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TARGETING CELL ENVELOPE SYNTHESIS OF STREPTOCOCCUS PNEUMONIAE AND MICROFLUIDIC DIAGNOSTIC TOOL DEVELOPMENT

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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For my family

POPULAR SCIENCE SUMMARY OF THE THESIS

The discovery of antibiotics not even a century ago was a sensation, that revolutionized the treatment of infectious diseases. Antibiotics exploit differences in the bacterial and human cells to specifically target structures in the bacterial intruders and selectively kill them without harming the human body. Unfortunately, the remarkable adaptation capabilities of bacteria to adverse conditions such as toxic chemicals came to play against our chemical defense strategies. Bacteria developed resistance to antibiotics, which endangers the effectivity of this treatment strategy. A loss of antibiotics as tools to treat bacterial infections poses a threat to the individual health and society in many ways. Therefore, we need new chemicals and innovative strategies to protect this valuable resource.

Work comprising this thesis aimed for the discovery of chemicals, that kill bacteria. The human respiratory tract pathogen *Streptococcus pneumoniae* served as model organism. Two molecules were characterized and evidence for their mechanism of action is provided. By employing medicinal chemistry, biochemical analyses and microbiological characterizations, we found, that they inhibit the synthesis of the bacterial cell envelope. The compounds constitute chemical scaffolds, that provide a starting point for further research to improve their characteristics. Our investigations also gave insight and raised further questions about metabolic processes in the bacteria. The compounds described in this thesis could be tools for further research on new antibiotics.

Sepsis is an acute bacterial blood infection and a life-threatening disease. The patient's life depends on the prompt administration of an antibiotic, which requires fast diagnostics. We have worked on a method with the aim to isolate bacteria from blood. First, we developed a protocol that lysed only blood cells while not harming bacteria. Then, we employed a microfluidic technique, which isolated the bacteria from the remaining particles of the blood lysate with the help of acoustic waves. We envision, that this sample preparation method could be of help for the acceleration of sepsis diagnosis.

In conclusion, the thesis hopes to contribute work serving research that aims to assure successful treatment of bacterial infections.

ABSTRACT

Evolving antibiotic resistance warrants the development of new therapeutic and diagnostic approaches as part of the strategies to secure future antibacterial therapies and preserve the compounds currently available. Work constituting the thesis characterized small molecules yielded from a screen for autolysis inducing compounds on *Streptococcus pneumoniae*. Two compound classes were characterized, and their targets identified.

The alkylated di-cyclohexyl carboxylic acid 2CCA-1 was identified as a fatty acid mimetic, that is incorporated into pneumococcal phospholipids via the polyunsaturated host fatty acid metabolism pathway. The formed 2CCA-1 containing lipids alter membrane fluidity, and treatment with 3 µM 2CCA-1 resulted in decreased pneumococcal viability and cell wall hydrolase mediated lysis. Deletion of the fatty acid binding protein FakB3 rendered pneumococci resistant to 2CCA-1, which could explain the inherent 2CCA-1 resistance of *Staphylococcus aureus* as FakB3 homologues are predominantly absent in bacteria of the Bacillales order. The involvement of the transcriptional repressor of the endogenous fatty acid synthesis machinery FabT in 2CCA-1 resistance, showed that FakB3 dependent host fatty acid incorporation is regulated depending on extracellular fatty acid availability.

The second compound class comprised analogs of 1-amino substituted Tetrahydrocarbazoles (THCz). THCz analogs are active in the low micromolar range against an array of gram-positive bacteria as well as mycobacteria, *Neisseria gonorrhoeae* and *Moraxella catarrhalis*. Mode of action studies identified the pyrophosphate moiety of undecaprenyl pyrophosphate as the minimal binding motif for THCz, which depended on the central diamino moiety for activity. THCz analogs consequently inhibited cell wall, teichoic acid and capsular biosynthesis. Reduction of the polysaccharide capsule increased pneumococcal tolerance to the compound, but resistant mutants could not be obtained.

Furthermore, we developed a microfluidic based sample preparation method for decomplexation of bacteria containing whole blood. First, blood cells were selectively lysed while preserving bacterial viability. For the reduction of the small, below micrometer sized debris, gradient acoustic focusing was developed, that allowed separation of bacteria from the blood lysate in a microfluidic channel. The so purified sample might facilitate further microfluidic downstream operations to accelerate antimicrobial susceptibility determination of the sepsis causing pathogen.

In conclusion, the thesis work identifies the target of two new compounds with bactericidal activity and presents a microfluidic based method for sample preparation as tool in sepsis diagnosis.

LIST OF SCIENTIFIC PAPERS

I. **Reithuber E**., Nannapaneni P., Rzhepishevska O., Lindgren AEG., Illchenko O., Normark S., Almqvist F., Henriques-Normark B., Mellroth P.
The bactericidal fatty acid mimetic 2CCA-1 selectively targets pneumococcal extracellular polyunsaturated fatty acid metabolism
mBio. 2020 Dec 15; 11(6):e03027-20. doi: 10.1128/mBio.03027-20.

II. **Reithuber E.**, Wixe T., Ludwig K.C., Müller A., Uvell H., Grein F., Lindgren AEG., Muschiol S., Nannapaneni P., Eriksson A., Schneider T., Normark S., Henriques-Normark B., Almqvist F., Mellroth P.
THCz – A small molecule with antimicrobial activity that blocks cell wall lipid intermediates
Manuscript

III. Van Assche D., **Reithuber E**., Qiu W., Laurell T., Henriques-Normark B., Mellroth P., Ohlsson P., Augustsson P.
Gradient acoustic focusing of sub-micron particles for separation of bacteria from blood lysate
Sci Rep, 2020 Feb 28; 10(1):3670. Doi: 10.1038/s41598-020-60338-2.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

Toepfner N., Herold C., Otto O., Rosendahl P., Jacobi A., Kräter M., Stächele J., Menschner L., Herbig M., Ciuffreda L., Ranford-Cartwright L., Gryzbek M., Coskun Ü., **Reithuber E.**, Garriss G., Mellroth P., Henriques-Normark B., Tregay N., Suttorp M., Bornhäuser M., Chilvers ER., Berner R., Guck J.
Detection of human disease conditions by single-cell morpho-rheological phenotyping of blood.
Elife. 2018 Jan 13;7:e29213. doi: 10.7554/eLife.29213.

CONTENTS

LIST OF ABBREVIATIONS

µTAS	Miniaturized total chemical analysis systems
2CCA	Alkylated di-cyclohexyl carboxylic acid
AATGal	2-acetamido-4-amino-2,4,6-trideoxy-D-galactose
AccABCD	Acetyl coenzyme A carboxylase
Acp	Acyl carrier protein
BHI	Brain heart infusion
C+Y	Casitone and Yeast extract
C55-P	Undecaprenyl-phosphate
C55-PP	Undecaprenyl-pyrophosphate
CAMHB	Cation-adjusted Mueller-Hinton broth
CBCS	Chemical Biology Consortium Sweden
Cbp	Choline binding protein
Cfu	Colony forming units
CHAP	Cysteine-& histidine-dependent aminohydrolases / peptidases
ChoP	Phosphorylcholine
Cib	Competence induced bacteriocins
Cls	Cardiolipin synthetase
CoA	Coenzyme A
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
EMA	European Medicines Agency
EMSA	Electrophoretic mobility shift assay
FabT	Fatty acid biosynthesis transcriptional regulator
Fak	Fatty acid kinase
FASII	Fatty acid synthesis type II
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FMM	Functional membrane microdomain
G3P	Glycerol-3-phosphate
GAF	Gradient acoustic focusing
GalGlc DAG	Galactosyl-glucosyl-diacylglycerol
GalNAc	N-acetyl-galactosaminyl
GlcDAG	Monoglycosyl diacylglycerol
GlcNAc	N-acetyl-glucosamine
HIV	Human immunodeficiency virus

IgA	Immunoglobulin A
JPIAMR	Joint Programming Initiative on Antimicrobial Resistance
LCP	LytR, CpsA, Psr phosphotransferase protein family
LRTI	Lower respiratory tract infections
LTA	Lipoteichoic acids
LysM	Lysin motif
MALDI-TOF	Matrix-associated laser desorption ionization-time of flight
MHB	Mueller-Hinton broth
MIC	Minimal inhibitory concentration
MlytC	Minimal lytic concentration
OD	Optical density
PAFR	Platelet activating factor receptor
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
Pgp	Phosphatidylglycerophosphate phosphatase
Pgs	Phosphatidylglycerophosphate synthase
Pht	Polyhistidine triad
Ply	Pneumolysin
PMEN	Pneumococcal Molecular Epidemiology Network
PPSV	Pneumococcal polysaccharide vaccine
PcsB	Protein required for cell separation
PspC	Pneumococcal surface protein C
RIF	Region of increased fluidity
RPMI	Roswell Park Memorial Institute
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SEDS	Shape, elongation, division and sporulation
TacL	Lipoteichoic acid ligase
TEM	Transmission electron microscopy
THCz	1-amino substituted tetrahydrocarbazoles
THY	Todd Hewitt medium with yeast extract
TSB	Tryptic soy broth
UDP	Uridine diphosphate
US	United States
WHO	World Health Organization
WTA	Wall teichoic acids

1 INTRODUCTION

1.1 ANTIBIOTICS AND ANTIBIOTIC RESISTANCE

Antibiotics are truly the magic bullets of modern medicine that Paul Ehrlich set out to discover (*1, 2*). The main classes of antibiotics currently used in the clinics are predominantly natural products derived from actinomycetes, bacteria or fungi (*3*) and selectively target prokaryotic but not eukaryotic cells.

Resistance development is an inherent and ubiquitous consequence when bacteria are exposed to toxic chemicals (*4*) since they can adapt to adverse changes in their environment with incredible speed. This is on account of their short generation time that allows quick evolutionary adaptation by mutational changes as well as their efficient strategies for the exchange of genetic material by horizontal gene transfer even between different bacterial species (*5, 6*).

Already when awarded with the Nobel Prize for the discovery of penicillin, Alexander Fleming warned that it is the under-dosage of bactericidal agents that provokes resistance development (*7*). Indeed, broad use and misuse of antibiotics tremendously speeded up the spread of resistance (*8*), and we have to deal with antibiotic resistant infections that were previously curable. This has been demonstrated by the spread of multi- and extended- antibiotic resistant bacteria especially in nosocomial infections, where even pan-drug resistant species have been identified (*9-11*).The World Health Organization (WHO) prioritized bacteria according to the importance for novel and innovative treatment regimes, emphasizing bacterial species in critical need for such, among them especially *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and Enterobacteriacea (*12*). If no or insufficient countermeasures are taken to decelerate the current trend, antimicrobial resistance is predicted to cause 10 million deaths in 2050, undoubtedly also because cornerstones of modern medicine such as cancer treatment and surgery are based on the use of antibiotics (*13*).

The Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) prioritizes six topics for global research campaigning against antimicrobial resistance (*14*) (**Figure 1**). Among them is the search for novel therapeutic approaches to treat bacterial infections, including the search for new antibacterial molecules and drug targets, the improvement of the pharmacological properties of existing and possibly neglected antibiotics or the identification of alternative approaches to treat bacterial infections (*14*). Furthermore, innovative and improved approaches for the diagnosis of bacterial infections and the pathogen`s antibiotic susceptibility determination are warranted (*14*).

Two major strategies are applied for the discovery of novel antibacterial or antivirulence (*15*) molecules. Target based discovery approaches explore or specifically design chemicals for certain intellectually chosen targets, whereas phenotypic screens aim to discover chemicals that provoke a desired phenotype. The major classes of antibiotics were discovered empirically in the beginning of the antibiotic era (*16*). Not only have they revolutionized medical care, they

also served as probes to study bacterial physiology (*17*). The first example thereof is the discovery of metabolite accumulation in penicillin treated *Staphylococcus aureus* in 1949 (*18*), which laid the foundation for the identification of peptidoglycan precursors (*17*). Screening hits that evoke an aspired phenotype and bind to a certain molecular target might serve as research tools to understand the target's biological role, explore or validate a drug target, and pave the way for drug development (*19*), provided they meet required characteristics of potency, selectivity and chemistry (*20, 21*).

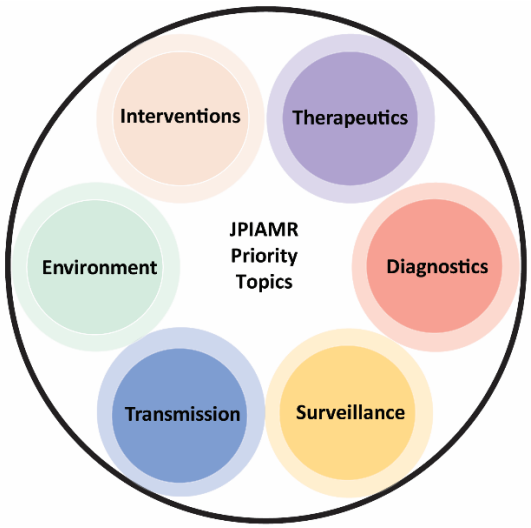

Figure 1 Research priority topics to combat antimicrobial resistance according to the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR). In the JPIAMR agenda for strategical coordination of global antimicrobial resistance research, the public health threat is tackled from a one health perspective, considering human, animal and environmental aspects of antimicrobial use and resistance development (*14*). Priority topics comprise therapeutics, diagnostics and research on transmission dynamics, including the unravelling of the environment´s role thereto. Furthermore, innovative intervention strategies for infection prevention and improvements of surveillance strategies are pronounced topics indispensable to curb antimicrobial resistance. The graphic is reproduced with permission from JPIAMR (*14*).

Innovations are needed for medical care of bacterial infections. None of the eight United States (US) Food and Drug Administration (FDA) or European Medicines Agency (EMA) approved antibacterial agents since 2017, and only four out of 25 that are currently in pre-clinical development have a new mechanism of action (*22*). However, in current pre-clinical research and development, more than halve of the ongoing projects focus on non-conventional approaches to treat bacterial infections, such as antivirulence (33 projects), phage (33 projects), immune supportive (12 projects) or microbiota modulating (21 projects) therapies (*23*). Moreover, 131 currently ongoing projects explore small antibacterial molecules with a new target, and 56 focus on already established targets, which together constitute 46 % of the current preclinical antibacterial therapy research and development (*23*).

