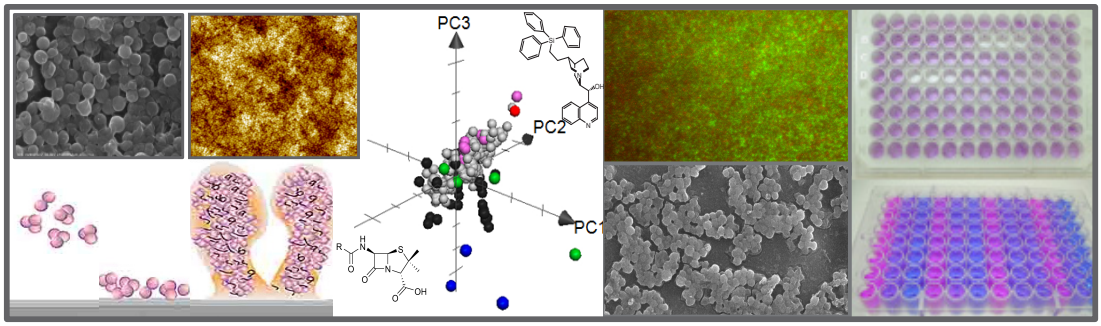
Åbo Akademi University

Malena Skogman

A PLATFORM FOR ANTI-BIOFILM ASSAYS

combining biofilm viability, biomass and matrix quantifications in susceptibility assessments of antimicrobials against *Staphylococcus aureus* biofilms



PHARMACEUTICAL SCIENCES
DEPARTMENT OF BIOSCIENCES
ÅBO AKADEMI UNIVERSITY
ÅBO, FINLAND

2012

A PLATFORM FOR ANTI-BIOFILM ASSAYS combining biofilm viability, biomass and matrix quantifications in susceptibility assessments of antimicrobials against *Staphylococcus aureus* biofilms

Malena Elise Skogman

Pharmaceutical Sciences
Department of Biosciences
Åbo Akademi University
Åbo, Finland
2012

Supervised by

Docent Adyary Fallarero
Pharmaceutical Sciences, Department of Biosciences
Åbo Akademi University
Turku, Finland

Professor Pia Vuorela
Pharmaceutical Sciences, Department of Biosciences
Åbo Akademi University
Turku, Finland

Reviewed by

Docent Mervi Vasänge
AstraZeneca
Research and Development | Innovative Medicines
Södertälje, Sweden

Professor Emeritus Kalevi Pihlaja
Department of Chemistry
University of Turku
Turku, Finland

Opponent

Assistant Research Professor Darla M. Goeres
Center for Biofilm Engineering
Montana State University
Bozeman, Montana, USA

Cover, from left: Biofilm formation including electron microscopy and atomic force microscopy images of untreated biofilms. Chemical space occupied by screened compounds and structures of the active antimicrobial agents, penicillin and 11-TPSCD. Electron microscopy and fluorescence microscopy images of treated biofilms. Plates stained with crystal violet and resazurin, respectively.

ISBN 978-952-12-2796-7
Painosalama Oy – Turku, Finland 2012

To Sebastian and Edward

Table of Contents

List of original publications

I. **SANDBERG M.**, MÄÄTTÄNEN A., PELTONEN J., VUORELA P., FALLARERO A. Automating a 96-well microtitre plate model for *Staphylococcus aureus* biofilms: an approach to screening of natural antimicrobial compounds. *International Journal of Antimicrobial Agents* 32(3):233-240, 2008.

II. **SANDBERG M.E.**, SCHELLMANN D., BRUNHOFER G., ERKER T., BUSYGIN I., LEINO R., VUORELA P.M., FALLARERO A. Pros and cons of using resazurin staining for quantification of viable *Staphylococcus aureus* biofilms in a screening assay. *Journal of Microbiological Methods* 78(1):104-106, 2009.

III. **SKOGMAN* M.E.**, VUORELA P.M., FALLARERO A. Combining biofilm matrix measurements with biomass and viability assays in susceptibility assessments of antimicrobials against *Staphylococcus aureus* biofilms. *Journal of Antibiotics,* 27 June 2012, doi:10.1038/ja.2012.49, 2012.

IV. CHEN J., FALLARERO A., MÄÄTTÄNEN A., **SANDBERG M.**, PELTONEN J., VUORELA P.M., RIEKKOLA M.L. Living cells of *Staphylococcus aureus* immobilized onto the capillary surface in electrochromatography: A tool for screening of biofilms. *Analytical Chemistry* 80(13):5103-9, 2008.

V. **SKOGMAN* M.E.**, KUJALA J., BUSYGIN I., LEINO R., VUORELA P.M., FALLARERO A. Evaluation of antibacterial and anti-biofilm activities of cinchona alkaloid derivatives against *Staphylococcus aureus. Natural Product Communications* 7(9):1173-1176, 2012.

* née Sandberg

Contribution of the author

The author has contributed to the publications in the thesis as following:

In Publication I the work was designed, performed and the manuscript was written by the author together with the supervisors. The AFM studies were performed by MSc Anni Määttänen.

In Publication II the work was designed, performed and the manuscript was written by the author together with the supervisors. The compounds used for the screening were synthesized by the groups of Professor Thomas Erker and Professor Reko Leino.

In Publication III the work was designed, performed and the manuscript was written by the author together with the supervisors. The FM studies were performed by MSc Janni Kujala.

In Publication IV the preparatory biofilm work was conducted by the author.

In Publication V the work was designed, performed and the manuscript was written by the author together with the supervisors. The compounds used for the screening were synthesized by the group of Professor Reko Leino. The FM studies were performed by MSc Janni Kujala.

Additional publications not included in the thesis

VARHIMO E., VARMANEN P., FALLARERO A., **SKOGMAN M.**, PYÖRÄLÄ S., IIVANAINEN A., SUKURA A., VUORELA P., SAVIJOKI K. Alpha- and β-casein components of host milk induce biofilm formation in the mastitis bacterium *Streptococcus uberis*. *Veterinary Microbiology* 149(3-4):381-9, 2011.

FALLARERO A., AINASOJA M., **SANDBERG M.**, TEERI T.H., VUORELA P.M. GT1-7 cell-based cytoxicity screening assay on 96-well microplates as a platform for the safety assessment of genetically modified *Gerbera hybrida* extracts. *Drug and Chemical Toxicology* 32(2):120-7, 2009.

Acknowledgements

This thesis project was carried out in the Pharmaceutical Science Laboratory, at the Department of Biosciences during the years 2007-2012. I would here like to express my gratitude towards the many people who have helped me one way or another over the years.

First of all I would like to thank professor Pia Vuorela for taking me on-board as a graduate student, for your endless encouragement and support along the way. Then an enormous gratitude goes to the best supervisor ever, docent Adyary Fallarero. My dearest Adyary, you have taught me more than you can imagine. You have an enormous scientific knowledge that you are able to passionately teach your students and you have an incredible ability to always be there and have time for us, scientifically or not.

I would like to thank docent Mervi Vasänge at AstraZeneca in Sweden and professor emeritus Kalevi Pihlaja at the University of Turku for reviewing my thesis, giving me constructive feed-back and suggestions to improve it. Professor Pihlaja is specially acknowledged for doing the reviewing so quickly under the unfortunate circumstances due to that the first appointed reviewer, professor Kielo Haahtela, unexpectedly passed away.

I would also like to acknowledge my co-authors professor Thomas Erker, Dr Gerda Brunhofer and Dr Denise Schellmann from University of Vienna as well as professor Reko Leino and Dr Igor Busygin from the Laboratory of Organic Chemistry, Åbo Akademi University for providing with the chemical libraries. Professor Jouko Peltonen and MSc Anni Määttänen are thanked for the AFM-related work for several of the publications and other experiments over the years. Professor Marja-Liisa Riekkola, Dr Jie Chen and MSc Janni Kujala are as well acknowledged as co-authors.

Professor Mark Johnson and coordinator Fredrik Karlsson from The National Graduate School of Informational and Structural Biology (ISB) are thanked for coordinating a very good graduate school, where I have been a member. I sincerely hope that you are able to continue the good work. Further, I would like to thank professor Jari Yli-Kauhaluoma for being a member of my thesis committee.

I am grateful for all the practical and technical help I have got from the personnel at the department, especially Jussi Meriluoto, Juha-Pekka Sunila, Elsmarie Nyman and Pirkko Luoma.

Then, I would also like to thank our collaborators, Dr Kirsi Savijoki and Dr Pekka Varmanen at the Faculty of Veterinary Medicine at the University of Helsinki for taking me into their lab and teaching me a lot about proteomics and providing with bacterial

strains. Dr Minna Männistö at Metla in Rovaniemi is thanked for providing with the bacterial strains and extracts from the ARMI collection.

I would also like to acknowledge all the people in the department, both biochemistry and pharmacy, for nice work environment. I am especially grateful to Daniela for all the endless discussions over and through the wall between our desks, for eminent travel company and delicious cakes! I would also like to thank everyone else in our research group, Pharmaceutical Sciences Laboratory, for nice times at work; Nora, Janni, Natalja, Olli, Gerda, Ana, Mohit, Hari, Dominik and Leena. Former students are thanked for work done in the lab. All the other people that I have shared the office with at some point are thanked for making the work hours very enjoyable with both scientific as well as less scientific discussions, to mention the most long-term; Jeanette, Anders, Shishir and Matts.

A huge appreciation goes to all my friends. My Åbo-friends (that are widely spread out nowadays), you understand me both personally and scientifically. My Borgå-friends are thanked for keeping me in the real world and making me think of something else than science. Thank you girls!

I would also like to thank my parents, Monica and Johan and my brother Joakim for all the support over the years.

At last, my greatest appreciation goes to my own lovely little family, my husband Sebastian and our son Edward for all the love; always and unconditionally!

This work was financially supported by Academy of Finland (BIOARMI, decision 128870), Finnish Cultural Foundation, Stiftelsens för Åbo Akademi forskningsinstitut, KAUTE - Niilo V. Santasalon ja Lauri N. Santasalon erikoisrahasto, Svenska Kulturfonden, Finnish Pharmaceutical Society, Waldemar von Frenckells stiftelse, K. Albin Johanssons stiftelse, Tor, Joe and Pentti Borgs minnesfond, Medicinska Understödsföreningen Liv och Hälsa rf. Rektors understöd, ISB and Systems Biology are additionally acknowledged for funding conference and research travels.

Åbo, September 5, 2012

Malena Skogman

Malena Skogman

Abbreviations

ADME-Tox	Absorption, distribution, metabolism, excretion, toxicity
AFM	Atomic force microscopy
APTES	3-aminopropyl-triethoxysilane
ARMI	Arctic Microbe Culture Collection
BGE	Background electrolyte
CEC	Capillary electrochromatography
CF	Cystic fibrosis
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase negative staphylococci
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
CV	Coefficient of variation
CVC	Central venous catheters
Da	Dalton
DMMB	Dimethyl methylene blue
DMSO	Dimethyl sulfoxide
eDNA	Extracellular DNA (deoxy ribonucleic acid)
EMA	European Medicines Agency
EOF	Electro osmotic flow
EPS	Extrapolymeric substance or extracellular polysaccharides
FDA	US Food and Drug Administration
HCS	High content screening
HTS	High throughput screening
IC_{50}	50% of the maximal inhibitory concentration
IMD	Indwelling medical device
logP	Octanol-water partition coefficient (lipophilicity)
MIC	Minimal inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MW	Molecular weight
PCA	Principal component analysis
PBS	Phosphate buffered saline
PGA	(poly) β-1,6-*N*-acetyl-d-glucosamine residues
PNAG	(poly) β-1,6-linked poly-*N*-acetyl glucosamine residues
PVC	Polyvinyl chloride
RAU	Relative absorbance units
RFU	Relative fluorescence units
SD	Standard deviation
S/B	Signal to background
S/N	Signal to noise

TSA Tryptic soy agar
TSB Tryptic soy broth
WGA Wheat germ agglutinin
WHO World Health Organization
XTT 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (tetrazolium salt)
9-TMSO-11-TPSCD 11 triphenylsilyl-9-*O*-trimethylsilyl-10,11 dihydrocinchonidine
11-TPSCD 11-triphenylsilyl-10,11-dihydrocinchonidine
96-mwp 96-micro well plate

Abstract

Bacteria can exist as planktonic, the lifestyle in which single cells exist in suspension, and as biofilms, which are surface-attached bacterial communities embedded in a self-produced matrix. Most of the antibiotics and the methods for antimicrobial work have been developed for planktonic bacteria. However, the majority of the bacteria in natural habitats live as biofilms. Biofilms develop dauntingly fast high resistance towards conventional antibacterial treatments and thus, there is a great need to meet the demands of effective anti-biofilm therapy.

In this thesis project it was attempted to fill the void of anti-biofilm screening methods by developing a platform of assays that evaluate the effect that screened compounds have on the total biomass, viability and the extracellular polysaccharide (EPS) layer of the biofilms. Additionally, a new method for studying biofilms and their interactions with compounds in a continuous flow system was developed using capillary electrochromatography (CEC). The screening platform was utilized with a screening campaign using a small library of cinchona alkaloids.

The assays were optimized to be statistically robust enough for screening. The first assay, based on crystal violet staining, measures total biofilm biomass, and it was automated using a liquid handling workstation to decrease the manual workload and signal variation. The second assay, based on resazurin staining, measures viability of the biofilm, and it was thoroughly optimized for the strain used, but was then a very simple and fast method to be used for primary screening. The fluorescent resazurin probe is not toxic to the biofilms. In fact, it was also shown in this project that staining the biofilms with resazurin prior to staining with crystal violet had no effect on the latter and they can be used in sequence on the same screening plate. This sequential addition step was indeed a major improvement on the use of reagents and consumables and also shortened the work time. As a third assay in the platform a wheat germ agglutinin based assay was added to evaluate the effect a compound has on the EPS layer. Using this assay it was found that even if compounds might have clear effect on both biomass and viability, the EPS layer can be left untouched or even be increased. This is a clear implication of the importance of using several assays to be able to find “true hits” in a screening setting.

In the pilot study of screening for antimicrobial and anti-biofilm effects using a cinchona alkaloid library, one compound was found to have antimicrobial effect against planktonic bacteria and prevent biofilm formation at low micromolar concentration. To eradicate biofilms, a higher concentration was needed. It was also shown that the chemical space occupied by the active compound was slightly different than the rest of the cinchona alkaloids as well as the rest of the compounds used for validatory screening during the optimization processes of the separate assays.

1 Review of the literature

1.1 Biofilms

Biofilms are defined in many ways, "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface", given by Costerton et al. (1999) is maybe the most explanatory definition. Every description of biofilms concludes similarly that a biofilm consists of the bacteria (single or multispecies), the matrix and the surface to which the whole structure is attached (Dunne 2002). Practically any surface, living or non-living can be targeted for bacteria colonization and, thus, biofilm formation. Surfaces can range from rocks in a water stream (Costerton et al. 1978, Henrici 1933), paper industry machinery (Kolari et al. 2001) to plants, human and animal lungs, noses, ears, and wounds etc. Biofilms have been known as such since the 1970s and 1980s when Costerton and his coworkers defined how bacteria stick to a surface and further studied the biofilm phenomenon (Costerton et al. 1978, Costerton et al. 1987). Prior to that, Henrici in the 1930s, came to the conclusion that bacteria in fresh water are not growing planktonically but in clusters attached to solid, even glass, surfaces (Henrici 1933). However, even earlier, Antonie van Leeuwenhoek, who was the first person to be able to study bacteria under a microscope, or animalculi as he referred to them in the 17th century, was actually studying biofilms because he scraped the microbes off his teeth. Biofilms in dental plaques are actually one of the most studied areas of biofilm research (Burmølle et al. 2010). Additionally, biofilm contamination of prosthetic devices is another area with an intense research focus (Schinabeck and Ghannoum 2006).

1.1.1 Formation of biofilms

Why are biofilms formed in the first place? At some point in the evolution of prokaryotes, biofilm formation was likely to be advantageous for survival. There are many theories as to why biofilms are the preferred lifestyle by so many bacteria. First, the surface provides stability in the growth environment by localizing the cells in close proximity. Secondly, the biofilm formation offers protection from a wide range of environmental challenges (Hall-Stoodley et al. 2004). It has also been widely discussed how nutrient availability affects initiation of biofilm formation. It has been seen that gram-negative bacteria favor biofilm formation in nutrient-rich media and in nutrient-deprived surroundings tend to return to the planktonic phase of living, based on the theory that the free floating mode helps in the search for fresh nutrients (O'Toole et al. 2000). However, the opposite has been seen for the gram-negative *Escherichia coli* where scarce conditions that tend to slow down bacterial growth actually favor biofilm formation (Adams and McLean 1999).

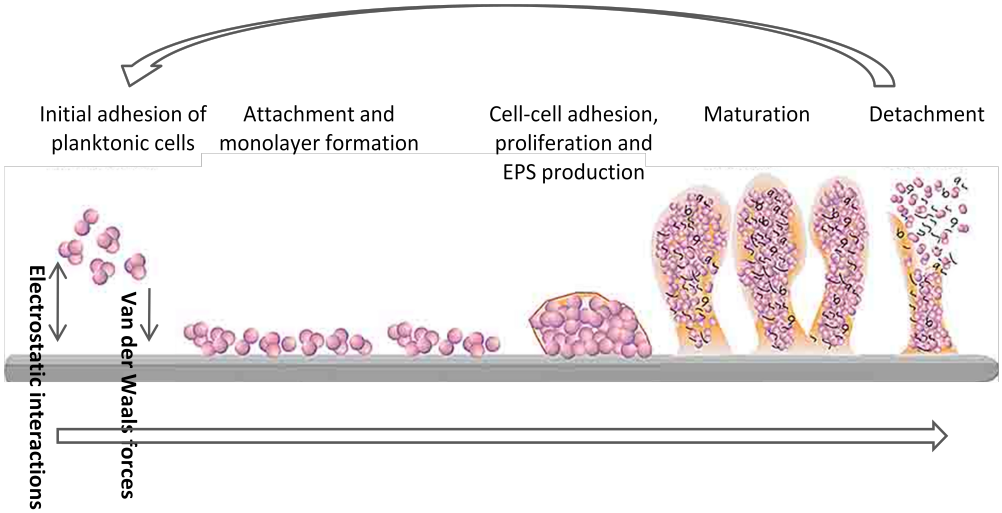


Figure 1. Formation of a model biofilm on a surface. The formation is divided into steps that include initial adhesion, attachment, proliferation, maturation and detachment. Modified from Otto (2004) and Pascual (2002).

1.1.1.1 Adherence and early maturation

Biofilm formation can be divided into several steps, presented above in figure 1. First, there has to be the opportunity for the bacteria to adhere to the surface. In a water stream or in a blood vessel with a continuous flow, this is usually not a limiting stage, but in surfaces less influenced by flow (i.e., medical implants) the bacteria have to be transported into close proximity of the substrate surface so that the attachment can take place. A distance of approximately 50 nm can be regarded as close enough for the bacteria to initially adhere (Gorman and Jones 2006). The attachment has been found to be mediated by electrostatic interactions and van der Waals forces (Pascual 2002).

Many structural features on the cell surface of bacteria affect the substrate's surface charge and hydrophobicity and, thus, contribute to the attachment step: fimbriae, the teichoic acids in the cell wall of gram-positive bacteria, and the liposaccharides in the outer cell membrane of gram-negative bacteria (Gorman and Jones 2006). Additionally, the environmental conditions and the characteristics of the surface may also affect the rate and the extent of attachment of the bacteria. In general, rougher and more hydrophobic materials will lead to more and faster development of biofilms (Donlan 2001). In contrast, less rough materials can also allow fast biofilm formation, due to a conditioning film that is formed of proteinaceous molecules on surfaces when they are placed into a liquid environment (can consist of anything between bloodstream and a freshwater lake) (Donlan 2001, Schinabeck and Ghannoum 2006). *Staphylococcus* spp. adherence is promoted by protein and glycoprotein components on the surface, the most

important being fibronectin, fibrinogen and fibrin, but also collagen, elastin, laminin, vitronectin and von Willebrand factors have been shown to be involved (Pascual 2002, Beenken et al. 2010). *Staphylococcus* spp. have several specific adhesion promoting proteins that comprehensively are called "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) (Patti et al. 1994). The most important proteins, fibronectin, fibrinogen and fibrin, have also been shown to induce adhesion for gram-negative bacteria as well as *Candida albicans* fungi (Schinabeck and Ghannoum 2006). Structural properties of the bacteria are also involved in the attachment phase. In biofilm-development of *Pseudomonas aeruginosa*, flagella and type-IV pili structures as well as lipopolysaccharides are involved in the initial phase (O'Toole et al. 2000). Similarly, type I pili are required for *E. coli* biofilm development on almost any surface (Pratt and Kolter 1998). Twitching motility, a form of bacterial translocation over a surface in a liquid environment, seems to be important in early development of biofilm structures (Mattick 2002). Gram-positive bacteria are considered non-motile and the initial attachment is established by cell surface protein adhesions and extrapolymeric substance or extracellular polysaccharides (both abbreviated to EPS) instead of motility factors (Pratt and Kolter 1999).

Following the initial rapid attachment, the cells proliferate, and the bacteria cluster together in microcolonies, which then mature into larger clusters if the conditions are suitable (Kiedrowski and Horswill 2011). This stage is considered to be an intermediate stage, often referred to as the accumulation stage. The biofilms then mature into a complex architecture of cells in multiple layers in three dimensions, with channel and pore structures (Stoodley et al. 2002). Due to being non-motile, gram-positive biofilms tend to be flatter with fewer three-dimensional features than their gram-negative counterparts. However, gram-positive biofilms can also form tower-like structures in certain conditions, for example under shear stress, such as the flow of the liquid or upon nutrient deprivation (Mann et al. 2009). It has also been shown that during the maturation stage of *P. aeruginosa* biofilms, more than half of the proteins are differently expressed or completely new in biofilm bacteria compared to their planktonic counterpart (Sauer et al. 2002). This is followed by irreversible attachment to the surface and EPS production by biofilm cells (Stoodley et al. 2002).

1.1.1.2 Extracellular matrix production

The EPS vary between and even within species, but mainly it has been shown to consist of polysaccharides, proteins and extracellular DNA (eDNA) (Steinberger and Holden 2005). The EPS is important for the biofilm since it provides structural stability and protection to the formed biofilm. It acts as a protective barrier against biocides or toxins and it sequesters nutrients from the environment, and therefore, is a part of the general bacterial strategy for persistence under extreme, non-favorable conditions (Donlan and

Costerton 2002). The EPS is also involved in virulence of many biofilm producing strains, especially in *Staphylococcus* spp. and *Pseudomonas* spp. (Jabbouri and Sadovskaya 2010).

In *P. aeruginosa* and other *Pseudomonas* spp. the EPS mainly consists of alginate, a polymer of uronic acid and guluronate (O'Toole et al. 2000, Gacesa 1998). Alginate monomers can be arranged in various polymeric compositions with different block structures and degrees of *O*-acetylation. Due to being negatively charged and having a high molecular mass, the alginate EPS is viscous and well hydrated (Gacesa 1998). The exact function of alginate varies but clearly, it plays an important role in biofilm maturation and is also an important virulence factor for many phytopathogenic *Pseudomonas* spp. among other exopolysaccharides (Gacesa 1998). *P. aeruginosa* alginate and, thus, the EPS is one of the key problems in persistent infections in the lungs of cystic fibrosis patients (Williams et al. 2010). In *E. coli* biofilms, the main polysaccharide in the EPS is called PGA and it is encoded by the *pga*ABCD operon (Cerca and Jefferson 2008).

The EPS of *Staphylococcus* spp. are represented by β-1,6-linked poly-*N*-acetyl-glucosamine residues (PNAG) which are encoded by the *ica*ADBC operon found in biofilm forming strains (Ziebuhr et al. 1999). PNAG is structurally and functionally very similar to the PGA of *E. coli*. For *Staphylococcus* spp., the main virulence factors are connected to the ability to form biofilms, possessing the *ica*ADBC operon and additionally to produce PNAG. A strain should, therefore, possess all of these to be considered pathogenic (Jabbouri and Sadovskaya 2010). Although it has been seen that biofilm-forming capacities are not essential for pathogens to cause infections, it has been shown that strains can change from being planktonic to biofilm formers depending on the surroundings and environmental circumstances (Chokr et al. 2007, O'Toole et al. 2000).

1.1.1.3 Detachment and the spread of infection

Biofilm development is a dynamic process and while continuously growing in size, individual cells or clumps of cells are also continuously detaching from the bulk. These detached bacteria can regain their planktonic status and move through the surrounding area to a new surface where a new biofilm can be formed. Three separate strategies have been identified for detachment of cells from a biofilm and can be divided into seeding dispersal (where individual cells are released), clumping dispersal (in which aggregates of cells are shed off the biofilm), and surface dispersal (in which the whole biofilm structure is moving along the surface it is adhered to) (Hall-Stoodley et al. 2004). In surroundings with continuous flow, i.e. water pipes or streams, blood vessels, or airways, the detachment is particularly clear and also best documented (Stoodley et al. 2001). Detachment of planktonic cells from biofilms is also one reason for the efficient spreading of biofilm-related infections (Hall-Stoodley and Stoodley 2005).

