hydrogel coatings with both silver particles and heparin embedded therein. Such formulations combine the well-known broad-spectrum antimicrobial features of silver with the anticoagulant activity of immobilized heparin. Previous work revealed that heparin augments antimicrobial activity of silver, while maintaining its anticoagulant function. This study set out to investigate the synergy of heparin and silver in more detail. Exit-challenge tests, experiments on bacterial killing and adherence, as well as *in vitro* challenge tests with three *S. aureus* strains (one reference strain, and two clinical isolates) consistently showed the synergistic effect. In addition, the impact of changing the coating's hydrophilicity, and changing the silver concentration in the coatings, were examined. The experimental results, taken together and combined with data from the literature, point out that synergy of heparin and silver is best explained by binding of Ag^+ ions to heparin within the swollen coating, followed by release of heparin- Ag^+ complexes upon immersion of the coatings in an aqueous environment such as blood. Possible implications of this work regarding the development of improved/safer CVCs are briefly discussed.

Introduction

Central venous catheters (CVCs) play a key-role in the treatment of critically ill patients. For example, CVCs are commonly applied for intravenous delivery of cytostatic agents, blood transfusion, hemodialysis, blood sampling, parenteral nutrition or plasmapheresis. Yet, the use of CVCs is associated with a high incidence of serious complications, particularly blood stream infection¹⁻⁶. Especially indigenous microorganisms such as coagulase-negative staphylococci (CoNSs) and the more virulent *Staphylococcus aureus* are involved^{7, 8}. Infections mostly occur from the port of entry along the CVCs surface⁸. Another important source of complications with CVCs is thromboembolism^{4, 9-11}, due to imperfect blood-compatibility of the blood-contacting interface of the CVC^{12, 13}.

Biomaterials science and engineering can contribute to further technical improvements of CVCs. Evidently, such endeavors must concentrate on the CVC surface. Ideally, the surface should inhibit bacterial adhesion and feature perfect or near-perfect hemocompatibility. In our previous work, we have reported on new synthetic hydrophilic biomaterials with both silver particles and heparin embedded therein¹⁴. These materials were applied as thin surface coatings, with the obvious aim to exploit both the broad-spectrum antimicrobial activity of silver¹⁵⁻¹⁷ and heparin's inhibitory effect on the intrinsic coagulation pathway. Our experiments revealed an unexpected synergistic effect: coatings containing both silver particles and heparin were much more effective in preventing bacterial adhesion, compared to coatings containing merely silver or heparin.

The objective of the present study was to further explore this synergy by: (i), examining different *Staphylococcus aureus* strains, including clinical isolates; (ii), studying the effect of silver particles and heparin on bacterial killing, and by performing exit-site and *in vitro* challenge tests; (iii), studying the effect of the hydrophilicity of the coating matrix; (iv), studying the effect of the silver content of the coating.

Materials and Methods

Materials

Hydrophilic SlipSkin[®] (SS) coating biomaterials were purchased from Interface BIOMaterials BV (Geleen, the Netherlands). Two versions were used: copolymer SS90:10, prepared from N-vinyl-2-pyrrolidinone (NVP) and n-butylmethacrylate (BMA) in a molar ratio 90:10, and the less hydrophilic copolymer SS70:30, prepared from NVP and BMA in a molar ratio 70:30. Sodium heparin was supplied by Celsus Laboratories (Cincinnati, OH, USA). Silver particles were purchased from Metalor SA (Neuchâtel, Switzerland). Two different types were used: Ag4E (stabilized by polyethylene glycol, PEG), and Ag6V (stabilized with polyvinylpyrrolidone, PVP). All experiments were done with Ag4E, in some cases parallel experiments with Ag6V were done as well. Data on Ag6V are described in the Supporting Information. Commercial silver-impregnated catheters were purchased from Vygon BV (Valkenswaard, the Netherlands). The Vygon[®] Multicath Expert 2 lumen catheter (ref. 8157.207)

was used throughout this work for benchmark referencing. The active antimicrobial constituent is the silver-based agent AgI_{ON}, which is not just a surface coating, but is incorporated into the material from which the tube is made.

Table 1. Composition of the seven solutions that were used in the preparation of the specimens for this study. Ag, refers to Ag4E silver particles

No.	Coating	SS90:10 / SS70:30 [mL]	Ag [g]	NMP [mL]	Sodium-Heparin [g]	Formamide [mL]
1	SS90:10	100	-	50	-	-
2	SS90:10-Hep	100	-	25	1.5	25
3	SS90:10-Ag	100	1.5	50	-	-
4	SS90:10-Ag-Hep	100	1.5	25	1.5	25
5	SS70:30-Ag	100	1.5	50	-	-
6	SS70:30-Ag-Hep	100	1.5	25	1.5	25
7	SS70:30-Ag2x-Hep	100	3.0	25	1.5	25

Formulation

Seven different coating solutions were prepared as is compiled in Table 1. First, 400 mL of a 10% solution of SS90:10 in N-methylpyrrolidone (NMP) was prepared. This solution was split into four equal parts. Secondly, 300 mL of a 10 % solution of SS70:30 in NMP was prepared; this solution was split into three equal parts. Thirdly, sodium heparin (6.00 g) was dissolved in 100 mL of formamide. This solution was split into four equal parts and these were mixed with two of the SS90:10 solutions (#s 2 and 4, Table 1) and two of the SS70:30 solutions (#s 6 and 7, Table 1). Fourthly, Ag4E silver particles (3.00 g) were dispersed in NMP (100 mL) through mechanical stirring, split into two equal parts, and combined with SS90:10 solution #3 and SS70:30 solution #5. Analogously, 3.00 g silver was dispersed in NMP (50 mL), split into two equal parts and combined with SS90:10 solution #4 and SS70:30 solution #6. Finally, silver (3.00 g) was dispersed in NMP (25 mL) and combined with SS70:30 solution #7. Note that in this case a double content of silver was achieved.

Analogously to the procedure described above for the Ag4E silver particles, two different coating solutions were prepared with Ag6V silver particles (with and without sodium heparin), as is compiled in Table 2.

All coating solutions were used in MCTec BV's procedure for the continuous and precisely controlled application of thin adherent polymer coatings on long metallic wires, as described previously¹⁸. Stainless steel wires with a diameter of 178 µm were used. The thickness of the coatings was 3–4 µm. Coated wires were coiled, using a rotating mandril of 600 µm diameter.

Table 2. Composition of the two solutions with Ag6V silver particles (with or without heparin) that were used in the preparation of the specimens for this study.

No.	Coating	SS90:10 [mL]	Ag6V [g]	NMP [mL]	Sodium-Heparin [g]	Formamide [mL]
1	SS90:10-Ag	100	1.5	50	-	-
2	SS90:10-Ag-Hep	100	1.5	25	1.5	25

Bacterial strains

The *Staphylococcus aureus* reference strain ATCC 29213, and two clinical *S. aureus* isolates (coined BF110 and D107) were tested. BF110 (meticillin-resistant, MRSA), previously shown to be a strong biomass producer¹⁹, with the genetic background associated with multi-locus sequence typing (MLST) clonal complex (CC)8. D107 (meticillin-susceptible, MSSA), was obtained from a patient with a CVC-related bloodstream-infection.

Exit site challenge

An *in vitro* method was adopted that determines the ability of antimicrobial coatings to prevent migration of bacteria along the external surface of a CVC after cutaneous colonization and invasion of the insertion site. Coils (30 mm length) were inserted half into 35-mm thick solidified 28 g/L nutrient (Oxoid) agar, which was in advance poured into containers (4 cm diameter). Afterwards, the surrounding surface of the insertion site was inoculated with *S. aureus* ATCC 29213, 10⁶ CFU/mL using a swab, mimicking the circumstances in which an insertion site on the skin is adequately disinfected before entrance of a CVC and the cutaneous port of entry later becomes re-colonized. To mimic not properly disinfected skin surface, coils were also inserted after inoculation of the agar surface, as described previously.²⁰ Migration of bacteria down the coils along the abluminal coating surface was assessed visually for up to seven days during incubation at 37°C.

Bacterial killing

Coils were segmented into 30-mm long test-samples by cutting under aseptic conditions. The length of the Vygon® catheter pieces was corrected for external and internal surface area. The specimen parts were added separately to 10-mL NaCl 0.9% tubes containing a bacterial inoculum of 10⁶ CFU/mL and incubated at 37°C. At t = 0, 2, 4, 8, 12, 24, 48 and 72 h samples of 100 µL were drawn to count viable bacteria. The lower limit of detection was 2.73 log₁₀ CFU/mL, using a spiralplater (Eddy Jet; IUL Instruments, Barcelona, Spain) as part of the enumeration procedure.

Bacterial adherence

Specimen pieces of 30 mm were separately exposed to bacterial suspensions to assess adherence of ATCC 29213, BF110 and D107 to the different coating surfaces. The length of

the Vygon[®] catheter pieces was corrected for external surface area. To mimic infusion through and/or flushing of CVC prior to colonization or infection, a part of the specimens was pre-washed in Milli-Q water or plasma (Sanquin, Amsterdam, the Netherlands). The duration of pre-wash varied between 0 and 72 h. The bacterial exposure time (10^5 , 10^6 or 10^7 CFU/mL in 1.5 mL NaCl 0.9%) varied between 1 and 8 h at 37°C. The inoculum was maintained at the same level by replacement of the inoculum each hour and was checked before and after each replacement.

Non-adhered bacteria were removed by vortexing each specimen for 1 s in fresh 1.5 mL NaCl 0.9% and transferring it to another tube with 1.5 mL NaCl 0.9%, which was kept in a shaking incubator at 37°C for 4h. Subsequently, specimens were rolled over Mueller-Hinton agar plates containing 5% defibrinated sheep blood, according to a roll-plate assay described previously.²¹ After overnight incubation at 37°C, the numbers of CFU per cm² of each roll track (33 cm²) were determined.

***In vitro* challenge**

In order to determine which coating provided the best anti-adhesive properties over a prolonged period of time, a repeating set-up of the adherence assay was used in which exposure to *S. aureus* ATCC 29213 (10^5 CFU/mL NaCl 0.9% at 37°C) was alternated with roll-plate assessment.²¹ The series was terminated after 25 challenges.

Statistical analysis

SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Independent t-tests were used to compare bacterial adherence data. A *P* value of ≤ 0.05 was considered to be statistically significant.

Figure 1. Scanning electron micrograph of a typical specimen, showed the coiled structure.

Results

Specimens

Preparation of the coiled specimens proceeded smoothly; Figure 1 shows a typical example. The advantage of this procedure is that numerous identical specimens can be produced. Reproducibility is an important issue, especially in comparative studies of surface coatings.

Figures 2A and B show scanning electron micrographs of the silver particles (Ag4E). Note that these particles are microparticles rather than nanoparticles, since their smallest dimension is approx. 1 μm . Figures 2C – F show light-microscopic images of our wires prior to coiling: SS90:10 (C), SS90:10-Hep (D), SS90:10-Ag (E), and SS90:10-Ag-Hep (F). Note the alignment of the rod-shaped silver particles at the wire's surface (E and F).

Figure 2. Scanning electron micrographs of the silver particles Ag4E, which were used throughout this work (A,B). Light-microscopic images of the coated wires SS90:10 (C), SS90:10-Hep (D), SS90:10-Ag (E), and SS90:10-Ag-Hep (F). Note that (i), the wires in C-F were photographed prior to coiling; (ii), the extrusion-like coating procedure results in some alignment of the silver particles in the direction of the wire (E,F).

Figure 3. Exit site challenge. First signs of ATCC 29213 migration extending along the coating surfaces of SS90:10 (A) and SS90:10-Hep (B), observed 24 h after inoculation of the surrounding agar surface at the insertion site, respectively. (C) No migration of ATCC 29213 along the coating surface (existing of SS90:10-Ag-Hep) was observed over 168 h. (D) Growth of inserted bacteria at the surface of SS90:10. (E) Vertical movement and/or growth of bacteria directed away from the coating surface (SS90:10-Ag-Hep) through the agar, when specimens were inserted after inoculation of the insertion site.

Exit site challenge

The set-up of this test essentially mimics the situation in which the insertion site on the skin is first disinfected, and later becomes invaded by bacteria after cutaneous recolonization. For the SS90:10 and SS90:10-Hep coatings, clear bacterial migration along the specimens was observed after 24 h (Figure 3A,B). Growth extended further down the coiled wires within 72 h. Coatings SS90:10-Ag and SS90:10-Ag-Hep did not show bacterial migration under the same conditions, indicating that the silver particles effectively inhibit movement and growth of bacteria. Still no bacterial invasion was noticed after 168 h of incubation (Figure 3C). Changing the matrix into the less hydrophilic version (SS70:30) influenced the outcome of the test. The surfaces SS70:30-Ag and SS70:30-Ag-Hep prevented bacterial migration within 48 h, but some signs of migration were observed after 72 h. The test was also performed in such a way that the agar surface was first inoculated with *S. aureus* ATCC 29213, as described previously.²⁰ Essentially, this would mimic the situation in which the skin surface was not properly disinfected before insertion.

For all specimens, it was observed that bacteria were transferred with the specimen into the agar. Subsequently, bacterial growth occurred in all cases, but there was a clear distinction between the reference coatings (SS90:10 and SS90:10-Hep) on one hand, and the silver-containing coatings on the other hand. The reference coatings showed bacterial growth along their surfaces (Figure 3D), whereas the silver-containing coatings directed

bacterial growth more away from the specimens, i.e. perpendicular to their surfaces (Figure 3E).

Figure 4.

Antibacterial activity of different coating compositions (containing Ag4E silver particles and/or heparin) over time against inocula of 10^6 CFU/mL *S. aureus* (A) ATCC 29213, (B) BF110 and (C) D107. Error bars indicate standard errors (SE).

Bacterial killing

Figure 4 shows data on survival of bacteria of the *S. aureus* reference strain ATCC 29213 (Figure 4A), and the clinical isolates BF110 and D107 (Figure 4B,C) upon incubation with the different test coatings, up to 72 h. Figure 4A reveals that SS90:10 and SS90:10-Hep did not influence the survival of ATCC 29213. Incorporation of silver (SS90:10-Ag) resulted in killing of a substantial portion of the bacteria, such that the number of bacteria dropped with 2–3 orders of magnitude. Embedding the same amount of silver in the less hydrophilic matrix (SS70:30) did not introduce a major difference. Strikingly, introduction of

silver *and* heparin led to almost complete killing of ATCC 29213 within 72 h. This effect was independent of the coating's hydrophilicity (SS90:10 vs. SS70:30). Doubling of the amount of silver (in SS70:30-Ag_{2x}-Hep) reduced the viable count similarly. Noteworthy, the commercial Vygon® catheter, which is claimed to have an antibacterial surface²², had no impact on the survival of ATCC 29213 within 72 h.

Figure 4-S. Antibacterial activity of different coating compositions (containing Ag6V silver particles and/or heparin) over time against inocula of 10⁶ CFU/mL *S. aureus* (A) ATCC 29213, (B) BF110 and (C) D107. Error bars indicate standard errors (SE).

The experiments with the *S. aureus* clinical isolates BF110 and D107 led to highly similar bacterial time-kill curves. Again, no variation in bacterial counts occurred upon incubation with SS90:10 or SS90:10-Hep. Furthermore, combining silver particles and heparin (SS90:10-Ag-Hep) substantially improved the bactericidal activities, achieving practically

complete kill (99.99%) within 24–48 h. Although eradication of the reference strain seemed independent of the amount of embedded silver and the coating hydrophilicity, substantial differences were seen regarding the clinical isolates BF110 and D107. Compared to SS90:10-Ag-Hep, SS70:30-Ag-Hep reduced the viable count of these isolates more slowly. However, whenever a double amount of silver particles was incorporated in the less hydrophilic coating (SS70:30-Ag_{2x}-Hep), similar reductions in viable count of BF110 were obtained as with SS90:10-Ag-Hep. Moreover, the fastest killing of D107 was observed with SS70:30-Ag_{2x}-Hep, which exceeded even the eradication time of SS90:10-Ag-Hep. No bactericidal effect was noticed when testing the Vygon[®] catheter pieces.

As shown in Figure 4-S, highly similar antimicrobial effects were realized for the PVP-stabilized silver particles. Generally, the biocidal effects of PEG-stabilized silver particles were slightly stronger than those of their PVP-stabilized counterparts.

Figure 5. Adherence of *S. aureus* ATCC 29213 to different specimens during incubation for 1–8 h in 10^5 CFU/mL (A). The same data expressed as normalized effect score (B). Adherence was assessed by roll-plate analysis. Asterisks denote statistically significant difference, (*) $P < 0.05$ and (**) $P < 0.01$.

Figure 6. Adherence of *S. aureus* ATCC 29213 to different specimens during incubation for 1 h in 10^6 CFU/mL after pre-wash of the specimens with milliQ water (MQ) for 12–72 h (A). The same data expressed as normalized effect score (B). Adherence was assessed by roll-plate analysis. Asterisks denote statistically significant difference, (*) $P < 0.05$ and (**) $P < 0.01$.

Bacterial adherence

Adherence to different specimens was examined after various washing periods and incubation times. Figure 5A shows data on the adherence of ATCC 29213 to different specimens after incubation in a constant inoculum of 10^5 CFU/mL for 1, 2, 4 or 8 h. The adherence to the SS90:10-coating was influenced by the incubation time; the longer the exposure the more adherence of ATCC 29213 was seen. Considerably more bacteria adhered to this coating compared to the other ones, which occurred already from on-set. Initial ad-

herence was much lower for coatings containing both (Ag4E) silver particles *and* heparin compared to silver particles alone. There was also a difference in initial adherence when the hydrophilicity of the coating was taken into account. The more hydrophilic coatings SS90:10-Ag and SS90:10-Ag-Hep adhered less bacteria compared to SS70:30-Ag and SS70:30-Ag-Hep, respectively. Especially the difference in initial adherence between the SS90:10-Ag and SS70:30-Ag coatings is noteworthy.

Figure 7. Adherence of *S. aureus* ATCC 29213 to different specimens during incubation for 1 h in 10^7 CFU/mL after pre-wash of the specimens with milliQ water (MQ) for 1–12 h or with human plasma (HP) for 1–12 h (**A**). The same data expressed as normalized effect score (**B**). Adherence was quantified by roll-plate analysis. Asterisks denote statistically significant difference, (*) $P < 0.05$ and (**) $P < 0.01$. Asterisks between brackets denote statistically significant difference of individual conditions versus all other circumstances (except SS90:10).

However, there was a tendency that the anti-adhesive effects on bacteria of the SS90:10-silver containing coatings deteriorated more quickly than their counterparts. The quicker reduction in anti-adhesive efficacy of the more hydrophilic coatings became more obvious when the data was presented as a normalized effect score, whereby the adherence within

1 h was set to an arbitrary value of 1.0 (Figure 5B). Although this relative increase was more pronounced for the more hydrophilic matrixes, absolute adherence was sustained at a slightly lower level for SS90:10-Ag-Hep compared to SS70:30-Ag-Hep. No influence of more embedded silver was observed since adherence to SS70:30-Ag-Hep and SS70:30-Ag_{2x}-Hep was maintained at a similar level.

