Staphylococcal response to daptomycin in implant-associated infections

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Abbreviations

agr	accessory gene regulator
AIP	autoinducing peptide
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
Ca^{2+}	calcium ions
$CaCl_2$	calcium chloride
CA-MRSA	community-associated methicillin-resistant *Staphylococcus aureus*
CAMP	cationic antimicrobial peptide
ClfA	clumping factor A
CLSI	Clinical and Laboratory Standard Institute
C_{max}	peak concentration
C_{min}	concentration after 24 hours
CO_2	carbon dioxide
CoNS	coagulase-negative *Staphylococcus* species
cSSSI	complicated skin and skin-structure infection
D-Ala	D-alanine
DAP	daptomycin
DNA	desoxyribonucleic acid
ECM	extracellular matrix
eDNA	extracellular genomic desoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid

EUCAST	European Committee on Antimicrobial Susceptibility Testing
Fc	fragment, crystallizable
FDA	Food and Drug Administration
FnBPA	fibronectin-binding protein A
FnBPB	fibronectin-binding protein B
g	gravity
GISA	glycopeptide-intermediate *Staphylococcus aureus*
H_2O_2	hydrogen peroxide
HA-MRSA	healthcare-associated methicillin-resistant *Staphylococcus aureus*
IM	intramuscular
IP	intraperitoneal
K^+	potassium ions
Lys	L-lysine
LTA	lipoteichoic acid
MBC	minimal bactericidal concentration
MBC_{log}	minimal bactericidal concentration under logarithmic growth phase condition
MBC_{stat}	minimal bactericidal concentration under stationary growth phase condition
MIC	minimal inhibitory concentration
MID	minimal infective dose
MprF	multiple peptide resistance factor
MRSA	methicillin-resistant *Staphylococcus aureus*

MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
MSSA	methicillin-sensitive *Staphylococcus aureus*
NaCl	sodium chloride
NCCLS	National Committee on Clinical Laboratory Standards
O_2	oxygen
O_2^-	superoxide
P	pressure
PBP	penicillin-binding protein
PBP2’	additional penicillin-binding protein
PBS	phosphate buffered saline
PG	phosphatidylglycerol
PIA	polysaccharide intercellular adhesin
PJI	prosthetic joint infection
PMN	polymorphonuclear leukocyte
PSM	phenol-soluble modulin
PVL	Panton-Valentine leukocidin
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature
SA	*Staphylococcus aureus*
SCC	staphylococcal cassette chromosome
SD	standard derivation

SE	*Staphylococcus epidermidis*
SCV	small colony variant
TDF	tissue cage fluid
TSB	trypticase soy broth
Units	CFU, colony forming unit
	°C, degree Celsius
	g, gram
	h, hour
	kg, kilogram
	L, liter
	M, molar
	mg, milligram
	min, minute
	mm, millimetre
	mM, milimolar
	mL, millilitre
	µg, microgram
	nm, nanometre
	%, percentage
VRE	vancomycin-resistant enterococci
wt	wild type

Summary

The use of medical devices carries the risk of infection. Due to the development of multi-resistant bacteria, missing microcirculation, and biofilm embedded bacteria these implant-associated infections are difficult to treat. For their successful treatment, drugs should act independently of the bacterial physiological state, penetrate the biofilm, and prevent further bacterial adherence to surfaces and formation of bacterial biofilm.

Daptomycin (DAP) exhibits concentration-dependent bactericidal activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). Its target is the bacterial membrane in which it forms pores resulting in membrane depolarization and subsequent cell death. Calcium ions (Ca^{2+}) are indispensable for its activity. DAP does not need cell division or active metabolism for bactericidal activity and is able to penetrate biofilms. Therefore, in this thesis we followed the question whether DAP is efficient in eradicating planktonic and adherent MRSA in an implant-associated infection.

In the first part, DAP was bactericidal against stationary grown planktonic MRSA *in vitro*. Because the physiology of biofilm embedded bacteria is similar to stationary grown planktonic bacteria, DAP alone or in combination with rifampin was applied to treat MRSA in a foreign-body infection model in guinea pigs. DAP in combination with rifampin showed the highest efficacy against planktonic and adherent MRSA compared to rifampin-containing combinations with vancomycin, linezolid and levofloxacin. Additionally, in this combination DAP prevented the emergence of rifampin resistance.

However, DAP alone was inefficient against attached and biofilm embedded MRSA in implant-associated infections.

Therefore, in the second part of this thesis various factors, which could explain treatment failure, were investigated by evaluating the effect of cell wall components, biofilm, adherence and Ca^{2+} *in vitro* and *in vivo* upon DAP activity. We found that the physiological state of the bacteria had an impact on the efficacy of DAP because DAP was only effective as prophylaxis, i.e. before bacterial adherence. Furthermore, DAP was not able to kill adherent staphylococci *in vitro*, independently of biofilm, nucleases, adhesins, autolysins, and alanyl-lipoteichoic acids. Resistance of adherent staphylococci was not due to mutations of adherent bacteria, since staphylococci became DAP-susceptible after detachment. Increasing DAP or Ca^{2+} concentrations partially enhanced killing of adherent staphylococci *in vitro* and in a murine tissue cage infection model.

In summary, DAP alone is not active in an implant-associated infection. This might be due to bacterial adhesion, which is associated with a change of their physiological state. We demonstrated in an implant-associated infection that DAP treatment is improved with Ca^{2+} and only successful with 2 repeated doses of DAP before and after infection or in combination with rifampin.

1 General Introduction

1.1 Bacterial infection

Bacteria are highly diverse and colonize nearly all habitats including mammals. It is estimated that more bacterial cells are in the gut (10^{14} bacteria) than human cells (in total 10^{13} cells) in the body (7). In the human body they occur as commensals or pathogens. They build up the normal human flora at the inner and outer surface of the body such as skin and mucosa of the intestinal tract. Usually, this is a symbiotic relationship, which often protects against invasion of opportunistic parasites, but sometimes these well-tolerated bacteria cause diseases (62).

The first step in the interaction between a bacterium and the host is bacterial adherence to epithelium. This colonization step involves binding to proteins or polysaccharides that are expressed at the surface of the host skin and mucosa. Due to virulence factors bacteria are able to overcome the host immune defence and enter the host tissue. The infection site and the mechanism that lead to a disease as well as the course of disease are extremely variable (62).

Depending on the host factors, such as genetic and immunological determinants, and the virulence, entry site and infection dosage of the bacteria, diseases can occur that require antimicrobial treatment to eradicate (62).

1.2 Staphylococci

Staphylococci are a genus of Gram-positive bacteria, which are arranged in short chains or in grape-like clusters. Species of this genus can be distinguished by their ability to produce coagulase, an enzyme that causes blood clot formation. *Staphylococcus aureus* (*S. aureus*) belongs to the coagulase-positive staphylococcus species, while *Staphylococcus epidermidis* (*S. epidermidis*) is a coagulase-negative staphylococcus.

Staphylococci are colonizer of the skin and mucosa (62). However, in case of a weakening of the mucoid-epithelial barrier or a skin lesion staphylococci are able to cause a wide range of diseases in human and other animals. Due to the presence of different virulence factors they penetrate through the skin in the body and produce toxins (62). In comparison to *S. epidermidis*, *S. aureus* has more virulence factors (31).

1.2.1 *Staphylococcus epidermidis*

Besides being a commensal, *S. epidermidis* is also seen as an important opportunistic pathogen causing nosocomial infections related to indwelling devices. These include endocarditis due to pacemakers and catheters, cardiovascular infections, infections of implants in joints, in the eye and ear (74). Infections with *S. epidermidis* are usually sub-acute or even chronic without signs of inflammation (62).

In general, *S. epidermidis* is not an enterotoxin producer. Its toxin production is mostly limited to phenol-soluble modulins (PSMs), which are short, amphipathic, and α-helical peptides with pro-inflammatory and sometimes cytolytic functions (74).

Due to its reduced repertoire of virulence factors, the success as pathogen has to be attributed to its ability to adhere to surfaces. It uses similar "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) as *S. aureus* in particular the fibrinogen-binding protein serine-aspartate repeat protein G (SdrG) (65, 86). To survive in a host, *S. epidermidis* forms a very thick, multilayered biofilm on the surface of catheters, prosthetic valves, and orthopaedic devices (74, 104).

1.2.2 *Staphylococcus aureus*

S. aureus colonizes mainly the nose. In the human population three pattern of nasal carriage can be distinguished: about 20% of humans are persistent carriers, ±60% harbour *S. aureus* intermittently, and a minor part of the population (±20%) almost never carry the pathogen (49). Due to similar nasal elimination kinetics and anti-staphylococcal antibody profiles of intermittent carriers and non-carriers the known pattern has to be reclassificated in persistent carrier and others (101). The permanent carriage of *S. aureus* in the nose is an important risk factor for infections, in particular for infections with methicillin-resistant *S. aureus* (MRSA) (49) and in patients with implant-associated infections (117).

S. aureus is able to break through epithelial and mucosal surfaces of the host and causes tissue inflammation and destruction. As opportunistic pathogen it is therefore extremely successful in causing infections including skin infections, abscesses, and life-threatening diseases such as endocarditis and bacteraemia (61).

1.2.3 *The cell wall of Staphylococcus aureus*

The cell wall of *S. aureus* (Figure 1.1) is composed of peptidoglycan, proteins, lipoproteins, and glycolipids. The strength and rigidity of the bacterial cell wall is due to a glycopeptide called peptidoglycan, which consists of glycan chains crosslinked by peptides. The glycan consists of alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid attached to each other via *β*-1,4 linkages. Attached to *N*-acetylmuramic acid is a tetrapeptide (L-alanine, D-glutamine, L-lysine, and D-alanine) that crosslinks the glycan chains via peptide bonds. Neighbouring tetrapeptides are linked by pentaglycine bridge peptides. Hence, the peptidoglycan forms a three-dimensional network surrounding the cell membrane (108).

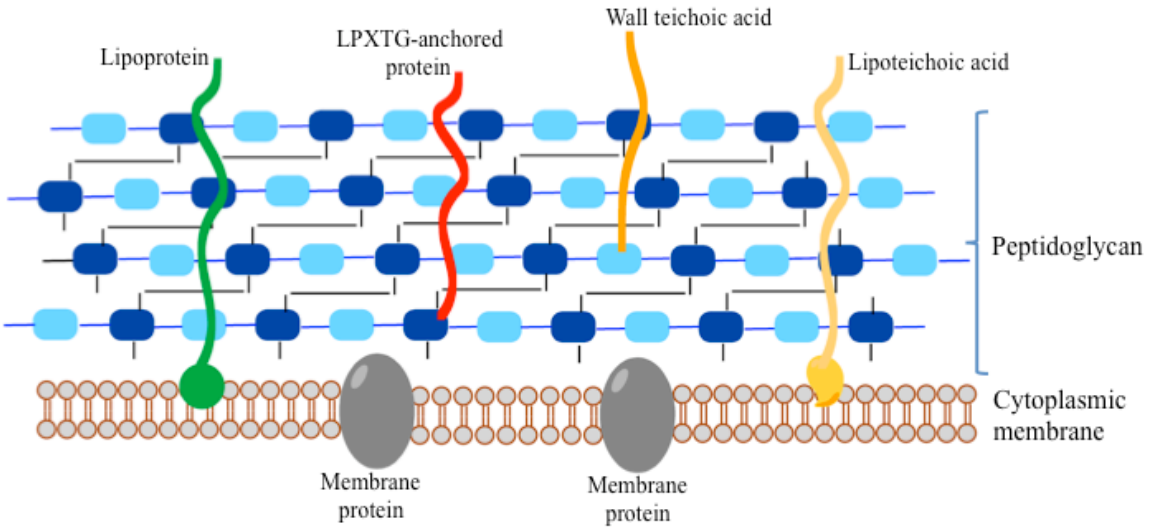

Figure 1.1: Schema of the cell wall envelope of *S. aureus*. The peptidoglycan consisting of alternating *β*-1,4 linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues surrounds the cell membrane. Attached to *N*-acetylmuramic acid residues is a tetrapeptide that crosslinks the glycan chains via pentaglycine bridges. Modifications include addition of teichoic acids, proteins, and lipoproteins.

The negatively charged teichoic acids carry divalent cations or provide a biophysical barrier for the diffusion of substances (111). While teichoic acid is covalently linked to peptidoglycan and binds to mannose-binding lectin (75), lipoteichoic acid (LTA) is membrane-anchored with a lipid that is bound to a linear polymer of phosphodiester-linked glycerol phosphate. This linear polymer extends into the cell wall. LTA is important for *S. aureus* survival under low-osmolarity conditions and due to its charge it governs the susceptibility of *S. aureus* to cationic antimicrobial peptides (CAMPs) (108, 111).

1.2.4 *Virulence factors of Staphylococcus aureus*

The ability to cause different diseases depends on the array of virulence factors (Figure 1.2). They play role in colonization of the host and are expressed according to the special growth conditions at the infection site (34). An important aspect in the pathogenesis of staphylococci is that one virulence factor may have several functions and several factors may have similar function (33).

Staphylococcal "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) are major virulence factors (27). They bind collagen, fibronectin, and fibrinogen and mediate adherence to host tissue (33). Most of them are covalently bound to the cell wall peptidoglycan, the linking is mediated by a membrane-bound enzyme called sortase. The most important one is sortase A. This enzyme recognizes a conserved amino acid motif (LPXTG) at the C-terminus of the surface protein sequence and mediates binding of the carboxyl group of threonine to the carboxyl group of glycine in the pentapeptide of peptidoglycan (27, 73). Relevant surface proteins are clumping factor

A (ClfA), fibrinogen-binding protein, and fibronectin-binding protein A and B (FnBPA and FnBPB) (76). They are important for the attachment to the extracellular matrix (ECM) of host or of foreign body implants (27). Due to an EF-hand motif ClfA contains a divalent cation-binding site (69). At calcium concentrations between 1 to 10 mM the interaction between ClfA and calcium is progressively inhibited. The calcium concentration in blood plasma is 1.3 mM and is strictly regulated at the threshold of the inhibitory range, i.e. around 1 mM (11, 27). Interestingly, ClfA is the dominant fibrinogen-binding protein present on the surface of stationary-phase *S. aureus* (26).

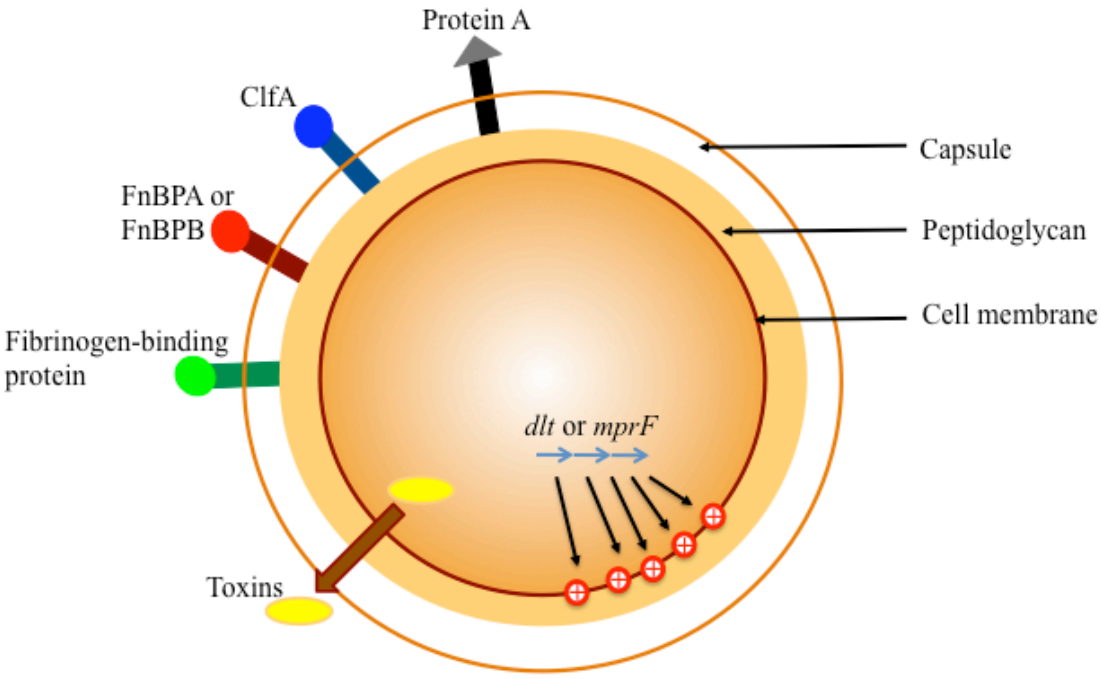

Figure 1. 2: Schema of staphylococcal virulence factors (adapted from (61, 67)). The mechanisms by which staphylococci subvert host innate immune defence are divers: Protein A decreases the effectiveness of IgG-opsonins. Clumping factor A (ClfA), fibrinogen-binding protein, and the fibronectin-binding proteins (FnBPA and FnBPB) are important for the attachment to ECM and implants. Toxins target the leukocyte membrane. Resistance to CAMPs is given by positive charge modifications of the cell wall. Capsules avoid the phagocytic uptake.

Expression of MSCRAMMs is strong during the logarithmic growth phase. During the stationary growth phase, *S. aureus* is able to secrete toxins that cause damage of the host membrane (alpha-hemolysin, Panton-Valentine leukocidine (PVL)) or that kill polymorphonuclear leukocytes (PMNs) (26).

Once attached, *S. aureus* has several mechanisms to resist uptake and killing by phagocytes. To avoid engulfment by neutrophils, *S.* aureus produces the cell wall-anchored protein A that binds the Fc (Fragment, crystallizable) proportion of antibodies and thereby decreases the effectiveness of IgG-opsonins. Additionally, capsules are expressed by many clinical *S. aureus* isolates and avoid the phagocytic uptake. Furthermore, *S. aureus* has the ability to inactivate special complement factors that are bound to the surface of opsonized bacterial cells resulting in reduction of phagocytic possibilities for neutrophils (26).

After engulfment by neutrophils *S. aureus* is well equipped with surface modifications for survival within the phagosome including modifications of the cell wall or membrane by positively charged components due to *dlt* or *mprF* genes. The *dlt* operon consists of *dltA*, *dltB*, *dltC*, and *dltD*. DltA activates D-alanine (D-Ala) in the cytoplasm via ATP hydrolysis. Activated D-Ala is coupled to the D-Ala carrier protein DltC. DltB transfers the D-Ala across the cytoplasmic membrane, where DltD catalyzes the introduction of the positive charged D-Ala in the otherwise negatively charged teichoic acids (78). The bifunctional integral membrane protein MprF (multiple peptide resistance factor) is able to catalyze the binding of L-lysine (Lys) to the negatively charged phosphatidylglycerol (PG) and to transfer the Lys-PG from the inner to the outer leaflet of the membrane (24). These modifications of negatively charged surface molecules reduce the affinity of

CAMPs that are secreted into the phagosome (16, 55). Additionally, to avoid the lethal effects of oxygen free radicals *S. aureus* expresses two superoxide dismutase enzymes that remove O_2^- formed during the respiratory burst (26).

Furthermore, non-phagocytic cells might take up *S. aureus*. Within these cells the bacterium can persist over a long time (26).

In conclusion, the described and many other mechanisms permit *S. aureus* to escape host defences, to adhere to cells and the tissue matrix, to spread within the host and to degrade cells and tissue.

1.2.5 *Regulation of virulence factors*

The expression of virulence factors is tightly regulated in response to cell density, energy availability, and environmental signals (68). Many Gram-positive bacteria, such as *S. aureus*, use peptide quorum sensing systems to control gene expression. The regulatory network recognizes environmental stimuli, which elicit the release of small autoinducing peptides (AIPs). They bind to a receptor, which by phosphorylating a response regulator leads to changes in gene expression of virulence factors. By the production of these AIPs bacteria are able to communicate with each other. The three major regulatory systems of biofilm are the two-component regulators system *agr* (accessory gene regulator), *luxS* (51), and the alternative transcription factor sigma B (58).

agr, as an essential global regulator of staphylococcal virulence, enhances the expression of many extracellular secreted proteins, such as toxins, and reduces surface associated virulence factors. It down-regulates biofilm (51, 68, 105). The two-component system encoded by *luxS* is required for AIPs synthesis, which negatively affects biofilm via

repression of *ica* gene transcription (112). Sigma B enhances biofilm by suppressing RNAIII, thus extracellular proteases and murein hydrolases, which are the target enzymes of proteases (58).

1.2.6 *Staphylococcal biofilm*

A biofilm is a sessile microbial community covered by a thin matrix (35, 93). The formation of a biofilm involves two steps (Figure 1.3): the attachment and the maturation phase. Non-specific factors, such as electrostatic and hydrophobic interactions, and specific factors, such as protein coated foreign bodies, play a role for the attachment step. *S. aureus* expresses MSCRAMMs on their surface that bind to human matrix (73). In addition, the autolysin family is able to facilitate attachment to plastic surfaces and has binding sites for human matrix proteins like fibronectin (37). Staphylococci have a strong ability to stick to plastic surfaces, a fact that has been used in the basic *in vitro* assays. However, during colonization of medical devices the additional interaction of MSCRAMMs and host proteins cause most likely a stronger attachment (73, 86).

The second phase, the maturation, includes the intercellular adhesion in an extracellular polysaccharide matrix. In staphylococci, the polysaccharide intercellular adhesin (PIA) is the main molecule responsible for intercellular adhesion. PIA is a linear β-1,6-linked *N*-acetylglucosaminoglycan (1). The biosynthesis of PIA is encoded by the *ica*-operon, which has an important role in the pathogenesis of implant-related *S. epidermidis* infections (70, 73). It is composed of regulator (*icaR*) and biosynthetic (*icaADBC*) genes, which consist of N-acetylglucosamine transferases (*icaA* and *icaD*), a PIA deacetylase (*icaB*), and a putative PIA exporter (*icaC*) (73, 103).