1.1.2 Resistance in biofilms

Ability to form biofilms, in itself, does not cause pathogenicity but the virulence for pathogenic bacteria is increased if they form biofilms (Dethlefsen et al. 2007). It is not clear what the reasons for the increased resistance toward anti-biofilm treatments of biofilms compared to planktonic bacteria could be. However, extensive research has been carried out during the last decade to clarify this issue. The most widely used explanation to resistance is target modification by mutation or enzymatic change, or complete change of target (Lewis 2007). Metabolically heterogeneous populations, with aerobically growing cells on the outer part of the biofilms and metabolic inactivation of cells inside the biofilm bulk due to nutrient or oxygen deprivation can also lead to increased antimicrobial resistance (Fux et al. 2004, Hall-Stoodley and Stoodley 2009, Bordi and de Bentzmann 2011). The EPS layer has been thought to be a physical barrier and, subsequently, able to restrict antibiotics diffusion. It has, however, been shown that it is rather a limitation of nutrients that results in oxygen limited zones in the biofilm and show increased antimicrobial tolerance (Borriello et al. 2006). On one hand, the matrix is also thought to retard the penetration of the antimicrobial molecules and to result in extra time for the cells deeper down to express new genes and establish resistance even before the antimicrobial reaches them (Jefferson et al. 2005). It has, on the other hand, been shown that even macromolecules penetrate through thick biofilms in minutes or even seconds, and consequentially, the penetration might not be the cause for the lower susceptibility to antibiotics (Takenaka et al. 2009). In addition, dead cells inside the biofilm have been regarded as important to the development of antimicrobial resistance among biofilms; they can act as a nutrient source for the surrounding live bacteria and increase their metabolic activity but may also function as a dilution gradient for the antimicrobial per cell basis (Mai-Prochnow et al. 2004, Hall-Stoodley and Stoodley 2009). Cell death within a biofilm can also lead to an increased dispersal rate of surviving cells and thereby indirectly cause spreading of the biofilm (Mai-Prochnow et al. 2006).

Persister cells are inactive cells within a bacteria population, planktonic or biofilm that are highly resistant toward antimicrobial therapies (Spoering and Lewis 2001). In a likely *in vivo* scenario, the metabolically active biofilm and planktonic cells of an infection would be killed by the antibiotic treatment, but leaving both planktonic and biofilm persisters alive (Lewis 2007). The planktonic persister cells are of no harm as they are effectively cleaned away by the immune system, but the biofilm persisters are encased within the EPS, which is non-penetrable for most of the immune cells and are thus not as easily removed by the immune system (Leid et al. 2002, Vuong et al. 2004). Yet, biofilms are not completely protected from the host defense, especially young or altered biofilms are not tolerant against host defense (Günther et al. 2009).

1.1.3 Biofilm related problems

1.1.3.1 Clinically severe biofilm related infections

Cystic fibrosis (CF) is a genetic disease, which is caused by a mutation in the cystic fibrosis transmembrane conductance regulator gene and leads to progressive dysfunctional secretion mainly in the respiratory system (Høiby 2011). Chronic colonization of the principal cystic fibrosis pathogen, *Pseudomonas aeruginosa*, results in progressive lung damage, respiratory failure and eventually death (Hall-Stoodley et al. 2004). Even with long-term antibiotic treatments, these infections remain chronic and this is most probably due to formation of resistant biofilms in the lung secret (Singh et al. 2000, Kirov et al. 2007). *P. aeruginosa* infections also induce immune system responses by the release of chemokines, cytokines and macrophage proteins related to inflammation. Nonetheless, instead of this being a response to eradicate the bacterial infection it is rather an increase in overall inflammation in the lung (Smith et al. 2002a, Smith et al. 2002b, Burmølle et al. 2010). In addition to *Pseudomonas* spp. infections, CF patients are more prone to *S. aureus* lung infections that are difficult to eradicate due to biofilm formation even with long-term anti-staphylococcal treatments (Goerke and Wolz 2010).

Otitis media is one of the most common diseases in young children; about 75% of all children under the age of three develop an otitis media infection at least once (Klein 1994, Tähtinen et al. 2011). The infection can be either acute or chronic and can be caused by a range of different bacteria *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Pneumococcus* spp. (Donlan and Costerton 2002). The chronic disease involves bacterial biofilm growth behind the tympanic membrane and in the middle ear. Antibiotic treatments are mostly used as treatment, but in the case of chronic infections, antibiotic treatment is usually ineffective and only contributes to resistant bacteria. Tympanostomy tubes are the suggested treatment for chronic infections, to relieve the pressure of the built-up fluids behind the eardrum and to increase ventilation to prevent further infections (Nguyen et al. 2010). However, the tubes themselves are also susceptible to biofilm attachment and make the treatment of the disease very troublesome (Donlan and Costerton 2002).

1.1.3.2 IMD-related infection

The number of indwelling medical device associated infections has risen in the last decades, mainly due to the increased use of IMDs that in turn depends on the advances in medical technology. Considering the advantages for patients acquiring a prosthetic device and the infection rate, which is usually low (around 1-10%) the risk is usually worth taking (Hall-Stoodley et al. 2004). The infection rate and the mortality risk in case of infection also need to be evaluated (Schinabeck and Ghannoum 2006). The material used in IMDs has been thoroughly investigated and the surface characteristics affecting the

adherence are texture, charge and hydrophobicity (Dunne 2002). It has been found that most materials can be colonized by biofilms; *in vitro* studies have shown that the order of the biomaterials that biofilms are prone to adhere to is: latex > silicone > PVC > Teflon > polyurethane > stainless steel > titanium (Dunne 2002, Gorman and Jones 2006).

The most frequently used IMDs are urinary and central venous catheters; they also have among the highest infection rates (10-30% and 3-8%, respectively) (Donlan 2001, Schinabeck and Ghannoum 2006). Central venous catheters (CVC) are essential devices for treating critically and chronically ill patients but they are also target for severe biofilm-related infections that cause nosocomial bloodstream infections if contaminated (Raad and Hanna 2002). The bacteria causing the infection either originates from the surrounding skin at the insertion site, moving along the outer or inner surface of the device, or from the health care personnel performing the insertion into the blood stream where the bacteria can proliferate and grow biofilms on the surface of the catheter (Raad 1998). Due to direct contact with the blood stream, the surface is conditioned with proteins (for example fibrinogen and fibronectin) that enhance biofilm formation on the catheter surface (Gorman and Jones 2006, Raad 1998). Pathogens that are the main cause of CVC-related infections are coagulase-negative staphylococci (CoNS - mainly *Staphylococcus epidermidis*), *S. aureus*, *P. aeruginosa* and other *Pseudomonas* spp., *Burkholderia cepacia*, *Acinetobacter baumannii*, *Stenotrophomonas* spp. and the fungal *Candida* spp. (Ferretti et al. 2003).

Urinary catheters are widely used during hospitalization and in nursing homes and are also connected with infections due to biofilm growth on the catheter surfaces. The number of urinary catheter related infections has increased during the last decade, and it is currently the second most commonly used indwelling device and has the highest related infection rate (Holá et al. 2010). The low mortality risk if the catheter has to be removed makes them widely used regardless (Schinabeck and Ghannoum 2006). The bacteria can originate from the gastrointestinal tract and enter the system at the time of insertion of the catheter, or they can stem from exogenous sources and ascend up the inner lumen of the catheter (Tenke et al. 2012). The most widely isolated species found in biofilms on urinary catheters are *Enterococcus faecalis*, *E. coli*, CoNS, *P. aeruginosa* and *Klebsiella pneumoniae* as well as *Candida albicans* biofilms (Holá et al. 2010).

Biofilm contamination on heart valves and pacemakers is the most problematic, with the high infectious rate (25-50%) and the highest mortality risk (> 25%) if removed (Schinabeck and Ghannoum 2006). Endocarditis can be caused by a native infection due to congenital heart defects, prosthetic heart valves or vascular grafts, probably due to the amassing of platelets and fibrin where biofilms can be assisted to attach and grow in the turbulent flow in the damaged tissue (Hall-Stoodley et al. 2004). The pathogens causing endocarditis have typically been found to be *Streptococcus* spp., *Staphylococcus* spp.,

especially CoNS and *S. aureus*, gram-negative coccobacilli and fungi (Donlan and Costerton 2002).

1.1.3.3 Severity of *Staphylococcus* spp. biofilms

Staphylococcus spp. are the cause of two thirds of all IMD-related infections, in particular *S. aureus* and *S. epidermidis* (Kiedrowski and Horswill 2011). *S. aureus,* is an especially difficult pathogen that is involved in a range of infections (Götz 2002). The main problem with *S. aureus* is the availability; it colonizes the nasal cavities of up to 40% of individuals having the infection and from there can easily be transferred to the skin, where damages are not uncommon and the infection can be spread to the blood stream (Higashi and Sullam 2006). The occurrence of metabolic zones and phenotypic diversity in *Staphylococcus* spp. biofilms favor division of the labor among the biofilm bacteria and contributes to higher resistance and persistence towards anti-biofilm treatments (Yarwood et al. 2007, Stewart and Franklin 2008).

1.1.3.4 Dental plaque

Dental plaque is one of the most well-known types of biofilm growth, as they are easy to study both *in vivo* and *in situ* at the right conditions (Burmølle et al. 2010). They are multispecies communities normally living side by side with the host. However, a shift in the balance in the oral surrounding or within the bacterial community can lead to diseases for the host, like caries and periodontal diseases (Burmølle et al. 2010, Takahashi and Nyvad 2011). Caries has been shown to be an endogenous disease that is due to a shift of dominance towards acidogenic and acidtolerant bacteria while peridontitis and gingivitis are caused by increased levels of gram-negative organisms (Takahashi and Nyvad 2011, Teles et al. 2012).

1.1.3.5 Other biofilms

Water pipes are another source of biofilm-related problems that become contaminated by biofilms. From these the water is then delivered in large doses into humans, in whom the bacteria can cause infections if the bacteria are pathogenic. Regular water supply systems deal with this problem all over the world (Szewzyk et al. 2000). All water environments are natural habitats for many water bacteria both in planktonic and biofilm stages that are of no harm, but in less controlled environments pathogenic bacteria can also end up in the drinking water supplies and cause severe outbreaks of infections, for example caused by *Vibrio cholerae* and *Salmonella typhi* (Szewzyk et al. 2000). Dental unit water lines at dentist practices constitute such a case, where the system of narrow tubes used and where the water flows irregularly, is prone to be contaminated with pathogenic bacteria, such as *P. aeruginosa*, *Legionella* spp. and even *S. aureus*. This water is then administered directly into human bodies and can in rare cases cause infections, at least in

immunocompromised individuals (O'Donnell et al. 2011). An additional hazardous place for contaminated water appears when dealing with dialysis fluid. The water used for this purpose is filtered by reverse osmosis and should remove 90-100% of all organic and inorganic matter, bacteria and pyrogens, but in rare cases errors in the filtration has been known to lead to bacteria in the end product then administered into humans (Lonnemann 2004).

1.1.4 Available treatments for biofilm infections

1.1.4.1 Antimicrobials

The first antimicrobial agent used in the purpose of treating biofilm infections was the cinchona tree bark infusion (*Cinchona calisaya*, *C. officinalis* and *C. succirubra*), during the 17th century in South America. Later, quinine and cinchonine were isolated in 1820 and used against malaria (Greenwood 2008). Penicillin was discovered in 1928 by Alexander Fleming and came into the market in the 1940s. However, already in the 1960s, penicillin-resistant staphylococcal infections were recognized as a medical problem (Greenwood 2008). The aminoglycoside antibiotic streptomycin was discovered in 1944 and was followed by chloramphenicol, tetracycline and macrolide antibiotics in the 1950s. Vancomycin was discovered in 1956 and the first quinolone antibiotic, nalidixic acid was introduced in 1962. Later in the 1960s, the cephem antibiotics were developed and divided into generations according to their improved antimicrobial properties. Carbapenem and monobactam antibiotics were developed in the early 1980s (Saga and Yamaguchi 2009). Following this development, no new classes of antibiotics were developed until the oxazolidinone compound linezolid was launched in 2000 (Norrby 2001). In the last couple of decades, only five new antibiotics actively in use against severe resistant bacteria have been approved: linezolid (in 2000), daptomycin (in 2003), tigecycline (in 2005), doripenem (in 2005) and the latest one, telavancin (in 2009) (Boucher et al. 2009, Moellering 2011). The antimicrobials available in Finland for the treatment of severe infections that can be related to biofilm growth are presented in Appendix 1.

Antibiotics can roughly be divided into three categories based on the targets of their mechanism of action. There are antibiotics that act on the biosynthesis of the cell wall, interfere with the protein biosynthesis and block DNA replication and repair (Walsh 2003). The first group is mainly represented by the beta-lactams; the penicillin and the cephalosporin compounds. The cell walls differ largely between gram-positive and gram-negative bacteria and, thus, the effect of the antibiotics also varies (Koebnik et al. 2000). Many antibiotic features related to cell wall synthesis are connected to the assembly and cross linking of the peptidoglycan layer, which is thicker and more multilayered in gram-positive bacteria (Walsh 2003). The second group consists of compounds that are able to interfere with the protein synthesis pathway of the bacteria; macrolides, chloramphenicol, tetracycline, aminoglycosides are a few examples (Schlünzen et al.

2001, Bulkley et al. 2010, Dunkle et al. 2010). Quinolone antibiotics are representatives of the third group of antibiotics, because their antimicrobial effect originates from the inhibition of DNA gyrase and topoisomerase IV, which are both type II bacterial topoisomerase enzymes that catalyze double strand DNA breakages in the bacteria (Chai et al. 2011). About half of the antimicrobial drugs on the market, those that have been used for a long time as well as those recently developed, are of natural origin or semi-synthesized, based on a natural origin structure. Thus, nature is still the major source for finding novel antimicrobial agents (Newman and Cragg 2012).

Most of the antibiotic compounds on the market are developed for planktonic bacteria. There are some promising agents with anti-biofilm activity that could be considered for drug development but are still in an early stage of development, mostly preclinical *in vitro* or *in vivo* studies (Moellering 2011). The cell membrane damaging porphyrin derivative XF-73 is one, and it has recently been shown to have anti-biofilm activity at low concentration levels (Ooi et al. 2010).

1.1.4.2 Available treatments of *Staphylococcus* spp. biofilm infections

There are not that many available drugs used for treatment of *Staphylococcus* spp. biofilm infections and most of the available ones have to be used as combination therapies. To prevent biofilm infections new materials that resist biofilm colonization are being developed (Lynch and Robertson 2008). Trials with medical devices emitting acoustic waves (Hazan et al. 2006), electric current (van der Borden et al. 2004) or pulsed ultrasound (Ensing et al. 2006) have been carried out on various materials (Lynch and Robertson 2008). Removal of the indwelling medical device, if possible, is often the best treatment if the infection is IMD-related (Schinabeck and Ghannoum 2006). In some cases, such as heart prosthetic devices, artificial joints or if the patient is not stable enough to undergo surgical procedures, this is not an option and there is need for effective anti-biofilm chemotherapies (Trampuz and Zimmerli 2006, Kiedrowski and Horswill 2011). Most often the treatments then required are extensive or even life-long. Antibiotic lock-therapies have been developed for prophylactic settings, lipopeptides and lipoglycopeptides have shown to be promising for use in these prophylactic methods (LaPlante and Mermel 2007).

Dalbavancin is a lipoglycopeptide used against catheter related infections caused by most gram-negative pathogens, including many MRSA strains (Raad et al. 2005). It has a very long half-life time and can be dosed intravenously once a week, which is cost saving and more convenient for the patient (Billeter et al. 2008). Daptomycin is a lipopeptide clinically used against severe infections mostly caused by gram-positive bacteria biofilms, for example bacteremia and endocarditis (Rehm et al. 2008, Cervera et al. 2009). Linezolid is the only oxazolidinone compound approved for clinical use (Ford et al. 2001). It has shown promising results during *in vitro* (Raad et al. 2007, Smith et al. 2009, Leite et

al. 2011a), *in vivo* (Oramas-Shirey et al. 2001) and clinical studies (Gómez et al. 2011) against various kinds of *Staphylococcus* spp. biofilm infections, including prostethic device associated. However, there is also evidence for linezolid-resistant *Staphylococcus* spp. strains isolated clinically as being effective (Dandache et al. 2009). Rifampicin has shown to be very active *in vitro* (Leite et al. 2011a) and *in vivo* against a variety of biofilm associated implant infections, in addition to showing promising results in clinical studies (Zimmerli et al. 1998). Due to the risk of resistance, rifampicin is always administered together with another antimicrobial, for example daptomycin (John et al. 2009), linezolid (Baldoni et al. 2009), tetracyclines (Rose and Poppens 2009) or dispersing agents, such as *N*-acetyl cysteine (Leite et al. 2011b). Tetracyclines, which include tetracycline, minocycline, doxycycline and the newest tigecycline, are mostly used for prevention of biofilm growth on catheters (Zhanel et al. 2004). Tigecycline has shown promising activity against gram-positive biofilms *in vivo* (Yin et al. 2005) and *in vitro* (Cafiso et al. 2010). Tigecycline together with vancomycin or rifampicin showed even an increased effect (Rose and Poppens 2009). Vancomycin is a glycopeptide that has shown *in vivo* and *in vitro* effect alone but is usually used in combination therapies with fosfomycin, rifampicin or tetracyclines against severe biofilm infections (Rose and Poppens 2009, Tang et al. 2011, Aybar et al. 2012). Unfortunately, resistance towards vancomycin has also been seen both *in vitro* and in clinical infections (Graninger et al. 2002, Antunes et al. 2011).

1.2 Assay development in antimicrobial drug discovery

1.2.1 Target identification and validation

In the search for novel drug compounds, the target for the desired pathological defect has to be identified (Zheng et al. 2006). Choosing a target is of great importance, as most of the drug failures are in the end probably due to a too vaguely chosen target for a drug candidate. The compound is then either ineffective or is proven unsafe (Brötz-Oesterhelt and Sass 2010). The targets can roughly be divided into genetic targets, where the goal is to find a drug exclusively targeting a disease associated gene or gene product, and mechanistic targets, where the drug is affecting a single molecular mechanism related to the course of the target disease (Sams-Dodd 2005). This, of course, requires more detailed information about the genetics and the mechanisms behind disease releasing and proceeding factors (Sams-Dodd 2005). In the 1990s, the antimicrobial drug discovery process was changed from targeting the phenotype of the bacteria into targeting the infection (Brown and Wright 2005). The ideal target displays well characterized functions and is conserved in significant pathogens as well as absent in humans (Brown and Wright 2005). It is, as well, of crucial importance for the target organism to be able to be handled in Petri dishes or micro well plates. The daunting problem of resistance that is evolving constantly would need completely new targets and active components targeting them, instead of moderately change the structure and quickly develop inactivation mechanisms and thus, resistance (Gwynn et al. 2010). The more unknown aspects there

are in the development process; the higher the risk of failure is. Therefore, there is less interest in investments. In fact, since the 1970s, only two new classes of antimicrobials have been developed and have reached the market; linezolid, an oxazolidinone compound (launched in 2000) and daptomycin, a lipopetide (launched in 2003) (Newman and Cragg 2012). However, the classical way of introducing new active compounds by improving old ones with reduced efficiency has given rise to important structures active against resistant organisms, for instance, rifampicin from rifamycin, and the semi-synthetic penicillins and the cephalosporins from penicillin (Chopra et al. 2002). There are almost 30 compounds in clinical trials for treatment of various bacterial infectious diseases pending for both FDA and EMA approvals. Most of the compounds are semi-synthetic derivatives and some are novel natural structures produced by microbes belonging to existing antimicrobial classes (Mishra and Tiwari 2011).

Many of the antimicrobial drug compounds on the market have been found to be active without the knowledge of the exact target or mechanism they act on from whole-cell screens (Baltz 2007). This strategy has re-emerged due to lack of activity on whole-cells for the active hits in these specific target based screenings (Gwynn et al. 2010). In this way, new active agents with unpredicted mechanisms on microbial whole-cell targets can be found, as well as being able to identify new mechanistic targets (Gwynn et al. 2010).

1.2.2 Screening toward lead identification

When a reliable target has been found, a suitable assay has to be chosen to be used in the screening for compounds against the target, and the first major choice to make is between using either a biochemical or a cell-based assay (Macarrón and Hertzberg 2002). Biochemical assays are *in vitro* based and have been the most frequently used in high throughput screening (HTS). The biochemical assays used in HTS today are very specific and easy to use. The most frequently used assays are so called mix-and-read assays with a relatively simple experimental procedure and an end point measurement based on absorbance, fluorescence, luminescence, optical, or scintillation readouts (Macarrón and Hertzberg 2011). Cell-based assays are one of the most dynamic area of modern HTS and they have evolved into the more physiological choice of assay that rather targets a pathway in the cell with complete regulatory system and feedback control, instead of just aiming for a single molecular target as in a biochemical approach (An and Tolliday 2010). Genomics has played an important role in the development of both cell based targets and screening assays (Johnston 2002).

The end point readings for biochemical and cell-based assays are based on a variety of detection technologies and can be divided into a single measurement per well and multiple measurements per well (An and Tolliday 2010). The single well measurements includes fluorescence, luminescence and spectrophotometric methods, with fluorescence based assays being the most widely used, with a wide variety of fluorescent labeled

molecules (Gribbon and Sewing 2003). High-content screening (HCS) is a generic name for using any technique to measure multiple parameters per sample well (Kümmel et al. 2010).

1.2.2.1 Assay development and optimization

The quality of the screening in an HTS process has to be particularly reliable compared to smaller scale screening areas. Variability has to be kept low and the signal to background (S/B) has to be as high as possible to gain optimal screening results throughout the whole screening campaign (Macarrón and Hertzberg 2002). To avoid the number of factors to optimize rising to impossible levels, knowledge about the system used is of great importance (An and Tolliday 2010). The reagents and consumables used in the assay have to stay stable during the whole process. The effect of the compound solvent, usually dimethyl sulfoxide (DMSO), has to be evaluated for a possible effect on the assay performance (Macarrón and Hertzberg 2002). Determination of cell density, incubation time with the compounds and exposure time with the probe are essential factors for optimization of the performance for cell-based assays (An and Tolliday 2010).

Miniaturization lowers the costs and consumables. Today the typical working platform for HTS is 384 micro well plates, but 96 well plates as well as 1535 well plates and even up to 3456 well plates are in use. The use of reagents is drastically lowered when using smaller wells, 96 well plates require 100-200 µl per well, 384 well plates 30-100 µl, 1536 well plates 2.5-10 µl and 3456 well plates require only 1-2 µl per well (Mayr and Bojanic 2009). The increased number of compounds that can be screened per day follows the assay miniaturization. In addition, the time used for the steps of the screening assay has to be thoroughly optimized in order to make the screening campaigns time-effective. Costs, along with the time and statistical robustness are the three pillars in which a successful HTS process relies on (Mayr and Bojanic 2009).

In an automated assay, the performance is improved by decreasing variability and higher throughput of screened compounds. The automation of assays allows saving labor, consumables and reagents due to higher accuracy of the performance (Tammela 2004). For automation of a HTS system, instrumentation for routine liquid-handling, robotic plate handling and sensitive detection is required. If the used assay requires more specialized instrumentation, there can be need of washing stations, incubators, plate sealer or piercer (Thiericke 2003). Modern screening systems are mostly integrated into large independent facilities with control systems that are not necessarily in need of constant human supervision (Michael et al. 2008).

1.2.2.2 The screening process

The initial source of drug compounds used to be nature and still almost half of all newly developed drugs are natural or based on natural scaffolds (Newman and Cragg 2012). The scaffold of chemical structures produced by nature has not and probably cannot be mimicked by synthetic concepts (von Nussbaum et al. 2006). The major source of drugs based on natural products is plants, followed by animals, bacteria and fungi, and about 30% of the total number of drugs consists of semi-synthetic analogues or derivatives (Harvey 2008). Despite the fact that plants are the major source of natural based drugs there are still no antimicrobial drugs on the market that are plant based (Gibbons 2005, Newman and Cragg 2012). About 70% of all natural compounds from bacterial sources originate from *Streptomyces* spp. and myxobacteria (Grabley and Sattler 2003). However, marine organisms are significantly more taxonomically diverse than terrestrial counterparts and possess completely new, highly complex structures that may possibly have potential to be active against various drug targets. So far, they are not as thoroughly investigated, but there is an increasing trend toward more interest in compounds from marine sources (Leal et al. 2012).

Combinatorial chemistry is a fast way to synthesize large scale libraries from known structures. It was revolutionary at its introduction in the 1990s and has been re-established as a widely used technique within all steps of modern drug discovery from high throughput screening campaigns to *in vitro* and *in vivo* drug metabolism studies and pharmacokinetic assays (Kennedy et al. 2008).

Chemoinformatics is a research area, which deals with the application of informatics methods to solve chemical or biological issues. It can be used for effectively designing the optimal library to be synthesized by combinatorial chemistry, which can then be used for HTS or to establish the relationships between structure and biological activity, among other problems. In a primary screening, the library used should cover a wide range of physicochemical properties and all the compounds should preferably be drug-like to make the search more biologically relevant. In targeted library design, the opposite needs to be utilized, because similarity of the compounds in the library is emphasized instead (Matter 2003, Engel 2006). Virtual screening is a complement to HTS where large libraries of compounds (even those that do not physically exist) can be tested using a variety of selection criteria. This approach mainly ranks the compounds before the *in vitro* testing of the compounds to prioritize the testing of the candidates most likely to be positive hits. This kind of *in silico* screening has been extensively applied for ligand-based targets where large databases are searched. Approaches typical for ligand-based virtual screening include pharmacophore, machine learning and similarity methods (Engel 2006, Dürig et al. 2010).

1.2.2.3 From hits to leads to drugs

A hit in a screening campaign is a compound showing activity against the target used at relatively low molarities, depending on the target (Wunberg et al. 2006). For the hit compound to become considered as a lead compound, it has to display other properties beyond potency, for instance some degree of selectivity and specific binding. Beneficial ADME-Tox properties (absorption, distribution, metabolism, excretion and toxicity) similarly play an important role in deciding the relevance of a hit compound. If a large number of hits are found in a screening, the accessibility of the compounds and how easily synthesizable they are can be of importance in the selection (Wunberg et al. 2006). Certain physicochemical properties in order to determine whether molecules are more likely to behave as lead compounds were introduced by Lipinski et al. (2001) (Table 1), with the rules of five and specified by Teague et al. (1999) to lead-likeness properties (MW < 350 Da, logP < 3) in the late 1990s. Lipinski's fifth rule, however, excludes the four first rules if the compound has a natural origin, because they do not apply to them and would require their own guidelines (Newman and Cragg 2012). The scenario seems to be truly complex as it has been shown in a study of effective natural products drugs that half of the compounds fell well within the rules of five, while the other half violated them radically (Ganesan 2008, Newman and Cragg 2012). Most of the available antimicrobial drug compounds are also exceptions to the rules of five, since they are usually larger and more hydrophilic than other drug classes (Payne et al. 2007, O'Shea and Moser 2008).