Figure 8. *In vitro* challenge results of all specimens with Ag4E silver particles. The roll-plate assay was used to assess adherence (CFU/cm²) of ATCC 29213 after each challenge, consisting of incubation in 10⁵ CFU/mL during 1 h at 37°C and removal of non-adhered bacteria afterwards. Error bars indicate standard errors (SE). The detection limit was approximately 80–100 CFU/cm².

Bacterial adhesion to the different matrixes was also assessed after preceding washing periods. This essentially mimics the situation whereby a CVC is routinely used for a certain period of time and later becomes contaminated with bacteria. As shown in Figure 6A, even after 72 h pre-wash, adherence of ATCC 29213 was more pronounced when only silver particles (without heparin) were incorporated in the coating. Pre-wash with milliQ water seemed to have no effect on the adherence of ATCC 29213 to the less hydrophilic coatings SS70:30-Ag and SS70:30-Ag-Hep. On the other hand, the anti-adhesive features of the coatings SS90:10-Ag and SS90:10-Ag-Hep declined when pre-wash was extended (Figure 6B). Remarkably, while the effect of pre-washing was negligible for the coatings having SS70:30 as the matrix, SS70:30-Ag_{2x}-Hep displayed a tendency towards more adherence of ATCC 29213 when a longer pre-wash program was applied.

To explore whether fouling a (thin) film of human plasma proteins would influence bacterial adherence to the specimens, milliQ water as a washing fluid was replaced by drug-free human plasma from healthy volunteer blood donors. It appeared that plasma incubated specimens containing heparin in its coating layer, i.e. SS90:10-Hep and SS90:10-Ag-Hep, displayed increased adherence of ATCC 29213 (Figure 7A,B). Still, adherence with SS90:10-Ag-Hep was far below the level achieved with the reference coatings SS90:10 and at the same level as the coating containing silver particles alone, SS90:10-Ag. The coatings

without heparin, i.e. SS90:10-Ag, lost repulsion activity when pre-washed during 12 h with milliQ water in the same order as when plasma was used. Surprisingly, there was a tendency for these specimens that pre-wash in plasma during 12 h resulted in less adherence of ATCC 29213 in comparison with 1 h pre-wash in plasma.

Figure 8-S. *In vitro* challenge results of all specimens with Ag6V silver particles. The roll-plate assay was used to assess adherence (CFU/cm²) of ATCC 29213 after each challenge, consisting of incubation in 10⁵ CFU/mL during 1 h at 37°C and removal of non-adhered bacteria afterwards. Error bars indicate standard errors (SE). The detection limit was approximately 80–100 CFU/cm².

***In vitro* challenge**

Upon multiple challenges, bacterial adherence to all specimens containing silver particles gradually increased over time, but remained relatively low, especially for the ones with heparin (Figure 8). In contrast, soon after the first few challenges, roll-plate analysis of SS90:10 and SS90:10-Hep displayed a strong rise in the number of adhered bacteria. Already after 4–5 challenges the limit of detection, i.e. 100 CFU/cm², was exceeded (Figure 8A). After eight challenges it was estimated that specimens SS90:10 and SS90:10-Hep achieved a maximum of adhered cells of approximately 230 CFU/cm² (data not shown). However, it has to be noticed that during the first four challenges it was clear that specimens containing heparin alone, SS90:10-Hep, demonstrated anti-adhesive properties on interaction with ATCC 29213. From the beginning, bacterial adherence to the Vygon[®] catheter segments was excessive compared to all silver-containing specimens. After the last challenge, adherence to the Vygon[®] segments was approximately six times more compared to all silver-containing specimens. Whereas SS90:10-Ag and SS90:10-Ag-Hep demonstrated low adherence from the on-set, SS70:30-Ag and Vygon[®] catheter segments

showed initially a higher adherence, while its anti-adhesive capacities improved soon thereafter (Figure 8B).

Compared to matrixes with both heparin and silver particles in them, adherence to silver-containing specimens remained slightly higher over multiple challenges. This observation was independent of the coating's hydrophilicity. The less hydrophilic coating with the double amount of silver particles, SS70:30-Ag_{2x}-Hep, displayed more decline in its anti-adhesive capacities than the one with the reference content, SS70:30-Ag-Hep.

Discussion

Our experimental data confirm that embedding of silver particles in hydrophilic surface coatings is a feasible strategy toward biocidal surfaces for medical devices such as catheters²³. The experiments on bacterial killing and adhesion showed strong synergistic effects, if silver particles and heparin were *co-embedded* in the coating. This is the key message of this work. The effect was found with three different *S. aureus* strains: a reference strain (ATCC 29213) and two clinical isolates.

The observed more-than-additive effect of silver particles and heparin on bacterial killing and adhesion clearly asks for an explanation. First, it is important to consider the mechanistic background of the antimicrobial activity of surfaces containing silver. It is well-known that silver particles expose Ag⁺ ions at their periphery²⁴. Furthermore, it is essential to realize that all our media contained chloride ions, and that the solubility of AgCl in aqueous media is extremely low. Hence, dissolution of Ag⁺ ions from the silver particles does – most probably – not play a major role in the observed biocidal effects. In our earlier work (see chapter 4), we have corroborated this through a series of titration experiments, using the soluble silver salt AgNO₃. We found that dissolved Ag⁺ ions only start to become biocidal if their concentration exceeds 100 nM²⁵. We hypothesized, therefore, that antimicrobial effects of our silver-containing surfaces can be attributed to *collisions* of bacteria with the surface. During such a collision, transfer of Ag⁺ ions can occur from the silver particles surface to the bacterium's membrane, e.g. by binding to negatively charged phosphatidyl serine moieties. These membrane-bound Ag⁺ ions can subsequently induce rupture of the bacterial wall, eventually killing the bacterium. In essence, this mechanism is heterogeneous, as the critical step is collision between suspended bacteria and a solid surface that exposes Ag⁺ ions at its surface.

The question now is how this mechanism will change if heparin is introduced in the surface coating, next to the silver particles. Clearly, collisions and transfer of Ag⁺ ions as described above can still occur. But in addition, there is release of heparin molecules from the coating's surface occurring, especially if the matrix is hydrophilic²⁶. Heparin molecules, escaping from the coating can drag silver ions with them; affinity of heparin for monovalent cations is well documented²⁷. So, in essence there is now a second mechanism by which Ag⁺ ions can reach the bacterium, based on dissolved heparin molecules working as a carrier for Ag⁺ ions. This second mechanism is *homogeneous*, and heparin/Ag⁺ complexes could bind to the outer surface of bacteria, or be internalized.

The present experimental data can not be used to prove our hypothesis right or wrong, but it is obvious that most of our observations are in line with it. Bacterial killing with the coatings SS90:10-Ag-Hep, SS70:30-Ag-Hep and SS70:30-Ag_{2x}-Hep against inocula of *S. aureus* was much stronger, in comparison with counterpart coatings that contained only silver particles or heparin (Figure 4). Analogously, adherence of *S. aureus* ATCC 29213 was much lower for the coatings containing both silver particles and heparin (Figure 5); pre-washing with MQ water or with human blood plasma did not change this picture (Figures 6, 7). The *in vitro* challenge tests also pointed out that the coatings SS90:10-Ag-Hep, SS70:30-Ag-Hep and SS70:30-Ag_{2x}-Hep outperformed their counterparts containing either silver particles or heparin. Interestingly, the adherence tests allowed us to compare the long term effects between SS90:10-Ag-Hep and SS70:30-Ag-Hep coatings (up to 72 h pre-wash). These coatings differ only regarding the hydrophilicity of the matrix. The less hydrophilic material SS70:30 is expected to have slower release of embedded heparin (and consequently augmented co-transport of silver particles), and –hence– a more sustained antimicrobial effect. This is exactly what is seen in Figure 6.

Conclusions

In summary, we found that embedding of silver particles in SlipSkin (NVP/BMA) hydrophilic surface coatings leads to pronounced antimicrobial effects in experiments with three strains of *S. aureus* (one reference strain and two clinical isolates). Heparin potentiates the biocidal activity of the silver particles. A tentative explanation for this effect of heparin is based on two premises: (i) the observed biocidal effects of silver *per se* relate to a collision mechanism, and (ii) silver ions are transported by heparin molecules that are released from the coating's surface. This work shows for the first time that it is possible to design surface coatings with strong antimicrobial and antithrombogenic features. Most probably, *rational* design of such coatings becomes possible if the mechanistic details are explored further. We expect that this work will open a new route to safer (percutaneous) blood-contacting medical devices such as CVCs, which have become indispensable in the treatment of critically ill patients.

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CHAPTER 6

Large-scale evaluation of mutations in *norA* gene and its promoter leading to overexpression of the multidrug efflux pump NorA in *Staphylococcus aureus*

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Introduction

To date, at least seven genes have been described in the chromosome of *Staphylococcus aureus* which encode multidrug efflux pumps (EPs): i.e. *mdeA*, *mepA*, *norA*, *norB*, *norC*, *sdrM* and *sepA*^{1, 2, 3, 4, 5, 6}. In addition, many other efflux pumps located on plasmids have been described as well, such as *qacA/B*, *qacC*, *qacD* and *qacG*^{7, 8, 9}. NorA has been one of the most well described EPs in *S. aureus*. Studies have shown that NorA is a protein containing twelve transmembrane segments, that uses proton motive force to export diverse drugs such as ethidium bromide, quaternary ammonium compounds and hydrophilic fluoroquinolones such as norfloxacin and ciprofloxacin^{3, 10, 11, 12}. Mutations in the promoter and the *norA* gene itself as well as mutations in *mgrA*, encoding a regulatory gene, are known to affect the expression of *norA*^{11, 13}. An increase in expression is linked to an increased resistance to its substrates such as ciprofloxacin³. NorA-related fluoroquinolone resistance is however low as compared to resistance caused by mutations in topoisomerase or gyrase genes, but there is evidence suggesting that *norA* mediated low-level resistance predisposes to development of high-level resistance¹⁴.

Several studies have been published describing different mutations which were associated with an increased expression of *norA*. Noguchi *et al.* published an overview of sequences of *norA* and its promoter of 20 strains, including five reference strains¹¹. The authors discerned three main alleles: *norA wt*, *norA1199* and *norAI β* ¹⁵. Most publications however, only investigated a limited number of strains, often providing even more limited or no gene expression data and to our knowledge, no studies have been performed looking at the link between the sequence of *norA* (and linked to that, overexpression of *norA*) and the genetic background of *S. aureus*. The goal of this study was to investigate a large group of *S. aureus* isolates, with different genetic backgrounds, both methicillin susceptible *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA), with respect to sequence and expression of *norA*.

Materials and Methods

Bacterial strains

The *S. aureus* strains (72 MRSA and 156 MSSA) investigated were isolated during 2005 to 2008 in the Maastricht University Medical Center, a tertiary 715-bed hospital, and originate from surveillance cultures (commensal flora) from individual patients, recovered from nasal swabs¹⁶. MRSA and/or MSSA strains associated with MLST CC1, CC5, CC8, CC22, CC30, CC45, CC7, CC12, CC15, CC25 and CC121, were randomly selected from our institutional collection. All MRSA strains were tested positive for the MRSA-specific *mecA* gene, by real-time PCR¹⁷. Antibiotic susceptibility data for an extensive panel of antibiotics including a.o. methicillin, oxacillin, ciprofloxacin, moxifloxacin, gentamicin, clindamycin, linezolid and vancomycin for the different strains was obtained by routine broth micro dilution susceptibility testing, according to CLSI guidelines.

Table 1. Primers used for sequencing of (promoter) *norA*, *grlA* and *gyrA*.

Primer	Sequence	T _m (°C)
NorAF4	GTCCGCTAAAAGTTTCATATGATTG	63
NorAF2	CAAATGTAGCAATGTTGTGAATAC	55
NorAF3	TTCACCAAGCCATCAAAAGC	53
NorAR2	TGGCATACGATGTGAAACTTC	55
NorAR3	TTGCAATTCATGCTAAATATCC	51
NorAR4	TTAATGAATTAGGTATGTGGATTGC	61
NorA LongF	AGCGAGATGTCCGCTAAAAG	55
NorA LongR	TAACGCACCTGCGATTAAAG	53
GyrF	TCGTGCATTGCCAGATGTTCCG	59
GyrR	TCGAGCAGGTAAGACTGACGG	61
GrlF	TGCCAGATGTTCTCGTGTGATGGT	67
GrlR	TCGAGCAGGTAAGACTGACGG	61

Characterization of the genetic background

Typing of the *spa* locus was carried out as described previously¹⁸. The *spa* types were assigned through the Ridom SpaServer <http://spaserver.ridom.de> and clustered into *spa*-CCs using the algorithm based upon repeat pattern (BURP) with Ridom StaphType 1.4 using the default settings^{19,20}. Although, *spa* typing alone sometimes lacks discriminatory power, due to related *spa* repeat patterns within different clonal lineages and the emergence of homoplasies among *spa* sequences²¹, it has been shown that *spa* typing/BURP results are often in agreement with results obtained by MLST^{20,22}. Therefore, the associated MLST CCs were allocated through the SpaServer. To confirm the association between MLST and *spa* typing, in combination with BURP, MLST was performed on a representative set of 16 strains of each major *spa* type and associated MLST CC^{23,24}.

norA, *grlA* and *gyrA* sequencing

For *norA* sequencing purposes, a number of PCRs were implemented. A dedicated PCR developed by Couto *et al.*, was used for sequencing of the *norA* promoter and the start of the *norA* coding region²⁵. A PCR amplifying a 1500bp amplicon covering most of the promoter and the *norA* gene was developed as part of this study. Within this amplicon, additional primers were developed for sequencing of internal regions (Table 1).

Briefly, the procedure was comprised of suspension of one colony picked from a blood agar plate in 100 µL of ultrapure water. Of this 1 µL was added to the PCR mixture. This mixture contained 0.4 µM of each primer, 0.2 units HotStarTaq Plus DNA polymerase (Qia-gen, Venlo, the Netherlands) 1× accompanying reaction buffer and 100 µM of each dNTP. The PCR protocol consisted of an initial denaturation step of 5 min. at 95°C, followed by 40 cycles of 30sec. at 95°C, 30sec. at 50°C and 2 min. at 72°C. After PCR, amplicons were purified using PCRapace spin columns (Invitex GmbH, Berlin, Germany). Subsequently sequence reactions were performed using 0.1 µM of each primer, 1 µL of 1.1 BigDye Termina-

tor reaction mix (Applied Biosystems, Carlsbad, CA, USA), 1.5 µL accompanying sequencing buffer and 1 µL of purified amplicon (in a final volume of 10 µL). Readout of the sequences was performed on a 3730 DNA sequencer (Applied Biosystems).

Ultimately, the different *norA* promoter sequences were aligned and compared to the three previously described *norA* alleles: *norA wt*³³, *norA1199*¹⁴ and *norAII*²¹. Afterwards, the different sequences were grouped, with each group carrying an identical *norA* promoter, and named after the most resembling allele.

Table 2. Overview of 24 strains which have been further investigated regarding *norA* expression of different promoter sequence groups and mutations in gyrase and topoisomerase.

<i>S. aureus</i> strain	Relative <i>norA</i> expression	CC	Sequence <i>norA</i> promoter	<i>norA</i> promoter – 10 region	MIC cipro (mg/L) (CLSI)	Topo-isomerase (GrlA codon 80)	Topo-isomerase (GrlA codon 84)	DNA gyrase (GyrA codon 84)	DNA gyrase (GyrA codon 88)
3	1	5	A (WT)	ataCAATata	0.25	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
137	0.5	5	A (WT)	ataCAATata	0.25	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
140	0.9	5	A (WT)	ataCAATata	0.25	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
29	11.8	5	B	ataCAATataG(Δ11bp)	16	TTC (Phe)	GAA (Glu)	GCA (Glu)	GAA (Glu)
11	1.5	22	C	ataCAATata	0.25	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
55	1.7	22	C	ataCAATata	0.25	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
135	14.6	5	D	ataCAAT(CAAT)ata	256	TAC (Tyr)	GGA (Gly)	TTA (Leu)	AAA (Lys)
139	32.1	5	D	ataCAAT(CAAT)ata	256	TAC (Tyr)	GGA (Gly)	TTA (Leu)	AAA (Lys)
82	2.2	8	F	ataCAATata	128	TTC (Phe)	AAA (Lys)	GTA (Val)	GAA (Glu)
69	1.9	45	I	ataCAATata	0.25	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
108	3.0	8	I	ataCAATata	0.25	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
95	5.0	45	K	ataCAATataGAAG	256	TTC (Phe)	GAA (Glu)	TTA (Leu)	GAA (Glu)
100	37.6	45	K	ataCAATataGAAG	256	TTC (Phe)	GAA (Glu)	TTA (Leu)	GAA (Glu)
101	46.0	45	K	ataCAATataGAAG	256	TTC (Phe)	GAA (Glu)	TTA (Leu)	GAA (Glu)
102	23.6	45	L	ataCAATataGAAG	512	TTC (Phe)	GAA (Glu)	TTA (Leu)	GAA (Glu)
16	43.0	30	N	ataCAAT(ATCAAT)ata	128	TTC (Phe)	GAA (Glu)	TTA (Leu)	GAA (Glu)
17	56.1	30	N	ataCAAT(ATCAAT)ata	128	TTC (Phe)	GAA (Glu)	TTA (Leu)	GAA (Glu)
90	81.2	30	N	ataCAAT(ATCAAT)ata	128	TTC (Phe)	GAA (Glu)	TTA (Leu)	GAA (Glu)
158	2.4	30	O	ataCAATata	0.25	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
160	2.2	30	O	ataCAATata	0.5	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
179	13.5	30	P	ataCAATata	0.5	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
207	1.1	121	Q	ataCAATata	0.25	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
212	2.2	121	Q	ataCAATata	0.25	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)
209	1.3	121	R	ataCAATataGAAG	0.5	TCC(Ser)	GAA (Glu)	TCA (ser)	GAA (Glu)

norA expression studies

Saturated overnight cultures of *S. aureus* strains ($n=24$) were diluted 1:20 in Mueller-Hinton (MH) and incubated for 1 hour at 37°C with constant shaking. 100 µL culture of each sample was pelleted (in triplicate) by centrifugation, the pellet was resuspended in 40 µL of cell suspension buffer (50 mM Tris, 50 mM EDTA; pH 8), 2 µL of RNase-inhibitor (Applied Biosystems) and 10 µL lysosthaphin solution (1 mg/mL) (Sigma) were added, mixed and incubated at 37°C until visible lysis of the cells (typically 5–10 min.) occurred. The lysate was extracted twice with phenol/CHCl₃ (1:1) and once with CHCl₃. Nucleic acids were ethanol precipitated, air-dried dissolved in DEPC treated water. Genomic DNA was removed by incubating the sample with 1–2 units of DNaseI (Fermentas GmbH) for 30 min. After DNase I digestion, the sample was extracted with phenol/CHCl₃, precipitated and dissolved in 50 µL DEPC treated water.