1.2 *STREPTOCOCCUS PNEUMONIAE*

Streptococcus pneumoniae is an extensively studied bacterial species, reflecting its role as a major human pathogen. Milestones in bacteriology, such as the development of the gram-stain, identification of natural genetic transformation and the first quorum sensing molecule as well as the discovery of the immunogenicity of capsular polysaccharide, have been set by studying *S. pneumoniae* (*24, 25*). It was also the first bacterium against which the first selectively acting

drug (optochin) was tested (*25*). *S. pneumoniae* moreover served as a model organism for the disclosure of deoxyribonucleic acid (DNA) as the carrier of genetic information (*26*), findings important beyond the research field of microbiology. Nevertheless, *S. pneumoniae*, which is commonly referred to as the pneumococcus, disguises many secrets, remains a widespread opportunistic pathogen and is one of the prioritized bugs for which novel antibiotics are needed (*12*).

In this thesis, the pneumococcus is employed as a model organism for bactericidal hit discovery and mode of action studies.

1.2.1 Epidemiology, colonization and disease

The discovery of *S. pneumoniae* took place already in the era during which the germ theory of disease was proven by Koch and Pasteur (*27, 28*). The first isolation of the pneumococcus in the laboratory from blood of rabbits that were subcutaneously infected with human saliva was reported by Sternberg and Pasteur in 1880 (*28*). The pneumococcus is a gram-positive organism comprising at least 98 serotypes with distinct polysaccharide capsules (*29*).

Even though the first isolation of pneumococci was made from experimentally infected rabbits, and mice were subsequently found to display even higher susceptibility to pneumococcal infections (*28*), these were mainly consequences of the artificial test situations, which still serve valuably as animal models to study pneumococcal infectious disease (*30*). The pneumococcus is a human adapted pathogen and displays several virulence factors that might account for its host tropism. It expresses for instance a protease to selectively inactivate human immunoglobulin A (IgA), displays the pneumococcal surface protein C (PspC) to specifically interact with constituents of the human immune system such as factor H, and can discriminatively sense the human version of sialic acid in the mucus of the respiratory airways (*31-33*). Nonetheless, pneumococcal colonization is reported also in other mammals living in close contact with humans (*34-36*).

S. pneumoniae commonly resides in the human nasopharynx as asymptomatic colonizer of up to 60 % of children and less than 10 % of the adult population (*37*), implying that worldwide around 1.9 - 5.8 billion people are colonized simultaneously (*38*). Transmission occurs via aerosol and droplets of nasal secretions through close person-to-person contact, as well as contact therewith from inanimate surfaces (*29, 39, 40*). In the majority of cases, pneumococcal colonization remains asymptomatic and of short duration (1-2 months), depending on the serotype and age of the host (*41*). The pneumococcus is adapted to a colonizing lifestyle and has evolved virulence strategies to successfully establish and maintain colonization as well as to promote transmission (**Figure 2**). Key determinants therefore are adherence strategies, subversion of the immune response, biofilm formation and the interaction with the residing microbiota. *S. pneumoniae* has to compete with other common colonizers of the nasopharyngeal niche, such as *Haemophilus influencae*, *Moraxella catharralis* or *Neisseiria meningitidis* (*42, 43*). The pneumococcus has the capacity for natural competence to acquire DNA from other pneumococcal strains that simultaneously colonize the nasopharynx or from

closely related *Streptococci* of the mitis group, such as *Streptococcus mitis* or *Streptococcus pseudopneumoniae* (*44, 45*). The resulting genome plasticity and vivid exchange of genetic material in the nasopharynx allow for the quick adaptation to environmental conditions, favoring persistence or transmission.

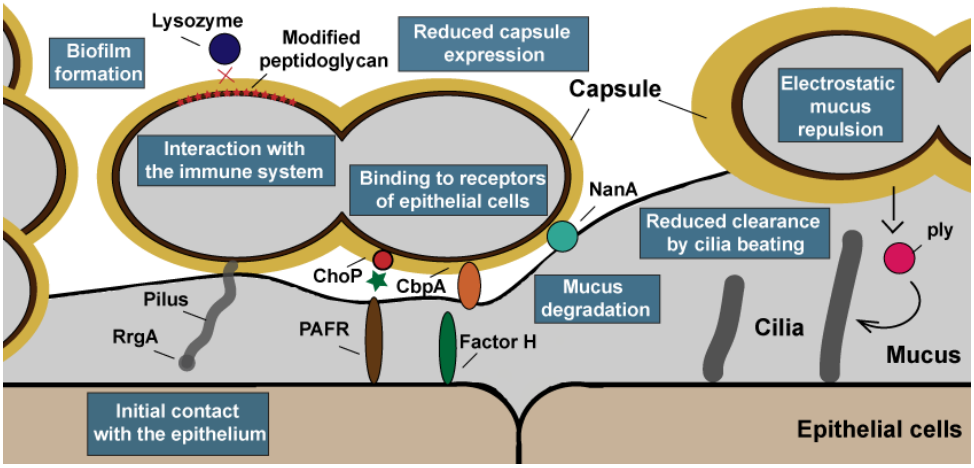

Figure 2 Selected factors needed for the establishment of pneumococcal colonization. After entering a new host, *S. pneumoniae* encounters a thick (0,5 – 10 µm) viscoelastic mucous layer containing glyco-, immune- and other proteins, inorganic salts and lipids (*38, 46, 47*). The expression of a thick, negatively charged capsule enables the pneumococcus to repel the negatively charged mucous (*29, 48*). The expression of the cytotoxin pneumolysin (Ply), damages epithelial cells, which leads to a reduction in beating cilia (*29*) and affects clearance via the oral-fecal route (*38, 46*). Additionally, the mucus is degraded by sialidases such as neuraminidase NanA, thereby reducing clearance, facilitating access to the epithelium and simultaneously sequestering valuable carbon sources (*33, 49, 50*). After paving the way closer to the epithelium, adhesins such as the ancillary pilus subunit RrgA, (UniProtKB A7KT66) on the tip of pili, choline binding protein CbpA or the cell wall component phosphorylcholine (ChoP), anchor the pneumococcus to epithelial cells (*29, 48*). ChoP interacts with the platelet activating factor receptor (PAFR) (*51*). Capsule expression was observed to be reduced in close contact to epithelial cells, likely favoring adherence (*52*). Additionally, the pneumococcus possesses an armor of strategies to ward off the immune response. An example is the modification of the peptidoglycan with reduced acetylation, which increased resistance to lysozyme (*53*). The formation of a biofilm, a pneumococcal community embedded in an extracellular matrix, strengthens colonization as it increases tolerance to antibiotics and factors of the immune response, and facilitates horizontal gene transfer (*54*).

S. pneumoniae can however spread from the nasopharynx to other, otherwise sterile tissues, and cause mild diseases like sinusitis and otitis media, but also severe invasive diseases such as pneumonia, sepsis, and meningitis (*37*). *S. pneumoniae* is the major causative pathogen of community acquired pneumonia (*55-57*). Sepsis can arise after pneumococcal invasion of the blood stream from the lungs in about one fourth of adult pneumonia cases (*56*), or in rare cases directly from the nasopharynx (occult bacteremia) (*48*). Colonization is a prerequisite for invasive disease but also for transmission, as pneumococcal pneumonia is not thought to be contagious (*50, 58*). Still, even though invasive disease is not the primary goal of pneumococcal evolution (*38*), *S. pneumoniae* is one of the most common causative bacterial agents of lower respiratory tract infections (LRTI) (*59*). LRTIs have been the 4th leading cause of death worldwide in 2010 (*60*), and remained the 8th leading cause of death in 2016 in high income countries (*61*). Pneumococcal LRTI claimed nearly 1.19 million deaths in 2016, among them 341 029 deaths of children below the age of 5 (29 % of total fatal cases) and 494 340 deaths of individuals above the age of 70 (42 % of fatal cases) (*62*).

The fate between colonization and invasive diseases relies on a finely tuned balance between pneumococcal virulence factors and the host immune system. Certain pneumococcal serotypes are more commonly isolated from colonized individuals, whereas others are associated with

invasive disease cases (*37*). Likewise, impairments of the immune system are known risk factors for the host to suffer from an invasive pneumococcal disease. Hence, young children with their immature immune system and individuals above the age of 65 with an aging immune system and comorbidities, constitute major risk groups (*63*). Furthermore, patients with immunosuppressive diseases (e.g. cancer, human immunodeficiency virus (HIV) infections), as well as individuals with underlying chronic malignancies (e.g. cardiovascular, chronic obstructive pulmonary diseases or diabetes mellitus), are at higher risk of invasive pneumococcal diseases (*63, 64*). Notably, a detrimental synergism of pneumococcal invasive diseases with viral co-infections of the influenza, rhino or respiratory syncytial viruses, is documented and accountable for disease severity (*57, 65, 66*). First observations for pneumococcal co-infections with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in critically ill patients have also been reported (*67*). Furthermore, viral infections facilitate pneumococcal transmission through increased shedding as well as colonization susceptibility of the new host (*29, 39, 68*).

Vaccines with different serotype coverage are available. The 23-valent pneumococcal polysaccharide vaccine (PPSV23) is recommended by the Centers for Disease Control and Prevention (CDC) for people above the age of 65 in the United States (US) and the 10- or 13-valent pneumococcal conjugate vaccines (PCV10 or PCV13) have been introduced in the childhood vaccination program in many countries (*69-72*)

1.2.2 Antibiotic therapy and antibiotic resistance

Standard treatment of *S. pneumoniae* relied mainly on β-lactam antibiotics such as penicillin (*73*). In the first roughly 20 years after the introduction of penicillin (1940s) (*73*), the pneumococcus was especially sensitive with a minimal inhibitory concentration (MIC) below 0.06 µg / mL (*74*). In 1967 however, the first non - susceptible strains (MIC ≥ 0.12 µg/mL) were detected (*74*). The pneumococcus achieves β-lactam resistance primarily by altering the targeted enzymes as they are penicillin binding proteins (PBP) to reduce their penicillin binding affinity (*73, 75*). Resistance can additionally be achieved by mutations in multiple genes, for example the peptidoglycan bridge formation alanyltransferase *murM* (UniProtKB Q9L447), resulting in changes of the peptidoglycan composition (further described in **chapter 2.1.2**) (*73, 75-77*). The overall extent of penicillin non-susceptibility in European pneumococcal invasive disease isolates from 2010 was reported to be 8.9 %, yet prevalently southern and eastern European countries bear penicillin resistance rates over 40 % (*78*). Their prevalence correlates with the antibiotic consumption (*79, 80*).

Macrolides, targeting the ribosome to impair protein synthesis have been the choice of treatment for upper and lower respiratory tract infections when β - lactam resistance emerged (*81*). Unfortunately, with the consequence of widespread global macrolide resistance (*81*), with resistant isolates constituting 20 – 40 % of pneumococcal isolates in the US (*82*). Clindamycin, a licosamide, is also indicated for therapy of β - lactam resistant pneumococcal infections and remains effective towards 78 % of isolates tested in 2011 in the US, despite the danger of cross – resistance with macrolides (*82*). Similar sensitivities are observed in the US for the

bacteriostatic protein synthesis inhibiting tetracyclins and trimethoprim – sulfametoxazole that are stalling the essential pneumococcal folic acid synthesis (*82*). DNA gyrase inhibiting fluoroquinolones prevail, with an inherent moderate activity, effective towards *S. pneumoniae*, but resistance development is following an increasing trend (*82*). Vancomycin is a last resort antibiotic to treat pneumococcal infections, however since long vancomycin tolerant strains have been registered (*74*).

Resistance is observed to expand due to the spread of successful clones. Their influence on antibiotic resistance infections is well-documented with the decline thereof in some areas after introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in the childhood vaccination program, which includes highly penicillin resistant serotypes (*83*).

1.3 SEPSIS AND SEPSIS DIAGNOSIS

Generally, blood is regarded as a sterile body fluid free of microorganisms. Yet, through the emergence of culture-independent analytical methods, presence of a dormant microbiome has been described in several chronic diseases (*84*). Viable bacteria from the own human microbiome are frequently passing the lesioned epithelial barrier resulting from daily activities such as tooth hygiene (*85*). These bacteria are usually defeated immediately by the host's immune system right at their entry side without noticeable symptoms (*85, 86*). When the fine balance between invading pathogen and the immune system is perturbed, a systemic inflammatory response can be provoked, which may progress to sepsis or septic shock (*85, 86*). Sepsis is seen as a life threatening dysregulated inflammatory response to an infection, leading to organ dysfunction (*86*).

Sepsis is associated with high mortality claiming 11 million deaths worldwide in 2017, imposing an immense global burden with an estimated incidence of about 49 million cases (*87*). Sepsis is most commonly caused by pneumonia (*88*). The earlier a diagnosis can be made enabling the initiation of a targeted therapy, the higher are the patient's chances to survive the acute disease (*89, 90*). However, currently no autonomous and culture-independent laboratory method is available to rapidly diagnose sepsis (*86, 91, 92*). In contrast, diagnostic standards rely on several time-consuming cultivation steps, starting with an enriching culture for the typically low abundant bacteria in a blood sample, followed by pathogen isolation, identification and antibiotic susceptibility determination (*86, 91*).

On average, a positive result is obtained after 10 hours incubation in an automated blood culture system, with nearly 90 % of positive cultures detected within the first 24 hours of cultivation (*93*). It could however take up to 48 to 72 hours to receive a positive blood culture and up to 96 hours for an antibiotic susceptibility profile (*93, 94*). Besides the expenditure of time, cultivation steps come with several disadvantages, which is reflected in the high percentage of sepsis cases, 30 %, with a negative blood culture (*95*). Especially slow growing and fastidious organisms are prone to cause false negative blood culture results (*92*). Furthermore, the blood volume of the cultures tremendously influences their sensitivity, which is especially critical for pediatric samples (*92, 96, 97*). Beyond that, poly-microbial infections might be misjudged due

to the possible outgrowth of one organism that is better adapted to the culturing conditions (*98*). One reason why a cultivation step is still not replaced by an alternative method is the low abundance of bacteria in a bacteremic blood sample, which might be as low as 0.5 to a few colony forming units (cfu)/mL in adult and pediatric samples (*97-99*).

Matrix-associated laser desorption ionization-time of flight (MALDI-TOF) is used for pathogen identification from a positive blood culture, and has also been implemented on whole blood without incubation (*91*). In combination with polymerase chain reaction (PCR) analysis, it has been shown to yield pathogen identification within 6 hours on whole blood without prior cultivation with 81 % sensitivity and 69 % specificity in comparison to conventional pathogen cultivation and identification (*95*). Another example for a rapid bacteria identification method from unincubated blood is the SeptiFast® multiplex qPCR assay from Roche. It delivers a result in 6 hours directly from 3 mL of blood with a sensitivity of 70 - 100 % for 30 cfu / mL and 50 – 100 % for 3 cfu / mL, depending on the investigated bacterial species (*100*).