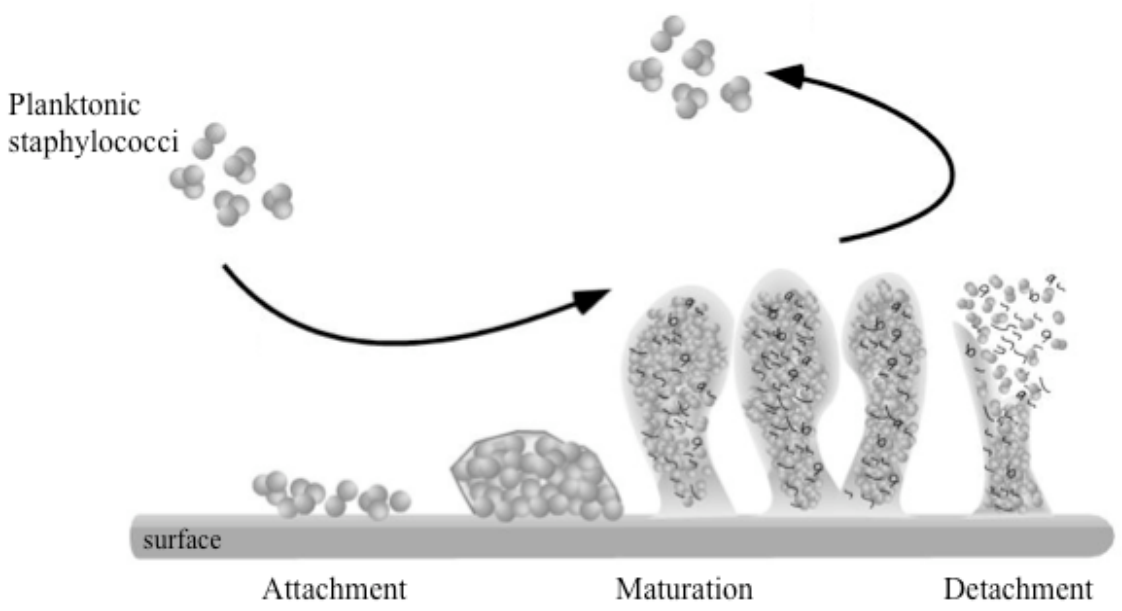

Figure 1.3: Phases of biofilm development in staphylococci (modified by (73)). The formation of biofilm includes different steps. It starts with attachment to the surface, followed by multilayered cellular proliferation and intracellular adhesion in an extracellular polysaccharide matrix (maturation). To colonize other sites, cell detachment is necessary.

The repressor *icaR*, which activity is influenced by environmental conditions, impairs expression of the *ica* genes and biofilm formation (73). Using PIA immunocytochemistry, Kristian et al. showed that planktonic- and stationary grown *S. aureus* 113 wild type (wt) were able to build up PIA, while the *ica*-mutant of this strain did not produce PIA (56). Bacteria lacking the *ica*-operon were unable to form a biofilm on titanium surfaces *in vitro*, but showed the ability for weak adherence (39). However, the course of an experimental implant infection model was similar with *S. epidermidis* wt and its isogenic *ica*-mutant (39, 56). This indicated that there are PIA-independent biofilm components. For example, the protein Aap causes a biofilm formation in 27% of biofilm-forming strains isolated from prosthetic joint infections (73, 80). Additionally, the cell wall bound surface protein Bap (biofilm associated protein) derived from bovine

mastitis isolates was shown to be involved as well in the adherence to polystyrene surfaces, intercellular adhesion, and biofilm formation of *S. aureus* isolates from animals (19, 73). *S. aureus* and *S. epidermidis* contain teichoic acids and LTAs, which increase via positive charge primary adhesion and via binding to fibronectin adhesion to matrix (41).

Furthermore, the biofilm matrix contains extracellular genomic DNA (eDNA) whose structural importance was first demonstrated in *Pseudomonas aeruginosa* (107). In *S. aureus* the *cidA* gene encoding a hydrolase was shown to promote cell lysis and the release of eDNA during biofilm formation. Accordingly, in the *cidA* mutant the biofilm was less adherent, contained more dead cells, and less genomic DNA (79). DNA is negatively charged and has within the biofilm matrix the ability to connect other molecules together similar to teichoic acids (73). On the other hand, a *nuc* mutant, which is inactivated in its thermonucleases, demonstrated higher levels of eDNA and thicker biofilms compared with the parental strain. The authors suggested that due to effective eDNA degradation by thermonuclease the lysis-mediated release of eDNA was balanced (63).

Due to concentration gradients of nutrients, signal compounds and bacterial waste within the biofilm it exhibits considerable structural, chemical and biological heterogeneity. Therefore, bacteria embedded in the biofilm are not only physiologically distinct from their planktonic counterpart, but also vary from each other (93). Bacteria within a biofilm are physiologically more similar to stationary than to exponential grown bacteria (29). The biofilm provides a structural and protective barrier and is a physiological niche in which the cells are more resistant against antimicrobial agents and against host defence.

Donlan demonstrated that biofilm embedded bacteria are 100 to 1,000 times less susceptible to antibiotics than their planktonic counterparts (21). Within a biofilm a small sub-population of cells still remains alive independent of the antibiotic concentration. These cells are non-dividing, dormant cells known as persister cells. When the antibiotic concentration is reduced, persisters are able to re-populate the biofilm (59).

Kristian and co-authors demonstrated that the biofilm formation of *S. epidermidis* leads to complement activation. The biofilm prevents complement deposition on bacteria and thereby biofilm embedded bacteria are protected from PMN-dependent killing (54).

To colonize other sites, cell detachment from the biofilm is necessary. Different factors are responsible for this process: these include mechanical forces and enzymes that destroy parts of the biofilm matrix, or lack of binding substrates for the biofilm. Biofilm dispersion is well controlled by the quorum-sensing system *agr* (73).

1.2.7 *Regulation of biofilm*

Quorum sensing allows the bacteria inside a biofilm structure to communicate with each other and to react to environmental conditions such as oxygen and iron limitation. MSCRAMMs are down-regulated by *agr* (51, 73, 76). Autolysins, e.g. murein hydrolase, are controlled by sigma B (58).

The regulation of PIA is well studied. PIA biosynthesis is up-regulated by low oxygen and enhanced by sub-inhibitory concentrations of antibiotics. By repressing *icaR* transcription the global stress response regulator sigmaB enhances the *ica* function in *S. epidermidis* (70). Furthermore, by reducing *ica* transcription, *luxS* has an influence on the biofilm formation. A mutant of *luxS* leads to a thicker and more compact biofilm than the

parental wt strain (51). In contrast, *agr* does not affect PIA-expression. However, *agr* regulates biofilm detachment by up-regulating of PSMs. As a consequence of this effect, *agr* mutants naturally have thicker biofilm (73, 74).

1.2.8 *Antibiotic resistance in staphylococci - Methicillin-resistant Staphylococcus aureus*

With increasing use of antibiotics resistance emerged fast. In the 1940s, the intense use of penicillin led to the formation of beta-lactamase-producing *S. aureus* isolates, which were resistant to penicillin. Methicillin was synthesized in the late 1950s and expresses methoxy instead of the phenol groups in the benzylpenicillin. These exchanged groups reduced the affinity for staphylococcal beta-lactamases. Unfortunately, soon after introduction of this new antibiotic, *S. aureus* strains resistant to methicillin appeared in the clinics (90). MRSA expresses an additional penicillin-binding protein (PBP2´) probably acquired from another strain. PBP2´ encoded by *mecA* is a peptidoglycan transpeptidase that is able to cross-link peptidoglycan even in the presence of beta-lactams, because it shows a low affinity for this antibiotic (60). *mecA* is part of a mobile genetic element named "staphylococcal cassette chromosome (SCC) *mec*". SSC*mec* is flanked by recombinase genes that permit intra-species transmission. MRSA evolved from 5 different regulated genotypes that spread world wide (33). It is still unknown why MRSA are so transmissible beyond resistance to multiple antibiotics. They may express specific virulence factors, which so far could not be identified (48).

MRSA infections have been classified based on where the patient acquires the bacteria. Healthcare-associated MRSA (HA-MRSA) are distinguished from community-associated

MRSA (CA-MRSA). They differ in their antibiotic resistant pattern and their virulence factors (33, 83).

1.3 Prosthetic joint-associated infections

In modern medicine indwelling devices are increasingly used to replace a damaged biological function or missing anatomical structure. Especially, orthopaedic implants represent the major procedure for patient with pain and compromised mobility or for fracture fixation. The risk of infection is increased by the presence of foreign material. However, less than 10% of these patients display implant-associated complications during their lifetime (61).

The most commonly cultured pathogens isolated from prosthetic joint-associated infections (PJIs) are *S. epidermidis* (30 to 43%) and *S. aureus* (12 to 23%), followed by mixed bacterial flora (10 to 11%), streptococci (9 to 10%), Gram-negative bacilli (3 to 6%), enterococci (3 to 7%), and anaerobes (2 to 4%) (117).

Recently, *S. aureus* small colony variants (SCVs), which have a slower metabolism, do not produce toxins, and are more resistant to antibiotics, have been denoted in PJIs. They represent a risk factor for treatment failure, persistence and relapse of infection (87).

PJIs occur by bacterial contamination of the surgical site during surgery or immediately thereafter, or they result from haematogenous seeding originating from distinct infections in the nose or on skin. According to their appearance PJIs are divided into three types: manifestation of an infection within 3 month after surgery (early), developing of an infection between 3 and 24 month after surgery (delayed), or those that develop after 2 years after surgery (late). Early PJIs occur perioperatively and are commonly caused by

virulent microorganisms, such as *S. aureus* and Gram-negative bacilli. Symptoms are persisting local pain, erythema and warmth at the implant site, and fever. Microorganisms of low virulence (coagulase-negative staphylococci, *Propionibacterium acnes*) are commonly responsible for delayed infections. The characteristics are persisting or increasing joint pain and early loosening, but clinical signs may be missing. Therefore, these kinds of infections are difficult to distinguish from aseptic failures. Both early and delayed PJIs are usually acquired during implantation, whereas late infections predominantly result from a distant haematogenously spread infection site (116). Haematogenous seeding originating from skin, respiratory, dental, and urinary tract infections may appear during the whole life, although the risk of infections is highest in the first years after implantation (96-98, 117).

1.3.1 *Pathogenesis*

All used foreign bodies are inert. Therefore, the host immune system responds with a localized inflammation. This leads first to the formation of a capsule-like membrane of fibrinogen, fibronectin and collagen on the surface (20). The extracellular proteins fibronectin and fibrinogen mediate bacterial adhesion to medical devices and host tissue. In the presence of serum it was shown that the level of *S. aureus* adherence to explanted coverslips was much higher compared to non-implanted coverslips (102). The localized inflammation causes secondly a phenomenon known as “frustrated phagocytosis”, which results from the granulocyte contact with the foreign body and causes a functional granulocyte defect with reduced oxygen burst and chemotaxis in the neighbourhood of foreign bodies. This may be partly responsible for the high susceptibility of implants to

infections (115). Indeed, Zimmerli and colleagues showed that in the presence of an implant the number of staphylococci required for a persistent infection is 1000-fold lower (≈100 to 1000 CFU) than in an absence (118). The pathogenesis of PJIs involves the above described interaction between the bacteria (biofilm formation), the implant (surface characteristics) and the host (inflammatory response). The initial phase involves the adherence of microorganisms to the surface by nonspecific factors (hydrophobicity and electrostatic forces) and by specific adhesins. It is followed by an accumulative phase that includes intercellular adherence and formation of biofilm mediated by PIA (96).

The surface of an implant has been found to trigger complement activation resulting in attraction of neutrophils and monocytes and in enhancing phagocytosis (42, 95). This inflammatory response may cause the aseptic loosening of a medical device (4) and its replacement becomes necessary. To avoid another surgical intervention a treatment algorithm and antimicrobial guidelines were developed (117).

1.3.2 *Treatment algorithm*

The goal of a PJI therapy is a long-term pain-free, functional joint and is achieved by elimination of the infection. But, PJIs are difficult to treat because foreign bodies remain devoid of a microcirculation, which is crucial for host defence and for the delivery of antibiotics (97). Five treatment approaches with a combination of both an appropriate surgical treatment and antimicrobial therapy are available. (I) Long-term antimicrobial therapy alone does not eradicate the infection. Additionally, clinical symptoms re-appear during or after antimicrobial breaks. Therefore, this treatment option is suitable only when surgery is contraindicated, when a functional prosthesis is not needed, or when the

patient refuses surgical procedure. (II) Debridement with retention of the prosthesis means removal of tissue around a stable implant for patients with an early postoperative or acute haematogenous infection (<3 weeks). Furthermore, an agent with activity against biofilm bacteria should be available. (III) and (IV) One- or two-stage revision includes removal of all devices, debridement, and replacement of a new prosthesis during the same procedure or after a period of time, respectively. (V) Finally, resection is defined as the permanent removal and debridement without replacement (97, 98, 117).

1.3.3 *Antimicrobial guidelines*

The choice of antibiotic is dependent on the isolated microorganisms and their susceptibility-resistance pattern. Antibiotics (Figure 1.4) can be distinguished based on the primary target they affect and additional whether they induce cell death (bactericidal drugs) or inhibit cell growth (bacteriostatic drugs) (50).

Quinolone/fluoroquinolone. The quinolone/fluoroquinolone class interferes with topoisomerase II (DNA gyrase) and topoisomerase IV resulting in the inhibition of DNA replication and therefore leading to the formation of double-stranded DNA breaks and cell death. Ciprofloxacin and levofloxacin belong to this bacteriostatic acting class (50). The emergence of resistance includes chromosomal mutations resulting in alteration of the target enzyme (gyrA), changes in cell-membrane porin channels, or drug efflux (110).

Rifamycin. Rifampin shows bactericidal activity against Gram-positive bacteria and belongs to the rifamycin group. It binds to the beta-subunit (encoded by *rpoB*) of the bacterial DNA-dependent RNA-polymerase. This leads to blocking of DNA transcription

into messenger RNA, and of the following protein synthesis (50). Resistance is associated with the complete loss of activity due to a point mutation in the *rpoB* gene (99).

Beta-Lactams. Beta-lactams are bactericidal and inhibit transpeptidation by binding to penicillin-binding proteins (PBPs) on maturing peptidoglycan strands. The beta-lactam is an analogue of the terminal D-alanyl-D-alanyl dipeptide of peptidoglycan and acts as substrate for the PBPs. The impaired peptidoglycan synthesis together with the action of autolysins that breaks down bonds within peptidoglycan leads to cell death with lysis. This antibiotic group includes penicillins and cephalosporins (50). Bacteria are often able to produce a resistance by synthesizing beta-lactamase, an enzyme that attacks the beta-lactam ring (109) or they acquired the *mecA* gene, which encodes a low affinity PBP (60).

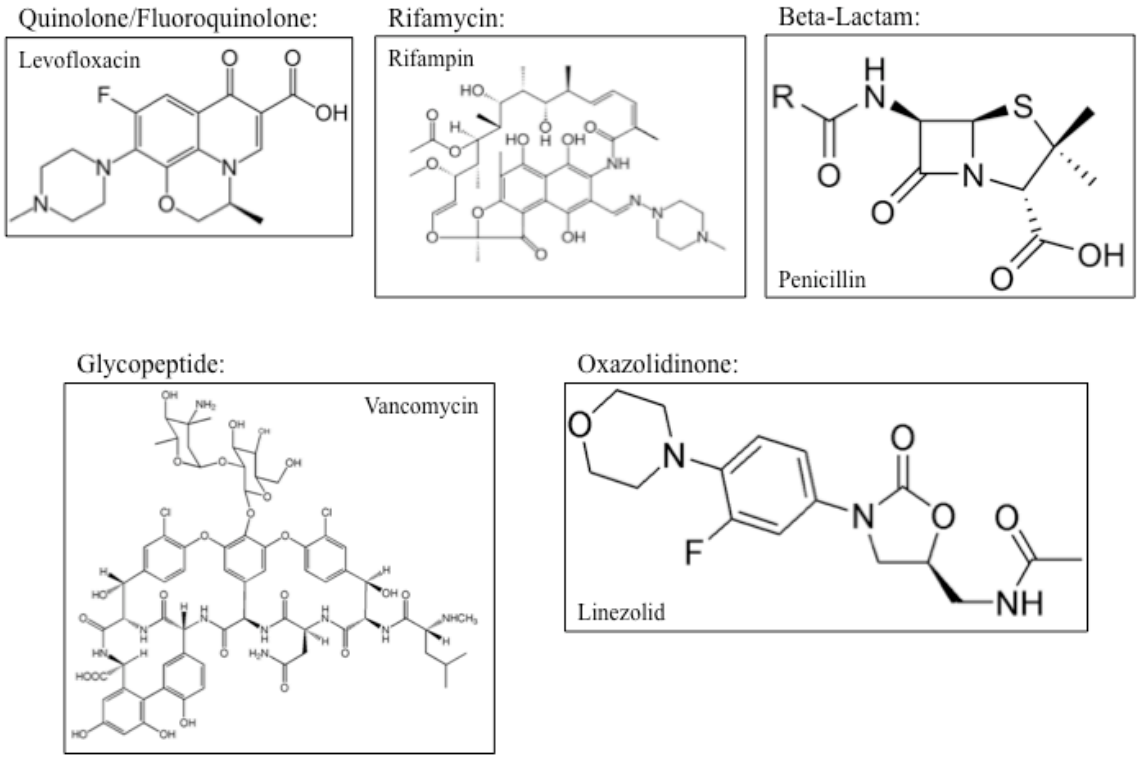

Figure 1.4: Structures of different antibiotics

Glycopeptides. The time-dependent bacteriostatic agents vancomycin and teicoplanin belong into this group. By binding the terminal D-alanyl-D-alanyl dipeptide and by blocking transglycosylase and PBP activity they inhibit the peptidoglycan synthesis. The development of glycopeptide resistance needed a long time. After 20 years of vancomycin use, a resistance mechanism, which was expressed in enterococci and includes 5 genes, was transferred into staphylococci. These genes encode a reprogramming of the peptidoglycan termini from high to low affinity by changing the D-alanyl-D-alanyl dipeptide to a D-alanyl-D-lactate (47).

Oxazolidinone. As member of the oxazolidinone class, linezolid is a dose-dependent bacteriostatic or bactericidal antibiotic against Gram-positive bacteria that binds to the 50S subunit of the bacterial ribosome via 23S ribosomal (r) RNA interaction. This binding causes the blocking of the protein synthesis. Resistances in *S. aureus* are typically associated with mutations in the 23 S rRNA gene (8).

Lipopeptides. The primary target for lipopetides is the cell membrane resulting in cell death without lysis (50). The first member of this antibiotic class is daptomycin, which is discussed below.

In view of the pathogenesis of PJIs the antimicrobial substance of choice should have bactericidal activity against surface-adhering, slow-growing, and biofilm-producing bacteria. Rifampin has been tested *in vitro*, in animal models, and in clinical studies and demonstrated good bactericidal activity against adherent and stationary-phase staphylococci. But due to the rapid emergence of resistance it should never be administrated as mono-therapy. However, the use of rifampin in a combination with other

antibiotics is well tolerated and cure infections without removal of the implant. For the treatment of methicillin-sensitive *S. aureus* (MSSA) a combination with beta-lactam, followed by rifampin with fluoroquinolone is recommended. In case of MRSA rifampin with glycopeptides, followed by fluoroquinolone is suggested (117, 119). Quinolones are excellent drugs because they have a good bioavailability and activity. In combination with rifampin new-generations of quinolones, such as levofloxacin, display better *in vitro* activity against staphylococci than ciprofloxacin, but levofloxacin alone was not able to eradicate adherent staphylococci *in vitro* as well as *in vivo* (85). In addition, MRSA are often resistant to quinolones (100, 117). As an alternative, glycopeptides could be used. Vancomycin showed lower antibacterial activity against Gram-positive bacteria than beta-lactams and should only be used for patients demonstrating hypersensitivity to beta-lactams. They are considered to be "the last resort" against MRSA, however diminished susceptibility emerged during the last decades (vancomycin-intermediate *S. aureus* and vancomycin-resistant *S. aureus*) reducing the efficacy of this drug (9). The potential of alternative antibiotic treatments, including linezolid and daptomycin, is under intensive supervision.

1.3.4 *Perioperative antimicrobial prophylaxis*

Besides the use of sterile conditions during surgery an antimicrobial prophylaxis remains one of the most effective method of reducing the prevalence of infection after joint replacement and should be directed against the most species commonly isolated from healthcare-acquired infections and taken into account their susceptibility-resistance pattern. For optimal efficacy an antimicrobial concentration that is able to inhibit

bacterial growth must be reached in tissue during the entire surgical procedure. In general, first- or second-generation cephalosporins are the first-choice antibiotics, when a patient is not allergic or when the risk of MRSA is minor. Otherwise vancomycin and teicoplanin can be used. For MRSA colonized patients with implants the decolonization of nose and skin is also possible to reduce the risk of secondary infection (97).

1.3.5 *Experimental models for PJI*

Over the last years, *in vivo* models that mimic the pathogenesis of PJIs have been developed, i.e. the catheter abscess model, knee arthroplasty, and the tissue cage model. With the murine model of peritonitis or catheter infection an abscess can be simulated by using a catheter intraperitoneally (113) or subcutaneously (81, 82), respectively. A knee arthroplasty involves the partial or total knee replacement in rabbits, which is reproducible and close to the human situation (5).

The tissue cage model of foreign-body infections best reproduces the clinical situation of patients with implanted prosthesis and helps to investigate the standard guidelines for an antimicrobial management. In this model, Teflon cylinder tubes (tissue cages) with an internal and external diameter of 8 mm and 10 mm, respectively, and a length of 32 mm perforated with 130 holes are implanted in the subcutaneous tissue of mice, rats, guinea pigs, rabbits, dogs, and calves. Guinea pigs are the most commonly used species because the tissue cage infection is very similar to the human device-associated infection. In addition, the minimal infective dose is low and spontaneous cure never occurs with *S. aureus* and coagulase-negative staphylococci. However, guinea pigs only tolerate short-term antimicrobial therapies because they die from diarrhoea and weight loss. They also

do not tolerate beta-lactams. Therefore, other animal species such as mice are preferred for prolonged studies or beta-lactam therapies (113).

During the healing period after surgery, the tissue cage becomes surrounded by fibroblasts and ECM with collagen and fibronectin (39) and interstitial fluid accumulates inside. The interstitial fluid differs significant in several values from serum, especially in a lower pH and higher P_{O2} and P_{CO2}. Furthermore, the fluid shows nearly half of the serum protein (g/L) concentration (113).

Usually, after complete healing and accumulation of non-hemorrhagic interstitial fluid the cages are infected by percutaneous injection (113). Only *S. epidermidis* infection is applied perioperatively because it is not persistent if bacteria are applied postoperatively (39). Zimmerli et al. and Kristian et al. demonstrated in guinea pigs and mice, respectively, that a persistent infection is inducible with a very low inoculum (≈100 to 1000 CFU). Before an infection the median granulocyte content in the cage fluid is about 8×10^5 PMNs/mL. After infection the number of PMNs is increasing proportional to the number of bacteria (55, 118).