Quantitative RT-PCR was performed using the Bio-Rad iScript™ One-Step RT-PCR kit according to the supplier's instruction with primers NorAF3 and NorAR2 (Table 1) and primers for 16S rDNA, which was used as a housekeeping reference gene²⁵. In summary, the template was diluted 1:100 for *norA* detection and 1:10000 for the endogenous control (16S rDNA). Amplification conditions were as follow: cDNA was synthesised at 42°C for 20 min. After a denaturing step at 95°C for 5 min., DNA was amplified in 42 cycles (95°C for 30 sec.; 50°C for 30 sec.; 72°C for 30 sec.) followed by a melting curve analysis (MyiQ™ Single-Color Real-Time detection system, Bio-rad). Negative controls and controls for genomic DNA contamination were included. Quantification (comparative Ct method²⁶) was performed using strain 3 as a reference strain (wild type for *norA*, *grlA* and *gyrA* genes).

Results

***norA* expression of different *norA* promotor sequence groups**

After sequencing of the *norA* promoter of 228 *S. aureus* isolates, the promoter sequences were aligned and 18 sequence groups, named A-R, could be differentiated (Figure 1, Table 2). Each group consisted of strains with identical sequences; i.e. when a point mutation was found, a new sequence group was designated. The sequences were compared to three reference sequences of the three known *norA* alleles as described by Noguchi *et al.*¹¹. The results show that sequence groups resembling all three known alleles were present in our collection. From the different promotor sequence groups A-R, 21 strains were selected for further investigation (Table 2). The selection of these strains was based on two points: i) from each sequence group consisting of > two strains, two strains were investigated, and ii) from each group containing mutations within the region -15 to +7, at least one strain was investigated. From each of the 21 strains, the expression of *norA* was monitored. Similar to earlier studies, a change of expression of ≥ 4 -fold was considered to be a significant change²⁷. Table 2 shows that representatives from six groups (B, D, K, L and N and P) demonstrated an increased expression of *norA* up to maximally ~80-fold. Representatives from all other groups showed an expression similar to a randomly chosen reference wt strain (i.e. strain 3). Interestingly, *norA* overexpression was seen within each of the three *norA* alleles (Figure 2).

For further analysis, all samples in each sequence group were assumed to have a similar expression profile. This assumption was tested by random choice of three more representative strains of respectively group A, K and N (in italics in Table 2). The *norA* expression of these strains confirmed our previous findings.

As high-level resistance to fluoroquinolones is traditionally a result of mutations in gyrase and topoisomerase, mutations in *gyrA* and *grlA* were monitored as part of this study. The results revealed that 22 of the 23 strains which were further investigated and representing groups B, D, K, L and N also showed mutations in these genes which are known to lead to resistance to fluoroquinolones (Table 2). Only the single strain representing group P showed mutations in group *norA* and no mutations in *grlA* and *gyrA*.


```

A TTTTACGAATATTTAGCATGAATTGCAATCTGTCGTGGAAAAGAAGATAACAGCTTTAA
B *****-----
C -----
D -----
E -----
F A-----
G -----
H -----
I -----A-----G--
J -----A-----G--
K -----A-----G--
L -----A-----G--
M -----A-----G--
N -----T-----A-----
O -----T-----A-----
P -----T-----A-----
Q -----A-----
R -----A-----

```

Start

```

A GCATGACATGGAGAAAAAA-GAGGTGAGCATATGAATAAACAGATTTTGTCTTATATTTT
B -----
C -----
D -----
E -----
F -----
G -----
H -----
I -AG--A-T-----A-----G-A-----
J -AG--A-T-----A-----G-A-----
K -AG--A-T-----A-----G-A-----
L -AG--A-T-----A-----G-A-----
M -AG--A-T-----A-----G-A-----
N -A-----
O -A-----
P -A-----*-----
Q -AG--A-T-----A-----G-T-----
R -AG--A-T-----A-----G-T-----

```

```

A AATATTTTCTTGATTTTTTTAGGTATCGGTTTAGTAATACCAGTCTTGCTGTTTATTTA
B -----
C -----
D -----
E -----
F -----
G -----
H -----
I -----A-----C-----G-----A-----
J -----A-----A-----C-----G-----A-----
K -----A-----C-----G-----A-----
L -----A-----C-----G-----A-----
M -----A-----C-----G-----A-----
N -----
O -----
P -----
Q -----A-A-----C-----G-----A-----
R -----A-A-----C-----G-----A-----

```

Figure 1: to be continued on the next page

Figure 1. Alignment of part of *norA* and its promoter. 18 different groups can be distinguished (A-R; *norA* overexpressing types as in bold and underlined). The -35 and -10 regions are marked and underlined. The transcription initiation site is marked with an arrow.

Figure 2. Distribution of the *norA* overexpressing *S. aureus* strains per *norA* allele.

Antibiotic susceptibility and *norA* overexpression

Of all 228 strains, resistance to the fluoroquinolones ciprofloxacin and moxifloxacin as well as to methicillin was investigated in relation to overexpression of *norA*. The results showed that there was a 95% correlation between strains in the *norA* overexpressing sequence groups (B, D, K, L, N and P) ($n=20$) also showing resistance to methicillin and ciprofloxacin. Only strain 179 in sequence group P was susceptible to both methicillin and ciprofloxacin. Regarding moxifloxacin, three overexpression strains were still susceptible to moxifloxacin,

but the other 84% was shown to be resistant to this antibiotic. For other antibiotics (such as vancomycin, gentamycin, clindamycin, linezolid, imipenem and rifampicin) no correlation with *norA* overexpression was found (data not shown).

Figure 3. (A) Distribution of the different MLST associated clonal complexes (CCs) per *norA* allele of the different examined *S. aureus* isolates. (B) The distribution of *norA* overexpressing strains per associated MLST-CC.

***norA* sequence and genetic background**

All strains were *spa*-typed after which associated MLST clonal complexes (CC's) were determined. Figure 3A shows the distribution of the different *norA* alleles within the different clonal lineages. Results show that with a maximum exception of one strain, the different CC's all contain only 1 *norA* allele. Figure 3B shows the distribution of the *norA* overex-

pressing strains over the different CC's. From the results, it is clear that *norA* overexpressing strains are only found in MLST associated CC's 5, 30 and 45. Specifically, these strains had *spa*-types t002 and t311 (CC5), t012 and t2854 (CC30) and t038 and t740 (CC45).

Discussion

Initial studies have shown that overexpression of the chromosomally encoded efflux pump NorA leads to resistance to a.o. fluoroquinolones, ethidium bromide and various biocides³. In several studies mutations in the *norA* gene and its promoter were presented, which are suspected to lead to overexpression of *norA*^{11, 25, 28, 29, 30}. However, most studies describe incidental findings of these mutations, with often a limited number of investigated strains or isolates. In this study we investigated a collection of 228 *S. aureus* strains, for mutations in the promoter of *norA*. Results showed that all three described alleles of *norA* were found within our collection. Notably, the *norA* allele was strongly correlated with the genetic background of *S. aureus*: i.e. all MLST associated CC30 strains except one contained a *norAll* allele, whereas CC45 and CC121 had the *norA1199* allele. All other CC's within the collection (CC1, 5, 7, 8, 12, 15, 22 and 25), except one exception in CC5, all contained the wt *norA* allele. To our knowledge, the *norA* allele has not been linked to the genetic background of *S. aureus* before. However, for example reference strain JCM2413 which was analysed by Noguchi *et al.* and shown to contain *norAll*, belongs to CC30, which is in agreement with our findings¹¹.

Looking at the *norA* sequences in more detail, we showed that within our collection, 16 different sequences could be distinguished. These sequences contained several previously described mutations, such as the T388→A mutation³⁰ and the insertions downstream of the -10 region in groups D and N³¹, but also new mutations such as the A→G mutation at the site of the start of transcription. Notably, the previously reported *flqB* mutation was not found in any of the 228 strains^{28, 32, 33}. In spite of the respectable number of studies reporting data regarding the sequence of *norA* and its promoter, only few studies combine this with data on the actual expression of the *norA* gene. In our study, we showed that strains with six *norA* sequence types demonstrated an increased expression of *norA* up to maximally ~80-fold. Both sequence types D and N showed an insert immediately downstream of the -10 region. Our overexpression data (between 15 and 80-fold) for those groups were in agreement with those previously reported by DeMarco *et al.* (4.8–50.6 fold) for strains with AAT or CAAT insertions downstream of the -10 region²⁷ and by Kosmidis *et al.* with an ~19-fold increase of expression for strains with an ATCAAT insertion at the same locus³¹. Our expression data were however markedly higher than for example the 4–5-fold increase in expression measured for strain TS69 with an TACAAT insert downstream of the -10 region¹¹. This is likely due to the difference in technique used for gene expression measurements, but could also be contributed to growth conditions at the time of expression measurements. As recommended by Kaatz *et al.*, we performed our experiments at mid-exponential phase³⁴. Sequence types K and L also showed *norA* overexpression (on average ~30-fold). Interestingly, the difference between for example group I, which expresses

norA at a wt level, and K is only one bp in the entire investigated area: i.e. A→G mutation at the site of the start of transcription. However, as the strain in group R contained this mutation and expressed *norA* at a wt level, future studies will have to reveal what the exact impact of this mutation on *norA* expression is. *norA* expression is known to be also regulated by *mgrA* and *norG*^{13, 32, 35}. Finally, group P (consisting of only one strain) showed an increased *norA* expression of around 13-fold. This strain was very similar to strains in group O, however the strain in group P contained a.o. a A→G mutation 2 bp upstream of the -10 region. To our knowledge this mutation has not been described before and similar to the mutation at the transcription start site, future studies will have to determine the impact of this mutation on the *norA* expression.

Our study clearly showed that overexpression of *norA* was associated with sequence types of all three known *norA* alleles. But, the overexpressing strains were limited to a few clonal lineages, and within the MLST associated clonal complexes to a few *spa*-types (*spa*-types between brackets): i.e. 5 (t002 and t311), 30 (t012 and t2854) and 45 (t038 and t740). These MLST associated clonal complexes are known to be associated with methicillin resistant *S. aureus* (MRSA)³⁶, and coincidentally, all the overexpressing strains, except for strain 179 in group P, found in our study were found to be resistant to methicillin. This is mostly in agreement with earlier results from DeMarco *et al.*, showing that the majority of the clinical isolates with efflux-related resistance in that study were MRSA³⁷. A potential limitation of our study was that all strains were collected in one geographical region/center (although spread over a period of three years) and thus a relatively large homogeneity between the strains in a clonal complex can be expected. Future studies will have to include strains collected from a larger geographical area.

NorA has long been associated with low-level resistance to hydrophilic fluoroquinolones³. In addition, high-level resistance to fluoroquinolones is caused by mutations in the genes encoding gyrase and topoisomerase (reviewed in³⁷) and it has been suggested that low-level *norA*-mediated resistance predisposes to high-level resistance¹⁴. In our study, all but one strain (strain 179, group P) overexpressing *norA* showed high-level resistance but also contained known mutations in the genes encoding gyrase and topoisomerase leading to this high-level expression. Strain 179 was the only *norA* overexpressing strain that was not resistant to fluoroquinolones with a ciprofloxacin MIC of 0.5 mg/L. However, previous studies demonstrated that strains overexpressing *norA* showed an increase in substrate MICs (amongst which fluoroquinolones) between 2 and 16-fold^{25, 38}. As from our data it cannot be excluded that the ciprofloxacin MIC of strain 179 has been raised slightly, more work will have to be performed to further clarify the impact of *norA* overexpression on fluoroquinolone MIC. Consequently, future work will also have to confirm expression data on a protein level.

In conclusion, our study has for the first time indicated that there seems to be a relation between the genetic background of *S. aureus* and the *norA* allele. Analysing 228 MSSA/MRSA strains from different genetic backgrounds revealed 16 different *norA* sequence types related to all three known *norA* alleles. Within the three *norA* alleles, six sequence types were associated with *norA* overexpression. Within the *norA* overexpressing strains, several novel mutations were found, especially in the -10 promoter region. These *norA* overexpressing strains were in this study in 95% found in MRSA strains from distinct

genetic backgrounds. All but one of the overexpressing strains were resistant to the fluoroquinolone ciprofloxacin, but all of these strains also contained mutations in genes encoding gyrase and topoisomerase, which are known to lead to fluoroquinolone resistance. Understanding the relationship between the genetic background of *S. aureus*, *norA* evolution, its overexpression and its contribution to resistance to its substrates such as hydrophilic fluoroquinolones, will ultimately contribute to the control of the emergence of antimicrobial resistant strains.

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CHAPTER 7

Inhibition of membrane-transporters as a potential approach against *Staphylococcus aureus* biofilm is hampered by adaptation to nutrient availability

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Abstract

Biofilm-embedded bacteria are often causative for persistent infections. Recently, inhibition of efflux pumps was considered as a manner to influence biomass formation of *Staphylococcus aureus*. The impact of efflux pump inhibitors (EPIs) was more pronounced to prevent biofilm formation than to reduce pre-existing mature biomass. The ineffectiveness of EPIs against the latter might be due to a penetration barrier of EPIs into the biofilm layers.

We exposed different clonal lineages to EPIs in the presence of 0.1% or 1% glucose to determine the effect on mature biofilm and biomass prevention. Our data confirm that at 0.1% glucose the EPI thioridazine effectively prevented biomass formation of strains particularly associated with multilocus sequence typing (MLST) clonal complex (CC)8, while at 1% glucose comparable results were obtained with strains associated with MLST CC30 and CC45. Reserpine was inferior to thioridazine in prevention of biomass accumulation. Remarkably, MLST CC8-associated strains elevated biomass production upon exposure to both EPIs at 1% glucose. We presume that EPIs have a non-specific affinity for a variety of transporters, including nutrient-importers. Blocking these importers at 0.1% glucose would result in suppression of biomass formation, especially among strains with a high carbohydrate uptake capacity. Conversion of abundantly imported amounts of carbohydrates, at 1% glucose, to acetate, would theoretically result in intracellular acidification. Concerning strains associated with MLST CC8 we hypothesized that blocking the efflux of acidic metabolites promotes – instead of prevents – biomass formation, while influx of glucose is not much affected.

Introduction

The native functions of trans-membrane transporters, such as efflux pumps, of bacteria like *Staphylococcus aureus* have not been fully unravelled yet. For multi drug resistance (MDR) pumps, extrusion of excessive amounts of (toxic) substances as part of intracellular waste management, is probably the most likely one^{1, 2}. These substances are often acidic by-products, originated from decomposition of metabolic pathways^{3, 4}. Another function includes the protection against toxic substances from the environment, which were influxed or diffused across the permeable cell wall. Besides the efflux mechanisms, trans-membrane transporters are also necessary to facilitate import of essential nutrients as glucose and amino acids^{3, 5, 6} or to maintain electrolyte and pH homeostasis^{2-4, 7}.

The expression of various trans-membrane transporters is positively (AbcA) or negatively (NorA, NorC and Tet38) regulated by the multiple gene regulator (*mgrA*)⁸⁻¹¹, except that of the NorB efflux pump, which is repressed by phosphorylated *mgrA*¹¹. Besides, *mgrA* is involved in the repression of biofilm formation¹². On one hand, this is the resultant of *mgrA*-induced upregulation of the accessory gene regulator (*agr*)¹², consequently ending up in (i) hindering microbial surface components recognizing adhesive matrix molecules (MSCRAMM) expression and (ii) improving the release of detachment factors or surfactant-like molecules such as phenol-soluble modulins (PSM) and *hla*-encoded α -toxin (α -hemolysin)¹³. On the other hand, *mgrA* inhibits *cidA*-related cell lysis triggered extracellular DNA (eDNA) release and stimulates the repression of autolysis by *IrgAB*¹².

From several points of view it has been postulated that efflux pumps are up-regulated during the transition from planktonic cells into sessile encapsulated ones^{1, 14}. First, the principle behind the well-known (remaining) recalcitrance of biofilm to normal concentration of antibiotics might be partly attributed to enhanced extrusion mechanisms^{1, 15}. Second, due to the cramped conditions in a biofilm, cells inside a biofilm need to exploit an efficient efflux system for endogenous waste products². Third, cell autolysis and thus eDNA release is affected by AbcA transporters¹⁶. Another ATP-binding cassette (ABC) transporter, the *grdS*-regulated *vraFG* efflux pump, is required for citrate-stimulated cell-to-cell interactions¹⁷. Finally, involvement of transporters in quorum sensing related extrusion of signalling molecules, also referred as autoinducers, has also been mentioned^{2, 15, 18}.

Although efflux pumps might play a vital role during biofilm development, their suggested overexpression does not fit with the *mgrA* repressor function of biomass formation and the opposite regulation on different efflux pumps, depending on the phosphorylation state of *mgrA*. Presumably, other regulators, such as *arlRS*, *sigB*, *rsbU*, *grdS* and *norG*, contribute in a crucial manner to the expression of efflux pumps^{10, 11, 15, 17}. Nevertheless, it has been recently demonstrated that efflux pump inhibitors (EPI) were effective in the prevention of biomass development¹. However, so far, EPIs were only tested against a limited number of strains and the effects of EPIs on mature *S. aureus* biofilms were unexplored. Therefore, we conducted a study in which we exposed mature biofilm of methicillin-susceptible *S. aureus* (MSSA) ($n=9$) and methicillin-resistant *S. aureus* (MRSA) ($n=15$) of

different clonal lineages to the EPIs thioridazine and reserpine. In addition, we assessed the capacity of the EPIs to prevent biomass formation of the same isolates.

Materials and Methods

Bacterial strains

As part of an ongoing surveillance of *S. aureus* nasal carriers, nine MSSA and fifteen MRSA strains from individual patients in the Maastricht University Medical Center, a tertiary 715-bed hospital, were evaluated (Table 1). The isolates were randomly selected from a group of MSSA and MRSA with distinct globally disseminated genetic backgrounds. The selected isolates were associated with multilocus sequence typing (MLST) clonal complex (CC)8, CC22, CC30 and CC45.

Characterization of the genetic background

Real-time amplification of the *spa*-locus, followed by sequencing, was performed as described earlier¹⁹. The *spa*-types were assigned through the Ridom SpaServer (<http://spaserver.ridom.de>) and clustered into *spa*-CCs using the algorithm based upon repeat pattern (BURP) with Ridom StaphType 1.5 using default settings²⁰. As *spa*-typing/BURP yields results consistent with typing outcomes obtained by MLST^{20, 21}, the associated MLST CCs were allocated through the Ridom SpaServer. To confirm the association between MLST and *spa* typing, in combination with BURP, MLST was performed on one isolate of each major *spa* type or associated MLST CC, as described previously^{19, 22}.

Susceptibility testing efflux pump inhibitors

The susceptibility of the strains to the EPIs was tested using a micro-dilution technique²³. In the biofilm experiments, the concentrations of EPIs used were between one-quarter and one-half the MICs in planktonic cultures. Thioridazine and reserpine (Sigma Aldrich, Zwijndrecht, the Netherlands) were used as EPIs^{24–26}. Reserpine is often used as benchmark compound during the development of novel EPIs^{15, 27, 28}. The highest final concentration of organic solvent for the EPIs, dimethyl sulphoxide (DMSO), which caused no inhibition of bacterial growth was <1% v/v.