Table 1. Rule of five properties for molecules in drug discovery and how the natural products and the antimicrobials are exceptions to these rules.

	Rules of five (Lead-likeness)	**Natural products**	**Antimicrobials**
Molecular weight	< 500 Da (< 350 Da)	ca 240-1600 Da	Usually < 600 Da, up to 1 kDa
Log P	< 5 (< 3)	Usually < 5	Usually < 5
H-bond donors	< 5	Usually < 5 up to 20	Usually < 5, up to 20
H-bond acceptors	< 10	Usually ca 10, up to 30	Usually ca 10, up to 40
References	(Lipinski et al. 2001, Teague et al. 1999)	(Ganesan 2008)	(O'Shea and Moser 2008)

For a compound to proceed in the drug development process, it also needs to have drug-like properties, such as good solubility, membrane permeability, proper half-life and pharmacophore properties to interact specifically with the target. For natural products, these properties are not always fulfilled. However, a natural product might more easily enter into a cell than a synthetic structure, but the natural compound might also be more susceptible to efflux pumps and other clearance methods (Ganesan 2008). Chemical optimization of the molecules can be carried out either at the lead discovery stage, by synthesizing analogues that possess improved properties to better fit the lead search, or

the discovered lead molecules can be optimized to have improved drug-likeness, in other words, drug optimization (Proudfoot 2002). For natural products, this can be a difficult task and often the compound is in its original form throughout the drug development (Ganesan 2008). This process can be carried out the other way around: first demanding the criteria and then searching for candidates. This probably increases the chance of finding positive hits within the scope of the search, but reduces the likelihood of discovering new drug classes (Muegge 2003). The design of natural product structures has had a biosynthetic purpose for the producer organism and is, hence, probably advantageous over synthetic molecules in bioactivity (Mishra and Tiwari 2011).

1.2.2.4 Statistical tools

Signal-to-noise (S/N) is a parameter distinguishing the real signal from the background noise (Macarrón and Hertzberg 2002). Another parameter used is signal to background which only distinguishes the maximal signal from the minimal signal, but no variation is taken into account. Signal-to-background (S/B) ratio is signal and equipment dependent and can be said to only be used within one assay to prove good plate-to-plate and day-to-day repeatability (Macarrón and Hertzberg 2002). This means that neither S/N nor S/B takes the variability and dynamic range of the signal simultaneously into account (Zhang et al. 1999).

The measuring of the relative variability of the signal or the background signal coefficient of variation (CV) is a useful parameter. The parameter indicates the stability and precision of the assay behavior, such as liquid handling and detection instrumentation (Macarrón and Hertzberg 2002).

The separation band is defined by the difference between the positive controls and the negative controls and measures the dynamic range of the assay (Figure 2) (Zhang et al. 1999). The *Z'*-factor is a dimensionless parameter able for comparison across methods. The parameter is defined as the ratio of the separation band to the signal range of the assay. In other words, it shows the relative separation of the maximal signal values from the background values (Zhang et al. 1999). It has a range from 0 to 1, where $Z'=0$ indicates the maximal and minimal signals overlap and $Z'=1$ indicates the ideal assay with infinite separation (Zhang et al. 1999). Usually, $Z'>0.5$ is considered to designate a properly performing assay. However, for cell based assay $Z'\geq 0.3\text{-}0.4$ is considered to be acceptable (Merten 2010). There is a correlation between *Z'*, S/B and CV: a *Z'* of 0.4 is equivalent to a CV of 10% and S/B of 3. A lower variation, accordingly, allows lower S/B, but due to CV, which hardly ever falls below 5%, an S/B of 2 is required to have an acceptable result for *Z'* (Macarrón and Hertzberg 2011). *Z'* should be used during validation of the screening assay but also throughout the screening process to be able to detect failed plates and exclude the results within them to avoid false results (Macarrón and Hertzberg 2002). To make a distinction between the tested compounds that do not

have any effect and those that show activity, a threshold value or a hit limit has to be set (Mayr and Bojanic 2009). In large screening campaigns, unbiased libraries of compounds are usually used and most of these are expected to show low or no activity and the activity histogram is usually close to be normally distributed. The hit limit can then be defined as SDs away from the mean of the control signal, 3 x SD is the most common choice (Zhang et al. 1999). However, the hit limit can also be empirically chosen depending on the assay, for example 50% activity, to retrieve reasonable numbers of hits to be handled in secondary screening assays. The determination of the hit limit is of utmost importance in order to end up with the best ratio of false negative and false positive results. The further away from the mean of the control the hit limit is set; the lower the risk of false positives. However, the risk of false negatives increases (Zhang et al. 1999). Compounds found close to the hit limit have some probability to cross over if retested and in the ideal situation very few points are found close to the hit limit; they are either clearly positive or clearly negative. This can be achieved by increasing the quality of the assay by lowering the variability (Zhang et al. 1999).

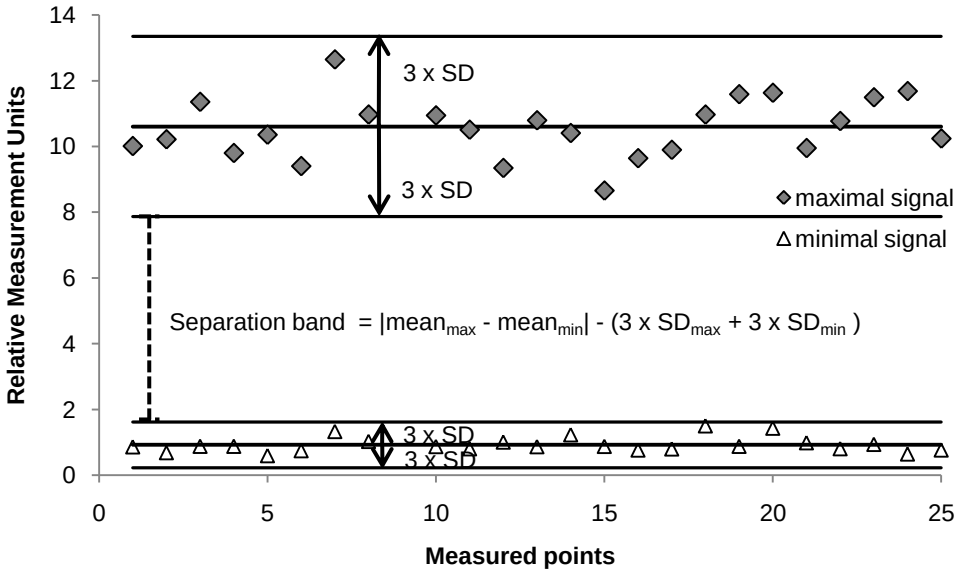


Figure 2. A schematic view of a model signal window and the equations of the statistical parameters. The values of the parameters are calculated from the Relative Measurement Units of the points.

1.2.3 Antimicrobial screening assays

There are many available methods that are standardized for testing compounds for antimicrobial activity against planktonic bacteria. These include: disk diffusion, agar dilution, antibiotic gradient disks, and broth micro dilution; they are appropriately optimized and have been used for decades (Jorgensen 1993, Amsler et al. 2010). Broth

micro dilution is the most widely used due to its simplicity and suitability for most bacterial strains (Jorgensen 1993, Rahman et al. 2004). The end point can be visualized or measured spectrophotometrically or using a colorimetric redox indicator (Rahman et al. 2004).

The corresponding situation is different when it comes to anti-biofilm screening. There are hardly any standardized methods for studying biofilms and especially not for screening for substances with anti-biofilm activity (Pettit et al. 2009). One assay for growing and treating a *P. aeruginosa* biofilm in a high throughput screening setting called the MBEC™ Assay has been standardized by the American Society for Testing and Materials (ASTM International 2011). Similarly, a few anti-biofilm screening assays have been developed using the most common biofilm forming species of *P. aeruginosa*, *E. coli*, *S. aureus* and *S. epidermidis*. These methods are mostly based on the 96 micro well plate format, with one exception being an anti-biofilm screening against *P. aeruginosa* biofilms in 384 well plates, using a BacTiter Glo based assay (Junker and Clardy 2007). Other methods use turbidity measurements (Ceri et al. 1999), crystal violet (Stepanovic et al. 2000), crystal violet together with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and log reduction (Pitts et al. 2003), or resazurin (Pettit et al. 2005, Pettit et al. 2009, Mariscal et al. 2009). Other staining methods have been developed for detecting biofilms or effects on biofilms, but not for screening purposes. Tetrazolium salts, such as XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) are reduced by metabolic activity to a colored formazan product that can be visually or spectrophotometrically detected, which measures cell viability in the biofilms (Adam et al. 2002, Peeters et al. 2008). Another viability probe is fluorescein diacetate, which is converted into highly fluorescent fluorescein by esterases found both intra- and extracellularly (Honraet and Nelis 2006). SYTO 9 has been widely used in microscopy approaches for detecting living cells, both eukaryotic and prokaryotic in a combination with a probe staining only the dead cells, i.e. a LIVE/DEAD staining kit (Karthikeyan and Beveridge 2002, Jefferson et al. 2005). SYTO 9 penetrates the cell membranes and binds to the DNA of living and dead cells, causing a green fluorescent signal (Honraet and Nelis 2006).

So far, no method has been developed that can measure all features desirable for anti-biofilm studies. The existing methods measure only one feature each. Viability can be measured by resazurin (O'Brien et al. 2000), total biomass can be measured by crystal violet (Christensen et al. 1985) and the effect on the EPS layer can be measured by wheat germ agglutinin linked to an Alexa fluorophore (Burton et al. 2007), or 1,9-dimethyl methylene blue (DMMB) (Toté et al. 2009).

Table 2. Methods for studying biofilms and effect of compounds on biofilms.

Staining probe	Detection method	Measured feature	Assay format	Key references
	Plate count	CFU	Agar plates	
	Turbidity	growth	96 mwp, pinlids	Ceri et al. 1999
Safranin or trypan blue stains	Visual detection	biomass	Test tube	Christensen et al. 1982
Crystal violet	Visual detection, absorbance	biomass	Tube test, 96-mwp	Christensen et al. 1985, Stepanovic et al. 2000
Tetrazolium salts (XTT, MTT, etc)	Absorbance	viability	96-mwp	Adam et al. 2002, Peeters et al. 2008
Fluorescein diacetate	Fluorescence	viability	Flow system	Honraet and Nelis 2006
Resazurin	Fluorescence, absorbance	viability	96-mwp	O'Brien et al. 2000, Mariscal et al. 2009, Pettit et al. 2005
SYTO 9	Fluorescence, microscopy	viable cells	Flow system, 96-mwp	Honraet and Nelis 2006, Jefferson et al. 2005
BacTiter Glo	Luminescence	Attachment vs detachment	384-mwp	Junker and Clardy 2007
WGA-Alexa fluorophore	Fluorescence, microscopy	EPS	96-mwp	Burton et al. 2007
Dimethyl methylene blue (DMMB)	Absorbance	EPS	96-mwp	Toté et al. 2009

2 Aims of the study

Most of the methods used in antimicrobial research have been developed for planktonic bacteria. However, bacteria in natural environments mainly exist in biofilm form and biofilms are more prone to cause severe infections than their planktonic counterparts. Additionally, since the infections caused by biofilms are often very difficult to treat, there is a need to find new active anti-biofilm agents. The aim of this research project has been to fill the void of screening assays that can be applied for the search of novel anti-biofilm compounds, using *Staphylococcus aureus* as a model strain. What distinguishes this study is the embraced strategy, as it is aimed to develop a platform of cell-based assays, which could measure the essential features of bacterial biofilms, most relevant from a drug discovery perspective. In this approach, the focus is on building high-information, multi-signal, screening-based assays that could permit the identification of true anti-biofilm hits.

The specific aims of the study were to:

- optimize the performance of the biomass based crystal violet assay with statistical tools, to be used for automated anti-biofilm screening of a natural compounds library **(I)**

- evaluate the use of the resazurin probe for viability detection on a fast anti-biofilm screening of a natural and naturally-derived chemical library **(II)**

- improve the anti-biofilm assay platform by addition of a third assay based on specific binding of a WGA-probe to the matrix and establish a platform of assays for screening of libraries against bacterial biofilms by targeting viability, biomass and the EPS-layer **(III)**

- develop an additional method using capillary electrochromatography (CEC) for immobilizing biofilm-forming bacteria and studying anti-biofilm effects of compounds **(IV)**

- apply the three optimized assays in a validatory antimicrobial and anti-biofilm screening using a library of cinchona alkaloids **(V)**

3 Materials and methods

3.1 Materials and equipment

Tryptic soy broth (TSB) and tryptic soy agar (TSA) were purchased from Fluka Biochemika (Buchs, Switzerland). Crystal violet (2.3% w/v), resazurin, dimethylsulfoxide (DMSO), 3-aminopropyltriethoxysilane (APTES), poly-L-lysine and Mueller-Hinton broth were from Sigma–Aldrich (Steinheim, Germany). Wheat germ agglutinin-Alexa 488 and LIVE/DEAD® *Bac*Light™ were from Molecular Probes, Inc. (Eugene, Oregon, USA). Phosphate buffered saline (PBS) was from Lonza (Verviers, Belgium). Hydrochloric acid was from Oy FF-Chemicals Ab (Yli-Ii, Finland). All the antibiotics used; penicillin G, ciprofloxacin, fusidic acid, methicillin, oxacillin, polymyxin B, rifampicin, streptomycin, and vancomycin were from Sigma–Aldrich (Steinheim, Germany).

The 96-well polystyrene micro well plates with Nunclon™ Δ surface were from Nunc (Roskilde, Denmark). The uncoated fused-silica capillaries were from Composite Metal Services Ltd. (Worcestershire, UK). The Varioskan Flash Multimode Plate Reader and the Multidrop® Combi dispenser were from Thermo Fisher Scientific Oy (Vantaa, Finland). The Biomek® 3000 liquid handling workstation was from Beckman Coulter, Inc. (Fullerton, California, USA). The Hewlett-Packard 3DCE system was from Agilent (Waldbronn, Germany). The Lauda Ecoline Re-104 water bath was from Lauda (Lauda-Königshofen, Germany). The Nanoscope IIIa scanning probe microscope equipped with a J-scanner was from Digital Instruments, Inc. (Santa Barbara, California, USA) and was used for imaging the sample surfaces. The silicon cantilevers (model NSC15/NoAl were from MicroMasch (Tallinn, Estonia). The AxioVert 200M fluorescence microscope was from Carl Zeiss MicroImaging GmbH (Munich, Germany).

3.1.1 Compound libraries

A chemical compound library of 686 compounds was provided by Professor Thomas Erker at the University of Vienna, Austria. The cinchona alkaloid library consisting of 24 compounds was provided by Professor Reko Leino at the Laboratory of Organic Chemistry, Åbo Akademi University, Finland. The library of 86 bacterial extracts from the Arctic Microbe culture collection (ARMI) was provided by Dr. Minna Männistö at the Finnish Forest Research Institute (Metla), Rovaniemi, Finland. An in-house collection of 123 natural compounds was also used, Department of Biosciences, Åbo Akademi University, Finland.

3.1.2 Bacterial strains (I-V)

The bacteria strains *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* Newman, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus epidermidis* RP62A (ATCC 35984) and *Escherichia coli* XL1 Blue were obtained from the Faculty of Pharmacy and the Faculty of Veterinary Medicine, University of Helsinki, Finland.

3.2 Methods

3.2.1 Bacterial culture (I-V)

All strains were stored in -70°C in tryptic soy broth (TSB) containing 20% glycerol. Fresh cultures were started from the glycerol stocks and pre-cultured in TSB over night in 37°C with 220 rpm shaking. The liquid cultures were prepared by diluting the pre-cultures 1000 times (*S. aureus*-strains, *E. coli*) or 100 times (*S. epidermidis*) in fresh TSB and under aerobic conditions at 37°C and 200 rpm to reach exponential growth. The concentration was routinely estimated by spectrophotometric turbidity measurement at 595 nm using a Varioskan Multimode Plate Reader. For determination of the concentrations, the cultures were serially diluted and plated on tryptic soy agar (TSA). The bacterial concentration was established as colony forming units per milliliter (CFU/ml).

3.2.2 Optimization of biofilm formation conditions (I-V)

Biofilms were always formed from fresh cultures, by diluting the exponentially grown culture to be approximately 10^6 CFU/ml. The biofilms were grown in sterile 96-microwell titer plates by dispensing 200 µl of the diluted bacterial culture per well. Biofilms were formed in 37°C, 200 rpm for 18 h. These conditions were optimized by testing various dilutions of the culture (from 10^3 to 10^8 CFU/ml) and incubation times (from 4 to 24 h). Crystal violet was used for the endpoint measurement.

3.2.3 Cryopreservation of *S. aureus* biofilms (I)

To store ready-made biofilms containing plates, 96-well polystyrene micro well plates with biofilms were prepared as described in 3.2.2. After 18 h of biofilm formation in optimal assay conditions, the planktonic suspension was removed and the plates containing biofilms were sealed and immediately frozen at −20°C for a period of up to 7 days. The bacterial concentration was compared in fresh and frozen plates made from the same culture by detaching the biofilms mechanically, diluting the bacteria in fresh TSB and performing counts on TSA plates. After 7 days, biofilm formation determined by crystal violet staining was also compared with freshly prepared biofilms. Similarly, the ability of the preserved biofilms to perform in a screening assay using model compounds was estimated and compared with freshly prepared biofilms. Briefly, plates were

removed from the deep-freezer 1 h prior to the experiments and were defrosted at room temperature. The selected compounds were added to the plates with fresh TSB and incubation was carried out in the same conditions used for screening (37 °C, 200 rpm, 2 h). The plates were stained with crystal violet stain assay.

3.2.4 Automation of the crystal violet staining protocol (I)

First, a manual performance of the crystal violet staining assay was optimized. The biofilms formed in a 96-well plate were washed once with 200 µl of MQ-water prior to the addition of 190 µl of the crystal violet solution. The plate was incubated for 5 minutes at room temperature (RT). The stain was removed and the plate was washed twice with MQ-water. After the last washing step the plate was left to dry and the remaining stain was dissolved in 96% ethanol. Absorbance was spectrophotometrically measured at 595 nm.

Automation of the assay was conducted by using the Multidrop Combi dispenser for sterile dispensing of the bacteria onto 96-well plates with an addition of the crystal violet stain. The Biomek® 3000 liquid handling workstation was used for the washing steps. The same staining protocol as was used in the manual method was utilized, except for an additional washing step after the removal of the stain. The programming of the dispensing and aspiration steps was optimized and the optimal settings were used for the experiments (dispensing speed 150 µl/s, aspiration steps conducted from the edge of the well bottom).

3.2.5 Optimization of the resazurin staining assay (II)

The resazurin stain was prepared in sterile water and kept sterile for further usage at +4°C during one month. For the staining procedure, the culture media and non-adhered cells were removed from the mature biofilms and replaced by resazurin diluted in PBS. Four concentrations of the stain were initially tested, ranging from 2 µM to 2 mM. The plates were incubated in darkness and at RT during six different ranges of time (15, 20, 30, 60, 120 and 240 minutes). As a final step, the fluorescence was measured using an excitation filter of 560 nm and an emission filter of 590 nm.

The relation between the fluorescent signal generated by the reduced resazurin and bacterial concentrations in the wells was studied for both bacterial suspensions and biofilms. In the first case, dilutions of an exponential phase bacteria culture (from 2.80 x 10^4 to 2.80 x 10^8 CFU/ml) were prepared in 96-well micro titer plates. Resazurin stain was added (20 µM) directly to the wells and the plates were incubated in darkness, RT for 20 minutes followed by the measurement of fluorescence as indicated above. In the second case, different biofilm concentrations were achieved by incubating suspensions

on the plate during different time periods ranging from 1 hour to 24 hours. For the staining, planktonic bacteria were removed and resazurin (20 µM) added as described above. To measure actual concentrations in the wells, biofilms from replicate wells were scraped and plated on TSA, as indicated before.

3.2.6 Wheat germ agglutinin staining (III)

The wheat germ agglutinin probe was kept in −20°C in 1 mg/ml stock solutions and always kept from redundant light exposure. The protocol used was according to the original description of the method by Burton et al. (2007). Briefly, the planktonic suspension was removed from the wells and the wells were washed once with sterile PBS. The stain was added (5 µg/ml) and the plate was incubated in darkness, +4°C for two hours. Unbound stain was washed off by washing the wells with 200µl of PBS three times, followed by air drying the plate for 15 minutes at RT. The bound stain was dissolved in 33% acetic acid and sonicated twice with an incubation period of one hour in between to detach the bound mass from the wells. The fluorescence was then measured at a Varioskan Multimode plate reader.

3.2.7 Capillary electrochromatography (IV)

Phosphate and acetate buffers (8 mM) were used as background electrolyte (BGE) solutions. Bacterial suspensions in BGE were prepared by centrifuging TSB suspensions at 2500*g* for 5 min, re-suspending the bacterial pellet in BGE, with the centrifugation step repeated twice.

The electrophoretic measurements were carried out with a Hewlett-Packard 3DCE system equipped with a diode array detector and an air-thermostating capillary. The capillary was coated with poly-L-lysine (1:10 v/v diluted in BGE) by flushing for 30 min with a sterile phosphate saline buffer, for 30 min with poly-L-lysine, and finally, the capillary was flushed once again with sterile PBS for 15 min to remove the extra poly-L-lysine not bound to the capillary surface. Following this, the capillary was treated for 17-20 h with bacteria. Briefly, the capillary was flushed for 30 min with *S. aureus* and left to stand filled with bacteria for 30 min. The whole 60 min treatment was repeated 17-20 times.

DMSO was used as EOF marker and worked as an indicator for surface charge changes during biofilm formation. The biofilm coated capillary was flushed for 2 min with the antibiotics, and then the antibiotics were left standing for 2 h. EOF was also monitored in the antibiotic/biofilm interaction studies by carrying out six successive runs. The experiments in the capillary and auto-sampler were always performed at 37°C. Before

every experiment, the coated capillary was flushed for 2 min with BGE. The time of the runs ranged from 5 to 20 min depending on the mobility of the EOF marker.

3.2.8 Antibiotics susceptibility testing (I, IV)

Minimal inhibitory concentrations (MIC) were established by using standard techniques according to the CLSI, with some modifications. To establish planktonic MIC for the antibiotics used, the compounds were serially diluted two-fold in plates (from $1.2x10^{-4}$ mg/ml to 1.024 mg/ml) and with bacteria added. The plates were incubated at 37°C and 200 rpm for 18 h and then stained using the crystal violet assay. Biofilm MIC values were established by adding the serially diluted antibiotics to mature biofilms and incubating in 37°C and 200 rpm for 24 h and then stained with crystal violet. The lowest concentration of the antibiotics able to prevent biofilm formation (planktonic MIC) or eradicate mature biofilms (biofilm MIC) and causing the crystal violet signal to be lower than the hit limit, was determined as MIC.

3.2.9 Screening of compound and extract libraries (I, II, V)

All compounds of the in-house library were prepared as dry DMSO stocks (20 mM) and stored at −20°C. Aliquots were further diluted in TSB to reach a final concentration of 40 µM and a DMSO final concentration of 0.25%. In cases of solubility problems no further dilution in TSB was used and maximal DMSO concentration used was 2%. The bacterial extracts were prepared to 30 mg/ml in dry DMSO and the final concentration tested on biofilms was 0.6 mg/ml. Screening was run in two different modes: prevention of biofilm formation and destruction of formed biofilms. In the biofilm prevention experiment, the compounds were added simultaneously with the bacterial suspension and were incubated in optimal biofilm-forming conditions (18 h, 37°C, 200 rpm). In the destruction mode, mature biofilms were grown for 18 h, the planktonic suspension was removed and replaced by fresh TSB along with the compounds and incubated in optimal biofilm-forming conditions for 2 or 24 h.

The effect of DMSO was established using the crystal violet assay by making two-fold serial dilutions to achieve final DMSO concentrations ranging from 0.001% to 20%.

3.2.10 Visualization using atomic force microscopy (AFM) (I, IV)

Bacterial biofilms formed on polystyrene pieces were used for AFM visualization. The pieces were taken from micro well plate bottoms (Nunclon™ Δ surface) and were small enough to fit the microscope sample area. Polystyrene pieces with biofilms formed during 0 h and 18 h, as well as polystyrene pieces with TSB during 0 h and 18 h, were prepared. In 0 h samples, the bacterial suspension (10^6 CFU/ml) or TSB was added to the

polystyrene plate and then immediately removed. Following incubation, the planktonic suspension was removed and the plates were left to air dry for 30 min in sterile conditions.

A Nanoscope IIIa scanning probe microscope equipped with a J-scanner was used for imaging the sample surfaces. The microscope was placed on an active vibration isolation table (MOD-1M; JRS Scientific Instruments, Zwillikon, Switzerland), which was further placed on a massive stone table to eliminate external vibrational noise. Silicon cantilevers (model NSC15/NoAl) were used for imaging. All images (512 × 512 pixels) were captured using the intermittent contact AFM mode in ambient conditions (RH = 34 ± 5%, T = 25 ± 2 °C) without filtering. The free amplitude of the oscillating cantilever (off contact) was ca. 60 ± 15 nm. A damping ratio (contact amplitude/free amplitude) of ca. 0.7–0.8 and a line frequency of 1.00 Hz was used for imaging. The Scanning Probe Image Processor (Image Metrology, Hørsholm, Denmark) software was used for the image analysis.

