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Intra- and intermolecular behavior of the Staphylococcus aureus virulence factor MprF and its effect on resistance against the antibiotic daptomycin

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Content

Abbreviations

A	alanine
aa	amino acid
aaPG	aminoacyl phosphatidylglycerol
aaPL	aminoacyl phospholipid
aatRNA	aminoacyl tRNA
AlaPG	alanyl phosphatidylglycerol
AMS	4-Acetamido-4'-Maleimidylstilbene-2,2'-Disulfonic Acid
AS	Aminosäure *bzw.* Aminosäuren
approx.	approximately
BM	basic medium
BSA	bovine serum albumin
β-ME	β-mercaptoethanol (also 2-mercaptoethanol, 2-ME)
C	cysteine
C. perfringens	*Clostridium perfringens*
ca.	circa
CAMP	cationic antimicrobial peptide
CM	cell membrane
C-terminus	carboxyl-terminus
CW	cell wall
D	aspartic acid
DAP	daptomycin
DAP-NS	daptomycin nonsusceptibility / -nonsusceptible
DAP-R	daptomycin resistance /-resistant (synonym to DAP-NS)
DAP-S	daptomycin susceptibility /-susceptible
DMSO	dimethylsulfoxid
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide triphosphates
ECC	electrocompetent cells
E. coli	*Escherichia coli*

EDTA	ethylendiaminetetraacetic acid
EMA	European Medicines Agency
Etest	Epsilometer test
e.g.	exempli gratia
FDA	Food and Drug Administration
Fig.	Figure
HPLC	high performance liquid chromatography
IP buffer	immunoprecipitation buffer
KAMP	kationisches antimikrobielles Peptid
L. monocytogenes	*Listeria monocytogenes*
LB	lysogeny broth
LTA	lipoteichoic acid
LysPG	lysyl phosphatidylglycerol
MHB	Mueller Hinton broth
MIC	minimum inhibitory concentration
MOPS	3-(N-morpholino)propanesulfonic acid
MPB	N*-(3-Maleimidylpropionyl)Biocytin
MprF	multiple peptide resistance factor
mRNA	messenger RNA
MRSA	methicillin-resistant *Staphylococcus aureus*
N-terminus	amino-terminus
OD_{λ}	optical density at wavelength λ (e.g. λ = 600 nm)
O/N	overnight
P	proline
P_i	Inorganic Phosphate
PAGE	polyacrylamide gel electrophoresis
PB	polymyxin B
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PGS	phosphatidylglycerol synthase
PMSF	phenylmethylsulfonyl fluoride

polyp	polyphosphate
RNA	ribonucleic acid
RT	room temperature (25 °C)
S	serine
S. aureus	*Staphylococcus aureus*
SDM	site directed mutagenesis
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
T	threonine
Tab.	Table
TAE	tris-acetate-EDTA
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween
TFB1&2	transformation buffer 1&2
TMD	transmembrane domain
TMS	transmembrane segment
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TSB	tryptic soy broth
UPLC	ultra performance liquid chromatography
UV	ultra violet
VISA	vancomycin-intermediate *Staphylococcus aureus*
VRSA	vancomycin-resistant *Staphylococcus aureus*
WT	wild type
WTA	wall teichoic acid

1 Introduction

Staphylococcus aureus, first described in 1884 and named for being arranged in grape-like clusters (Greek σταφυλή (staphylé) – grape), round (Greek κόκκος (kókkos) – corn) and of yellow colour (Latin aureus – golden), belongs to facultative aerobic Gram-positive bacteria [58, 74]. As part of the flora and in 30-50 % of mankind colonizing human nares, it is an opportunistic commensal and a major human pathogen [1, 15, 33, 91]. A diversity of diseases is caused by *S. aureus*, both community-acquired and nosocomial infections, e.g. skin and soft tissue infections, bacteraemia, infective endocarditis, pulmonary, catheter-related, and osteoarticular bone and joint infections [3, 42, 80, 92]. To protect from such invasive infections, human evolution bore different mechanisms of the human innate immune system, such as cationic antimicrobial peptides (CAMPs) like defensins, bacteriolytic enzymes like lysozyme, and many more [48]. Defensins are mostly produced by neutrophils, and all three types of defensins (α, β, θ) mainly act via membrane permeabilisation, are active both intra- and extracellularly, and are chemoattractants for macrophages and neutrophils [26]. *S. Aureus* infections can be treated by an assortment of antibiotics with different targets, namely β-lactams (penicillins, cephalosporins and carbapenems), aminoglycosides, glycopeptides, sulfonamides, tetracyclines, and others [25, 51]. One of them is daptomycin (DAP), a cyclic lipopeptide antibiotic against Gram-positives with an anionic molecule core, while the net charge becomes positive when in complex with Ca^{2+} (Fig. 1) [41, 88].

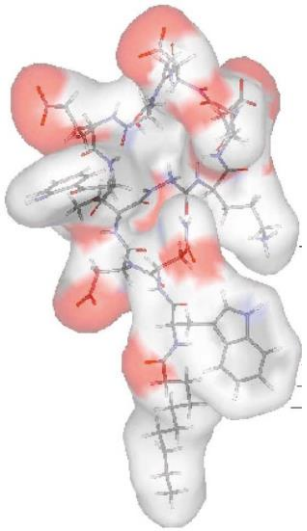

Fig. 1: Structure of daptomycin showing the distribution of charge (colour coded). The structure was determined via nuclear magnetic resonance spectroscopy. Here, the anionic molecule core apo-daptomycin without Ca^{2+} is shown. Negative charge is indicated as red, neutral as white, and positive as blue. From Ball et al. [6].

On the one hand, DAP targets the cell membrane (CM) binding to phosphatidylglycerol and leading to membrane depolarisation due to a loss of cytoplasmic potassium ions, and, on the other hand, it interacts with proteins synthesising lipoteichoic acids (LTAs) and thereby inhibits cell wall (CW) synthesis [29, 37, 72, 82]. It is a last resort antibiotic, having gained approval in the US by the Food and Drug Agency (FDA) in 2003 and in Europe by the European Medicines Agency (EMA) in 2006 [46]. DAP offers an alternative therapy to vancomycin which has been the standard antibiotic of last resort for treatment of staphylococcal infections. Such alternatives become more and more necessary due to emergence of broad-spectrum antimicrobial resistant strains, such as in methicillin-resistant or vancomycin-intermediate *S. aureus* (MRSA, VISA) [16, 60]. *In vivo*, DAP resistance (DAP-R, synonym for DAP-NS, term for ease of presentation) has frequently been reported in recent years [9, 19, 21, 24, 34, 37, 39, 44, 45, 57, 81, 94]. Bacteria can express a variety of resistance mechanisms to human CAMPs, for example (a) proteolytic degradation of CAMPs, (b) extracellular CAMP-capturing and -inactivating molecules, (c) active extrusion of CAMPs, and (d) reduction of the affinity of the bacterial cell envelope for CAMPs or repelling them via altering the bacterial

surface net charge, i.e. reducing or exchanging anionic charges of the cell surface and thereby neutralising the former negative net charge (Fig. 2) [67].

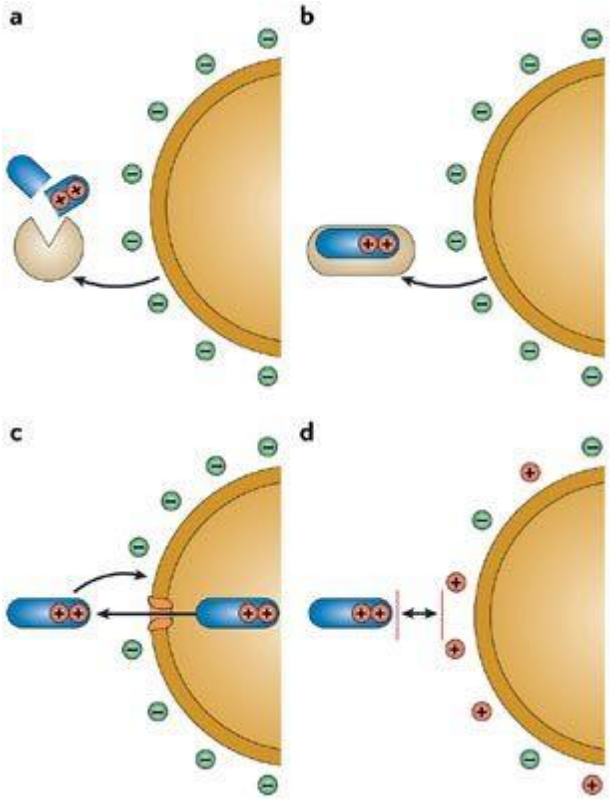

Fig. 2: Bacterial resistance mechanisms against CAMPs. (a) Proteolytic degradation, (b) capturing and inactivating, (c) export, (d) altering the bacterial cell surface net charge and thereby reducing affinity, respectively, repelling. From Peschel et al. **[67].**

Resistances as these, in turn, emerge upon genetic mutations [13, 17, 20, 68, 69, 96]. Especially under selective pressure due to antibiotic treatment, as through vancomycin or daptomycin, such mutations can be – even simultaneously – induced via multiple evolutionary pathways, e. g. *yccH* and *mprF*, and are able to influence cross resistances to glycopeptides, such as vancomycin or teicoplanin [17, 22]. Nonetheless, resistances can occur at different genes leading to heteroresistances and do not have to be permanent [28, 43, 87]. A very blatant example is a reported evolution of a MRSA strain during a protracted clinical infection lasting 115 days. In one patient, diverse mutations appeared at different time points, and several different morphotypes with reduced antibiotic susceptibilities have been detected. After non selective growth, the genomes restored to their original

susceptibility profile [35]. There have been reports of MRSA strains spontaneously reverting to a subsequent methicillin-susceptible *S. aureus* (MSSA), and of DAP-NS MRSA strains having reverted to DAP-S [43]. Through discontinuation of the therapy with DAP a reversion of resistance was detected and the above mentioned enhanced findings decreased back to the same level as in DAP-S strains [43]. On the contrary, clinical DAP-NS strains have been described that did not revert their resistance and showed stable MICs even after two years of storage without DAP selective pressure [87]. Therefore, resistance proves to be a highly complex and multifaceted topic.

Different DAP resistance mechanisms have been proposed. Most of them suggest alterations concerning different aspects of the cell wall, such as its composition, ionic charge and membrane fluidity [37]. While DAP-NS isolates can show more positive or similar charges of the cell surface as in DAP-S ones, DAP-NS is usually accompanied by higher thickness of the CW, higher mRNA levels (overexpression) of several two-component-systems (TCSs), higher amount of wall teichoic acids (WTAs), higher proportion of D-alanylated WTAs, and upregulated transcription of CW biosynthesis genes [14, 43]. In contrast, there is also data suggesting that DAP-R does not always correlate with positive net charge or thickness of the cell wall [61].

Since CAMPs and numerous antibiotics target the bacterial membrane or have to pass it to take effect, both understanding interactions between CAMPs and the cell surface, and understanding the basic cell surface mechanisms is of great importance [2]. The bacterial cell surface is made up of a mesh of peptidoglycan teichoic acids that covers and spans out the lipid cell membrane. In many Gram-negative bacteria and only some Gram-positive bacilli the lipid membrane is composed of zwitterionic phosphatidyletholamine (PE), whereas several Gram-positive bacteria, like staphylococci, have mostly anionic phosphatidylglycerol (PG) and diphosphatidylglycerol (also known as cardiolipin, CL) [31, 70]. Moreover, numerous bacteria modify their membrane lipids with amino groups creating aminoacyl phospholipids (aaPL), in particular aminoacyl phosphatidylglycerols (aaPGs) as lysyl or alanyl phosphatidylglycerol

(LysPG, AlaPG). Addition of such amino acid (aa) moieties to membrane lipids increases the overall negative net charge of bacterial membranes by integrating cationic or zwitterionic charges. aaPLs are usually found in Gram-positives, especially LysPG which is synthesised by MprF proteins. While *E. faecalis* and other bacteria can have several types of aaPGs, in *S. aureus* there is only LysPG [4, 36, 67]. By converting PG to LysPG, the net charge of PG is changed from -1 to +1 [2]. Furthermore, as a major post-translational modification, N-succinyl-lysyl-PG has also been reported, which presumably forms a complete loop of charge reversal back to -1 [4]. The distribution and composition of phospholipids in the two leaflets of the membrane is not static but changes according to environmental challenges [48, 49]. Whereas in Gram-negative bacteria, having an inner cytoplasmic membrane and an outer periplasmic membrane, some types of lipids are likely to be found exclusively within the outer compartment (Fig. 2 and 3) [55].