PCR based techniques for empirical therapy decision making can deliver additional information about presence of investigated resistance genes (*91*). Due to the high variety of possible and emerging antibiotic resistance mechanisms and different degrees of phenotypic susceptibility in presence of a genomic resistance marker, molecular biological methods for resistance determination are however not a sufficient basis for clinical treatment decisions (*92, 101*). One major advantage of culture-based methods is therefore the delivery of an antibiotic susceptibility profile which could not yet be replaced by a rapid detection method (*92*), albeit being crucial information because appropriate antibiotic treatment of bloodstream infections is associated with lower mortality (*102*). Furthermore, PCR based methods are sensitive to contamination and interference with the abundant human DNA (*91, 92*).

1.4 RELEVANT CONCEPTS IN MICROFLUIDICS

The advent of microfluidics appeared about 30 years ago with the first miniaturized total chemical analysis systems (µTAS) (*103, 104*) wherefore developments in microelectronics, molecular biology and analytical methods paved the way (*105*). Microfluidic systems manipulate small volumes typically in the microliter to attoliter range, that are confined in glass or polymer channels with cross sectional dimensions in the low micrometer range (*105, 106*).

In this small sized world, where surface dominates volume, phenomena other than these we experience in our macroscopic world prevail (*107, 108*). For fluids in microfluidic systems, which are usually operated at low Reynolds numbers (**Figure 3A**) (*109*), this means that inertia is becoming negligible whereas viscosity dominates (*107*). Fluid and particle movement is therefore attributable to very immediate forces and not to resulting momentums of those previously applied (*108*). This results in a laminar flow pattern, where liquids are following defined streamlines without mixing in a turbulent manner (**Figure 3B**). This in turn means that precise manipulation of fluids and particles is possible which is exploited by microfluidic techniques.

Yet, one important prerequisite for precise particle manipulation must be met with a high enough convection to overcome the effects of diffusion, i.e. a sufficiently high Péclet number (**Figure 3C**) must be assured (*110*).

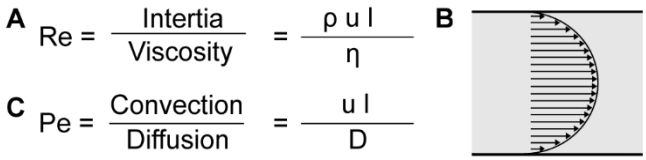

Figure 3 Characteristics of a microfluidic system. (A) Reynolds number (*110*). **(B)** Laminar flow profile is characterized by maximal velocity in the channel center and zero velocity at the channel walls. Flow lines are shown. **(C)** Péclet number (*110*) **(A, C)** Two dimensionless numbers for the characterization of microfluidic systems with ρ…fluid density, u…velocity, l…channel length, η…dynamic viscosity, D…diffusivity.

Microfluidic systems enable a miniaturization of reactions with the advantage of using minute amounts of samples and reagents (*104, 105, 111*). These systems have the potential to be a fully integrated lab on a chip, offering fast diagnosis for an affordable price without the requirements of expert personal and high-end laboratories (*111, 112*). This makes microfluidic diagnostic assays highly relevant for point of care diagnosis especially for applications in the developing world, where the high demands of the resource-limited environments constitute a remaining challenge (*111, 112*).

Particles suspended in a liquid can be displaced through the impact of an acoustic radiation force (**Figure 4A**), evoked by a sound wave (**Figure 4B**). Already in 1787, the physicist Ernst Chladni demonstrated particle movement in response to acoustic vibration with sand pictures on an agitated plate (**Figure 4C-J**) (*113*). In acoustic microfluidic systems the force of an ultrasonic standing acoustic wave is used to move particles within the generated pressure field. This happens due to only intrinsic, physical properties, e.g. size, shape, compressibility and density of the particles and the surrounding fluid (*114*) (**Figure 4A**).

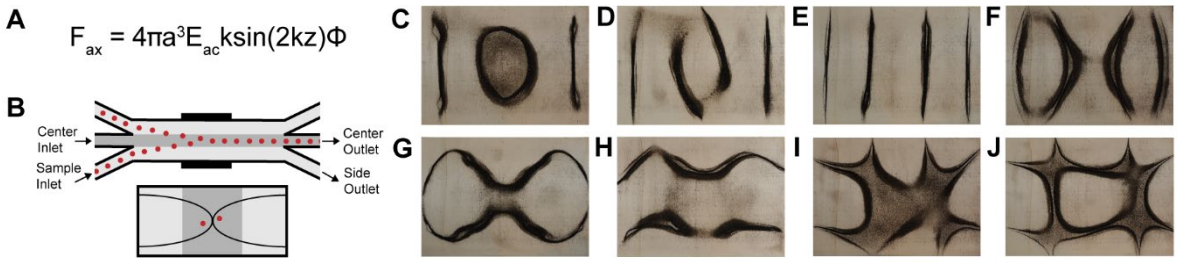

Figure 4 Acoustofluidics. (A) Formula of the acoustic radiation force F_{ax} with a…particle radius, E_{ac}…acoustic energy density, k…wavenumber as a function of the applied frequency divided by the speed of sound in the fluid, z…distance from the pressure anti-node, ϕ…acoustic contrast factor is determined by the relation of density and compressibility of the particle and the suspending medium (*115*). **(B)** Acoustic standing wave used to move particles into the center of the microfluidic channel. **(C - J)** Adapted Chladni figures with sand on a wooden plate actuated with different frequencies **(C)** 112 Hz **(D)** 125 Hz **(E)** 150 Hz **(F)** 163 Hz **(G)** 175 Hz **(H)** 188 Hz **(I)** 212 Hz, **(J)** 225 Hz.

2 LITERATURE REVIEW: THE PNEUMOCOCCAL CELL ENVELOPE AND CELL WALL HYDROLYSIS

2.1 THE CELL ENVELOPE OF *STREPTOCOCCUS PNEUMONIAE*

The bacterial cell envelope defines an individual living prokaryote and fulfills a multitude of purposes. It must function as a strong and tight cover to withstand the bacterial turgor pressure and serve as a shield to protect the organism from hostile environments. Simultaneously to providing strength, the shield needs to adapt flexibly to everchanging environments that differ for example in osmotic conditions, temperature, and concentrations of harmful compounds. In addition, the cell envelope is in constant turnover during growth and is therefore a very variable structure. Furthermore, the cell envelope must be permeable for example to nutrients or ions, whose gradient is important for the maintenance of the ion motif force (*116*). The cell envelope is therefore essential for the cellular energy balance. Overall, the pneumococcal cell envelope is negatively charged. The surface charge is mainly constituted by the usually negative charge of the polysaccharide capsule (*117*), teichoic acids (*118*), and the cell membrane (*119-121*). It is advantageous for example to establish colonization by avoiding trapping and subsequent clearance in the positively charged airway mucous and to evade phagocytosis through electrostatic repulsion (*29, 48, 117*) (**Figure 2**). The host innate immune system also exploits this surface property to establish electrostatic interactions between the negatively charged bacterial surface and positively charged antimicrobial peptides (*122-124*).

The following paragraphs describe selected components of the pneumococcal cell envelope, that are especially relevant for the thesis, starting with the cell membrane and continuing with the cell envelope structures that depend on undecaprenyl pyrophosphate (bactoprenyl) carriers for their synthesis: peptidoglycan, teichoic acids and the polysaccharide capsule. How cell envelope structures are exploited as antibacterial targets is discussed in **chapter 2.2**. Surface exposed proteins, other very important components of the cell envelope, are only mentioned briefly here and few other sections of the thesis, but **chapter 2.3** deals with selected surface exposed proteins, that are cell wall hydrolases in more detail.

Pneumococcal surface proteins can be membrane associated (lipoproteins), non-covalently bound for example to choline residues in teichoic acids, or covalently bound to the peptidoglycan with an LPxTG anchor (*125-127*). Furthermore, non-classical surface proteins constitute secreted proteins such as the polyhistidine triad (Pht), and other moonlighting proteins that associate with the pneumococcal surface in a to date unknown way (*125, 126, 128*). Surface exposed proteins constitute major virulence factors and are of high interest as candidates for vaccine antigens to mount an antibody response (*48*). Their function is diverse and includes adhesins, enzymes, transporters, channels, and environmental sensors such as two-component systems (*129*).

2.1.1 The pneumococcal cell membrane

The main lipid species in the pneumococcal membrane are the anionic phosphatidylglycerol, cardiolipin, neutral glycolipids such as diacylglycerol, and the zwitterionic phosphatidylcholine (*130-132*). Studies on the membrane composition in pneumococci are rare and therefore knowledge about selected aspects of membrane biology generated through investigations of other bacteria are compiled. In gram-positive bacteria, the surface exposed membrane bilayer is more negatively charged than those of human cell membranes, where anionic lipids are predominantly found in the inner leaflet of the membrane (*119-121*). The properties of lipids are important for the topology, folding and consequently the function of membrane proteins (*132, 133*). The degree of freedom for the movement of phospholipid head groups influences membrane fluidity and viscosity, whereas the packing of the lipids is determined by the lipid head group and saturation degree of their constituting fatty acids (*134, 135*). The biophysical properties of the membrane are carefully monitored, as important associated functions such as protein-protein interactions and membrane permeability are influenced by them (*134*). The adjustments of the bacterial phospholipids to changes in temperature, osmotic conditions or pH are referred to as homeoviscous adaptation (*134, 136, 137*).

Within a bilayer, lipids are not randomly distributed. Lateral lipid organization can be determined by the lipid head group (*135*). Cardiolipins, for example were found enriched at the division septum and the poles of *Escherichia coli* and *Bacillus subtilis* (*138-140*). Likewise, non-bilayer forming glycolipids are localized in areas of increased membrane curvature (*131, 141, 142*). Anionic lipid microdomain formation by phosphatidylglycerol and cardiolipins have been described to determine the topology of protein secretion in the gram-positive coccus *Streptococcus pyogenes* (*143*). Furthermore, the bacterial membrane is compartmentalized in regions of increased fluidity (RIF) (*144*) and in functional membrane microdomains (FMM) that are characterized by a lower fluidity in comparison to the surrounding membrane (*135, 145*). The arrangement of the lipids and associated membrane proteins into RIFs in the rod-shaped *B. subtilis* is determined by the bacterial actin homologue cell-shape determining protein MreB (UniProtKB Q01465) (*144*), a protein family absent in the coccoid *S. pneumoniae* (*146*). Membrane fluidity homeostasis is furthermore controlled by bacterial flotillins (*147*). The compartmentalization of the membrane is important for protein localization (*148*) and proper function of cell membrane associated processes, such as peptidoglycan synthesis (*147, 149, 150*).

The building blocks of membrane lipids are fatty acids. Bacterial fatty acid synthesis type II (FASII) relies on a multitude of enzymes that elongate the fatty acid chain by two carbons in one cycle and thereby differs from eukaryotic fatty acid synthesis type I, which is solely accomplished by one multifunctional fatty acid synthase (**Figure 5A, B**) (*151-153*). The pneumococcal fatty acid synthesis genes are organized in one operon with two binding sites for the fatty acid biosynthesis transcriptional regulator FabT (**Figure 5A**) that regulates fatty acid synthesis through feedback inhibition of the pathway`s end product: long chain fatty acids

bound to acyl carrier protein 1 (Acp1) (*154, 155*). Fatty acid synthesis is initiated by the acetyl coenzyme A (CoA) carboxylase AccABCD that converts acetyl-CoA to malonyl-CoA (**Figure 5B**) (*153, 156*). This step is also the target for biochemical inhibition of the FASII system in presence of enough host fatty acids in addition to the transcriptional repression by FabT and co-repressor acylated acyl carrier protein 2 (acyl-Acp2) (*157-160*). Host fatty acids can be taken up by the pneumococcus via a fatty acid kinase (Fak) composed of a fatty acid binding protein FakB and the ATP binding protein FakA (*159, 161, 162*) (**Figure 5C**). *S. pneumoniae* contains three FakB homologues for the acquisition of the whole fatty acid spectrum of saturated, unsaturated and polyunsaturated fatty acids available in the human host (*159*). Synthesis of unsaturated fatty acids in *S. pneumoniae* is carried out by the trans-2, cis-3-decenoyl-ACP isomerase FabM, and the ratio of endogenously synthesized saturated and unsaturated fatty acids is determined by the balance between FabM and the enoyl-ACP reductase II FabK activity (*154, 163, 164*).