3.2.11 Visualization using Fluorescence Microscopy (III, V)

Mature (18 h) biofilms were imaged for viability and EPS production using Fluorescence Microscopy (FM). Viability imaging was undertaken with the commercial bacterial viability kit LIVE/DEAD® *Bac*Light™ that contains SYTO 9 (stains viable cells green) and propidium iodide (stains dead cells red). Final concentrations of the probes (added as a mixture, 6 µl/well) were 5 µM and 30 µM, respectively. For EPS imaging, the WGA probe was added and incubated for 2 h at 4°C in darkness, as previously described. FM pictures were taken after removing the unbound dye. Images were captured with an AxioVert 200M fluorescence microscope, using a FITC filter (SYTO 9, WGA) or a TRITC filter (propidium iodide).

3.2.12 Data processing and statistical analysis (I-V)

For characterizing the assays, plates containing positive (bacteria, maximal signal) and negative (TSB, minimal signal) control wells were made, so that plate-to-plate and day-to-day variations could be established. The plate-to-plate variability was established by comparing the mean of the maximal signal in three plates made the same day, whilst the day-to-day variability was made by comparing the mean of the maximal signal of three plates made on separate days. In all cases, coefficients of variations of the maximal signal were calculated. Statistical parameters characterizing the performance of the screening assay were calculated and used to monitor the assay optimization process. The parameters signal window coefficient Z'-factor, signal-to-noise (S/N), signal-to-background (S/B), separation band, and coefficient of variations (CV) of the signals were calculated using the corresponding formulae indicated below. The hit limits used in the screening experiments were calculated according to equation 6. In all equations, SD_{min},

X_{min} and SD_{max}, X_{max} represent the standard deviations and means of the minimal (min) and maximal (max) signals, respectively.

The Kolmogorov-Smirnov test was used to assess the signals' binominal distribution. One-way ANOVA comparisons and Tukey post tests were applied to the original fluorescence and absorbance data points. In the case of paired comparisons, unpaired t-test with Welch's correction was used, where $p<0.05$ was considered statistically significant. GraphPad Prism software, v. 5.0 for Mac OS (La Jolla, CA, US) was used for the entire span of calculations.

$$Z' = 1 - \frac{(3*\mathrm{SD_{max}} + 3*\mathrm{SD_{min}})}{|\mathrm{X_{max}} - \mathrm{X_{min}}|} \quad \text{Eq. 1}$$

$$\mathrm{S/N} = \frac{\mathrm{X_{max}} - \mathrm{X_{min}}}{\sqrt{\mathrm{SD_{max}}^2 - \mathrm{SD_{min}}^2}} \quad \text{Eq. 2}$$

$$\mathrm{S/B} = \frac{\mathrm{X_{max}}}{\mathrm{X_{min}}} \quad \text{Eq. 3}$$

$$\text{Separation band} = |\mathrm{X_{max}} - \mathrm{X_{min}}| - (3 * \mathrm{SD_{max}} + 3 * \mathrm{SD_{min}}) \quad \text{Eq. 4}$$

$$\mathrm{CV} = 100 * \frac{\mathrm{SD_{max}}}{\mathrm{X_{max}} - \mathrm{X_{min}}} \quad \text{Eq. 5}$$

$$\text{Hit limit} = \mathrm{X_{max}} - 3 * \mathrm{SD_{max}} \quad \text{Eq. 6}$$

4 Results

4.1 Optimization of biofilm formation conditions (I, IV)

To determine the optimal conditions for *S. aureus* biofilm formation in 96-well micro well plates ten different time ranges (2 to 24 h) and six different bacterial concentrations (10^3 to 10^8 CFU/ml) were tested and stained using the manual crystal violet assay. Previously, a wide variety of conditions for biofilm formation in 96-well micro well plates have been reported using initial bacteria concentrations from 10^5 to 10^8 CFU/ml and incubation times from 4 up to 24 h (Amorena et al. 1999, Stepanović et al. 2001, Giacometti et al. 2005). The optimal biofilm formation according to statistical performance was detected using 10^6 CFU/ml as the initial bacterial concentration. At that concentration, a significant biofilm formation was registered when bacteria were incubated between 12 and 18 h in 37°C and 200 rpm. For practical convenience in performing the experiments overnight, 18 h of biofilm formation was further selected. However, biofilms also seem to survive longer without refreshing of nutrients in 96-micro well plates as well as in the CEC system (IV), as biofilms were kept in the capillary for two days after formation.

To confirm biofilm formation in these optimal conditions, imaging experiments using AFM were conducted. A clean surface was obtained in the negative control wells indicating the absence of biofilms when incubating the plates only with TSB (Figure 2A in Publication I). Moreover, no biofilm was detected in samples in which bacteria were added and then removed immediately afterward (0 h sample). The image of the sample treated with bacteria for 18 h showed a homogeneous surface of round bacteria capsules (Figure 2B in Publication I).

4.2 Screening assay platform (I-III)

4.2.1 The crystal violet assay (I)

4.2.1.1 Optimization and detection limit (I)

The manual performance of the crystal violet staining assay was optimized by modifying previously published protocols (Stepanovic et al. 2000, Kolari et al. 2001). This was carried out in order to improve the performance without endangering the statistical quality of the assay. Modifications to the protocol consisted of reducing the number of washings steps that were conducted: one washing before adding the stain was carried out instead of two, and two washing steps to remove unbound stain were undertaken instead of three washing steps after the staining. These modifications did not affect the statistical performance of the assay, as the parameters were similarly good, but they allowed for cutting down the time required and decreasing the labor involved in testing. Although the crystal violet assay has been extensively applied, the detection limit has not been emphasized. This is probably due to the fact that the readout signal measures both living

and dead cells (that together with the EPS generally is referred to as the biomass). However, we attempted to measure the detection limit, as it can still provide information on the linear dependence range of the assay. If this assay is used in combination with other assays, this information is relevant to understand whether the assays are able to quantify biofilm formation in a similar fashion. The lowest biofilm concentration resulting in a signal was $2x10^7$ CFU/ml (Figure 3) which coincides well with bacterial densities (10^7 CFU/cm^2) normally reached in micro plate wells (Pitts et al. 2003).

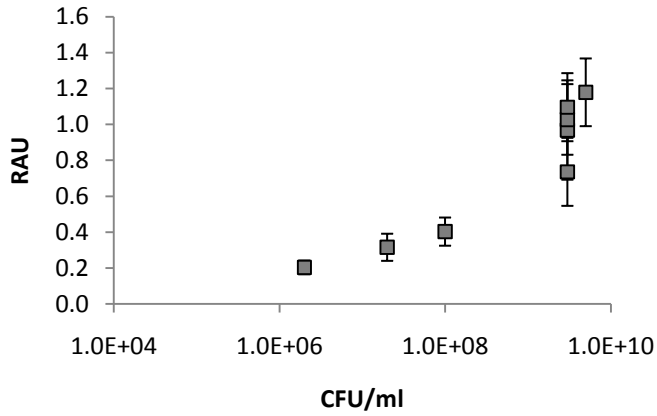


Figure 3. Relation between bacterial concentration and relative absorbance units of biofilms stained with crystal violet.

4.2.1.2 The crystal violet assay; manual vs automated performances (I)

After optimization of the manual protocol it was then transferred into an automated environment by introducing a Biomek® liquid handling robot during all the steps of the assay, with the exception of the cellular dispensing and the crystal violet addition steps that were performed with a Multidrop dispenser. A comparison was made between the manual and the automated assays in terms of the signal window, which is defined as the separation of the maximal signals (biofilm samples) and the minimal signals (TSB controls) (Figure 4). In addition, the assay performance in terms of statistical parameters was compared for the manual and the automated assay (Table 2 in Publication I). In the automated approach, the mean of the maximal signal decreased but the dispersion of the maximal signal points was also lower, resulting in similar *Z'* values for both assays. The obtained *Z'* values for both assays (> 0.4) can be regarded as an indicator of a well performing cell-based screening assay. Although the separation band was narrower in the automated assay due to the lower maximal signal and slightly higher minimal signal (Figure 4), a significant improvement in the repeatability measures was registered. This was due to that the plate-to-plate variability decreased from 3.50% to 0.64% and day-to-

day variability was reduced from 35.2% to 8.90% when the automated performance was utilized.

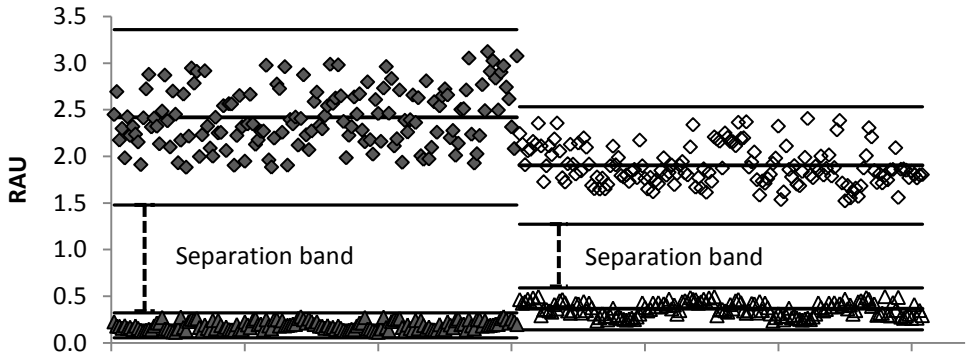


Figure 4. Signal window graph of crystal violet methods, filled symbols represent the manual assay and empty symbols the automated assay. Squares represent biofilm controls and triangles TSB controls. 3 x SDs of the controls are shown and the separation bands of the assays are the dashed lines.

4.2.1.3 Validatory screening and antimicrobial susceptibility (I)

The performance of the automated crystal violet assay was further tested by investigating the susceptibility to eight different antibiotics with previously reported effect on *S. aureus* bacteria (Table 2 in Publication I) and running a validatory screening campaign using a small in-house library of natural, commercially available, low molecular weight molecules (123 compounds, Figure 4 in Publication I).

Planktonic bacteria were more susceptible to treatment than the biofilms, when using the most active antibiotic compounds. None of the antibiotics was able to kill more than 50% of the biofilms and thus, MIC_{50} for biofilms as well as both MIC_{50} and MIC_{90} for planktonic bacteria were established for the antimicrobials. The MIC_{50} ratios ranged from being equally effective to 1000 times more effective against planktonic bacteria than biofilms (Table 1 in Publication I). Rifampicin was found to be the most active against *S. aureus* biofilms followed by fusidic acid, penicillin G and oxacillin. For the planktonic counterparts, the most active agents were the same but in a slightly different order: rifampicin > penicillin G > oxacillin > fusidic acid. Polymyxin B, streptomycin and vancomycin had only minimal effect on biofilms and only a slightly higher effect on planktonic bacteria than on biofilms.

The compounds found as positive hits in both prevention and destruction screening of the natural products collection, farnesol and lauryl gallate, have previously been reported to have antimicrobial and anti-biofilm activity against *S. aureus* bacteria (Kubo et al. 2002,

Jabra-Rizk et al. 2006, Unnanuntana et al. 2009). None of the other compounds in the screened library has been reported to be active against *S. aureus* biofilms, indicating that the assay was not giving rise to neither false positive nor false negative hits.

4.2.1.4 Cryopreservation of biofilms for faster screening (I)

To additionally improve the efficiency of the screening assay, a trial was performed using cryopreserved biofilms on plates. The typical biofilm assay testing takes four days when including all of the steps from preculturing the bacteria to staining the destruction experiment plate (see scheme in Figure 1 in Publication III). The use of cryopreserved biofilms would shorten the whole screening process from 4 to 2 days, as the biofilms could be grown in larger batches, stored and be used when needed without the need of re-culturing the bacteria and forming the biofilms.

To validate this approach, the cryopreserved biofilms were compared with freshly prepared biofilms. Bacterial viability, as measured by CFU counts on TSA plates, was slightly decreased (although not statistically significantly) in 7 days-cryopreserved biofilms. In terms of assay performance, a slight increase was observed in the dispersion of the data points when using cryopreserved biofilms. This resulted in a decrease of Z' (from 0.49 to 0.34) as well as S/B. Then again, the plate-to-plate variability was kept at about 3% when performing the assay with cryopreserved biofilms, but the day-to-day variability significantly decreased from 35% to 13%, when compared to the manual assay. In addition, the activity of eight model antibiotics was tested in cryopreserved and fresh biofilms and the results showed similar activities of the compounds on both biofilm types.

4.2.2 The resazurin assay (II)

4.2.2.1 Optimization of the performance

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) is non-fluorescent and blue in its oxidized state and can be reduced by metabolically active cells to resofurin, which is highly fluorescent and pink (Guerin et al. 2001). The resofurin molecule can be further reduced to non-fluorescent and colorless hydroresofurin and thus, the staining time and the concentration used of the probe had to be optimized to find stable conditions for measuring the fluorescence. During the optimization experiments, 4 different stain concentrations as well as 6 different incubation times with the probe were tested. We found that 20 min incubation time using 20 μM resazurin was the shortest possible incubation time and lowest possible resazurin concentration providing statistically good performance results (Z'=0.66, S/N=9.41, S/B=10.56). Incubation time periods between 30 and 120 min correspondingly provided statistically satisfactory signals, but they implicated longer duration assays with no significant benefits from the screening point of view. When the incubation was extended to 240 minutes, a reduction in the detectable

fluorescence signal was observed, which decreased the assay quality (from *Z'*>0.6 to *Z'*<0.4). This behavior was likely due to the accumulation of the colorless, non-fluorescent hydroresofurin.

4.2.2.2 Planktonic calibration curve and detection limits

The resazurin assay can also be directly applied to planktonic bacteria and it had previously been suggested that planktonic calibration curves could be used to estimate the number of bacteria in the stained biofilms (Toté et al. 2008). To be able to conduct a better interpretation of resazurin fluorescent signals and to establish the detection limit of the assay, the relation between planktonic and biofilms bacterial concentration was studied in closer detail (Figure 5). In both cases, a sigmoidal behavior of the fluorescent signal with increasing bacterial concentrations (as measured by actual CFU/ml in planktonic or biofilms) was registered. However, a preliminary comparative analysis of the curves reveals that similar resazurin fluorescent signals cannot be related to equivalent bacterial concentration in suspensions and in biofilms. The actual bacterial concentration associated with a certain fluorescent signal, is higher for cells in biofilms compared to in planktonic suspensions, i.e. at 100 RFU the actual bacterial concentration (in CFU/ml) was 59% higher for planktonic bacteria than for biofilm bacteria. A similar bacterial concentration generates a higher fluorescent signal when cells are in suspension, compared to biofilms at the similar cell concentration, i.e. at $1x10^8$ CFU/ml the RFU for planktonic bacteria was 32% higher than for biofilm bacteria. A plausible explanation for this result is that it is indeed more difficult for the probe to be evenly distributed among the more densely packed biofilms cells than among suspended single bacterial cells. The metabolic activity among the cells in a biofilm varies and metabolically inactive cells can be present in the cellular core of the biofilm, causing a reduction of the fluorescent signal.

The observation was made that the detection limit of the probe is high; concentrations higher than 5×10^7 CFU/ml are required of *S. aureus* bacteria in the well, to reach significant resazurin fluorescent signals for biofilm cells. However, such as in the crystal violet assay, the concentration required corresponds well to reported bacterial densities (10^7 CFU/cm^2) in micro plate wells (Pitts et al. 2003). A linear increase-phase of the fluorescent signal was registered within a concentration range of 6×10^6 - 3×10^8 CFU/ml. Under the optimal conditions identified in our assay, the fluorescent signal in the maximal signal wells (bacteria controls) is found at the end of this linear phase.

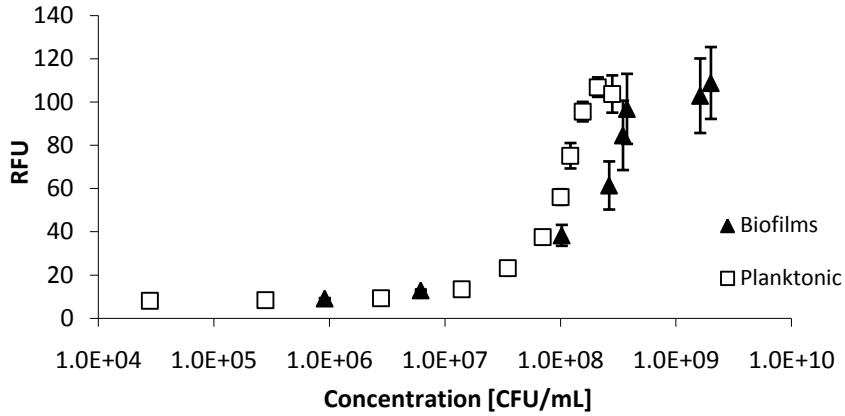


Figure 5. Relation between bacterial concentration (in CFU/ml) and relative absorbance units versus biofilms stained with resazurin.

4.2.2.3 Assay performance

The maximal (only biofilms) and minimal (only TSB) fluorescent control signals of this screening assay were normally distributed and clearly separated. Moreover, the average *Z'*-factor value calculated for the assay (0.66) as well as S/N (9.41), S/B (10.56) and CV (14.06%) indicated that the assay performs well, especially when taking into account that it is a cell-based method. The well-to-well, plate-to-plate and day-to-day variabilities were in all cases lower than 13%, which also support the assay repeatability as high.

The resazurin assay consists of one very simple addition step. Performing the assay in manual conditions does not demand high labor efforts. Using an automated protocol would actually have increased the labor and prolonged the assay procedure. The procedure comprised about 30 seconds to manually add 200 µl per well in a 96-well plate, while in comparison, using the liquid handling robot it acquired more than 2 minutes (using an 8-tip head multipipette, without changing the tips between columns). Furthermore, the robot had to be loaded with tips and reagents, which prolonged the time needed for the assay procedure. Automating the assay would not have represented a key necessity in order to screen libraries of moderate sizes.

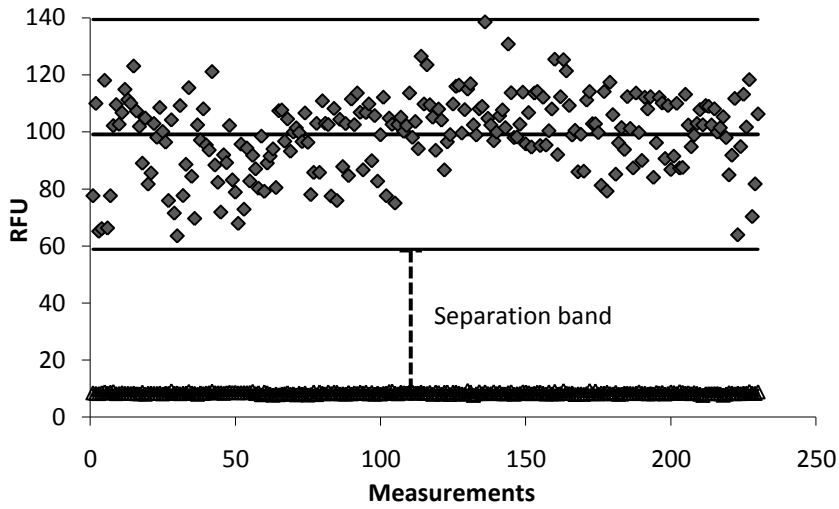


Figure 6. Signal window of the resazurin assay. 3 x SD are shown for both maximal (squares) and minimal (triangles) signals (the SD of the minimal signal is very low and symbols exceed the lines). The separation band is shown with a dashed line.

4.2.2.4 Validatory screening and antimicrobial susceptibility

To evaluate the performance of the resazurin assay in a real setting, a validatory screening campaign of a chemical library was performed (Figure 7). A set of 6 conventional antibiotics with known effects on *S. aureus* biofilms were randomly distributed within the screened plates to evaluate the assay reliability. Same antibiotics that showed some effect (fusidic acid, oxacillin, penicillin G, rifampicin and vancomycin) on the biofilms in the crystal violet assay were used in addition to methicillin.

The library used in the validatory screening for the crystal violet assay was extended with low-weight compound derivatives, spanning through different chemical classes such as benzanilides, chalcones, imidazoles, thiophenes and thienothiazepines. In this screening campaign, the effects on the 18 h old biofilms were measured and no hits were found, other than the active antibiotics (listed above) to have an effect. The two compounds found positive in the first screening campaign, lauryl gallate and farnesol, were not reconfirmed here and apparently do not have an effect on the viability of the biofilms at the 40 µM concentration used in the screening. Neither farnesol nor lauryl gallate have been reported to be able to eradicate mature biofilms at this low concentration (Gomes et al. 2009, Kubo et al. 2003).

In addition, the suitability of the assay to perform screening with natural extracts was also tested. The bacterial extracts from the ARMI collection were screened for destruction

of mature biofilms. In the initial screening, three extracts showed anti-biofilm activity. The complex composition of the bacterial extracts could make them more prone to interfere with the resazurin signal and appear as false negatives in the screening. However, the extracts showed no effect on the reduction of the resazurin probe. This proved the usefulness of the assay, as it can be suitable for pure compounds as well as for extracts.

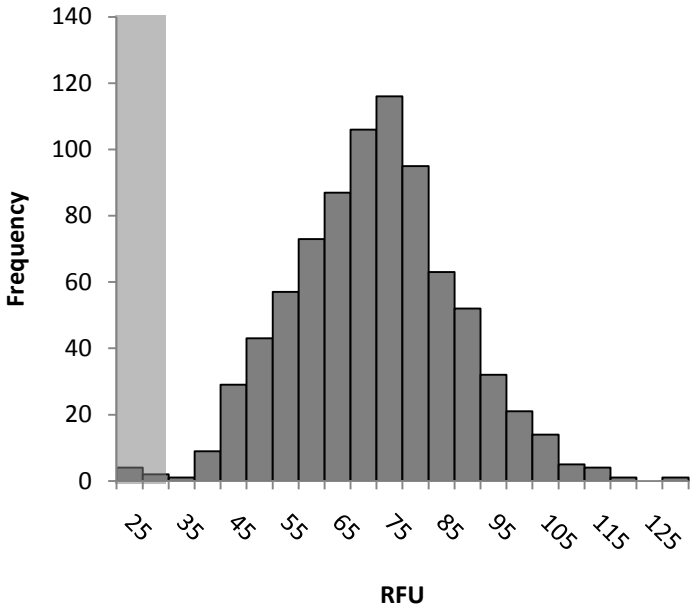


Figure 7. Screening graphs of 815 pure compounds using resazurin staining in destruction of mature biofilms. The shadowed area indicates positive hits in the screening. The hit limit is calculated as mean of control – 3 x SD.

4.2.3 The wheat germ agglutinin assay (III)

4.2.3.1 Optimization and detection limit

This protocol was originally proposed by Burton et al. (2007) and it was based on the specific binding of the wheat germ agglutinin-Alexa Fluor 488 fluorescent conjugate (WGA) to poly-*N*-acetylglucosamine (PNAG) residues present in the typical EPS of *Staphylococcus* spp. biofilms (Burton et al. 2007). The original protocol proved a statistically well performing assay: several conditions were tested and in the end minor modifications were undertaken. Different concentrations (ranging from 0.01 to 10 µg/ml) of the WGA-Alexa 488 probe were tested and the original 5 µg/ml resulted in the best statistical performance and therefore it was considered optimal for use. The transfer of

the dissolved sample onto a new plate before measuring the fluorescence did not improve the assay and was hence removed from the protocol.

To prove that the WGA assay was able to detect biofilms at the concentrations achieved in micro plate assays, the detection limit was established and compared with the previously established methods of the crystal violet and resazurin assays (Figure 8). If responses from the same compounds using the same three assays differ in terms of the detection limits, then conditions for bacterial biofilm formation need to be optimized again as comparisons between them could be limited. In this study, it was seen that the WGA assay could detect biofilm concentrations above $2x10^7$ CFU/ml, which coincide well with the other two methods (Figures 3 and 5). Untreated biofilms reach up to 10^9-10^{10} CFU/ml, so a 3-log reduction could be measured using all of the above mentioned methods. The EPS has been reported to consist of extracellular DNA (eDNA) and proteins in addition to the polysaccharides (Steinberger and Holden 2005). To consider the activity of other molecules in the EPS, the biofilms were treated with DNase I and proteinase K to evaluate the effect of eDNA and extracellular proteins, respectively. DNase I did not show any effect on the biofilm at all. The treatment with proteinase K killed 23.9 ± 9.1% of the biofilms after 6 h treatment and 13.7 ± 8.4% after 22 h treatment. This indicated that neither eDNA nor proteins were a major constituent of the matrix of *S. aureus* biofilms.

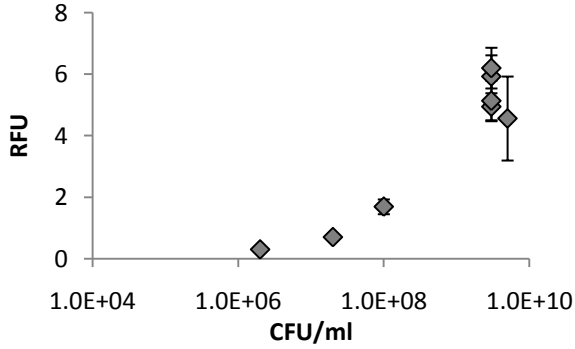


Figure 8. Relation between bacterial concentration and relative absorbance units versus biofilms stained with WGA.

4.2.3.2 Performance and suitability

The performance of the WGA assay made it amenable for screening purposes. The average *Z'*-values from carried out assays (both prevention and destruction) exceeded 0.4. The maximal signal (biofilm controls) varied between 3 and 12 RFUs, but within the same experiment, the SD of the signal was kept low and the statistical parameters were good. Figure 9 shows the controls from six separate experiment plates, with the average of the statistical parameters as *Z'*=0.44±0.07, S/N=5.7±0.80, S/B=17.8±9.9.

Due to the wheat germ agglutinin assay being very laborious, requiring at least 3 h for the whole assay and using a relatively expensive probe, this assay was regarded to not be suitable for the primary screening stage. It is, however, important to take into account the effect of possible active compounds on the EPS as biofilm infections tend to re-emerge if matrix is left after treatment (Toté et al. 2008). This assay is therefore to be used in follow up studies of active screening hits.