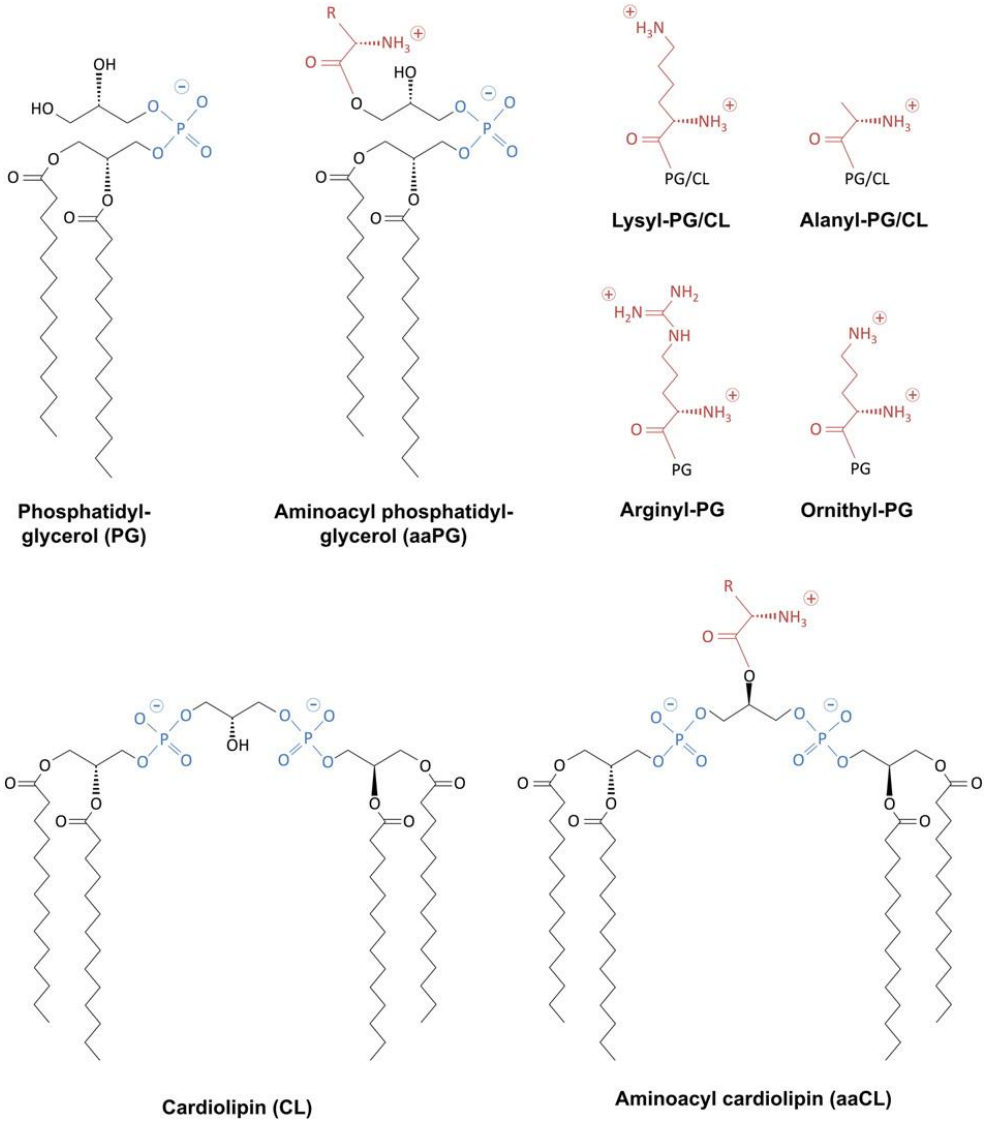

Fig. 3: Chemical structures of the common phospholipids, PG and CL, and derived aaPG variants, changing their net charge from anionic to zwitterionic or cationic. R – residue, referring to the amino acid side chain. From Slavetinsky et al. [83].

Considering the alteration of the surface net charge as a resistance mechanism to CAMPs, there are two important and often described bacterial gene *loci*, the *dlt* operon and the multiple peptide resistance factor (*mprF*) [11, 18, 95].

The gene products of the *dltABCD*-operon are responsible for the D-alanylation of WTAs, resulting in a relatively increased net charge [95]. With the consequent altered electrostatic properties of the surface, the cell envelope is more tolerant to CAMPs [63, 95] and has reduced autolysis. Overexpression of *dltA*, which has been found in different strains, i. e. DAP-R clinical isolates or VISA [17, 18], resulted in reduced autolysis while biofilm formation remained

intact [63]. The overexpression of *dltA* is a common pathway of resistance, leading to an increase of D-alanylated WTA [18, 61]. Due to a point mutation in *pitA* (an inorganic phosphate transporter), the upregulation of the *dlt* operon is induced (not yet clear whether directly or indirectly) by intracellular accumulation of inorganic phosphate (P_i), while resistance is not triggered by the accumulation of polyphosphate (polyP), and the GraXRS TCS (an activator of the *dlt* operon and of *mprF*) is not involved [59]. In contrast, mutants lacking *dlt* gene products show increased affinity to CAMPs while the ability to bind to endothelial cells is decreased, leading to an attenuated virulence and reduced metastatic infections, although binding to fibronectin remains unchanged [95].

In 2001, *mprF* was discovered as a gene locus in *S. aureus* which, when knocked out, led to high susceptibility of *S. aureus* to many CAMPs – hence the name 'multiple peptide resistance factor'. It was shown that *mprF* knockout mutants, in comparison to the wildtype (WT), were killed significantly faster by neutrophils, had increased susceptibility to CAMPs, and possessed attenuated virulence in mouse and rabbit infection models. In one study, tested with one distinct type of human cells, *MprF* knockout mutants did not show reduced adherence to fibronectin and, in contrast to *dltA* knockout mutants, did not have a reduced binding to endothelial cells or reduced metastatic infections [66, 95]. Later, other species than *S. aureus* were found to have MprF homologues as well, with attenuated virulence when knockout mutants were tested [56, 90]. For instance, *Bacillus anthracis* gets hypersusceptible to CAMPs when *mprF* is knocked out due to impaired biosynthesis of LysPG [79]. Among prokaryotes, MprF and its nearly 350 known homologues, all being aminoacyl phosphatidylglycerol synthases (aaPGSs), belong to a highly conserved family of membrane proteins, which are found among 93 bacterial genera and three archaeal species [76, 77]. In *S. aureus*, MprF catalyses the synthesis of LysPG [66], and when trans-expressed in WT *E. coli* cells, LysPG synthesis can be detected [64]. The amount of aaPLs depends on environmental conditions and species, suggesting that biosynthesis of MprF is regulated [47, 56]. MprF regulation in *S. aureus* is conducted by the Aps/GraRSX TCS systems, which besides *mprF* activates other CAMP resistance genes such as the *dlt* operon,

when exposed to CAMPs [31, 52, 53]. A *graS* knockout mutant exhibits impaired *mprF* and *dltA* expression, resulting in increased sensitivity to CAMPs and DAP [23]. The expression of *mprF* can also be induced by antibiotics such as DAP [17]. Prolonged exposure to antibiotics such as DAP, vancomycin and telavancin were found to influence different gene regulations and expressions causing adaptations to the effects of glycol- and lipopeptide antibiotics [73]. The *mprF* gene has a length of 2520 base pairs and encodes a membrane protein consisting of 840 amino acids with a size of 93 kDa, forming twelve N-terminal transmembrane segments (TMSs) with an enlarged cytosolic loop between TMS 6 and 7, and a large cytosolic C-terminus (Fig. 4) [30, 83].

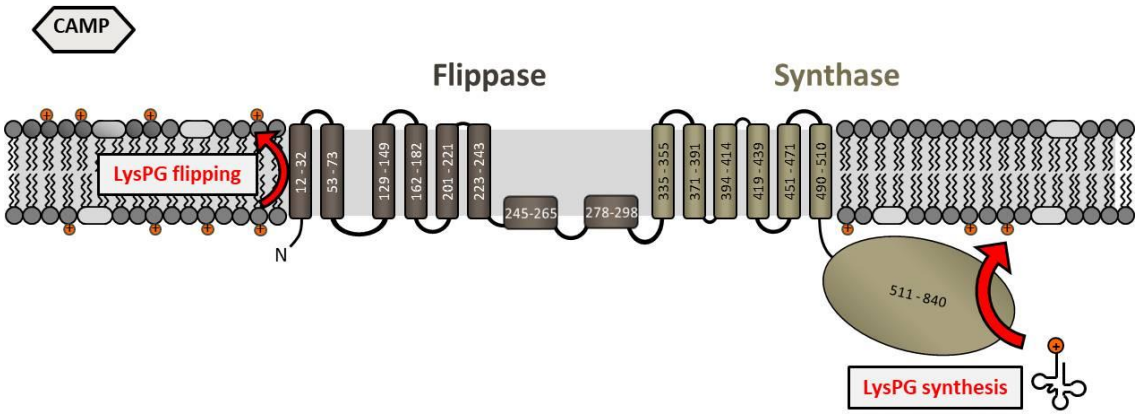

Fig. 4: Proposed topology model of MprF. Derived from a lysyl tRNA, the lysyl group (orange) is transferred to PG by the synthase domain. Translocation from the inner to the outer leaflet of the membrane of a LysPG is conducted via the flippase domain, building up a more positive net charge that repulses CAMPs. CAMP – cationic antimicrobial peptide, PG – phosphatidylglycerol, LysPG – lysyl PG. Modified after Slavetinsky et al. **[83].**

This large integral membrane protein possesses two distinct functions via its two separable functional sub-domains. One is the synthase domain, consisting of the large cytosolic C-terminus and the six C-terminal TMSs, producing LysPG in the inner leaflet of the bacterial cytoplasmic membrane. The other is the flippase domain, which is composed of the six N-terminal TMSs, translocating LysPG from inner to outer membrane leaflet, which is crucial for the resistance of *S. aureus* to CAMPs (Fig. 5) [2, 30, 83].

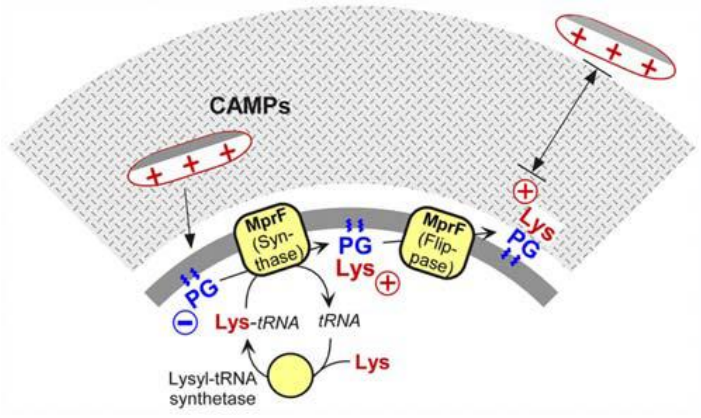

Fig. 5: Mode of *S. aureus* MprF-mediated bacterial resistance. From Ernst et al. [30].

The synthase domain acts uniquely like a transesterase, covalently linking a lysine residue derived from a lysyl tRNA to PG, hereby producing an aaPG [47, 86]. The homologues of MprF can utilize different amino acid residues from different aminoacyl tRNAs (aatRNAs) to form different aaPGs [75]. Some MprF homologues possess a relaxed substrate specifity, such as *E. faecalis* MprF2 which synthesises not only LysPG but also AlaPG and ArgPG, while these aminoacylations lead to increased resistances against CAMPs as well. Though, *mprF1* from *E. faecalis* is not involved in that synthesis, only *E. faecalis mprF2* [8, 83, 85]. Other homologues of MprF are restricted to only one type of aaPG, such as *S. aureus* specifically synthetizes LysPG [83, 85]. Furthermore, in *Clostridium perfringens* two homologues of MprF are encoded, one facilitating LysPG synthesis, the other one producing AlaPG. Although only the synthase domain for LysPG is fused to a flippase domain while the synthase domain for AlaPG is not, both LysPG and AlaPG can be translocated by the same flippase domain and confer CAMP resistance at a similar level. This resistance is achieved as long as the flippase domain and at least one of the two types of synthase are present. There is no need for both types of synthase to be present simultaneously. [84]. This indicates that MprF consists of two distinct and interacting synthase and flippase sub-domains, which both have to be present but do not have to be covalently linked for full MprF activity [31, 32].