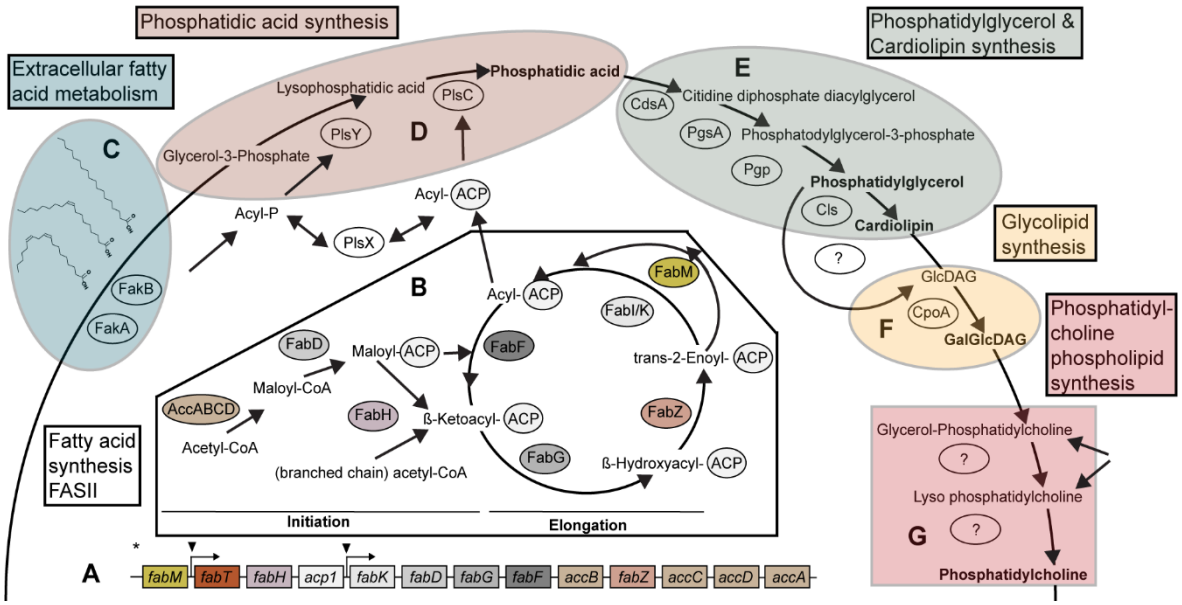

Figure 5 Membrane biogenesis in *S. pneumoniae*. **(A)** Pneumococcal fatty acid synthesis operon organization. Promoter regions are indicated by an arrow, experimentally validated FabT binding sites (▼) (*154, 155, 160*) and a proposed binding site (*) (*163*) are indicated. **(B)** Fatty acid synthesis type II is initiated by the tetrameric AccABCD with malonyl CoA as product, that malonyl-CoA:acyl carrier protein transacylase FabD further converts to manoyl- ACP, which serves as substrate for the β-ketoacyl-ACP synthase III FabH and β-ketoacyl-ACP synthase II FabF (*134, 153, 156*). Straight and branched chain fatty acid synthesis from acetyl CoA is initiated by FabH to initiate the elongation cycle of the FASII system (*134, 153, 156, 165*). Fatty acid elongation is continued by β-ketoacyl-ACP reductase FabG and β-hydroxyacyl-ACP dehydratase FabZ (*156*). For the reduction of trans-2-enoyl-ACP to acyl-ACP, *S. pneumoniae* contains two non-homologous enzymes the enoyl-ACP reductases FabI and FabK, which make it refractory to FabI inhibitors such as triclosan (*134, 166*). Unsaturated fatty acid synthesis is carried out by FabM that competes for the trans-2-enoyl-ACP substrate of at least 10 carbons in length with FabK (*154, 163, 164*). **(C)** Host fatty acids can be bound by FakB proteins and after phosphorylation with the help of FakA, serve as substrate for the acyl-phosphate-dependent glycerol-3-phosphate acyltransferase PlsY or the phosphate:acyl-ACP acyltransferase PlsX, that synthesizes acyl-ACP, which is used by the 1-acyl-glycerol-3-phosphate acyltransferase PlsC to produce phosphatidic acid (*159, 161, 162, 167, 168*). **(D)** Phospholipid synthesis from glycerol-3-phosphate (G3P) is initiated by G3P acylation through PlsY with acyl-phosphate as substrate. The resulting lysophosphatidic acid is further acylated from acyl-ACP acyl donors by PlsC to synthesize phosphatidic acid (*167, 168*). **(E)** Phosphatidic acid can be further modified to phosphatidylglycerol through enzymatic conversion by the phosphatidate citidylyltransferase CdsA, phosphatidylglycerophosphate synthase PgsA and the phosphatidylglycerophosphate phosphatase Pgp (*130, 169*). Cardiolipin (diphosphatidylglycerol) is synthesized by the cardiolipin synthetase Cls from two phosphatidylglycerol species (*130*). **(F)** Glycolipid synthesis is catalyzed by a yet unidentified glycosyltransferase for monoglycosyl diacylglycerol (GlcDAG) synthesis, while galactosyl-glucosyl-diacylglycerol (GalGlc DAG) is synthesized by the GalGlc DAG synthase CpoA (*131, 142*) **(G)** Phosphatidylcholine presence in the pneumococcal membrane has only recently been described and shown to commence at the host derived metabolites glycerophosphocholine or lysophosphatidylcholine that are acylated by yet uncharacterized acyltransferases (*130*).

The pneumococcus utilizes the among gram-positive bacteria widespread PlsX / PlsY / PlsC route for phosphatidic acid synthesis from glycerol-3 phosphate (**Figure 5D**) (*167, 168*). Phospholipid head group diversity is established by subsequent enzymatic actions (**Figure 5E-F**), except for phosphatidylcholine biosynthesis, where extracellular glycerophosphocholine and lysophosphatidylcholine are acylated by yet unidentified acyltransferases (**Figure 5G**) (*130*).

2.1.2 The pneumococcal cell wall

The pneumococcus appears typically in the shape of a diplococcus of about 1 µm in diameter, but is also found as a single coccus or in chains (*170*), which provides advantages in systemic infection or colonization respectively (*171, 172*). The bacterial morphology is determined by the peptidoglycan architecture. Atomic force microscopy of group B *Streptococci* revealed a net-like structure of the coccoid peptidoglycan, with parallel oriented glycan strains crosslinked to a varying degree with peptides resulting in bands and pores (*173*). However, the peptidoglycan net is not rigid, but rather a dynamic hydrogel that resists the turgor pressure of the bacterial cell and responds to changes in osmotic and pH conditions (*174, 175*). Recent observations in the rod-shaped *B. subtilis* and spherical *S. aureus* revealed a difference in peptidoglycan architecture in the plane perpendicular to the cell membrane, where the newly formed peptidoglycan is of higher density and organization than the mature peptidoglycan on the cell surface (*175*). The typical ovococcal shape of the pneumococcus is maintained through adjacent septal and peripheral peptidoglycan synthesis during cell division (*176*). The current model proposes that proteins responsible for elongation (elongasome) and division (divisome) form a highly coordinated super-complex at the pneumococcal mid-cell (*76*). Cell wall hydrolases are involved in the remodeling of the newly synthesized peptidoglycan, whereas in contrary to rod-shaped bacteria like *B. subtilis*, the old peptidoglycan of the inherited hemisphere in pneumococcal daughter cells is not subjected to extensive turnover (*177*).

The main biosynthesis steps of pneumococcal peptidoglycan are conserved in gram positive bacteria (**Figure 6A**), and so are the building blocks such as the glycan backbone and amino acids in D- and L-conformation of the crosslinking peptides (**Figure 7A**) (*178, 179*). There are however many variations to the peptidoglycan fine structure, depending on the species, its growth state and prevailing living conditions (*179*). With a minimal length of 25 disaccharide units of the glycan backbone, the pneumococcus has much longer polymers of alternating N-acetylmuramic acid (MurNAc) and N-acetyl glucosamine (GlcNAc) (*178*) than *S. aureus* where six disaccharide units are majorly found (*174*). The pneumococcal cell wall was found to be 16 nm to 25 nm thick (*180*). Crosslinking of the glycan chains with peptides is important for a proper function of the cell wall. During exponential growth about 50 % of the characterized muropeptide fraction of pneumococcal peptidoglycan (71.4 %) were cross-linked to dimers or trimers (*178*), which indicates a lower degree of crosslinking than typically found in *S. aureus* (*174, 179, 181*). Crosslinking can be achieved directly via peptide bonds or di-peptide bridges of L-Ser-L-Ala or L-Ala-L-Ala (**Figure 7A**), which are abundantly found in the peptidoglycan of penicillin resistant strains (*77, 179, 182*). A prerequisite for peptide

crosslinking and proper cell function is lipid II amidation in the cytoplasm (*183, 184*) (**Figure 6A**). The peptides of the pneumococcal peptidoglycan prevail as tripeptides, as the result of cell wall hydrolase function (**Figure 7A**), but are also rarely found as tetrapeptides and pentapeptides (*178*). After synthesis, the degree of acetylation of the glycan backbone can be modified, thereby for example conferring resistance to the abundant mucosal cell wall hydrolase lysozyme, which constitutes an immune evasive strategy of the pneumococcus (**Figure 2**) (*53*).

As for most gram-positive bacteria, the pneumococcal cell wall is decorated with teichoic acids (wall teichoic acids, WTA), which can also be linked to a lipid anchor in the cell membrane (lipoteichoic acids, LTA). In comparison to teichoic acids of other gram-positive bacteria, pneumococcal teichoic acids have a more complex composition (*118, 185*) (**Figure 6B**). WTA and LTA are synthesized in the same pathway and only differ in their cell envelope anchor, and they contain ChoP (*186*). *S. pneumoniae* incorporates ChoP in the cell envelope and is auxotroph for choline (*186-188*). Choline can be replaced by the structurally similar amino alcohol ethanolamine to support pneumococcal growth (*189*). However, those growth conditions result in several physiological alterations in the pneumococcus, such as the formation of long chains or the loss of typical lysis phenotypes (**Figure 7B**) (*186, 189*). Phenotypes also observed in mutants which lost the nutritional requirement for choline and the ability to decorate teichoic acids with ChoP (*190-192*). Choline independence can be achieved through the acquisition of a point mutation in the teichoic acid flippase TacF (**Figure 6B**), which confers extended substrate specificity also for non - ChoP containing teichoic acids, showing that teichoic acid synthesis independent of their ChoP content is essential (*190, 193*). Even though absence of choline can provide growth permissive conditions *in vitro*, incorporation of choline into teichoic acids has been shown to substantially reduce pneumococcal virulence (*194*). ChoP for example enables the binding to the PFAR, as it constitutes a part of the natural ligand, representing a strategy for epithelial cell invasion (**Figure 2**) (*51*). Interestingly, other common opportunistic pathogens that colonize the nasopharynx, like *N. meningitidis* and *H. influenzae,* also incorporate choline in their cell envelope, indicating the relevance of choline in the niche (*195, 196*). It is however only *S. pneumoniae* and closely related streptococcal species from the mitis group that incorporate choline in their teichoic acids, which is important for example to assure substrate access of choline binding cell wall hydrolases (*197*). Also, known virulence factors are among the pneumococcal choline binding proteins, for instance CbpA (**Figure 2**) or the major autolysin LytA (**Figure 7**) (*48*).

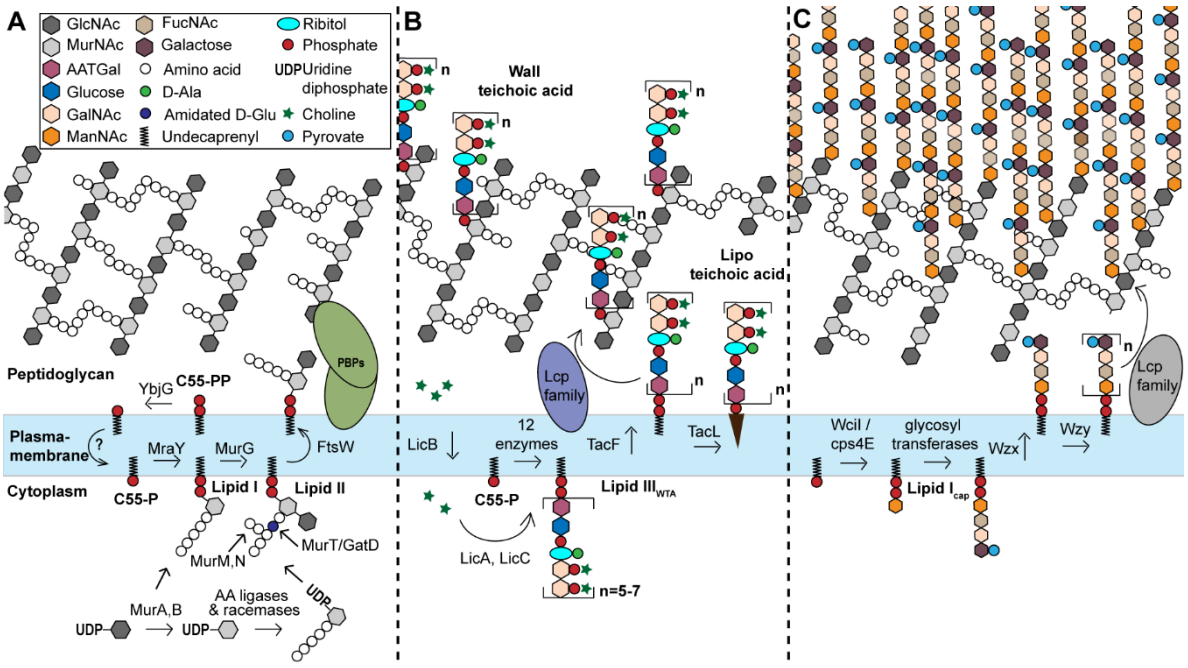

Figure 6 Cell envelope biosynthesis pathways that rely on undecaprenyl pyrophosphate carriers. (A) Peptidoglycan synthesis commences with the two-step enzymatic conversion of uridine diphosphate (UDP) GlcNAc to UDP-MurNAc catalyzed by the UDP-acetylglucosamine-enolpyrovyl-transferase MurA and the UDP-acetylenolpyruvylglucosamine reductase MurB (*198, 199*). Through the combined action of amino acid ligases and racemases, a pentapeptide is added to UDP-MurNAc (*146*). The integral membrane enzyme phospho-N-acetylmuramoyl-pentapeptide transferase MraY catalyzes the addition of UDP-MurNAc-pentapeptide to undecaprenyl-phosphate (C55-P) to form lipid I (*199*). Lipid II is formed when UDP-GlcNAc is attached by the undecaprenyldiphospho-muramoylpentapeptide β-N-acetylglucosaminyltransferase MurG (*199*). Subsequent amidation of Lipid II by the lipid II isoglutamyl synthase MurT / GatT (UniProtKB Q8DNZ9 / AOAOH3JN63), is crucial for efficient peptidoglycan crosslinking (*183, 184*). Diamino branches for the formation of peptide bridges in the peptidoglycan are added by MurM and the aminoacyltransferase MurN (UniProtKB: Q9L446) to the lipid II stempeptide (*76, 200*).The molecule is flipped to the outer leaflet of the plasma membrane by the cell division protein FtsW, that is proposed to facilitate transmembrane diffusion of Lipid II (*146, 201*). On the outer membrane leaflet, Lipid II becomes a substrate for class A bifunctional PBPs that mediate transglycosylation as well as transpeptidation or class B PBPs that mediate transpeptidation of septal and peripheral peptidoglycan (*76, 176*). Recycling of the undecaprenyl pyrophosphate (C55-PP) is mediated by phosphatases such as YbjG, and the C55-P carrier is translocated to the cytoplasmatic side of the membrane by a to date unknown mechanism (*146*). Here one model for the gram-positive peptidoglycan with glycan chains oriented perpendicular to the cell membrane is depicted (*202, 203*). **(B) Teichoic acid synthesis** is catalyzed on the undecaprenyl phosphate carrier by a manifold of enzymatic synthesis steps in the cytoplasm and the membrane, some of which have not yet been biochemically characterized (*185*). Pneumococcal teichoic acids consist of typically 4 to 7 repeating units of the unique sugar 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (AATGal), followed by one glucose molecule as well as ribitol that can be modified with a D-alanine to increase surface charge, and two N-acetyl-galactosaminyl (GalNAc) residues that are decorated with ChoP residues (*76, 118, 185, 204, 205*). Choline from the host is imported through the transmembrane protein LicB and activated by the kinase LicA and cytidyltransferase LicC (*185*). The complex teichoic acid polymer is translocated to the outer leaflet of the membrane by the transmembrane teichoic acid repeat unit transporter TacF (UniProtKB B0BEX9) (*185*). Synthesis of wall teichoic acids (WTA), by attachment of the TA polymer to 15 - 30 % of MurNAc residues of the peptidoglycan (*200*) are likely accomplished by phosphotransferases of the LCP (LytR, CpsA, Psr) family (*185*). Attachment of the TA polymer to the glycolipid anchor to form lipoteichoic acids is likely mediated by the putative lipoteichoic acid ligase TacL (*206*). **(C) Capsular polysaccharide synthesis** principal steps are shared among serotypes that assemble their capsule utilizing polysaccharide polymerase Wzy polymerization (*207*). Capsular biosynthesis commences with the transfer of a sugar moiety to the undecaprenyl phosphate carrier by the initiating glycosyl transferase (Tigr4: capsular polysaccharide biosynthesis protein Cps4E / WciI, UniProtKB Q7WVW9) (*208, 209*) to form lipid I_{cap} (*210*). Continuing glycosyltransferases synthesize one serotype specific polysaccharide unit in the cytoplasm. The repeat unit transporter Wzx transfers the polysaccharide unit to the outer membrane leaflet and Wzy catalyzes the polymerization of those polysaccharide units (*208*). LCP family proteins or Wzy are proposed to covalently ligate the polymer to the peptidoglycan (*211, 212*). The capsular polysaccharide of serotype 4 is depicted (*213*).