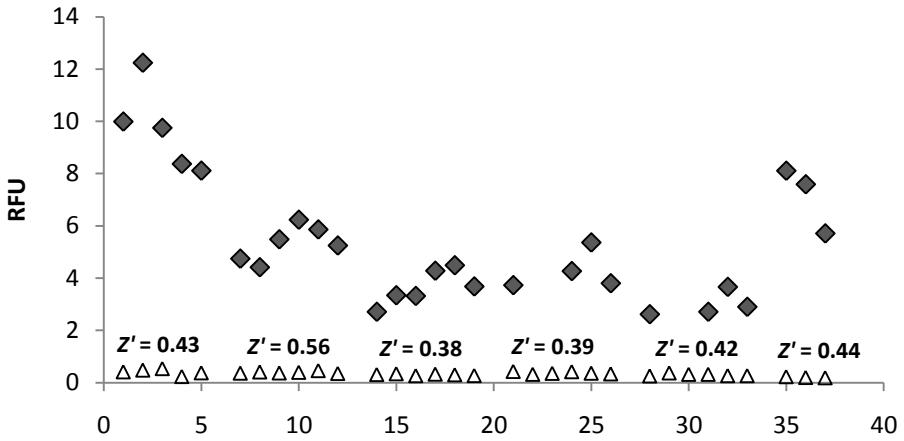


Figure 9. Control samples from six experimental plates, with individual *Z'*-factor values included. Filled symbols represent maximal signals (biofilm controls) and empty symbols represent minimal signals (TSB controls).

4.3 Capillary electrochromatography –an additional biofilm method (IV)

4.3.1 Optimization of the assay

So far CEC has only been used for separation and analysis of planktonic bacteria (Desai and Armstrong 2003, Kłodzińska et al. 2009). As a novel approach, biofilms were immobilized in the capillary and the effect of various compounds on the biofilms could be studied. For the formation of biofilms in fused-silica capillaries, the first step involved the binding of the bacteria cells to the silica surface, which in the conditions used was negatively charged. This was conducted by growing biofilms on silica disks in Petri dishes, staining using the crystal violet assay and applying the statistically well performing conditions to the actual CEC apparatus. No biofilms could be formed on uncoated silica surface, due to the highly negative charge that is not present in polystyrene surfaces where the *S. aureus* bacteria adhere without problems (Gross et al. 2001). Therefore, there was a need for a positively charged coating agent to increase the *S. aureus* binding to the inner surface of the capillary. Poly-L-lysine provided the surface with positively-charged amino groups forming an excellent base for the bacteria to adhere onto. The best biofilm attachment was achieved by coating the silica with 0.01%

of poly-L-lysine (Figure 2 in Publication IV). This concentration also provided the most stable surface in the capillary and was hence chosen for coating the inner surface of the capillary. The same results were also proven by imaging the biofilms formed on the coated silica and the capillary using AFM, and both the coated silica and the capillary showed bacterial aggregations of more than 300-500 nm, which confirmed the presence of biofilms (Figure 3 in Publication IV). The size of the biofilms matched previously reported AFM-studies (Tollersrud et al. 2001, Jonas et al. 2007).

The stability was evaluated by measuring the mobility of the EOF during six successive runs. In order to evaluate the stability of the formed biofilm in the capillary, the mobility of the EOF was measured after first preconditioning the capillary and coating it with *S. aureus* biofilm corresponding to 17-20 h of biofilm formation in the capillary. The EOF mobility did not vary remarkably among the different coating times (Table 2 in Publication IV). The relative standard deviation values were, however, the lowest at 18 h and this incubation time was also used in the micro well plate approach and was chosen for use in optimal biofilm coating of the capillary in the future. To ensure the stability of the biofilm over time in the capillary, the EOF mobility was measured with 12 successive runs per day on 2 consecutive days. The results showed that the biofilm coating of the capillary was kept perfectly stable for at least the two-day period.

4.3.2 Antimicrobial susceptibility

The susceptibility of five of the same antibiotic compounds as in the crystal violet section was estimated in uncoated and biofilm coated electrochromatography capillaries using retention factors and reduced mobilities. The retention of the antibiotics by the biofilms was seen to decrease in the following order: penicillin > fusidic acid > oxacillin > vancomycin > rifampicin. The order of the interactions between biofilm and antibiotics followed the order for retention factor calculations; penicillin had the strongest interaction, followed by fusidic acid and oxacillin. Both vancomycin and rifampicin seemed to have very little interaction and were almost without effect on the biofilms. The rifampicin result was the only one that was contradictory to what was found in the micro well plate assay. The order of the antibiotics according to the biofilm MIC values in micro plates (stained by crystal violet, Publication I) was rifampicin (0.031 µg/ml) > fusidic acid (0.063 µg/ml) > penicillin (0.13 µg/ml) > oxacillin (0.25 µg/ml) > vancomycin (1 µg/ml).

4.4 Applying the screening platform

4.4.1 Combination of assays in a validatory study: a third method added (III)

4.4.1.1 Resazurin and crystal violet assays performed in sequence

First the performance of the screening platform was improved by combining resazurin and crystal violet measurements to one plate in a sequential workflow. Resazurin was proven not to have any effect on the following crystal violet staining, for crystal violet

staining of biofilms in the same plate as resazurin RAU was 1.52 ± 0.25 compared to crystal violet stained biofilms in a separate plate gave RAU = 1.57 ± 0.29. No statistically significant change was detected (p=0.4149). A scheme of the whole experimental sequence is visualized in Figure 1 in Publication III.

4.4.1.2 Susceptibility studies using known antimicrobial compounds

The combination of the three assays in a susceptibility study of three model compounds was performed using ciprofloxacin and penicillin G as active compounds and rosmarinic acid as a negative control (Figure 10). Ciprofloxacin and penicillin G significantly reduced the viability, the biomass and the EPS layer in the prevention approach. Rosmarinic acid is a weak antimicrobial agent and only slightly reduced the viability, but had no effect on the total biomass and the EPS. When the compounds were added to mature biofilms, the rosmarinic acid did not show any effect. Both ciprofloxacin and penicillin G were able to reduce the biomass and the viability significantly but in the case of ciprofloxacin unchanged WGA signal was detected compared to the control. Biofilms treated with penicillin showed a drastically elevated WGA signal that was more than 200% of the control.

In addition, fluorescence microscopy was used to confirm the results obtained with the WGA and resazurin assays (Figure 4 in Publication III). For that purpose, images were taken in samples stained with WGA and the LIVE/DEAD probe that provides a functional response distinguishing living and dead cells, in a way comparable to the resazurin signal. Penicillin G (400 µM) gave a nearly 50%-reduction of the viable cells compared to untreated cells (G/R ratio) visualized by the LIVE/DEAD stained sample. In contrast, the surviving cells clearly produced more EPS and increased the green fluorescence in the treated sample compared to the control sample (Figure 4 in Publication III).

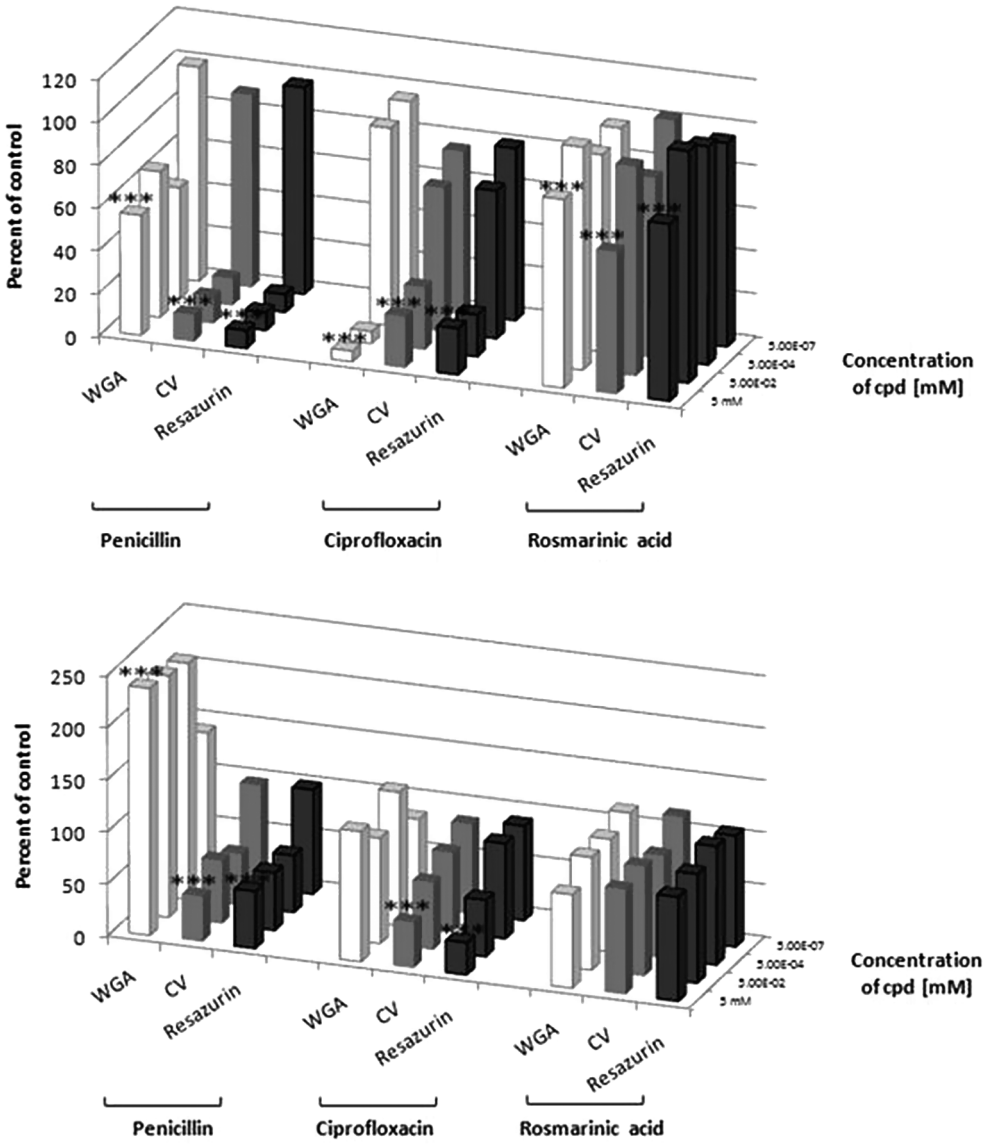


Figure 10. The effects of three model compounds in (A) prevention of and (B) destruction of *S. aureus* biofilms utilizing the combination of the three assays of the platform (resazurin - black bars, crystal violet - grey bars and WGA - white bars), are shown here. One-way ANOVA comparisons and Tukey post tests were applied to the original data points but for better clarification, only the results of the comparisons between the untreated biofilms and the highest concentration of the two compounds are presented here. *** indicates a high statistical significance in the difference from the control, $p<0.001$.

By investigating the effect of the treatments on other biofilm-forming strains, this phenomenon could be excluded to be a strain-specific artifact. For *S. aureus* Newman strain and *E. coli* (XL1 Blue) the same increase of the WGA signal was measured for penicillin G treated biofilms. The EPS of *S. epidermidis* RP62A was not affected by any of the antibiotics. *S. epidermidis* ATCC 12228, on the contrary, was not showing reliable WGA-signals (even negative *Z'*-values were received). As a result it seems like that the WGA-assay is dependent on PNAG production, as this strain is lacking the *ica*ADBC operon and is not producing any PNAG.

4.4.2 A pilot study (V)

4.4.2.1 The screening campaign for anti-biofilm activity

A cinchona alkaloid library containing the natural cinchona alkaloids cinchonidine and cinchonine as well as 22 of their analogues and derivatives was initially screened for investigating the effect on planktonic bacteria (Figure 11A). The entire library was also screened for activity in preventing biofilm formation (Figure 11B) and destruction of mature biofilms (Figure 11C) using the initial screening approach with staining the biofilms sequentially with resazurin and crystal violet. The same active compound was also able to prevent biofilm formation, but had no effect on mature biofilms at the concentration used in the screening.

No antimicrobial effect was found for the original compounds cinchonidine or cinchonine, whereas one derivative inhibited the growth completely, 11-triphenylsilyl-10,11-dihydrocinchonidine (11-TPSCD). The most distinctive feature of this molecule is the triphenylsilyl group in position 11, which is absent in all the other molecules of the library, except for compound 9-TMSO-11-TPSCD (11-triphenylsilyl-9-*O*-trimethylsilyl-10,11-dihydrocinchonidine (Figure 12). However, 11-TPSCD has a free OH group in position 9 that is blocked in 9-TMSO-11-TPSCD by a trimethylsilyl group. Thus, these two structural features seem to be crucial for high activity of 11-TPSCD, in contrast to the other cinchonidine derivatives from the library.

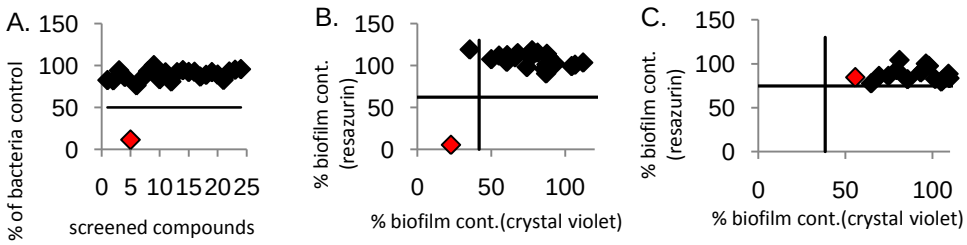


Figure 11. Screening graphs for planktonic, prevention, and destruction assay results. The active compound, 11-TPSCD is marked in red in all the graphs.

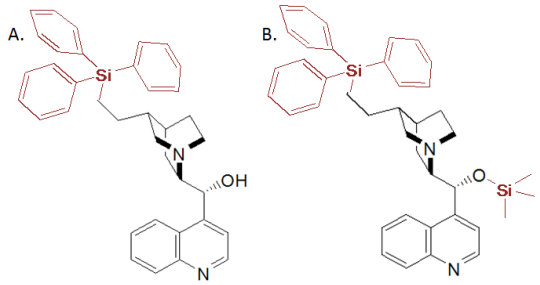


Figure 12. The structures of the active hit compound 11-TPSCD (A) and the structurally related 9-TMSO-11-TPSCD (B). The cinchonidine part of the structures is indicated in black and the substitution parts are marked in red.

4.4.2.2 Follow up studies

Potencies (IC_{50}s) were determined for the active cinchonidine derivative against planktonic bacteria as well as biofilms in prevention and destruction. For planktonic bacteria and the prevention of biofilms, the IC_{50}s were low, 6.11 µM (planktonic), 6.56 µM and 2.21 µM (prevention of biofilms resazurin and crystal violet, respectively). For destruction of mature biofilms, the potency values as measured by IC_{50} values were 212 µM and 225 µM for resazurin and crystal violet staining, respectively.

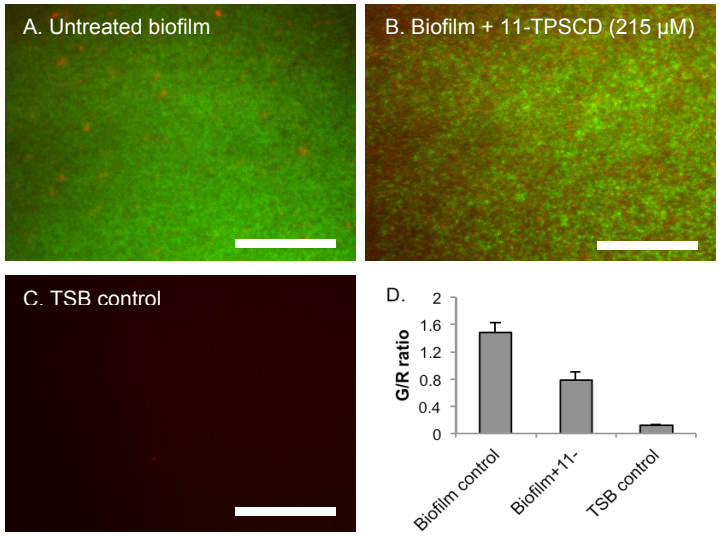


Figure 13. Fluorescence microscopy images of untreated biofilms (A), biofilm treated with 11-TPSCD (B) and TSB control (C). The imaging was conducted with the fluorescent LIVE/DEAD® *Bac*Light™ commercial kit. Alive cells are stained green with SYTO 9 and dead or ruptured cells are stained red with propidium iodide. The ratios of green-to-red fluorescence (G/R ratio) quantified from parallel samples are shown in D. The scale bars equal 100 µm.

Imaging studies were carried out in parallel to confirm the anti-biofilm activity of 11-TPSCD by fluorescence microscopy (Figure 13). An untreated biofilm control sample, a TSB control sample and a biofilm sample treated with 215 μM 11-TPSCD were imaged. The ratio between the green and the red fluorescence in the samples were calculated.

The reduction of the biofilm amount by treatment with the active compound was quantified using log reduction. In the prevention assay, the log R values (the difference in logCFU/ml in treated and untreated samples) were 2.1 at 10 μM for biofilms and 3.9 for the planktonic phase. Log R values in the destruction assay were at 200 μM, 2.7 for biofilm bacteria and 4.9 for the planktonic bacteria.

The effect of the active compound on the EPS layer was also determined by the WGA assay. In prevention, a slight decrease in the signal could be observed at concentrations ranging from 5 to 10 μM (14-31% of inhibition), higher concentrations (20-100 μM), however, increased the WGA-signal to 118-165% of the control. In destruction, no effect compared to untreated biofilms could be observed. This indicates that the compound is not fully able to remove the biofilms. However, in combination with a dispersing agent, the compound could be effective, by killing planktonic bacteria and preventing biofilm formation.

4.4.2.3 Screened compound locations in chemical space

To explore the chemical space occupied by the active compound 11-TPSCD, the antibiotics and the non-active compounds from the cinchona alkaloid library, as well as all the other compounds used in the validatory screening campaigns, a comparative study was performed using the ChemGPS-NP tool. ChemGPS is a Principal Component Analysis (PCA)-based chemical space navigation tool applicable to natural products (Larsson et al. 2007, Rosén et al. 2009).

In Figure 14, it is demonstrated that the cinchona alkaloid derivatives populate a fairly similar chemical space when compared to the other screened libraries. The cinchona alkaloids were characterized by low molecular sizes (given by PC1), high aromaticity (given by PC2) and restricted flexibility (given by PC4, not shown here). The compounds were quite evenly distributed between hydrophilic or hydrophobic compounds (PC3). The antibiotics were the ones most widely spread over the chemical space presented here. The largest antibiotics (amphotericin B, polymyxin B, rifampicin and vancomycin) represent both active (green dots) and non-active antibiotics (blue dots) and were the most distant from the rest of the compounds. The rest of the antibiotics are low molecular weight compounds and were distributed among the rest of the compounds. The non-active antibiotics were the least aromatic. The active hit compound, 11-TPSCD (red dot), was found to be located in a slightly more lipophilic region than the rest of the compounds together with its non active analogue, 9-TMSO-11-TPSCD. It appears like

there is no specific space for antimicrobial compounds; they vary in all three dimensions. The hit compound 11-TPSCD supports the fact that also compounds with anti-biofilm activity can be found outside the drug- and lead-like space defined by Lipinski (2001) and Teague (1999).

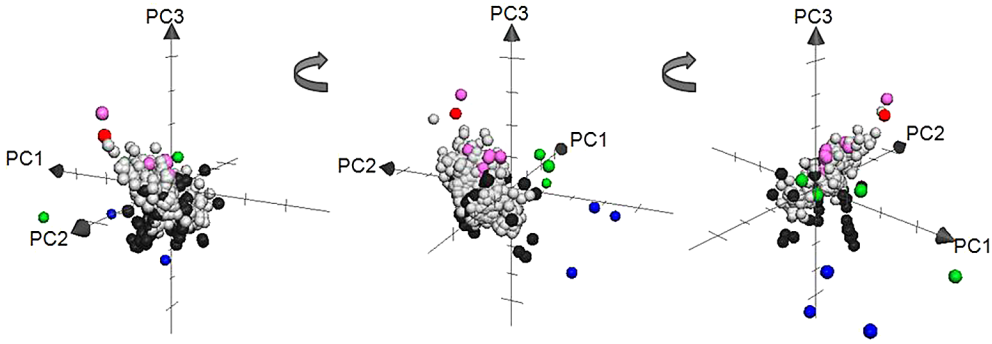


Figure 14. Three-dimensional representation of the chemical space occupied by all of the compounds (829 compounds) screened for anti-biofilm activity using the PCA-based navigation tool ChemGPS-NP, as described in publication V. The cinchona alkaloid library is represented by the purple dots, the hit compound 11-TPSCD by a red dot, the natural product library is represented by black dots and the chemical library by grey dots. The active antibiotics are marked in green and the non-active antibiotics in blue.

5 Discussion

5.1 Antimicrobial and anti-biofilm research and screening against biofilms

Antimicrobial resistance is an evolving problem and there is a lack of effective agents. In spite of this, antimicrobial drug discovery is not a favored area for pharmaceutical companies today (Moellering 2011). The reasons are understandable as the promised techniques introduced about 20 years ago by genome sequencing of micro-organisms and combinatorial chemistry for gene target based high throughput screening campaigns have not provided the much sought after results (Payne et al. 2007). However, there are multifactorial reasons explaining the failure of screening campaigns. Regarding only the targets and the libraries used; the need of validation of targets in every species used, *in vivo* validation, rapid resistance development, and the libraries used for screening are often containing very few compounds with the physical-chemical properties suitable for antimicrobials are reasons for failure (Brötz-Oesterhelt and Sass 2010). The fact that if an effective antibiotic is found, it most probably would not be a high-profit product for the company investing in developing it as it has to be used with caution to avoid the occurrence of resistance (ECDC/EMEA 2009, Fox 2006). One advantage antimicrobial drug compounds have over many other drug compounds is the high predictive value of the animal models that are used for *in vivo* studies. This is due to the fact that most of the pathogens causing problematic infections in humans also infect animals (Payne et al. 2007). In many cases, the same strains have been detected in humans and animals, and this is also forging a new thinking in veterinary use of antimicrobials to prevent the evolving of more resistant strains due to overuse of antimicrobials to treat animal infections (ECDC/EMEA 2009). Nevertheless, economically feasible or not, the problem remains and progress as, biofilms are currently regarded as the cause of a majority of all severe infections (Kiedrowski and Horswill 2011). Until other effective antibiotics are found, currently existing moderate compounds will be needed to keep the pathogen problems under control and in order to do that, effective assays for screening will be required (Fox 2006).

Whole-cell screening as opposed to target-based screening has re-emerged into the antibacterial drug discovery (Gwynn et al. 2010). This is due to the fact that targets have been problematic. Single specific gene targets have been prone to resistance development and multiple targets would be beneficial instead. Complex mechanisms rather than simple enzyme inhibition functions have given better results as well (Brötz-Oesterhelt and Sass 2010). The drawback of whole-cell primary screening is the unspecific mode of action of potential hits (Brötz-Oesterhelt and Sass 2010). Many of the existing antimicrobial agents are naturally produced or semisynthetic derivatives of natural products. Erythromycin, vancomycin and daptomycin from actinomycetes or fungi are

examples of compounds that have been found by whole-cell screening methods, even without knowing the exact mechanism (Baltz 2007). However, quorum sensing (QS) targeting screening has, for instance, been suggested to be an effective anti-biofilm screening approach (Landini et al. 2010). For gram-negative *P. aeruginosa* biofilms there are several well-established QS-targets, such as the *las* and the *rhl* systems (Sintim et al. 2010). In *S. aureus* biofilms, there is the autoinducing peptide (AIP) based *agr* and the *luxS* systems that have been shown to play a role in virulence of *S. aureus* biofilms (Kuehl et al. 2009). It has been shown that the susceptibility of gram-positive and gram-negative biofilms to antibiotics might be enhanced by suppression of the QS-system, even though the QS-suppression itself was not enough (Brackman et al. 2011). Therefore, the impact of quorum sensing for biofilm formation and virulence does not seem to be a straight forward approach. The fact that, with the exception of a few QS-suppressor-mimicking molecules, the targets of the anti-biofilm agents found are not known (Sintim et al. 2010). The applicability of the lead molecules found by target-based screening has been limited by the lack of activity when they are tested in cell-based assays (Baltz 2007). This has prompted to favor the use of whole-cell based assays during the initial chemical screening to identify lead molecules, followed by target specific assays in a secondary screening phase, to establish the mode of action of tentative leads (Payne et al. 2007). For this reason, in this study, it was chosen to develop a platform of whole-cell based screening assays against *S. aureus* biofilms.

S. aureus is a clinically relevant strain causing severe infections and can currently be nosocomial and community-acquired. The infections are difficult to treat, probably due to biofilm formation (Kiedrowski and Horswill 2011). Biofilms show multidrug resistance that can even be increased if the initial treatment has been too mild to eradicate the biofilm completely (Hall-Stoodley and Stoodley 2009). Additionally, it is difficult to diagnose a severe infection as to have been caused by biofilms and thereby be able to administer the correct treatment (Hall-Stoodley et al. 2004). There are diagnostic criteria for biofilm infections including demands of being surface attached, showing microbial aggregates in localized infections with resistance to antibiotics with or without culturing the bacteria, as well as ineffective host clearance, but they are hard to determine in a timely fashion (Parsek and Singh 2003, Hall-Stoodley and Stoodley 2009). Poor diagnostics lead to requirements of rapid treatments with effective broad spectrum drugs (Brötz-Oesterhelt and Sass 2010). However, there is no diagnostic method or specific biofilm marker for *Staphylococcus* spp. biofilm infections and they remain challenging to treat (Bordi and de Bentzmann 2011). The *S. aureus* ATCC 25923 strain has been a widely used reference strain for antimicrobial research (Kronvall 1982, Ozturk et al. 2008), i.e., as well as for biofilm research (Zmantar et al. 2010, Singh et al. 2010), i.e.