The membrane topology of MprF is not yet completely identified. Via X-ray crystallography, the structures of the cytosolic parts of the AlaPG synthase

domain (AlaPGS) from *Pseudomonas aeruginosa* MprF and of the LysPG synthase domain (LysPGS) from *Bacillus licheniformis* MprF were examined. It was displayed that the hydrophobic PG and the hydrophilic aatRNA opposingly access the catalytic site via a continuous tunnel. The main determinants to recognise the aaPG substrate have shown to be the tRNA acceptor stem, the aminoacyl moiety, and the hydrophilic head group of PG [40]. First assumptions about the topology of MprF had been made by the membrane protein topology prediction algorithm SOSUI [30]. Later, TOPCONS, another web server, was employed. The underlying algorithm of TOPCONS combines and quantifies topology predictions of five different algorithms in one consensus prediction, including OCTOPUS, a prediction method using a dataset of sequences with known and experimentally validated structures [12, 93]. In comparison, these predictions for the TMS topology of MprF by SOSUI and TOPCONS had essential differences from one another [32]. To reconnoitre the contrarieties, an experiment using marker enzymes had been conducted. Truncated variants of MprF were fused at several regions which were predicted loop regions to the marker enzymes galactosidase (LacZ) or alkaline phosphatase (PhoA). LacZ shows enzymatic activity only when it is in the cytoplasm. In opposition to that, intracellular PhoA is enzymatically inactive and shows activity only when translocated into the periplasm where intrachain disulphide bonds can be formed [32, 38]. These findings suggested that TOPCONS is superior to SOSUI considering predictions of the MprF membrane structure, as they were mostly conform to the prediction of TOPCONS, whilst it showed discrepancies to the prediction by SOSUI [32]. A research group around Andreas Peschel has created a monoclonal antibody which binds to that extended loop between TMS 6 and TMS 7 at amino acids 259 – 272. Originally, it was expected not to bind and thereby to confirm that the loop is cytoplasmic. But surprisingly, binding could be detected. In line with the results of an enzymatic experiment, data suggests that this loop can be both exposed to the cytoplasm and to the outer surface (Fig. 6) (Slavetinsky et al., unpublished).

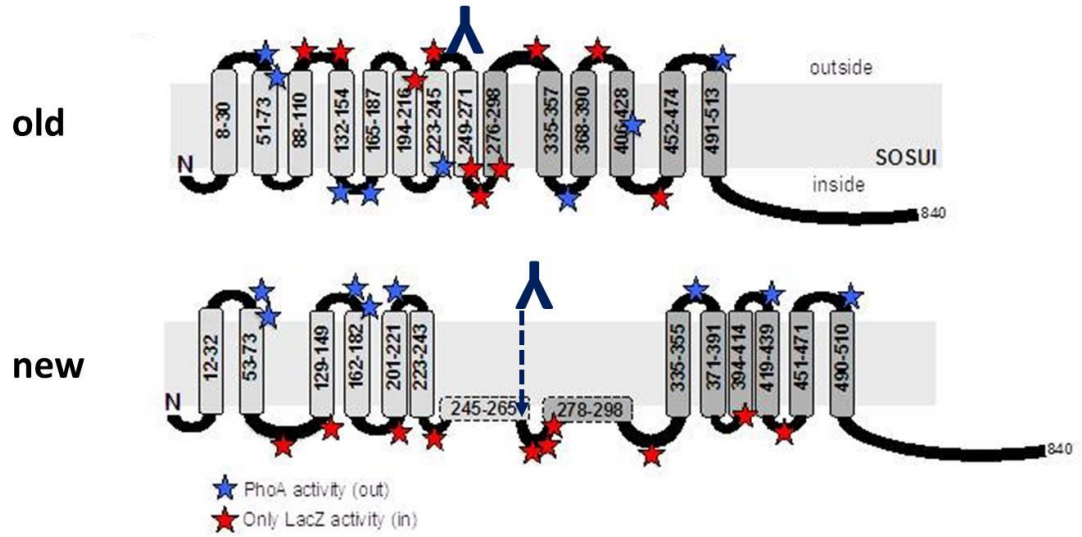

Fig. 6: Comparison of the old and new topology analyses of MprF. Predicted MprF topology by SOSUI (top panel, labelled "old") and actual topology based on the results of Ernst et al., largely following predictions by TOPCONS (bottom panel, labelled "new"). Determined activities of LacZ and PhoA via fusion constructs are displayed as red (LacZ) and blue (PhoA) asterisks. TMSs associated with the synthase domain are shown in dark grey, those with the flippase domain in light grey. Two putative α-helices as part of the extended loop are shown with dashed lines. The antibody's specific binding to amino acids 259 – 272 within the extended loop (Slavetinsky et al., unpublished) is indicated as dashed arrow. Modified from Ernst et al. [32].

Therefore, it seems likely that in the course of flipping aaPGs from the inner to the outer leaflet of the bacterial membrane, this extended loop itself changes its location from intra- to extracellular, suggesting that this loop could possibly serve as an active enzymatic center, respectively, as a binding pocket.

Better understanding of the MprF mechanism could be of great help, facing the increasing number of *S. aureus* strains that became spontaneously resistant to DAP during therapy, being thus resistant to virtually almost all available antibiotics [7, 62, 81, 94]. The adaptive mechanisms leading to DAP-R, at least partially, seem to be multi-factorial and strain specific [61]. Within those isolates showing spontaneous resistance, mutations in several genes (e. g. *rpoB*, *rpoC*, *yycG, cls2, pgsA*) and changes in the expression as well as mutations of *dltABCD* have been reported [7, 65]. Mutations of *mprF* (S295L, T345I), in turn, have been shown to play a role in an increased transcription of *dltA* [18]. In comparison to DAP-S MRSA, DAP-R MRSA strains have shown dysregulations of *dltA* and *mprF*, e. g. conferring an increased cell membrane fluidity or

reduced susceptibility to CAMPs [61]. There is a report of a clinical MRSA strain isolated from one patient during therapy, describing different genetic mutations. A mutation in *rpoB* led to a higher tolerance of DAP and other antibiotics due to pleiotropic changes conducting to a thicker CW but also less virulence traits and slower growth. Subsequently, loss-of-function of another locus, *clpX*, accelerated bacterial growth to some extent without effecting the susceptibility to antibiotics. Only after acquisition of a mutation in *mprF*, DAP-R was observed [5]. Other clinical DAP-R isolates lacking prior exposure to DAP and without mutations leading to amino acid substitutions in MprF have been reported [68]. Nonetheless, the major part of DAP-R *S. aureus* strains were found to have specific single nucleotide polymorphisms (SNPs) in *mprF* causing resistance to CAMP-like antibiotics, such as DAP [31]. Since *mprF*-knockout-mutants lacking MprF are hypersusceptible to DAP, MprF itself is very unlikely to be the target of DAP [30]. Those point mutations leading to resistance occur within the open reading frame (ORF) of *mprF* and usually show a phenotype with gain-of-function considering either synthesis or flipping of LysPG, and a thicker cell wall, as electromagnetic and lipid membrane composition analyses revealed, while the SNPs appear to be not merely biomarkers but casually related to that phenotype. However, these findings have been merely shown in clinical isolates but not yet in a defined genetic background [61, 65, 97]. Different *mprF* mutations have been characterised for their effect on the phospholipid content and the proportion of LysPG to PG, and it has been shown that not all but some of them have effects on that content and that these effects differ from each other [78]. In clinical isolates of MRSA, there have been reported SNPs within the *mprF* in DAP-S strains, however, none of these SNPs were in the known hotspots for resistance to daptomycin but dispersed along the genome. In contrast, in DAP-R MRSA isolates, all SNPs were reported to be found in known hot spots (L341S or L826F). These isolates showed increased expression of *mprF*, gain-of-function of MprF, higher survival against CAMPs, and less binding of DAP to the cell membrane [10].

The aims of this work are to further investigate the influence of the *dlt* operon and MprF on the bacterial resistance against CAMPs and CAMP-like antibiotics

such as DAP, to ultimately contribute to the better understanding of the mechanism of resistances via MprF, and to further explore the putative flipping mechanism of the large predicted intracellular loop within MprF.

Bacterial resistance is based on a plethora of reasons and genes [13, 17, 20, 68, 69, 96]. Amongst these, especially for DAP-R, *mprF* has been identified as the first genetic hotspot for mutations in clinical isolates or passage experiments [31]. These mutations occur mainly in the transition region from the synthase to the flippase domain. One of them is the missense mutation T345A associated with a DAP-R phenotype [62]. It shall be shown that the mediated resistance to DAP is operated via the cell wall. Therefore, the MICs, determined via Etest, of DAP have been compared to those of another antibiotic, bacitracin, which does not target the cell wall, in three strains of *S. aureus* Newman with the WT. The former is an anionic cyclic lipopeptide forming cationic complexes with calcium which mainly targets the CM and leads to its depolarisation [29, 37, 41, 72, 82, 88]. The latter is a cyclic dodexylpeptide antibiotic impeding cell wall polymer biosynthesis due to inhibition of the dephosphorylation of undecaprenyl pyrophosphate (Upp) via the Upp phosphatase (UppP) which is required to recycle the lipid carrier [54, 71, 89]. When binding Upp, bacitracin completely envelopes the pyrophosphate group of the ligand [27].

In a second step, it was examined whether DAP-R correlates with less binding of DAP to the CW. While it is known that in *S. aureus*, MprF serves as LysPGS and catalyses the synthesis of LysPG out of PG, the exact resistance mechanism to DAP is not clear [47, 66, 86]. Two major hypotheses have been suggested. One is that resistance to CAMPs and CAMP-likes is due to increased charge repulsion through positive charged LysPG. Other hypotheses consider that by synthesising more LysPG, there is less PG left for antibiotic agents targeting PG, such as DAP, to possibly interact with [83].

Furthermore, having been involved in previous work from Sebastian Kuhn [50], other proteins of the synthesis apparatus of membrane lipids have been screened for steric closeness to MprF, implying possible interaction with MprF. The DltABCD system and MprF, respectively the overexpression of their genes,

are both common pathways of resistance [18, 61]. So another aim is to analyse the effects of those two resistance factors alone and in combination.

Finally, as MprF's structure and topology have been predicted by different software tools and, over the time, due to additional examinations and data these predictions had to be revised and adapted [12, 30, 32, 38, 40, 93], this work aims to investigate on this topic, namely, to show that there is a loop located both cyto- and periplasmic, indicating a flipping mechanism.

2 Material and Methods

2.1 Material

2.1.1 Primers and Sequencing

PCR primers were synthesized by MWG-Biotech / Eurofins Genomics. The primer constructions were first checked for self-pairing or hybridization or hairpin formation via the free software “oligocalc”.

DNA samples for sequencing were sent to GATC.

2.1.2 Strains

Table 1: Strains

Species	Strain	Genotype / Characteristics	Ref.
Staphylococcus aureus	Newman	Wild type (WT)	
Staphylococcus aureus	Newman *ΔmprF*	Deletion of *mprF*.	
Staphylococcus aureus	SA113	Wild type (WT)	
Staphylococcus aureus	SA113 *ΔmprF*	Deletion of *mprF* leading to no synthesis of LysPG. Erm^R.	[66]
Staphylococcus aureus	RN4220		
Escherichia coli	BTH101		
Escherichia coli	XL-10 gold	Used as ultra-competent cells for transformation.	

2.1.3 Vectors

Table 2: pRBs

Plasmid	Size [kb]	Characteristics	Host	Resistance marker	Ref.
pRB474	5.9	Empty plasmid.	*S. aureus*, *E. coli*.	Cm^R in *S. aureus* (10 µg/ml), Amp^R in *E. coli* (100 µg/ml).	[30]
pRB474*mprF*	8.4	*mprF* of *S. aureus.*	*S. aureus*, *E. coli*.	Cm^R in *S. aureus* (10 µg/ml), Amp^R in *E. coli* (100 µg/ml).	[30]
pRB474*mprF345*	8.4	T345A, increasing tolerance to CAMPs and CAMP-likes.	*S. aureus*, *E. coli*.	Cm^R in *S. aureus* (10 µg/ml), Amp^R in *E. coli* (100 µg/ml).	
pRB474*mprF(syn)*	7.6	Synthase domain of *S. aureus* MprF, reaching from aa 274 – 840.	*S. aureus*, *E. coli*.	Cm^R in *S. aureus* (10 µg/ml), Amp^R in *E. coli* (100 µg/ml).	[30]
pRB474*cpmprF1*	7.6	*cpmprF1* of *C. perfringens*. AlaPGS.	*S. aureus*, *E. coli*.	Cm^R in *S. aureus* (10 µg/ml), Amp^R in *E. coli* (100 µg/ml).	[84]
pTX16	6.7	Empty plasmid.	*S. aureus*, *E. coli*.	Tet^R in *S. aureus* (nn µg/ml).	
pTX*dlt*	11.4	*dlt*	*S. aureus*, *E. coli*.		
pTX1-393	7.8	Plasmid with aa 1 to 393 of *mprF*	*S. aureus*, *E. coli*.		
pTX1-393(E206A)	7.8		*S. aureus*, *E. coli*.		
pTX*Ybhn*	7.7		*S. aureus*, *E. coli*.		
pTX*cpmprF2*	8.4		*S. aureus*, *E. coli*.		
pT10	5.6	Empty plasmid.	*E. coli*.	Kan^R in *E. coli* (nn µg/ml).	
pT10*mprF*	7.8	*mprF* of *S. aureus*	*E. coli*.		
pT10*mprF(267)*	7.8	*mprF* of *S. aureus* with a cysteine P267C	*E. coli*.		
pT10*mprF(267,731)*	7.8	P267C, D731A	*E. coli*.		