Biofilm formation

To find out the effect of EPIs on the destruction of mature biofilms, the *S. aureus* strains were allowed to establish biofilms during 24 h in 96-well flat-bottomed non-tissue culture treated polystyrene microtiter plates (Greiner Bio-One, Frickenhausen, Germany) at 37°C on a rocking platform at 25 oscillations min⁻¹. Individual wells were filled with 100 µL of an overnight culture (10⁸ CFU/mL in Trypticase Soy Broth (TSB) without dextrose) (Becton Dickinson, Le Pont de Claix, France), and supplemented with 0.1% or 1% glucose. After 24 h biomass formation, growth medium with planktonic cells was discarded and replaced by fresh broth containing EPIs at ¼ - ½ × planktonic MIC, except for controls, where medium

and/or organic solvent (DMSO) were added only. The plates were incubated for an additional 24 h. Subsequently, the wells were washed three times with 200 μ L NaCl 0.9%, and the amount of biomass was quantified.

Table 1. Overview of *S. aureus* strains included in this study.

<i>S. aureus</i> strain	associated MLST CC	<i>spa</i> type	MSSA (S) or MRSA (R)	<i>mecA</i>
BF82	8	t008	R	+
BF106	8	t052	R	+
BF109	8	t052	R	+
BF110	8	t052	R	+
BF111	8	t052	R	+
BF143	8	t008	R	+
BF144	8	t008	R	+
BF11	22	t223	R	+
BF55	22	t005	S	-
BF151	22	t223	R	+
BF153	22	t223	R	+
BF236	22	t1433	S	-
BF237	22	t005	S	-
BF238	22	t005	S	-
BF244	22	t005	S	-
BF87	30	t012	R	+
BF88	30	t012	R	+
BF246	30	t483	S	-
BF254	30	t021	S	-
BF92	45	t445	S	-
BF94	45	t038	R	+
BF98	45	t038	R	+
BF256	45	t230	S	-
BF265	45	t772	R	+

In order to examine prevention of biomass formation by the EPIs, the experiments were carried out by adding the EPIs from onset to the growth medium. After 24 h of biomass formation in the presence of EPIs, planktonic cells were removed and the amount of biomass was determined.

Biomass formation was quantified using the crystal violet adherence assay¹⁹ and performed in six fold. Influence of EPIs on biofilm formation was calculated as follows:

$$\left(\frac{A_{590}^{\text{EPI + DMSO}} - A_{590}^{\text{DMSO}}}{A_{590}^{\text{control}}} \right) \times 100\%$$

Statistical analysis

SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. After verifying that the assumption of normality was met, a paired two-tailed sampled *t* test for the independent experiments was used. $P < 0.05$ was considered statistically significant.

Results

Prevention of biomass formation

Biofilm formation was prevented when *S. aureus* strains associated with MLST CC8 were grown in the presence of 0.1% glucose plus thioridazine (0–24 h) from onset. Similar results were obtained with reserpine (Figure 1). However, at 1% glucose the opposite was noticed with both compounds separately, i.e. enhanced biomass formation compared to control (Figure 1 and 2A).

Figure 1. Prevention of biomass formation by strains associated with MLST CC8 upon exposure to the efflux pump inhibitors thioridazine (T) and reserpine (R) at 0.1% and 1% glucose.

In addition, *S. aureus* strains from other clonal lineages were tested. Both at 0.1% and 1% glucose concentration, reserpine exposure from 0–24 h did not prevent biomass formation by strains associated with MLST CC22, CC30 and CC45. Moreover, it promoted biomass formation at 1% glucose (CC22 and CC45). In contrast to strains associated with MLST CC8,

thioridazine (0–24 h) inhibited biomass formation similarly (CC45) or slightly more (CC30 and CC22) at a glucose concentration of 1% instead of at 0.1% (Figure 2B, C and D).

Figure 2. Influence of thioridazine and reserpine on biofilm formation by *S. aureus* strains associated with MLST (A) CC8, (B) CC22, (C) CC30 and (D) CC45, displayed as bar plots. Indicated above each bar plot is the percentage of glucose present in the growth medium, the period of exposure to the efflux pump inhibitors (EPIs) and the time frame of biomass formation (A_{590}). EPIs were added from onset (0–24h) when prevention of biomass formation was examined, while exposure started later on (24–48h) during monitoring of mature biofilm destruction. The A_{590} values of the controls in the absence of EPIs and/or solvents, which are directly proportional to the amount of biomass produced, are also shown (line plots with symbols: •). Asterisks denote statistically significant difference (**) $P < 0.01$, (*) $P < 0.05$, as determined by a paired two-tailed *t*-test for the independent experiments).

Destruction of existing (mature) biofilm matrix

Addition of reserpine during 24–48 h to a 24 h pre-developed biofilm did not reduce the amount of biomass produced, but augmented biomass formation of several strains, independent of the tested clonal lineages and especially at 0.1% glucose. Compared to reserpine, thioridazine was somewhat more successful in destruction of mature biofilms. Against mature biofilms of strains associated with MLST CC8, thioridazine added during 24–48 h achieved a reduction (~ 12%) at 0.1%, but not at 1% glucose (Figure 2A).

However, this reduction in biomass was far beyond the level achieved by thioridazine when testing prevention of biomass accumulation (~ 39%). Thioridazine did not have the capability to destruct mature biofilms at 0.1% glucose of the other clonal lineages (associated with MLST CC22, CC30 and CC45). However, in contrast to our observations with strains associated with CC8, the other clonal lineages showed more pronounced reductions in biomass at 1% glucose (Figure 2B, C and D).

The amount of biomass formed by strains associated with MLST CC8 increased more quickly at 0.1% glucose compared to the other clonal lineages, as could be deduced from the higher A_{590} -values: 0.78 vs. an average of 0.46 during 0–48 h (Figure 2). At 1% glucose within 24 h, strains associated with CC30 and CC45 demonstrated a considerable increase in the amount of biomass produced (A_{590} -values of 3.52 and 2.03 after 24 h, respectively). After 48 h the amount of biomass produced by strains associated with MLST CC22 was (still) the lowest (A_{590} -value of 1.97 vs. an average of 2.79). Linked to the impression that biofilm maturation was the fastest for CC8 and the slowest for CC22, it was noticeable that thioridazine was able to prevent biomass formation at 0.1% glucose of the former and could destruct pre-formed biomass at 1% glucose of the latter.

Discussion

Various serious persistent infections have been associated with biofilm embedded micro-organisms attached to indwelling devices. Eradication of biofilm by antibiotics is difficult and leads often to surgical removal of the foreign body. Attempts to prevent biofilm formation, such as by anti-adhesive coatings or catheter lock solutions are under broad-scaled investigation^{29, 30}. Still, under particular circumstances biofilm formation could so far not be prevented. Therefore, an innovative concept for the development of a new class of agents to prevent biomass formation and eradicate existing biofilm is of paramount importance.

This study demonstrated that the EPIs thioridazine and reserpine did not fully meet this criterion, especially regarding the inefficient destruction of mature biofilms. We observed that the glucose availability was important for the effects of EPIs on the prevention of biomass formation. At 0.1% glucose thioridazine was only able to reduce biomass formation of strains associated with MLST CC8, while at 1% glucose it only partly diminished biomass formation of the other clonal lineages CC22, CC30 and CC45. Differences in the genetic background may have accounted for these divergent results. Taken together,

thioridazine prevented biomass formation more effectively than reserpine, independent of the glucose availability.

Based on the assumption that various efflux pumps might be overexpressed during the biofilm mode of growth, EPIs were put forward as potential anti-biofilm agents¹. Indeed, we observed that both thioridazine and reserpine were able to prevent biomass formation at 0.1% glucose of strains associated with MLST CC8, as shown earlier for *S. aureus* 8325-4 (*spa*-type t211, MLST CC8)¹. However, the opposite was observed when the glucose concentration was raised to 1%, to simulate circumstances with large amounts of nutrients. The inability to prevent biomass formation at 1% glucose, with even stimulation of biomass formation as a consequence, suggest that glucose availability and/or metabolism influences the outcome. Based on data from the literature we propose the following model for biofilm-associated pathways (Figure 3), which enabled us to obtain premises to support our observations.

When rapidly catabolizable carbon sources are abundantly present (as is in the presence of 1% glucose), it is known that the tricarboxylic acid (TCA) cycle is repressed³¹. As a consequence this is followed by the accumulation of acetate and/or lactate. As proposed in Figure 3, increasing concentrations of acetate and lactate enhance (by intracellular pH lowering) the catabolite control protein A (*ccpA*) triggered polysaccharide intracellular adhesin (PIA) formation and *cidA* related eDNA release³. Thus, blocking the efflux of acetate might stimulate biofilm formation of strains associated with MLST CC8, as observed at 1% glucose concentration. The reduction in biomass formation by EPIs at a lower glucose (~0.1 – 0.3%) concentration has previously been attributed to the accumulation of waste products¹, resulting in toxification. However, this does not clarify our results at 1% glucose. Another explanation for the observations at 0.1% might be that EPIs have affinity for other membrane-based transporters, besides efflux pumps. Inhibition of glucose-influx by EPIs might prohibit biomass accumulation. A deficiency in intracellular amount of glucose would lead to TCA cycle activation, enhancement of TCA cycle intermediates c.q. metabolites and consequently inhibition of *icaABCD* and upregulation of the *mgrA* biofilm-repressor function¹² via activated *graRS* (Figure 3). Although *graRS* might also upregulate *rot*³², which acts as a negative transcription regulator of α -toxin and protease expression and as a positive regulator on certain MSCRAMMs³³, we expect that *graRS* exerts a stronger effect on *mgrA* than *rot*. This expectation is based on a transcriptome analysis³² and a described negative feedback on *rot* via *mgrA* and *agr*³⁴ and is in agreement with the presented data as well. We presume that due to an excessive amount of glucose at 1%, more competition exist for the transporter, resulting in an incomplete inhibition of the glucose importer function by the EPI.

Furthermore, we observed that the EPIs in the presence of 0.1% glucose were less effective in destruction of pre-existing established biofilms, compared to prevention of biomass production. This could be clarified by different assumptions. First, the recalcitrance to antibiotics is explained by a penetration barrier caused by the densely adherent biofilm matrix³⁵, consisting of proteins, eDNA and/or PIA. It is plausible that such a diffusion problem also exist for EPIs, resulting in subinhibitory concentrations locally present inside the biofilm. Such concentrations are then below the minimum biofilm eradication concentration (MBEC). Subinhibitory concentrations of certain antibiotics and probably also other

compounds were able to augment biomass formation of all different types of bacteria^{36, 37}. Additionally, it has been shown that after a shift to (semi)-anaerobic conditions, the amount of lactate accumulates since the conversion of pyruvate into acetyl-CoA requires oxygen³¹. As shown in Figure 3, (partially) blocking lactate extrusion would improve acidification leading to increased *ccpA* expression and thus biomass expansion. Cells inside mature biofilms surrounded by a nutrient depleted environment might also start to produce autoinducing peptides (AIP), as part of quorum sensing intercellular communication². Reducing the extrusion of AIP by EPIs would prohibit dispersal and result in the conservation of biomass, with consequently more biomass compared to control.

Figure 3. Simplified overview of (postulated) biofilm related pathways in *S. aureus*. It is clearly noticeable that the multiple gene regulator *mgrA* plays a central role as a regulator of transmembrane transporters, capsular polysaccharide formation and biomass formation. *Abbreviations* – *agr*: accessory gene regulator; *ccpA*: catabolite control protein A; ECP: extracellular proteases; eDNA: extracellular DNA; MSCRAMMs: microbial surface components recognizing adhesive matrix molecules; PIA: polysaccharide intracellular adhesion; PSM: phenol-soluble modulins; *sarA*: staphylococcal accessory regulator; *spoVG*: stage V sporulation protein G; *rot*: repressor of toxins.

Interestingly, at 1% glucose, differences between prevention of biofilm formation and destruction of established biofilms by EPIs were also observed. Compared to the elevated

biomass forming capacity of strains associated with MLST CC8 provoked by the EPIs during the prevention period (0–24 h), the inducible effect of EPIs (24–48 h) on mature biofilms was less pronounced (Figure 2A). Probably, after 24h of biomass formation in the presence of high glucose concentration, the cells are trying to counteract the produced large amounts of lactic acid, acetic acid and formic acid by upregulating urease activity (Figure 3). This would result in ammonium and/or ammonia formation^{8, 38}. Excess of ammonia is normally expelled since it is toxic for bacterial cells. Thus, blocking the extrusion of ammonia would break down biomass. In addition, at the post-exponential biofilm phase, (intracellular) glucose is almost completely consumed and oxygen becomes limited in the deeper layers of the biofilm^{39, 40}. As a survival strategy, (some) TCA cycle enzymes could get upregulated due to oxygen limitation and/or a too-high acidification to permit better metabolism and/or consumption of excreted fermentation products^{7, 17, 31, 39}. Thus blocking the influx of previously excreted fermentation products like acetate³¹ would reduce the intracellular acetate concentration more quickly in order to create more TCA intermediates, and consequently depletes biomass.

The influence of thioridazine and reserpine on biomass was slightly different for strains belonging to the clonal lineages associated with MLST CC22, CC30 and CC45, which were not previously tested¹. These variations might be attributed to other biomass-related metabolic changes or different expression levels of membrane-based transporters. We confirmed that the amount of biomass produced during a certain time period depends on the genetic backgrounds¹⁹, indicating that nutrient import capacities are variable as also the rate of metabolic alterations. However, the effects of EPIs on biomass of strains associated with the related MLST lineages CC30 and CC45⁴¹ were practically similar.

In general, the glucose and oxygen availability within microbial biofilm communities is changing over time, which concomitantly modifies the TCA cycle activity of the embedded bacteria. As a result, based on fluctuations in indirect *mgrA* stimulation by TCA cycle intermediates^{17, 32}, the expression of efflux pumps and other trans-membrane transporters of these bacteria changes. Therefore, it was not surprising that inhibition of trans-membrane transporters by EPIs as a tool to hinder biofilm formation was only effective under certain circumstances, depending on the environmental glucose availability and the already pre-existing amount of biomass. Since the latter differed per clonal lineage and variations among their trans-membrane transporters are likely, the genetic background of the strain is of importance in whether an EPI could be a valuable tool to prevent biomass formation or to eradicate mature biofilm.

In summary, we demonstrated with *in vitro* experiments that at 0.1% glucose the EPIs thioridazine and reserpine were predominantly able to prevent biomass accumulation of strains associated with MLST CC8, while at 1% glucose almost the same was achieved with thioridazine only for strains associated with MLST CC30 and CC45. Destruction of mature biofilms was in general ineffective. Only biofilm produced by strains associated with MLST CC22 were slightly affected. Based on biofilm-related pathways described in the literature, we discussed our findings regarding the inconsistent and inefficient anti *S. aureus* biofilm characteristics of EPIs.

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CHAPTER 8

Diminished *in vitro* antibacterial activity of oxacillin against clinical isolates of borderline oxacillin resistant *Staphylococcus aureus*

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Abstract

Since it is unknown whether β -lactam antimicrobial agents can be used effectively against borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) with oxacillin MIC ≥ 4 mg/L, the *in vitro* bactericidal activity and pharmacodynamic effect of oxacillin against clinical BORSA isolates was evaluated. Time-kill experiments with oxacillin were performed and compared with those of vancomycin, daptomycin and linezolid against BORSA with oxacillin MIC ≥ 4 mg/L and BORSA with oxacillin MIC ≤ 2 mg/L. Furthermore, the effect of β -lactamase production and plasmid profile analysis were used to clarify responses to oxacillin.

The oxacillin killing activity was attenuated against BORSA compared to ATCC 29213, since the pharmacodynamic parameters revealed that the potency of oxacillin was markedly reduced (approximately 10 $\times$) against BORSA with oxacillin MIC ≥ 4 mg/L. pBORa53-like plasmid containing BORSA with oxacillin MIC ≤ 2 mg/L showed markedly more regrowth.

In conclusion, oxacillin displayed non-effective eradication of either (i) BORSA that possesses oxacillin MIC ≥ 4 mg/L or (ii) β -lactamase hyperproducing BORSA (MIC ≤ 2 mg/L). Further investigation into β -lactam dosing strategies against different BORSA strains is warranted in order to avoid putative therapy failure.

Introduction

In addition to methicillin-sensitive (MSSA) and -resistant *S. aureus* (MRSA), borderline oxacillin-resistant *S. aureus* (BORSA) have been described. BORSA strains exhibit oxacillin MIC values enclosing the European Committee on Antimicrobial Susceptibility Testing (EUCAST) oxacillin break-point of >2 mg/L, since BORSA strains with oxacillin MIC below or above the break-point have been described^{1,2}. BORSA strains lack the MRSA-specific *mecA* gene on the Staphylococcal Cassette Chromosome *mec* (SCC*mec*), and, consequently, do not express the low-affinity penicillin-binding protein (PBP) 2a or 2'. The borderline phenotype has been attributed to (i) the hyperproduction of *blaZ*-encoded penicillinase and/or (ii) the production of an inducible, plasmid-mediated, membrane-bound methicillinase²⁻⁵ and/or (iii) different modifications in the PBPs 1, 2 and 4 genes, due to spontaneous amino acid substitutions in the transpeptidase domain^{2,6,7}. Unfortunately, a single trait that differentiates BORSA strains from susceptible or resistant *S. aureus* isolates has not yet been identified. Outbreaks of BORSA infections were reported at an estimated incidence of 5%^{8,9}. Treatment of early experimental endocarditis and *in vitro* data suggest efficacy of β -lactamase-resistant penicillins against BORSA strains with oxacillin MIC of 2 mg/L by enhancement of the dosage^{10,11}. Whether BORSA with higher oxacillin MIC (≥ 4 mg/L) can be treated effectively with β -lactamase-resistant penicillins is unknown.

The aim of the present study was to examine the antibacterial activity of oxacillin compared to vancomycin, daptomycin and linezolid, against three *mecA*-negative BORSA isolates, M002, M008 and M015, with oxacillin MIC ≥ 4 mg/L and compared with two BORSA, M007 and VU94, with oxacillin MIC ≤ 2 mg/L, using time-kill analysis.

Materials and methods

Bacterial strains

S. aureus ATCC 29213 and VU94 were used as MSSA and BORSA reference strain, respectively^{4,12}. BORSA M015, which was investigated in more detail, was a clinical isolate from a patient admitted to the Maastricht University Medical Center. Fourteen other BORSA isolates, including M002, M007 and M008, were isolates from general practice patients.