1.4 Daptomycin

Daptomycin (DAP) was discovered in the early 1980s and first used as an intravenous antibiotic agent for serious Gram-positive infections. Early clinical trails in which DAP at 2 mg/kg/day or at 3 mg/kg/12h was given were stopped due to unexpected treatment failures. Later clinical studies with increasing dosages (4 mg/kg/12h) to improve the clinical efficacy were suspended due to musculoskeletal toxicity (36). Further studies with DAP were ceased. But, due to the strong increase of bacterial resistance among

Gram-positive microorganisms in the 1990s, new efforts to study and develop DAP were favoured (84). In 2000 it turned out that toxicity to skeletal muscle in dogs was lower if DAP was given only once per day compared with a twice-daily administration (71). Subsequently, clinical studies showed rare skeletal muscle toxicity with a once daily regimen of DAP (36). Finally, successful outcomes in studies of patients with complicated skin and skin-structure infection (cSSSI) associated with susceptible strains of Gram-positive microorganisms led to the approval of DAP at 4 mg/kg/day in the US by the Food and Drug Administration (FDA) in 2003 (23). Today 4 mg/kg/day is approved for the treatment of cSSSI in Europe and 6 mg/kg/day for the treatment of *S. aureus* bacteraemia and right-sided infective endocarditis in the US and in Europe (18).

1.4.1 *In vitro potency of daptomycin*

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) defines the breakpoint of susceptibility as a minimal inhibitory concentration (MIC) of ≤1 μg/ml (25).

DAP shows potential bactericidal activity against a wide range of aerobic and anaerobic Gram-positive bacteria including multiple antibiotic-susceptible and -resistant strains. The spectrum ranges from MSSA and MRSA to glycopeptide-intermediate *S. aureus* (GISA), methicillin-resistant coagulase-negative *S.* ssp. (CoNS), and vancomycin-resistant enterococci (VRE). Furthermore, it is active against linezolid-resistant *S. aureus*, *E. faecium*, and a variety of streptococcal groups such as *S. pyrogenes*, *S. agalatiae*. Among the anaerobic bacteria DAP is efficient against *Clostridium perfringes*, *C. difficile*, and *P. acnes* (91).

Probably due to the presence of an outer membrane DAP is ineffective against Gram-negative organisms (89).

1.4.2 *Pharmacokinetics*

Today, the FDA-approved dose of DAP is 4 to 6 mg/kg/day. However, for some serious infections clinicians use up to 8 or 10 mg/kg/day (3, 30, 52). Due to the emergence of side effects a once daily administration is recommended. Benvenuto and colleagues showed that DAP pharmacokinetics remain linear at doses up to 12 mg/kg (6).

The half-life of 8-9 h is relatively long. The distribution volume is very low and mostly limited to serum and the interstitial fluid. Tissue penetration in human blister fluid is 68% of that in the plasma (23). DAP is eliminated primarily by the kidneys (18).

DAP is 90 to 93% bound to serum proteins, primarily albumin. This binding is weak and reversible in contrast to its binding to the bacterial membrane. The addition of serum proteins to DAP *in vitro* leads to an increase in MIC because of altered free calcium concentration. However, within the human body physiological mechanisms highly regulate free extracellular calcium concentrations (range of 1.15 to 1,31 mM) (23).

The post-antibiotic effect, i.e. the suppression of bacterial growth that persists after exposure of microorganisms to antibiotic, is long for DAP (23).

1.4.3 *Structure*

DAP is a fermentation product from *Streptomyces roseosporus*. It belongs to the lipopeptide class that is characterized by a peptide chain and an attached fatty acid side

chain. The lipopeptides classified into linear and cyclic lipopeptides. According to their biological activities the cyclic lipopeptides are divided into four groups: active against Gram-negative and Gram-positive bacteria, against fungi and mycobacteria (12).

DAP, formerly Ly-146032, belongs to the cyclic lipopeptides with high bactericidal activity against Gram-positive bacteria (12). It is composed of a 13-member amino acid water-soluble (hydrophilic) core and a lipid soluble (lipophilic) 10-carbon tail (Figure 1.5). An ester bond links the ten-membered ring. One site of the molecule includes hydrophobic moieties, while neutral polar and anionic residues are localized at the other site, resulting into amphipathicity of the molecule. This amphiphilic character is important for its unique mechanism of action in which the large hydrophobic part interacts with the acyl-chain region of the bacterial membrane (23).

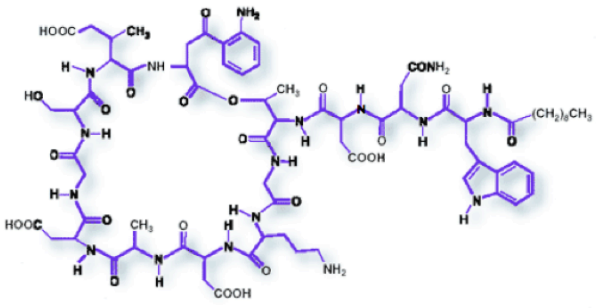

Figure 1.5: Chemical structure of daptomycin (from (18))

1.4.4 *Mechanism of action*

DAP is able to penetrate the staphylococcal biofilm (92) and, in contrast to most antibiotics, it does not require cell division or active metabolism for its bactericidal

activity (64). It seems that the bactericidal activity of DAP is similar to that of CAMPs, which mainly act on bacterial membranes (94).

Based on their action the following multistep model was developed (Figure 1.6). First, DAP and calcium ions (Ca^{2+}) at a molar ratio of 1:1 aggregate to a micelle of between 14 and 16 monomers (38). This is accompanied by a change in conformation that locks DAP into the active version. Jung and colleagues demonstrated that in the absence of Ca^{2+} the minimal inhibitory concentration (MIC) of DAP is greater than 64 µg/mL while with increasing Ca^{2+} concentrations the MIC is gradually decreasing.

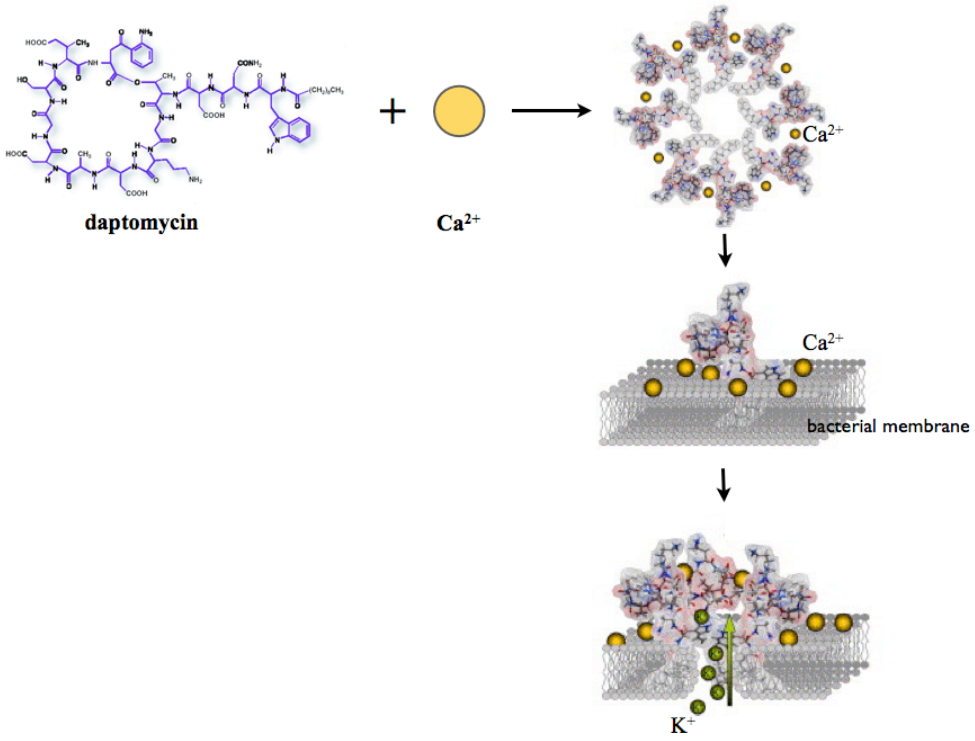

Figure 1.6: Daptomycin mechanism of action (modified by (94)). Daptomycin binds to the cytoplasmic membrane of Gram-positive bacteria in a calcium (Ca^{2+})-dependent manner. This leads to membrane depolarization, release of intracellular potassium (K^+) and subsequent cell death without lysis.

The interaction with Ca^{2+} is absolutely necessary because they neutralize the anionic charges. Therefore, the binding with Ca^{2+} increases the amphiphilic property and the solvent-exposed hydrophobic surface of DAP and promotes its association with the membrane head groups (45). Once DAP comes into close proximity with the membrane of Gram-positive bacteria, the micelle formation dissociates and DAP inserts into the bilayer. This step is accompanied by a second structural transition that needs both the presence of Ca^{2+} and lipids with negatively charged head groups. Within the membrane, oligomerization may occur, followed by disruption of the functional integrity of the bacterial membrane with formation of pores. This triggers the release of intracellular potassium ions, which leads to membrane depolarization and therefore rapid cell death (84, 89, 94). Using transmission electron microscopy and fluorometric methods it was shown that the rapid cell death does not involve cell lysis (17).

It is still unclear whether DAP arrests DNA, RNA and protein synthesis. In addition, a dual mechanism of action for DAP involving cell wall and cell membrane targets was suggested after transcriptome analysis of *S. aureus* (66).

1.4.5 *Mechanism of resistance*

It is often documented that due to the unique mechanism of action the risk of bactericidal resistance against DAP is low. However, during the last years clinical *S. aureus* strains with an increase in the MIC were reported in association with DAP therapy (28, 44). These resistances arise at low frequencies and result in small MIC changes. *In vitro*, resistant mutants can be generated after more than 20 passages in presence of DAP indicating a low adaptation rate to DAP (46, 88). However, individual mutations resulting

in increased resistance were associated with alterations in *mprF*, *rpoB* and *rpoC*, and *yycG* (2).

The MprF protein is a large bifunctional integral membrane protein catalyzing first the binding of Lys to the negatively charged lipid PG at the inner leaflet of the membrane and second the translocation of Lys-PG to the outer leaflet of the membrane. This results in neutralization of the membrane surface and provides CAMP resistance (24). A mutational insertion into this gene leads to more susceptibility to CAMP (77). DAP resistant strains with a mutation in this gene have membranes with higher Lys-PG to PG ratios and bind less DAP than the wt (28, 44).

It is still unknown how mutations in the genes *rpoB* and *rpoC* influence the susceptibility to DAP, but it is believed that due to interactions between RNA polymerase and one or more sigma factors transcription of one or more key genes is altered (*mprF* or *yycG*) (2).

YycG is a membrane spanning sensor/histidine kinase of the two-component system YycFG. In *S. aureus* an enhanced expression of YycFG leads to increased peptidoglycan biosynthesis and biofilm formation, while the depletion causes cell death without lysis (22). It is proposed that DAP blocks signal transduction by binding to YycG resulting in histidine kinases with altered DAP affinities (2).

1.4.6 *Safety and tolerability*

Usually, DAP is well tolerated. However, some patients develop side effects in skeletal muscles. Former studies with a twice-daily administration lead to skeletal myopathy resulting in the stop of clinical trails. However, muscle degenerations were found to be reversible and related more to the interval between doses than to the given concentration

(71). Over long time, no data exist which clarified the exact mechanism responsible for these side effects. Previously, histochemical and immunohistochemical analysis of skeletal muscle of rats demonstrated that a twice-daily dose of DAP leads to infiltration of multiple muscle fibers with macrophages. In contrast, a one-day dose showed no effect on skeletal muscles. The authors suggested that the loss of sarcolemmal integrity might be the trigger for the effect of DAP on skeletal muscles. The breaching of plasma membrane integrity causes a Ca^{2+} leakage from the extracellular compartment into the myofibers resulting in cell death (53).

2 Aim of the thesis

With the increasing number of joint replacements in the growing older population, prevention and treatment of implant-associated infections has become priority. But these infections are difficult to treat because implants remain devoid of a microcirculation, which is crucial for host defence and for the delivery of antibiotics. Microorganisms growing in biofilms typically cause implant infections. The biofilm confesses protection to bacteria against antimicrobial agents and host defence. Therefore, an antimicrobial active against implant infections should have bactericidal activity against surface-adhering, slow-growing, and biofilm-producing bacteria. Rifampin fulfils all requirements for staphylococci, but is not useful as mono-therapy. Consequently, other drugs or combinations of different drugs are needed. Therefore, the question arose whether the lipopeptide daptomycin, whose target is the bacterial membrane of Gram-positive bacteria, is a promising candidate for the treatment of implant-associated MRSA infections.

The first aim of this thesis was to assess the efficacy of daptomycin against MRSA *in vitro* and *in vivo*. Because rifampin-containing antimicrobial regimes were able to eradicate staphylococcal biofilms *in vitro* and *in vivo*, the activity of daptomycin in combination with rifampin in a tissue cage-associated infection model was evaluated and compared to the efficacy of daptomycin alone and to three other antibiotics commonly used against MRSA.

Because *in vitro* data of daptomycin did not predict *in vivo* success of the mono-therapy in the implant-associated infection model, in the second part we intended to understand

the mechanism of daptomycin resistance in staphylococci *in vitro* and *in vivo*. The question arose whether the *in vivo* daptomycin efficacy is influenced by time and inoculum of infection. Therefore, we evaluated whether a shorter infection time and a lower inoculum of MRSA lead to the eradication of bacteria. A special interest was to investigate the *in vitro* efficacy of daptomycin against adherent staphylococci. In addition, the necessity of calcium ions for the eradication of adherent staphylococci was examined.

3 Results

3.1 Part 1: Efficacy of daptomycin in implant-associated infection due to methicillin-resistant *Staphylococcus aureus*: importance of combination with rifampin

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Efficacy of Daptomycin in Implant-Associated Infection Due to Methicillin-Resistant *Staphylococcus aureus*: Importance of Combination with Rifampin[▽]

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Limited treatment options are available for implant-associated infections caused by methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA). We compared the activity of daptomycin (alone and with rifampin [rifampicin]) with the activities of other antimicrobial regimens against MRSA ATCC 43300 in the guinea pig foreign-body infection model. The daptomycin MIC and the minimum bactericidal concentration in logarithmic phase and stationary growth phase of MRSA were 0.625, 0.625, and 20 μg/ml, respectively. In time-kill studies, daptomycin showed rapid and concentration-dependent killing of MRSA in stationary growth phase. At concentrations above 20 μg/ml, daptomycin reduced the counts by >3 $\log_{10}$ CFU/ml in 2 to 4 h. In sterile cage fluid, daptomycin peak concentrations of 23.1, 46.3, and 53.7 μg/ml were reached 4 to 6 h after the administration of single intraperitoneal doses of 20, 30, and 40 mg/kg of body weight, respectively. In treatment studies, daptomycin alone reduced the planktonic MRSA counts by 0.3 $\log_{10}$ CFU/ml, whereas in combination with rifampin, a reduction in the counts of >6 $\log_{10}$ CFU/ml was observed. Vancomycin and daptomycin (at both doses) were unable to cure any cage-associated infection when they were given as monotherapy, whereas rifampin alone cured the infections in 33% of the cages. In combination with rifampin, daptomycin showed cure rates of 25% (at 20 mg/kg) and 67% (at 30 mg/kg), vancomycin showed a cure rate of 8%, linezolid showed a cure rate of 0%, and levofloxacin showed a cure rate of 58%. In addition, daptomycin at a high dose (30 mg/kg) completely prevented the emergence of rifampin resistance in planktonic and adherent MRSA cells. Daptomycin at a high dose, corresponding to 6 mg/kg in humans, in combination with rifampin showed the highest activity against planktonic and adherent MRSA. Daptomycin plus rifampin is a promising treatment option for implant-associated MRSA infections.

Implants are increasingly used in modern medicine to replace a compromised biological function or missing anatomical structure. Periprosthetic infections represent a devastating complication, causing high rates of morbidity and consuming considerable health care resources. Implant-associated infections are caused by microorganisms growing adherent to the device surface and embedded in an extracellular polymeric matrix, a complex three-dimensional structure called a microbial biofilm (8). Bacterial communities in biofilms cause persistent infection due to increased resistance to antibiotics and the immune system and the difficulty with eradicating them from the implant (6).

Staphylococcus aureus is one of the leading pathogens causing implant-associated infections. Successful treatment requires the use of bactericidal drugs acting on surface-adhering microorganisms, which predominantly exist in the stationary growth phase. Previous in vitro, experimental, and clinical studies demonstrated that rifampin (rifampicin)-containing antimicrobial regimens were able to eradicate staphylococcal biofilms and cure implant-associated infections (23, 25). Quinolones are often used in combination with rifampin in order to prevent the emergence of rifampin resistance (4, 19, 21). However, methicillin (meticillin)-resistant *S. aureus* (MRSA) strains are often resistant to quinolones. In addition, MRSA strains were recently shown to have decreased susceptibility to vancomycin, reducing the efficacy of this drug. Therefore, alternative drugs for use in combination with rifampin against implant-associated infections are needed (12, 20).

Daptomycin is a negatively charged cyclic lipopeptide with bactericidal activity against gram-positive organisms, including MRSA (17). The drug inserts into the bacterial cytoplasmic membrane in a calcium-dependent fashion, leading to rapid cell death without lysis, and causing only minimal inflammation (15). Daptomycin has been well tolerated in healthy volunteers dosed with up to 12 mg/kg of body weight intravenously for 14 days (2). Only limited data on the use of daptomycin in combination with rifampin against staphylococcal implant-associated infections are available.

In this study, we investigated the activity of daptomycin against MRSA ATCC 43300 in vitro. In addition, we evaluated

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the activity of daptomycin in combination with rifampin in a cage-associated infection model in guinea pigs and compared the efficacy of the treatment with the efficacies of three other antibiotics commonly used against MRSA, vancomycin, linezolid, and levofloxacin (alone and in combination with rifampin).

(Part of the results of the present study were presented at the 48th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 24 to 29 October 2008 [abstr. B-1000].)

MATERIALS AND METHODS

Study microorganisms. *S. aureus* strain ATCC 43300, which is resistant to methicillin and which is susceptible to rifampin, vancomycin, linezolid, and levofloxacin, was studied. For the testing of rifampin resistance, rifampin-resistant clinical *S. aureus* strain T4050 and rifampin-susceptible laboratory *S. aureus* strain ATCC 29213 were used. The strains were stored at −70°C by using the cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). For preparation of the inoculum, single beads were transferred to 1 ml of sterile trypticase soy broth (TSB; Becton Dickinson and Company, Le Pont de Claix, France) and incubated for 7 h at 37°C. This preculture was then diluted 1:100 in fresh TSB and incubated overnight at 37°C without shaking. The bacteria were washed twice and resuspended in sterile and pyrogen-free 0.9% saline to the desired concentration. Bacterial concentrations were determined by plating of aliquots from appropriate dilutions on agar, followed by colony counting after 24 h of incubation at 37°C.

Antimicrobial agents. Daptomycin for injection was supplied by Novartis Pharma Schweiz AG (Bern, Switzerland). A stock solution of 50 mg/ml was prepared in sterile and pyrogen-free 0.9% saline. All other solutions were prepared in sterile water. Rifampin (Sandoz AG, Steinhausen, Switzerland) was prepared as a 60-mg/ml stock solution. Levofloxacin hemihydrate injectable solution (5 mg/ml) was purchased from Aventis Pharma AG (Zurich, Switzerland). Vancomycin was supplied by Teva Pharma AG (Aesch, Switzerland), and a stock solution of 50 mg/ml was prepared. Linezolid was provided as a purified powder from the manufacturer (Pfizer AG, Zurich, Switzerland), and a stock solution of 2.5 mg/ml was prepared.

In vitro antimicrobial susceptibility. A standard inoculum of 1×10^5 to 5×10^5 CFU/ml of MRSA strain ATCC 43300 was used. The MIC and the minimal bactericidal concentration (MBC) in the logarithmic growth phase (MBC_{log}) were determined by using twofold dilutions of antimicrobial agents in Mueller-Hinton broth supplemented with 50 mg/liter calcium ions ($CaCl_2$), according to the CLSI (formerly the NCCLS) guidelines (3). This concentration of calcium is necessary for the antimicrobial activity of daptomycin to be exhibited (1). The MIC was the lowest drug concentration that inhibited visible bacterial growth. The MBC_{log} was defined as the lowest antimicrobial concentration which killed ≥99.9% of the initial bacterial count (i.e., ≥3 $\log_{10}$ CFU/ml) in 24 h (10). In addition, the MBC was determined also in the stationary (nongrowing) growth phase (MBC_{stat}), reflecting the characteristics of microorganisms causing implant-associated infections. MBC_{stat} was determined by using overnight cultures of *S. aureus* in nutrient-limited medium (0.01 M phosphate buffered saline [PBS], pH 7.4) containing 0.1% glucose and 50 mg/liter calcium ions. In this medium, the bacterial counts remained stable for up to 48 h. MBC_{stat} was defined as the lowest concentration which reduced the inoculum by ≥99.9% in 24 h. The experiments were performed in triplicate.

Time-kill study in stationary growth phase. Glass tubes containing 10 ml PBS supplemented with 50 mg/liter calcium ions and 0.1% glucose were incubated with daptomycin at concentrations representing 4×, 8×, 16×, 32×, 64×, and 128× the MIC of the test strain at 37°C without shaking. Bacterial survival in the antimicrobial-free culture served as a control. To determine whether the inoculum size affects the killing activity of daptomycin, a low initial inoculum (3×10^5 CFU/ml) and a high initial inoculum (5×10^6 CFU/ml) were tested. For the high-inoculum assays, PBS with 50 mg/liter calcium ions was supplemented with 0.001% TSB to keep the bacterial counts in the antimicrobial-free culture stable for at least 24 h. Colony counts were determined immediately before addition of daptomycin (0 h) and after 2, 4, 6, 8, and 24 h of incubation with daptomycin at the appropriate concentrations. Before sampling of the probes, the tubes were gently vortexed and colony counts were determined by plating aliquots of appropriate dilutions on Mueller-Hinton agar. A bactericidal effect was defined as a ≥3-$\log_{10}$ (≥99.9%) reduction of the initial bacterial count (11). The experiments were performed in triplicate.