2.2 Culture media, growth conditions and strain maintenance

2.2.1 Culture media

Culture media were prepared with deionized water and autoclaved (20 min, 2 bar, 120 °C). To prepare agar plates, agar (EU-Agar) was added to the

medium (15g / 1l) before autoclaving. For soft agar, a lesser amount of agar (4g / 1l) was used.
Where required, antibiotics were added after autoclaving and after cooling down the medium to approx. 60°C.

Basic medium (BM)

- Caseinhydrolysate peptone 10 g
- Yeast extract 5 g
- NaCl 5 g
- Glucose 1 g
- $K_2HPO_4 \cdot 3\ H_2O$ 1 g
- H_2O ad 1 l
- pH 7.2

Luria Bertrani medium (LB)

- Caseinhydrolysate peptone 10 g
- Yeast extract 5 g
- NaCl 5 g
- H_2O ad 1 l
- pH 7.2

Mueller Hinton Broth (MHB)

- Cattle bouillon 2 g
- Caseinhydrolysate peptone 17.5 g
- Starch 1.5 g
- H_2O ad 1 l
- pH 7.4

Tryptic Soy Broth (TSB) and Casein-Soja-Pepton-Bouillon (CASO)

- Casein peptone (enzymatic digest of casein) 17 g
- Dipotassium hydrogen phosphate 2.5 g
- Glucose (CASO contains no glucose) 2.5 g

- Sodium chloride 5 g
- Soya peptone (enzymatic digest of soybean) 3 g
- H_2O ad 1 l
- pH 7.2

2.2.2 Growth conditions

To grow cultures, appropriate media were chosen considering their requirements (i.e. strain, genotype, antibiotic resistance). For liquid cultures, depending on the volume needed, test tubes with 10 ml medium or Erlenmeyer flasks with e.g. 25 ml or 100 ml medium, were inoculated overnight (37°C, 160rpm). These overnight starter cultures were generally used to inoculate main cultures to an initial OD_{600} 0.1.

Bacteria streaked on suitable agar plates were inoculated overnight in the incubator at 37°C.

2.2.3 Strain maintenance

Over short times (4-6 weeks), bacterial strains were kept on appropriate agar plates at 4°C. For long term storage, 1ml overnight culture was mixed with 1ml freez medium (65% glycerine; 0.1M $MgSO_4$; 25mM Tris/HCl; pH 8) in a CryoTube and frozen at -80°C.

2.3 Molecular genetic methods

2.3.1 Isolation of plasmid DNA (Miniprep)

For quick isolation of small amounts of plasmid DNA, the peqGOLD Plasmid Miniprep Kit I was used according to the manufacturer's instructions. By doing so, the bacterial components get separated by silica columns: plasmid DNA is bound; unwanted proteins get eluted by different buffers. In the end, plasmid DNA is eluted with dd H_2O or elution buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0).

Isolation of DNA from *S. aureus* include an extra lysis with lysostaphin which was added with Buffer P1 and incubated approx. 30min at 37°C to lyse more effectively.

2.3.2 Isolation of plasmid DNA (Midiprep)

The Quiagen QIA Filter Plasmid Midiprep Kit was used to obtain higher amounts of plasmid DNA. The procedure is also based on alkaline lysis.

2.3.3 Polymerase Chain Reaction (PCR)

To synthesise specific short DNA sequences for the construction of plasmids, desired DNA fragments were amplified via Polymerase Chain Reaction (PCR). Therefore, a standardized approach and program was used varying the annealing temperature according to the congruence of the primers with the template, and the extension time according to length of the intended amplicon. For denaturation, the temperature required for the Phusion Hot Start Polymerase was used.

Standard approach (total 50 µl)

- H_2O (bidest.) 37.5 µl
- 5 x Phusion HF reaction buffer 10 µl
- dNTPs (10 mM of each dNTP) 1 µl
- Primer 1 (10 pmol/µl) 2.5 µl
- Primer 2 (10 pmol/µl) 2.5 µl
- DNA template (genomic DNA 4 ng/µl) 1 µl
- Phusion Hot Start Polymerase (2 U/µl) 0.5 µl

Table 3: PCR programme

	Denaturation	Denaturation	Annealing	Extension	Final extension	End
Cycles	1	18			1	-
Temp.	95°C	95°C	60°C	68°C	68°C	4°C
Duration	60 s	50 s	50 s	60 s/kb plasmid length	7 min	∞

Generally, an annealing temperature of Tm ± 5°C was chosen. If the primers had a high tendency to hybridize and the PCR resulted in many unspecific PCR products, a higher annealing temperature was chosen. Lower annealing temperatures increased the output.

In this study, mostly lower temperatures were set.

2.3.4 Site Directed Mutagenesis (SDM)

By Site Directed Mutagenesis (SDM) point mutations can be inserted at defined locations in DNA molecules. This way, specific exchanges of base pairs resulted in an exchange of specific amino acids. Two complementary primers carrying the particular base pair to be exchanged were used to replicate whole double stranded (ds) plasmids via PCR.

Standard approach

- 10 x reaction buffer 5 µl
- dNTPs (10 mM of each dNTP) 1.5 µl
- Primer 1 (125 ng) x µl
- Primer 2 (125 ng) x µl
- DNA template (plasmid DNA 5-10 ng) y µl
- PfuUltra HF DNA polymerase (2.5 U/µl) 1 µl
- H_2O (bidest.) ad 50 µl total volume

Table 4: PCR programme for SDM

	Denaturation	Denaturation	Annealing	Extension	Final extension	End
Cycles	1	18			1	-
Temp.	95°C	95°C	60°C	68°C	68°C	4°C
Duration	60 s	50 s	50 s	60 s/kb plasmid length	7 min	∞

Following the PCR standard approach, 1.5 µl of DpnI was added to the product and digested for 4-6 h at 37°C to remove template plasmids since they are methylated in contrast to the synthetic ones. The amplified plasmids were then transformed into chemically competent *E. coli* cells.

2.3.5 Restriction digestion of DNA

Each digest approach was adapted from the manufacturer's advices as different restriction enzymes require different optimal conditions for full catalytic activity.

Standard approach (10 µl)

- DNA sample x µl
- 10 x restriction buffer 1 µl
- Restriction enzyme 0.5 – 1 µl
- H_2O (bidest.) ad 10 µl

Digestions with ordinary restriction enzymes were incubated at 37 °C for approximately 6 h as 1 U enzyme is predicted to digest 1 µg DNA within an hour. To digest 1 µg DNA by “Fast digest” enzymes (Fermentas), DNA samples were incubated with 1 U recombinant enzymes at 37 °C for 10-15 min.
For quality control of plasmids, “Fast digest” enzymes were used, and the product sizes were controlled via preparative agarose gel.

2.3.6 Separation of DNA fragments via agarose gel electrophoresis

Buffers and enzymes

- 50 x TAE buffer:
  2 M Tris/HCl; 0.1 M EDTA; 1 M acetic acid; pH 8.2 adjusted with glacial acetic acid; before use TAE buffer was diluted to 1-fold concentration (1 x) with H_2O (bidest.)
- 10 x Fast digest loading buffer
- Agarose gel:
  0.5-1 % (w/v, according to requirements) agarose in 1 x TAE buffer
- Size marker (1 kb ladder)
- SYBR® Safe DNA gel stain solution (10 000-fold diluted)
- Methylene blue (0.1 % in H_2O)

Agarose gels were prepared shortly before use (e.g. 1 g agarose in 100 ml 1 x TAE buffer to get 1 % agarose gel). Gels with high percentage of agarose (up to 3 %) were used to separate smaller DNA fragments (200-1 000 bp). To separate larger fragments (6-10 kb) low percentage gels (< 1 %) were used.
For the visualisation of DNA bands, SYBR® Safe was added 1 : 1 000 after cooling down below 60 °C (10 µl SYBR® Safe in 100 ml agarose gel) before pouring. Having applied a sample (10 : 1 mixed with 10 x loading buffer), the

electrophoresis was run with 140 V for 40 min and analysed with the gel documentation system BioDocAnalyse under photonic excitation at 312 nm (UV).

Electrophoresis of preparative gels ran with 140 V for 1-2 h. Then they first got stained with methylene blue for 10-20 min and afterwards destained with H_2O (both shaking) until distinct bands were visible. On a light table, the desired band could be cut out.

2.3.7 Extraction of DNA fragments from agarose gels

For isolation of DNA fragments from agarose gels the peqGOLD Gel Extraction Kit was used according to the manufacturer's instructions. The gel carrying the wanted DNA fragment was solubilised, and the DNA was isolated with a silica column.

2.3.8 Purification of DNA after PCR or enzymatic modification

With the aid of the peqGOLD Cycle-Pure Kit DNA was quickly purified from proteins and enzymes, according to the manufacturer's advices.

2.4 Transformation

2.4.1 Heat shock transformation of of *E. coli* cells

Preparation of chemically competent *E. coli* DH5α cells (for heat shock)

Buffers and enzymes

TFB1 buffer (pH 5.8):

- RbCl 100 mM
- $MnCl_2$ 50 mM
- $KC_2O_2H_3$ 30 mM
- $CaCl_2$ 10 mM
- Glycerol 15 %

TFB2 buffer (pH 8.0):

- MOPS 10 mM
- RbCl 10 mM

- $CaCl_2$ 75 mM
- Glycerol 15 %

Procedure

1. Inoculate 100 ml LB with an O/N culture to an initial OD_{600} = 0.1, shake at 37 °C for approx. 2 h with 160 rpm.
2. Transfer cells at OD_{600} = 0.5 in two sterile Falcon tubes (50 ml), incubate them briefly (5 min) on ice and pelletize them 10 min with 4600 rpm at 4 °C
3. Discard supernatant and keep the cells on ice
4. Resuspend cells with cold (4 °C) TFB1 buffer (30 ml) and incubate for 90 min on ice
5. Pelletize cells (4600 rpm, 5 min, 4 °C) and discard supernatant
6. Resuspend cells with cold (4 °C) TFB2 buffer (4 ml)
7. Aliquot 100-200 µl of culture in sterile ERTs and store at -80 °C

Heat shock transformation of competent *E. coli* DH5α

Procedure

1. Thaw 50 µl of competent *E. coli* DH5α cells on ice
2. Place appropriate amounts of the ligation or SDM reaction mixture in ERT
3. Add cells to DNA (up to 50 µl), mix gently and incubate for 15 min on ice
4. Expose cells for 70-90 s to a heat shock at 42 °C in the Thermocycler
5. Add 950 µl BM immediately and shake cells for 20-40 min at 37 °C for phenotypical expression of antibiotic resistance genes
6. Plate culture on LB agar plates prepared with the appropriate selective antibiotic

2.4.2 Electroporation of *S. aureus* cells

Preparation of electrocompetent *S. aureus*

Buffers and enzymes

- Glycerine solution 10 %

Procedure

1. Inoculate 100 ml LB with an O/N culture to an initial OD_{600} = 0.1, grow at 37 °C for approx. 2 h in the shaker with 160 rpm.
2. Transfer cells at OD_{600} = 0.5 into two sterile Falcon tubes (50 ml), incubate them briefly (5 min) on ice and pelletize them 10 min with 4600 rpm at 4 °C
3. From now on, work always on ice. Wash cells 3 times with 30 ml sterile, ice cold glycerine solution, pelletizing the cells after each washing step for 12 min with 4 600 rpm at 4 °C and discarding the supernatant
4. Resuspend cells with 200 µl glycerine solution (v/v) per Falcon tube
5. Aliquot 50 µl of cell suspension in ERTs and store at -80 °C

Electroporation of competent *S. aureus*

Procedure

1. Thaw 50 µl of electrocompetent *S. aureus* cells at 4 °C
2. Place appropriate amounts (2-10 µg) of plasmid in ERT (do not exceed a volume of 50 µl H_2O with plasmids)
3. Add cells to DNA, mix gently and incubate for 30 min at RT (25 °C)
4. Transfer DNA cell mixture in a electroporation cuvette (0.2 cm cuvette)
5. Adjust Eppendorf Multiporation apparatus and electroporate with the following conditions:
   Pulse: U = 2.0 kV; C = 25 µF; R = 100 Ω
   Optimal time constant: 2.5 ms
6. Add 950 µl BM immediately and shake cells for 1.5-2 h at 37 °C for phenotypical expression of antibiotic resistance genes
7. Plate approach on BM or TSB agar plates with the appropriate antibiotic and incubate overnight at 37 °C

2.5 Transduction

2.5.1 Propagation of phage

Solid propagation of phage Φ11

Buffers and enzymes

- BM soft agar

Procedure

1. Dilute a fresh O/N culture of the propagation strain (RN4220 for Φ11) to OD_{600} = 0.1 and transfer 90 µl into a 15 ml Falcon tube
2. Add 1 ml of the phage lysate to be propagated
3. Incubate for 5 to 10 min at RT (25 °C)
4. Add 5 ml BM soft agar pre-warmed to 47 to 49 °C
5. Several times (3 - 5 x), vortex thoroughly and invert
6. Plate mixture on TSA plate avoiding bubbles, incubate O/N at 37 °C; the next day, there should not be seen any CFUs, but if so, less propagation strain is necessary
7. Carefully, scratch off the soft layer into a 50 ml Falcon tube, wash the plate with 1 ml TSB and add it carefully into the tube
8. Centrifuge for 10 min with 4 600 rpm at RT, transfer the supernatant into a 1.5 ml ERT
9. Centrifuge for 10 min with 13 000 rpm, transfer supernatant in fresh ERT
10. Store propagated phage lysate at 4 °C