2.1.3 The pneumococcal polysaccharide capsule

The capsule is a polysaccharide structure that defines the more than 98 pneumococcal serotypes (*29*). It is composed of a variety of sugar moieties and additional constituents such as pyruvate, ribitol and for serotypes 15F, 24A, 27, 28A, 28F, 32A and 32F also choline (*124, 208*). The differences in polysaccharide composition are reflected in variations of the

capsular biosynthesis gene locus. Except for serotype 3 and 37, which synthesize their capsular polysaccharide in a synthase dependent way, most other capsules are synthesized through Wzy polymerization (*124, 214, 215*) with the principal biosynthetic steps depicted in **Figure 6C**. The entire capsular genetic locus for all serotypes, except of serotype 37 (*215*), is embedded between the same genes (*207*). Wzy- dependent capsular biosynthesis genetic loci contain a common cluster of four genes *cpsA-D*, with *cpsB-D* regulating capsule production (*208*). The fifth gene of the locus encodes for the initiating sugar transferase (Serotype 4: *cps4E / wciI*) which is, as the following genes in the capsular locus, serotype specific (*207*). The capsule can be 200 to 400 nm thick and therefore makes it the most dominant cell envelope structure of the pneumococcus (*180*). Pneumococcal capsules are usually negatively charged, only a few are of nearly neutral charge (*117*). The capsule is a major virulence factor of the pneumococcus. Loss of the capsular polysaccharide through enzymatic degradation, markedly reduced *S. pneumoniae* serotype 3 pathogenicity in a mouse model for invasive disease (*216*). The capsule facilitates evasion of phagocytosis, shields antigens, reduces complement deposition and prevents receptor interaction of deposited complement (*48*). It has also been reported to increase tolerance to cell wall synthesis inhibiting antibiotics (*217*).

2.2 MEMBRANE AND UNDECAPRENYL PYROPHOSPHATE TARGETING ANTIBIOTICS

Detergents interact with the membrane through amphiphilic interactions, with no distinction between prokaryotic or eukaryotic membranes. Essentially, the surfactant is inserting into the membrane and accumulates to a threshold concentration when mixed micelles of membrane lipids and surfactant are formed (*218*). Membrane targeting antibiotics however can specifically alter physiological properties of the bacterial membrane, such as lateral lipid organization, membrane curvature, as well as fluidity by causing aberrant lipid packing or clustering (*119*). The effect can be exerted either directly or indirectly by binding to a lipid species (*119*).

An example for direct lipid binding is the plant derived acylphloroglucinol rhodomyrtone, that exerts antibacterial activity towards gram-positive bacteria by binding to the headgroups of phospholipids (*219*). This transient interaction causes a distortion of the phospholipid headgroup packing, which provokes overall membrane fluidization, permeability alteration and increased curvature, which ultimately leads to vesicle formation (*219*). Rhodomyrtone is the first described membrane targeting molecule that exerts activity without integrating into the membrane (*219*). How specificity towards prokaryotic membranes is established remains to be studied, but rhodomyrtone displays low eukaryotic cell toxicity *in vitro* on erythrocytes (*220, 221*), as well as *in vivo* in caterpillars, zebrafish and mice (*220*), and was effective in a zebrafish model for pneumococcal infection (*219*).

Cationic antimicrobial peptides are a component of the innate immune response that target the bacterial cell membrane. Specificity is provided by the electrostatic interaction between

negatively charged membranes and amphipathic cationic antimicrobial peptides, where some of them undergo conformational changes after insertion into the lipid environment (*121*). Insertion of a critical amount of peptides destroys bacterial membrane integrity (*121*).

Moreover, most antimicrobial peptides also bind to bactoprenyl carried cell envelope precursors in the bacterial membrane (*222*). Similarly, antibacterial agents such as nisin, teixobactin, and daptomycin interact with undecaprenyl pyrophosphate coupled cell envelope precursors and the bacterial membrane. The lantibiotic nisin binds to the pyrophosphate moiety of lipid II and lipid III_{WTA}, and requires the first sugar moiety to establish high affinity interactions (*223*). Accumulation of nisin, bactoprenyl carried cell envelope precursor complexes, results in membrane pore formation (*222, 223*). Teixobactin, which was isolated with the iChip technology for under laboratory conditions uncultivable soil bacteria, anchors in the membrane and binds to the pyrophosphate moiety of lipid II, but also interacts with the adjacent sugar moiety of lipid II or lipid III_{WTA} (*224, 225*). However, those interactions have been shown to be rather weak in anionic membranes, and bactericidal activity is mainly exerted through clustering and consequential sequestration of cell envelope synthesis precursors (*225*). Complex formation has also been shown for Ca^{2+} mediated daptomycin oligomers, which interact with phosphatidylglycerol and bactoprenyl coupled cell envelope precursors to stall cell envelope synthesis (*149*). Furthermore, membrane integrity interference was described for the lipoglycopeptides oritavancin and telavancin that, as the glycopeptide vancomycin, bind to the di-alanine moiety of the stem peptide of lipid II, but additionally anchor in the membrane via a lipophilic moiety (*222, 226, 227*). To conclude, targeting bactoprenyl carried cell wall precursors can result in pleiotropic effects beyond those resulting from target sequestration, such as interference with membrane integrity and membrane protein localization (*223*).

Through cessation of the supply of building blocks, membrane synthesis can be inhibited by FASII inhibitors. Nearly all enzymes of the pathway (**Figure 5B**) can be inhibited (*134, 153, 228*), and a new FabI inhibitor (Afabicin) is currently in clinical development (*22*).

2.3 CELL WALL HYDROLASES AND LYSIS PHENOMENA OF *STREPTOCOCCUS PNEUMONIAE*

Cell wall hydrolases are ubiquitous in bacteria, as they are essential for growth and cell division (**Figure 7**) (*229, 230*). For *S. pneumoniae* several peptidoglycan hydrolases are described to date (**Figure 7A**) (*200*), among them the major pneumococcal autolysin LytA. The N-acetylmuramoyl-L-alanine amidase LytA is involved in the various autolysis phenomena of pneumococci (**Figure 7B**), but also contributes to daughter cell separation (*231-233*), which is mainly accomplished by the non-autolytic peptidoglycan hydrolase LytB (*232, 233*) and the vaccine candidate PcsB (*234-237*). LytB deficient strains form long chains (*233*), and PcsB activity is essential for cell division (*234, 235, 238*). Proper division in the pneumococcal mid-cell during growth also requires the action of the carboxypeptidases PBP3 (DakA) (*239*) and DakB (*240*). The tripeptide containing peptidoglycan, formed by the consecutive action of PBP3 and DakB, is a prerequisite for pneumococcal morphology, vancomycin resistance and

invasive disease potential (*76, 200, 241*). Aberrant transpeptidation resulting from an altered substrate availability in absence of carboxypeptidase activity, might be the cause of the anomalous peptidoglycan architecture (*239-241*).

Furthermore, several other putative cell wall hydrolases with a suspected role in cell division have been identified, but are not yet fully characterized (*235, 240, 242*). Among them is a putative N-acetylmuramidase (locus tag SPD_1874) or a putative shape, elongation, division and sporulation (SEDS) family protein (locus tag SPD_0703) (*240*). Additionally, non-covalent GlucNAc binding lysin motif (LysM) containing proteins are found among bacterial cell wall hydrolases (*243, 244*), that supposedly substitute the enzymatic actions of pneumococcal PcsB in cell division, as their augmented expression has been found in *pcsB* knockout strains (*235*). The newly identified lytic transglycosylase MltG indicates that LysM motif containing proteins might also play a role in peripheral peptidoglycan synthesis (*242, 245*).

It is common in bacteria to express multiple cell wall hydrolases with similar activities at which redundancy emphasizes their importance for cell physiology (*229, 230, 240*). A large share of to date characterized pneumococcal cell wall hydrolases include choline binding proteins that associate with the cell wall through non - covalent binding to ChoP (*246, 247*). The presence of ChoP in teichoic acids is therefore important for their function in cell separation and lysis (*189*). However, besides redundancy in enzymatic activity, diversity in cell envelope binding is conceivable for maintenance of cell wall hydrolase mediated peptidoglycan remodeling. The above-mentioned putative cell wall hydrolases, with the among bacteria widely distributed non-covalent peptidoglycan binding motif LysM (*243, 244*), might be an example thereof.

During *in vitro* growth, pneumococcal cultures undergo spontaneous autolysis in the nutrient depleted stationary growth phase (**Figure 7B**). This is proposed to be a phenomenon of altruistic suicide, which is undergone by a subpopulation of bacterial cells to provide nutrients and reduce the cell number, so that a small subpopulation of cells can survive a longer period of nutritional stress (*248, 249*). This might be especially important in the nutrient scarce nasopharynx (*250*), and *S. pneumoniae* shares the phenotype of spontaneous stationary phase lysis with common niche colonizers *N. meningitidis* and *H. influenzae* (*37*). Growth inhibition evoked by cell wall targeting antibiotics such as penicillin (**Figure 7B**) also triggers the onset of autolysis (*251*), in contrary to erythromycin and tetracycline that inhibit protein synthesis, or the RNA synthesis inhibiting rifampicin (*252*). Antibiotic induced autolysis in early logarithmic phase could constitute an altruistic stress response akin to the stationary phase triggered lysis (*249*). Here, benefits of altruistic autolysis apart from nutrient homeostasis might take effect, as removal of damaged cells can be beneficial for the population, analogous to the programmed cell death in eukaryotes (*249, 253*). Furthermore, it can be a way of removing especially fast-growing mutants as they are most susceptible to growth inhibitors (*249*) in favor for stress resistant persister cells to secure population survival (*254*).

Stress conditions that infer DNA damages can provoke pneumococcal lysis due to the activation of temperate phages, which have been integrated in the pneumococcal chromosome and entered the lytic cycle (*255*). Phages lyse their host pneumococcal cells by a choline binding N-acetylmuramoyl-L-alanine amidase that closely resembles the bacterial LytA, which is exported by two phage genome encoded holins (*255*).

The effects of the multicellular behavior of bacteria in the case of lysis are however not only of altruistic nature. During the state of natural transformability, competent pneumococci commit fratricide on non-competent sibling cells in the population (*248, 256*). Fratricidal allolysis is mediated by the endopeptidase CbpD, but also the activity of LytA and LytC from the assassinated cells are required for their lysis (**Figure 7A**) (*257*). Competent bacteria express an immunity protein ComM that protects them from committing suicide (*258*). How CbpD activity is inhibited by ComM is not yet understood, nevertheless modulation of peptidoglycan is suspected as cells overexpressing ComM displayed aberrant cell morphology (*259*). Additionally, two lipophilic competence induced peptide bacteriocins CibAB initiate allolysis, likely by inserting into a non-competent sibling membrane upon close contact (*248, 260*). Competent cells are protected by the CibC immunity factor (*260*).

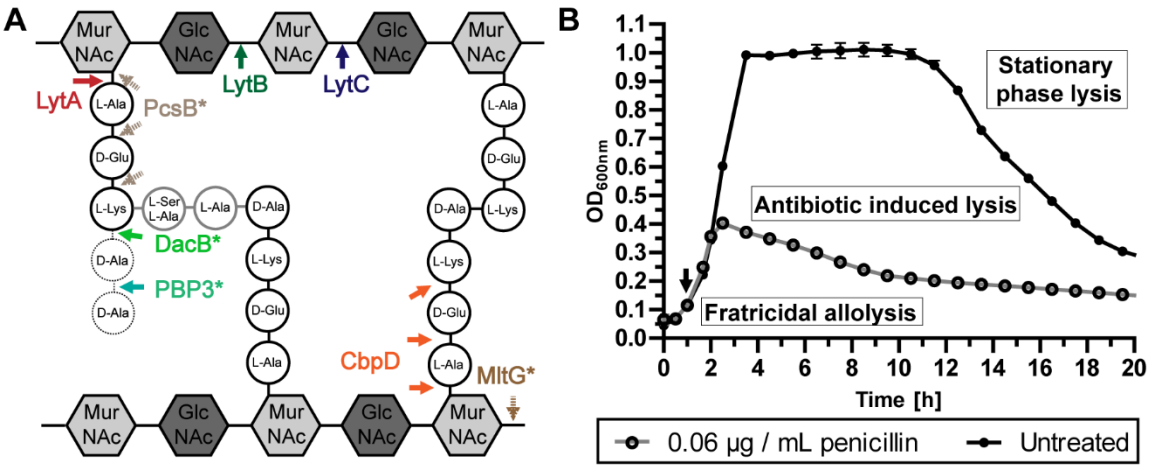

Figure 7. Peptidoglycan hydrolases and lytic events in the *in vitro* growth phases of *Streptococcus pneumoniae*. (A) The glycosidic bonds of the glycane backbone (MurNAc, GlcNAc) are hydrolyzed by cell wall glycosidases (*261*). In *S. pneumoniae* two cell wall glycosidases are known: the endo-β-N-acetylglucosaminidase LytB that cleaves the polysaccharide after GlcNAc (*230, 232*) and the N-acetyl-β-D-muramidase / lysozyme LytC which hydrolyzes the glycosidic bond after MurNAc (*230, 262*). An additional putative glycosidase, the lytic transglycosylase MltG, has been identified (*242, 245*). Lytic transglycosylases also cleave the glycosidic bond after MurNAc but unlike lysozyme, hydrolysis results in a terminal anhydro-ring modified MurNAc residue (*230, 261*). The N-acetylmuramyl-L-alanine amidase LytA cleaves the amide bond between L-alanine and MurNAc (*230, 252, 263*). Both choline binding protein CbpD and the protein required for cell separation PcsB contain a cysteine- and histidine-dependent aminohydrolases / peptidases (CHAP) domain, a feature of amidases and endopeptidases (*230, 236, 237, 264, 265*). For CbpD, cell wall hydrolase activity has been demonstrated (*265*), whereas no such could be measured *in vitro* for PcsB (*235*), likely because of the requirement for activation through the cell division protein membrane bound ABC transporter FtsXE complex (*200, 236, 264*). The penicillin binding protein PBP3 (also called D, D-carboxypeptidase DacA) and the L, D carboxypeptidase DacB are carboxypeptidases that trim the newly synthesized pentapeptide to a tetrapeptide (PBP3) and tripeptide (DacB) (*240, 241*). Except for those marked with an asterix, pneumococcal cell wall hydrolases are choline binding proteins (*246, 247*). **(B)** The typical growth curve of *S. pneumoniae* Tigr4 strain in *in vitro* culturing conditions in the laboratory. At the beginning of the exponential growth phase the bacteria are naturally competent for a limited time (*248*). Allolysis due to fratricide is occurring during the competence window (*248*), the lytic changes are not recordable with the resolution of the spectrophotometric optical density (OD) determination. Autolysis is induced in response to cell wall synthesis inhibiting antibiotics such as penicillin, that have been administered in the early logarithmic growth phase (indicated by an arrow) and is readily recordable with a decrease in OD. In stationary phase, pneumococci undergo autolysis, which onset is dependent on the serotype and growth conditions.