5.2 The chosen assays in comparison with other biofilm assays

Crystal violet is a widely used method for studying bacteria in biofilms (Djordjevic et al. 2002, Li et al. 2003, Peeters et al. 2008). The method was first used in tubes and was later optimized for micro well plates (Christensen et al. 1985, Stepanovic et al. 2000). The assay is based on the use of available, inexpensive reagents, with a simple absorbance endpoint reading. Staining biofilms with the crystal violet assay requires several washing steps and when dealing with screening campaigns on several plates this signifies a great number of dispensing and aspiration steps performed by hand. This issue was solved in publication **I** by using automation with a liquid handling robot that easily manages dispensing and aspiration.

The most serious drawback of the method is the fact that it only measures total biomass and is, therefore, not applicable to viability measurements (Li et al. 2003, Pitts et al. 2003). This drawback is targeted by the addition of the viability measuring resazurin assay to the primary screening platform (Publication **II**).

Resazurin is a very fast (single-addition) and non-laborious method based on a single addition step of a non-fluorescent probe, which is reduced by metabolic activity to a fluorescent compound (Pettit et al. 2005). However, this method requires optimization for every separate strain it is used with. An example of optimization of the performance of this assay was presented in publication **II**. A wide variety of experimental protocols had been used according to the literature and both the concentration of the probe and the incubation time for staining the *S. aureus* biofilms in these experiments had to be validated. It was demonstrated from that study that 20 μM final concentration of the probe and 20 min incubation time were the fastest optimal conditions found for the screening purpose. The importance of optimization when taking a new strain into use was seen in publication **III** when we used additional biofilm strains for the combination of the assays. For the two *Staphylococcus epidermidis* strains and *S. aureus* strain Newman used, the same conditions as for the original strain could be conducted. In contrast, staining *E. coli* biofilms with resazurin, required a longer incubation time with the probe to give an appropriate signal. Peeters et al. (2008) optimized the resazurin assay for several different strains and came to the conclusion that 1 h was an appropriate incubation time for all the strains (yeast, gram-positive and gram-negative bacteria) except for *S. aureus* that required shorter incubation (30 minutes). Toté et al. (2008) similarly used 30 minutes for *S. aureus* biofilms. Pettit et al. (2005, 2009) used 1 h incubation for *S. epidermidis*.

Another important factor that was found in this study during the resazurin optimization was the difference in responses when the resazurin assay is applied to the staining of planktonic and biofilm bacteria. Previously it had been suggested that bacteria

concentration of biofilms could easily be estimated by using a titration curve done with planktonic bacteria (Toté et al. 2008). This, however, is clearly not the case. It was seen that higher concentrations of biofilm bacteria compared to planktonic bacteria were required to achieve similar resazurin signal (Figure 3).

Other viability probes such as XTT, FDA and SYTO 9 have also been successfully optimized for biofilm staining. In a comparison study with different biofilm forming bacteria and fungi, resazurin and FDA were found to be the most favorable assays (Peeters et al. 2008). Both XTT and SYTO 9 are performing-wise usable but they are expensive and XTT is also much more laborious to use than the resazurin and FDA assays (Peeters et al. 2008). The most cost-effective reagent is resazurin (according to concentrations used and prices provided by Sigma Aldrich) and is clearly not toxic to the biofilms.

There is no single method that can measure the effect of a compound on viability, biomass and the EPS layer at the same time. Therefore, there is a need for combining assays to be able to detect an effect on all three features already at a primary screening stage. Because the resazurin compound is not toxic to the cells, crystal violet could be performed on the same sample after concluding the resazurin assay without interfering with the outcome (O'Brien et al. 2000). In the present study, this was a remarkable improvement of the workflow and it lowered costs and time needed for the experiments. The assay platform was also shown to be reliable from the validatory screening campaigns performed for each assay separately, as only compounds with reported antimicrobial and/or anti-biofilm activity were detected.

Biofilms are built not only from the viable bacteria but of the self-produced extracellular polysaccharide matrix surrounding them as well. To measure the production of this matrix, a third assay was used based on the fluorescence staining with WGA. A comparison of the three biofilm quantification assays showed that they have similar detection limits (10^7 CFU/ml), which correspond well with concentrations generally measured for biofilms in micro well plates (Pitts et al. 2003). This also enables the assays to be used together without need of thorough optimizations of biofilm formation conditions.

The combination of the three assays optimized in this thesis, allowed an overall picture of the anti-biofilm effects of compounds in terms of biomass, viability and EPS production. It had previously been noticed that certain antibiotics could apparently have positive effects on biofilms by causing a significant decrease of biomass and viability, but still leave the EPS unaffected (Toté et al. 2009). Based on these results, Toté et al. proposed a classification of antibiotics in connection to their effects on biofilm bacteria and matrix. We proposed in publication III that a sixth category of compounds could be added to the list (Table 1). These types of compounds, exemplified in our case with penicillin G, have a

significant effect on decreasing the viability and the total biomass, but the EPS are simultaneously increased upon compound exposure. The other model antibiotic used, ciprofloxacin, was able to decrease the viability and the biomass, but had no effect on the EPS layer of *S. aureus* biofilms (Publication **III**). Ciprofloxacin could as a consequence be added into the fourth category proposed by Toté et al. (2009) along with kanamycin that displays similar effects against *P. aeruginosa* biofilms (Toté et al. 2009). The same effect on the EPS levels caused by penicillin G was also proven to occur in another *S. aureus* (Newman strain) and *E. coli* (XL1 Blue) biofilms, confirming that the registered results were not exclusive to the model strain used here. Leaving the EPS can be the cause of a re-emerging biofilm infection, as there can be latent cells left protected in the slime or the pre-existing slime layer can enhance attachment of bacteria and promote biofilm formation (Toté et al. 2008). This would ultimately affect the long-term chemotherapeutical effects of antibiotics against biofilms.

Table 3. Antibiotics divided into categories based on their effect on the biofilm bacteria and the matrix. Modified from Toté et al. (2009).

Category	Effect on bacteria[a]	Effect on matrix[a]	Example antibiotic (target biofilm[c])	Reference
1	none	none	ampicillin (SA)	(Toté et al. 2009)
2	none	–	cefalotin (SA)	(Toté et al. 2009)
3	–	none	polymyxin (SA)	(Toté et al. 2009)
4	–	–	kanamycin (PA) ciprofloxacin (SA)	(Toté et al. 2009) Publication III
5[b]	– (**+**)	–	doxycycline (PA)	(Toté et al. 2009)
6	–	**+**	penicillin G (SA, EC)	Publication III

[a] none; no effect, –; decreased, **+**; increased

[b] includes compounds having an increased effect at sub-MIC levels.

[c] biofilm strains, SA – *S. aureus*, PA – *P. aeruginosa*, EC – *E. coli*.

Toté and colleagues (2009) used dimethylmethylene blue (DMMB) that has been used for quantification of sulphated polysaccharides because it binds glycosaminoglycans (GAG) in biological samples (Barbosa et al. 2003). The similarity of GAG to the polysaccharide intercellular adhesin (PIA) that is a major component in *Staphylococcus* spp. biofilms, makes the DMMB probe suitable for quantification of the biofilm matrix (Toté et al. 2008). The method used in publication III similarly targets the PIA of the *Staphylococcus* spp. biofilm matrix but is based on the specific binding of wheat germ agglutinin to poly-*N*-acetylglucosamine PNAG residues in the biofilm matrix (Burton et al. 2007). PNAG is also involved in biofilm formation by both gram-positive and negative strains. The *ica*ADBC-operon has been shown to be required in the genome for PNAG production in *Staphylococcus* spp. (Gerke et al. 1998), and the *pga*ABCD-operon in *E. coli* (Cerca and Jefferson 2008). The importance of PNAG was seen with the *S. epidermidis* ATCC 12228

strain, which lacks the *ica*-locus (Zhang et al. 2003) and the WGA-assay was not applicable to that strain (Publication **III**). There was no replicability and the signal variation was too high, even negative *Z'*-values were received. The WGA assay for quantification of the biofilm matrix was originally described as an enzyme-linked lectin-sorbent assay (ELLA) by Thomas et al. (1997) and was further optimized by Burton et al. (2007) using the wheat germ agglutinin Alexa Fluor 488-conjugate. The Alexa Fluor 488 conjugation enables fluorometric readout, which simplifies the assay (Burton et al. 2007).

The CEC-method (Publication **IV**) allows for a completely novel way of studying biofilms and the effect of complex mixtures on biofilms. The growth conditions are simple to change because the flush of bacteria is constant. It can be compared to methods using constant flow for the growing environments, such as the Robbins device where biofilms are formed on a surface in a flow of nutrients and fresh bacteria (Linton et al. 1999). The CEC-method proved in the present study to be reliable for growing biofilms when compared to the crystal violet micro plate based assay. The effect of the antibiotics was similar with the exception of rifampicin. In the micro plate assays (also resazurin) it was shown to be the most active agent against biofilms. In the CEC, however, rifampicin did not show any interaction with the biofilm in the capillary in contrast to the other active agents and was considered to be the least active compound against biofilms (Figures 4 and 5 in Publication **IV**). Previous research has shown that antibiotics might bring about varying results when the assay format is changed (Tammela et al. 2004). In another study of the impact of different anti-biofilm agents on the viscoelastic properties of the biofilm structure, rifampicin was shown to alter the mechanical properties of the biofilm (Jones et al. 2011). The rifampicin might have caused physical changes in the biofilms subsequently causing the discrepant signal in the CEC.

5.3 Screening for new chemical structures and bacterial extracts

In the screening of the cinchona alkaloid library, the primary screening platform of resazurin and crystal violet staining was used (Publication **V**). Only one compound (11-TPSCD) was able to reduce the viability and the biomass in screening for prevention of biofilm formation. Using the crystal violet assay, two compounds (9-TMSO-11-TPSCD and 11-TPSCD) were found to be beneath the hit limit. 9-TMSO-11-TPSCD, however, had no effect on the viability of the biofilms and was not considered for further studies. The active 11-TPSCD and the non-active 9-TMSO-11-TPSCD compounds were found to be occupying a slightly different area in the chemical space than the rest of the screened compounds. However, only one of them was active leading to the conclusion that the physico-chemical properties cannot be the only explanation for the activity. The active as well as and the non-active antibiotics were widely spread over the chemical space, implying that biofilms are complicated to eradicate and that active anti-biofilm compounds can be found in different locations in chemical space. This approach of using

small targeted libraries for a single aim is not only the proceeding of small academic laboratories, but of large pharmaceutical companies, like GSK, where they have tried this approach when large multi-target screening campaigns had failed (Payne et al. 2007). The active compound does not fall within all the Lipinski's rule of five (slightly larger and more lipophilic) but it has been stated that many natural products as well as antimicrobials are exceptions to the rule (Coates and Hu 2007, Newman and Cragg 2007). Maybe there would be requirements for new rules for the screening of antimicrobials or when using natural products. As proposed by i.e. Payne at al. (2007), the screening campaigns could be concentrated to fewer compounds (i.e. small natural products libraries). Interesting compounds from these small libraries could then be chemically modified, if needed. However, the amount of unexplored natural chemical space is so wide that the right structure for activity most probably does exist; the difficult part is the strategy to find it. Thus, the development of effective methods compatible for testing compounds, including natural products, against chosen targets is of great importance. One future direction is to use natural products for drug development; especially in field like antimicrobials where the usual compound demands are not the most suitable. The use of crude extracts is also an interesting strategy, especially combined with fast and effective methods for separation and identification of the pure substituents (Vuorela et al. 2004).

A library of bacterial extracts was also used for validatory screening of the resazurin method and two extracts were displaying clear anti-biofilm effects in both prevention and destruction of biofilms (unpublished results). Screening libraries of natural product extracts have been revisited since many large screening campaigns with pure compounds have failed (Payne et al. 2007). Earlier extract screening was the primary source for drug screening, but was then considered to be too laborious and abandoned for pure compound libraries in the era of synthetic chemistry (Grabley and Sattler 2003). Nevertheless, with modern technologies extracts can be efficiently microfractionated and identified (Vuorela et al. 2004). Especially in the field of antimicrobial agents, the natural product extracts have shown interesting results. Additionally, the physico-chemical properties of natural products seem to coincide quite well with antimicrobial and targeted libraries with natural product scaffolds (Payne et al. 2007, Brötz-Oesterhelt and Sass 2010). It is also possible that there is not one single active component, but rather a combination of several which provides the activity. Indeed, the first mixture of compounds was approved for drug use by the FDA in 2007 and in Europe in 2010 (Mishra and Tiwari 2011). Bacteria clearly represent the largest biodiversity (Pace 1997, Pace 2009). The Arctic Microbes culture collection (ARMI) used in the screening comprises bacteria isolated from arctic environments and they most likely possess interesting components for survival in the harsh conditions they were isolated from (Männistö and Häggblom 2006). Micro-organisms from marine environments are also yet unexplored to a large extent and are of present interest for the drug discovery research (Leal et al. 2012).

6 Conclusions and future perspectives

Biofilms are the cause of very severe infections and *S. aureus*, especially, has been a troublesome pathogen in nosocomial infections. The dauntingly rapid development of resistance among these pathogenic bacteria increases the necessity of novel effective antibiotics. The majority of all bacteria are able to form biofilms and it is currently believed that existing in biofilm format is actually the preferred bacterial lifestyle. However, the existing antibiotics as well as the methods for studying bacteria have been developed for planktonic bacteria, which basically make them unusable for biofilm studies.

In the project at hand, the overall aim was to fill the void of biofilm assays suitable for screening. For this purpose, a statistically robust platform of three assays amenable for anti-biofilm screening was developed. Three very important features of biofilms, essential from a drug discovery perspective, were targeted using three separate assays carried out in sequence succeeding each other. We propose that the effects that the screened compounds have on biomass (Publication **I**) and viability (Publication **II**) can be studied at a primary screening stage using crystal violet and resazurin, respectively. Positive hits could then be followed up by investigating the effects on the EPS-layer (Publication **III**), in order for true hits, with positive effects on viability, biomass and matrix could be identified. The CEC-method is providing an additional way of studying interaction between compounds and biofilms in a continuous flow system (Publication **IV**). Smaller institutions, such as academic groups or small companies, could utilize this platform of methods for anti-biofilm screening. They are perhaps interested in screening a specific library or against a certain biofilm strain, but lack the interest or knowledge in developing the assays.

Large pharmaceutical companies have failed in finding new antimicrobial drugs in their large screening campaigns using compounds that fulfill the drug-like properties established by Lipinski (2001). Consequently, natural products have been left out in some cases from large screening libraries due to not fulfilling the drug-like properties. Both antibiotics and natural products are mostly larger and more hydrophilic and it has been suggested that targeted libraries of natural products-like compounds could be successful in the search for new antimicrobials.

A total of 820 small molecular, mostly naturally derived compounds, were screened during the validation steps of the current study's assays. In the pilot study (Publication **V**), where the whole platform was utilized using a small library of cinchona alkaloids, one novel compound, 11-TPSCD - a derivative of cinchonidine - was found to have antimicrobial activity against planktonic bacteria. The compound was also able to prevent biofilm formation at a low micromolar concentration and eradicate mature biofilms at a

higher concentration. Still, no effects were found on removing the EPS layer using the WGA-assay.

Nature is to a wide extent still unexplored, marine and extreme environments in particular. Thus, there are certainly natural products to find with interesting activities in antimicrobial and anti-biofilm research. The difficulties lie in finding them and economically motivate the research, while with the knowledge that if a potential compound is found and developed into a drug it has to be saved for seriously threatening situations, such as risks of pandemics. This, however, makes antimicrobial research an increasingly important field and to be able to find the active compounds new strategies are required, especially in the field of anti-biofilm compounds.

This project could be continued by further screening additional libraries, including targeted natural products and extract libraries. Another interesting input would be to optimize the platform for other biofilm-forming strains. Gram-negative strains are the new threat for pandemic outbreaks, for example the multi-resistant EHEC-bacteria (WHO, 2011). Also other dangerous pathogens that are known to form biofilms could be used as target in this screening platform.

References

Adam, B., G. S. Baillie & L. J. Douglas (2002) Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. *J Med Microbiol,* 51**,** 344-9.

Adams, J. L. & R. J. McLean (1999) Impact of rpoS deletion on *Escherichia coli* biofilms. *Appl Environ Microbiol,* 65**,** 4285-7.

Aguinaga, A., M. L. Francés, J. L. Del Pozo, M. Alonso, A. Serrera, I. Lasa & J. Leiva (2011) Lysostaphin and clarithromycin: a promising combination for the eradication of *Staphylococcus aureus* biofilms. *Int J Antimicrob Agents,* 37**,** 585-7.

Amorena, B., E. Gracia, M. Monzón, J. Leiva, C. Oteiza, M. Pérez, J. L. Alabart & J. Hernández-Yago (1999) Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed in vitro. *J Antimicrob Chemother,* 44**,** 43-55.

Amsler, K., C. Santoro, B. Foleno, K. Bush & R. Flamm (2010) Comparison of broth microdilution, agar dilution, and Etest for susceptibility testing of doripenem against gram-negative and gram-positive pathogens. *J Clin Microbiol,* 48**,** 3353-7.

An, W. F. & N. Tolliday (2010) Cell-based assays for high-throughput screening. *Mol Biotechnol,* 45**,** 180-6.

Antunes, A. L., J. W. Bonfanti, L. R. Perez, C. C. Pinto, A. L. Freitas, A. J. Macedo & A. L. Barth (2011) High vancomycin resistance among biofilms produced by *Staphylococcus* species isolated from central venous catheters. *Mem Inst Oswaldo Cruz,* 106**,** 51-5.

ASTM. 2011. Standard test method for testing disinfectant efficacy against *Pseudomonas aeruginosa* biofilm using the MBEC assay. ASTM Standard E2799 – 11.

Aybar, Y., R. Ozaras, K. Besirli, E. Engin, E. Karabulut, T. Salihoglu, B. Mete, F. Tabak, A. Mert, G. Tahan, M. H. Yilmaz & R. Ozturk (2012) Efficacy of tigecycline and vancomycin in experimental catheter-related *Staphylococcus epidermidis* infection: microbiological and electron microscopic analysis of biofilm. *Int J Antimicrob Agents*, 39**,** 338-42.

Bala, A., R. Kumar & K. Harjai (2011) Inhibition of quorum sensing in *Pseudomonas aeruginosa* by azithromycin and its effectiveness in urinary tract infections. *J Med Microbiol,* 60**,** 300-6.

Baldoni, D., M. Haschke, Z. Rajacic, W. Zimmerli & A. Trampuz (2009) Linezolid alone or combined with rifampin against methicillin-resistant *Staphylococcus aureus* in experimental foreign-body infection. *Antimicrob Agents Chemother,* 53**,** 1142-8.

Baltz, R. H. (2007) Antimicrobials from Actinomycetes: Back to the future. *Microbe,* 2**,** 125-31.

Barbosa, I., S. Garcia, V. Barbier-Chassefière, J. P. Caruelle, I. Martelly & D. Papy-García (2003) Improved and simple micro assay for sulfated glycosaminoglycans quantification in biological extracts and its use in skin and muscle tissue studies. *Glycobiology,* 13**,** 647-53.

Beenken, K. E., L. N. Mrak, L. M. Griffin, A. K. Zielinska, L. N. Shaw, K. C. Rice, A. R. Horswill, K. W. Bayles & M. S. Smeltzer (2010) Epistatic relationships between sarA and agr in *Staphylococcus aureus* biofilm formation. *PLoS One,* 5**,** e10790.

Billeter, M., M. J. Zervos, A. Y. Chen, J. R. Dalovisio & C. Kurukularatne (2008) Dalbavancin: a novel once-weekly lipoglycopeptide antibiotic. *Clin Infect Dis,* 46**,** 577-83.

Blango, M. G. & M. A. Mulvey (2010) Persistence of uropathogenic *Escherichia coli* in the face of multiple antibiotics. *Antimicrob Agents Chemother,* 54**,** 1855-63.

Bordi, C. & S. de Bentzmann (2011) Hacking into bacterial biofilms: a new therapeutic challenge. *Ann Intensive Care,* 1**,** 19.

Borriello, G., L. Richards, G. D. Ehrlich & P. S. Stewart (2006) Arginine or nitrate enhances antibiotic susceptibility of *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Chemother,* 50**,** 382-4.

Boucher, H. W., G. H. Talbot, J. S. Bradley, J. E. Edwards, D. Gilbert, L. B. Rice, M. Scheld, B. Spellberg & J. Bartlett (2009) Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis,* 48**,** 1-12.

Brackman, G., P. Cos, L. Maes, H. J. Nelis & T. Coenye (2011) Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics in vitro and in vivo. *Antimicrob Agents Chemother,* 55**,** 2655-61.

Brooun, A., S. Liu & K. Lewis (2000) A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother,* 44**,** 640-6.

Brown, E. D. & G. D. Wright (2005) New targets and screening approaches in antimicrobial drug discovery. *Chem Rev,* 105**,** 759-74.

Brötz-Oesterhelt, H. & P. Sass (2010) Postgenomic strategies in antibacterial drug discovery. *Future Microbiol,* 5**,** 1553-79.

Bulkley, D., C. A. Innis, G. Blaha & T. A. Steitz (2010) Revisiting the structures of several antibiotics bound to the bacterial ribosome. *Proc Natl Acad Sci U S A,* 107**,** 17158-63.

Burmølle, M., T. R. Thomsen, M. Fazli, I. Dige, L. Christensen, P. Homøe, M. Tvede, B. Nyvad, T. Tolker-Nielsen, M. Givskov, C. Moser, K. Kirketerp-Møller, H. K. Johansen, N. Høiby, P. Jensen, S. J. Sørensen & T. Bjarnsholt (2010) Biofilms in chronic infections - a matter of opportunity - monospecies biofilms in multispecies infections. *FEMS Immunol Med Microbiol,* 59**,** 324-36.

Burton, E., N. Yakandawala, K. LoVetri & M. Madhyastha (2007) A microplate spectrofluorometric assay for bacterial biofilms. *J Ind Microbiol Biotechnol,* 34**,** 1-4.

Cafiso, V., T. Bertuccio, D. Spina, S. Purrello & S. Stefani (2010) Tigecycline inhibition of a mature biofilm in clinical isolates of *Staphylococcus aureus* : comparison with other drugs. *FEMS Immunol Med Microbiol,* 59**,** 466-9.

Cai, Y., Y. Fan, R. Wang, M. M. An & B. B. Liang (2009) Synergistic effects of aminoglycosides and fosfomycin on *Pseudomonas aeruginosa* in vitro and biofilm infections in a rat model. *J Antimicrob Chemother,* 64**,** 563-6.

Cerca, N. & K. K. Jefferson (2008) Effect of growth conditions on poly-*N*-acetylglucosamine expression and biofilm formation in *Escherichia coli*. *FEMS Microbiol Lett,* 283**,** 36-41.

Ceri, H., M. Olson, C. Stremick, R. Read, D. Morck & A. Buret (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol,* 37**,** 1771-6.

Cervera, C., M. Almela, J. A. Martínez-Martínez, A. Moreno & J. M. Miró (2009) Risk factors and management of Gram-positive bacteraemia. *Int J Antimicrob Agents,* 34 Suppl 4**,** S26-30.

Chai, Y., M. L. Liu, K. Lv, L. S. Feng, S. J. Li, L. Y. Sun, S. Wang & H. Y. Guo (2011) Synthesis and in vitro antibacterial activity of a series of novel gatifloxacin derivatives. *Eur J Med Chem,* 46**,** 4267-73.

Chokr, A., D. Leterme, D. Watier & S. Jabbouri (2007) Neither the presence of ica locus, nor in vitro-biofilm formation ability is a crucial parameter for some *Staphylococcus epidermidis* strains to maintain an infection in a guinea pig tissue cage model. *Microb Pathog,* 42**,** 94-7.

Chopra, I., L. Hesse & A. J. O'Neill (2002) Exploiting current understanding of antibiotic action for discovery of new drugs. *J Appl Microbiol,* 92 Suppl**,** 4S-15S.

Christensen, G. D., W. A. Simpson, A. L. Bisno & E. H. Beachey (1982) Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect Immun,* 37**,** 318-26.

Christensen, G. D., W. A. Simpson, J. J. Younger, L. M. Baddour, F. F. Barrett, D. M. Melton & E. H. Beachey (1985) Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol,* 22**,** 996-1006.

Coates, A. R. & Y. Hu (2007) Novel approaches to developing new antibiotics for bacterial infections. *Br J Pharmacol,* 152**,** 1147-54.

Costerton, J., P. Stewart & E. Greenberg (1999) Bacterial biofilms: A common cause of persistent infections. *Science,* 284**,** 1318-22.

Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta & T. J. Marrie (1987) Bacterial biofilms in nature and disease. *Annu Rev Microbiol,* 41**,** 435-64.

Costerton, J. W., G. G. Geesey & K. J. Cheng (1978) How bacteria stick. *Sci Am,* 238**,** 86-95.

Dandache, P., P. A. Moise, J. Orsini, M. Montecalvo & G. Sakoulas (2009) Reduced biofilm production associated with increasing linezolid MICs among linezolid-resistant staphylococci. *J Antimicrob Chemother,* 64**,** 1114-5.

del Pozo, J. L., M. S. Rouse, J. N. Mandrekar, M. F. Sampedro, J. M. Steckelberg & R. Patel (2009) Effect of electrical current on the activities of antimicrobial agents against *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis* biofilms. *Antimicrob Agents Chemother,* 53**,** 35-40.

del Prado, G., V. Ruiz, P. Naves, V. Rodríguez-Cerrato, F. Soriano & M. del Carmen Ponte (2010) Biofilm formation by *Streptococcus pneumoniae* strains and effects of human serum albumin, ibuprofen, *N*-acetyl-*l*-cysteine, amoxicillin, erythromycin, and levofloxacin. *Diagn Microbiol Infect Dis,* 67**,** 311-8.

Desai, M. J. & D. W. Armstrong (2003) Separation, identification, and characterization of microorganisms by capillary electrophoresis. *Microbiol Mol Biol Rev,* 67**,** 38-51.

Dethlefsen, L., M. McFall-Ngai & D. A. Relman (2007) An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature,* 449**,** 811-18.