Animal model. We used a guinea pig model of foreign-body infection which was established by Zimmerli et al. (24). Guinea pigs (Charles River, Sulzfeld, Germany) were kept in the Animal House of the Department of Biomedicine, University Hospital Basel. The animal experiments were performed according to the regulations of Swiss veterinary law. In brief, four sterile polytetrafluoroethylene (Teflon) tubes (10 by 30 mm) perforated with 130 holes (Angst + Pfister AG, Zurich, Switzerland) were aseptically implanted into the flanks of male guinea pigs weighing at least 500 g. The animals were anesthetized with an intramuscular injection of ketamine (20 mg/kg; Parke-Davis, Zurich, Switzerland) and xylazine (4 mg/kg; Gräub, Bern, Switzerland). The experiments were started after complete wound healing (i.e., approximately 2 weeks after surgery). Before each experiment, the cages were checked for sterility by culturing the aspirated cage fluid. The guinea pigs were weighed daily to monitor their well-being during the experiment and to adjust the antibiotic doses.

Pharmacokinetic study. Pharmacokinetic studies were performed with sterile tissue cages. A single dose of 20, 30, and 40 mg/kg daptomycin was injected intraperitoneally (three animals and 12 cages per dose group). Cage fluid was aspirated by percutaneous cage puncture at 1, 2, 4, 6, 8, 10, 12, and 24 h after drug administration. For each drug dose, fluid from six cages per time point (two cages per time point and animal) was collected. Aliquots of 150 μl of cage fluid were transferred to tubes containing 15 μl of filter-sterilized 5% polyanetholsulfonic acid sodium salt (Sigma-Aldrich, Buchs, Switzerland), mixed by hand, and centrifuged at 2,100 × *g* for 7 min. The supernatant was stored at −20°C until further analysis.

(i) High-performance liquid chromatography assay, followed by mass spectrometry. Daptomycin standards were prepared in cage fluid by spiking cage fluid from untreated animals with daptomycin solution in water-methanol (1/1) to give concentrations in the range of 0.2 to 150 μg/ml. Two hundred microliters of precipitation solution (methanol, acetonitrile, 1 mM zinc sulfate) containing 2 μg of CB183253 (internal standard) was added to 50 μl of each of the standards, samples, and controls. After vortexing of the samples and centrifugation, 100 μl of the supernatant was diluted with water-methanol (1/1) and 10 μl was injected into the liquid chromatography-mass spectrometry apparatus (TSQ; Thermo Fisher Scientific). Separation of the components was performed on a C_{18} column (Uptisphere; particle size, 5 μm; 125 by 2 mm) by using acetonitrile and 0.1% formic acid as the mobile phase. Daptomycin was quantified by analyzing *m/z* 811 → 341, and the internal standard was quantified by analyzing *m/z* 837 → 393. The daptomycin concentrations were calculated by linear regression of the peak ratios between daptomycin and the internal standard.

(ii) Pharmacokinetic parameters. Individual concentration-time data were analyzed by using the WinNonlin software package (Pharsight Corp., Mountain View, CA). For each time point, the mean fluid concentration of the six cages was used. Mean ± standard deviation (SD) values of the peak (maximum) concentration (C_{max}), the time required to reach C_{max} (T_{max}), the trough (minimum) concentration at 24 h after dosing (C_{min}), the half-life ($t_{1/2}$), and the area under the concentration-time curve (AUC) from time zero to 24 h (AUC_{0-24}) were calculated.

Antimicrobial treatment study. Cages were infected with the MRSA test strain by percutaneous injection of 200 μl bacterial suspension containing 4×10^6 CFU (day 0). The establishment of an infection was confirmed by quantitative culture of cage fluid 3 days later, immediately before the start of treatment. Three animals were randomized into each of the following 10 treatment groups: saline (control), rifampin at 12.5 mg/kg alone, linezolid at 50 mg/kg plus rifampin at 12.5 mg/kg, levofloxacin at 10 mg/kg plus rifampin at 12.5 mg/kg, vancomycin at 15 mg/kg alone and in combination with rifampin at 12.5 mg/kg, and daptomycin at 20 mg/kg and 30 mg/kg alone and in combination with rifampin at 12.5 mg/kg. The antimicrobial agents were given intraperitoneally for 4 days. The dosing interval was 12 h for all drugs except daptomycin, which was given every 24 h.

(i) Efficacy of treatment against planktonic bacteria. Bacterial counts (median and interquartile range) were determined before the start of treatment (i.e., day 3), during treatment (i.e., day 5), and 5 days after the completion of treatment (i.e., day 12). The efficacy of each treatment against planktonic bacteria in cage fluid was expressed as the difference in the bacterial counts ($\Delta\log_{10}$ CFU/ml) before and 5 days after the completion of treatment and the clearance rate (in percent), defined as the number of cage fluid samples without growth of MRSA divided by the total number of cages in the individual treatment group.

(ii) Efficacy of treatment against adherent bacteria. Five days after the end of treatment (i.e., day 12), the animals were sacrificed and the tissue cages were removed under aseptic conditions and incubated at 37°C in 5 ml TSB. After 48 h of incubation, 100 μl of the cage culture was spread on Columbia sheep blood agar plates (Becton Dickinson) and analyzed for bacterial growth. A positive culture of MRSA was defined as a treatment failure. The efficacy of treatment against adherent bacteria was expressed as the cure rate (in percent), defined as

TABLE 1. In vitro susceptibility of MRSA ATCC 43300

Antibiotic[a]	MIC (μg/ml)	MBC_{log} (μg/ml)	MBC_{stat} (μg/ml)	MBC_{stat}/MBC_{log} ratio
DAP	0.625	0.625	20	32
RIF	0.01	0.08	2.5	31
VAN	1	2	32	16
LZD	2.5	>20	>20	NA[b]
LVX	0.16	0.63	>20	>32

[a] DAP, daptomycin; RIF, rifampin; VAN, vancomycin; LZD, linezolid; LVX, levofloxacin.
[b] NA, not applicable.

the number of cages without growth divided by the total number of cages in the individual treatment group.

Emergence of antimicrobial resistance in vivo. Positive cultures of samples from explanted cages were screened for the in vivo emergence of resistance to rifampin, vancomycin, and daptomycin. In addition, all positive cultures of samples from cage fluid were screened for rifampin resistance. Colonies were collected from subcultures on agar; suspended in saline to the turbidity of a McFarland 0.5 standard; and spread on Mueller-Hinton agar plates containing 2 μg/ml of daptomycin, 1 μg/ml of rifampin, or 16 μg/ml of vancomycin. The plates were incubated at 37°C and screened for growth after 24 h.

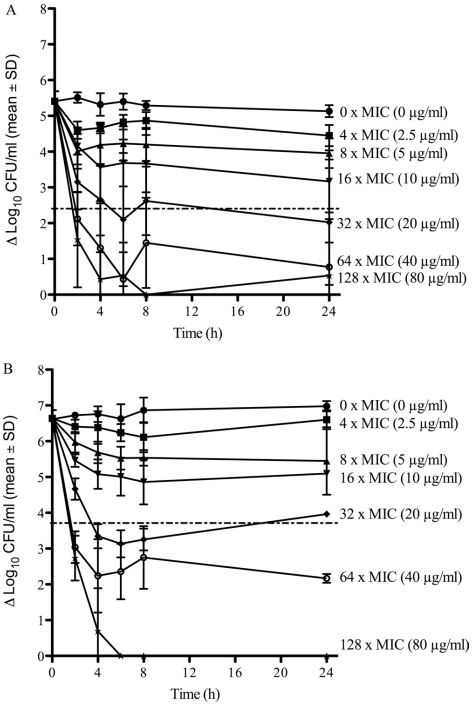

FIG. 1. Time-kill curve of a low inoculum (3×10^5 CFU/ml) (A) and a high inoculum (5×10^6 CFU/ml) (B) of MRSA in stationary growth phase exposed to increasing daptomycin concentrations (2.5 μg/ml to 80 μg/ml) corresponding to 4× to 128× MIC. Values are means ± SDs. The experiments were performed in triplicate. The horizontal dotted lines indicates a 3-log_{10} reduction of the numbers of CFU/ml from the initial inoculum.

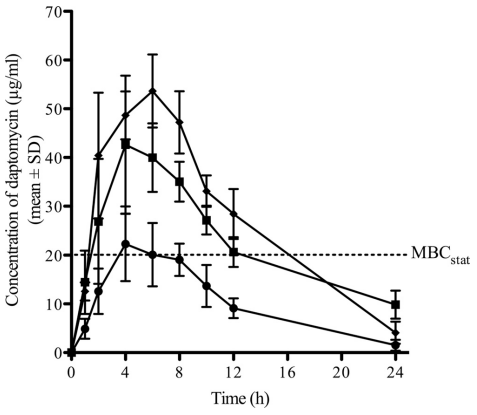

FIG. 2. Pharmacokinetics of daptomycin in sterile cage fluids after administration of single intraperitoneal doses of daptomycin at 20 mg/kg (circles), 30 mg/kg (squares), and 40 mg/kg (diamonds). Values are means ± SDs. The horizontal dotted line indicates the MBC_{stat} of MRSA for daptomycin.

Evaluation of antimicrobial toxicity. To evaluate the potential toxicity of daptomycin (20 mg/kg) administered with or without rifampin (three animals per group), histopathologic analysis of liver, kidney, and skeletal muscle tissues was performed. The corresponding organs of the saline-treated animals served as controls. The organs were fixed overnight in 4% buffered formalin, rinsed with PBS, and embedded into paraffin immediately after the animals were killed. Sections of 3 to 4 μm were mounted on slides and dried overnight at 37°C. The specimen sections were stained with hematoxylin-eosin and inspected by light microscopy.

Statistical calculations. Comparisons were performed by the Mann-Whitney U test for continuous variables and the two-sided χ^2 test or Fisher's exact test for categorical variables, as appropriate. For all tests, differences were considered significant when P values were <0.05. The graphs in the figures were plotted with Prism (version 5.0a) software (GraphPad Software, La Jolla, CA).

RESULTS

In vitro antimicrobial susceptibility. Table 1 summarizes the in vitro susceptibility of MRSA ATCC 43300. Of the antibiotics tested, rifampin showed the lowest MBC_{stat} (2.5 μg/ml), followed by daptomycin (20 μg/ml) and vancomycin (32 μg/ml), whereas linezolid and levofloxacin did not kill MRSA in the stationary growth phase. The MBC_{stat} was at least 16-fold higher than the MBC_{log} for all agents (except linezolid, which had only a bacteriostatic effect).

In vitro time-kill study in stationary growth phase. In the low-inoculum and the high-inoculum studies, the bacterial counts remained within ±5% of the initial inoculum in the antimicrobial-free culture for 24 h. The time-kill curves in Fig. 1 demonstrate that daptomycin had rapid and concentration-dependent bactericidal activity against stationary-phase MRSA with a low inoculum (Fig. 1A) as well as a high inoculum (Fig. 1B). At 20 μg/ml (32× MIC), which corresponded to the MBC_{stat}, daptomycin reduced the counts by ≥3 log_{10}

TABLE 2. Pharmacokinetic parameters of daptomycin in cage fluid after a single intraperitoneal dose and linked to the in vitro susceptibility parameters of the MRSA strain tested[a]

Dose (mg/kg)	C_{max} (μg/ml)	C_{min} (μg/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC_{0-24} (μg · h/ml)	C_{max}/MIC	C_{max}/MBC_{log}	C_{max}/MBC_{stat}	AUC > MBC_{stat}/AUC_{0-24}
20	23.1 ± 7.0	1.5 ± 1.1	6.0 ± 2.0	4.8 ± 1.7	247 ± 52	36.9 ± 11.2	36.9 ± 11.2	1.2 ± 0.3	0.05 ± 0.04
30	46.3 ± 8.8	9.8 ± 2.9	4.7 ± 1.2	8.7 ± 0.5	548 ± 65	74.0 ± 14.1	74.0 ± 14.1	2.3 ± 0.4	0.26 ± 0.04
40	53.7 ± 1.3	4.1 ± 2.3	6.0 ± 0.0	4.5 ± 1.2	662 ± 10	85.8 ± 2.1	85.8 ± 2.1	2.7 ± 0.1	0.39 ± 0.01

[a] Values represent means ± SDs.

CFU after 4 to 6 h. At concentrations above 20 μg/ml, daptomycin reduced the counts by >3 log_{10} CFU/ml in 2 to 4 h.

Pharmacokinetic study. Figure 2 shows the concentration-time curves in sterile cage fluid after the administration of a single intraperitoneal dose of 20, 30, or 40 mg/kg daptomycin. Table 2 summarizes the values of the pharmacokinetic parameters calculated. For all three doses administered, the peak C_{max}s were above the MBC_{stat}s, whereas the concentrations of daptomycin after 24 h (C_{min}) remained above the MIC and MBC_{log} but not above the MBC_{stat}. The AUC_{0-24} increased with the dose from 247 to 662 μg · h/ml. The ratio of the AUC > MBC_{stat} to AUC_{0-24} increased in a dose-dependent manner from 5% (at 20 mg/kg) to 26% (at 30 mg/kg) and 39% (at 40 mg/kg).

Antimicrobial treatment study. Three days after inoculation, the bacterial counts surpassed the initial inoculum two- to threefold in all infected animals (data not shown). The planktonic bacterial counts (median ± interquartile range) in the cage fluid of the control group (treated with saline) increased by 1.4 ± 0.1 log_{10} CFU/ml (Fig. 3); no bacterial clearance (Fig. 4A) or spontaneous cure (Fig. 4B) was observed in the untreated group.

(i) Efficacy of treatment against planktonic bacteria. Figure 3 shows the killing of planktonic bacteria in cage fluid 5 days after the completion of therapy (compared to the bacterial counts before treatment start). By the use of monotherapy, the planktonic bacterial counts increased by <1 log_{10} CFU/ml with vancomycin or daptomycin at 20 mg/kg and decreased by 0.3 log_{10} CFU/ml with daptomycin at 30 mg/kg. In combination

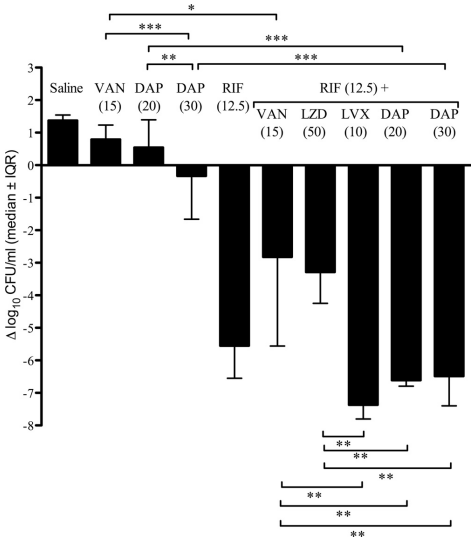

FIG. 3. Killing of planktonic MRSA in cage fluid 5 days after the completion of therapy. Positive values on the *y* axis denote the net growth and negative values denote the net killing. Values are medians ± interquartile ranges. The numbers in parentheses indicate the dose (in mg/kg) administered twice daily for all drugs except daptomycin, which was administered once daily. DAP, daptomycin; RIF, rifampin; VAN, vancomycin; LZD, linezolid; LVX, levofloxacin; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

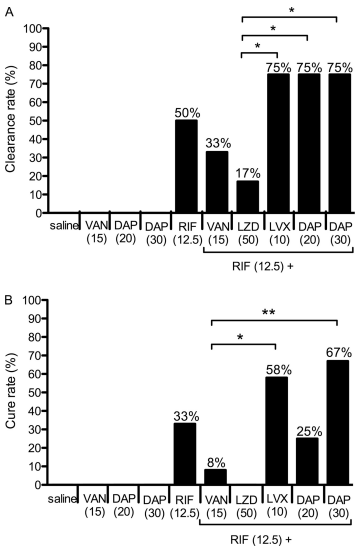

FIG. 4. Clearance rate of planktonic MRSA (A) and cure rate of adherent MRSA in explanted cages (B). The numbers in parentheses indicate the dose (in mg/kg) administered twice daily for all drugs except daptomycin, which was administered once daily. DAP, daptomycin; RIF, rifampin; VAN, vancomycin; LZD, linezolid; LVX, levofloxacin; *, $P < 0.05$; **, $P < 0.01$.

TABLE 3. Rates of emergence of rifampin resistance in cage fluid during and after treatment (planktonic bacteria) and in culture from explanted cages (adherent bacteria)

Treatment (dose)[a]	Planktonic bacteria[b]		Adherent bacteria[c] after treatment (day 12)
	During treatment (day 6)	After treatment (day 12)	
RIF (12.5)	2/12 (17)	2/12 (17)	3/12 (25)
VAN (15) + RIF (12.5)	4/12 (33)	5/12 (42)	7/12 (58)
LZD (50) + RIF (12.5)	0/12 (0)	0/12 (0)	1/12 (8)
LVX (10) + RIF (12.5)	0/12 (0)	0/12 (0)	0/12 (0)
DAP (20) + RIF (12.5)	0/12 (0)	0/12 (0)	2/12 (17)
DAP (30) + RIF (12.5)	0/12 (0)	0/12 (0)	0/12 (0)

[a] The doses are in mg/kg and were administered every 12 h for all drugs except daptomycin, which was administered every 24 h. RIF, rifampin; VAN, vancomycin; LZD, linezolid; LVX, levofloxacin; DAP, daptomycin.
[b] The data represent the number of cage fluid specimens with rifampin-resistant colonies/total number of all cage fluids (percent).
[c] The data represent the number of cage cultures with rifampin-resistant colonies/total number of cage cultures (percent).

with rifampin, levofloxacin and daptomycin at 20 and 30 mg/kg killed planktonic MRSA more efficiently (7.4 $\log_{10}$, 6.6 $\log_{10}$, and 6.5 $\log_{10}$ CFU/ml, respectively) than linezolid or vancomycin (3.3 $\log_{10}$ and 2.8 $\log_{10}$ CFU/ml, respectively) ($P < 0.01$ for all groups). In comparison to monotherapy, vancomycin plus rifampin was significantly more active against planktonic bacteria ($P = 0.019$). Similarly, daptomycin performed significantly better in combination with rifampin ($P < 0.0001$) in a manner that was independent of the dose administered.

Figure 4A shows the rate of clearance of planktonic bacteria in cage fluid. Vancomycin and daptomycin monotherapy were unable to clear planktonic MRSA. In combination with rifampin, levofloxacin and daptomycin showed higher clearance rates (all 75%) than linezolid (17%), vancomycin (33%), and rifampin (50%) alone.

(ii) Efficacy of treatment against adherent bacteria. Figure 4B shows the efficacy of treatment against adherent bacteria. Vancomycin and daptomycin (at both doses) were unable to cure any cage-associated infection when they were given as monotherapy, whereas rifampin alone cured the infections in 33% of the cages. In combination with rifampin, levofloxacin (58%) and daptomycin at 30 mg/kg (67%) cured significantly more infected cages than vancomycin (8%) and linezolid (0%).

Emergence of antimicrobial resistance in vivo. Table 3 shows the rates of emergence of rifampin resistance in planktonic MRSA during and after rifampin monotherapy (both 17%) as well as in adherent MRSA after treatment (25%). Rifampin resistance emerged more often during therapy with vancomycin plus rifampin (58%) than during therapy with linezolid plus rifampin (8%) or daptomycin at 20 mg/kg plus rifampin (17%). Levofloxacin plus rifampin and daptomycin at 30 mg/kg plus rifampin completely prevented the emergence of rifampin resistance in planktonic as well as adherent bacteria. No MRSA strain in cage fluid cultures from animals treated with daptomycin or vancomycin alone or in combination with rifampin developed resistance to daptomycin or vancomycin (data not shown).

Evaluation of antimicrobial toxicity. In animals treated with daptomycin (20 mg/kg), no acute lesions in the kidneys, liver, or skeletal muscles, such as acute muscle fiber necrosis (rhabdomyolysis), were observed. In animals treated with daptomycin and rifampin, liver histology showed mild inflammation.

DISCUSSION

Daptomycin was highly bactericidal in the logarithmic growth phase as well as in the stationary growth phase of MRSA ATCC 43300. These in vitro studies suggested that daptomycin may be efficacious in eradicating MRSA implant-associated infections. We used the cage-associated infection model in guinea pigs, which has been validated for use for the evaluation of drug activity against implant-associated infections (7, 9, 25). In contrast to the cage model in mice and rats (14), no spontaneous cure of infected cages occurs in guinea pigs, which resembles the situation in humans. Assuming an approximately 50% penetration into cage fluid, daptomycin doses of 20, 30, and 40 mg/kg in guinea pig correspond to human doses of 4, 6, and 8 mg/kg, respectively (2, 5, 22). Therefore, daptomycin was used at 20 and 30 mg/kg in subsequent treatment studies with guinea pigs.

In the treatment studies, none of the monotherapy regimens tested (except rifampin monotherapy) cleared planktonic MRSA or eradicated adherent MRSA from the cages. It might be possible that the concentrations of daptomycin administered were not sufficiently high to eradicate biofilm-associated MRSA. In a recent study, daptomycin at a concentration of 64 μg/ml had improved activity against staphylococci embedded in a biofilm (16). Therefore, a higher concentration of daptomycin corresponding to human doses above 6 mg/kg should be examined in future studies with animals.

In contrast, when levofloxacin or daptomycin at a high dose (30 mg/kg) were combined with rifampin, they showed high degrees of efficacy against the adherent bacteria. These data suggest that addition of rifampin to quinolones or lipopetides is important for the eradication of staphylococcal implant-associated infections. Interestingly, in combination with rifampin, vancomycin and linezolid, both first-line drugs used against MRSA, had lower cure rates. Furthermore, a higher daptomycin dose (30 mg/kg versus 20 mg/kg) in combination with rifampin was associated with a higher cure rate. The importance of rifampin-containing regimens was also demonstrated in vitro, when rifampin in combination with daptomycin was significantly more effective in eliminating MRSA from the biofilm than daptomycin alone (13).

In a previous study (18), levofloxacin alone was unable to eradicate methicillin-susceptible *S. aureus*, even though quinolone monotherapy cured about half of the staphylococcal implant-associated infections in the clinical setting (25). This reflects the stringent experimental conditions which were applied in the present experiments, in which a high infecting inoculum, a lack of debridement of the infected cages, and a short duration of antibiotic treatment (4 days) were used. These conditions were chosen in order to better discriminate the differences in efficacies of the antibiotics tested and to determine the risk of emergence of rifampin resistance. Antimicrobial regimens effective in the present animal model will probably also be effective in the clinical setting.

Rifampin resistance emerged in adherent MRSA from cage cultures with rifampin monotherapy; the rate of resistance was higher with addition of vancomycin and lower with addition of

daptomycin at 20 mg/kg or linezolid. Addition of levofloxacin and daptomycin at a high dose completely prevented the emergence of rifampin resistance. These data show the importance of combining rifampin with an effective antibiofilm drug administered at a sufficient dose.