Liquid propagation of Φ11

Buffers and enzymes

- $CaCl_2$ stock solution

Procedure

1. Dilute a fresh O/N culture of the propagation strain (RN4220 for Φ11) with BM to OD_{600} = 0.1 and transfer 100 µl into a 15 ml Falcon tube
2. Add 2 ml of the phage lysate to be propagated (V_{phage})
3. Add 3 ml BM (V_{BM})
   (when dealing with higher volumes, V_{BM} should not exceed V_{phage})

4. Add sterile $CaCl_2$ solution to a final concentration of 4 mM
5. Incubate with shaking for 3 h at 37 °C, until mixture is clear
6. Centrifuge in ERTs for 10 min with 13 000 rpm
7. Sterile filter the supernatant to a fresh Falcon tube
8. Store at 4 °C

Spot assay for quantifying phage concentration

Buffers and enzymes

- BM soft agar

Procedure

1. Dilute a fresh O/N culture of the propagation strain (RN4220 for Φ11) with BM to OD_{600} = 0.1 and transfer 100 µl into 5 ml BM soft agar; the soft agar should be previously melted and used at approx. 50 °C
2. Pour the bacteria-containing soft agar onto a BM agar plate, leave it for 5 min
3. Prepare a serial dilution of the phage lysate in BM up to 10^{-8}
4. Spot 10 µl of each dilution onto the plate in a distinct pattern
5. Incubate the plate O/N at 37 °C facing up
6. Count spots (n), multiply by 10^2 as for getting from 10 µl to the concentration per ml, multiply by $10^{|x|}$, with x being the exponent of the step of dilution the spots are counted in:

$$c_{phage\ lysate} = n \cdot 10^2 \cdot 10^{|x|} \frac{1}{ml};$$

a good titre should be between 10^9 and 10^{11}

2.5.2 Phage transduction

Buffers and enzymes

- $CaCl_2$ stock solution 400 mM
- Phage buffer
  - $CaCl_2$ 4 mM
  - $MgSO_4$ 1 mM
  - Tris-HCl pH 7.8 50 mM
  - NaCl 100 mM

- Gelatine 0.1%

→ Autoclave

Procedure

1. To load the phage with plasmid of a donor strain, dilute a fresh O/N culture of the donor strain to OD_{600} ≈ 0.5 in phage lysate to a final volume of 1 ml, add $CaCl_2$ to a final concentration of 4 mM (i. e. 10 µl stock solution)
2. Incubate for 30 min at 37 °C without shaking, then incubate for 3 h at 30 °C with low agitation (Thermomix 300 rpm)
3. Centrifuge for 10 min at 5 000 g, sterile filter (0.2 µm) the supernatant; if necessary, store at 4 °C
4. Dilute a fresh O/N culture of the recipient strain to OD_{600} ≈ 0.5 in BM
5. Pelletize 200 µl, resuspend the pellet in 200 µl Phage buffer, add 100 µl of the loaded phage lysate
6. Incubate for 15 min at 37 °C with slight agitation (Thermomix 300 rpm)
7. Plate onto TSA agar plates prepared with the selective antibiotic of the plasmid (Clarithromycin for pRB, and Spectromycin according to the resistance gene within the gene cassette of *S. aureus* SA113*ΔdltA*)
8. Incubate O/N at 37 °C

2.6 PC based sequence analysing methods

2.6.1 Sequence analysis

Sequences of nucleic acids or proteins were analysed with SeqBuilder (Lasergene 7 of DNASTAR). Thereby, sequence alignments, primer constructions and control of GATC sequences could be performed. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to analyse complete genome sequences.

2.6.2 Data analysis, creation of diagrams and pictures

Data was analysed via GraphPad Prism 7, where mentioned, unpaired t tests were carried out, figures were edited with Adobe Photoshop CC 2014.

2.7 Analytical methods

2.7.1 Determination of susceptibility to antimicrobial peptides

In order to determine how susceptible the different strains are to antimicrobial peptides, the MICs of some specific antimicrobial agents were identified via the Epsilometer test (Etest). The MIC describes the lowest concentration of an antibiotic to prevent visible growth of a microorganism after being incubated overnight. Strains, at a defined OD_{600} plated on an agar plate to gain a bacterial lawn, were exposed to different specific concentrations of antimicrobials, incubated overnight (20-22 h) and their growth examined. All working steps occurred under sterile conditions.

Determination of MICs via Etest

Buffers and enzymes

- MHB
- MHB agar plates with appropriate induction source (e.g. 0.5 % xylose)
- Etest stripes

Procedure

1. Adjust 1 500 µl of a fresh overnight culture to OD_{600} = 0.05 in a 2 ml ERT: Measure the OD_{600} of the O/N culture and dilute appropriately with medium (MHB without antibiotics)
2. Dip a sterile cotton swab into the ERT for approx. 20 s, carefully twirling until enough culture is absorbed
3. Smear the culture onto MHB agar plates: first do three diameter like strokes in different directions (usually ca. 0°, 45°, 90°), then spread them smearing orthogonally to each of them until the plate is completely covered with bacteria and not wet anymore
4. Carefully place an Etest stripe onto the plate, avoid pressure or shifting
5. Turn the plate upside down and incubate for 20-22 h at 37 °C

2.7.2 Determination of daptomycin binding via UPLC

To determine how much Daptomycin binds to the bacterial surface of different strains, different amounts of bacteria were required. Bacteria loads were quite

high, as on the one hand very little Daptomycin binds to the surface and on the other hand a possible difference needs to be large enough to be clearly detectable by UPLC. Those high density cultures were incubated with a specific amount of Daptomycin (i.e. specific volume with specific concentration). Analysing the supernatant for free Daptomycin ("left-over") allows the calculation of the amount of Daptomycin bound.

Preparing for UPLC

Buffers and enzymes

- MHB BD + 50 µg Ca^{2+}/ml
- Daptomycin stock solution 200 µg/ml

Procedure

1. With an O/N culture (20 ml MHB), inoculate a main culture to OD_{600} = 0.1 in 250 ml MHB without supplements and incubate with shaking at 37 °C until reaching 0.7 < OD_{600} < 1.0
2. Pelletize the cells 15 min, 4 000 rpm, RT (25 °C)
3. Resuspend pellet in 1 ml MHB BD supplemented with 50 µg Ca^{2+}/ml
4. Measure OD_{600} (therefore, dilute 10 µl in 990 µl MHB, take 100 µl of that and dilute with 900 µl MHB)
5. Adjust 600 µl to OD_{600} = 200 in an ERT (2 ml)
6. Make a serial dilution with n dilutions (here n = 3 for OD_{600} = {200; 100; 50; 25}
    a. Provide n ERTs (2 ml) with 300 µl MHB BD + 50 µg Ca^{2+}/ml
    b. Transfer 300 µl of the suspension into the first ERT, mix thoroughly
    c. Transfer 300 µl of ERT no. (n-1) into no. (n), mix thoroughly, take out and discard 300 µl
    d. Transfer 200 µl each into a fresh ERT (1.5 ml)
7. Prepare standard: 2 ERTs (1.5 ml) with 200 µl MHB BD + 50 µg Ca^{2+}/ml
8. Incubate 15 min, 37 °C, 400 rpm in the Thermocycler

9. Add Daptomycin to a resulting concentration of 100 µg/ml
   Stock solution: c = 200 µg/ml => 200 µl added
   Finally resulting OD_{600} = {100; 50; 25; 12.5}
10. Incubate 10 min, 37 °C, 400 rpm
11. Pelletize the cells: 12 min, 14 000 rpm, RT (25 °C)
12. Sterile filter the supernatant (here used 0.2 µm)
13. UPLC

2.7.3 Labelling single cysteine in loops of transmembrane proteins

To determine the position of a specific domain of MprF, a single cysteine was inserted via SDM. *E. coli* or *S. aureus* cultures (in 50 ml LB or BM with supplements added if needed for induction of the plasmid, e. g. rhamnose for pT10) were grown to the exponential phase (OD_{600} = 0.5). The culture was divided in 2 aliquots, pelletized, and resuspended in Buffer A. For one part, the extracellular side was labelled with MPB (700 µl suspension + 7.5 µl 10 mM MPB adds up to a final concentration of approx. 0.1 mM MPB), the other half was blocked with AMS. After bead milling, the first half was left unprocessed, the other half labelled on the cytoplasmic side with MPB. The supernatants were pelletized via ultracentrifugation at 38 000 x g.
With these an immunoprecipitation was performed to extract the FLAG-tagged proteins containing the single cysteine with the aid of magnetic FLAG beads. These were separated by SDS PAGE and visualised via Western Blot by using three antibodies (Streptavidin 800nm + anti-FLAG; anti-mouse 700nm). The proteins were detected with LICOR (using two channels, 700 nm and 800 nm).

Buffers and enzymes

- N*-(3-Maleimidylpropionyl)Biocytin (MPB) 10 mM in DMSO
- 4-Acetamino-4‘-Maleimidylstilbene-2,2’-Disulfonic Acid (AMS) 20mM in DMSO
- Buffer A (best, if already high-conc. stock solutions there to mix)
    - HEPES 100 mM
    - Sucrose 250 mM

    - $MgCl_2$ 25 mM
    - KCl 0.1 mM

    → pH 7.5 with KOH (ca. 6 ml)

    → sterile filter, store at 4 °C
- IP buffer 1 / 2
    - Tris-HCl 50 mM
    - NaCl 150 mM / 1 mM
    - EDTA 1 mM
    - Thesit 2 %
    - SDS 0.4 %

    → pH 8.1 with HCl

    → sterile filter, store at 4 °C
- Solubilisation buffer
    - Tris-HCl 50 mM
    - EDTA 1 mM
    - SDS 2 %
- Rhamnose 100 mM
- $MgSO_4$ 1 M (or $MgCl_2$)
- EDTA 0.5 M, pH 7.5
- β-ME 2 M (β-Mercaptoethanol)
- Glycin HCl 0.1 M, pH 2.2
- Tris-HCl 1 M, pH 9
- PMSF 50 mM in isopropanol (Phenylmethylsulfonyl fluoride)

<u>Main culture and labelling</u>

1. With an O/N culture, inoculate
   50 ml LB + Rha 100 µM (for *E. coli*) or BM (for *S. aureus*) + AB;
   Incubate at 37 °C to $OD_{600} \approx 0.5$ (if too much degradation, 30°C)
2. After this step, work always on ice.
   Pelletize cells in two Falcon tubes (50 ml, containing each 25 ml culture)
   (Meanwhile the glass beads for step 6 can be prepared)

3. Resuspend in 700 µl Buffer A supplemented with $MgSO_4$ (to 1 mM, or $MgCl_2$), lysozyme, protease inhibitor, DNase, EDTA (to 1 mM), pH 7.5;
   Do this carefully, avoid bubbles (they would damage the membrane)
4. Incubate 30 – 60 min on ice
5. Labelling / Blocking extracellular
   a. Labelling extracellular:
      add 7.5 µl MPB (10 mM); incubate 20 min;
      quench reaction by adding 7.5 µl β-ME (2 M)
   b. Blocking extracellular:
      add 3.75 µl AMS (20 mM); incubate 20 min;
      do not quench reaction
6. Add samples to 0.5 ml pre-cooled glass beads in 1.5 ml screw cap tubes
7. Labelling cytoplasm
   a. Not labelling cytoplasm (having labelled extracellular):
      -
      bead mill for 30 s with 6 m/s
      -
   b. Labelling cytoplasm (steps should be done within 2 min):
      add 7.5 µl MPB (10 mM);
      bead mill for 30 s with 6 m/s;
      quench reaction after max. 2 min by adding 7.5 µl β-ME (2 M)
8. Centrifuge 1 min, 1 000 x g, 4 °C;
   transfer supernatants into fresh ERTs (1.5 ml),
   avoid taking out beads as much as possible
9. Centrifuge 2 min, 10 000 x g, 4 °C
   transfer equal volumes (e.g. 450 µl) supernatant into ultracentrifugation tubes (1.5 ml), (leave 1-2 mm in ERTs to be sure of not carrying beads);
   make sure to have exact the same amount in every ultracentrifugation tube;
   tare tubes on precision scale
10. Ultracentrifuge 30 min, 38 000 x g, 4 °C

11. Aspirate supernatant with (vacuum) pipet;
    pellet should be mostly yellow/translucent, few black dots are okay;
    if pellet is big and mostly white, steps 9-10 should be performed more carefully next time
12. Freeze pellet at -80 °C (-20 °C is sufficient only if proceeding the next day)

Immunoprecipitation (IP)