VU94 and M007 were BORSA with oxacillin MIC ≤ 2 mg/L and M002, M008 and M015 were BORSA with oxacillin MIC ≥ 4 mg/L. The *S. aureus* protein A (*spa*) types were t3033, t078, t127, t002 and t091, respectively, associated with multilocus sequence typing (MLST) clonal complexes (CC)25, CC25, CC1, CC5 and CC7. The remaining eleven strains were subjected only to susceptibility testing, using the Phoenix system as well as a microdilution technique. All BORSA strains were tested positive for the *S. aureus*-specific *femA* gene and negative for the MRSA-specific *mecA* gene¹³. ATCC 29213 and 25923 served as positive and negative β -lactamase producing controls, respectively^{14,15}.

Antibiotics

Daptomycin and linezolid were kindly supplied by the manufacturers (Novartis, Basel, Switzerland and Pfizer, Groton, CT, respectively). Oxacillin and vancomycin were purchased (Sigma-Aldrich, Zwijndrecht, the Netherlands). Prior to each experiment stock solutions were prepared.

Susceptibility testing

Susceptibility testing was performed by microdilution techniques according to CLSI guideline¹⁶. Daptomycin MICs were determined using E-test-strips (AB Biodisk, Solna, Sweden). Additionally, oxacillin MICs were determined using the Phoenix system (Becton Dickinson, Sparks, MD). Tolerance was defined as a MBC/MIC ratio ≥ 32 ¹⁷.

Time-Kill experiments

Time-Kill studies were done according to Isenberg's guidelines¹⁸ and performed in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, Hampshire, England). When testing daptomycin, Mueller-Hinton broth II (BBL™, Becton Dickinson, Le pont de Claix, France) was supplemented with calcium (50 mg/L)¹⁹. Bacteria from an overnight culture were grown until late logarithmic phase and diluted to 1×10^6 CFU/mL. Antibiotics were added (100 μ L per 10 mL medium) to obtain concentrations of $\frac{1}{2}$, 1, 2, 4 and $8 \times$ MIC. Samples were taken at $t=0, 2, 4, 6, 8, 10, 24$ and 48 h of incubation at 37°C, serially diluted (10^{-1} to 10^{-6}) to minimize carryover effects, plated (37.3 μ L) using a spiralplater (Eddy Jet, IUL instruments, Barcelona, Spain) and incubated at 37°C for 18–24 h. CFU were counted with a colony counter (Flash & Go, IUL instruments). The lower limit of detection was 2.73 $\log_{10}$ CFU/mL. Experiments with M015 and ATCC 29213 were carried out in duplicate to eightfold to test reproducibility. Bactericidal activity (99.9% kill) was defined as a $\geq 3\text{-log}_{10}$ CFU/mL decrease in colony count at 24 h compared to the initial inoculum.

Isolation and restriction of plasmid DNA

To analyse the presence of β -lactamase encoding plasmids, such as pBORa53, plasmid DNA was isolated and digested with *HindIII*, essentially as described previously²⁰.

Beta-lactamase quantitation

The crude β -lactamase was prepared as described previously²¹. Oxacillin was used as the inducer at a concentration of 0.375 mg/L. Uninduced cultures were also included. ATCC 29213 and 25923 were induced with 0.125 and 0.0625 mg/L oxacillin ($\frac{1}{2} \times$ MIC), respectively. The specific activity was calculated by dividing the total activity by the protein content.

Statistical analysis

Differences between concentration profiles in $\log_{10}$ CFU/mL at 24 h were assessed by analysis of variance with Tukey's test for multiple comparisons, using SPSS 14.0. A *P* value of < 0.05 was considered statistically significant.

Determination of pharmacodynamic parameters

An uncomplicated $\epsilon_{\max}$ -model can describe the change in CFU over time during the initial phase of cell killing, using the growth rate constant (λ) in the absence of antibiotic pressure and on the concentration depending kill rate constant (ϵ), as shown in Equation 1.

$$\frac{dN}{dt} = \left(\lambda - \frac{\epsilon_{\max} C^\gamma}{\epsilon C_{50}^\gamma + C^\gamma} \right) \times N \quad (1)$$

The maximum kill rate constants $\epsilon_{\max}$ (h^{-1}) and concentration necessary to achieve half of the maximum kill rate ϵC_{50} (mg/L) were obtained as described previously²².

However, this model can be inaccurate to estimate pharmacodynamic parameters since effect-time profiles are often not log-linear over a longer period of time. In order to cover the overall effect over 24 h, the log difference viable count was used as the difference between growth in the presence and absence of an antibiotic. For each concentration, the effect (*E*) was defined as the area under the curve (AUC_{0-24}) of the log difference curves (Equation 2), equal to the area between the curves (ABC_{0-24}), and calculated via the trapezoidal rule method in Prism 4.0 (GraphPad Inc).

$$\text{AUC} \left[\log_{10}(\text{CFU}_{\text{growth control}}) - \log_{10}(\text{CFU}_{\text{antibiotic}}) \right]_{0-24} \quad (2)$$

The maximum effect ($E_{\max}$) was determined by fitting the data to the Hill equation. To carry out a parallel line assay²³, the logarithms of $E/E_{\max} - E$ were plotted against the logarithm of the concentration (mg/L) according to the Hill plot (Equation 3)²⁴.

$$\log[E/(E_{\max} - E)] = \gamma \log[C] + K \quad (3)$$

The EC_{50} represents the concentration at which 50% of the maximum effect is obtained and is therefore considered as a potency marker. The EC_{50} is equal to $10^{-K/\gamma}$.

Results

Susceptibility testing

Similar MICs of daptomycin and vancomycin were observed for BORSA with a MIC oxacillin ≥ 4 mg/L (M002, M008 and M015) or ≤ 2 mg/L (M007 and VU94) (Table 1). Tolerance to oxacillin was noted for M002 and M008¹⁷. Persisters were observed when determining the MBC of M015²⁵. All BORSA, including eight isolates with oxacillin MICs of 4 and 8 mg/L, tested on the Phoenix system were classified as susceptible for oxacillin (as shown for five strains in Table 1).

Table 1. *In vitro* activity and pharmacodynamic parameters of oxacillin, vancomycin, daptomycin and linezolid against BORSA.

Bacterial strains and antibiotics ^a	MIC (mg/L)	MIC ^b (mg/L)	MBC (mg/L)	Time to 99.9% kill (h)			E _{max}	γ	EC ₅₀ (mg/L)	γ ^{∗c}	E _{max,0.01} ^d (h ⁻¹)			EC ₅₀ (mg/L)	γ	
				2×	4×	8×										
				MIC	MIC	MIC					I ^e			II ^e		
29213 OXA	0.25	ND	0.5	21.36	18.31	15.09	121.03	2.28	0.20	2.20	0.94 / 2.00			0.19	1.66	
M015 OXA	4	2	8 [†]	26.61	27.38	25.59	95.88	1.85	2.01		0.52 / 1.50			2.22	2.59	
M008 OXA	4	0.5	>64	44.32	47.96	44.30	80.22	2.29	1.89		0.75 / 1.93			1.58	2.05	
M002 OXA	8	2	>64	NA	NA	NA	89.04	1.57	1.92		0.74 / 1.88			1.89	2.60	
VU94 OXA	0.75	0.5	1	NA	NA	NA	100.40	1.67	0.42		1.06 / 2.05			0.31	2.03	
M007 OXA	2	0.5	4	NA	NA	18.89	75.02	2.07	0.61	1.05	0.75 / 1.67			0.49	2.94	
29213 DAP	1	ND	ND	4.92	3.63	2.10	176.52	0.85	0.66		2.05 / 2.82			0.57	0.88	
M015 DAP	0.5	ND	ND	9.15	6.82	6.10	131.47	1.64	0.26		1.18 / 1.82			0.14	1.55	
M008 DAP	0.5	ND	ND	7.42	5.21	4.39	147.40	1.09	0.21		1.28 / 2.02			0.24	1.42	
M002 DAP	0.75	ND	ND	4.03	4.88	3.48	147.80	1.03	0.08		1.27 / 2.10			0.29	1.36	
VU94 DAP	0.75	ND	ND	5.76	5.73	5.39	147.90	1.40	0.33	2.16	1.39 / 2.09			0.49	1.47	
M007 DAP	0.75	ND	ND	5.61	5.12	4.64	142.80	1.79	0.41		1.04 / 1.89			0.28	1.40	
29213 LZD	4	ND	ND	NA	NA	NA	82.54	5.36	1.79		4.13	0.53 / 1.59			1.32	3.27
M015 LZD	1	ND	ND	NA	32.49	27.63	99.68	1.98	0.73			0.71 / 1.69			0.80	1.02
29213 VAN	1	ND	ND	NA	24.36	24.24	98.82	4.31	1.48	0.66 / 1.72			0.57	6.51		
M015 VAN	1	ND	ND	NA	22.01	22.20	100.07	3.58	1.60	0.56 / 1.54			0.62	5.82		
M008 VAN	1	ND	ND	NA	22.52	29.18	97.45	4.37	1.37	0.83 / 2.01			0.95	6.58		
M002 VAN	1	ND	ND	NA	17.24	16.12	114.80	3.98	1.28	4.13	1.01 / 2.16			0.87	3.04	
VU94 VAN	1	ND	ND	NA	21.97	20.00	102.20	2.83	1.34		0.84 / 1.84			0.63	5.85	
M007 VAN	1	ND	ND	NA	20.85	17.24	103.90	4.14	1.83		0.77 / 1.69			1.27	3.92	

^a DAP, daptomycin; LZD, linezolid; OXA, oxacillin; VAN, vancomycin

^b MIC values determined with the Phoenix system

^c common sigmoidicity coefficients for strains ATCC 29213 and BORSA M015 when concentration-effect lines were forced to be in parallel

^d observed (o) and intrinsic (i) maximum kill rate constants ($E_{max,i} = E_{max,o} + \lambda$); the generation times ($gt = t / ((\log 2)^{-1} \times \log (N_t/N_0))$) and $\lambda = \ln 2 / gt$) were in BHI or CAMHB (DAP): 0.70 and 0.90; 0.70 and 0.99; 0.65 and 1.08; 0.61 and 0.83; 0.75 and 0.81 and 0.58 and 0.93 (h) for respectively ATCC 29213, VU94, M015, M002, M007 and M008.

^e method I, effect expressed as the ABC_{0-24} ; method II, effect expressed as the kill rate (ϵ)

† persisters detected up to 64 mg/L

ND, not determined

NA, not achieved within 48 h

Time-Kill experiments

ATCC 29213 and all BORSA showed similar growth characteristics: there were no considerable differences in the effects of vancomycin, linezolid and daptomycin (Figure 1, Table 1). The EC_{50} and εC_{50} were in the same size of order, except for vancomycin. Regrowth was especially observed with vancomycin, even at $2\times$ MIC for all strains.

Figure 1. The pharmacodynamic effect, expressed as the difference in log viable count over a time period of 24 h, and denoted by the area between the curves (ABC_{0-24}), versus the concentration in mg/L for (A) oxacillin, (B) vancomycin and (C) daptomycin against BORSA strains M002, M007, M008, M015 and VU94 and MSSA ATCC 29213.

Oxacillin was significant less effective at 4 and $8\times$ MIC against M015, compared to ATCC 29213, in reducing the bacterial viable counts over 24 h ($P < 0.05$ and $P < 0.001$), as was the time to achieve 99.9% killing at 2, 4 and $8\times$ MIC (5–10 h longer) (Table 1). This was consistent with the lower ε_{max} . Both, M002, M008 and M015 did not achieve 99.9% kill within 24 h, even not at $8\times$ MIC. The maximum effect of oxacillin was considerably reduced for all BORSA compared to ATCC 29213 (Figure 1A). Furthermore, markedly regrowth at 2 and $4\times$ MIC was observed with BORSA M007 and VU 94 (oxacillin MIC ≤ 2 mg/L), while no regrowth was seen with BORSA with oxacillin MIC ≥ 4 mg/L (M002, M008 and M015) (Figure 2).

Figure 2. Antibacterial activity of oxacillin (OXA) against (A) *S. aureus* ATCC 29213 (white symbols and dotted lines) versus the average data of BORSA strains M002, M008 and M015 (MIC OXA ≥ 4 mg/L) (black symbols and straight lines) and (B) *S. aureus* ATCC 29213 (white symbols and dotted lines) versus the average data of BORSA strains M007 and VU94 (MIC OXA ≤ 2 mg/L) (black symbols and straight lines) at concentrations corresponding to $\frac{1}{2}\times$ (circles), $1\times$ (triangles downwards), $2\times$ (squares), $4\times$ (diamonds) and $8\times$ MIC (triangles upwards) in comparison to growth controls (hexagon). Error bars represent standard errors of the mean.

Beta-lactamase activity and plasmid profile analysis

The β -lactamase activity and plasmid profile analysis were performed to clarify the differences in regrowth observed with oxacillin between BORSA M007 and VU94, and BORSA M002, M008 and M015, at the same multiples of the MIC. Although, BORSA M015, M008 and M002, with oxacillin MIC ≥ 4 mg/L, showed a twice as high induction ratio than BORSA M007 and VU94, with oxacillin MIC ≤ 2 mg/L, the latter produced considerably higher amounts of β -lactamase after induction (Figure 3A). The four plasmid DNA fragments of BORSA M007 and VU94 were similar in size to that of plasmid pBORa53 (Figure 3B), previously described as a pBW15-like β -lactamase plasmid²⁶. In the other BORSA, pMW2-like plasmids were identified, a penicillinase-encoding plasmid²⁷.

Pharmacodynamic comparison

For each antibiotic a potency ratio was calculated between ATCC 29213 and M015, using the parallel line assay²³. The difference in potency is visualized by the horizontal shift of the concentration-effect curve (Figure 4). Oxacillin was approximately $10\times$ more potent against ATCC 29213 than against M015. The same result was found for the other BORSA, M002 and M008 with oxacillin MIC ≥ 4 mg/L. These strains showed similar concentration – effect profiles (Figure 1A), resulting in consistent EC_{50} . On the other hand, the potency estimates for ATCC 29213 versus M007 and VU94 were $2\times$ and $3\times$, respectively.

Figure 3. (A) The effect of β -lactamase induction by oxacillin on the nitrocefin-hydrolyzing activities of BORSA strains. **(B)** BORSA M007 and VU94, contain a BORSA-related, pBORa53-like plasmid. The image shows an ethidium bromide-stained agarose gel containing *Hind*III-digested plasmids from M007 (lane 1), VU94 (lane 2), M002 (lane 3), M008 (lane 4) and M015 (lane 5). The *Hind*III-digested DNA fragment sizes predicted from the plasmids pBORa53 (GenBank Accession Nr. AY917098) and pMW2 (Genbank Accession Nr. AP004832) are indicated on the left- and right-hand side, respectively.

Discussion

This study revealed that oxacillin was not effective to eradicate, *in vitro*, neither BORSA with MIC oxacillin ≥ 4 mg/L, nor BORSA with MIC oxacillin ≤ 2 mg/L. Actual concentrations of oxacillin to eradicate *in vitro* BORSA with oxacillin MIC ≥ 4 mg/L (M002, M008 and M015) were notably higher than can be attained with normal dosing strategies, while M007 and VU94 (MIC oxacillin ≤ 2 mg/L), showed early regrowth with oxacillin, possibly due to a pBORa53-like plasmid related hyperproduction of β -lactamases.

The change in killing rate over time is a reflection of the influence of (i) degradation of antibiotics, (ii) adaptation and (iii) heterogeneous subpopulations²⁸. Consequently, regrowth can occur. Furthermore, the growth rate is not constant over time, since the replication of microorganisms is influenced by the amount of nutrients in the medium. Therefore, an alternative method, which incorporates all available time-kill data, was used to obtain concentration-effect parameters. In contrast to the method of Tsuji *et al.*²⁹, who used the log ratio based on the proportion between growth or killing in the absence and presence of an antibiotic, our method is based on the absolute difference between these characteristics.

Because the kill rates were obtained from the log linear initial phase of the survival curve, $\varepsilon_{\max}$ was affected only minimally by regrowth. In contrast, the reduced efficacy as a result of regrowth was manifested in $E_{\max}$, since the variation of the survival curve over a longer period of time was taken into account. This might clarify the discrepancy between EC_{50} and εC_{50} values (Table 1). The vancomycin effect is overestimated by the εC_{50} .

Figure 4. The logarithm of $(E/E_{\max} - E)$ versus the logarithm of the concentration in mg/L for *S. aureus* ATCC 29213 (solid lines) and BORSA M015 (dashed lines). The corresponding EC_{50} were determined from the points where the dotted horizontal line ($y = 0$) intersects the different concentration-effect curves. The differences in potency are visualized by the left or right shift of the curves. The corresponding Hill coefficients (γ') are equal to the slopes of the curves. The dotted vertical lines indicate the MIC of the represented antibiotics. The abbreviations DAP, OXA, LZD and VAN represent daptomycin and oxacillin, linezolid and vancomycin, respectively.

The decreased EC_{50} for oxacillin against BORSA with oxacillin MIC ≥ 4 mg/L (M002, M008 and M015) is accompanied by a reduction in $E_{\max}$. Usually, target site alterations are reflected by a reduced $E_{\max}$. BORSA resistance mechanisms have been related, among others, to target site alterations, such as amino acid substitutions in PBP2, which might explain the reduced $E_{\max}$ ^{2, 6}. Upregulation of β -lactamases could be responsible for the increased EC_{50} . However, the production of β -lactamases by these strains was only slightly enhanced after induction, compared to ATCC 29213 (Figure 3A). Although the data do not allow to establish a specific mechanism, other resistance mechanisms, that have not been elucidated so far, might be involved. On the other hand, VU94, and particularly M007 (BORSA with oxacil-

lin MIC ≤ 2 mg/L and both belonging to the predominant MLST CC25 lineage²), showed a considerably higher production of β -lactamases, after induction. This is in accordance with the plasmid profile analysis, since pBW15-like plasmids, such as pBORa53, are associated with an inducible hyperproduction of β -lactamases. These plasmids encode very likely for methicillinase¹⁵. Two β -lactamases have been detected in the membrane of VU94 previously, and one of them being a methicillinase⁴. For this reason, VU94 and M007 were probably able to hydrolyze oxacillin very efficiently, with regrowth within 12 h as a consequence at 4 $\times$ and 8 $\times$ MIC, respectively. This indicates the need for high dosing strategies of penicillinase-resistant penicillins for β -lactamase hyperproducing BORSA. Unfortunately, oxacillin in combination with a β -lactamase inhibitor is not commercially available.

Although, oxacillin against BORSA with oxacillin MIC ≥ 4 mg/L (M002, M008 and M015) reduced the bacterial counts at 4 $\times$ and 8 $\times$ MIC, the actual concentrations, i.e. 16–32 mg/L, were high. Concentrations above 4 $\times$ MIC are considered clinically effective for time-dependent antibiotics, such as oxacillin²⁸. For β -lactams, the pharmacodynamic index that correlates best with clinical outcome is the time (t) that serum concentrations of the unbound fraction exceeds the MIC (t>MIC) and values of 40–50% or greater are considered necessary for effective treatment of *S. aureus* infections³⁰. Therefore, it is very unlikely that for a drug with a high protein binding affinity (94%) and an unfavourable EC₅₀, a free plasma concentration of ≥ 4 –8 mg/L (corresponding to the MIC of M002, M008 and M015) for approximately 50% of the dosing interval (t>MIC) can be attained with current dosage strategies (IV 1–12 g/24 h).