Knowledge about the regulation of pneumococcal cell wall hydrolases is scarce. The best studied example for regulation in *S. pneumoniae* is the major autolysin LytA. Interestingly, pneumococcal cells are protected from the action of LytA during exponential growth, which only induces lysis upon growth inhibition of antibiotics or in stationary phase (*252, 266*). In exponential phase LytA is primarily located intracellularly and cell surface bound concentrations only increase before the onset of stationary phase autolysis (*252, 266*). LytA requires access to a big substrate interface and is proposed to act on the newly synthesized peptidoglycan (*252, 267, 268*). This is supported by the observation that LytA binds to nascent teichoic acids at the mid cell (*266*). Access to the substrate in exponential phase is hindered by the sequestration of LytA from the cell wall through binding to lipoteichoic acids (*269*). Furthermore the acetylation of peptidoglycan could exclude the concurrent attachment of a teichoic acid to the acylated MurNAc and may promote peptidoglycan maturation through efficient transpeptidation (*270*). In stationary phase the balance of lipoteichoic acid to wall teichoic acid synthesis is skewed as TacL is degraded by the membrane anchored ATP dependent zinc metalloprotease FtsH, which can facilitate substrate access for LytA (*269*). Furthermore, the lack of MurNAc O- acetylation has been shown to sensitize *S. pneumoniae* to penicillin and LytA actions during exponential growth (*270*).

How substrate access for LytA is regulated is not yet elucidated, although observations have been made. One of them is the involvement of Vex3 of the Vex123 ABC transporter in vancomycin tolerance through reduced autolysis (*271*), which has been proposed to export a peptide Pep^{27} that functions as quorum sensing molecule for cell density (*272*). The mechanism of autolysis regulation is however not clear as reported observations (*272, 273*) have been affected by erythromycin in the growth medium (*271, 274*). Furthermore, as programmed cell death, stationary autolysis has been proposed to be regulated by toxin – antitoxin systems (*248*). Also, two-component systems might regulate autolysis activation in response to environmental signals. The cell envelope stress sensing two-component system LiaSFR has a protective effect on the pneumococci during hydrolase (CbpD, LytC and LytA) provoked cell wall stress in the course of competence (*275*). Absence of the two-component system CiaRH increased pneumococcal sensitivity to cell wall synthesis inhibitor induced lysis (*276*). Independent of the exact regulatory mechanism of stationary phase autolysis activation, antibiotic induced autolysis in exponential growth phase might be short circuiting the regulatory mechanism (*271*).

3 RESEARCH AIMS

The research efforts included in the thesis aimed in the broadest sense for a contribution to the armors race in combating antibiotic resistance in the areas of therapeutic and diagnostic interventions.

Paper I and II describe the characterization of two bactericidal chemical classes respectively, that were discovered in a phenotypic screen. The studies aimed to elucidate the bacterial target of the chemicals and describe their mechanism of bactericidal action.

Paper III aspired to develop a sample preparation process employing microfluidic label free particle operations for the decomplexation of bacteria containing blood with the aim to facilitate and accelerate sepsis diagnosis.

4 MATERIALS & METHODS

4.1 BACTERIA AND GROWTH CONDITIONS

The thesis is relying on *S. pneumoniae* D39 (serotype 2) and Tigr4 (T4, serotype 4) wildtype strains as model organisms. A variety of D39 and T4 derivative mutant strains were constructed, where a gene of interest was replaced with an antibiotic resistance cassette. Additionally, THCz sensitivity of multi–antibiotic resistant pneumococci from the Pneumococcal Molecular Epidemiology Network (PMEN) (*277*) was determined in **paper II**.

S. pneumoniae was cultured on Columbia blood agar plates over night at 37 ºC and 5 % CO_2. The standard growth and assay medium for the pneumococcus was Casitone and Yeast extract (C+Y) medium, supplemented with 9 % glucose bouillon and 1 % horse serum, which provides optimized conditions for the fastidious organism. Todd Hewitt medium with 0.5 % yeast extract (THY) was employed when required, such as for comparing THCz sensitivity of *S. pneumoniae* with other bacterial species in **paper II.** Those media were adapted to establish specialized growth conditions, for example defined extracellular fatty acid concentrations in **paper I** or selective media for obtaining THCz-tolerant mutants in **paper II**. For the construction of pneumococcal mutant strains, transformation was carried out in Tryptic Soy broth (TSB) with additional glycerol (10 %) and $CaCl_2$ (0.01 %) (*252*).

Other gram-positive and gram-negative bacterial species were grown in Mueller-Hinton broth (MHB) or cation-adjusted Mueller-Hinton broth (CAMHB), TSB, Brain Heart Infusion (BHI) medium or THY medium with adjustments as stated in the respective papers. CAMHB is the standard medium for MIC determination defined by the Clinical and Laboratory Standards Institute (CLSI) and requires addition of lysed horse blood to ensure good growth of *S. pneumoniae (278)*. Both investigated screening hits (described in **paper I** and **paper II**) did however display a degree of plasma protein binding (*279*) and therefore, the standard media for compound characterization was kept to supplemented C+Y medium, as it was used in the screening campaign.

4.2 PHENOTYPIC SCREEN FOR THE DISCOVERY OF BACTERICIDAL ANTIBIOTICS

The phenotype employed in the screen was cell wall hydrolase mediated lysis of *S. pneumoniae* assessed by turbidimetry. Measurement of the optical density (OD) of a bacterial culture at 600 nm provided an easy and scalable readout for the evaluation of a compound`s effect on the bacteria. Cell wall synthesis inhibition induces pneumococcal autolysis (*251, 252*). As the cell wall is degraded, osmotic lysis causes a release of the intracellular content into the medium, resulting in a measurable decrease in OD of the culture. As detergents such as deoxycholate or Triton X-100 may also activate autolysin mediated lysis (*189, 252*), it is important to exclude compounds with such undesired properties. The screen is described in more detail in **paper II**.

4.2.1 Characterization of screening hits

Determination of cell wall hydrolase mediated lysis

For the confirmation of cell wall hydrolase involvement in bacterial lysis and the corresponding OD decrease, choline chloride concentration in the growth medium of a *lytA* gene replacement mutant was increased. Thereby all choline binding cell wall hydrolases were decoyed from binding to the choline residues on the pneumococcal teichoic acids (*280*). Consequently, they could not execute their enzymatic activity on the cell wall and the resulting absence of a prominent decrease in OD was attributed to the involvement of choline binding cell wall hydrolases in pneumococcal lysis upon chemical and antibiotic treatment. A complementary assessment of viability through counting colony forming units after compound treatment allows for the judgement of the cell wall hydrolase independent activity of the compound. Penicillin for example does then, albeit considerably weakened, still exert bactericidal activity (*281*). Furthermore, transmission electron microscopy (TEM) was employed to confirm the involvement of cell wall hydrolases in THCz mediated lysis in **paper II**.

Minimal inhibitory and minimal lytic concentration (MIC/ MlytC)

In the thesis two key figures, MIC and MlytC, for antibacterial activity of the screening hit compounds and their analogs were determined for characterization and structure activity relationship studies. The key differences between the methods are the inoculum size as they are ~ $5x10^5$ colony forming units (cfu) / mL for the MIC and ~ $3x10^7$ cfu / mL for MlytC determination of *S. pneumoniae* D39. Furthermore, bacteria for MlytC assays were at the start of their exponential growth phase, whereas for MIC determination the chemicals were added directly after dilution of the pre-culture, likely catching the bacteria in the lag phase of their growth. The duration of incubation differs from about one hour for MlytC to 20 hours for MIC determination. For both assays the result is measured optically, with a spectrophotometric turbidity recording at 600 nm for MlytC and visual judgement by the eye for MIC determination. While the MIC is a standard value in microbiology, we have introduced MlytC for the characterization of 2CCA-1 in **paper I**, to account for the lysis inducing response in exponential growth phase, which would have been disguised in the incubation period of MIC assays due to the exceptionally fast resistance development.

Identification of membrane interactions

To determine effects of 2CCA-1 treatment on membrane fluidity in **paper I**, generalized polarization of the membrane probe laurdan was recorded. The fluorescence properties of laurdan are sensitive to changes in the number and agitation of water molecules in the membrane-liquid interphase, which gives information about the packing of phospholipid head groups that also reflects the saturation degree of their fatty acid chains and thus membrane fluidity (*150, 219, 282, 283*). The organic solvent benzylalcohol is a known membrane fluidizer and was used as control treatment, along with dimethylsulfoxid (DMSO) as solvent control (*284*). As 2CCA-1 needed actively growing cells to exert activity, which would not have been assured in the staining buffer (PBS with 2 % glucose), we performed an end-point measurement

of laurdan generalized polarization. Bacteria were stained with laurdan after about 30 minutes incubation with the chemicals, shortly before lysis occurred. However, benzyl alcohol and 2CCA-1 induced membrane fluidity changes might differ in their kinetics, which cannot be recorded in the employed experimental setup. Organic solvents cause an abrupt change in membrane fluidity which bacteria counteract with rapid modifications of membrane phospholipids e.g. cis-trans isomerization of unsaturated fatty acids (*134, 150*). In comparison, slower kinetics are expected for changes in membrane fluidity exerted by *de-novo* synthesized phospholipids, as it could apply to 2CCA-1 (*134, 150*).

Compound challenge of non-growing bacteria in their stationary phase was performed to assess membrane homeostasis perturbation. Bactericidal activity of daptomycin for non-growing stationary phase *S. aureus* was shown (*285*) and the phenotype has been exploited in a screen for membrane targeting chemicals in *B. subtilis* (*286*). Membrane pore formation, as caused by the lantibiotic nisin (*222*), was examined through the quantification of cellular potassium release in **paper II**.

Eukaryotic cell toxicity

Toxicity of THCz screening hit compounds and analogs on eukaryotic cells were assessed *in vitro* on A549 lung epithelial cells. The assay was conducted in the cell growth medium Roswell Park Memorial Institute (RPMI) supplemented with 10 % fetal bovine serum (FBS) with cells seeded to a concentration of $1x10^5$ cells / mL or $1x10^6$ cells / mL. For initial structural toxicity relationship estimation, analogs that displayed activity against *S. pneumoniae* were tested with a standard concentration range of 100 µM, 50 µM, 25 µM and 12.5 µM. An adjusted and more narrow concentration range was subsequently tested for a selection of compounds to determine the 50 % inhibitory concentration IC_{50}. Viability of A549 cells was judged through the capacity of metabolically active cells to reduce resazurin to resorufin, causing a measurable color change.

4.2.2 Target identification and mechanism of action description

Pneumococcal mutant strains

Spontaneous resistant 2CCA-1 mutants were isolated after over-night incubation of plated cultures, that were challenged with 2CCA-1 in the early exponential growth phase (**paper I**). Isolation of THCz resistant mutants in **paper II** was attempted through continuous sub-cultivation of *S. aureus* in a broth microdilution assay with sub-inhibitory THCz-1 concentrations adapted from Ling et. al. for teixobactin resistance development (*224*). Furthermore, THY plates were developed that allowed both the growth of the fastidious *S. pneumoniae* and activity of THCz-1. Continuous cultivation on THY plates containing readily incrementing concentrations of THCz-1 was performed at 37 ºC and 5 % CO_2. Genomic DNA was isolated from resistant or tolerant strains and whole genome sequencing was performed. Gene replacement mutants were constructed to confirm the underlying genotype of resistant mutants and guide target identification.

Total lipid analysis

Lipidomic analysis was performed on 2CCA-1 treated wildtype and a spontaneous resistant mutant strain to determine the mechanism of action of the screening hit (**paper I**). Isolated lipids were analyzed in ultra-high-performance liquid chromatography and subsequent tandem mass spectrometry. The lipid profile was subjected to targeted analysis and the molecular features unique to the 2CCA-1 treated sensitive pneumococci, were subjected to targeted tandem mass spectrometry for further characterization.

Gene expression and protein-DNA interaction determination

Gene expression was determined via reverse transcriptase quantitative PCR to explain the 2CCA-1 resistance profile in **paper I**. For the examination if the regulation of the transcriptional regulator FabT is exerted through direct binding to the promoter of *fakB3*, an electrophoretic mobility shift assay (EMSA) with radioactively labelled probes was performed.

Assays for THCz target identification

Whole cell experiments and biochemical assays were performed for THCz target identification. *B. subtilis* reporter strains were employed in a β-galactosidase assay to investigate macromolecular synthesis pathway (cell wall, nucleic acid and protein synthesis) inhibition by THCz. This was complemented with a microscopical approach to examine *B. subtilis* cell wall integrity after THCz treatment in comparison to known antibiotics. The concentration of UDP-MurNAC pentapeptide was determined upon THCz treatment of *S. aureus* to assess if early, cytoplasmic steps of peptidoglycan synthesis were inhibited. Furthermore, the response of the LiaRS two-component system, which is sensitive to lipid II and C_{55}-PP targeting antibiotics (*287*), was recorded with a luciferase reporter *B. subtilis* strain. Antagonization of THCz with cell envelope precursors was performed in an adapted MIC assay with *Micrococcus luteus*. Furthermore, THCz inhibition of PBP2 mediated peptidoglycan transglycosylation and YbjG mediated C_{55}-PP dephosphorylation were examined in *in vitro* with purified enzymes and substrates. Determination of capsular polysaccharide amount of THCz-1 tolerant pneumococci was performed in a dot blot assay with an antibody raised against type 4 capsule which was purified for additional surface expressed antigens through incubation with the unencapsulated isogenic T4R strain.