1. Thaw samples on ice (1 h; 30 min is enough if from -20 °C)
2. Add 100 µl buffer A pH 7.5 supplemented with β-ME (to 0.02 M); vortex 1 h, 1 400 rpm, 4 °C (meanwhile you can prepare magnetic FLAG beads as in step 7)
3. Add 100 µl solubilisation buffer, vortex 30 min, 1 400 rpm, 4 °C
4. Keep at 37 °C for 30 min
5. Vortex 30 min, 1 400 rpm, 4 °C
6. Add 300 µl IP buffer 1; centrifuge 10 min, max speed, 4 °C
7. Prepare magnetic FLAG beads (keep beads and rack on ice):
    a. Take a total of 5 µl FLAG bead slurry per sample;
       pipette slowly, leave tip in slurry and stir
    b. Pipette into ERT (1.5 ml) inserted to magnetic rack (or hold a magnet to the ERT), angle tip, so beads are pulled out, take off liquid
    c. Wash 2 x with twice as much volume of IP buffer as slurry
    d. Take off buffer, add half as much volume of IP buffer 1 as slurry
    e. Put 5 µl per sample into ERTs (1.5 ml) on magnetic rack, angle tip, so beads are pulled out; take tubes out of magnetic rack (or put magnet away)
8. Add supernatant from step 6 onto prepared magnetic beads in ERTs (1.5 ml);
    incubate 1-2 h rotating at 4 °C
9. Wash 3 x with 100 µl IP buffer (last time with IP 2);
    make sure to take tubes out of the rack and rinse the beads off the wall;

leave beads 5 min in IP buffer before removing it again;
get as much buffer as possible off after last washing step; take tubes out of magnetic rack

10. Elution: add 50 µl glycine HCl (0.1 M, pH 2.2 or 3.5);
    incubate 15 min at RT (25 °C, possibly Thermocycler with minimum shaking);
    if degradation, lower temperature (to 10 or 4 °C)
11. Prepare ERTs (1.5 ml) with 3 µl Tris-HCl (1 M, pH 9)
    (later, if the stains on the preparative gel are blue, this step was done alright;
    later, if the stains are yellow, probably the mixture got too acidic in this step)
    Transfer 40 µl eluate to prepared tubes; add 10 µl SDS buffer;
12. if necessary, store at 4 °C

<u>SDS PAGE</u>

Buffers and enzymes

- 5 x SDS sample buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 30% (v/v) Glycerol, 10 mM DTT, 0.05% (w/v) Bromophenol Blue)

Procedure

1. Pipette 20 µl of the sample and 5 µl 5 x SDS sample buffer into an ERT (1.5 ml)
   and incubate 5 min at 37 °C
2. Apply 10 µl onto a 10 % SDS gel
3. Start electrophoresis for 15 min with 20 mA,
   then increase to 40 mA for 30-60 min

<u>Western Blot</u>

Buffers and enzymes

- 10 x Towbin buffer
  - Trizmabase 250 mM
  - Glycin 1 920 mM

→ pH should be 8.3 (do not adjust)

- SDS 3.7 g/l (wear mask, stir slowly to avoid bubbles)

- 1 x Towbin buffer
  - 10 x Towbin 1:10
  - H_2O 7:10 (before Methanol to avoid precipitating)
  - Methanol 2:10
- 10 x TBS
  - Trizmabase 500 mM
  - NaCl 1 500 mM

  → pH 7.5 with HCl
- 1 x TBS
- 1 x TBST
  - Tween 0.05 %
- 1 x TBST + 3 % BSA (0.3 g for 10 ml)

Antibodies

1. Streptavidin 800 1:10 1000 + anti-FLAG 1:10 1000 (each 1 µl in 10 ml TBST)
2. anti-mouse 700 1:10 000 (1 µl in 10 ml TBST)

Procedure

1. Activate membrane (Immobilion FLPVDF), for each side 10 s by immersion in methanol; then put in an extra box with Towbin buffer
2. Layer (from white bottom to black top):
   1. Sponge soaked in 1 x Towbin buffer
   2. Whatman paper 5 mm soaked in 1 x Towbin buffer
   3. Activated membrane, add some 1 x Towbin buffer
   4. SDS gel (incubated in 1 x Towbin buffer)
   5. Whatman paper soaked in 1 x Towbin buffer, roll out bubbles with test tube
   6. Sponge soaked in 1 x Towbin buffer, add some 1 x Towbin buffer on top

3. Blotting: 1 h, 15 V
4. Take off gel (should be empty)
5. Block: Incubate membrane in lightproof or dark box with 1 x TBST + 3 % BSA (do not use milk) for 1 h
6. Wash 2-3 x 5 min with 1 x TBST
7. Incubate 1 h with 1st antibody:
   Streptavidin 800 1:10 000 + anti-FLAG 1:10 000 (each 1 µ in 10 ml TBST)
8. Wash 3 x 5 min with 1 x TBST
9. Incubate with 2nd antibody: anti-mouse 700 1:10 000 (1 µl in 10 ml TBST)
10. Wash 1-2 x 5 min with 1 x TBS (not TBST)

- Visualise Membrane with the Odyssey Imaging System of LI-COR (Lambda Instruments Corporation): 2 channels, 700 and 800 nm

3 Results

3.1 Comparison of daptomycin binding to *S. aureus*

3.1.1 Daptomycin susceptibility of *S. aureus* knockout mutants

The main objective of this experiment was to compare the effects of the point mutation T345A to the WT in regards to their antibiotic susceptibility.

For this, the MICs of two antibiotics, daptomycin and bacitracin, were measured (see 2.7.1). The following five stems were used: (a) the WT, (b) the *mprF*-knockout-mutant, (c) knockout mutants with the plasmid pRB being empty, (d) pRB containing *mprF* as complementation, and (e) pRB containing *mprF* with the DAP-R point mutation T345A leading to DAP resistance.

The *mprF* knockout mutant bearing the empty vector plasmid pRB showed the same susceptibility to both antibiotics as the *mprF* knockout-mutant without plasmid. Complemented with pRB*mprF*, the MICs of both antibiotics were on the level of the WT. Mutants bearing the missense mutation T345A, however, tolerated much higher concentrations of DAP, while susceptibility to bacitracin was not altered. This shows the point mutation T345A mediates resistance specifically against DAP. Furthermore, it implies that the mechanism of additional resistance through the point mutation T345A is different from the general resistance.

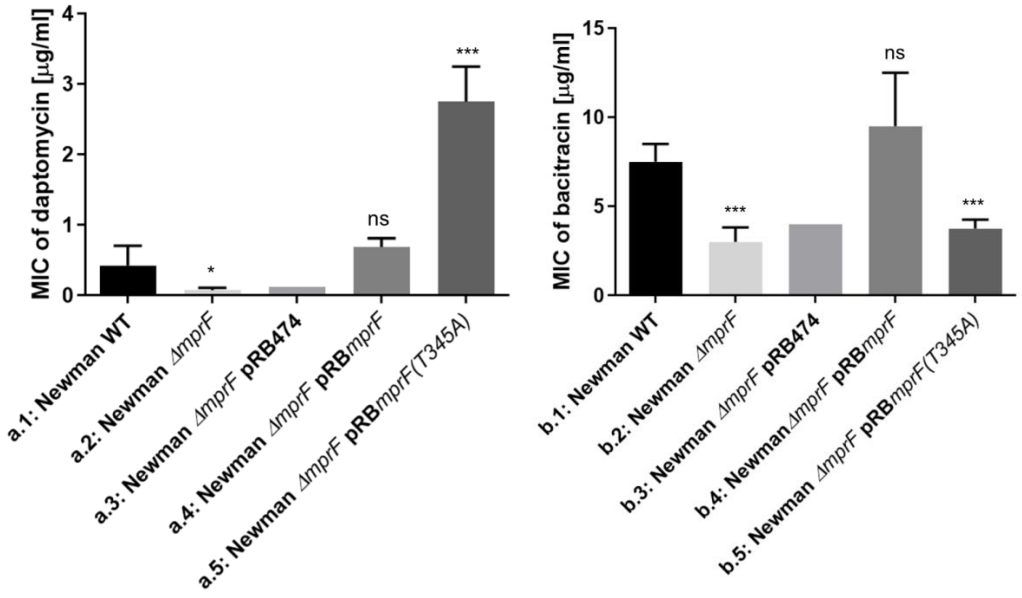

Fig. 7: MICs of DAP and bacitracin (a, b) for five strains of *S. aureus* Newman: WT (1), *mprF*-knockout-mutant (2), the knockout mutant with an empty plasmid (3), the knockout mutant complemented (4), and the knockout mutant complemented with the DAP-R mutation T345A (5). Biological replicates n = 4. P values of unpaird t tests are: a.1 vs. a.2 0,0473; a.1 vs. a.4 0,1396; a.1 vs. a.5 0,0002; b.1 vs. b.2 0,0004; b.1 vs. b.4 0,2528; b.1 vs. b.5 0,0005.

3.1.2 Quantitative determination of daptomycin binding to *S. aureus* via HPLC

In order to analyse binding of DAP to *S.* aureus in DAP-S versus DAP-R strains, the quantity of DAP in the supernatant of strains with different genotypes (knockout, complementation, resistance mutation T345A) was analysed via UPLC after a defined incubation of DAP with respective strains in medium endued with Calcium (see 2.7.2). The different concentrations of the bacterial suspensions were adjusted based on their CFUs, specifically their OD_{600}. Then they were incubated with a specific amount of DAP and Calcium for a defined time. Finally, free DAP released to the supernatant was quantified. The difference of DAP before and after incubation was assumed to be the portion of DAP bound to the cell surface.

The results showed that the less bacteria in suspension are exposed to DAP, the higher is the concentration of free DAP, suggesting that the binding is proportional to the amount of bacteria. However, no difference could be seen when comparing the complementation with native *mprF* and the mutant

mprF345. In fact and contrary to expectations, the *mprF* knockout mutant and its complementation did not seem to differ in DAP binding neither.

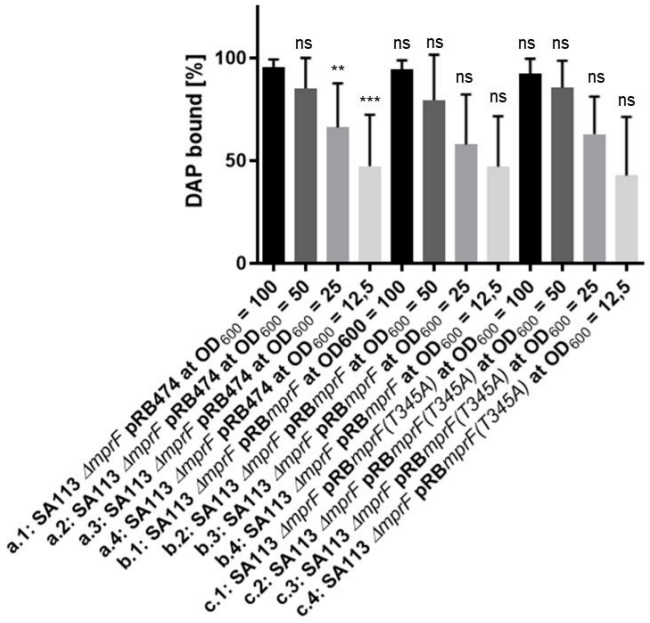

Fig. 8: Percentage of cell-bound DAP to different strains determined via UPLC. Three strains of *S. aureus mprF*-knockout mutants with (a) the blank pRB, (b) the complementation pRB*mprF*, and (c) the resistance mutation pRB*mprF345* each in condensed suspensions with final ODs_{600} of 100, 50, 25, and 12.5 (a.1 – a.4, b.1 –b .4, c.1 –c.4) were incubated with DAP and Ca^{2+}. Via UPLC, the supernatant was analysed for DAP. The differences of the concentrations of DAP before and after incubation was interpreted as DAP bound to the bacteria. Biological replicates n = 8. P values of unpaired t tests are: a.1 vs. a.2 0,6040; a.1 vs. a.3 0,0066; a.1. vs. a.4 0,0006; a.1 vs. b.1 0,6040; a.2 vs. b.2 0,5547; a.3 vs. b.3 0,4798; a.4 vs. b.4 0,9771; a.1 vs. c.1 0,3486; a.2 vs. c.2 0,0905; a.3 vs. c.3 0,7557; a.4 vs. c.4 0,7342.

Therefore, it can be confirmed that daptomycin binds to the cell surface. However, there seems to be no difference in DAP binding, whether MprF is present or not. Consequently, it seems unlikey that the resistance mechanism of MprF is based on charge repulsion. Likewise, the DAP-R mutation SNP T345A in *mprF* does not lead to an increased repulsion of DAP into the supernatant, which suggests that the increase in DAP-R via the SNP T345A is due to another effect.