A recent case report highlights the fact that patients infected with non- β -lactamase producing BORSA, with oxacillin MIC ≥ 4 mg/L, and treated with β -lactams may lead to treatment failure³¹. The case report describes a patient with endocarditis, due to BORSA with oxacillin MIC ≥ 4 mg/L, who failed to respond to cloxacillin. This confirms our findings that treatment of infections caused by such BORSA with β -lactams is full of risk.

Since this study showed that BORSA with oxacillin MICs ≥ 4 mg/L are difficult to eradicate *in vitro* with β -lactam antibiotics, it is important to identify BORSA isolates by routine susceptibility testing. However, sixteen BORSA, including eight isolates with oxacillin MICs of 4 and 8 mg/L, tested with the Phoenix system were all considered MSSA. Apparently, this method is not suitable to differentiate between fully susceptible *S. aureus* strains and BORSA. BORSA isolates will thus be missed by routine susceptibility testing and as a consequence, the (empiric) antibiotic therapy choice, i.e. oxacillin, is not useful for the eradication.

In summary, out of the four antibiotics tested, oxacillin was, *in vitro*, non-effective against both BORSA with oxacillin MIC ≥ 4 mg/L and pBORa53-like plasmid containing BORSA with oxacillin MIC ≤ 2 mg/L. Further investigation into β -lactam dosing strategies against different BORSA strains is warranted in order to avoid treatment options consistent for MRSA infections.

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CHAPTER 9

General Discussion

General Discussion

Staphylococcus aureus continues to be a predominant cause of community-acquired and nosocomial associated infections¹. It is the most prevalent cause of endovascular infections, including catheter sepsis and infective endocarditis^{2,3}, and the second most common cause of bacteraemia⁴. There are a number of factors that contribute to the success of *S. aureus* in evading host immunity and in establishing (persistent) infections. It is partly due to its ability to form biofilm^{5,6}. Biofilm formation often prolongs the duration of debilitating infections since it restricts the access of many conventional antimicrobial agents to sessile bacterial communities and because of its recalcitrance to the innate⁷, as well as adaptive⁸, immune response. Several studies were undertaken to get a better understanding of how biofilm formation can be prohibited and whether mature biofilm can be disassembled.

***Staphylococcus aureus* biofilm formation in relation to the genetic background**

In a natural environment, most bacteria grow aggregated to each other and to solid surfaces⁹. Therefore, biofilm communities are considered the predominant mode of bacterial life¹⁰. Cells within a biofilm are embedded by extracellular polysaccharides (EPS), which provides protection against a variety of environmental stresses, such as pH shifts, osmotic shock, desiccation and UV radiation¹¹. It has been demonstrated that the *S. aureus* biofilm matrix could consist of different components, including polysaccharide intercellular adhesin (PIA) or poly- β (1,6)-N-acetyl-D-glucosamine (PNAG), extracellular DNA (eDNA), surface-anchored microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and proteins. In order to find strategies to tackle or to prevent biofilm associated infections, it is necessary to get insight in the mechanisms of biofilm formation. Evidently, the principles behind the capacity to form a biofilm are strain dependent. So far, it remains unelucidated which strains are more prone to become embedded by biomass. Under static conditions, we demonstrated in **chapter 2** that the number of clonal lineages associated with multilocus sequence typing (MLST) clonal complex (CC)8 that displayed excessive biomass attachment to polystyrene surfaces was higher compared to a variety of isolates with other genetic backgrounds. Although the majority of MLST CC8 associated strains tested were classified as strong biofilm formers, 40% of these strains were not. This means that there is another underlying predisposing factor for strong biofilm formation that is not unravelled yet. Although all CC8 sublineages originate from the same progenitor¹², differences in the presence and expression of regulatory loci between subclones could be expected due to evolutionary events. As postulated, also the major components of biofilm matrices might be essential for the formation of robust biofilm, since it was found that matrices containing more PIA/PNAG were predominantly associated with resolving instead of persistent bacteraemia⁶. Most of the CC8 isolates did not demonstrate a dry crystalline colony morphology on Congo red agar (CRA), which was considered indicative for PIA/PNAG formation¹³.

The importance of accessory gene regulator (*agr*) genotype for biofilm formation was recently discussed by Cafiso *et al.*¹⁴ and Kawamura *et al.*¹⁵ using strains isolated from patients with orthopaedic device-related infections. Based on their observations that *agr*-II genotype, *spa*-type t002, MLST CC5 associated strains were stronger biofilm formers than *agr*-I and *agr*-III strains, they stressed the importance of this *agr* genotype for biomass accumulation. However, in both studies the variety of clonal lineages of the isolates was limited or even unknown and not uniformly distributed compared to our examination. We observed at 0.1% glucose only strong biofilm formers among *agr*-II, *spa*-type t002, MLST CC5 associated strains and not in strains associated with MLST CC12 or CC15. All of their *agr*-I strains belonged to *spa*-type t1767, which is affiliated to MLST CC8, and were not included in our study. We are therefore convinced that the genetic background is a more appropriate predisposing factor for biofilm formation than *agr*-type.

All together, these results suggest that especially the MLST CC8 (*agr*-I) genetic background deserves special attention in future biofilm exploratory studies as well as *spa*-type t002 isolates (*agr*-II) for orthopaedic infections.

Biofilm formation among MLST CC8 isolates

It is known that the CC8 lineage, including USA300, disseminated successfully¹⁶ and caused serious biofilm-associated infections^{17–19}. Beside the ability to form biofilm, the extraordinarily success as a pathogen of various MLST CC8 subclones including the community-associated methicillin-resistant *S. aureus* (CA-MRSA) USA300 has been attributed to their highly active *agr* quorum-sensing system^{12,20}. Thus, the enhanced invasive potential is the consequence of abundant expression levels of virulence determinants, in particular α -toxin and α -type PSMs^{21,22}. Based on the observation that both a biofilm can be established as well as toxins can be excreted by USA300, it is likely to assume that large changes in activity of global regulatory systems (i.e. *agr* and *sarA*) in the majority of MLST CC8 associated strains occur. If the turnover from repression to activation of *agr* is relatively quick, fast biomass accumulation followed by rapid dispersal of bacteria could be expected.

It would be interesting to evaluate whether slow-developing but long-lasting biofilm communities do exist as well and to compare their (dis)advantages in terms of defence against host immune responses and antimicrobials. With our data this question remained unanswered, since biofilm formation was only assessed until 48 h. Although, *S. aureus* biofilm are generally considered fully mature within 24–48 h^{23,24}, it would be possible that it takes longer for certain clones²⁵. This sounds plausible since differences in the rapidity of *agr* RNAIII (de)activation have been noticed²⁶.

Biofilm formation among MLST CC5 isolates

The strong biofilm producing capacity of some strains with *spa*-type t002, belonging to the MLST CC5 clone, could be clarified by a high percentage of dysfunctional *agr* as previously observed by Schweizer *et al.*²⁷. In their study, 19 out of 190 (10%) USA300 blood isolates vs. 78 out of 234 (33%) MLST sequence type (ST)5 strains were dysfunctional in *agr*, respectively. This was higher than the overall percentage among blood stream isolates: 181 out

of 814 (22%) isolates. The prevalence of *agr* dysfunction among colonizing *S. aureus* strains was previously indicated as roughly 9%²⁸.

In general, *agr*-dysfunction (i), has been associated with a prolonged duration of bacteremia^{27,29}; (ii), has been attributed to prior antibiotic administration (including β -lactams and fluoroquinolones)³⁰; (iii), has been correlated with slow-growing small colony variants³¹; (iv), has been linked with reduced killing by innate host thrombin-induced platelet microbicidal proteins (tPMPs) and consequently a strong ability to establish endovascular infection such as endocarditis³²; (v), is less common among CA-MRSA versus healthcare-associated (HA)-MRSA³³; and (vi), showed a tendency toward higher mortality among patients infected with *agr*-dysfunctional *S. aureus*²⁷. These observations were consistent with *in vitro* studies showing that knocking out *agr* enhances biomass accumulation by the mutants^{34,35}.

However, recently, doubts have arisen concerning the determination of *agr*-dysfunction, since it was discovered that even though the lack of delta-hemolysin production is a phenotypic marker for a nonfunctional *agr* locus, RNAlII transcription was not absent but rather late²⁶. Although the *agr*-II genotype correlated clinically with vancomycin treatment failure³⁶, it was found that *agr* function, transcription and sequence did not influence vancomycin outcomes in an infective endocarditis model²⁶. Therefore, it remains undefined as to how *agr* activity impacts treatment outcome within the *agr*-II (CC5) group.

Another question to be answered is whether initial adhesion and biofilm formation of the various clonal lineages on polystyrene are in the same order of magnitude to other surfaces. Using flow-cells to grow biofilm on abiotic surfaces as glass, polycarbonate and titanium, USA300 showed only minor differences in film thickness, total biomass and surface coverage³⁷. Also, MLST CC8 strains easily adhered to human tissue such as airway epithelial cells^{38,39}.

Finally, there is a limitation in the quantification of biofilm employed by ourselves and others. Although the microtiter plate assay has been worldwide adopted as a useful biofilm performance test^{23,40}, its relevance for the clinical setting, i.e. device-related attachment of bacteria, has never been indisputably proven⁴¹. Moreover, a non-invasive diagnostic tool to detect biofilm *in vivo* is currently not available^{42,43}, but would be highly advantageous. Infected implant or indwelling device removal, necessary to isolate the causative micro-organism is not always possible or desirable⁴⁴ but often mandatory⁴⁵, and many replaced catheters become reinfected⁴⁶. Strains could under circumstances where preservation of the biomaterial is desirable only be indirectly isolated when quorum-sensing induced dispersal is followed by spread via the bloodstream⁴⁷. In case of device removal, screening for biofilm can be performed by a range of standardised methods. Beside microscopic imaging techniques, the role plate assay⁴⁸, sonication and scraping methods are available^{49,50}. Although the roll-plate assay is not appropriate for endoluminal evaluation of central venous catheters (CVCs), the roll-plate assay remains useful as a reference standard for catheter tip culture. We therefore chose this method for bacterial adherence analysis when testing new anti-microbial adhesive and anti-thrombogenic coatings.

Prevention of biofilm formation

Prevention of initial adherence of bacteria to indwelling devices is of unprecedented importance to prohibit biofilm formation and consequently bacteraemia on the long term. Further improvements in biomaterials are essential since the interventions to prevent complications with CVCs are often unsuccessful^{51,52}. In **chapter 4** and **5** we assessed the dual application of silver particles and heparin in hydrophilic surface coatings in order to overcome bacterial adherence on and thrombotic occlusions of catheters.

Advantages of silver as anti-adherent component in coatings are broad-spectrum antimicrobial activity, a slow appearance of resistance⁵³ and the lack of cross-resistance to antibiotics⁵⁴. A major concern about the clinical application of silver coatings is a general safety issue. Although silver is considered less toxic to human cells than other metals^{55,56}, the potential hazard to human health of long-term exposure is not fully investigated⁵⁷. Most research focuses on silver nanoparticles (1 – 110 nm)⁵⁸, since nanoparticles were considered to prolong antimicrobial activity because of their large surface to volume ratio^{59,60}. It accounts for a sustained release of Ag⁺ ions at the coating-tissue interface⁶¹. It could be argued that slightly larger silver particles, i.e. microparticles of approximately 1 µm, maintain strikingly refractory to microbial colonization whereas particles distribution throughout the body would be limited⁶². Since, it was demonstrated *in vivo* by Lankveld *et al.* that nanoparticles of smaller sizes were distributed more extensively to various kinds of tissues than the larger silver particles⁶². It is plausible that size-dependent distribution is coupled to size-dependent toxicity. Silver nano-particles have been demonstrated to bind to and transfer across cell membranes of lung fibroblasts, glioblastoma cells⁶³, keratinocytes and macrophages⁶⁴. Their internalisation and oxidative nature induces inflammatory, genotoxic, and cytotoxic characteristics⁵⁷. Some suggest that the window between minimum concentrations lethal to microbes and those toxic to the host is narrow⁶⁵, since the intensity of toxicity seems strongly associated with the local available concentration^{57,64}. Therefore the release of silver ions from the biomaterial should be kept under control, which will be the ultimate challenge for further studies. Our silver containing coatings remained antimicrobial after several stress-tests with high bacterial inocula under vigorously shaking conditions and many refreshments of medium to remove released silver ions. However, as shown in **chapter 5**, coatings containing only heparin lost their anti-adherent properties after only a few challenges, in concordance with a previously shown short-term controlled release of heparin from comparable coatings⁶⁶. In contrast, silver and heparin synergistically maintained the adherence at a lower level than silver alone during all the consecutive challenges. Altogether, this suggests that the release of heparin from the coating is retarded by the presence of the silver particles, and that simultaneously the release of silver ions is augmented by co-transport upon binding to heparin molecules. To prove this novel concept, the release of heparin and silver from all coating compositions should be analytically measured over time.

Although the addition of heparin to the silver coatings was remarkably successful in prevention of *in vitro* adherence of *S. aureus*, the effect of heparin is considerably unpredictable *in vivo*. On one hand, heparin can specifically bind certain proteins that may possess antimicrobial activity^{67,68}. On the other hand, heparin might stimulate biofilm formation when initial adherence could not be fully prohibited⁶⁹. In a meta-analysis of different

CVCs, heparin-bonded catheters were associated with a significantly lower rate of catheter related blood stream infections (CRBSI) compared with standard catheters, but no reduction in colonisation was shown⁷⁰. Since CVC colonization can be a precursor of CRBSI, it would be possible that heparin prohibited dispersal of attached bacteria. Silver-containing CVCs performed disappointingly in another meta-analysis not demonstrating any significant reduction in colonization or CRBSI compared to standard non-surface modified polyurethane or silicone catheters⁷¹. However, many studies included in the meta-analysis suffered from substantial shortcomings in methodological quality. The fact that the formation of a fibrin sleeve and CVC-related infection are interrelated⁷² stresses the importance of designing biomaterials that suppress both initial attachment of micro-organisms and the deposition of fibrin and platelets. The preventive capacity of heparin or silver particles alone within a coating might be highly divergent from their combined counterparts. Before clinical assessment of our identified NVP/BMA coating formulations that effectively combine biocidal and anticoagulant features could be performed, they should meet the requirements of negligible silver losses in order that patients are not exposed to avoidable risk. Medical device regulation is currently a matter of debate since variable standards for device regulatory approval exist and post-marketing surveillance is poorly organized^{73,74}.

Another way to approach prevention of biofilm formation is the prophylactic permanent treatment with drugs that prohibit the initial attachment of bacteria. In **chapter 7** we evaluated thioridazine and reserpine, which have proven functionality as an efflux pump inhibitor. Trans-membrane transporters, including efflux pumps, have been suggested to play a functional role during the biofilm maturation and disassembly process^{75,76}. We concluded that thioridazine and reserpine could only hinder biofilm accumulation of MLST CC8-associated *S. aureus* strains at 0.1% glucose, probably due to differences in the trans-membrane transporter capacity among the tested strains with different MLST-associated genetic backgrounds, i.e. CC5, CC22 and CC30. Fluctuations in nutrient and oxygen levels inside the biofilm and the availability in the surroundings could lead to clonal lineage-specific modification of tricarboxylic acid (TCA) cycle activity and consequently multiple gene regulator (*mgrA*) expression. It seems that *mgrA* plays a central role in efflux pump expression, but it also directly interferes with biofilm formation via *agr* and autolysis associated regulators, i.e. *cidA* and *IrgAB*. Our observations of the influences of EPIs on biomass require further research focussing on gene expression of genes involved in biofilm formation and efflux and influx pump activity to confirm our presumptions. It is also worthwhile to evaluate the simultaneous exposure to EPIs and antibiotics or to combine two or more types of EPIs affecting an extended range of *S. aureus* trans-membrane transporters.

One of the main efflux transporter proteins in *S. aureus* is NorA. As shown in **chapter 6**, we demonstrated that *norA* overexpression was restricted to a few clonal lineages associated with MLST CC5, CC30 and CC45. We did not find a correlation between *S. aureus* with mutations in the promoter region of *norA* and the level of efficacy of EPIs against the same strains under biofilm conditions. The discordance could be explained in terms of a weak correlation between mutations in the promoter region of *norA* and the actual expression on a protein level or by the presence of other trans-membrane transporters that play a

more crucial role in adjusting to nutrient availability and in the release of toxic molecules from the cytosol.

Disassembly of the biofilm matrix

The observed dormancy of bacteria in the deeper parts of the biofilm hinders the killing by antibiotics like β -lactams and glycopeptides that require an active life-cycle or cell-wall turnover to exert their efficacy. Under planktonic conditions, β -lactams covalently modify the active site of penicillin binding proteins (PBPs), enzymes that synthesize and alter bacterial peptidoglycan⁷⁷, and consequently inhibit the cross-linking of linear strands of peptidoglycan into a fishnet-like polymer that surrounds the bacterial cell and confers osmotic stability. Furthermore, β -lactams interfere in the balance between peptidoglycan synthesis and autolysis. Activation of the cell division machinery seems to be required for β -lactam induced cell lysis⁷⁷. It is a plausible scenario that nutrient deprivation deep within the biofilm limits cell division, an aspect that deserves further research attention. Besides slow cell division, the biofilm matrix provides a diffusion barrier to most antimicrobial compounds⁷⁸. This would lead to subinhibitory concentrations deep inside a biofilm. As a result, β -lactamase plasmid-related (hyper)induction might be the consequence. Accumulation of the overproduced enzyme in the polysaccharide matrix in the immediate surrounding vicinity of the bacteria impairs the penetration and efficacy of β -lactams even more. As shown in **chapter 8**, the pBORa53-like plasmid-containing borderline oxacillin resistant *S. aureus* (BORSA) are more prone to β -lactamase induction. Although these strains were not capable to produce large amounts of biomass (data not shown), multi-species biofilm or multiclonal aggregates containing BORSA could potentially further increase the biofilm recalcitrance to beta-lactams. Previously observed high levels of β -lactamase inside biofilm were believed to originate from lysed bacteria or membrane vesicles liberated from embedded bacteria⁷⁹.

This highlights the fact that conventional resistance mechanisms might contribute to the bacterial survival inside biofilm. Simultaneous exposure to matrix destructive agents seems to be essential for efficacy of penetration-limited antibiotics. We evaluated in **chapter 3** whether rifampicin could serve as a pore-forming agents as adjunct to oxacillin. We argued that if rifampicin is able to destruct the biofilm matrix, then rifampicin should also be active against pre-formed biofilm of isogenic rifampicin-resistant mutants. Using an *in vitro* static, microtiter plate biofilm assay, we demonstrated that the addition of rifampicin to oxacillin neither reduced the viable count nor affected the amount of biomass. Moreover, the efficacy of rifampicin against biofilm established by the rifampicin-susceptible parent isolates was highly variable. So for this reason of unpredictability, it seems necessary to re-evaluate rifampicin as standard adjunctive agent to empirically initiated therapy in patients with *S. aureus* infections with clinical suspicion of biofilm involvement.