In conclusion, daptomycin at a high once-daily dose, corresponding to 6 mg/kg in humans, in combination with rifampin showed the highest activity against planktonic and adherent MRSA and prevented the emergence of rifampin resistance. The cure rate achieved with this combination was comparable to that achieved with levofloxacin plus rifampin but higher than the one with vancomycin plus rifampin, which could not prevent emergence of rifampin resistance. This raises concern about vancomycin combination therapy. Since health care-associated MRSA strains are increasingly resistant to quinolones, daptomycin in combination with rifampin presents a promising treatment option for implant-associated staphylococcal infections.

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REFERENCES

1. **Andrew, J. H., M. C. Wale, L. J. Wale, and D. Greenwood.** 1987. The effect of cultural conditions on the activity of LY146032 against staphylococci and streptococci. J. Antimicrob. Chemother. **20:**213–221.
2. **Benvenuto, M., D. P. Benziger, S. Yankelev, and G. Vigliani.** 2006. Pharmacokinetics and tolerability of daptomycin at doses up to 12 milligrams per kilogram of body weight once daily in healthy volunteers. Antimicrob. Agents Chemother. **50:**3245–3249.
3. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 7th ed. CLSI document M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Drancourt, M., A. Stein, J. N. Argenson, R. Roiron, P. Groulier, and D. Raoult.** 1997. Oral treatment of *Staphylococcus* spp. infected orthopaedic implants with fusidic acid or ofloxacin in combination with rifampicin. J. Antimicrob. Chemother. **39:**235–240.
5. **Dvorchik, B. H., D. Brazier, M. F. DeBruin, and R. D. Arbeit.** 2003. Daptomycin pharmacokinetics and safety following administration of escalating doses once daily to healthy subjects. Antimicrob. Agents Chemother. **47:**1318–1323.
6. **Fux, C. A., J. W. Costerton, P. S. Stewart, and P. Stoodley.** 2005. Survival strategies of infectious biofilms. Trends Microbiol. **13:**34–40.
7. **Giulieri, S. G., P. Graber, P. E. Ochsner, and W. Zimmerli.** 2004. Management of infection associated with total hip arthroplasty according to a treatment algorithm. Infection **32:**222–228.
8. **Hall-Stoodley, L., J. W. Costerton, and P. Stoodley.** 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol. **2:**95–108.
9. **Laffer, R. R., P. Graber, P. E. Ochsner, and W. Zimmerli.** 2006. Outcome of prosthetic knee-associated infection: evaluation of 40 consecutive episodes at a single centre. Clin. Microbiol. Infect. **12:**433–439.
10. **Murray, P. R., E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.).** 2003. Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, DC.
11. **National Committee for Clinical Laboratory Standards.** 1999. Methods for determining bactericidal activity of antimicrobial agents. NCCLS document M26-A. National Committee for Clinical Laboratory Standards, Villanova, PA.
12. **Perlroth, J., M. Kuo, J. Tan, A. S. Bayer, and L. G. Miller.** 2008. Adjunctive use of rifampin for the treatment of *Staphylococcus aureus* infections: a systematic review of the literature. Arch. Intern. Med. **168:**805–819.
13. **Raad, I., H. Hanna, Y. Jiang, T. Dvorak, R. Reitzel, G. Chaiban, R. Sherertz, and R. Hachem.** 2007. Comparative activities of daptomycin, linezolid, and tigecycline against catheter-related methicillin-resistant *Staphylococcus* bacteremic isolates embedded in biofilm. Antimicrob. Agents Chemother. **51:**1656–1660.
14. **Schaad, H. J., M. Bento, D. P. Lew, and P. Vaudaux.** 2006. Evaluation of high-dose daptomycin for therapy of experimental *Staphylococcus aureus* foreign body infection. BMC Infect. Dis. **6:**74.
15. **Silverman, J. A., N. G. Perlmutter, and H. M. Shapiro.** 2003. Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47:**2538–2544.
16. **Smith, K., A. Perez, G. Ramage, C. G. Gemmell, and S. Lang.** 2009. Comparison of biofilm-associated cell survival following in vitro exposure of meticillin-resistant *Staphylococcus aureus* biofilms to the antibiotics clindamycin, daptomycin, linezolid, tigecycline and vancomycin. Int. J. Antimicrob. Agents **33:**374–378.
17. **Steenbergen, J. N., J. Alder, G. M. Thorne, and F. P. Tally.** 2005. Daptomycin: a lipopeptide antibiotic for the treatment of serious gram-positive infections. J. Antimicrob. Chemother. **55:**283–288.
18. **Trampuz, A., C. K. Murphy, D. M. Rothstein, A. F. Widmer, R. Landmann, and W. Zimmerli.** 2007. Efficacy of a novel rifamycin derivative, ABI-0043, against *Staphylococcus aureus* in an experimental model of foreign-body infection. Antimicrob. Agents Chemother. **51:**2540–2545.
19. **Trebse, R., V. Pisot, and A. Trampuz.** 2005. Treatment of infected retained implants. J. Bone Joint Surg. Br. **87:**249–256.
20. **Tverdek, F. P., C. W. Crank, and J. Segreti.** 2008. Antibiotic therapy of methicillin-resistant *Staphylococcus aureus* in critical care. Crit. Care Clin. **24:**249–260, vii-viii.
21. **Widmer, A. F., A. Gaechter, P. E. Ochsner, and W. Zimmerli.** 1992. Antimicrobial treatment of orthopedic implant-related infections with rifampin combinations. Clin. Infect. Dis. **14:**1251–1253.
22. **Wise, R., T. Gee, J. M. Andrews, B. Dvorchik, and G. Marshall.** 2002. Pharmacokinetics and inflammatory fluid penetration of intravenous daptomycin in volunteers. Antimicrob. Agents Chemother. **46:**31–33.
23. **Zimmerli, W., R. Frei, A. F. Widmer, and Z. Rajacic.** 1994. Microbiological tests to predict treatment outcome in experimental device-related infections due to *Staphylococcus aureus*. J. Antimicrob. Chemother. **33:**959–967.
24. **Zimmerli, W., F. A. Waldvogel, P. Vaudaux, and U. E. Nydegger.** 1982. Pathogenesis of foreign body infection: description and characteristics of an animal model. J. Infect. Dis. **146:**487–497.
25. **Zimmerli, W., A. F. Widmer, M. Blatter, R. Frei, P. E. Ochsner, et al.** 1998. Role of rifampin for treatment of orthopedic implant-related staphylococcal infections: a randomized controlled trial. JAMA **279:**1537–1541.

3.2 Part 2: Ca^{2+}-sensitive reversible daptomycin resistance of adherent staphylococci in an implant infection model

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Keywords: daptomycin, *Staphylococcus aureus*, biofilm, adherence, calcium, implant infection

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Abstract

Daptomycin (DAP) is bactericidal against methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro*, but it failed to eradicate MRSA in an experimental model of implant-associated infection. We therefore investigated various factors, which could explain treatment failure, by evaluating DAP activity, including the role of different cell wall components, adherence, biofilm, and calcium ions (Ca^{2+}) *in vitro* and *in vivo*.

In the tissue cage infection model, DAP was only active prophylactically and against low inocula. To identify the mechanisms of treatment failure against adherent microorganisms, the activity of DAP against *S. aureus* and *S. epidermidis* mutants differing in their capacity of biofilm formation and adherence, was determined. For planktonic staphylococci, the MIC was 0.625 µg/mL. It increased by alanylated lipoteichoic acid (LTA), since the Δ*dltA* mutant was more susceptible ($p<0.05$). For adherent staphylococci, DAP reduced biofilm at 30 µg/mL. However, it did not kill adherent bacteria up to 500 µg/mL, independent of biofilm biosynthesis (*ica*), nuclease (*nuc1*/*nuc2*), LPXTG-anchored adhesin (*srtA*), autolysin (*atl*) or alanyl-LTA (*dltA*). Resistance of adherent staphylococci was not due to mutations of adherent bacteria, since staphylococci became DAP-susceptible after detachment. Phenotypic resistance was not explained by inactivation of DAP or inability of initial Ca^{2+}-DAP complex formation. However, addition of up to 100 mg/L (2.5 mmol/l) Ca^{2+} gradually improved bactericidal activity towards adherent staphylococci *in vitro* and increased the prevention rate in the cage-model from 40% to 60%. In summary, adherent staphylococci resist to DAP killing unless Ca^{2+} is supplemented to physiologic concentrations.

Introduction

Prosthetic joints are increasingly used to maintain life quality of patients with damaged joints. Hip and knee implants have good long-term results (21). However, they carry the risk of bacterial infection. Staphylococci (*Staphylococcus* (*S.*) *epidermidis* (30-43%) and *S. aureus* (12-23%)) are the most frequent causes of periprosthetic hip and knee infections (45). Immediately after implantation, extracellular plasma proteins cover the implant surface. Staphylococci adhere to these proteins through their microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Subsequently, staphylococci aggregate in an extracellular matrix, called biofilm, which consists mainly of polysaccharide intercellular adhesin (PIA) (12), extracellular DNA (33) and a few proteins (29). The biofilm matures into a three-dimensional structure and undergoes quorum sensing-controlled dispersion at its surface (29). The biosynthetic enzymes of PIA are encoded by the *ica* operon. This is controlled by global regulatory networks, which suppress virulence factor gene expression, and thereby maintain this special mode of growth (12, 17, 26, 29). The gene changes, which stabilize staphylococci in stationary phase in biofilm, may also explain the limited activity of antibiotics that target growing cells against bacteria in biofilm (6, 12, 29, 30). The biofilm further confers resistance against innate host defence by preventing bacterial complement binding and reducing phagocytosis (18, 42).

For successful treatment of device-related infections, drugs with bactericidal effect on surface-adhering, slow-growing, and biofilm-producing microorganisms are needed. These antimicrobial compounds should penetrate the biofilm, act independently of the bacterial physiological state, and prevent further biofilm formation. So far, anti-biofilm

drugs such as dispersin, have been tested in vitro. However, none of the compounds significantly eradicated biofilms when applied alone (22, 29).

A promising candidate might be the cyclic lipopeptide daptomycin (DAP). Despite its high affinity for proteins, it exhibits concentration-dependent bactericidal activity against Gram-positive organisms, including methicillin-resistant *S. aureus* (MRSA) (37). It leads to rapid calcium-dependent cell death due to membrane depolarization (16, 36, 39). The bacterial membrane is the only target for DAP. It has been previously shown that DAP does not require cell division or active metabolism for bactericidal activity, although it is more active against growing staphylococci (13, 23). However, we and others could previously show that DAP was not able to eradicate adherent staphylococci in an implant-associated infection model at clinically relevant doses (13, 28). We therefore investigated the mechanism of phenotypic resistance of adherent staphylococci to DAP *in vitro* and *in vivo* in the present study.

Genotypic DAP resistance has been observed previously and was attributed to individual sequential mutations, which lead to modest increases in the MIC (2, 9). Single point mutations in *mprF, rpoB* and *yycG* were identified by comparative genome sequencing of clinical isolates and of laboratory-derived *S. aureus* with decreased susceptibility to DAP (7, 9). We found that DAP treatment failed to eliminate adherent staphylococci independently of biofilm formation. However, with increased Ca^{2+} concentration, efficacy of DAP increased against adherent bacterial cultures *in vitro* and in the implant model *in vivo*.

Material and Methods

Bacterial strains and growth conditions. The following staphylococcal strains were used: *Staphylococcus aureus* (SA) ATCC 43300, a clinical isolate resistant to methicillin (MRSA), SA113 wild type (wt) (ATCC 35556) and its isogenic mutants Δ*ica*, Δ*nuc1/nuc2*, Δ*srtA*, Δ*atl*, and Δ*dltA* (kindly provided by F. Götz and Andreas Peschel), *S. epidermidis* (SE) 1457 wt and its isogenic mutant Δ*luxS::ermB* (kindly provided by M. Otto). For the analysis of DAP concentrations in the tissue cage fluid, *Kocuria rhizophila* (ATCC 9341), formerly known as *Micrococcus luteus*, was used as indicator organism in the bioassay.

The strains were stored at -70°C in a cryovial bead preservation system (Microbank, Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). For preparation of the inoculum, a bead was incubated in 1 mL of trypticase soy broth (TSB, Becton Dickinson and Company, Allschwil, Switzerland) for 7 h at 37°C, diluted 1:100 in fresh TSB and incubated overnight at 37°C without shaking. The overnight culture was diluted 1:100 and further incubated 5-6 h at 37°C to reach the logarithmic growth phase. Afterwards, bacteria were washed twice with 0.9% saline (Bichsel, Interlaken, Switzerland) and diluted to the needed inoculum. Bacterial numbers were determined by plating aliquots from appropriate dilutions on agar, followed by colony counting after 24 h of incubation at 37°C.

Antimicrobial agents. Daptomycin was supplied by Novartis Pharma Schweiz AG (Bern, Switzerland). Stock solutions were prepared in pyrogen-free 0.9% saline.

Animal model. We used a mouse model of foreign-body infection established by Kristian et al. (19). The method was approved by the review board of the "Kantonale Veterinaeramt Basel-Stadt" (permit #1710). Experiments were conducted according to the regulations of the Swiss veterinary law. Nine to 11-week-old male C57BL/6 mice (Harlan Laboratories, Switzerland), kept in the Animal House of the Department of Biomedicine, University Hospital Basel, were anesthetized via intraperitoneal (IP) injection of 65 mg/kg ketamine (Ketalar, Warner-Lambert) and 13 mg/kg xylazium (Xylasol, Graeub). A sterile tissue cage (Angst + Pfister AG, Zurich, Switzerland) containing 8 sinter glass beads (Sikuf, Schott Schleifer, Muttenz, Switzerland) was implanted subcutaneously. After surgery, mice were treated with 0.05 mg/kg buprenorphine (Temgesic, Essex Chemie AG, Luzern) to treat postoperative pain. After complete wound healing (2 weeks), cages were tested for sterility by culturing the aspirated tissue cage fluid (TCF).

Pharmacokinetic study. A single IP dose of 30 and 40 mg/kg DAP was injected (6 mice per group). The pharmacokinetic levels of DAP were investigated in TCF of uninfected mice at various time points (2, 4, 6, 8, and 24 h after drug administration). TCF was collected by cage puncture and centrifuged at 2,100×g for 7 min. The supernatant was stored at -20°C until further analysis.

The concentration of DAP was evaluated by a previously described bioassay method (34). Briefly, 4-5 × 10^3 CFU/mL of a 6 h- *K. rhizophila* (ATCC 9341) culture were added to antibiotic medium 11 (Difco, Becton Dickinson and Company, Allschwil, Switzerland) and filled into Bioassay dishes (Fisher Scientific, Wohlen, Switzerland). Samples in duplicate were applied in punched holes. A standard curve was established with a range

from 1 to 128 µg/mL DAP in phosphate buffered saline (PBS, 0.01M, pH 7.4) supplemented with one volume of sterile TCF. To determine the DAP concentration in TCF over time, the diameters of the inhibition zones of the standard probes were plotted against the logarithm of the concentrations.

Minimal infective dose (MID). To evaluate the MID for MRSA, 10^2 to 10^5 CFU were injected into the tissue cage (3 mice per group). At different time points TCF was collected in 1.5% EDTA (in 0.45% NaCl, pH 7.3) and bacterial numbers were determined by plating. The MID was defined as CFU/tissue cage which was required to induce a persistent infection (15 days) in 100% of the tissue cages.

Prophylaxis study: 40 mg/kg DAP (5 mice per group) was IP administered 6 h before injection of 3×10^2 CFU MRSA with or without 50 mg/L (1.25 mM) calcium ions (Ca^{2+}) ($CaCl_2$). In a second approach, an additional IP dose of 40 mg/kg DAP was administered 6 h after injection of MRSA. Saline served as control. TCF was collected in EDTA and tissue cages were explanted 24 h after inoculation. TCF was used to determine the efficacy of DAP against planktonic bacteria by plating aliquots from appropriate dilutions on blood agar plates. The efficacy was expressed as the difference in bacterial counts ($\Delta \log_{10}$ CFU/mL) between inoculation and 24 h later. The tissue cages were incubated in TSB for 48 h at 37°C to determine the prevention rate by plating the supernatant. The prevention rate is defined as number of cages without growth divided by the total number of inoculated cages.

Treatment study (1-day infection): Mice were infected with 4×10^4 and 3×10^2 CFU MRSA, respectively. After 24 h, TCF was aspirated and bacterial numbers were determined by plating to proof an established infection. For therapy, infected mice were

treated with saline (control group) and 40 mg/kg DAP IP every 24 h for 4 days before sacrifying the animals 5 days later. On day 9, TCF was collected to quantify planktonic bacteria and tissue cages were explanted to determine the efficacy of DAP against adherent MRSA (cure rate). The efficacy of DAP against planktonic bacteria was quantified as the difference in bacterial counts (Δlog_{10} CFU/mL) before and 5 days after the end of treatment. The cure rate was defined as number of cages without growth divided by the total number of cages in the individual treatment group.

Biofilm assay. MRSA at 10^5 CFU/mL were seeded into flat-bottom 96-well plates (Becton Dickinson and Company, Allschwil, Switzerland) and treated with 30 µg/mL and 500 µg/mL. After 24 h incubation at 37°C, biofilm was stained using crystal violet as previously described (20). Briefly, supernatants were removed by dropping and plates were washed twice with PBS. The biofilm was fixed by incubating plates for 60 min at 60°C and stained with 100 µL of a 0.5% crystal violet solution for 20 min at room temperature (RT). After washing under running tap water, 100 µL of 33% acetic acid was added, and the optical density was measured at 590 nm using a Molecular Devices Reader (Applied Biosystems, Rotkreuz, Switzerland).

***In vitro* susceptibility.** According to the CLSI (formerly the NCCLS) guidelines, a standard inoculum of 1-5 × 10^5 CFU/mL was used. The MIC (lowest DAP concentration that inhibits visible bacterial growth) and the minimal bactericidal concentration in the logarithmic growth phase (MBC_{log}) (lowest DAP concentration, which kills ≥99.9% of

the initial bacterial count in 24 h) (24) were determined by using two-fold dilutions of DAP in Mueller-Hinton broth supplemented with 50 mg/L Ca^{2+} (25).

Daptomycin effects on adherent and detached staphylococci. MRSA, SA113 wt and its isogenic mutants, SE1457 wt and its isogenic mutant were incubated at 10^5 CFU/mL in TSB supplemented with 0.5% glucose for 18 h at 37°C in uncoated or 50% plasma-precoated (2 h, RT) flat-bottom 96-well plate. After washing away non-adherent bacteria with PBS, adherent bacteria were treated with DAP at 0 µg/mL, 30 µg/mL, and 500 µg/mL supplemented with 50 mg/L Ca^{2+} for 24 h at 37°C. Then biofilm was stained using crystal violet as described above and adherent bacterial numbers were determined by plating after detachment. To avoid cell cluster, adherent bacteria were detached carefully by pipetting up and down.

To investigate the efficacy of DAP against detached bacteria, adherent MRSA were detached by pipetting up and down after 18 h seeding. Detached MRSA were treated with DAP at 0 µg/mL and 30 µg/mL supplemented with 50 mg/L Ca^{2+} for 24 h and optical density was measured at 590 nm. DAP efficacy was also tested against dispersed MRSA ex vivo. To that aim tissue cages without sinter glass beads of the 1-day infection study were incubated in TSB for 48 h at 37°C. Dispersed MRSA (10^4 CFU/mL) were incubated in a 96-well plate with 0 µg/mL, 0.625 µg/mL, or 30 µg/mL DAP supplemented with 50 mg/L Ca^{2+}. After incubation for 24 h at 37°C, the optical density at 590 nm was measured. TSB without MRSA and DAP served as negative control.

To evaluate the activity of DAP after 24 h treatment of adherent MRSA, the supernatant was collected, sterile filtered and added to 10^5 CFU/mL fresh planktonic MRSA. After 24 h at 37°C the optical density at 590 nm was measured.

Calcium-competition assay. Adherent MRSA were treated with non-pre-incubated or Ca^{2+}-pre-incubated DAP (30 µg/mL with 50 mg/L Ca^{2+} for 2 h at RT). After 24 h incubation at 37°C, adherent bacterial numbers were determined after detachment.
To confirm the effect of additional Ca^{2+}, adherent MRSA were treated with 30 µg/mL DAP supplemented with either 75 mg/L (1.8 mmol/L) or 100 mg/L (2.5 mmol/l) Ca^{2+}. The difference in adherent bacterial counts before and after 24 h incubation was plotted.

Statistical analysis. Treatment effects were analyzed with the Mann-Whitney U test. A two-way ANOVA test was used for statistical analysis of the in vitro data. A *p* value of <0.05 was considered statistically significant. Statistical analysis was done with 5.0a Prism (GraphPad Software).

Results

Daptomycin treatment in a mouse tissue cage infection model. We previously reported that DAP mono-therapy was not successful against implant-associated MRSA-infection in guinea pigs (13). The aim of the present study was to elucidate this treatment failure by evaluating DAP in the tissue cage model in mice. This model is suitable to study phenotypic staphylococcal resistance against antimicrobial treatment (11, 20). We first performed pharmacokinetic studies in sterile tissue cage fluid (TCF) of mice after intraperitoneal (IP) administration of 30 and 40 mg/kg DAP. The peak concentrations (C_{max}) for the 30 and 40 mg/kg dose were 25 and 35 µg/mL, respectively. For both doses C_{max} was reached after 6 h and was above the MBC_{stat} of 20 µg/mL (13) during 4 to 8 h. DAP concentration after 24 h (C_{min}) remained above the MIC and MBC_{log} of 0.625 µg/mL (13) (Figure 3.1).

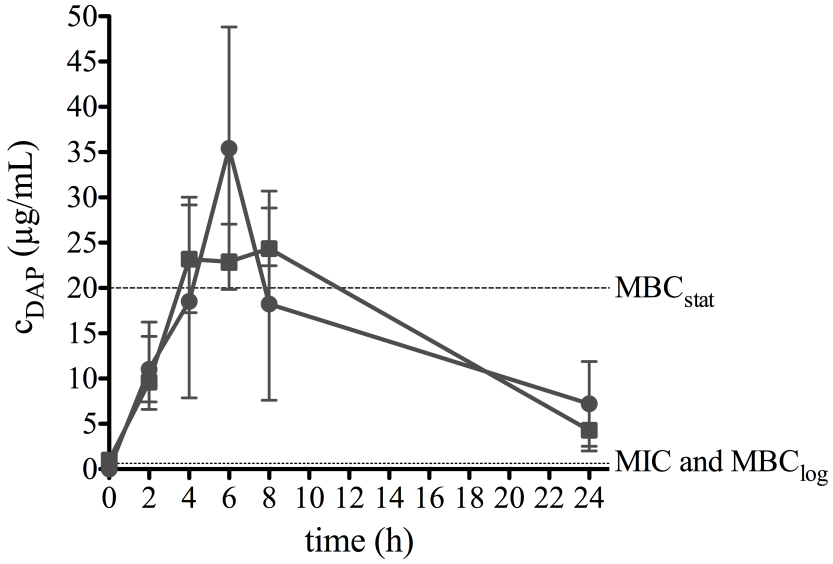

Figure 3.1. Pharmacokinetics of daptomycin (DAP) in sterile cage fluid of mice after a single intraperitoneal administration of a 30 mg/kg (squares) and 40 mg/kg (circles) dose. Values are means ± SDs. The horizontal dotted lines indicate the MIC and MBC_{log} (below) and MBC_{stat} (above) of MRSA 43300 for DAP.