4.3 LYSIS BUFFER EVALUATION AND MICROFLUIDIC SETUP

Lysis buffer evaluation

Combinations of agents described in the literature were explored for the selective lysis of blood cells with the aim to not harm bacterial cells. Blood cell lysis was aspired to be as complete as possible in order to reduce the presence of large intact blood cells or debris, that would interfere with subsequent size-based label free particle sorting. For the purpose of lysis protocol characterization, a flow cytometry protocol was developed, that allowed the size estimation of

the lysis debris. Bacterial viability of *S. pneumoniae*, *S. aureus* and *E. coli* in lysed blood was compared to their viability in whole blood.

Gradient acoustic focusing (GAF)

Acoustic focusing was previously limited to a critical particle diameter of about 2 µm, as for particles of a lower diameter acoustic streaming dominated the acoustic radiation force, making it impossible to manipulate particles below the critical size. GAF utilizes the stabilizing forces of an implemented acoustic impedance gradient to suppress acoustic streaming (*288, 289*). The acoustic impedance of a fluid is characterized by its density and speed of sound. Subjecting fluids differing in those characteristics in the center and side inlet of the microfluidic device (**Figure 4B**) creates the acoustic impedance gradient along the channel width. The laminar flow pattern (**Figure 3B**) ensures that the fluids remain in the center and side channels throughout the device and can be collected in the respective outlets (**Figure 4B**). Particle containing samples are injected through the side inlets of the chip (**Figure 4B**). In GAF, the center buffer is of higher acoustic impedance than the sample buffer. Upon application of an acoustic standing wave, particles move to the pressure node in the center of the microfluidic channel. With suppressed acoustic streaming in GAF, particles below the critical diameter can now be manipulated with the acoustic standing wave and focused to the microfluidic channel center, allowing the subsequent collection in the center outlet.

4.4 ETHICAL CONSIDERATIONS

The papers included in the thesis did not rely on animal experiments. For method setup and evaluation in **paper III**, small, anonymized blood samples from healthy volunteers were used. For those, no ethical application was required according to the regional ethical board at Lund university.

5 RESULTS & DISCUSSION

5.1 HIT DISCOVERY – PHENOTYPIC SCREEN

17 500 chemicals from the Chemical Biology Consortium Sweden (CBCS) primary screening set collection (*290*), comprising hit like molecules of a wide chemical space, were screened on cultures of the unencapsulated T4R strain and it´s parental T4 wildtype strain. 156 chemicals (0.9 %) induced lysis in T4R whereof only 71 (0.4 %) induced lysis in the encapsulated T4 strain. Higher sensitivity of unencapsulated pneumococci could result from reduced negative surface charge in absence of the capsule. This might allow access of more negatively charged molecules to the cell surface, which could otherwise be repelled in the encapsulated wildtype strain. Effect of bacterial surface charge on sensitivity towards antibacterial factors is reported for cationic antimicrobial peptides, however with opposite effect (*123*). The negatively charged capsule attracts more cationic peptides, shields modifications on teichoic acids (D-alanylation that increases the positive surface charge, **Figure 6B**) (*123*) and when shed from the cell surface acts as a decoy of antimicrobial peptides (*291*). The 71 screening hits were clustered by chemical properties and two chemical classes thereof were investigated in the thesis: the alkylated di-cyclohexyl carboxylic acid (2CCA-1) in **paper I** and the 1-amino substituted tetrahydrocarbazoles (THCz) in **paper II**.

5.1.1 Paper I

The bactericidal fatty acid mimetic 2CCA-1 selectively targets pneumococcal extracellular polyunsaturated fatty acid metabolism

2CCA-1 is an alkylated di-cyclohexyl carboxylic acid and posseses the typical amphiphatic properties of a fatty acid, with a hydrophilic carboxylic acid moiety and a hydrophobic extension. The structural difference to the twelve carbon lauric acid are however two cyclohexyl residues in the hydrophobic core. Structure activity relationship investigation showed that the carboxylic acid moiety as well as the cyclohexyl rings and an aliphatic chain of maximal five carbons were indispensable for the potent bactericidal action on *S. pneumoniae*. The suspected decoy for amphiphatic structures is the bacterial cell membrane, as it is shown for antibacterial free fatty acids (*292, 293*). They excert their bactericidal activity in a pleiotropic manner by interfearing with membrane physiology and disrubt membrane associated processes (*292, 293*). Even though antibacterial free fatty acids find recognition in topical applications such as the curation of skin infections, their potential for use as therapeutics is currently limited (*292*). This is on the one hand due to the eukaryotic cell toxicity of free fatty acids (*292*) and on the other hand because binding to carrier proteins opposes their antibacterial function *in vivo*. However, an innovative delivery strategy with liposome carried linoleic acid was shown to be efficiaous in treating *Heliobacter pylori* gastrointestinal infections after oral administration in a mouse model without epithelial cell toxicity (*294*).

A notable difference to the bactericidal action of free fatty acids, was the potency of 2CCA-1. Already approximately 30 times lower concentrations than those of free unsaturated and polyunsaturated fatty acids induced cell wall hydrolase mediated lysis. When compared to the lysis inducing capacity of the unsaturated twelve carbon chain lauric acid or the saturated eighteen carbon chain stearic acid, 2CCA-1 was even around 167 times more potent. Moreover, 2CCA-1 did not induce premature autolysis in non-growing cultures in stationary phase, even at 16 fold the MlytC concentration. This would have been indicative for a membrane associated interaction that is independent of the bacterial metabolism, as we observed for polyunsaturated fatty acids, nisin or daptomycin. In contrary, 2CCA-1 seems to need actively growing cells to excert its function. A clear correlation between growth and activity has long been established for the cell wall synthesis inhibitor penicillin (*295*).

2CCA-1 also induces cell wall hydrolase mediated lysis in *S. pneumoniae*, a typical response to cell wall synthesis inhibiting antibiotics (*251, 252*). Daptomycin inhibits cell wall synthesis by binding and sequestering lipid II, but also interferes with membrane physiology (*149, 150*). Treatment of *S. pneumoniae* with daptomycin induces cell wall hydrolase mediated lysis (**Figure 8**). The influence of membrane fluidity on cell wall synthesis at high growth rates was described for *B. subtilis* (*147*). Albeit evoked through an undesired, unspecific membrane interaction, detergents also induce LytA mediated lysis (*189, 252*). Autolysis activation does therefore not exlude the pneumococcal membrane as target for 2CCA-1.

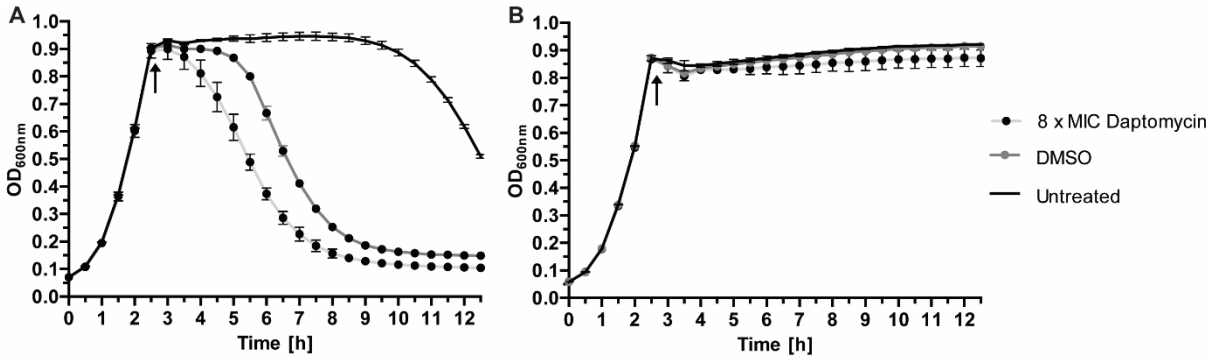

Figure 8 Daptomycin induces cell wall hydrolase mediated lysis. (A) T4 wildtype grown in supplemented C+Y medium with added Ca^{2+} (50 µg/mL). **(B)** T4Δ*lytA* a major cell wall hydrolase gene replacement mutant grown in the same medium as Tigr4 which additionally contained an elevated concentration of choline chloride (110 mM). Both strains were treated with daptomycin (8 µg / mL, 8 x MIC) after reaching stationary phase. The arrows indicate the timepoint of treatment addition. Average and standard deviations of triplicate treatments from a representative experiment are shown.

An important step in target identification and mechanism of action discovery was the analysis of 2CCA-1 resistant mutants. Whole genome sequencing revealed that 2CCA-1 resistance required inactivation of either the extracellular polyunsaturated fatty acid binding protein FakB3 (*159*), or the transcriptional regulator of the endogenous fatty acid synthesis machinery FabT (*154*). 2CCA-1 resistance of the *fakB3* spontaneous and gene replacement mutant suggests that uptake of 2CCA-1 was required for the compund to excert activity. An additional observation indicating that 2CCA-1 was not excerting antipneumococcal activity through unspecific membrane interaction, but rather required active metabolization to show activity.

Gram positive bacteria do not use extracellular fatty acids as energy source through catabolic β-oxidation but utilize them as membrane building blocks instead (*156, 167, 296*). Indeed, metabolomic analysis revealed the presence of unique molecular masses in the 2CCA-1 treated wildtype pneumococcus, which could be modeled as 2CCA-1 containing lysophosphatidic and phosphatidic acid respectively. Those species were not found in the untreated wildtype, nor in the 2CCA-1 treated spontaneous resistant *fakB3* mutant strain BHN852. Combined, the 2CCA-1 structure as well as activity characteristics, the resistant pattern and the unique molecular lipid species suggest that the compound is metabolized to a toxic phospholipid species.

2CCA-1 induced a dose-dependend change in membrane fluidity upon 2CCA-1 treatment, which could be attributable to the short hydrophobic tail of 2CCA-1 containing lipids, and the cyclohexyl rings which demand an untypical amount of space directly beneath the lipid head group. This constitutes a notable difference to typical cis-unsaturated fatty acids in the pneumococcal membrane, which are 16 to 18 carbons long with a double bond ealiest a the 7^{th} carbon atom, but more abudant between the 9^{th} and 10^{th} or 11^{th} and 12^{th} carbon atom (*134, 154, 157*). Due to the indistuingishability of laurdan generalized polarization between the membrane fluidizer benzyl alcohol from the DMSO solvent control, the explanatory power of our experiments as to if 2CCA-1 incorporation results in membrane fluidization or rigidification, was limited. However, when compared to published experiments, the measured increase in laurdan generalized polarization points to a decrease of membrane fluidity after 2CCA-1 treatment (*150, 219*). Membrane rigidification was also observed in *B. subtilis* after daptomycin

treatment, caused by lipid clustering after insertion of the bulky lipopeptide in RIFs (*150*). Similarly, the natural product rhodomyrtone attracted fluid lipids to the site of insertion resulting in an increased rigidity of the residual membrane (*219*). One could hypothesize, that 2CCA-1 containing lipids are localized in RIFs, as those are rich in lipids with short, branched and unsaturated fatty acids (*144, 150*). Similarly as for daptomycin (*150*), fluid lipids could be attracted to 2CCA-1 lipids to compensate for membrane distortions caused by the spaceous dicyclohexyl group adjacent to the lipid head group, resulting in abberant fluid lipid distribution and overal membrane rigidification. Complementary experiments are needed to describe 2CCA-1 induced changes in the pneumococcal membrane and the nature of cell envelope synthesis inhibition that results in cell wall hydrolase mediated lysis.

Expression analysis of resistant mutants revealed, that extracellular polyunsaturated fatty acid incorporation and endogenous fatty acid synthesis are interdependendly regulated in response to extracellular fatty acid availability. The conditions under which we isolated the 2CCA-1 resistant *fabT* mutant strain likely represent those of low extracellular polyunsaturated fatty acid concentration, during which *fakB3* expression was lower when compared to the wildtype strain. Loss of FabT under those conditions, could lead to a decreased uptake of 2CCA-1 and therefore increased pneumococcal tolerance to the compound. Correspondingly, the *fakB3* mutant tolerated higher concentrations of 2CCA-1 than the *fabT* mutant, which indicated the residual expression of some FakB3 protein for 2CCA-1 uptake in the *fabT* mutant. Low fatty acid concentrations established in the expression studies were a 0.1 mM equimolar mixture of saturated, unsaturated and polyunsaturated 18 carbon chain fatty acids. Those could resemble the free fatty acid concentrations in the nasal fluid, reported to be in the range of 0.03 - 0.2 mM (*47*). In contrary, fatty acid concentrations of 1 mM established in the expression analysis, could resemble the serum concentration of the 18 carbon fatty acid of different sturation degree, which was measured to be on average around 5 mM in citizens of the fishing area in the japanese town Kumihama (*297*). We found that FabT repressed *fakB3* expression under those conditions of high extracellular fatty acid availability, which could ensure membrane homeostasis by limiting the phospholipid polyunsaturated fatty acid content.

The expression analysis performed in **paper I** is limited to the FakB3 homologue of the extracellular fatty acid binding proteins, as it was the 2CCA-1 target. Extension of the extracellular fatty acid metabolism to FakB1 and FakB2, incorporating saturated and unsaturated fatty acids respectively (*159*), can help to draw a more general picture of the regulation of extracellular fatty acid supply and membrane homeostasis. Furthermore, it will be interesting to identify mediators of the transcriptional regulation, as we found that FabT binding to the *fakB3* promoter did not occur. FabT must therefore indirectly influence *fakB3* expression.

An interesting observation was the natural 2CCA-1 resistance of *S. aureus*, which does not encode a FakB3 homologous protein. Bioinformatic analysis revealed that FakB3 homologues are with one exception absent in species of the Bacillales order, but widespread in members of

the Lactobacillales order. The only species within the Bacillales order where FakB3 homologues were found, were of the *Gemella* genus, for which a vivid genetic exchange with other members of the oral microbiota such as *Streptococci* has been described (*298*). This could indicate a selective bactericidal activity of 2CCA-1 to *S. pneumoniae* and related species harboring a FakB3 homologue or a FakB homologue with a similar binding pocket (*159*).

A limitation of 2CCA-1 in the antibacterial treatment perspective is the exceptionally fast adaptation of the pneumococcus to the toxic compound. The possibility of recovering resistant mutants already as shortly as two hours after compound exposure, indicates that 2CCA-1 exerts a strong selective pressure on the pneumococcus.

Paper I characterizes the small fatty acid like compound 2CCA-1 with a new mechanism of action, that provides insight in pneumococcal polyunsaturated fatty acid metabolism regulation. 2CCA-1 might serve as tool compound to develop narrow spectrum antibiotics as well as to gain further insight in pneumococcal metabolism of fatty acids, the essential membrane lipid building blocks.