Closing remarks

Scientific progress in identifying the key bacterial and fungal biofilm associated (genetic) elements and unravelling the biofilm related pathways provides a platform for the development of novel therapeutic agents against already established biofilm. An abundance of biofilm modifying or disruptive substances has already been tested *in vitro* against mature biofilm. It seems to be only a matter of time before the first intravenous applicable anti-biofilm agent will get market approval. At the same time the development of improved biomaterials for (permanent) indwelling devices, such as heart valves, CVCs and prosthetic joints, continues to provide a better basis for the prevention of bacterial adherence and consequently biofilm formation. This is of ultimate importance since biofilm-associated infections remain inherently difficult to treat and device removal is still the mainstay of clinical management.

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CHAPTER 10

Summary

Summary

During recent decades it has been discovered that many serious chronic and/or recurrent infections including endocarditis, osteomyelitis and CVC-related bacteremia are biofilm associated. Upon initial adherence to a surface, bacteria consequently become embedded in a selfproduced protective matrix consisting of a mixture of bacterial components and substances extracted from the environment. The biofilm matrix forms a surrounding protective barrier against host defences and antibiotic treatment. Unravelling the principles behind this survival strategy contribute to the goal-oriented design and application of preventive anti-biofilm technologies in biomaterial science and can lead to novel ways to reinforce the destruction of pre-existing mature biofilm.

In contrast to *Staphylococcus epidermidis* biofilm, most *Staphylococcus aureus* biofilm are extracellular polysaccharides (mostly referred as PIA, PNAG or slime) independent, as we found when evaluating 254 *S. aureus* clinical isolates by means of Congo red agar (CRA) screening. On CRA plates, only 9% of the strains displayed a distorted morphology, associated with slime formation (**chapter 2**). CRA-screening is therefore not useful as a high-throughput screening assay for *S. aureus* biofilm formation. To find out whether the genetic background is a predisposing factor for biofilm development, the total accumulated amount of biomass in polystyrene micro-titre plates within 24 h was related to the genetic background. At the physiologic blood glucose concentration (0.1%), more than 60% of the *S. aureus* strains associated with multilocus sequence typing (MLST) clonal complex (CC)8 produced large amounts of biomass compared to 0–7% for various other clonal lineages. The strong biofilm forming capacity of the CC8 associated strains was observed both for bloodstream isolates as well as for their commensal counterparts (**chapter 2**). Accessory gene regulator (*agr*) type II has been previously put forward as predisposing factor for promoting abundant biofilm formation. We did not find this correlation, since strains with *agr*-II genotype includes also genetic backgrounds that did not produce large amounts of biomass, such as CC15, CC12, and *spa*-types associated with MLST CC5. However, it was confirmed that various strains with *spa*-type t002 were indeed strong biofilm formers. In general, strains with *spa*-type t002 have been frequently isolated from patients with orthopaedic device-related infections.

It is known that bacteria embedded in biofilm are far more recalcitrant to antibiotics than their planktonic counterparts. Nevertheless, rifampicin is often used as adjunctive agent to empirically initiated therapy in situations of clinical suspicion of biofilm involvement. The rationale for recommending rifampicin administration is based on the ability to penetrate into biofilm and a presumed activity against dormant bacteria. Rifampicin has been extensively investigated for prosthetic device-related infections and found beneficial in terms of clinical and/or bacteriological cure rates. However, the number of studies for other indications is limited and the outcome often contradictory. Moreover, rifampicin resistant strains have emerged during treatment. As rifampicin diffuses more quickly into the biofilm than the companion agent, it would be logical that without proper destruction of biomass

rifampicin resistant subpopulations emerge. These could be responsible for persistent or re-occurring infections. It was hypothesized that if rifampicin possesses pore-forming or matrix destructive capabilities, rifampicin with a companion agent should be effective against rifampicin-resistant embedded bacteria. Using a static *in vitro* biofilm assay, this concept was evaluated in **chapter 3** with oxacillin alone or in combination with rifampicin against biofilm formed by rifampicin-susceptible or isogenic rifampicin-resistant methicillin-susceptible *S. aureus* (MSSA). Rifampicin (1 µg/mL) added to oxacillin enhanced the reduction in biomass produced by most rifampicin-susceptible isolates compared to oxacillin alone. However, the greater reductions achieved were highly variable, ranging from 17 to 54% compared to 4% for oxacillin alone. Strains embedded by more biomass at the moment of initial exposure showed a diminished reduction in viable count. None of the biofilm formed by isogenic rifampicin-resistant mutants yielded a synergistic reduction in biomass and viable count upon rifampicin addition to oxacillin. Rifampicin as adjunctive agent is therefore unwarranted against biofilm formed by rifampicin-resistant strains and is unpredictable in case of unknown rifampicin susceptibility. Especially since rifampicin resistance can be easily induced in the absence of a companion agent, which might be the result of slower diffusion into the biofilm.

Substances suitable for intravenous application that specifically destruct preformed mature biofilm are under investigation but are not or rarely available on the market yet. A more straight-forward approach is to prevent biofilm formation. Since biofilm formation mostly starts with initial attachment to a surface, this step should be hindered to prevent biofilm formation. As adhesion and thrombus formation are interrelated we aimed in **chapter 4** and **5** to develop coatings for central venous catheters (CVCs) that are both anti-microbial and anti-thrombogenic as well. For this purpose, hydrophilic bifunctional biocompatible coatings containing sodium heparin and silver particles were systematically developed. The coatings were examined in different *in vitro* test settings. Blood compatibility was assessed through thrombin generation and thrombocyte adherence assays, while exit-site experiments and long-term challenge tests were employed to investigate bacterial adherence and killing. Adhesion of both reference strain (ATCC 29213) and clinical isolates of *S. aureus* was strongly inhibited by silver alone, but surprisingly, heparin augmented the antimicrobial activity of silver, while maintaining its anticoagulant function. The effect of changing the coating's hydrophilicity, the type of silver stabilization or the silver content was also assessed. The less hydrophilic SlipSkin® material (SS70:30), with a NVP/BMA molar ratio of 70/30, was expected to have slower release of embedded heparin, and –hence– a more sustained antimicrobial effect due to a long-lasting improved mobility of silver particles. Our expectations were confirmed with the pre-washing experiments, demonstrating that the longer the pre-wash with milliQ water, the more *S. aureus* cells adhered to SS90:10-Ag-Hep at the moment of bacterial exposure, while the number of adhered bacteria remained constant over time when SS70:30-Ag-Hep specimens were more intensively pre-washed. Doubling the silver content (SS70:30-Ag_{2x}-Hep) did not improve the long-term prevention of adherence of *S. aureus* and reduced the viable count in the time-kill assays to a similar extent as the specimens containing the standard content of silver (SS70:30-Ag-Hep).

All experimental results taken together with observations described in the literature pointed out that antimicrobial synergy of heparin and silver is best explained by binding of Ag^+ ions to heparin within the swollen coating, consequently followed by release of Ag^+ -heparin ligand-carrier complexes upon immersion of the coatings in an aqueous environment such as blood.

It is generally accepted that the recalcitrance to antibiotics, and also the inability of the host's immune system to clear *S. aureus* biofilm, cannot be solely attributed to the diffusion barrier formed by the matrix. Due to the cramped conditions inside the deeper parts of the biofilm, it is likely that locally a waste disposal problem exists. As described by others, the matrix structure is partially interspersed with channels that are considered essential for nutrition and oxygen support and waste drainage. Still, the supply of nutritious substances is believed to be insufficient, which may clarify the dormancy of embedded bacteria. The high consumption of nutrients, necessary to produce extracellular polymeric substances (EPS) for the matrix, very likely exacerbates the waste management difficulty. Enhanced extrusion of toxic components from the cytosol via upregulation of transmembrane transporters has been suggested as a manner to help pass off toxins.

In first instance we investigated whether certain clonal lineages of *S. aureus* were more prone to mutations in the promoter region of the *norA* gene. NorA is one of the main efflux transporter proteins in *S. aureus*. As notified in **chapter 6**, we showed that the overexpression of *norA* was associated with sequence types of all three known NorA alleles, i.e. *norA* wt, *norA1199* and *norAII*. But the overexpressing strains were restricted to a few clonal lineages including (mainly methicillin resistant) *S. aureus* strains associated with MLST CC5, CC30 and CC45. The question remained whether these mutations would lead to augmentation of efflux pump expression whenever these strains become embedded into a biofilm. Recent observations already pointed out that efflux pump inhibitors (EPIs) like reserpine or thioridazine could prevent *in vitro* biofilm formation of a single tested CC8-associated *S. aureus* strain. We incorporated strains associated with MLST CC8, CC22, CC5 and CC30 in our *in vitro* experiments to test whether reserpine and thioridazine could prohibit initial attachment. We confirmed that under 0.1% glucose conditions thioridazine effectively prevented biomass formation of CC8 associated strains (**chapter 7**). Interestingly, this genetic background was not linked to *norA* overexpression, since no mutations that could lead to *norA* hyperexpression were detected among our collection of CC8 isolates ($n=41$). There was no effect of EPIs on pre-mature biofilm of CC22 associated strains. Biomass production of CC30 and CC45 associated strains was specifically prevented by thioridazine at a higher supplemental glucose concentration, i.e. 1%. Independent of the genetic background, reserpine was inferior to thioridazine in the prevention of biomass accumulation at both tested glucose concentrations. Remarkably, at 1% glucose, biomass formation of MLST CC8-associated strains was elevated upon exposure to both EPIs. We presume that thioridazine and reserpine have a non-specific affinity for a variety of transporters, including nutrient-importers and toxin exporters. This would consequently implicate that glucose availability and/or metabolism influences the effects of EPIs on biofilm formation. Blocking glucose importers at 0.1% glucose (extracellular concentration) would result in suppression of biomass formation, especially among strains with a high carbohydrate

uptake capacity, since a deficiency in intracellular amount of glucose would lead to tricarboxylic acid (TCA) cycle activation. Enhancement of TCA cycle intermediates is known to correlate with biofilm repression. Abundantly imported amounts of carbohydrates at 1% glucose would theoretically result in intracellular acidification due to an inactive TCA cycle and the conversion to acetate. Concerning the CC8-associated strains, we hypothesized that blocking the efflux of acidic metabolites, such as acetate, promotes – instead of prevents – biomass formation, while influx of glucose is not much affected by the excessive availability in the environmental medium.

In conclusion, the glucose and oxygen presence within microbial biofilm communities is changing over time, which concomitantly modifies the TCA cycle activity of the embedded bacteria. Unique accumulation molecules and metabolic heterogeneity have been observed on a large-scale basis by others. We hypothesized that the biofilm life-cycle turnover is clonal lineage specific, as is the expression of transmembrane transporters during the different consecutive stages of biofilm formation. This makes EPIs inappropriate in the prevention of early biomass accumulation.

The impact of EPIs on pre-existing mature biofilm was also evaluated and biofilm disassembly was less pronounced (CC8) or even absent (CC22, CC30 and CC45) compared to the prevention of biomass formation. It seems that the penetration barrier of mature biofilm for most antibiotics also clearly exist for the EPIs themselves.

The biofilm matrix could retain waste materials by improper functioning of down-regulated transmembrane transporters, but could also keep excreted substances necessary for their defence in their immediate surrounding vicinity. As noticed previously, entrapment and accumulation of β -lactamases inside the biofilm occurs. Borderline oxacillin-resistant *S. aureus* (BORSA) are potentially interesting in this context, since these strains have been associated with the hyperproduction of β -lactamases (penicillinases and/or methicillinases). In **chapter 8** we investigated a collection of BORSA isolates in order to unravel whether they could be killed under planktonic conditions with a standard β -lactam antibiotic, i.e. oxacillin. Time-kill curves analysis was also performed with comparative alternatives of oxacillin, such as vancomycin, daptomycin and linezolid. Plasmid profile analysis revealed that a part of our collection of BORSA strains, with oxacillin MIC ≤ 2 mg/L and including the BORSA reference strain VU94, possesses a pBORa53-like plasmid that encodes for β -lactamases including methicillinases. In the other BORSA, with oxacillin MIC ≥ 4 mg/L, pMW2-like penicillinase-encoding plasmids were identified. The pBORa53-like plasmid containing BORSA showed markedly more regrowth in the time kill curve studies when testing in the presence of oxacillin. The oxacillin killing activity was also attenuated against BORSA with oxacillin MIC ≥ 4 mg/L compared to ATCC 29213 (MSSA reference strain), since the pharmacodynamic parameter (EC_{50} or ϵC_{50}) revealed that the potency of oxacillin was markedly reduced (approximately 10 $\times$). Since these BORSA do not produce methicillinases upon induction by oxacillin, it was anticipated, but yet not proven, that these BORSA obtained modifications in the penicillin-binding proteins (PBPs) 1, 2 and/or 4 genes. Further investigation into β -lactam dosing strategies against different type of BORSA strains is warranted in order to avoid putative therapy failure. We discovered that not all BORSA are equally interesting for biofilm related research. Especially the pBORa53-

like plasmid-containing BORSA should be evaluated in biofilm experimental settings, since they are the most sensitive to β -lactamase (methicillinase) induction. However, a recently performed pilot experiment (data not shown) revealed that our pBORa53-like plasmid-containing BORSA, which belonged to the associated MLST clonal lineage CC25 were not capable to produce large amounts of biomass at 0.1% glucose, when compared to the MSSA and MRSA isolates associated with MLST CC8. Nevertheless, it could be interesting to introduce BORSA strains in multi-species biofilm.

Additional to the main purpose of these findings, we examined an alternative method to evaluate time-kill data. When performing time-kill analysis, it should be recommended to take pharmacodynamic parameters into account as efficacy markers in addition to traditional end-points, like “time to 99.9% kill” or presenting the number of remaining CFU/mL at a certain time point. These traditional methods to present efficacy data do not properly reflect all available time-kill data, including e.g. regrowth or alterations in the growth rate over time. By taking the absolute difference in $\log_{10}$ viable count between growth or killing in the absence and presence of an antibiotic, i.e. the area between the curves over 24h (ABC_{0-24h}), all available time-kill data could be incorporated in the concentration-effect parameters.

Nederlandse samenvatting

Samenvatting

In de laatste decennia heeft men ontdekt dat chronische en recidiverende infecties waaronder endocarditis, osteomyelitis en centraal-veneuze catheter (CVC)-gerelateerde bacteriëmiën biofilm geassocieerd zijn. Na initiële aanhechting van bacteriën aan een oppervlak zorgen deze er vervolgens voor dat ze zich inkapselen in een zelfgeproduceerde matrix bestaande uit bacteriële componenten en substanties die zijn onttrokken uit hun nabije omgeving. Deze biofilm matrix waarin de bacteriën zich bevinden biedt bescherming tegen het menselijke afweersysteem en antimicrobiële therapieën. Het ontrafelen van de mechanistische principes van deze overlevingsstrategie van bacteriën, zal bijdragen aan doelgerichte anti-biofilm toepassingen om al gevormde biofilm te kunnen afbreken en aan het ontwikkelen van biomaterialen die biofilm vorming kunnen voorkomen.

In tegenstelling tot *Staphylococcus epidermidis* biofilm bestaat de matrix van *Staphylococcus aureus* biofilm zelden uit extracellulaire polysaccharides, ook wel aangeduid als bacterieel slijm of PNAG (Poly- β (1,6)-N-acetyl-D-glucosamine). Dit is overeenkomstig onze bevindingen met de Congo rood agar (CRA) test van 254 klinische *S. aureus* isolaten. Bij de beoordeling van de kolonies op de agar platen bleek dat slechts 9% van de stammen een afwijkende morfologie, kenmerkend voor slijmvormende stammen, vertoonden (**hoofdstuk 2**). De CRA screenings test is om deze reden niet geschikt als diagnostische bepalingmethode om biofilmvorming van *S. aureus* mee aan te tonen. Om na te gaan of de genetische achtergrond van de bacterie een predisponerende factor is voor biofilmvorming, werd de totale hoeveelheid biomassa die in 24 uur werd geproduceerd gerelateerd aan de genetische achtergrond van de betreffende bacterie. Bij een fysiologische glucose concentratie van 0,1% was 60% van de *S. aureus* stammen met een multilocus sequence typing (MLST) klonaal complex (CC)8 geassocieerde genetische achtergrond in staat een bovengemiddeld grote hoeveelheid biomassa te genereren terwijl dit voor stammen met andere genetische achtergronden maar in 0-7% van de gevallen gold. De goede biofilm vormende eigenschap van de CC8 geassocieerde stammen werd zowel bij bloedstroom als commensale isolaten waargenomen (**hoofdstuk 2**). Recent werd verondersteld dat het accessory gene regulator (*agr*) type II een predisponerende factor zou kunnen zijn voor het bevorderen van overmatige biofilm vorming. Deze correlatie hebben wij niet kunnen bevestigen. Immers stammen met genetische achtergronden die geen sterke mate van biomassa vorming vertoonden, zoals *spa*-typen gerelateerd aan MLCT CC15, CC12 en CC5 behoren tot *agr* type II. Echter verschillende stammen met *spa* type t002 gerelateerd aan CC5 bleken wel degelijk sterke biofilm vormende eigenschappen te bezitten. Dit is overeenkomstig de bevindingen in de dagelijkse praktijk: stammen met *spa* type t002 worden veelvuldig geïsoleerd bij orthopedische patiënten met kunstmateriaal geassocieerde infecties.