We asked the question whether DAP efficacy was better with a lower inoculum, or when given prophylactically, i.e. before establishment of a biofilm. The minimal infective dose (MID) of MRSA for induction of a persistent infection in tissue cages of C57BL/6 mice was 3×10^2 CFU/cage. Therefore, all experiments were performed with at least this inoculum.

First, the efficacy of a 4-day-treatment of DAP (40 mg/kg) against planktonic and adherent MRSA was assessed in a 1-day infection study with an inoculum of either $\sim 10^4$ or $\sim 10^2$ CFU/cage (Figure 3.2). One day after infection, planktonic bacterial numbers were more than two-fold increased as compared to the initial inoculum (data not shown). Five days after the end of treatment, planktonic bacteria were not significantly reduced (0.6 $\log_{10}$ CFU/mL) and DAP failed to cure any tissue cages infected with the high inoculum (Figure 3.2A, B). In contrast, DAP killed 3 $\log_{10}$ CFU/mL planktonic bacteria and cured 33% of tissue cage associated infections in mice with the low inoculum (Figure 3.2A, B). As expected, untreated mice showed an increase of 2.7 $\log_{10}$ planktonic CFU/mL and no spontaneous cure (Figure 3.2A, B).

To study whether DAP prevents colonization of implants, we administered 40 mg/kg DAP prophylactically 6 h before infection with the MID inoculum of 3×10^2 CFU. One day after infection, untreated mice showed an increase of 1.3 $\log_{10}$ CFU/mL (Figure 3.2A). DAP reduced the planktonic bacteria by 2.3 $\log_{10}$ CFU/mL to minimal CFU numbers, and it was able to prevent 40% tissue cage associated infections (Figure 3.2B).

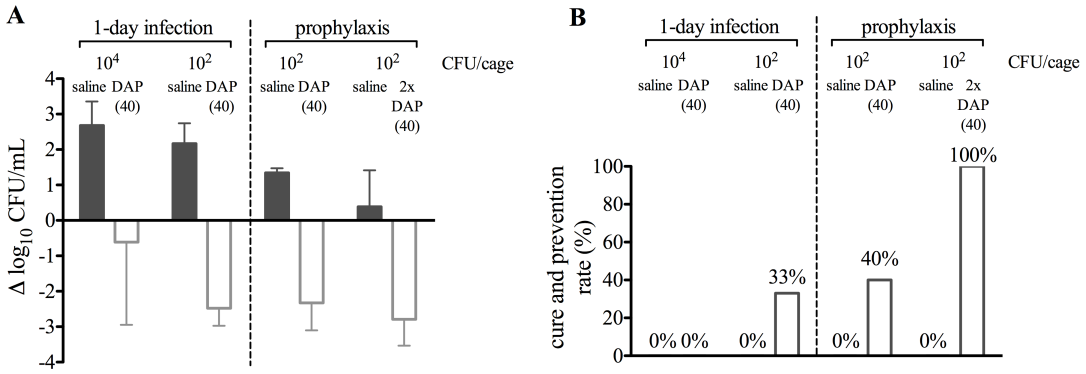

Figure 3.2. Influence of infection time and inoculum on the efficacy of daptomycin at 40 mg/kg (open bars) in a 1-day infection- and a prophylaxis study against planktonic (A) and adherent (B) MRSA. Inocula of the 1-day infection and prophylaxis study were 4 × 10^4 CFU/cage and 3-4 × 10^2 CFU/cage, respectively. Saline (gray bars) served as control. In (A) positive values denote growth and negative values killing. In (B) efficacy against adherent MRSA was expressed as cure or prevention rate.

In a second series of experiments a second 40 mg/kg DAP dose was applied 6 h after infection in order to achieve prolonged bactericidal drug levels in the cage. Under these conditions, CFU numbers were reduced by 3 $\log_{10}$ CFU/mL to a few counts within 24 h and DAP prevented infection in all tissue cages (Figure 3.2B).

Taken together, DAP was partially efficacious in the 4-day therapy only when using a low inoculum. Complete prevention of cage-associated infection was only reached with 2 consecutive doses of DAP before and after inoculation.

Daptomycin susceptibility of planktonic and adherent staphylococci *in vitro*. Since DAP did not eradicate tissue cage-associated infection when treatment was started one day after inoculation, we asked whether the biofilm prevented killing. In addition, the effect of cell wall modifications on DAP susceptibility was assessed in order to link

phenotypic resistance of adherent staphylococci to defined molecules participating in adherence.

Therefore, we compared the susceptibility of planktonic wt and mutant staphylococci to DAP *in vitro* (Table 3.1). All tested SA113 and SE1457 strains had a MIC of 0.625 µg/mL and MBC_{log} of 1.25 µg/mL except for the Δ*dltA* mutant, lacking alanylation of LTA, which was more sensitive.

Table 3.1 *In vitro* susceptibility to daptomycin.

Strain	MIC[a] (µg/mL)	MBC_{log}[b] (µg/mL)
SA113 wt	0.625	1.25
SA113 Δ*ica*	0.625	1.25
SA113 Δ*nuc1/nuc2*	1.25	1.25
SA113 Δ*srtA*	0.625	1.25
SA113 Δ*atl*	0.625	1.25
SA113 Δ*dltA*	0.157	0.313
SE1457 wt	0.625	1.25
SE1457 Δ*luxS*	0.625	1.25

[a], minimal inhibition concentration

[b], minimal bactericidal concentration under logarithmic growth phase condition

DAP was similarly efficient against planktonic staphylococci independently of biofilm (Δ*ica*) or biofilm regulation (Δ*luxS*), of undegraded extracellular DNA (Δ*nuc1/nuc2*), of LPXTG-anchored cell wall molecules (Δ*srtA*) and of autolysins (Δ*atl*). These results

suggest that only the positive charge conferred by alanine to LTA affected DAP efficacy on planktonic staphylococci.

In addition, the effect of DAP on adherent staphylococci was assessed. Both, the DAP effects on biofilm and on adherent staphylococci were quantified 24 h after incubation with 30 and 500 µg/mL DAP. Biofilm was moderate in untreated SA113 wt, lower in the Δ*ica*, Δ*atl*, and Δ*dltA* mutants and 3-fold higher in Δ*nuc1/nuc2* (Figure 3.3A). DAP eliminated biofilm in all *S. aureus* strains except in Δ*nuc1/nuc2*, where it remained unchanged with 30 µg/mL DAP (Figure 3.3A) and was only decreased after 500 µg/mL DAP (data not shown).

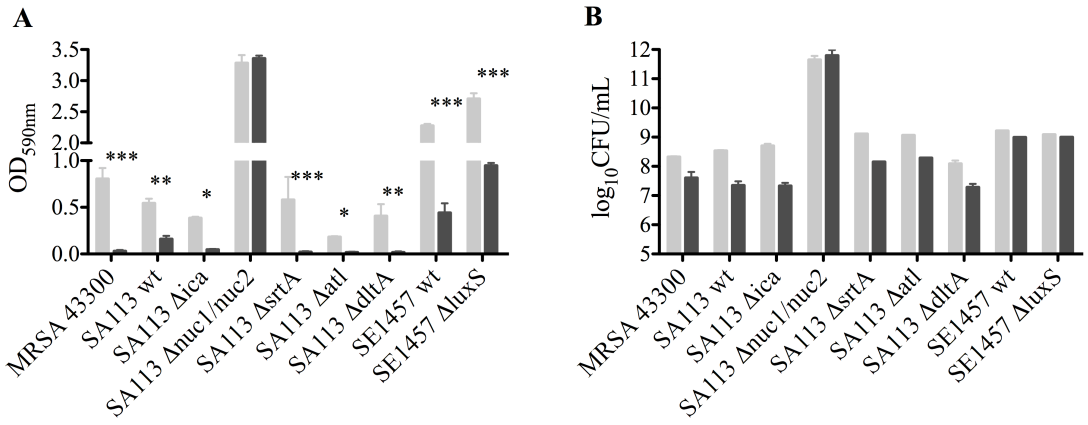

Figure 3.3. Efficacy without (gray bars) and with 30 µg/mL daptomycin (black bars) on adherent staphylococci in vitro. After daptomycin exposure biofilm formation (A) using crystal violet staining and adherent bacterial numbers (B) using plating after detachment were determined. Values are means ± SD of 3 independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.01$.

Untreated SE1457 wt and its Δ*luxS* mutant produced very strong biofilm, which was reduced two-to threefold by DAP in both strains (Figure 3.3A).

Adherent staphylococci numbered 10^7 to 10^8 CFU per well except for Δ*nuc1/nuc2*, which showed higher numbers of 10^{11} CFU/well. DAP did not significantly lower CFU counts independently of biofilm, nucleases, adhesins, autolysins, and alanyl-LTAs (Figure 3.3B). Pre-coating of the plates with plasma proteins did also not allow a significant eradication of adherent staphylococci (data not shown).

These surprising results suggest, that despite an effect on the biomass, DAP was not bactericidal on adherent cells. To investigate whether DAP resistance of adherent staphylococci was reversible, the efficacy of 30 µg/mL DAP against detached MRSA was determined. DAP was found bactericidal against detached MRSA 24 h after incubation (Figure 3.4A).

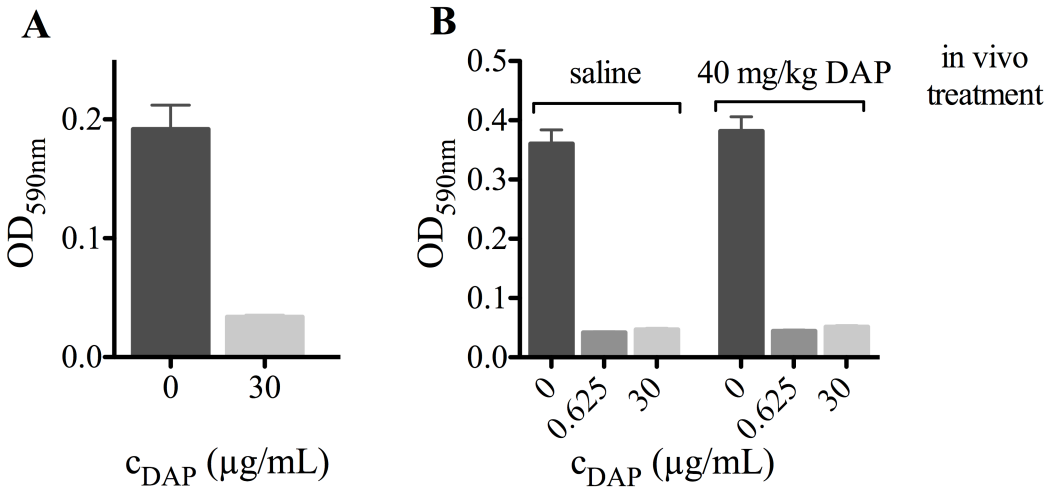

Figure 3.4. Efficacy of daptomycin (DAP) against detached MRSA in vitro (A) and against dispersed MRSA derived from untreated and treated tissue cages of the 1-day infection study, (B). Values are means ± SD of 3 experiments.

To confirm these *in vitro* data, dispersed MRSA from untreated (saline) or treated (40mg/kg DAP) tissue cages of the 1-day infection study were treated with 0.625 µg/mL (corresponding to MIC) and 30 µg/mL DAP *ex vivo* (Figure 3.4B). DAP was bactericidal at both concentrations DAP and the effect was independent of a previous *in vivo* DAP treatment. These data indicate that adherent bacteria were not genotypically resistant to DAP, but resistance was linked to the physiological status of the bacteria and therefore fully reversible after detachment.

To determine whether adherent bacteria inactivated DAP, the bactericidal activity of a supernatant of adherent, treated MRSA was tested with fresh planktonic MRSA. DAP in the supernatant was able to inhibit growth of planktonic MRSA indicating that adherent bacteria did not inactivate DAP (Figure 3.5).

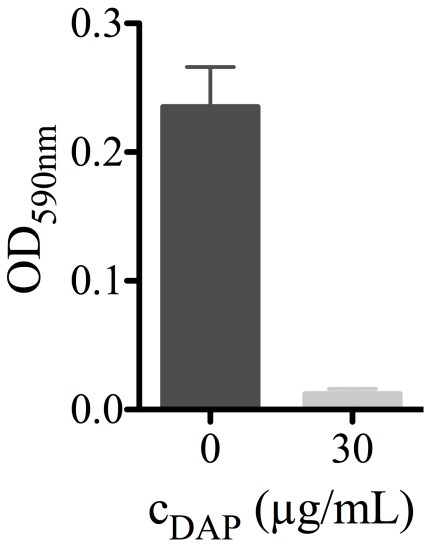

Figure 3.5. Efficacy of daptomycin (DAP) in sterile-filtrated supernatant, derived from adherent DAP-treated MRSA against fresh planktonic 10^5 CFU/mL MRSA. Values are means ± SD of 3 experiments.

Calcium-competition between daptomycin and staphylococci. Adherent staphylococci did not inactivate DAP, but might restrict accessible calcium ions (Ca^{2+}) required for killing by DAP. Therefore, we asked whether there was a calcium competition between DAP and staphylococci. To answer this question, 30 µg/mL DAP was pre-incubated with 50 mg/L Ca^{2+} for 2 h to lock DAP in its active conformation. This time period was considered sufficient for the formation of the active DAP-conformation because time kill studies showed bactericidal activity against stationary grown staphylococci above 20 µg/mL of DAP within 1-2 h (13). The efficacy of Ca^{2+}-pre-incubated DAP against adherent MRSA was similar to non-pre-incubated DAP since numbers of adherent MRSA were similar (9.8×10^6 and 1.7×10^7 CFU/mL, respectively).

Increasing Ca^{2+} may enhance bactericidal activity on adherent bacteria. Therefore, the influence of a Ca^{2+}-concentration above 50 mg/L on DAP efficiency against adherent MRSA was determined. Increasing Ca^{2+}-concentrations added to 30 µg/mL DAP progressively reduced adherent bacterial numbers; 100 mg/L Ca^{2+} lead to a significant reduction of adherent MRSA by 2.5 $\log_{10}$ CFU/mL (Figure 3.6A). Ca^{2+} alone had no effect on bacterial survival (data not shown). To confirm these *in vitro* data, we investigated the effect of DAP treatment in a tissue cage MRSA infection with increased Ca^{2+}-concentrations. One day after infection DAP with additional 50 mg/L Ca^{2+} reduced the planktonic MRSA by 2.7 $\log_{10}$ CFU/mL (Figure 3.6B). Furthermore, DAP was able to prevent 60% of the cage-associated infections (Figure 3.6C). These data indicate that with increasing Ca^{2+} the phenotypic resistance of adherent bacteria can be partially overcome.

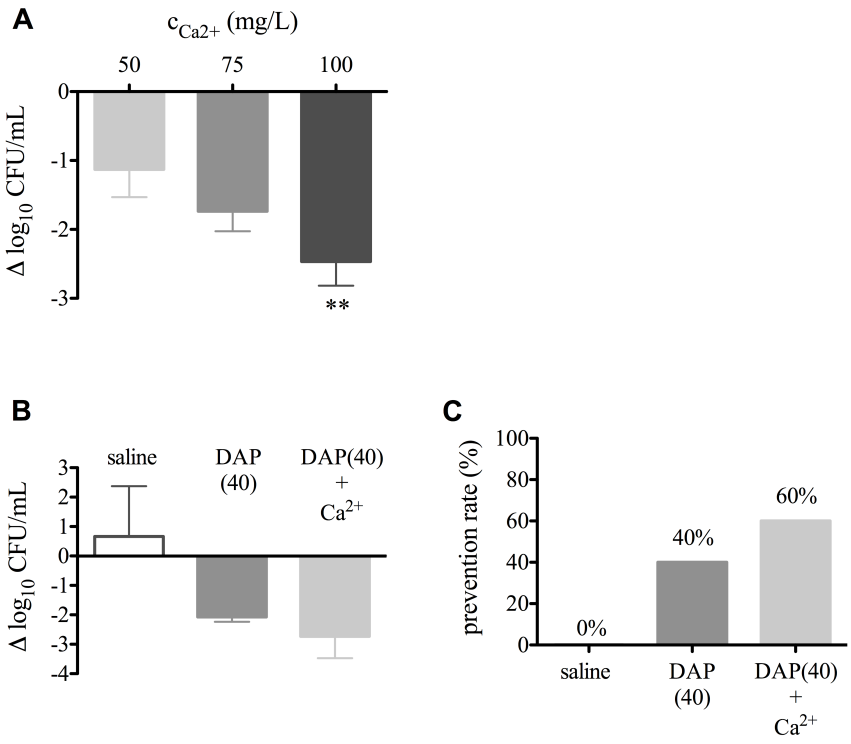

Figure 3.6. Influence of increasing calcium concentrations on efficacy of 30 µg/mL daptomycin (A). The difference in adherent MRSA numbers before and after daptomycin exposure is shown. Negative values denote killing. Values are means ± SD of 6 experiments. **, p<0.01. Influence of additional calcium ions (50 mg/L) on daptomycin (DAP) efficiency (bright gray bars) against planktonic (B) and adherent MRSA (C) *in vivo*. Inocula were 6 × 10^2 CFU/cage. Saline (open bars) and DAP without additional calcium (dark gray bars) served as controls. In (B) positive values denote growth and negative values killing. Values are means ± SD. In (C) efficacy against adherent MRSA was expressed as prevention rate.

Discussion

Daptomycin (DAP) has potent activity against a wide range of Gram-positive bacteria including beta-lactam- and vancomycin-resistant strains (37). It is approved for skin infections, bacteraemia and endocarditis (8). However, in experimental implant-associated infection DAP mono-therapy failed for unknown reasons (13).

Earlier results showed that stationary phase bacteria had a 16-fold higher MBC for DAP than bacteria in logarithmic growth (23). The fraction of stationary phase and adherent bacteria increases during the time after inoculation, resulting in a time dependent failure rate of DAP-treatment (13). In the present study, only the prophylactic application of DAP was highly efficacious against implant-associated infection. This suggests that treatment failure might be due to the high quantity and/or the physiological state of the bacteria in the tissue cage. In this model, staphylococci change from the planktonic to the adherent phenotype and are increasingly embedded in a biofilm. Therefore, we investigated the effect of adherence and of biofilm on DAP responsiveness.

We observed the expected strong biofilm in *S. epidermidis* wt and even more so in the Δ*luxS* mutant, which lacks the quorum system with autoinducer 2-mediated *ica* repression (43). We confirmed low biofilm formation by all *S. aureus* strains except for the Δ*nuc1/nuc2* mutant, which lacks extracellular DNase and therefore accumulates more DNA-containing biofilm (3). Independent of the amount of biofilm in untreated cells, DAP strongly reduced the biofilm in concentrations above the MBC_{log} except for the Δ*nuc1/nuc2* mutant, which was more resistant against DAP. It was shown earlier that DAP is able to enter a biofilm (38); it may lead to biofilm dispersion via modulation of the cell membrane. By reducing the membrane potential (15) it may cause an altered

redox status and a stress response with reactive oxygen and nitrogen species; in this context it is interesting to know that H_2O_2 as well as free iron, which arises during oxidative stress, and nitrite inhibit biofilm formation (10, 14, 35). Alternatively, DAP may favor biofilm detachment by activating proteases or detergent-like molecules - like phenol-soluble modulins (PSMs) - that disrupt hydrophobic interactions between cationic exopolysaccharides and anionic bacterial surface structures (29, 30). However, PSMs are under strict *agr* control and since SA113, which is a natural *agr*-deletion mutant, showed the same biofilm decrease in response to DAP, PSM regulation by DAP is unlikely.

Our data show that the adherent growth mode and not the extracellular polysaccharide matrix formation was responsible for the DAP resistance to killing. A similar phenomenon was shown before for other antibiotics (32, 40). These authors described a high resistance against oxacillin, vancomycin, teicoplanin, ciprofloxacin and rifampicin of adherent biofilm-positive and biofilm-negative *S. epidermidis* and of *S. aureus* strains, which were sensitive to all antibiotics in their planktonic state. Based on these results, the resistance to DAP in our study was likely independent of the antibiotic structure and mechanism of action. Accordingly, we found no evidence for inactivation of DAP by staphylococci. Furthermore, the reversibility of the resistant phenotype upon detachment excluded the genetic perturbations, which had been previously associated with DAP resistance (2). However, it appeared that adherent staphylococci adapted to DAP and became tolerant resulting in treatment failure. This may be due to their metabolic status with a higher net positive charge, which accompanies all adherence (41). Support for this hypothesis is provided by two observations, namely an enhanced DAP susceptibility of the Δ*dltA* mutant, which has a lower positive charge and thus adheres less to polystyrene

(31), and from a DAP-resistant clinical isolate, which showed enhanced Δ*dltA* expression (44).

Furthermore, adherence is accompanied by fermentative processes resulting from low oxygenation, from an active nitrosative pathway, and, importantly, from activation of cation ATPases, which help neutralizing the progressive acidification. Among the latter, the high affinity potassium (K^+) transport system encoded by the *kdp* operon is induced by high extracellular K^+ and low osmolarity in the cell to restore pH homeostasis and intracellular K^+ (1). DAP most likely inactivates the Kdp and other K^+ transport systems in planktonic cells and thereby leads to cell death (2). In *S. aureus* biofilms, three genes of the *kdp* operon are strongly induced (4), it is conceivable that adherence is sufficient to increase expression of K^+ transport systems, and thus offers resistance to DAP action.

Adherence in a polystyrene plate does not mirror biofilm formation in a medical device, because in the first case hydrophobicity and *atl* (5) play a role, while *in vivo* the interaction between MSCRAMMS and host extracellular matrix molecules initiates biofilm (29). We used both adherence settings with and without plasma coating and the *in vitro* assays fairly predicted the *in vivo* effects. Interestingly, we could overcome DAP resistance of adherent bacteria *in vitro* and *in vivo* by increasing Ca^{2+} concentrations. This effect was not due to early DAP-Ca^{2+} complex formation but it may have altered the ionic forces involved in adhesion. A likely explanation is that *S. aureus* adhesion was inhibited by additional Ca^{2+}, which blocks the Ca^{2+}-binding sites of clumping factor A (ClfA) involved in extracellular matrix adhesion (27).