5.1.2 Paper II

THCz - A small molecule with antimicrobial activity that blocks cell wall lipid intermediates

Paper II describes analogs of the 1-amino- substituted tetrahydrocarbazole (THCz) class, that induced early logarithmic phase autolysis in the high throughput screen. The screening hit THCz-1 and selected analogs displayed activity in the low micromolar range against *S. pneumoniae* and other gram-positive bacteria as well as mycobacteria. Gram-negative bacteria with an outer membrane more permeable to hydrophobic compounds, such as *Neisseria gonorrhoeae* and *M. catarrhalis* (*299, 300*), and an outer membrane hyper-permeable *E. coli* mutant, were also inhibited by THCz analogs. No activity was recorded against *Pseudomonas aeruginosa* or *E. coli* wildtype strains, suggesting that THCz cannot permeate the intact outer membrane.

Structure activity relationship studies revealed that the central diamino motif was essential for activity, however also responsible for the eukaryotic toxicity of THCz analogs. Furthermore, a sterically demanding group and a correctly spaced linking carbon chain at the 1-amino substituted position of the tetrahydrocarbazole core was required for bactericidal activity. Bactericidal activity as well as cytotoxicity could be modulated by substitutions on the central 1-amino substituted tetrahydrocarbazole scaffold. The investigated THCz molecules were a racemic mixture, the enantiomers of selected analogs did however display similar activity.

THCz analogs induced rapid bactericidal action and cell wall hydrolase mediated lysis in early logarithmic phase. Cell wall synthesis inhibition upon THCz treatment was furthermore confirmed with the macromolecular synthesis inhibition assay and the induction of membrane blebbing and autolysis in *B. subtilis* characteristic for cell wall synthesis inhibitors. The target within cell wall biosynthesis was narrowed down to lipid II and undecaprenyl pyrophosphate as THCz induced the LiaRS two-component system. Indeed, the pyrophosphate moiety of the undecaprenyl carrier was determined as minimal binding motif of THCz, and we could show that THCz bind to lipid I, lipid II, lipid III_{WTA} and lipid I_{cap}. THCz therefore inhibit peptidoglycan, teichoic acid and capsular biosynthesis and YbjG mediated C_{55}-P recycling. As for nisin (*223*) and teixobactin (*224, 225*), interaction with the first sugar of the cell envelope precursors was beneficial for the binding affinity of THCz-1 in *in vitro* biochemical assays. The positive charge of THCz-1 at neutral pH supports a model for compound - target interaction between the pyrophosphate moiety and the diamino group of the THCz molecules.

Molecules that target undecaprenyl-pyrophosphate carried cell envelope precursors such as nisin, or daptomycin also interfere with membrane integrity. We show though, that THCz analogs did not form membrane pores such as the lantibiotic nisin. The rapid killing of THCz analogs might nonetheless indicate that membrane interaction could contribute to the antibacterial activity, as it was described for telavancin (*227*). For the lipid II targeting lipoglycopeptide with membrane interaction, bactericidal action was observed to occur more rapidly in comparison to the glycopeptide vancomycin that solely binds to the extracellular part

of lipid II (*227, 301*). Further investigations are needed to explore possible alterations of membrane physiology upon THCz treatment.

As observed for lipid II and pyrophosphate moiety targeting antibiotics (*223, 224, 302*), resistance to THCz-1 did not easily develop. We could not obtain THCz-1 resistant *S. aureus* strains through continuous incubation in sub-inhibitory concentrations of THCz-1. Also, multi-antibiotic resistant pneumococcal isolates retained wildtype sensitivity to THCz-1 and the analog THCz-40. This was also seen for methicillin- resistant and vancomycin- intermediate *S. aureus* strains as well as vancomycin-resistant *Enterococcus faecium*, suggesting that evolved resistance strategies do not confer cross-protection to THCz.

Interestingly, *S. pneumoniae* mutants with increased THCz-1 tolerance were obtained after THCz-1 exposure on THY plates. Whole genome sequencing of an isolate of increased THCz-1 tolerance and subsequent characterization of THCz-1 sensitivity of individual gene replacement knockouts of genes with point mutations, revealed that a mutation in *cps4E / wciI* conferred the phenotype. Unlike a complete gene replacement mutant in the first glycosyltransferase in capsular biosynthesis, or previously described mutations (*209*), the herein obtained mutation in $cps4E^{G394C}$ resulted in a decreased but not abolished capsular polysaccharide production. Decreased THCz-1 sensitivity of the unencapsulated T4R strain confirmed the role of the capsular polysaccharide in THCz-1 sensitivity, which could be a result of increased availability of the bactoprenyl carrier for cell envelope synthesis other than the polysaccharide capsule (*210*). The capsule is one of the major virulence factors of the pneumococcus and chemical induced reduction of the capsular polysaccharide amount might help the immune system to clear the infections.

The THCz analogs synthesized for the untargeted structure activity relationship studies in paper II also displayed toxicity towards eukaryotic cells, in a similar concentration range as for prokaryotic cells. THCz molecules analogous to the one presented in **paper II** were reported to have antifungal activity (*303*). Gene replacement mutants of the P-type ATPase homologs in *S. pneumoniae*, which have been identified as fungal targets, retained however wildtype sensitivity, possibly, due to the low sequence homology of the eukaryotic and prokaryotic target. Together with the available information on the THCz binding interface to the eukaryotic target (*303*), this opens the possibility for further targeted compound design. The synthetic accessibility of THCz allows for comparably easy modifications of the molecule in comparison to complex natural lipid II targeting products, enabling further exploration and tailoring of the bactericidal and cytotoxic properties of the compound.

Combined, **paper II** presents a small molecule that binds to undecaprenyl coupled cell envelope precursors and inhibits all pathways of cell envelope synthesis.

5.2 MICROFLUIDIC DIAGNOSTIC TOOL DEVELOPMENT

5.2.1 Paper III

Gradient acoustic focusing of sub-micron particles for separation of bacteria from blood lysate

Paper III presents a sample preparation method for bacteria containing complex samples, such as whole blood.

First, blood cells were selectively lysed without compromising bacterial viability. The lysis step utilized the different susceptibility of eukaryotic and prokaryotic cell envelope structures to stress conditions. An example is the cholesterol dependent membrane disruption of saponins, protecting the bacterial cholesterol free membrane from the detergent action (*304*). Detergent sclerosants and lipophilic vitamin K (menadione) have been described to favor platelet lysis (*305, 306*) and utilization of moderate concentrations preserved bacterial viability. The evolvement of quick stress response mechanisms in bacteria might also favor their survival in environments that provoke eukaryotic blood cell lysis. An access of choline chloride in the lysis buffer for reduction of autolysis of *S. pneumoniae* has been shown to benefit isolation of viable bacteria from blood samples (*307*).

The lysis step in the sample preparation method presented in **paper III**, was not yet integrated in a microfluidic setup, which would be beneficial for contamination prevention. As microfluidic solutions therefore have been proposed (*308, 309*), the integration of a lysis step in a microfluidic compartment could possibly be also applied to the herein presented setup. Chemical blood cell lysis of the selected buffer 9 for gradient acoustic focusing (GAF), reduced the number of large blood cells and platelet sized particles and did not produce more of about 1 µm sized particles in the bacteria size range per milliliter of lysate compared to whole blood. Small sized lysis debris of a size equivalent to 0.5 µm and below however increased about 2-log in concentration in lysed blood.

Therefore, GAF was employed as a second step purify the bacteria from small sized lysis debris and perform a buffer exchange. In **paper III** the methodological development of GAF is described. The GAF principle was tested on polystyrene particles as well as *S. pneumoniae*, *S. aureus* and *E. coli*, bacterial species that frequently cause septic shock (*89*). 97.0 ± 0.9 % (Average ± standard deviation) of *S. aureus*, 80.0 ± 2.7 % of *E. coli* and 70.4 ± 4.2 % of *S. pneumoniae* collected in the device outlets were obtained in the center outlet, in comparison to 98.24 ± 0.66 % of 1 µm and 37.67 ± 3.22 % of 0.5 µm polystyrene particles. The difference in focusing yield between bacterial species might be due to their difference in morphology, yet uncharacterized compressibility and the size distribution within a population. However, when the device is operated with media of matched acoustic impedance ($\Delta Z=0$, homogenous media) in the center and side channel, 63.7 ± 9.4 % of 1 µm particles and 59.9 ± 12.0 % of *S. aureus* were obtained in the center outlet. This shows that with GAF small micrometer sized particles can be focused, while they could previously only be mixed efficiently due to acoustic streaming.

For validation of the GAF principle, the device particles and bacteria were dissolved in a BSA containing aqueous buffer with low viscosity and a high acoustic impedance difference to the center medium. Fluids laminated for GAF need to have an acoustic impedance difference ΔZ of more than 1 % so that acoustic streaming is sufficiently suppressed. The acoustic impedance of the center buffer was therefore adjusted with the hydrophilic polysaccharide Ficoll to establish those conditions in respect to the blood lysate. GAF enabled the isolation of 92.19 ± 1.11 % of *S. aureus* in the center outlet of the microfluidic device from three times diluted lysate, while lysis debris was reduced by about 70 %. Furthermore, bacteria were transferred to a different buffer, judged by the low hemoglobin concentration (20 %) in the center outlet. This might also reduce the concentration of lysis agents, and antimicrobial compounds of the blood sample, presupposed their diffusion coefficients are comparable to the one of hemoglobin. Importantly, the application of GAF allowed the solute exchange and concentration dilution without an increase in sample volume.

A rapid antibiotic susceptibility profile is of high importance for the therapeutic success and antibiotic stewardship. Standard antibiotic susceptibility testing in clinical microbiology laboratories, such as disk diffusion methods on agar plates (*310*), require augmentation of bacteria through several cultivation steps. Faster methods for the targeted identification of genotypic resistance markers cannot fully replace the phenotypic antibiotic susceptibility determination for clinical treatment decisions (*92, 101*). The microfluidic setup based on GAF of bacteria from a blood lysate presented in **paper III,** provides a possibility for label free sample decomplexation and preparation of viable bacteria. The lysis step comes with an additional advantage as to bacteria that were in the process of being phagocytosed might be able to escape the toxic environment (*98*). This could be one reason for the decrease in time until the detection (*311, 312*) and antibiotic susceptibility determination (*313*) of the sepsis etiologic agent with the lysis centrifugation method, that was invented in the 1970s. Furthermore, the size-based separation of bacteria from lysed blood rather than the separation of larger blood cells from plasma could favor detection sensitivity, as bacteria can adhere to blood cells (*314-318*) and would otherwise be sorted out (*319*).

To summarize, the setup for sample decomplexation presented in **paper III** allows isolation of viable bacteria with an unselective, label free method and the potential to be operated in continuous flow in a sealed, contamination preventing microfluidic environment. The sample contains a reduced amount of blood cells and debris with the aim to facilitate downstream label free microfluidic operations. Continuous operation and the potential to multiplex the microfluidic channels, opens the possibility to process a higher sample volume to increase detection sensitivity. The preservation of bacterial viability might allow a faster microfluidic solution for the phenotypic antibiotic susceptibility determination of the sepsis causing pathogen for a faster diagnosis of acute bloodstream infections.

6 CONCLUSIONS & POINTS OF PERSPECTIVE

Antibiotic resistance is an emerging threat to public health. Infections with antibiotic resistant bacteria pose a challenge to our society and healthcare systems. Multifaceted approaches and innovations are needed to face and resolve the public health threat. In the light of the effective and targeted use of antibiotics and successful implementation of innovative treatment approaches, it is crucial that novel treatment strategies and diagnostics concurrently evolve.

Paper I and **II** characterize hits from a screen for bactericidal chemicals and describe their mode of action. Interestingly, those hits differ in their activity spectrum. Activity of compound 2CCA-1 described in **paper I** depends on the presence of a fatty acid binding protein homologous to the pneumococcal FakB3 with a binding affinity for polyunsaturated fatty acids (*159*). The lack of FakB3 homologues in species belonging to the Bacillales order, provides an explanation for the tolerance of *S. aureus* to 2CCA-1. *S. pneumoniae*, belonging to the Lactobacillales order, displays exceptional sensitivity to the compound and incorporates 2CCA-1 in its membrane phospholipids. The structural features of the compound and measured 2CCA-1 induced membrane fluidity alterations, suggest the disruption of membrane homeostasis as cause of the bactericidal effect.

While we recorded a rather narrow spectrum for 2CCA-1, the THCz compound class described in **paper II** possesses a comparably broad activity spectrum. THCz analogs were active against gram-positive *S. pneumoniae*, *S. pyogenes, S. aureus*, *B. subtilis*, and *Enterococcus faecalis* to name a selection, and to mycobacteria as well as gram-negative *N. gonorrhoeae* and *M. catarrhalis* in low micromolar concentrations. This is attributed to the conservation of the THCz target among prokaryotes. In **paper II** we identify the THCz target to be undecaprenyl pyrophosphate carried cell envelope precursors lipid I, lipid II, lipid III_{WTA} and Lipid I_{cap} with undecaprenyl pyrophosphate as minimal binding partner. THCz analogs thereby inhibit all major branches of cell envelope synthesis with low resistance development propensity.

The responsible use of antibiotics requires a fast and precise diagnosis. It is even more indispensable for the development and use of narrow spectrum antibiotics, which impact the microbiota with less collateral damage and might therefore inherently decelerate the rate of resistance development. When a novel treatment is to be in place, readily available, affordable and precise diagnostics will be crucial to help serving the ethical responsibility of best treatment for an individual patient that is only administered if absolutely necessary to serve societies need for the preservation of a valuable resource.

Microfluidic operations provide the possibility to handle fluids on a single bacteria level an offer huge potential for innovations that meet the need for fast, precise and comparably cheap diagnosis. This is especially true for microfluidic solutions that offer the possibility for a “lab on a chip”, which can be employed on the bedside, in households and rural environments.

The microfluidic approach for sample decomplexation of bacteria containing blood, presented in **paper III** could be a first step of an integrated microfluidic solution for antibiotic

susceptibility determination of the sepsis causing pathogen. The setup described in **paper III** approaches the complex sample through the reduction of the excess of blood cells and isolation of viable bacteria. This might allow faster identification and susceptibility profile characterization with microfluidic downstream operations, providing a shortcut to the time-consuming cultivation steps for an accelerated diagnosis and targeted treatment of sepsis.

Future research is needed to inquire if 2CCA-1 is effective as antibacterial agent *in vivo* and can be formulated to improve pharmacological properties. Further medicinal chemistry efforts are required to decrease the toxicity of THCz analogs and investigate, if and how THCz interact with the bacterial membrane. Microfluidic operations downstream of the setup presented in **paper III** need to be developed or integrated and the setup needs to be validated on clinical sepsis specimens.

In conclusion, the thesis hopes to contribute work that may serve information for the development of innovations to combat antibiotic resistance.

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