Djordjevic, D., M. Wiedmann & L. A. McLandsborough (2002) Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl Environ Microbiol,* 68**,** 2950-8.

Domínguez-Herrera, J., F. Docobo-Pérez, R. López-Rojas, C. Pichardo, R. Ruiz-Valderas, J. A. Lepe & J. Pachón (2012) Efficacy of daptomycin versus vancomycin in an experimental model of foreign-body and systemic infection caused by biofilm producers and methicillin-resistant *Staphylococcus epidermidis*. *Antimicrob Agents Chemother,* 56**,** 613-7.

Donlan, R. & J. Costerton (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev,* 15**,** 167-193.

Donlan, R. M. (2001) Biofilm formation: a clinically relevant microbiological process. *Clin Infect Dis,* 33**,** 1387-92.

Dunkle, J. A., L. Xiong, A. S. Mankin & J. H. Cate (2010) Structures of the *Escherichia coli* ribosome with antibiotics bound near the peptidyl transferase center explain spectra of drug action. *Proc Natl Acad Sci U S A,* 107**,** 17152-7.

Dunne, W. M. (2002) Bacterial adhesion: seen any good biofilms lately? *Clin Microbiol Rev,* 15**,** 155-66.

Dürig, A., I. Kouskoumvekaki, R. M. Vejborg & P. Klemm (2010) Chemoinformatics-assisted development of new anti-biofilm compounds. *Appl Microbiol Biotechnol,* 87**,** 309-17.

ECDC/EMEA. 2009. The bacterial challenge: time to react. http://www.ema.europa.eu/docs/en_GB/document_library/Report/2009/11/WC500008770.pdf. (12.9.2012)

Engel, T. (2006) Basic overview of chemoinformatics. *J Chem Inf Model,* 46**,** 2267-77.

Ensing, G. T., D. Neut, J. R. van Horn, H. C. van der Mei & H. J. Busscher (2006) The combination of ultrasound with antibiotics released from bone cement decreases the viability of planktonic and biofilm bacteria: an in vitro study with clinical strains. *J Antimicrob Chemother,* 58**,** 1287-90.

Ferretti, G., M. Mandala, S. Di Cosimo, C. Moro, G. Curigliano & S. Barni (2003) Catheter-related bloodstream infections, part II: specific pathogens and prevention. *Cancer Control,* 10**,** 79-91.

Field, T. R., A. White, J. S. Elborn & M. M. Tunney (2005) Effect of oxygen limitation on the in vitro antimicrobial susceptibility of clinical isolates of *Pseudomonas aeruginosa* grown planktonically and as biofilms. *Eur J Clin Microbiol Infect Dis,* 24**,** 677-87.

Ford, C. W., G. E. Zurenko & M. R. Barbachyn (2001) The discovery of linezolid, the first oxazolidinone antibacterial agent. *Curr Drug Targets Infect Disord,* 1**,** 181-99.

Fox, J. L. (2006) The business of developing antibacterials. *Nat Biotechnol,* 24**,** 1521-8.

Fux, C. A., S. Wilson & P. Stoodley (2004) Detachment characteristics and oxacillin resistance of *Staphyloccocus aureus* biofilm emboli in an in vitro catheter infection model. *J Bacteriol,* 186**,** 4486-91.

Gacesa, P. (1998) Bacterial alginate biosynthesis recent progress and future prospects. *Microbiology,* 144 (Pt 5)**,** 1133-43.

Ganesan, A. (2008) The impact of natural products upon modern drug discovery. *Curr Opin Chem Biol,* 12**,** 306-17.

Gerke, C., A. Kraft, R. Süssmuth, O. Schweitzer & F. Götz (1998) Characterization of the *N*-acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. *J Biol Chem,* 273**,** 18586-93.

Giacometti, A., O. Cirioni, R. Ghiselli, F. Orlando, F. Mocchegiani, C. Silvestri, A. Licci, M. De Fusco, M. Provinciali, V. Saba & G. Scalise (2005) Comparative efficacies of quinupristin-dalfopristin, linezolid, vancomycin, and ciprofloxacin in treatment, using the antibiotic-lock technique, of experimental catheter-related infection due to *Staphylococcus aureus*. *Antimicrob Agents Chemother,* 49**,** 4042-5.

Gibbons, S. (2005) Plants as a source of bacterial resistance modulators and anti-infective agents. *Phytochem Rev,* 4**,** 63-78.

Goerke, C. & C. Wolz (2010) Adaptation of *Staphylococcus aureus* to the cystic fibrosis lung. *Int J Med Microbiol,* 300**,** 520-5.

Gomes, F. I., P. Teixeira, J. Azeredo & R. Oliveira (2009) Effect of farnesol on planktonic and biofilm cells of *Staphylococcus epidermidis*. *Curr Microbiol,* 59**,** 118-22.

Gorman, S. P. & D. S. Jones. 2006. Medical device composition and biological secretion influences on biofilm formation. In *Biofilms, infection and antimicrobial therapy,* eds. J. L. Pace, M. E. Rupp & R. G. Finch, 51-70. Boca Raton, FL, USA: Taylor & Francis Group.

Grabley, S. & I. Sattler. 2003. Natural products for lead identification: Nature is a valuable resource for providing tools. In *Modern Methods of Drug Discovery,* eds. A. Hillisch & R. Hilgenfeld, 87-107. Basel, Switzerland: Birkhäuser Verlag.

Graninger, W., O. Assadian, H. Lagler & M. Ramharter (2002) The role of glycopeptides in the treatment of intravascular catheter-related infections. *Clin Microbiol Infect,* 8**,** 310-5.

Greenwood, D. 2008. *Antimicrobial Drugs*. Oxford, United Kingdom: Oxford University Press.

Gribbon, P. & A. Sewing (2003) Fluorescence readouts in HTS: no gain without pain? *Drug Discov Today,* 8**,** 1035-43.

Gross, M., S. E. Cramton, F. Götz & A. Peschel (2001) Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. *Infect Immun,* 69**,** 3423-6.

Guerin, T. F., M. Mondido, B. McClenn & B. Peasley (2001) Application of resazurin for estimating abundance of contaminant-degrading micro-organisms. *Lett Appl Microbiol,* 32**,** 340-5.

Gwynn, M. N., A. Portnoy, S. F. Rittenhouse & D. J. Payne (2010) Challenges of antibacterial discovery revisited. *Ann N Y Acad Sci,* 1213**,** 5-19.

Gómez, J., E. Canovas, V. Baños, L. Martínez, E. García, A. Hernández-Torres, M. Canteras, J. Ruiz, M. Medina, P. Martínez, A. Canovas, A. Soriano & M. Clavel (2011) Linezolid plus rifampin as a salvage therapy in prosthetic joint infections treated without removing the implant. *Antimicrob Agents Chemother,* 55**,** 4308-10.

Götz, F. (2002) Staphylococcus and biofilms. *Mol Microbiol,* 43**,** 1367-78.

Günther, F., G. H. Wabnitz, P. Stroh, B. Prior, U. Obst, Y. Samstag, C. Wagner & G. M. Hänsch (2009) Host defence against *Staphylococcus aureus* biofilms infection: phagocytosis of biofilms by polymorphonuclear neutrophils (PMN). *Mol Immunol,* 46**,** 1805-13.

Haddadin, R. N., S. Saleh, I. S. Al-Adham, T. E. Buultjens & P. J. Collier (2010) The effect of subminimal inhibitory concentrations of antibiotics on virulence factors expressed by *Staphylococcus aureus* biofilms. *J Appl Microbiol,* 108**,** 1281-91.

Hajdu, S., A. Lassnigg, W. Graninger, A. M. Hirschl & E. Presterl (2009) Effects of vancomycin, daptomycin, fosfomycin, tigecycline, and ceftriaxone on *Staphylococcus epidermidis* biofilms. *J Orthop Res,* 27**,** 1361-5.

Hall-Stoodley, L., J. W. Costerton & P. Stoodley (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol,* 2**,** 95-108.

Hall-Stoodley, L. & P. Stoodley (2005) Biofilm formation and dispersal and the transmission of human pathogens. *Trends Microbiol,* 13**,** 7-10.

Hall-Stoodley, L. & P. Stoodley (2009) Evolving concepts in biofilm infections. *Cell Microbiol,* 11**,** 1034-43.

Harvey, A. L. (2008) Natural products in drug discovery. *Drug Discov Today,* 13**,** 894-901.

Hazan, Z., J. Zumeris, H. Jacob, H. Raskin, G. Kratysh, M. Vishnia, N. Dror, T. Barliya, M. Mandel & G. Lavie (2006) Effective prevention of microbial biofilm formation on medical devices by low-energy surface acoustic waves. *Antimicrob Agents Chemother,* 50**,** 4144-52.

Henrici, A. T. (1933) Studies of freshwater bacteria: I. A direct microscopic technique. *J Bacteriol,* 25**,** 277-87.

Herrmann, G., L. Yang, H. Wu, Z. Song, H. Wang, N. Høiby, M. Ulrich, S. Molin, J. Riethmüller & G. Döring (2010) Colistin-tobramycin combinations are superior to monotherapy concerning the killing of biofilm *Pseudomonas aeruginosa*. *J Infect Dis,* 202**,** 1585-92.

Higashi, J. M. & P. M. Sullam. 2006. *Staphylococcus aureus* biofilms. In *Biofilms, infection and antimicrobial therapy,* eds. J. L. Pace, M. E. Rupp & R. G. Finch, 81-108. Boca Raton, FL, USA: Taylor & Francis Group.

Holá, V., F. Ruzicka & M. Horka (2010) Microbial diversity in biofilm infections of the urinary tract with the use of sonication techniques. *FEMS Immunol Med Microbiol,* 59**,** 525-8.

Honraet, K. & H. J. Nelis (2006) Use of the modified robbins device and fluorescent staining to screen plant extracts for the inhibition of S. mutans biofilm formation. *J Microbiol Methods,* 64**,** 217-24.

Huang, Q., H. J. Yu, G. D. Liu, X. K. Huang, L. Y. Zhang, Y. G. Zhou, J. Y. Chen, F. Lin, Y. Wang & J. Fei (2012) Comparison of the effects of human β-defensin 3, vancomycin, and clindamycin on *Staphylococcus aureus* biofilm formation. *Orthopedics,* 35**,** e53-60.

Hurdle, J. G., R. Yendapally, D. Sun & R. E. Lee (2009) Evaluation of analogs of reutericyclin as prospective candidates for treatment of staphylococcal skin infections. *Antimicrob Agents Chemother,* 53**,** 4028-31.

Høiby, N. (2011) Recent advances in the treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis. *BMC Med,* 9**,** 32.

Jabbouri, S. & I. Sadovskaya (2010) Characteristics of the biofilm matrix and its role as a possible target for the detection and eradication of *Staphylococcus epidermidis* associated with medical implant infections. *FEMS Immunol Med Microbiol,* 59**,** 280-91.

Jabra-Rizk, M. A., T. F. Meiller, C. E. James & M. E. Shirtliff (2006) Effect of farnesol on *Staphylococcus aureus* biofilm formation and antimicrobial susceptibility. *Antimicrob Agents Chemother,* 50**,** 1463-9.

Jang, C. H., H. Park, Y. B. Cho, C. H. Choi & I. Y. Park (2009) The use of piperacillin-tazobactam coated tympanostomy tubes against ciprofloxacin-resistant Pseudomonas biofilm formation: an in vitro study. *Int J Pediatr Otorhinolaryngol,* 73**,** 295-9.

Jefferson, K. K., D. A. Goldmann & G. B. Pier (2005) Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. *Antimicrob Agents Chemother,* 49**,** 2467-73.

John, A. K., D. Baldoni, M. Haschke, K. Rentsch, P. Schaerli, W. Zimmerli & 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.

Johnston, P. 2002. Cellular assays in HTS. In *High throughput screening, methods and protocols,* ed. W. P. Janzen, 107-116. Totowa, New Jersey, USA: Humana Press Inc.

Jonas, K., H. Tomenius, A. Kader, S. Normark, U. Römling, L. Belova & Ö. Melefors (2007) Roles of curli, cellulose and BapA in Salmonella biofilm morphology studied by atomic force microscopy. *BMC Microbiology,* 7**,** 1-9.

Jones, W. L., M. P. Sutton, L. McKittrick & P. S. Stewart (2011) Chemical and antimicrobial treatments change the viscoelastic properties of bacterial biofilms. *Biofouling,* 27**,** 207-15.

Jorgensen, J. H. (1993) Selection criteria for an antimicrobial susceptibility testing system. *J Clin Microbiol,* 31**,** 2841-4.

Junker, L. M. & J. Clardy (2007) High-throughput screens for small-molecule inhibitors of

Pseudomonas aeruginosa biofilm development. *Antimicrob Agents Chemother,* 51**,** 3582-90.

Karthikeyan, S. & T. J. Beveridge (2002) *Pseudomonas aeruginosa* biofilms react with and precipitate toxic soluble gold. *Environ Microbiol,* 4**,** 667-75.

Kennedy, J. P., L. Williams, T. M. Bridges, R. N. Daniels, D. Weaver & C. W. Lindsley (2008) Application of combinatorial chemistry science on modern drug discovery. *J Comb Chem,* 10**,** 345-54.

Kiedrowski, M. R. & A. R. Horswill (2011) New approaches for treating staphylococcal biofilm infections. *Ann N Y Acad Sci,* 1241**,** 104-21.

King, P., O. Lomovskaya, D. C. Griffith, J. L. Burns & M. N. Dudley (2010) In vitro pharmacodynamics of levofloxacin and other aerosolized antibiotics under multiple conditions relevant to chronic pulmonary infection in cystic fibrosis. *Antimicrob Agents Chemother,* 54**,** 143-8.

Kirov, S. M., J. S. Webb, C. Y. O'may, D. W. Reid, J. K. Woo, S. A. Rice & S. Kjelleberg (2007) Biofilm differentiation and dispersal in mucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Microbiology,* 153**,** 3264-74.

Klein, J. O. (1994) Otitis media. *Clin Infect Dis,* 19**,** 823-33.

Koebnik, R., K. P. Locher & P. Van Gelder (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol,* 37**,** 239-53.

Kolari, M., J. Nuutinen & M. S. Salkinoja-Salonen (2001) Mechanisms of biofilm formation in paper machine by Bacillus species: the role of *Deinococcus geothermalis*. *J Ind Microbiol Biotechnol,* 27**,** 343-51.

Koseoglu, H., G. Aslan, N. Esen, B. H. Sen & H. Coban (2006) Ultrastructural stages of biofilm development of *Escherichia coli* on urethral catheters and effects of antibiotics on biofilm formation. *Urology,* 68**,** 942-6.

Kronvall, G. (1982) Analysis of a single reference strain for determination of gentamicin regression line constants and inhibition zone diameter breakpoints in quality control of disk diffusion antibiotic susceptibility testing. *J Clin Microbiol,* 16**,** 784-93.

Ku, T. S., S. K. Palanisamy & S. A. Lee (2010) Susceptibility of *Candida albicans* biofilms to azithromycin, tigecycline and vancomycin and the interaction between tigecycline and antifungals. *Int J Antimicrob Agents,* 36**,** 441-6.

Kubo, I., K. Fujita & K. Nihei (2003) Molecular design of multifunctional antibacterial agents against methicillin resistant *Staphylococcus aureus* (MRSA). *Bioorg Med Chem,* 11**,** 4255-62.

Kubo, I., P. Xiao & K. Fujita (2002) Anti-MRSA activity of alkyl gallates. *Bioorg Med Chem Lett,* 12**,** 113-6.

Kuehl, R., S. Al-Bataineh, O. Gordon, R. Luginbuehl, M. Otto, M. Textor & R. Landmann (2009) Furanone at subinhibitory concentrations enhances staphylococcal biofilm formation by luxS repression. *Antimicrob Agents Chemother,* 53**,** 4159-66.

Kümmel, A., H. Gubler, P. Gehin, M. Beibel, D. Gabriel & C. N. Parker (2010) Integration of multiple readouts into the *Z'*-factor for assay quality assessment. *J Biomol Screen,* 15**,** 95-101.

Kłodzińska, E., M. Szumski, K. Hrynkiewicz, E. Dziubakiewicz, M. Jackowski & B. Buszewski (2009) Differentiation of *Staphylococcus aureus* strains by CE, zeta potential and coagulase gene polymorphism. *Electrophoresis,* 30**,** 3086-91.

Landini, P., D. Antoniani, J. Burgess & R. Nijland (2010) Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. *Appl Microbiol Biotechnol,* 86**,** 813-23.

LaPlante, K. L. & L. A. Mermel (2007) In vitro activity of daptomycin and vancomycin lock solutions on staphylococcal biofilms in a central venous catheter model. *Nephrol Dial Transplant,* 22**,** 2239-46.

LaPlante, K. L. & S. Woodmansee (2009) Activities of daptomycin and vancomycin alone and in combination with rifampin and gentamicin against biofilm-forming methicillin-resistant *Staphylococcus aureus* isolates in an experimental model of endocarditis. *Antimicrob Agents Chemother,* 53**,** 3880-6.

Larsson, J., J. Gottfries, S. Muresan & A. Backlund (2007) ChemGPS-NP: tuned for navigation in biologically relevant chemical space. *J Nat Prod,* 70**,** 789-94.

Leal, M. C., J. Puga, J. Serôdio, N. C. Gomes & R. Calado (2012) Trends in the discovery of new marine natural products from invertebrates over the last two decades--where and what are we bioprospecting? *PLoS One,* 7**,** e30580.

Lee, J. Y., K. S. Ko, K. R. Peck, W. S. Oh & J. H. Song (2006) In vitro evaluation of the antibiotic lock technique (ALT) for the treatment of catheter-related infections caused by staphylococci. *J Antimicrob Chemother,* 57**,** 1110-5.

Leid, J. G., M. E. Shirtliff, J. W. Costerton & P. Stoodley (2002) Human leukocytes adhere to, penetrate, and respond to *Staphylococcus aureus* biofilms. *Infect Immun,* 70**,** 6339-45.

Leite, B., F. Gomes, P. Teixeira, C. Souza, E. Pizzolitto & R. Oliveira (2011a) In vitro activity of daptomycin, linezolid and rifampicin on *Staphylococcus epidermidis* biofilms. *Curr Microbiol,* 63**,** 313-7.

Leite, B., F. Gomes, P. Teixeira, C. Souza, E. Pizzolitto & Oliveira R. (2011b) *Staphylococcus epidermidis* biofilms control by *N*-acetylcysteine and rifampicin. *Am J Ther*. 2011 Apr 23. [Epub ahead of print].

Lewis, K. (2007) Persister cells, dormancy and infectious disease. *Nat Rev Microbiol,* 5**,** 48-56.

Li, X., Z. Yan & J. Xu (2003) Quantitative variation of biofilms among strains in natural populations of *Candida albicans*. *Microbiology,* 149**,** 353-62.

Liaqat, I., F. Sumbal & A. N. Sabri (2009) Tetracycline and chloramphenicol efficiency against selected biofilm forming bacteria. *Curr Microbiol,* 59**,** 212-20.

Linton, C. J., A. Sherriff & M. R. Millar (1999) Use of a modified Robbins device to directly compare the adhesion of *Staphylococcus epidermidis* RP62A to surfaces. *J Appl Microbiol,* 86**,** 194-202.

Lipinski, C. A., F. Lombardo, B. W. Dominy & P. J. Feeney (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev,* 46**,** 3-26.

Lonnemann, G. (2004) When good water goes bad: how it happens, clinical consequences and possible solutions. *Blood Purif,* 22**,** 124-9.

Lynch, A. S. & G. T. Robertson (2008) Bacterial and fungal biofilm infections. *Annu Rev Med,* 59**,** 415-28.

Macarrón, R. & R. P. Hertzberg (2002) Design and implementation of high throughput screening assays. In *High throughput screening, methods and protocols,* ed. W. P. Janzen, 1-29. Totowa, New Jersey, USA: Humana Press Inc.

Macarrón, R. & R. P. Hertzberg (2011) Design and implementation of high throughput screening assays. *Mol Biotechnol,* 47**,** 270-85.

Mai-Prochnow, A., F. Evans, D. Dalisay-Saludes, S. Stelzer, S. Egan, S. James, J. S. Webb & S. Kjelleberg (2004) Biofilm development and cell death in the marine bacterium *Pseudoalteromonas tunicata*. *Appl Environ Microbiol,* 70**,** 3232-8.

Mai-Prochnow, A., J. S. Webb, B. C. Ferrari & S. Kjelleberg (2006) Ecological advantages of autolysis during the development and dispersal of *Pseudoalteromonas tunicata* biofilms. *Appl Environ Microbiol,* 72**,** 5414-20.

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 & K. W. Bayles (2009) Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. *PLoS One,* 4**,** e5822.

Mariscal, A., R. Lopez-Gigosos, M. Carnero-Varo & J. Fernandez-Crehuet (2009) Fluorescent assay based on resazurin for detection of activity of disinfectants against bacterial biofilm. *Appl Microbiol Biotechnol,* 82**,** 773-83.

Matter, H. 2003. Computational approaches towards the quantification of molecular diversity and design of compound libraries. In *Modern methods of drug discovery,* eds. A. Hillisch & R. Hilgenfeld, 125-156. Basel, Switzerland: Birkhäuser Verlag.

Mattick, J. S. (2002) Type IV pili and twitching motility. *Annu Rev Microbiol,* 56**,** 289-314.

Mayr, L. M. & D. Bojanic (2009) Novel trends in high-throughput screening. *Curr Opin Pharmacol,* 9**,** 580-8.

McConeghy, K. W. & K. L. LaPlante (2010) In vitro activity of tigecycline in combination with gentamicin against biofilm-forming *Staphylococcus aureus*. *Diagn Microbiol Infect Dis,* 68**,** 1-6.

Merten, C. A. (2010) Screening Europe 2010: an update about the latest technologies and applications in high-throughput screening. *Expert Rev Mol Diagn,* 10**,** 559-63.

Miceli, M. H., S. M. Bernardo & S. A. Lee (2009) In vitro analyses of the combination of high-dose doxycycline and antifungal agents against *Candida albicans* biofilms. *Int J Antimicrob Agents,* 34**,** 326-32.

Michael, S., D. Auld, C. Klumpp, A. Jadhav, W. Zheng, N. Thorne, C. P. Austin, J. Inglese & A. Simeonov (2008) A robotic platform for quantitative high-throughput screening. *Assay Drug Dev Technol,* 6**,** 637-57.

Minardi, D., O. Cirioni, R. Ghiselli, C. Silvestri, F. Mocchegiani, E. Gabrielli, G. d'Anzeo, A. Conti, F. Orlando, M. Rimini, L. Brescini, M. Guerrieri, A. Giacometti & G. Muzzonigro (2011) Efficacy of tigecycline and rifampin alone and in combination against *Enterococcus faecalis* biofilm infection in a rat model of ureteral stent. *J Surg Res*, 176**,** 1-6. Epub 2011 May 31.

Mishra, B. B. & V. K. Tiwari (2011) Natural products: an evolving role in future drug discovery. *Eur J Med Chem,* 46**,** 4769-807.

Moellering, R. C. (2011) Discovering new antimicrobial agents. *Int J Antimicrob Agents,* 37**,** 2-9.

Monzón, M., C. Oteiza, J. Leiva, M. Lamata & B. Amorena (2002) Biofilm testing of *Staphylococcus epidermidis* clinical isolates: low performance of vancomycin in relation to other antibiotics. *Diagn Microbiol Infect Dis,* 44**,** 319-24.

Morrow, B. J., D. Abbanat, E. Z. Baum, S. M. Crespo-Carbone, T. A. Davies, W. He, W. Shang, A. M. Queenan & A. S. Lynch (2011) Antistaphylococcal activities of the new fluoroquinolone JNJ-Q2. *Antimicrob Agents Chemother,* 55**,** 5512-21.

Moskowitz, S. M., J. C. Emerson, S. McNamara, R. D. Shell, D. M. Orenstein, D. Rosenbluth, M. F. Katz, R. Ahrens, D. Hornick, P. M. Joseph, R. L. Gibson, M. L. Aitken, W. W. Benton & J. L. Burns (2011) Randomized trial of biofilm testing to select antibiotics for cystic fibrosis airway infection. *Pediatr Pulmonol,* 46**,** 184-92.

Moskowitz, S. M., J. M. Foster, J. Emerson & J. L. Burns (2004) Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. *J Clin Microbiol,* 42**,** 1915-22.

Muegge, I. (2003) Selection criteria for drug-like compounds. *Med Res Rev,* 23**,** 302-21.

Männistö, M. & M. Häggblom (2006) Characterization of psychrotolerant heterotrophic bacteria from Finnish Lapland. *Syst Appl Microbiol,* 29**,** 229-43.

Newman, D. J. & G. M. Cragg (2007) Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products,* 70**,** 461-477.

Newman, D. J. & G. M. Cragg (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Prod*, 75**,** 311-35.

Nguyen, C. T., H. Tu, E. J. Chaney, C. N. Stewart & S. A. Boppart (2010) Non-invasive optical interferometry for the assessment of biofilm growth in the middle ear. *Biomed Opt Express,* 1**,** 1104-16.

Norrby, R. (2001) Linezolid--a review of the first oxazolidinone. *Expert Opin Pharmacother,* 2**,** 293-302.

O'Brien, J., I. Wilson, T. Orton & F. Pognan (2000) Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cytotoxicity. *Eur J Biochem,* 267**,** 5421-26.

O'Donnell, M. J., M. A. Boyle, R. J. Russell & D. C. Coleman (2011) Management of dental unit waterline biofilms in the 21st Century. *Future Microbiol,* 6**,** 1209-26.

O'Shea, R. & H. E. Moser (2008) Physicochemical properties of antibacterial compounds: implications for drug discovery. *J Med Chem,* 51**,** 2871-8.

O'Toole, G., H. B. Kaplan & R. Kolter (2000) Biofilm formation as microbial development. *Annu Rev Microbiol,* 54**,** 49-79.