3.2 Analysis of an assumed interaction between DltABCD and MprF

3.2.1 Screening for interactions between MprF and other proteins of the synthesis apparatus for membrane lipids

A screening using the bacterial adenylate cyclase two-hybrid (BACTH) method was performed to show steric closeness and to find interaction partners for MprF among proteins of the LTA synthesis machinery. Transformed *E. coli* BTH101 with constructs expressing MprF fused to the T18 fragment of the adenylate cyclase, and proteins from the LTA synthesis machinery (DltA, DltB, DltC, DltD – D-alanylation of polyanionic cell walls and lipoteichoic acids; LysS – lysine-tRNA-ligase; Cls1, Cls2 – synthesis of cardiolipids; YpfP – synthesis of diglucosyldiacylglycerol; LtaA – translocase of diglucosyldiacylglycerol; LtaS – synthesis of lipoteichoic acids) fused to the T25 fragment allowed to study interaction between two proteins. Where the two adenylate cyclase fragments fused to a functional complementation, colonies would appear blue indicating interaction between the two proteins (Fig. 9) [50].

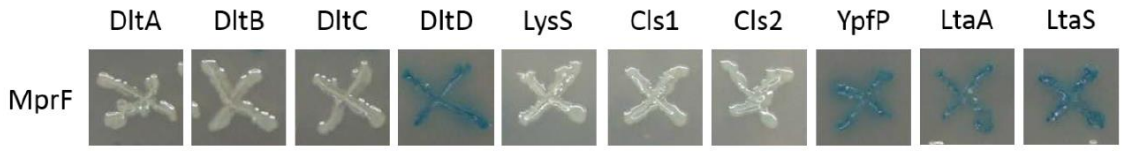

Fig. 9: BACTH analysis of interactions between MprF and other proteins. Interaction is indicated by blue strains, no interaction appears white. After Kuhn et al. [50].

Interaction has been detected with DltD, YpfP, LtaA and LtaS [50].

To investigate the previously obtained interactions more specifically, the flippase domain (aa 1-320) and the synthase domain (aa 328-840) respectively were fused to the T18 fragment. Another screening with the BACTH method was carried out. For both domains, this screening resulted in blue colonies for the glycolipid anchor synthase YpfP, the assumed LTA anchor flippase LtaA, the LTA synthase LtaS, and the D-alanine transferase DltD. This implies protein interaction of MprF with the LTA synthesis apparatus and with the LTA and WTA alanylation system (Fig. 10).

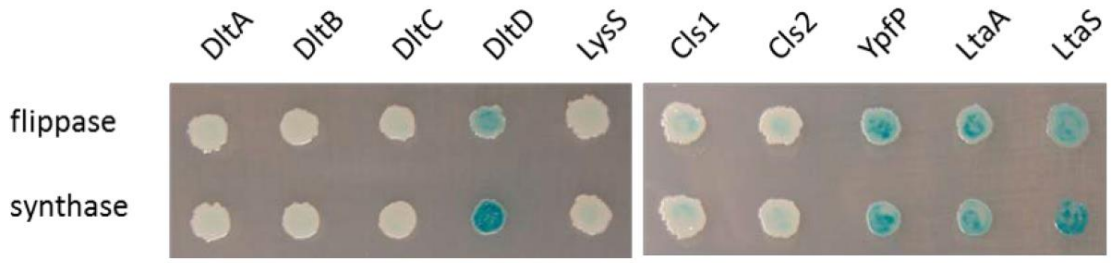

Fig. 10: BACTH analysis of interactions between the two domains of MprF and other proteins. Interaction is indicated blue, no interaction appears white. From Kuhn et al. [50].

3.2.2 Analysis of a connection between the DltABCD system and MprF in regards to daptomycin resistance

MprF and *dlt* are both known to be hot spots for SNPs leading to DAP-R. To further elucidate the effects of the two resistance factors, DltABCD and MprF, undermentioned strains have been constructed. It should be determined how each protein by itself affects DAP-R. Furthermore, it should be explored how MprF and the DltABCD system in synergy affect DAP-R or hypersusceptibility to DAP, and whether the *mprF* point mutation T345A alters their interaction in some way.

In a first step, in addition to the previously described constructed strains – with *mprf* knocked out and with pRB bearing *mprF* as WT or with the resistance SNP – *dltA*-knockout-mutations as well were supplied with those pRB variants via electroporation. Thus, the double knockout mutant of *mprF* and the *dlt* system has been compared to both the complementations with *mprF* and the DAP-R mutation T345A of *mprF*. Per agarose gel electrophoresis it was checked, whether the genes have been constructed successfully. Then the MICs of DAP and polymyxin B (PB) were determined via Etests. As expected, without *mprF* the non-susceptibility to DAP was decreased, and with the SNP T345A significantly increased compared to the WT. Without *dltA*, resistance to DAP was decreased and the resistance mutation in *mprF* showed an equally low tolerance level. Interestingly, without *dltA* the presence or absence of *mprF* seemed to have no relevance for tolerance of PB, and was consistently on a low level, even lower than when lacking only *mprF* (Fig. 11).

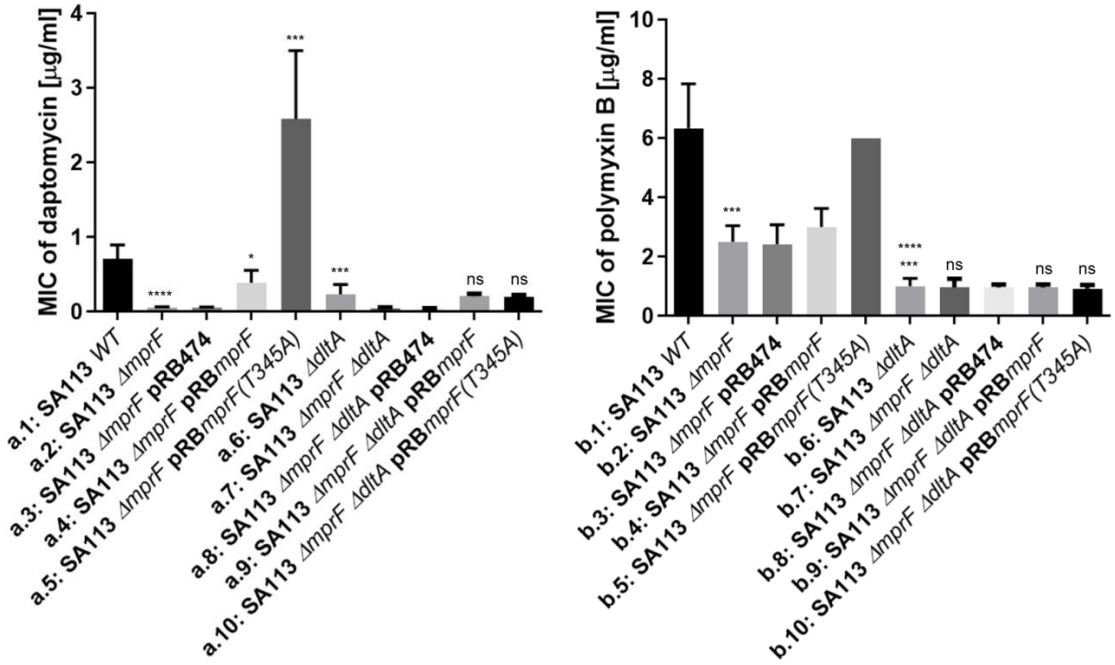

Fig. 11: MICs of DAP and PB (a, b) for ten strains of S. aureus SA113. The strains used were S. aureus SA113 WT (1), *ΔmprF* (2), *ΔmprF* with pRB (3), *ΔmprF* with pRB*mprF* (4), *ΔmprF* with pRB*mprF345* (5), *ΔdltA* (6), the double knockout *ΔdltA ΔmprF* (7), and the double knockout with pRB (8), pRB*mprF* (9), or pRB*mprF345* (10), likewise. Biological replicates n = 6. P values of unpaired t tests are: a.1 vs. a.2 < 0,0001; a.1 vs. a.4 0,8187; a.1 vs. a.5 0,0006; a.1 vs. a.6 0,0005; a.6 vs. a.9 0,7191; a.6 vs. a.10 0,5925; b.1 vs. b.2 0,0002; b.1 vs. b.6 < 0,0001; b.2 vs. b.6 0,0001; b.6 vs. b.7 0.8040; b.6 vs. b.9 0,7342; b.6 vs. b.10 0,5155.

In the second step, those variants of *dltA* knockout mutants were complemented via phage transduction with the plasmid pTX containing the *dlt* operon. The complemented *dltA* knockout and the complemented double knockout both showed approximately the same level of resistance to DAP as the WT. The *dlt*-complemented double knockout with the *mprF345*, however, showed neither WT level of resistance nor increased DAP-R but remained highly susceptible (Fig. 12).

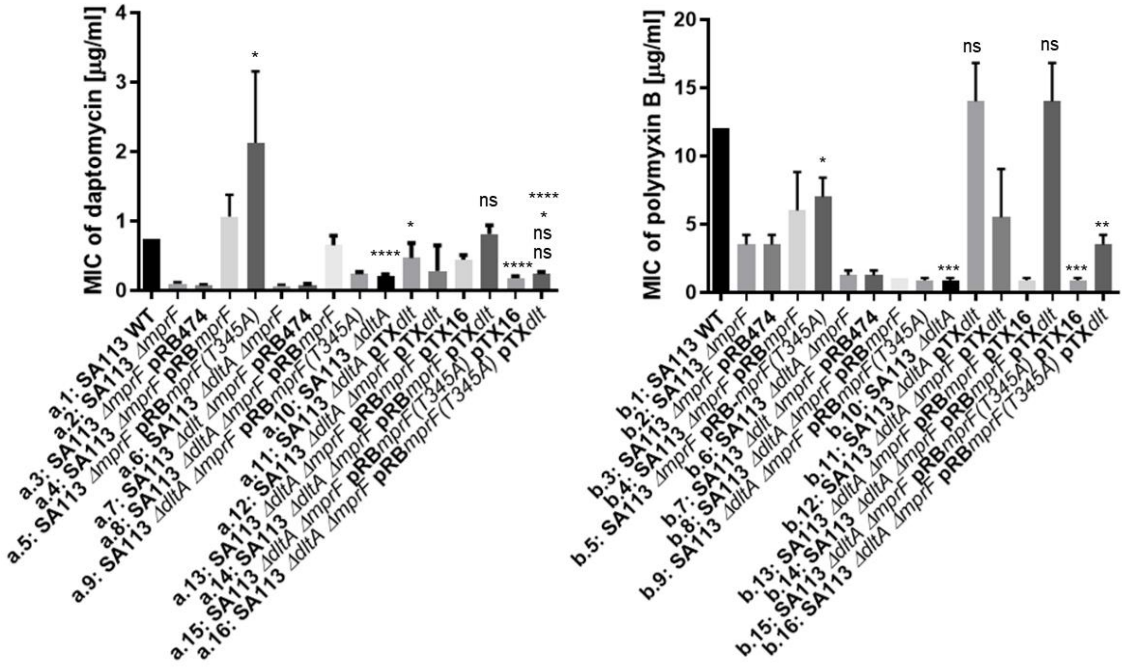

Fig. 12: MICs of DAP and PB (a, b) for 16 strains of *S. aureus SA113*. Biological replicates n = 4 for DAP and n = 2 for PB. Not depicted are the MICs of three other antibiotics (colistin, linezolid, oxacillin) that were tested as well with four replicates and of two antibiotics (bacitracin, vancomycin) with two replicates. P values of t tests are: a.1 vs. a.5 0,0371; a.1 vs. a.10 < 0,0001; a.1 vs. a.11 0,0390; a.1 vs. a.14 0,3559; a.1 vs. 1.15 < 0,0001; a.1 vs. 1.16 < 0,0001; a.5 vs. a.16 0,0105; a.10 vs. a.16 0,2070; a.15 vs. a.16 0,0390; b.1 vs. b.5 0,0377; b.1 vs. b.10 0,0001; b.1 vs. b.11 0,4226; b.1 vs. b.14 0,4226; b.1 vs. b.15 0,0001; b.1 vs. b.16 0,0034.

This result suggests that the *dlt* operon cannot express its whole function when encoded on a plasmid, and might need other factors for full expression.

3.3 Membrane localisation of predicted peptide loops from MprF by insertion and detection of cysteine residues at a defined amino acid position

3.3.1 Localisation of antibody binding sites to MprF

To further examine the structure and function of MprF, especially of its flippase domain, a biochemical approach was used to localise the orientation of a certain peptide loop within the membrane.

Maleimid is a highly specific marker for the amino acid cysteine. Hence, the extended loop – which was presumed to be flipping from cyto- to periplasmic and back again – was marked by exchanging an amino acid to a cysteine. In doing so, the amino acid to be substituted should be as similar to cysteine as possible, the charge and behaviour should be maintained, so that the exchange to cysteine would not alter the protein. Thus, out of different exchanges (T263C, V266C, P267C, L273C) proline 267 became the amino acid of choice (Fig. 13), as in the cysteine localisation assay it produced the strongest and clearest signals. So the other exchanges were not pursued.