Bacteriën omgeven door een biofilm zijn vele malen meer recalcitrant voor antibiotica dan wanneer deze in planktonische omstandigheden verkeren. Desalniettemin, wordt rifampicine vaak als additief toegevoegd aan reeds empirisch geïnitieerde antibiotische therapie,

met name in situaties met klinische verdenking op biofilm betrokkenheid. De reden voor de toevoeging van rifampicine is gebaseerd op de eigenschap van goede penetratie in biofilm en een veronderstelde activiteit tegen bacteriën in “ruste”. De toepassing van rifampicine bij prothese-gerelateerde infecties is uitvoerig bestudeerd en zowel ten aanzien van de kans op klinische verbetering als op vermindering van het aantal bacteriën is de meerwaarde aangetoond. Echter, het aantal uitgevoerde onderzoeken met rifampicine voor andere indicaties is beperkt en/of de uitkomsten zijn tegenstrijdig. Ook is gebleken dat rifampicine resistente subpopulaties kunnen ontstaan tijdens rifampicine behandeling. Aangezien rifampicine vermoedelijk sneller diffundeert in de biofilm dan het antibioticum waaraan het is toegevoegd, is het logisch dat zonder goede afbraak van biomassa rifampicine resistente subpopulaties ontstaan. Deze kunnen vervolgens aanleiding geven tot persisterende of recidiverende infecties. Indien rifampicine in staat zou zijn om poriën te vormen in de biofilm, of de matrix zou kunnen afbreken, dan zou rifampicine toegevoegd aan empirische therapie in staat moeten zijn om biofilm gevormd door rifampicine-resistente *S. aureus* stammen te verwijderen. Deze hypothese werd in **hoofdstuk 3** getest middels een statische *in vitro* biofilm opstelling waarbij biofilm, gevormd door rifampicine gevoelige of isogene rifampicine-resistente methicilline gevoelige *S. aureus* stammen, werden blootgesteld aan oxacilline alleen en in combinatie met rifampicine. Rifampicine (1 mg/L) toegevoegd aan oxacilline leidde bij rifampicine-gevoelige stammen in vrijwel alle gevallen tot een sterkere afname van de geproduceerde biomassa in vergelijking met blootstelling aan alleen oxacilline. De additionele reductie in biomassa varieerde voor de combinatie tussen de 17% en 54% in vergelijking met een afname van gemiddeld 4% voor oxacilline alleen. Stammen die initieel omgeven werden door meer biomassa waren minder gevoelig voor behandeling met rifampicine en oxacilline, zoals bleek uit het feit dat het aantal levende bacteriën per biofilm minder afnam. Een synergistische reductie in biomassa of in aantal levende bacteriën per biofilm ten gevolge van oxacilline en rifampicine blootstelling werd niet bereikt bij isogene rifampicine resistente mutanten. Om deze reden is rifampicine niet geschikt als additieve strategie tegen biofilm gevormd door rifampicine resistente stammen. Tevens is de meerwaarde van rifampicine onvoorspelbaar wanneer de rifampicine gevoeligheid onbekend is. Bovendien kan rifampicine resistentie op eenvoudige wijze worden geïnduceerd in de biofilm in lokale afwezigheid van een ander antibioticum. De kans hierop is aanwezig aangezien andere antibiotica langzamer diffunderen in de biofilm dan rifampicine. Daarom wordt in de kliniek gewacht met het toevoegen van rifampicine tot de bacteriële load verlaagd is.

Verschillende stoffen die een reeds gevormde volgroeide biofilm kunnen aantasten of afbreken, en die intraveneus kunnen worden toegediend, zijn nog niet of nauwelijks op de markt beschikbaar. Een alternatieve manier om biofilm vorming te lijf te gaan is het voorkomen van biofilm vorming door middel van preventieve maatregelen. Aangezien biofilm vorming start met aanhechting aan een oppervlak, is het duidelijk dat deze initiële stap verhinderd moet worden. Zoals beschreven in **hoofdstuk 4** en **5** zijn adhesie en trombus vorming aan elkaar gerelateerd. Om die reden werd een coating ontwikkeld voor centraal veneuze katheters (CVCs) die zowel antimicrobieel als antitrombogene is. Om dit te bereiken werden op systematische wijze hydrofiele bifunctionele biocompatibele coatings

ontwikkeld die zowel natriumheparine als zilver deeltjes bevatten. De coatings werden getest met behulp van verschillende *in vitro* meetopstellingen. De compatibiliteit met bloed werd onderzocht door middel van trombine generatie metingen en door microscopisch naar de aanhechting van trombocyten te kijken. Bacteriële aanhechting en afdoding werden geanalyseerd met behulp van langdurige blootstellingsexperimenten en de zogenaamde katheter-tip test. De adhesie van zowel een *S. aureus* referentie stam (ATCC 29213) als die van klinische isolaten werd in hoge mate verhinderd door coatings met alleen zilver. Het effect van zilver werd versterkt indien ook heparine in de coating was opgenomen. De anticoagulerende werking van heparine werd niet tenietgedaan door de aanwezigheid van zilver. Het effect van aanpassing van de hydrofiliciteit van de coating, de wijze waarop het zilver was gestabiliseerd en het veranderen van de hoeveelheid zilver per coating werd eveneens onderzocht. Het was de verwachting dat de minst hydrofiele samenstelling van het coatingsmateriaal SlipSkin® (SS70:30), met een NVP/BMA molaire ratio van 70/30, zou leiden tot een tragere afgifte van heparine. Dit zou dan bijdragen aan een verlengd antimicrobieel effect vanwege het feit dat de zilverdeeltjes onder invloed van heparine langduriger en tevens gelijkmatiger uit de coating zullen vrijkomen. Dit werd bevestigd met een experiment waarbij de coatings werden voorgewassen met milliQ water om langdurig gebruik te simuleren. Aangetoond werd met de SS90:10-Ag-Hep materialen, dat hoe langer er werd voorgewassen, des te meer *S. aureus* bacteriën konden aanhechten zodra de coatings werden blootgesteld aan bacteriën. Daarentegen bleef het aantal aangehechte bacteriën constant indien de SS70:30-Ag-Hep coatings intensiever werden voorgewassen. Het verdubbelen van de geïncorporeerde hoeveelheid zilver (SS70:30-Ag_{2x}-Hep) had geen effect op de lange termijn preventie ter voorkoming van *S. aureus* aanhechting. Tevens bleek het aantal bacteriën dat werd gedood per tijdseenheid gelijk aan de coatings met de standaard hoeveelheid zilver (SS70:30-Ag-Hep).

Wanneer we alle resultaten van de afzonderlijke experimenten gezamenlijk bekijken en deze vergelijken met de literatuur dan kan de waargenomen antimicrobiële synergie tussen heparine en zilver het best verklaard worden door binding van de zilverionen aan de heparinemoleculen in de opgezwollen coating gevolgd door het vrijkomen van de gevormde complexen na immersie van de coating in een waterige omgeving zoals bloed.

De algemeen geaccepteerde opvatting betreffende de recalcitrantie van *S. aureus* biofilm tegen antibiotica en het lichaamseigen afweersysteem is dat dit niet volledig kan worden toegeschreven aan de diffusiebarrière die gevormd wordt door de biofilmmatrix. De dichte opeenstapeling van bacteriecellen in de diepere lagen van de biofilm zouden er voor kunnen zorgen dat lokaal de afvalstoffen niet goed kunnen worden afgevoerd. Anderen beschreven dat de matrix kanalen bevat die als essentieel worden beschouwd voor de aanvoer van voedingsstoffen en zuurstof en de drainage van afvalstoffen. Desondanks wordt de beschikbaarheid van voedingssubstanties als ontoereikend beschouwd en vormt daarmee de gangbare verklaring voor de latentie van de omgeven bacteriecellen. De grote interne consumptie van nutriënten tijdens de opbouw- en/of uitbreidingsfase van de biofilm om voldoende extracellulaire polymere substantie (EPS) te vormen zorgt hoogstwaarschijnlijk voor een exacerbatie van het afvalstoffenprobleem. Er wordt verondersteld

dat via upregulatie van transmembraan transporters toxische componenten uit het cytosol verwijderd kunnen worden.

In eerste instantie onderzochten wij of bepaalde *S. aureus* klonale complexen vatbaarder zijn voor mutaties in de promotorregio van het *norA* gen. NorA is één van het meest voorkomende efflux transporter eiwitten in de celwand van *S. aureus*. In **hoofdstuk 6** is beschreven dat *norA* overexpressie voorkomt bij stammen van alle drie de bekende sequentie typen van het *norA* allel, namelijk *norA wt*, *norA1199* en *norAll*. Echter overexpressie kwam slechts bij een beperkt aantal klonale complexen voor. Dit waren voornamelijk methicilline-resistente *S. aureus* geassocieerd met MLST CC5, CC30 en CC45. De cruciale vraag die resteert is of de mutaties in de promotor regio van het *S. aureus norA* gen tot overexpressie van efflux pompen zou leiden indien deze stammen omgeven worden door een biofilm. Recent is met een enkele CC8-geassocieerde *S. aureus* stam waargenomen dat efflux pomp inhibitoren (EPIs) zoals reserpine of thioridazine preventief *in vitro* biofilm vorming konden verhinderen. Om te onderzoeken of initiële aanhechting van andere stammen ook door reserpine en thioridazine voorkomen kan worden werden verscheidene MLST CC8, CC22, CC5 en CC30 geassocieerde stammen getest met behulp van *in vitro* opstellingen. Zoals beschreven in **hoofdstuk 7**, kon bevestigd worden dat bij een glucose concentratie van 0,1% thioridazine inderdaad in staat is om biomassa vorming door CC8 geassocieerde stammen te verminderen. Opvallend is dat bij deze genetische achtergrond overexpressie van *norA* onwaarschijnlijk is aangezien in de gehele collectie van CC8 isolaten ($n=41$) geen mutaties in de promotorregio van *norA* werden gevonden die gerelateerd zijn aan *norA* overexpressie. De EPIs hadden geen effect op reeds volgroeide biofilm van stammen geassocieerd met MLST CC22. De biomassa productie van stammen geassocieerd met MLST CC30 en CC45 werd specifiek geremd bij een hogere glucose concentratie van het medium, namelijk 1%. Onafhankelijk van de genetische achtergrond bleek dat bij beide geteste glucose concentraties reserpine inferieur was ten opzichte van thioridazine in de preventie van biomassa vorming. Opmerkelijk is dat bij 1% glucose de biomassa vorming van de MLST CC8 geassocieerde isolaten bevorderd werd zodra deze werden blootgesteld aan de EPIs. Mogelijk hebben thioridazine en reserpine een niet specifieke activiteit voor een diversiteit aan transporters, zoals voor het opnemen van voedingsstoffen en het uitscheiden van toxische componenten. Derhalve impliceert dit dat de beschikbaarheid van glucose en/of het metabolisme ervan de effecten van EPIs op biofilm vorming beïnvloedt. Het blokkeren van de glucose toevoerkanalen bij een extracellulaire glucose concentratie van 0,1% zou moeten resulteren in een vermindering van de biofilmvorming, met name bij stammen met een grote opnamecapaciteit van koolhydraten, aangezien een intracellulaire deficiëntie in glucose zal leiden tot activatie van de tricarboxylzuur (TCA) cyclus. Het is bekend dat verhoging van TCA cyclus intermediären gecorreleerd is aan biofilm repressie. Overvloedige influx van koolhydraten bij een glucose concentratie van 1% zou theoretisch resulteren in een intracellulaire verzuring ten gevolge van een inactieve TCA cyclus en de conversie naar acetaat. Betreffende de CC8-geassocieerde stammen kwamen we tot de hypothese dat het blokkeren van efflux pompen voor het verwijderen van zure metabolieten zoals acetaat, zou resulteren in een bevordering – in plaats van de preventie – van biomassa vorming, terwijl de influx van gluco-

se niet veel beïnvloed zal worden door de overvloedige beschikbaarheid van glucose in het omgevingsmedium.

Ten gevolge van fluctuatie in de tijd van de hoeveelheid glucose en zuurstof in microbiële populaties in biofilm verandert de activiteit van de TCA cyclus van de ingebedde bacteriën ook voortdurend. Unieke accumulatie moleculen en metabole heterogeniteit zijn reeds veelvuldig beschreven in de literatuur. Wij veronderstelden dat de overgang van de opeenvolgende fasen van biofilmontwikkeling specifiek zijn per genetische achtergrond en daarmee tevens ook de expressie van de afzonderlijke transmembraan transporters tijdens de verschillende fasen. Hierdoor zijn EPIs ongeschikt als preventief middel tegen de initiële accumulatie van biomassa.

Het effect van EPIs op reeds bestaande volgroeide biofilm werd ook bestudeerd. De afbraak en/of het uiteenvallen van volgroeide biofilm was minder uitgesproken (CC8) of zelfs volledig afwezig (CC22, CC30 en CC45) in vergelijking met de effecten die zijn bereikt ter preventie van biomassa vorming. Ogenscheinlijk lijkt de penetratiebarrière van volgroeide biofilm die bestaat voor diverse antibiotica ook te bestaan voor de EPIs zelf.

Indien de transmembraan transporters disfunctioneel of downgereguleerd zijn kan de biofilm matrix afvalmaterialen vasthouden evenals uitgescheiden substanties die noodzakelijk zijn voor zelfbescherming van de bacterie in de directe leefomgeving. Zo kunnen β -lactamases (penicillinases en/of methicillinases) opgesloten raken en vervolgens accumuleren in de biofilm. In dat verband zijn "borderline" oxacilline resistente *S. aureus* (BORSA) isolaten potentieel interessant aangezien deze stammen geassocieerd zijn met een overproductie van β -lactamases. **Hoofdstuk 8** beschrijft de resultaten van afdodingsexperimenten van een aantal BORSA isolaten onder planktonische condities met een standaard β -lactam-antibioticum zoals oxacilline en met andere vergelijkbare antibiotica zoals daptomycine, vancomycine en linezolid. Met behulp van een plasmide profiel analyse is gebleken dat de BORSA stammen met een oxacilline MIC van ≤ 2 mg/L en de BORSA referentiestam VU94, een pBORa53-achtig plasmide heeft dat codeert voor β -lactamases inclusief methicillinases. Bij BORSA isolaten met een oxacilline MIC van ≥ 4 mg/L werd een pMW2-achtig plasmide geïdentificeerd. De pBORa53-achtige plasmide bevattende BORSA stammen vertoonden meer hergroei in de afdodingscurves in een oxacilline bevattend groeimedium. Ook bij de andere groep BORSA isolaten met een oxacilline MIC van ≥ 4 mg/L was de afdodingscapaciteit drastisch verminderd in vergelijking met de MSSA referentie stam ATCC 29213, zoals bleek uit de farmacodynamische parameter (EC50 of ϵ C50) die sterk gereduceerd was (ongeveer $10\times$). Aangezien deze BORSA stammen geen methicillinases produceren werd gesuggereerd dat in deze isolaten modificaties in de genen van de penicilline-bindende peptiden (PBPs) 1, 2 en/of 4 zijn opgetreden. Meer onderzoek naar de doseringsstrategie van β -lactam-antibiotica bij verschillende BORSA stammen lijkt noodzakelijk om mogelijk therapiefalen te voorkomen. We hebben vastgesteld dat niet alle BORSA's even interessant zijn voor biofilm gerelateerd onderzoek. Met name de pBORa53-achtige plasmide bevattende BORSA zouden verder geëvalueerd moeten worden onder biofilm condities, aangezien deze stammen het meest gevoelig zijn voor β -lactamase (methicillinase) inductie. Echter, een recent uitgevoerd pilot experiment liet zien dat de pBORa53-achtige plasmide bevattende BORSA uit onze collectie, welke allemaal geassocie-

eerd zijn met MLST CC25, niet in staat zijn grote hoeveelheden biomassa bij 0,1% glucose te genereren in vergelijking met de MLST CC8 geassocieerde MSSA en MRSA isolaten. Desondanks kan het relevant zijn om biofilmonderzoek met BORSA stammen uit te voeren, aangezien deze ook kunnen voorkomen in multi-species biofilm.

Naast de hoofddoelstelling van dit onderzoek is een alternatieve methode geëvalueerd voor het analyseren van data verkregen uit afdodingscurves. Onze aanbeveling is dat aan de al bestaande traditionele eindpunten, als markers voor effectiviteit, zoals “de tijd totdat 99,9% van de bacteriën is afgedood”, of het aantal CFU/mL na een bepaalde tijdsperiode, nieuwe pharmacodynamische parameters toegevoegd zouden moeten worden. De traditionele eindpunten om effectiviteit in uit te drukken zijn geen goede weergave van het totaal aan data afkomstig van afdodingsexperimenten. Processen zoals hergroei of veranderingen in de groeisnelheid van de bacteriën tijdens het experiment worden niet verdisconteerd in de traditionele parameters. Door het absolute verschil in $\log_{10}$ eenheden levende bacteriën te nemen tussen de groeicurve zonder antibioticum en de afdodingscurve in aanwezigheid van een antibioticum kan de oppervlakte tussen beide curven over 24 uur berekend worden (ABC_{0-24h}) en daarmee alle beschikbare data verwerkt worden in een concentratie-effect parameter.

List of abbreviations

List of abbreviations

aaa	Autolysin adhesion protein
AgNP	Silver nanoparticle
<i>agr</i>	Accessory gene regulator
AIP	Auto inducing peptide
ATCC	American type culture collection
aur	Metalloprotease aureolysin
bap	Biofilm-associated protein
bbp	Bone sialoprotein-binding protein
BHI	Brain heart infusion (broth)
BMA	n-butyl methacrylate
BORSA	Borderline oxacillin resistant <i>Staphylococcus aureus</i>
BSI	Blood stream isolates
BURP	Based upon repeat pattern
CAMBH	Cation adjusted Mueller Hinton Broth
CA-MRSA	Community-associated methicillin/multidrug-resistant <i>Staphylococcus aureus</i>
CC	Clonal complex
ccpA	catabolite control protein A
c-di-GMP	3'-5'-cyclic diguanylic acid
can	Collagen-binding adhesin
CF	Cystic fibrosis
ClfA/B	Clumping factor A and B
CoNS	Coagulase-negative staphylococci
CLSI	Clinical laboratory and standards institute
CPS	Counts per second (intensity)
CRA	Congo red agar
CRBSI	Catheter-related bloodstream infections
CTAD	Citrate-theophylline-adenosine-dipyridamole
CVC	Central venous catheter
Eap	Extracellular adherence protein
<i>ebh</i>	Extracellular matrix bindinghomologue
ebpS	Elastin-binding protein
ECP	Extracellular proteases
eDNA	Extracellular deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
emp	Extracellular matrix binding protein
eno	Laminin binding protein
EPI	Efflux pump inhibitor
EPS	Extracellular polymeric substance / Exopolysaccharides
EUCAST	European committee on antimicrobial susceptibility testing
FbpA	Fibrinogen binding protein
FnbA/B	Fibronectin-binding protein A and B
HA-MRSA	Healthcare/Hospital-associated methicillin-resistant <i>Staphylococcus aureus</i>
hld	δ -hemolysin, PSM- δ
LDH	lactate dehydrogenase
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
MLST	Multi Locus Sequence Typing
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin Susceptible <i>Staphylococcus aureus</i>
MRSA	Methicillin/Multi- Resistant <i>Stahylococcus aureus</i>
NMP	N-methylpyrrolidone

PBP	Penicillin-binding protein
PEG	Polyethylene glycol
PIA	Polysaccharide intercellular adhesin
PNAG	Poly- β (1,6)-N-acetyl-D-glucosamine
PRP	Platelet-rich blood plasma
PSM	Phenol soluble modulín
PVL	Panton-Valentine leucocidin
PVP	Polyvinylpyrrolidone
<i>rot</i>	Repressor of toxins
RT/rt-PCR	Reverse transcription polymerase chain reaction or Real time polymerase chain reaction
<i>sarA</i>	Staphylococcal accessory regulator
<i>SasG</i>	Surface protein G
<i>SCCmec</i>	Staphylococcal cassette chromosome mec
<i>scpA/B</i>	Cysteine proteases staphopain A/B
SCV	Small Colony Variant
<i>sdrC/D/E</i>	Serine-aspartate repeat-containing proteins C, D and E
<i>spa</i>	Staphylococcal protein A
<i>spoVG</i>	Stage V sporulation protein G
SS	SlipSkin [®] (coating)
<i>sspA</i>	V8 serine protease
ST	Sequence type
TCA	Tricarboxylic acid (cycle)
TSB	Trypticase soy broth
UTI	Urinary tract infection
XPS	X-ray photoelectron spectroscopy

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