Olson, M., H. Ceri, D. Morck, A. Buret & R. Read (2002) Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Canadian Journal of Veterinary Research,* 66**,** 86-92.

Ooi, N., K. Miller, C. Randall, W. Rhys-Williams, W. Love & I. Chopra (2010) XF-70 and XF-73, novel antibacterial agents active against slow-growing and non-dividing cultures of *Staphylococcus*

aureus including biofilms. *J Antimicrob Chemother,* 65**,** 72-8.

Oramas-Shirey, M. P., L. V. Buchanan, C. L. Dileto-Fang, C. F. Dailey, C. W. Ford, D. H. Batts & J. K. Gibson (2001) Efficacy of linezolid in a staphylococcal endocarditis rabbit model. *J Antimicrob Chemother,* 47**,** 349-52.

Otto, M. (2004) Virulence factors of the coagulase-negative staphylococci. *Front Biosci,* 9**,** 841-63.

Ozturk, A. M., A. Y. Tabak, C. N. Aktekin, M. Altay, E. Erdemli, S. Karahuseyinoglu & F. Korkusuz (2008) Alendronate enhances antibiotic-impregnated bone grafts in the treatment of osteomyelitis. *Int Orthop,* 32**,** 821-7.

Pace, N. R. (1997) A molecular view of microbial diversity and the biosphere. *Science,* 276**,** 734-40.

Pace, N. R. (2009) Mapping the tree of life: progress and prospects. *Microbiol Mol Biol Rev,* 73**,** 565-76.

Parsek, M. R. & P. K. Singh (2003) Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol,* 57**,** 677-701.

Pascual, A. (2002) Pathogenesis of catheter-related infections: lessons for new designs. *Clin Microbiol Infect,* 8**,** 256-64.

Patti, J. M., B. L. Allen, M. J. McGavin & M. Höök (1994) MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu Rev Microbiol,* 48**,** 585-617.

Payne, D. J., M. N. Gwynn, D. J. Holmes & D. L. Pompliano (2007) Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat Rev Drug Discov,* 6**,** 29-40.

Peeters, E., H. Nelis & T. Coenye (2008) Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J Microbiol Methods,* 72**,** 157-65.

Pereira, S. G., J. Paixão, R. Leitão & O. Cardoso (2011*) Pseudomonas aeruginosa* in a hydropathic facility: diversity, susceptibility and imipenem resistance mutation. *Lett Appl Microbiol,* 53**,** 518-24.

Pettit, R., C. Weber & G. Pettit (2009) Application of a high throughput Alamar blue biofilm susceptibility assay to *Staphylococcus aureus* biofilms. *Ann Clin Microbiol Antimicrob,* 8**,** 28.

Pettit, R. K., C. A. Weber, M. J. Kean, H. Hoffmann, G. R. Pettit, R. Tan, K. S. Franks & M. L. Horton (2005) Microplate Alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. *Antimicrob Agents Chemother,* 49**,** 2612-7.

Pitts, B., M. Hamilton, N. Zelver & P. Stewart (2003) A microtiter-plate screening method for biofilm disinfection and removal. *J Microbiol Methods,* 54**,** 269-76.

Pratt, L. & R. Kolter (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Molecular Microbiology,* 30**,** 285-93.

Pratt, L. A. & R. Kolter (1999) Genetic analyses of bacterial biofilm formation. *Curr Opin Microbiol,* 2**,** 598-603.

Proudfoot, J. R. (2002) Drugs, leads, and drug-likeness: an analysis of some recently launched drugs. *Bioorg Med Chem Lett,* 12**,** 1647-50.

Raad, I. (1998) Intravascular-catheter-related infections. *Lancet,* 351**,** 893-8.

Raad, I., R. Darouiche, J. Vazquez, A. Lentnek, R. Hachem, H. Hanna, B. Goldstein, T. Henkel & E. Seltzer (2005) Efficacy and safety of weekly dalbavancin therapy for catheter-related bloodstream infection caused by gram-positive pathogens. *Clin Infect Dis,* 40**,** 374-80.

Raad, I., H. Hanna, Y. Jiang, T. Dvorak, R. Reitzel, G. Chaiban, R. Sherertz & 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**,** 1656-60.

Raad, I. I. & H. A. Hanna (2002) Intravascular catheter-related infections: new horizons and recent advances. *Arch Intern Med,* 162**,** 871-8.

Rahman, M., I. Kühn, B. Olsson-Liljequist & R. Möllby (2004) Evaluation of a scanner-assisted colorimetric MIC method for susceptibility testing of gram-negative fermentative bacteria. *Appl Environ Microbiol,* 70**,** 2398-403.

Rehm, S. J., H. Boucher, D. Levine, M. Campion, B. I. Eisenstein, G. A. Vigliani, G. R. Corey & E. Abrutyn

(2008) Daptomycin versus vancomycin plus gentamicin for treatment of bacteraemia and endocarditis due to *Staphylococcus aureus*: subset analysis of patients infected with methicillin-resistant isolates. *J Antimicrob Chemother,* 62**,** 1413-21.

Riera, E., M. D. Macià, A. Mena, X. Mulet, J. L. Pérez, Y. Ge & A. Oliver (2010) Anti-biofilm and resistance suppression activities of CXA-101 against chronic respiratory infection phenotypes *of Pseudomonas aeruginosa* strain PAO1. *J Antimicrob Chemother,* 65**,** 1399-404.

Rodríguez-Martínez, J. M., S. Ballesta & A. Pascual (2007) Activity and penetration of fosfomycin, ciprofloxacin, amoxicillin/clavulanic acid and co-trimoxazole in *Escherichia coli* and *Pseudomonas aeruginosa* biofilms. *Int J Antimicrob Agents,* 30**,** 366-8.

Rose, W. E. & P. T. Poppens (2009) Impact of biofilm on the in vitro activity of vancomycin alone and in combination with tigecycline and rifampicin against *Staphylococcus aureus*. *J Antimicrob Chemother,* 63**,** 485-8.

Rosén, J., A. Lövgren, T. Kogej, S. Muresan, J. Gottfries & A. Backlund (2009) ChemGPS-NP(Web): chemical space navigation online. *J Comput Aided Mol Des,* 23**,** 253-9.

Saber, S. l.-D. & S. A. El-Hady (2012) Development of an intracanal mature Enterococcus faecalis biofilm and its susceptibility to some antimicrobial intracanal medications; an in vitro study. *Eur J Dent,* 6**,** 43-50.

Saga, T. & K. Yamaguchi (2009) History of antimicrobial agents and resistant bacteria. *JMAJ,* 52**,** 103-108.

Sams-Dodd, F. (2005) Target-based drug discovery: is something wrong? *Drug Discov Today,* 10**,** 139-47.

Sauer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton & D. G. Davies (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol,* 184**,** 1140-54.

Schinabeck, M. K. & M. A. Ghannoum. 2006. Biofilm-related indwelling medical device infections. In *Biofilms, infection and antimicrobial therapy,* eds. J. L. Pace, M. E. Rupp & R. G. Finch, 39-50. Boca Raton, FL, USA: Taylor & Francis Group.

Schlünzen, F., R. Zarivach, J. Harms, A. Bashan, A. Tocilj, R. Albrecht, A. Yonath & F. Franceschi (2001) Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. *Nature,* 413**,** 814-21.

Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh & E. P. Greenberg (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature,* 407**,** 762-4.

Singh, R., P. Ray, A. Das & M. Sharma (2009) Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated Staphylococcus aureus: an in vitro study. *J Med Microbiol,* 58**,** 1067-73.

Singh, R., P. Ray, A. Das & M. Sharma (2010) Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *J Antimicrob Chemother,* 65**,** 1955-8.

Sintim, H. O., J. A. Smith, J. Wang, S. Nakayama & L. Yan (2010) Paradigm shift in discovering next-generation anti-infective agents: targeting quorum sensing, c-di-GMP signaling and biofilm formation in bacteria with small molecules. *Future Med Chem,* 2**,** 1005-35.

Smith, K., A. Perez, G. Ramage, C. G. Gemmell & 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**,** 374-8.

Smith, R. S., S. G. Harris, R. Phipps & B. Iglewski (2002a) The *Pseudomonas aeruginosa* quorum-sensing molecule N-(3-oxododecanoyl) homoserine lactone contributes to virulence and induces inflammation in vivo. *J Bacteriol,* 184**,** 1132-9.

Smith, R. S., R. Kelly, B. H. Iglewski & R. P. Phipps (2002b) The Pseudomonas autoinducer N-(3-oxododecanoyl) homoserine lactone induces cyclooxygenase-2 and prostaglandin E2 production in human lung fibroblasts: implications for inflammation. *J Immunol,* 169**,** 2636-42.

Spoering, A. L. & K. Lewis (2001) Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol,* 183**,** 6746-51.

Steinberger, R. E. & P. A. Holden (2005) Extracellular DNA in single- and multiple-species unsaturated biofilms. *Appl Environ Microbiol,* 71**,** 5404-10.

Stepanovic, S., D. Vukovic, I. Dakic, B. Savic & M. Svabic-Vlahovic (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods,* 40**,** 175-9.

Stepanović, S., D. Vuković, P. Jezek, M. Pavlović & M. Svabic-Vlahović (2001) Influence of dynamic conditions on biofilm formation by staphylococci. *Eur J Clin Microbiol Infect Dis,* 20**,** 502-4.

Stewart, P. S. & M. J. Franklin (2008) Physiological heterogeneity in biofilms. *Nat Rev Microbiol,* 6**,** 199-210.

Stoodley, P., K. Sauer, D. G. Davies & J. W. Costerton (2002) Biofilms as complex differentiated communities. *Annu Rev Microbiol,* 56**,** 187-209.

Stoodley, P., S. Wilson, L. Hall-Stoodley, J. D. Boyle, H. M. Lappin-Scott & J. W. Costerton (2001) Growth and detachment of cell clusters from mature mixed-species biofilms. *Appl Environ Microbiol,* 67**,** 5608-13.

Szewzyk, U., R. Szewzyk, W. Manz & K. H. Schleifer (2000) Microbiological safety of drinking water. *Annu Rev Microbiol,* 54**,** 81-127.

Takahashi, N. & B. Nyvad (2011) The role of bacteria in the caries process: ecological perspectives. *J Dent Res,* 90**,** 294-303.

Takenaka, S., B. Pitts, H. M. Trivedi & P. S. Stewart (2009) Diffusion of macromolecules in model oral biofilms. *Appl Environ Microbiol,* 75**,** 1750-3.

Tammela, P. (2004) Screening methods for the evaluation of biological activity in drug discovery. PhD Thesis, *Viikki Drug Discovery Technology Center, Division of Pharmacognosy, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland.*

Tammela, P., J. Alvesalo, L. Riihimäki, S. Airenne, M. Leinonen, P. Hurskainen, K. Enkvist & P. Vuorela (2004) Development and validation of a time-resolved fluorometric immunoassay for screening of antichlamydial activity using a genus-specific europium-conjugated antibody. *Anal Biochem,* 333**,** 39-48.

Tang, H. J., C. C. Chen, W. C. Ko, W. L. Yu, S. R. Chiang & Y. C. Chuang (2011) In vitro efficacy of antimicrobial agents against high-inoculum or biofilm-embedded meticillin-resistant Staphylococcus aureus with vancomycin minimal inhibitory concentrations equal to 2 μg/mL (VA2-MRSA). *Int J Antimicrob Agents,* 38**,** 46-51.

Teague, S. J., A. M. Davis, P. D. Leeson & T. Oprea (1999) The design of leadlike combinatorial libraries. *Angew Chem Int Ed Engl,* 38**,** 3743-8.

Teles, F. R., R. P. Teles, N. G. Uzel, X. Q. Song, G. Torresyap, S. S. Socransky & A. D. Haffajee (2012) Early microbial succession in redeveloping dental biofilms in periodontal health and disease. *J Periodontal Res,* 47**,** 95-104.

Tenke, P., B. Köves, K. Nagy, S. J. Hultgren, W. Mendling, B. Wullt, M. Grabe, F. M. Wagenlehner, M. Cek, R. Pickard, H. Botto, K. G. Naber & T. E. Bjerklund Johansen (2012) Update on biofilm infections in the urinary tract. *World J Urol,* 30**,** 51-7.

Thiericke, R. 2003. High-throughput screening technologies. In *Modern Methods of Drug Discovery,* eds. A. Hillisch & R. Hilgenfeld, 71-85. Basel, Switzerland: Birkhäuser Verlag.

Thomas, V., B. Sanford, R. Moreno & M. Ramsay (1997) Enzyme-linked lectinsorbent assay measures *N*-acetyl-D-glucosamine in matrix of biofilm produced by *Staphylococcus epidermidis*. *Curr Microbiol,* 35**,** 249-54.

Tollersrud, T., T. Berge, S. R. Andersen & A. Lund (2001) Imaging the surface of *Staphylococcus aureus* by atomic force microscopy. *APMIS,* 109**,** 541-5.

Toté, K., D. V. Berghe, M. Deschacht, K. de Wit, L. Maes & P. Cos (2009) Inhibitory efficacy of various antibiotics on matrix and viable mass of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. *Int J Antimicrob Agents,* 33**,** 525-31.

Toté, K., D. Vanden Berghe, L. Maes & P. Cos (2008) A new colorimetric microtitre model for the detection of *Staphylococcus aureus* biofilms. *Lett Appl Microbiol,* 46**,** 249-54.

Trampuz, A. & W. Zimmerli (2006) Antimicrobial agents in orthopaedic surgery: Prophylaxis and treatment. *Drugs,* 66**,** 1089-105.

Tré-Hardy, M., H. Traore, N. El Manssouri, F. Vanderbist, M. Vaneechoutte & M. J.

Devleeschouwer (2009) Evaluation of long-term co-administration of tobramycin and clarithromycin in a mature biofilm model of cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Int J Antimicrob Agents,* 34**,** 370-4.

Tähtinen, P. A., M. K. Laine, P. Huovinen, J. Jalava, O. Ruuskanen & A. Ruohola (2011) A placebo-controlled trial of antimicrobial treatment for acute otitis media. *N Engl J Med,* 364**,** 116-26.

Unnanuntana, A., L. Bonsignore, M. E. Shirtliff & E. M. Greenfield (2009) The effects of farnesol on *Staphylococcus aureus* biofilms and osteoblasts. An in vitro study. *J Bone Joint Surg Am,* 91**,** 2683-92.

van der Borden, A. J., H. van der Werf, H. C. van der Mei & H. J. Busscher (2004) Electric current-induced detachment of *Staphylococcus epidermidis* biofilms from surgical stainless steel. *Appl Environ Microbiol,* 70**,** 6871-4.

von Nussbaum, F., M. Brands, B. Hinzen, S. Weigand & D. Häbich (2006) Antibacterial natural products in medicinal chemistry--exodus or revival? *Angew Chem Int Ed Engl,* 45**,** 5072-129.

Vuong, C., J. M. Voyich, E. R. Fischer, K. R. Braughton, A. R. Whitney, F. R. DeLeo & M. Otto (2004) Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell Microbiol,* 6**,** 269-75.

Vuorela, P., M. Leinonen, P. Saikku, P. Tammela, J. P. Rauha, T. Wennberg & H. Vuorela (2004) Natural products in the process of finding new drug candidates. *Curr Med Chem,* 11**,** 1375-89.

Walsh, C. 2003. *Antibiotics; actions, origins, resistance*. Washington DC, USA: ASM Press.

WHO Fact sheet no 125, December 2011. http://www.who.int/mediacentre/factsheets/fs125/en/ (12.9.2012)

Williams, B. J., J. Dehnbostel & T. S. Blackwell (2010) *Pseudomonas aeruginosa*: host defence in lung diseases. *Respirology,* 15**,** 1037-56.

Wu, E. C., R. P. Kowalski, E. G. Romanowski, F. S. Mah, Y. J. Gordon & R. M. Shanks (2010) AzaSite® inhibits *Staphylococcus aureus* and coagulase-negative Staphylococcus biofilm formation in vitro. *J Ocul Pharmacol Ther,* 26**,** 557-62.

Wunberg, T., M. Hendrix, A. Hillisch, M. Lobell, H. Meier, C. Schmeck, H. Wild & B. Hinzen (2006) Improving the hit-to-lead process: data-driven assessment of drug-like and lead-like screening hits. *Drug Discov Today,* 11**,** 175-80.

Yarwood, J. M., K. M. Paquette, I. B. Tikh, E. M. Volper & E. P. Greenberg (2007) Generation of virulence factor variants in *Staphylococcus aureus* biofilms. *J Bacteriol,* 189**,** 7961-7.

Yassien, M. & N. Khardori (2001) Interaction between biofilms formed by *Staphylococcus epidermidis* and quinolones. *Diagn Microbiol Infect Dis,* 40**,** 79-89.

Yassien, M., N. Khardori, A. Ahmedy & M. Toama (1995) Modulation of biofilms of *Pseudomonas aeruginosa* by quinolones. *Antimicrob Agents Chemother,* 39**,** 2262-8.

Yin, L. Y., L. Lazzarini, F. Li, C. M. Stevens & J. H. Calhoun (2005) Comparative evaluation of tigecycline and vancomycin, with and without rifampicin, in the treatment of methicillin-resistant *Staphylococcus aureus* experimental osteomyelitis in a rabbit model. *J Antimicrob Chemother,* 55**,** 995-1002.

Zhanel, G. G., K. Homenuik, K. Nichol, A. Noreddin, L. Vercaigne, J. Embil, A. Gin, J. A. Karlowsky & D. J. Hoban (2004) The glycylcyclines: a comparative review with the tetracyclines. *Drugs,* 64**,** 63-88.

Zhang, J., T. Chung & K. Oldenburg (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen,* 4**,** 67-73.

Zhang, Y. Q., S. X. Ren, H. L. Li, Y. X. Wang, G. Fu, J. Yang, Z. Q. Qin, Y. G. Miao, W. Y. Wang, R. S. Chen, Y. Shen, Z. Chen, Z. H. Yuan, G. P. Zhao, D. Qu, A. Danchin & Y. M. Wen (2003) Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). *Mol Microbiol,* 49**,** 1577-93.

Zheng, C. J., L. Y. Han, C. W. Yap, Z. L. Ji, Z. W. Cao & Y. Z. Chen (2006) Therapeutic targets: progress of their exploration and investigation of their characteristics. *Pharmacol Rev,* 58**,** 259-79.

Ziebuhr, W., V. Krimmer, S. Rachid, I. Lössner, F. Götz & J. Hacker (1999) A novel mechanism of phase variation of virulence in *Staphylococcus epidermidis*: evidence for control of the polysaccharide intercellular adhesin synthesis by

alternating insertion and excision of the insertion sequence element IS256. *Mol Microbiol,* 32**,** 345-56.

Zimmerli, W., A. F. Widmer, M. Blatter, R. Frei & 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.

Zmantar, T., B. Kouidhi, H. Miladi, K. Mahdouani & A. Bakhrouf (2010) A microtiter plate assay for *Staphylococcus aureus* biofilm quantification at various pH levels and hydrogen peroxide supplementation. *New Microbiol,* 33**,** 137-45.

Appendix 1

Antibiotics with reported anti-biofilm activity against the most common biofilm producing strains. Trade names for compounds used clinically for severe infections in Finland.

Antibiotic	Year[a]	Target organism	Biofilm MIC	Origin[b]	References[c]
amoxicillin **AMORION** **AMOXICILLIN** **AMOXIN**	1972	*Streptococcus pneumoniae*	8 mg/l	NPD	del Prado et al. 2010
+ clavulanic acid **AMORION COMP** **AUGMENTIN**	1980	*E. coli*	5-6 mg/l		Rodríguez-Martínez et al. 2007
ampicillin **A-PEN**	1961	*S. aureus*	128 mg/l	NPD	Olson et al. 2002
amikacin **BIKLIN**	1970 s	*S. aureus* *P. aeruginosa*	 4-64 mg/l	NPD	Singh et al. 2009 King et al. 2010
azithromycin **AZITHROMYCIN** **ZITHROMAX**	1988	*P. aeruginosa* *S. aureus* *Candida albicans*		NPD	Bala et al. 2011 Wu et al. 2010 Ku et al.2010
aztreonam **AZACTAM**	1984	*P. aeruginosa*		NPD	Moskowitz et al. 2004, Field et al. 2005, Høiby 2011
cefalexin **KEFALEX** **KEFEXIN**	1967	*S. aureus*	8 mg/l	NPD	Haddadin et al. 2010
ceftazidime **CEFTAZIDIM** **GLAZIDIM**	1990	*P. aeruginosa*		NPD	Riera et al. 2010
ceftriaxone **CEFONOVA** **CEFTRIAXON** **ROCHEPHALIN**	1982	*S. epidermidis*	600-2400 mg/l	NPD	Hajdu et al. 2009
cefuroxime **CEFUROXIM** **ZINACEF** **ZINNAT**	1987	*E. coli*	1.3 mg/l (prevention)	NPD	Koseoglu et al. 2006
ciprofloxacin **CIPROFLOXACIN** **CIPROMED** **CIPROXIN** **SIPIRON**	1986	*P. aeruginosa*		Synthetic	Brooun et al. 2000
clarithromycin **CLARITHROMYCIN** **KLACID** **ZECLAR**	1990	*S. aureus* *P. aeruginosa*	4 mg/l 200 mg/l	NPD	Aguinaga et al. 2011 Tré-Hardy et al. 2009
clindamycin **CLINDAMYCIN** **CLINDOXYL** **DALACIN**	1970	*S. aureus*		NPD	Huang et al. 2012

cloxacillin **CLOXACILLIN** **STAFLOCIL**	1960	*S. aureus*	512 mg/l	NPD	Olson et al. 2002
daptomycin **CUBICIN**	2003	*S. aureus* *S. epidermidis*		Natural	Domínguez-Herrera et al. 2012, Leite et al. 2011a
doxycycline **DOXIMED** **DOXIMYCIN** **DOXITIN**	1967	*C. albicans* *Enterococcus faecalis*	256 mg/l	NPD	Miceli et al. 2009 Saber and El-Hady 2012
erythromycin **ABBOTICIN** **ERMYSIN**	1988	*S. pneumoniae*		NPD	del Prado et al. 2010
gentamicin **GENSUMYCIN** **GENTAMICIN B** **SEPTOMAL**	1963	*S. aureus* *P. aeruginosa*		Natural	McConeghy and LaPlante 2010, LaPlante and Woodmansee 2009 Cai et al. 2009
imipenem **IMIPENEM** **TIENAM**	1985	*P. aeruginosa*	2-4 mg/l	NPD	Pereira et al. 2011
levofloxacin **LEVOFLOXACIN** **TAVANIC**	1993	*S. aureus* *P. aeruginosa*	1-64 mg/l 1-2 mg/l	Synthetic	Cafiso et al. 2010 King et al. 2010
linezolid **ZYVOXID**	2000	*S. aureus* *S. epidermidis*	0.5-64 mg/l 18 mg/l	Synthetic	Cafiso et al. 2010 Leite et al. 2011a
meropenem **MERONEM** **MEROPENEM**	1994	*P. aeruginosa*	16 mg/l	NPD	Moskowitz et al. 2011
moxifloxacin **AVELOX** **VIGAMOX**	1999	*S. aureus*	0.06-4 mg/l	Synthetic	Morrow et al. 2011
mupirocin **BACTROBAN**	1985	*S. aureus,* *S. epidermidis*	0.06-0.12	Natural	Hurdle et al. 2009
nitrofurantoin **NITROFUR-C**	1978	*E. coli*		Synthetic	Blango and Mulvey 2010
norfloxacin **NORFLOXACIN**	1983	*S. epidermidis* *P. aeruginosa*	50 mg/l 3.125 mg/l	Synthetic	Yassien and Khardori 2001 Yassien et al. 1995
ofloxacin **TARIVID**	1985	*S. epidermidis* *P. aeruginosa*	6.25 mg/l 12.5 mg/l	Synthetic	Yassien and Khardori 2001 Yassien et al. 1995
rifampicin **RIMAPEN**	1959	*S. aureus* *S. epidermidis*	1-16 mg/l 10 mg/l	NPD	Cafiso et al. 2010 Leite et al. 2011a

tazobactam – piperacillin **PIPERACILLIN/ TAZOBACTAM** **TAZOCIN**	1992	*P. aeruginosa*		NPD	Jang et al. 2009
teicoplanin **TARGOCID**	1988	*S. aureus,* *S. epidermidis*	0.5 mg/l	Natural	Lee et al. 2006
tetracycline **APOCYCLIN** **ORICYCLIN**	1945	*S. epidermidis* *P. aeruginosa*		Natural	Monzón et al. 2002 Liaqat et al. 2009
tigecycline **TYGACIL**	2005	*S. aureus* *E. faecalis* *Candida albicans*	0.06-1 mg/l 4 mg/l 512 mg/l	NPD	Cafiso et al. 2010 Minardi et al. 2011 Ku et al. 2010
trimethoprim-sulfamethoxazole **COTRIM**	1960	*S. aureus* *S. epidermidis*		Synthetic	del Pozo et al. 2009
tobramycin **TOBI** **TOBRAMYCIN B** **TOMYCIN**	1975	*S. aureus* *P. aeruginosa*	2-128 mg/l	Natural	Cafiso et al. 2010 Herrmann et al. 2010
vancomycin **VANCOMYCIN** **VANCOSAN**	1956	*S. aureus,* *S. epidermidis*	2 mg/l 2 mg/l	Natural	Aybar et al. 2012, Rose and Poppens 2009, Tang et al. 2011, Giacometti et al. 2005

[a]year developed or launched (can be an old drug launched for a new purpose).
[b]NPD – natural product derivative
[c]References for the reported anti-biofilm activity of the compounds.

MALENA SKOGMAN (née Sandberg) was born on March 24, 1982 in Porvoo, Finland. She graduated from Åbo Akademi University in 2006 with a Master of Science in Biochemistry. This PhD thesis project in Pharmaceutical Sciences has taken place 2007-2012 under the supervision of Docent Adyary Fallarero and Professor Pia Vuorela at the Department of Biosciences.

Painosalama Oy | ISBN 978-952-12-2796-7