In conclusion, our data revealed that DAP is inefficient in experimental implant-associated infections, and showed that this effect is independent of biofilm but influenced by modulations of extracellular Ca^{2+}.

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References

1. **Ballal, A., B. Basu, and S. K. Apte.** 2007. The Kdp-ATPase system and its regulation. J Biosci **32:**559-68.
2. **Baltz, R. H.** 2009. Daptomycin: mechanisms of action and resistance, and biosynthetic engineering. Curr Opin Chem Biol **13:**144-51.
3. **Bayles, K. W.** 2007. The biological role of death and lysis in biofilm development. Nat Rev Microbiol **5:**721-6.
4. **Beenken, K. E., P. M. Dunman, F. McAleese, D. Macapagal, E. Murphy, S. J. Projan, J. S. Blevins, and M. S. Smeltzer.** 2004. Global gene expression in *Staphylococcus aureus* biofilms. J Bacteriol **186:**4665-84.
5. **Biswas, R., L. Voggu, U. K. Simon, P. Hentschel, G. Thumm, and F. Gotz.** 2006. Activity of the major staphylococcal autolysin Atl. FEMS Microbiol Lett **259:**260-8.
6. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318-22.
7. **Cui, L., T. Isii, M. Fukuda, T. Ochiai, H. M. Neoh, I. L. Camargo, Y. Watanabe, M. Shoji, T. Hishinuma, and K. Hiramatsu.** An RpoB mutation confers dual heteroresistance to daptomycin and vancomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother **54:**5222-33.
8. **Eisenstein, B. I.** 2004. Lipopeptides, focusing on daptomycin, for the treatment of Gram-positive infections. Expert Opin Investig Drugs **13:**1159-69.
9. **Friedman, L., J. D. Alder, and J. A. Silverman.** 2006. Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother **50:**2137-45.
10. **Glynn, A. A., S. T. O'Donnell, D. C. Molony, E. Sheehan, D. J. McCormack, and J. P. O'Gara.** 2009. Hydrogen peroxide induced repression of icaADBC transcription and biofilm development in *Staphylococcus epidermidis*. J Orthop Res **27:**627-30.
11. **Gordon, O., T. Vig Slenters, P. S. Brunetto, A. E. Villaruz, D. E. Sturdevant, M. Otto, R. Landmann, and K. M. Fromm.** Silver coordination polymers for prevention of implant infection: thiol interaction, impact on respiratory chain enzymes, and hydroxyl radical induction. Antimicrob Agents Chemother **54:**4208-18.
12. **Gotz, F.** 2002. Staphylococcus and biofilms. Mol Microbiol **43:**1367-78.
13. **John, A. K., D. Baldoni, M. Haschke, K. Rentsch, P. Schaerli, W. Zimmerli, and A. Trampuz.** 2009. Efficacy of daptomycin in implant-associated infection due to methicillin-resistant *Staphylococcus aureus*: importance of combination with rifampin. Antimicrob Agents Chemother **53:**2719-24.
14. **Johnson, M., A. Cockayne, P. H. Williams, and J. A. Morrissey.** 2005. Iron-responsive regulation of biofilm formation in *Staphylococcus aureus* involves fur-dependent and fur-independent mechanisms. J Bacteriol **187:**8211-5.
15. **Jones, T., M. R. Yeaman, G. Sakoulas, S. J. Yang, R. A. Proctor, H. G. Sahl, J. Schrenzel, Y. Q. Xiong, and A. S. Bayer.** 2008. Failures in clinical treatment of *Staphylococcus aureus* Infection with daptomycin are associated with

alterations in surface charge, membrane phospholipid asymmetry, and drug binding. Antimicrob Agents Chemother **52:**269-78.

16. **Jung, D., A. Rozek, M. Okon, and R. E. Hancock.** 2004. Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin. Chem Biol **11:**949-57.
17. **Kong, K. F., C. Vuong, and M. Otto.** 2006. Staphylococcus quorum sensing in biofilm formation and infection. Int J Med Microbiol **296:**133-9.
18. **Kristian, S. A., T. A. Birkenstock, U. Sauder, D. Mack, F. Gotz, and R. Landmann.** 2008. Biofilm formation induces C3a release and protects *Staphylococcus epidermidis* from IgG and complement deposition and from neutrophil-dependent killing. J Infect Dis **197:**1028-35.
19. **Kristian, S. A., X. Lauth, V. Nizet, F. Goetz, B. Neumeister, A. Peschel, and R. Landmann.** 2003. Alanylation of teichoic acids protects *Staphylococcus aureus* against Toll-like receptor 2-dependent host defense in a mouse tissue cage infection model. J Infect Dis **188:**414-23.
20. **Kuehl, R., S. Al-Bataineh, O. Gordon, R. Luginbuehl, M. Otto, M. Textor, and R. Landmann.** 2009. Furanone at subinhibitory concentrations enhances staphylococcal biofilm formation by luxS repression. Antimicrob Agents Chemother **53:**4159-66.
21. **Learmonth, I. D., C. Young, and C. Rorabeck.** 2007. The operation of the century: total hip replacement. Lancet **370:**1508-19.
22. **Lee, J. H., J. B. Kaplan, and W. Y. Lee.** 2008. Microfluidic devices for studying growth and detachment of *Staphylococcus epidermidis* biofilms. Biomed Microdevices **10:**489-98.
23. **Mascio, C. T., J. D. Alder, and J. A. Silverman.** 2007. Bactericidal action of daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells. Antimicrob Agents Chemother **51:**4255-60.
24. **Murray, P. R., Baron, E.J., Jorgensen, J.H., Pfaller, M.a., Yolken, R.H. (ed.).** 2003. Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, DC.
25. **National Committee for Clinical Laboratory Standards.** 1999. Methods for determining bactericidal activity of antimicrobial agents. NCCLS documents M26-A. National Committee for Clinical Laboratory Standards, Villanova, PA.
26. **Novick, R. P., and E. Geisinger.** 2008. Quorum sensing in staphylococci. Annu Rev Genet **42:**541-64.
27. **O'Connell, D. P., T. Nanavaty, D. McDevitt, S. Gurusiddappa, M. Hook, and T. J. Foster.** 1998. The fibrinogen-binding MSCRAMM (clumping factor) of *Staphylococcus aureus* has a Ca2+-dependent inhibitory site. J Biol Chem **273:**6821-9.
28. **Olson, M. E., S. R. Slater, M. E. Rupp, and P. D. Fey.** Rifampicin enhances activity of daptomycin and vancomycin against both a polysaccharide intercellular adhesin (PIA)-dependent and -independent *Staphylococcus epidermidis* biofilm. J Antimicrob Chemother **65:**2164-71.
29. **Otto, M.** 2008. Staphylococcal biofilms. Curr Top Microbiol Immunol **322:**207-28.

30. **Otto, M.** 2009. *Staphylococcus epidermidis*--the 'accidental' pathogen. Nat Rev Microbiol **7:**555-67.
31. **Peschel, A., M. Otto, R. W. Jack, H. Kalbacher, G. Jung, and F. Gotz.** 1999. Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem **274:**8405-10.
32. **Qu, Y., A. J. Daley, T. S. Istivan, D. A. Rouch, and M. A. Deighton.** 2010. Densely adherent growth mode, rather than extracellular polymer substance matrix build-up ability, contributes to high resistance of *Staphylococcus epidermidis* biofilms to antibiotics. J Antimicrob Chemother **65:**1405-11.
33. **Rice, K. C., E. E. Mann, J. L. Endres, E. C. Weiss, J. E. Cassat, M. S. Smeltzer, and K. W. Bayles.** 2007. The cidA murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. Proc Natl Acad Sci U S A **104:**8113-8.
34. **Schaad, H. J., M. Bento, D. P. Lew, and P. Vaudaux.** 2006. Evaluation of high-dose daptomycin for therapy of experimental *Staphylococcus aureus* foreign body infection. BMC Infect Dis **6:**74.
35. **Schlag, S., C. Nerz, T. A. Birkenstock, F. Altenberend, and F. Gotz.** 2007. Inhibition of staphylococcal biofilm formation by nitrite. J Bacteriol **189:**7911-9.
36. **Silverman, J. A., N. G. Perlmutter, and H. M. Shapiro.** 2003. Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. Antimicrob Agents Chemother **47:**2538-44.
37. **Steenbergen, J. N., J. Alder, G. M. Thorne, and F. P. Tally.** 2005. Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. J Antimicrob Chemother **55:**283-8.
38. **Stewart, P. S., W. M. Davison, and J. N. Steenbergen.** 2009. Daptomycin rapidly penetrates a *Staphylococcus epidermidis* biofilm. Antimicrob Agents Chemother **53:**3505-7.
39. **Straus, S. K., and R. E. Hancock.** 2006. Mode of action of the new antibiotic for Gram-positive pathogens daptomycin: comparison with cationic antimicrobial peptides and lipopeptides. Biochim Biophys Acta **1758:**1215-23.
40. **Trafny, E. A.** 1998. Susceptibility of adherent organisms from *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains isolated from burn wounds to antimicrobial agents. Int J Antimicrob Agents **10:**223-8.
41. **Van Oss, C. J.** 1995. Hydrophobic, hydrophilic and other interactions in epitope-paratope binding. Mol Immunol **32:**199-211.
42. **Vuong, C., J. M. Voyich, E. R. Fischer, K. R. Braughton, A. R. Whitney, F. R. DeLeo, and M. Otto.** 2004. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell Microbiol **6:**269-75.
43. **Xu, L., H. Li, C. Vuong, V. Vadyvaloo, J. Wang, Y. Yao, M. Otto, and Q. Gao.** 2006. Role of the luxS quorum-sensing system in biofilm formation and virulence of *Staphylococcus epidermidis*. Infect Immun **74:**488-96.
44. **Yang, S. J., B. N. Kreiswirth, G. Sakoulas, M. R. Yeaman, Y. Q. Xiong, A. Sawa, and A. S. Bayer.** 2009. Enhanced expression of dltABCD is associated with the development of daptomycin nonsusceptibility in a clinical endocarditis isolate of *Staphylococcus aureus*. J Infect Dis **200:**1916-20.

45. **Zimmerli, W., A. Trampuz, and P. E. Ochsner.** 2004. Prosthetic-joint infections. N Engl J Med **351:**1645-54.

3.2.1 Additional results

Minimal infective dose (MID):

Description of the material and methods see above in the manuscript.

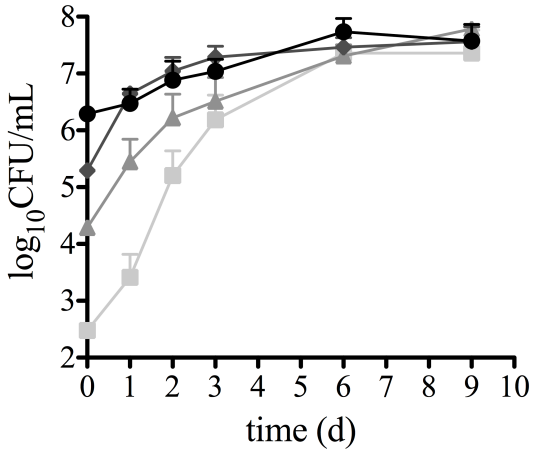

Figure 3.7: Determination of the minimal infective dose of MRSA 43300 in C57BL/6 mice. Mice were infected with 10^2 CFU/cage (squares), 10^3 CFU/cage (triangles), 10^4 CFU/cage (diamonds), and 10^5 CFU/cage (circles), respectively. Values are means ± SD of 3 mice.

For MRSA 43300 the MID was 3×10^2 CFU/cage (Figure 3.7). Within 9 days infections with 10^2 to 10^5 CFU/cage reached a plateau of 10^7-10^8 CFU/mL. These data confirm the results from Kristian et al. and Zimmerli et al. that a low staphylococcal inoculum is required to establish a persistent infection (55, 118).

Daptomycin at sub-inhibitory concentrations:

MRSA at 10^5 CFU/mL were seeded into flat-bottom 96-well plates and treated with DAP concentrations below the MIC of 0.625 µg/mL (13). After 24 h incubation at 37°C, biofilm was stained using crystal as described above (Material and Methods).

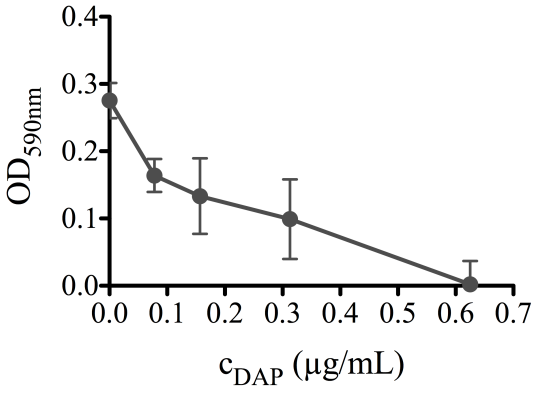

Figure 3.8: Influence of sub-inhibitory daptomycin concentrations on biofilm formation. The MIC for MRSA 43300 was 0.625 µg/mL. Biofilm formation was determined using crystal violet staining. Values are means ± SD of 3 experiments.

DAP concentrations below the MIC did not increase biofilm formation in MRSA (Figure 3.8). At the MIC, DAP eliminated the biofilm. These data indicate that DAP did not demonstrate a stress response at sub-inhibitory concentrations resulting in a staphylococcal biofilm formation, like furanone (57) and vancomycin (14).

4 Perspectives

Over the last years more and more bacterial genera developed resistance against commonly used antibiotics. For *S. aureus* antibiotic resistance started very early after introduction of penicillin, it was followed by the development of MRSA strains shortly after introduction of beta-lactamase resistant methicillin. In the meantime, infections caused by MRSA strains are increasing all over the world in both healthcare and community settings. Nasal carriage of *S. aureus* is a main risk factor for invasive diseases and carriers are assumed to be a key source of *S. aureus* strains that spread among individuals (15). Further, in *S. aureus* carriers who have medical devices the prevalence for an infection is increased (117).

Besides the problem of antibiotic resistance, implant-associated infections are difficult to treat due to missing microcirculation (97). In implants bacteria are able to adhere to the surface and build up a protection layer known as biofilm (73). Biofilm-embedded microorganisms are slow-growing and/or non-dividing (29) and less susceptible against antibiotics than their planktonic counterparts (21). However, most used antibiotics are able to inhibit or kill growing bacteria. For the treatment of adherent bacteria in implants antimicrobials targeting the bacterial membrane appear therefore suitable (40).

One promising candidate is the lipopeptide daptomycin (DAP) that damages the bacterial membrane of Gram-positive bacteria including MRSA by pore formation resulting in membrane depolarization and cell death without lysis (89). However, despite our promising *in vitro* data, DAP alone failed to eradicate an implant-associated MRSA infection in a guinea pig tissue cage model (43). In line with our results in another study

using the guinea pig tissue cage DAP was unable to eliminate an *S. epidermidis* infection (72). Treatment failure was independent of biofilm (72, 106). A possible explanation might be that adherent bacteria with or without biofilm may differ in the composition of membrane lipids (114), which might confer less susceptibility to DAP. Further studies should address the composition of the membrane between stationary grown planktonic and adherent bacteria. Membrane lipids and their modification as well as the expression of proteins in different physical states of staphylococci during DAP treatment should be investigated. A helpful tool would be gene expression analysis, which will further elucidate the differences caused by growth and interactions of bacteria in a DAP exposed bacterial population with or without biofilm.

Further, inadequate concentrations of either active DAP or Ca^{2+} in the tissue cage might have caused the treatment failure. Indeed, DAP alone was effective in higher doses in a tissue cage infection model in rats (30), and we could prevent MRSA infection in the mouse tissue cage model with increasing DAP concentrations.

The other limiting factor in DAP activation might be the availability of Ca^{2+}. Adequate Ca^{2+} levels are important to induce DAP activity through a conformational change (89). Perhaps, enhanced Ca^{2+} delivery has to occur at the site of implant infection. Indeed, increasing Ca^{2+} concentrations *in vivo* slightly enhanced the killing of adherent bacteria suggesting that Ca^{2+} is a limiting factor in our model. However, increased Ca^{2+} by application at the site of implant might have toxic side effects on the muscles (53). Ca^{2+} binding to DAP at selected amino acids has been found essential with NMR for bactericidal activity (38). Structural modification of the DAP might be a possibility to

alter Ca^{2+} binding and a stronger binding might increase Ca^{2+} levels at the implant infection site and enhance DAP activity.

The clinical use of DAP alone is limited to complicated soft skin infections, bacteraemia and endocarditis (18). To widen the potential clinical application to implant infections, we tested DAP in combination with other antibiotics, which have different targets. Indeed, we could show that DAP in combination with rifampin (43), which targets the DNA-dependent RNA polymerase (50), was able to cure guinea pigs from staphylococcal implant-associated infections. Levofloxacin inhibiting replication (50) and vancomycin targeting the peptidoglycan synthesis (47) were less efficient to eradicate planktonic and adherent MRSA when combined with rifampin. These latter antibiotics require active bacterial growth, which seemed to limit their efficacy in the treatment compared to DAP. Furthermore, in the combination with rifampin DAP was able to prevent the emergence of rifampin-resistant MRSA strains, while rifampin-resistance developed with a higher frequency in the vancomycin-rifampin combination (43).

The effective combination of DAP together with rifampin delays the development of resistant strains for a certain period. However, when multidrug resistant strains appear, new antimicrobial drugs are required. The research for new antimicrobials has been decreasing during the last years (10). Yet, the identification of new targets, which are unique to bacteria and help the host to overcome an infection, is needed. In that context, inactivation of enzymes in the metabolism may provide an attractive target for antimicrobials due to their role in both the growth of pathogens and in control of the host. Proteome analysis of pathogens *ex vivo* and of the corresponding host tissue will give a

close-up view of important aspects in the fight between host and bacteria and will further close some knowledge gaps in host metabolism (13).

The introduction of implants immobilized with antimicrobial substances is another strategy to overcome implant infections. Substances preventing adherence of bacteria to an implant surface or that are released in the presence of bacteria are under investigation. In our group furanone and silver were investigated for the purpose of this therapeutic application. Despite bactericidal and biofilm-modulating activity of free furanone, furanone-coated implants were colonized, since furanone acts on quorum sensing and needs to penetrate bacteria (57). Silver is bactericidal against *S. epidermidis* by a mechanism that inactivates key enzymes of the respiratory chain leading to a rapid de-energization. Silver, which was slowly released from polymers, was bactericidal in the tissue cage model (32). However, soluble furanone and silver showed also cytotoxic effects against mammalian cells in a dose range, which was not far from the MIC against staphylococci, which limits their potential clinical application (32, 57).

Before bacteria adhere to the surface, DAP is efficient in eradication of *S. aureus*. Whether DAP can be used for coating of implants has not been addressed yet. Another approach would be the linkage of DAP to the surface via ligands or substrates of enzymes produced by bacteria such as autolysins. This may allow a well-tuned local preventive action of the antibiotic.

In the second part of the thesis, we found that adhesion to surfaces renders MRSA resistant to DAP. Surprisingly, DAP treatment reduced the biofilm but bacteria remained viable even when production and release of PIA, nuclease, adhesins, autolysins, and

alanyl-LTAs were absent. Since resistance was reversible with detachment of bacteria and thus genetic modifications of bacteria could be excluded, the question arises why DAP was inactive against adherent bacteria. In future, this reduced susceptibility of adherent staphylococci has to be explored in depth. It will be important to understand whether similar effects are observed with different bacterial genera and different biomaterials.

Furthermore, the improved knowledge of how and where antimicrobial peptides are active will open new approaches for DAP treatment. The knowledge from CAMPs is helpful to identify resistance mechanisms against DAP. The *dlt* mutant (78) or *mprF* (24) mutant are more susceptible to CAMPs and DAP indicating that one mechanism of resistance is the repulsion of positively charged complexes by changing the net charge of the cell envelope. It remains to be studied whether adherence in our model is associated with a higher net positive charge.

Similar to CAMPs, eDNA, if abundant, limited the treatment efficacy of DAP as shown by the stronger resistance of the Δ*nuc1/nuc2* mutant against DAP. For effective DAP treatment of implant-associated infections, the interaction between DAP and eDNA as part of biofilm should be identified. It could be that DAP binds to the negatively charged DNA, or that the required Ca^{2+} is sequestered by DNA, which prevents the attack of the membrane. Whether DAP captured by DNA is again active after cleavage of DNA by nucleases is another interesting experiment. This recycling of DNA by bacteria may allow activation of DAP and favour their killing.

In conclusion, DAP is most efficient applied against implant infections either in a combination therapy or when given as mono-therapy before bacteria are able to adhere to a surface.

5 References

1. **Agarwal, A., K. P. Singh, and A. Jain.** 2010. Medical significance and management of staphylococcal biofilm. FEMS Immunol Med Microbiol **58:**147-60.
2. **Baltz, R. H.** 2009. Daptomycin: mechanisms of action and resistance, and biosynthetic engineering. Curr Opin Chem Biol **13:**144-51.
3. **Bassetti, M., E. Nicco, F. Ginocchio, F. Ansaldi, D. de Florentiis, and C. Viscoli.** 2010. High-dose daptomycin in documented *Staphylococcus aureus* infections. Int J Antimicrob Agents **36:**459-61.
4. **Bauer, T. W., and J. Schils.** 1999. The pathology of total joint arthroplasty.II. Mechanisms of implant failure. Skeletal Radiol **28:**483-97.
5. **Belmatoug, N., A. C. Cremieux, R. Bleton, A. Volk, A. Saleh-Mghir, M. Grossin, L. Garry, and C. Carbon.** 1996. A new model of experimental prosthetic joint infection due to methicillin-resistant *Staphylococcus aureus*: a microbiologic, histopathologic, and magnetic resonance imaging characterization. J Infect Dis **174:**414-7.
6. **Benvenuto, M., D. P. Benziger, S. Yankelev, and G. Vigliani.** 2006. Pharmacokinetics and tolerability of daptomycin at doses up to 12 milligrams per kilogram of body weight once daily in healthy volunteers. Antimicrob Agents Chemother **50:**3245-9.
7. **Berg, R. D.** 1996. The indigenous gastrointestinal microflora. Trends Microbiol **4:**430-5.
8. **Besier, S., A. Ludwig, J. Zander, V. Brade, and T. A. Wichelhaus.** 2008. Linezolid resistance in *Staphylococcus aureus*: gene dosage effect, stability, fitness costs, and cross-resistances. Antimicrob Agents Chemother **52:**1570-2.
9. **Boneca, I. G., and G. Chiosis.** 2003. Vancomycin resistance: occurrence, mechanisms and strategies to combat it. Expert Opin Ther Targets **7:**311-28.
10. **Boucher, H. W., G. H. Talbot, J. S. Bradley, J. E. Edwards, D. Gilbert, L. B. Rice, M. Scheld, B. Spellberg, and J. Bartlett.** 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis **48:**1-12.
11. **Brown, E. M., P. M. Vassilev, and S. C. Hebert.** 1995. Calcium ions as extracellular messengers. Cell **83:**679-82.
12. **Bryskier, A.** 2005. Antimicrobial Agents-Antibacterials and Antifugals. ASM Press.
13. **Bumann, D.** 2010. Pathogen proteomes during infection: A basis for infection research and novel control strategies. J Proteomics **73:**2267-76.
14. **Cargill, J. S., and M. Upton.** 2009. Low concentrations of vancomycin stimulate biofilm formation in some clinical isolates of *Staphylococcus epidermidis*. J Clin Pathol **62:**1112-6.

15. **Chambers, H. F., and F. R. Deleo.** 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol **7:**629-41.
16. **Collins, L. V., S. A. Kristian, C. Weidenmaier, M. Faigle, K. P. Van Kessel, J. A. Van Strijp, F. Gotz, B. Neumeister, and A. Peschel.** 2002. *Staphylococcus aureus* strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. J Infect Dis **186:**214-9.
17. **Cotroneo, N., R. Harris, N. Perlmutter, T. Beveridge, and J. A. Silverman.** 2008. Daptomycin exerts bactericidal activity without lysis of *Staphylococcus aureus*. Antimicrob Agents Chemother **52:**2223-5.
18. **Cubicin, N.** 2007. Product Monograph cSSTI/SAB/RIE, Basel, Switzerland.
19. **Cucarella, C., C. Solano, J. Valle, B. Amorena, I. Lasa, and J. R. Penades.** 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol **183:**2888-96.
20. **Darouiche, R. O.** 2001. Device-associated infections: a macroproblem that starts with microadherence. Clin Infect Dis **33:**1567-72.
21. **Donlan, R. M.** 2000. Role of biofilms in antimicrobial resistance. ASAIO J **46:**S47-52.
22. **Dubrac, S., I. G. Boneca, O. Poupel, and T. Msadek.** 2007. New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. J Bacteriol **189:**8257-69.
23. **Eisenstein, B. I.** 2004. Lipopeptides, focusing on daptomycin, for the treatment of Gram-positive infections. Expert Opin Investig Drugs **13:**1159-69.
24. **Ernst, C. M., P. Staubitz, N. N. Mishra, S. J. Yang, G. Hornig, H. Kalbacher, A. S. Bayer, D. Kraus, and A. Peschel.** 2009. The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. PLoS Pathog **5:**e1000660.
25. **EUCAST,** 2006. EUCAST Technical Note on daptomycin. Clin Microbiol Infect **12:**599-601.
26. **Foster, T. J.** 2005. Immune evasion by staphylococci. Nat Rev Microbiol **3:**948-58.
27. **Foster, T. J., and M. Hook.** 1998. Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol **6:**484-8.
28. **Friedman, L., J. D. Alder, and J. A. Silverman.** 2006. Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother **50:**2137-45.
29. **Fux, C. A., J. W. Costerton, P. S. Stewart, and P. Stoodley.** 2005. Survival strategies of infectious biofilms. Trends Microbiol **13:**34-40.
30. **Garrigos, C., O. Murillo, G. Euba, R. Verdaguer, F. Tubau, C. Cabellos, J. Cabo, and J. Ariza.** 2010. Efficacy of usual and high doses of daptomycin in combination with rifampin versus alternative therapies in experimental foreign-body infection by methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **54:**5251-6.
31. **George, E. A., and T. W. Muir.** 2007. Molecular mechanisms of agr quorum sensing in virulent staphylococci. Chembiochem **8:**847-55.

32. **Gordon, O., T. Vig Slenters, P. S. Brunetto, A. E. Villaruz, D. E. Sturdevant, M. Otto, R. Landmann, and K. M. Fromm.** 2010. Silver coordination polymers for prevention of implant infection: thiol interaction, impact on respiratory chain enzymes, and hydroxyl radical induction. Antimicrob Agents Chemother **54:**4208-18.
33. **Gordon, R. J., and F. D. Lowy.** 2008. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. Clin Infect Dis **46 Suppl 5:**S350-9.
34. **Gotz, F.** 2002. Staphylococcus and biofilms. Mol Microbiol **43:**1367-78.
35. **Hall-Stoodley, L., J. W. Costerton, and P. Stoodley.** 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol **2:**95-108.
36. **Hawkey, P. M.** 2008. Pre-clinical experience with daptomycin. J Antimicrob Chemother **62 Suppl 3:**iii7-14.
37. **Heilmann, C., M. Hussain, G. Peters, and F. Gotz.** 1997. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. Mol Microbiol **24:**1013-24.
38. **Ho, S. W., D. Jung, J. R. Calhoun, J. D. Lear, M. Okon, W. R. Scott, R. E. Hancock, and S. K. Straus.** 2008. Effect of divalent cations on the structure of the antibiotic daptomycin. Eur Biophys J **37:**421-33.
39. **Hudetz, D., S. Ursic Hudetz, L. G. Harris, R. Luginbuhl, N. F. Friederich, and R. Landmann.** 2008. Weak effect of metal type and ica genes on staphylococcal infection of titanium and stainless steel implants. Clin Microbiol Infect **14:**1135-45.
40. **Hurdle, J. G., A. J. O'Neill, I. Chopra, and R. E. Lee.** 2011. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol **9:**62-75.
41. **Hussain, M., C. Heilmann, G. Peters, and M. Herrmann.** 2001. Teichoic acid enhances adhesion of *Staphylococcus epidermidis* to immobilized fibronectin. Microb Pathog **31:**261-70.
42. **Janatova, J., A. K. Cheung, and C. J. Parker.** 1991. Biomedical polymers differ in their capacity to activate complement. Complement Inflamm **8:**61-9.
43. **John, A. K., D. Baldoni, M. Haschke, K. Rentsch, P. Schaerli, W. Zimmerli, and A. Trampuz.** 2009. Efficacy of daptomycin in implant-associated infection due to methicillin-resistant *Staphylococcus aureus*: importance of combination with rifampin. Antimicrob Agents Chemother **53:**2719-24.
44. **Jones, T., M. R. Yeaman, G. Sakoulas, S. J. Yang, R. A. Proctor, H. G. Sahl, J. Schrenzel, Y. Q. Xiong, and A. S. Bayer.** 2008. Failures in clinical treatment of *Staphylococcus aureus* infection with daptomycin are associated with alterations in surface charge, membrane phospholipid asymmetry, and drug binding. Antimicrob Agents Chemother **52:**269-78.
45. **Jung, D., A. Rozek, M. Okon, and R. E. Hancock.** 2004. Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin. Chem Biol **11:**949-57.
46. **Kaatz, G. W., T. S. Lundstrom, and S. M. Seo.** 2006. Mechanisms of daptomycin resistance in *Staphylococcus aureus*. Int J Antimicrob Agents **28:**280-7.

47. **Kahne, D., C. Leimkuhler, W. Lu, and C. Walsh.** 2005. Glycopeptide and lipoglycopeptide antibiotics. Chem Rev **105:**425-48.
48. **Karauzum, H., T. Ferry, S. de Bentzmann, G. Lina, M. Bes, F. Vandenesch, M. Schmaler, B. Berger-Bachi, J. Etienne, and R. Landmann.** 2008. Comparison of adhesion and virulence of two predominant hospital-acquired methicillin-resistant *Staphylococcus aureus* clones and clonal methicillin-susceptible *S. aureus* isolates. Infect Immun **76:**5133-8.
49. **Kluytmans, J., A. van Belkum, and H. Verbrugh.** 1997. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev **10:**505-20.
50. **Kohanski, M. A., D. J. Dwyer, and J. J. Collins.** 2010. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol **8:**423-35.
51. **Kong, K. F., C. Vuong, and M. Otto.** 2006. Staphylococcus quorum sensing in biofilm formation and infection. Int J Med Microbiol **296:**133-9.
52. **Kosmidis, C., and D. P. Levine.** 2010. Daptomycin: pharmacology and clinical use. Expert Opin Pharmacother **11:**615-25.
53. **Kostrominova, T. Y., C. A. Hassett, E. P. Rader, C. Davis, L. M. Larkin, S. Coleman, F. B. Oleson, and J. A. Faulkner.** 2010. Characterization of skeletal muscle effects associated with daptomycin in rats. Muscle Nerve **42:**385-93.
54. **Kristian, S. A., T. A. Birkenstock, U. Sauder, D. Mack, F. Gotz, and R. Landmann.** 2008. Biofilm formation induces C3a release and protects *Staphylococcus epidermidis* from IgG and complement deposition and from neutrophil-dependent killing. J Infect Dis **197:**1028-35.
55. **Kristian, S. A., M. Durr, J. A. Van Strijp, B. Neumeister, and A. Peschel.** 2003. MprF-mediated lysinylation of phospholipids in *Staphylococcus aureus* leads to protection against oxygen-independent neutrophil killing. Infect Immun **71:**546-9.
56. **Kristian, S. A., T. Golda, F. Ferracin, S. E. Cramton, B. Neumeister, A. Peschel, F. Gotz, and R. Landmann.** 2004. The ability of biofilm formation does not influence virulence of *Staphylococcus aureus* and host response in a mouse tissue cage infection model. Microb Pathog **36:**237-45.
57. **Kuehl, R., S. Al-Bataineh, O. Gordon, R. Luginbuehl, M. Otto, M. Textor, and R. Landmann.** 2009. Furanone at subinhibitory concentrations enhances staphylococcal biofilm formation by luxS repression. Antimicrob Agents Chemother **53:**4159-66.
58. **Lauderdale, K. J., B. R. Boles, A. L. Cheung, and A. R. Horswill.** 2009. Interconnections between Sigma B, agr, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. Infect Immun **77:**1623-35.
59. **Lewis, K.** 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol **5:**48-56.
60. **Livermore, D. M.** 2000. Antibiotic resistance in staphylococci. Int J Antimicrob Agents **16 Suppl 1:**S3-10.
61. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N Engl J Med **339:**520-32.
62. **Mandell, G. L., Bennett, J.E., Dolin, R.** 2000. Principles and Practice of Infectious Diseases, 6th ed, vol. 2. Elsevier.

63. **Mann, E. E., K. C. Rice, B. R. Boles, J. L. Endres, D. Ranjit, L. Chandramohan, L. H. Tsang, M. S. Smeltzer, A. R. Horswill, and K. W. Bayles.** 2009. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS One **4:**e5822.
64. **Mascio, C. T., J. D. Alder, and J. A. Silverman.** 2007. Bactericidal action of daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells. Antimicrob Agents Chemother **51:**4255-60.
65. **McCrea, K. W., O. Hartford, S. Davis, D. N. Eidhin, G. Lina, P. Speziale, T. J. Foster, and M. Hook.** 2000. The serine-aspartate repeat (Sdr) protein family in *Staphylococcus epidermidis*. Microbiology **146 (Pt 7):**1535-46.
66. **Muthaiyan, A., J. A. Silverman, R. K. Jayaswal, and B. J. Wilkinson.** 2008. Transcriptional profiling reveals that daptomycin induces the *Staphylococcus aureus* cell wall stress stimulon and genes responsive to membrane depolarization. Antimicrob Agents Chemother **52:**980-90.
67. **Nizet, V.** 2007. Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets. J Allergy Clin Immunol **120:**13-22.
68. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol **48:**1429-49.
69. **O'Connell, D. P., T. Nanavaty, D. McDevitt, S. Gurusiddappa, M. Hook, and T. J. Foster.** 1998. The fibrinogen-binding MSCRAMM (clumping factor) of *Staphylococcus aureus* has a Ca2+-dependent inhibitory site. J Biol Chem **273:**6821-9.
70. **O'Gara, J. P.** 2007. ica and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. FEMS Microbiol Lett **270:**179-88.
71. **Oleson, F. B., Jr., C. L. Berman, J. B. Kirkpatrick, K. S. Regan, J. J. Lai, and F. P. Tally.** 2000. Once-daily dosing in dogs optimizes daptomycin safety. Antimicrob Agents Chemother **44:**2948-53.
72. **Olson, M. E., S. R. Slater, M. E. Rupp, and P. D. Fey.** 2010. Rifampicin enhances activity of daptomycin and vancomycin against both a polysaccharide intercellular adhesin (PIA)-dependent and -independent *Staphylococcus epidermidis* biofilm. J Antimicrob Chemother **65:**2164-71.
73. **Otto, M.** 2008. Staphylococcal biofilms. Curr Top Microbiol Immunol **322:**207-28.
74. **Otto, M.** 2009. *Staphylococcus epidermidis*--the 'accidental' pathogen. Nat Rev Microbiol **7:**555-67.
75. **Park, K. H., K. Kurokawa, L. Zheng, D. J. Jung, K. Tateishi, J. O. Jin, N. C. Ha, H. J. Kang, M. Matsushita, J. Y. Kwak, K. Takahashi, and B. L. Lee.** 2010. Human serum mannose-binding lectin senses wall teichoic acid Glycopolymer of *Staphylococcus aureus*, which is restricted in infancy. J Biol Chem **285:**27167-75.
76. **Patti, J. M., B. L. Allen, M. J. McGavin, and M. Hook.** 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu Rev Microbiol **48:**585-617.
77. **Peschel, A., R. W. Jack, M. Otto, L. V. Collins, P. Staubitz, G. Nicholson, H. Kalbacher, W. F. Nieuwenhuizen, G. Jung, A. Tarkowski, K. P. van Kessel,**

and J. A. van Strijp. 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. J Exp Med **193:**1067-76.

78. **Peschel, A., M. Otto, R. W. Jack, H. Kalbacher, G. Jung, and F. Gotz.** 1999. Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem **274:**8405-10.
79. **Rice, K. C., E. E. Mann, J. L. Endres, E. C. Weiss, J. E. Cassat, M. S. Smeltzer, and K. W. Bayles.** 2007. The cidA murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. Proc Natl Acad Sci U S A **104:**8113-8.
80. **Rohde, H., C. Burdelski, K. Bartscht, M. Hussain, F. Buck, M. A. Horstkotte, J. K. Knobloch, C. Heilmann, M. Herrmann, and D. Mack.** 2005. Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Mol Microbiol **55:**1883-95.
81. **Rupp, M. E., J. S. Ulphani, P. D. Fey, K. Bartscht, and D. Mack.** 1999. Characterization of the importance of polysaccharide intercellular adhesin/hemagglutinin of *Staphylococcus epidermidis* in the pathogenesis of biomaterial-based infection in a mouse foreign body infection model. Infect Immun **67:**2627-32.
82. **Rupp, M. E., J. S. Ulphani, P. D. Fey, and D. Mack.** 1999. Characterization of *Staphylococcus epidermidis* polysaccharide intercellular adhesin/hemagglutinin in the pathogenesis of intravascular catheter-associated infection in a rat model. Infect Immun **67:**2656-9.
83. **Rybak, M. J., and K. L. LaPlante.** 2005. Community-associated methicillin-resistant *Staphylococcus aureus*: a review. Pharmacotherapy **25:**74-85.
84. **Sauermann, R., M. Rothenburger, W. Graninger, and C. Joukhadar.** 2008. Daptomycin: a review 4 years after first approval. Pharmacology **81:**79-91.
85. **Schwank, S., Z. Rajacic, W. Zimmerli, and J. Blaser.** 1998. Impact of bacterial biofilm formation on in vitro and in vivo activities of antibiotics. Antimicrob Agents Chemother **42:**895-8.
86. **Sellman, B. R., Y. Timofeyeva, J. Nanra, A. Scott, J. P. Fulginiti, Y. V. Matsuka, and S. M. Baker.** 2008. Expression of *Staphylococcus epidermidis* SdrG increases following exposure to an in vivo environment. Infect Immun **76:**2950-7.
87. **Sendi, P., M. Rohrbach, P. Graber, R. Frei, P. E. Ochsner, and W. Zimmerli.** 2006. *Staphylococcus aureus* small colony variants in prosthetic joint infection. Clin Infect Dis **43:**961-7.
88. **Silverman, J. A., N. Oliver, T. Andrew, and T. Li.** 2001. Resistance studies with daptomycin. Antimicrob Agents Chemother **45:**1799-802.
89. **Silverman, J. A., N. G. Perlmutter, and H. M. Shapiro.** 2003. Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. Antimicrob Agents Chemother **47:**2538-44.
90. **Stapleton, P. D., and P. W. Taylor.** 2002. Methicillin resistance in *Staphylococcus aureus*: mechanisms and modulation. Sci Prog **85:**57-72.

91. **Steenbergen, J. N., J. Alder, G. M. Thorne, and F. P. Tally.** 2005. Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. J Antimicrob Chemother **55:**283-8.
92. **Stewart, P. S., W. M. Davison, and J. N. Steenbergen.** 2009. Daptomycin rapidly penetrates a *Staphylococcus epidermidis* biofilm. Antimicrob Agents Chemother **53:**3505-7.
93. **Stewart, P. S., and M. J. Franklin.** 2008. Physiological heterogeneity in biofilms. Nat Rev Microbiol **6:**199-210.
94. **Straus, S. K., and R. E. Hancock.** 2006. Mode of action of the new antibiotic for Gram-positive pathogens daptomycin: comparison with cationic antimicrobial peptides and lipopeptides. Biochim Biophys Acta **1758:**1215-23.
95. **Tang, L., L. Liu, and H. B. Elwing.** 1998. Complement activation and inflammation triggered by model biomaterial surfaces. J Biomed Mater Res **41:**333-40.
96. **Trampuz, A., and A. F. Widmer.** 2006. Infections associated with orthopedic implants. Curr Opin Infect Dis **19:**349-56.
97. **Trampuz, A., and W. Zimmerli.** 2006. Antimicrobial agents in orthopaedic surgery: Prophylaxis and treatment. Drugs **66:**1089-105.
98. **Trampuz, A., and W. Zimmerli.** 2005. Prosthetic joint infections: update in diagnosis and treatment. Swiss Med Wkly **135:**243-51.
99. **Tupin, A., M. Gualtieri, F. Roquet-Baneres, Z. Morichaud, K. Brodolin, and J. P. Leonetti.** 2010. Resistance to rifampicin: at the crossroads between ecological, genomic and medical concerns. Int J Antimicrob Agents **35:**519-23.
100. **Tverdek, F. P., C. W. Crank, and J. Segreti.** 2008. Antibiotic therapy of methicillin-resistant *Staphylococcus aureus* in critical care. Crit Care Clin **24:**249-60, vii-viii.
101. **van Belkum, A., N. J. Verkaik, C. P. de Vogel, H. A. Boelens, J. Verveer, J. L. Nouwen, H. A. Verbrugh, and H. F. Wertheim.** 2009. Reclassification of *Staphylococcus aureus* nasal carriage types. J Infect Dis **199:**1820-6.
102. **Vaudaux, P. E., F. A. Waldvogel, J. J. Morgenthaler, and U. E. Nydegger.** 1984. Adsorption of fibronectin onto polymethylmethacrylate and promotion of *Staphylococcus aureus* adherence. Infect Immun **45:**768-74.
103. **Vuong, C., S. Kocianova, J. M. Voyich, Y. Yao, E. R. Fischer, F. R. DeLeo, and M. Otto.** 2004. A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. J Biol Chem **279:**54881-6.
104. **Vuong, C., and M. Otto.** 2002. *Staphylococcus epidermidis* infections. Microbes Infect **4:**481-9.
105. **Vuong, C., H. L. Saenz, F. Gotz, and M. Otto.** 2000. Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. J Infect Dis **182:**1688-93.
106. **Weiss, E. C., A. Zielinska, K. E. Beenken, H. J. Spencer, S. J. Daily, and M. S. Smeltzer.** 2009. Impact of sarA on daptomycin susceptibility of *Staphylococcus aureus* biofilms in vivo. Antimicrob Agents Chemother **53:**4096-102.
107. **Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick.** 2002. Extracellular DNA required for bacterial biofilm formation. Science **295:**1487.

108. **White, D.** 2000. The Physiology and Biochemistry of Prokaryotes, 2nd ed. Oxford University Press.
109. **Williams, J. D.** 1999. Beta-lactamases and beta-lactamase inhibitors. Int J Antimicrob Agents **12 Suppl 1:**S3-7; discussion S26-7.
110. **Wimer, S. M., L. Schoonover, and M. W. Garrison.** 1998. Levofloxacin: a therapeutic review. Clin Ther **20:**1049-70.
111. **Xia, G., T. Kohler, and A. Peschel.** 2010. The wall teichoic acid and lipoteichoic acid polymers of *Staphylococcus aureus*. Int J Med Microbiol **300:**148-54.
112. **Xu, L., H. Li, C. Vuong, V. Vadyvaloo, J. Wang, Y. Yao, M. Otto, and Q. Gao.** 2006. Role of the luxS quorum-sensing system in biofilm formation and virulence of *Staphylococcus epidermidis*. Infect Immun **74:**488-96.
113. **Zak, O., Sande M.A.** 1999. Handbook of Animal Models of Infection. Academic Press.
114. **Zhang, Y. M., and C. O. Rock.** 2008. Membrane lipid homeostasis in bacteria. Nat Rev Microbiol **6:**222-33.
115. **Zimmerli, W., P. D. Lew, and F. A. Waldvogel.** 1984. Pathogenesis of foreign body infection. Evidence for a local granulocyte defect. J Clin Invest **73:**1191-200.
116. **Zimmerli, W., and P. E. Ochsner.** 2003. Management of infection associated with prosthetic joints. Infection **31:**99-108.
117. **Zimmerli, W., A. Trampuz, and P. E. Ochsner.** 2004. Prosthetic-joint infections. N Engl J Med **351:**1645-54.
118. **Zimmerli, W., F. A. Waldvogel, P. Vaudaux, and U. E. Nydegger.** 1982. Pathogenesis of foreign body infection: description and characteristics of an animal model. J Infect Dis **146:**487-97.
119. **Zimmerli, W., A. F. Widmer, M. Blatter, R. Frei, and P. E. Ochsner.** 1998. Role of rifampin for treatment of orthopedic implant-related staphylococcal infections: a randomized controlled trial. Foreign-Body Infection (FBI) Study Group. JAMA **279:**1537-41.

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