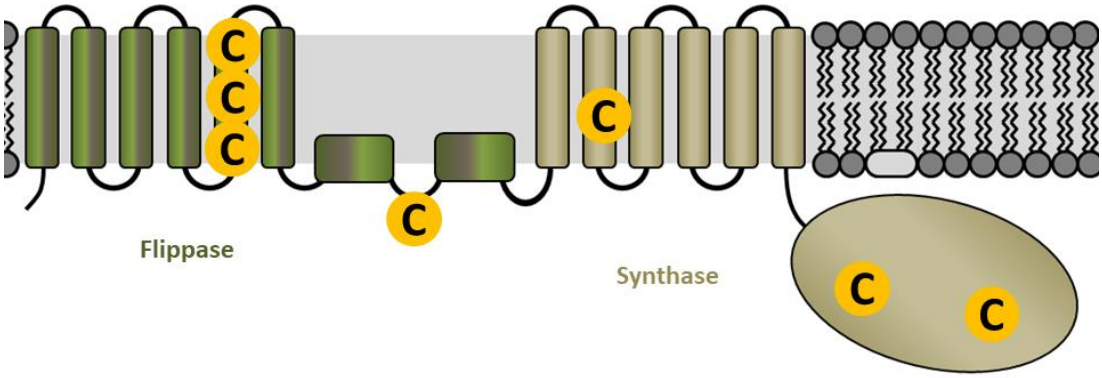

Fig. 13: Schematic figure of MprF with an amino acid exchange P267C in the putative loop. The naturally present cysteines (C199, C204, C217, C380, C526, C717) are depicted as well. Grey depicted is the lipid bilayer, green depicted is the flippase domain, beige depicted is the synthase domain, as yellow circles depicted are amino acids, C - cysteine, P - proline.

Exchange was performed using site directed mutagenesis (see 2.3.4) within *mprF* inserted in the plasmid pT10. Initially, the strain used for cloning was *E. coli*, as it is easier to maintain and transform.

Furthermore, cysteine is naturally found in MprF on amino acid positions 199, 204 (on the extracellular side), 217, 380 (in transmembrane segments), 526 and

717 (on the intracellurlar side) (Fig. 13). To avoid detection of these cysteins, they also should have been exchanged to similar amino acids (C199S, C204S, C217A, C380A, C526S, C717S) (Fig. 14). Maleimid can only access the outer side of the intact cell membrane. However, during the labelling assay (see 2.7.3), the membrane is damaged and the cytoplasmic cysteines become accessible as well.

The cysteine exchanges should have been performed out of precaution, so that they would not produce a polluting signal.

Unfortunately, exchange of those naturally occurring cysteines, especially C717S, presented more difficult and time consuming. Thus, it was abandoned, as not all of them could be successfully exchanged within the scope of this work.

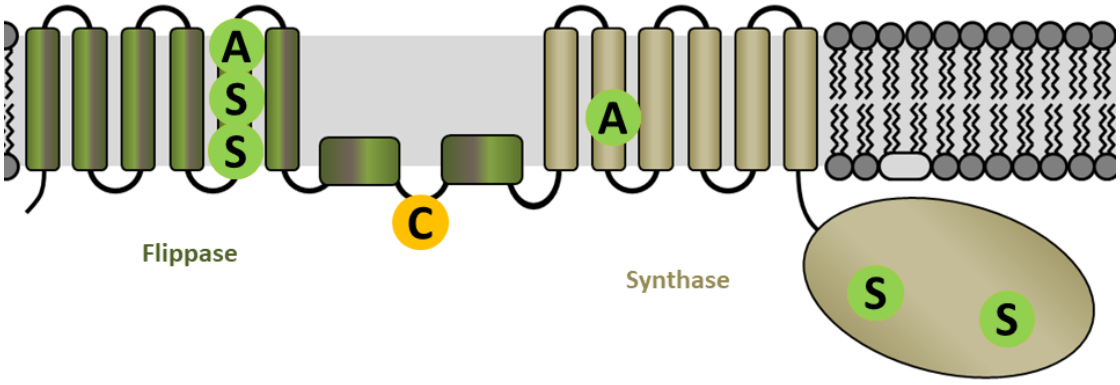

Fig. 14: Schematic figure of MprF with P267C (in putative loop) and attempted but not succeeded exchanges of cysteine: C199S, C204S, C217A, C380A (in TMDs), C526S, C717S (intracellular). Grey depicted is the lipid bilayer, dark green depicted is the flippase domain, beige depicted is the synthase domain, as light green circles depicted are attempted but not succeeded amino acid exchanges, as yellow circle depicted is an amino acid, A - alanine, C - cysteine, P - proline, S - serine.

To verify that the mutation P267C was successful DNA samples for sequencing were sent to GATC. Additionally, the MprF protein was provided with FLAG-tag, so that the MprF could later be extracted with magnetic FLAG beats and detected with an anti-FLAG-antibody.

Preparing for the labelling assay, strain cultures were split in two halves and processed differently. For marking cysteine only on the outer surface, MBP was added and the reaction was quenched after a short time. In order to mark only intracellular cysteine, cysteine on the outer surface was blocked with AMS, cell

lysis was achieved with glass beads, so that the intracellular cysteine is exposed, and subsequently, MBP was added.

For immunoprecipitation, magnetic FLAG beads were used, to separate MprF from cell debris. Via electrophoresis and Western Blot, the membrane proteins were transferred to a blot membrane (polyvinylidene fluoride).

To detect and visualise MprF on the one hand, and the MPB labelled cysteine on the other hand, specific antibodies were applied. The antibodies used to index MprF were anti-FLAG (mouse) and anti-mouse 700 (with emission maxima of approximate 700 nm). For tracing the MPB marked cysteine in the putative flipping loop of MprF, a Streptavidin antibody (with emission maxima of approximate 800 nm) was added, which binds to MPB.

Finally, antibody binding was measured with the Odyssey Imaging Systemof LI-COR (Lambda Instruments Corporation).

The strains used for cloning were *E. coli XL-10 gold* ultra-competent cells harbouring versions of the FLAG-tagged plasmid pT10, namely (a) the empty plasmid, (b) pT10 with the original *mprF*, (c) pT10 bearing *mprF* marked with the amino acid change P267C, and (d) pT10 with *mprF* P267C and additionally the point mutation D731A disabling the synthase domain from LysPG synthesis as a control. For later analysis, *S. aureus SA113 ΔmprF* was used.

For the strain with the “empty” pT10 plasmid, LI-COR analysis displayed no signal at 700 nm for MprF (more precisely for the anti-mouse-antibody binding to the anti-FLAG-antibody indicating the FLAG-tag of MprF), neither in the outer nor in the inner leaflet (see Fig. 15). The other three strains complemented with variants of *mprF* showed signals at 700 nm both on the peri- and the cytoplasmic side (see Fig. 15), thus, indicating presence of MprF. The bacteria containing pT10*mprF* had an additional signal at 800 nm indicating the presence of cysteine only on the inner side but not on the outer side (see Fig. 15). This is concordant with the current topology model of MprF, having no cysteine on the outer side but only in TMDs (C199, C204, C217) and on the inner side (C526, C717) (see Fig. 13).

However, the strain with pT10*mprFP267C* showed a 800 nm signal on the inner as well as the outer side (see Fig. 15). This confirms that the loop of interest is

on the periplasmic side. Whether this loop is also on the cytoplasmic side cannot be deduced, as the signal maxima at 800 nm for the inner side could also originate from the original cytoplasmic cysteines (C526, C717).
With the SNP D731A, which disables the synthase domain, there was no more indication of a cysteine at the outer side but only on the inner side. This suggests, that the loop with P267C, which has been detected at the outer side, is no more ot the outer side, implying it is on the inner the inner leaflet (Fig. 15).

Fig. 15: Western blot of the single cysteine labelling assay. Left: Showing, the signals of 700 nm (anti-mouse binding to anti-FLAG binding to FLAG) showing the presence of MprF, and of 800 nm (streptavidin coupling to MPB coupling to cysteine) showing the marked loop of interest for the periplasmic (upper two) and cytoplasmic (lower two) side. **Right:** Overlay of the signals for MprF (indicated red) and the loop of interest (indicated green) for the periplasmic (upper) and cytoplasmic (lower) side. A - alanine, C - cysteine, D - aspartic acid, P - proline.

The fact that the position of the extended loop – whether on the inner or on the outer side – depends on the presence of a substrate implies a flipping function as in the absence of LysPG there is no detection of the loop on the outer side or as to say no flipping of the loop to the periplasmic side.

4 Discussion

MprF is known to be a major bacterial virulence factor that possesses the function of a LysPG synthase and is important for resistance against many CAMPs and CAMP-like antibiotics. The resistance is assumed to be based on an increased positive net charge of the bacterial cell surface. Furthermore, additionally to that so to say basic resistance, SNPs in *mprF* lead to increased tolerance or resistance to daptomycin without cross-resistance to other antibiotics. Whereupon the mechanism of this additional resistance is based on remains not clear. MprF is a flippase transferring the synthesised LysPG from the inner to the outer lipid leaflet. The exact transfer mechanism is not yet clear. Interestingly, most of the mutations enhancing resistance are located in the transient area from the synthase to the flippase domain in or near an extended loop, which is thought to be essential for the flipping mechanism.

In this work it could be confirmed that *mprF* is indeed a potent resistance factor against CAMPs and that modification of DAP binding to the cell surface plays a vital role in its mechanism. Furthermore, it could be revealed that enhanced resistance to DAP through a specific SNP within *mprF* (T345A) is not due to less binding and more repulsion of DAP, as so far supposed. In contrast, it might possibly rely on the altered interaction between DAP and PG as a result of increased L-PG and decreased PG content of the bacterial membrane [11, 61, 83]. In addition, the results show that both domains of MprF physically interact with other proteins of the LTA synthesis machinery and with the teichoic acid D-alanylation apparatus [50]. Of note, when deprived of the DltABCD system, MprF seems to be insufficient to confer resistance on its own against polymyxin B. However, no distinct connection could be detected between the interaction of MprF with the DltABCD system and DAP. Furthermore, the topology and function of MprF has been explored in more detail via a cysteine labelling assay. We provide evidence that the extended cytoplasmic loop (aa 245-298) of the flippase domain can flip to the outer side only when the synthase domain is intact. Therefore this loop is supposedly dependent on the presence of its substrate LysPG.

Moreover, the originally contained cysteines of MprF will also be exchanged to other amino acids to improve the quality of the signal outcome of the cysteine assay. Again, the properties of the amino acids have to be taken into account. Needless to say, the function of the mutated MprF has to be verified by measuring the levels of resistance via Etest MICs of DAP and other antibiotics.

Our results help to understand the exact mechanism of how MprF operates and contributes to bacterial resistance against CAMPs and CAMP-like antibiotics. Our finding that the flipping loop is probably essential and therefore may represent the active center of the flippase, could deliver a target for a new class of therapeutic agents. These agents will not be antibiotics in a classical sense of being bacteriostatic or bactericidal but rather supporting known antibiotics. Their support might potentially be based on impeding bacterial resistance mechanisms, for instance antibodies binding to the flipping loop, inactivating its function, and thereby restoring the susceptibility of the cell to antibiotics or the human innate immune system.

During this work, several difficulties, bottlenecks and some possible sources of error have been encountered. It became very apparent that, although using the same medium type (e. g. MHB or LB) with the same ingredients and recipes, growth of bacterial cultures and MIC analyses can show very different results when different providers (e. g. Roth, BD, Sigma-Aldrich) or even batches are used. Therefore, to ensure the reproducibility of data it has especially been seen to that medium and other materials (e. g. Etest stripes) were used from only one label and, when possible, from only one batch for one experimental setup. For future research, the single cysteine detection assay could be further improved. One aspect is suitability of the internal control. Here, only at the beginning of the experimental setup the OD_{600} was measured and adjusted. For better comparability of the final signals, adjustments could be integrated also at a later point. Another topic is protein degradation causing multiple bands in the Western Blot. It might be that this problem could already be solved by performing the experiment in *S. aureus*, which is the natural host for MprF rather than *E. coli*.

5 Abstract / Zusammenfassung

Abstract

An important virulence factor for bacterial pathogenicity is the Multiple Peptide Resistance Factor (MprF). This membrane protein produces the positively charged phospholipid lysyl phosphatidylglycerol (LysPG) that confers bacterial resistance against cationic antimicrobial peptides (CAMPs), CAMP-like antibiotics and the human immune system.

This work served to better clarify MprF's role in interaction with other proteins of the synthesis apparatus for membrane lipids. Furthermore, the function of MprF should be examined more in detail to possibly find new potential targets for antivirulent therapeutics. Such inhibitors, in contrast to antibiotics, could make bacterial pathogens more vulnerable for our immune system and therefore provide an alternative therapeutic option.

Three aspects of MprF were looked into.

For one thing, resistance conferred by *mprF* and *mprFT345A* was compared. It could be shown that the enhanced tolerance of *S. aureus* to DAP via MprF is not based on less binding of DAP, which implies that this is not due to an increased charge repulsion. Additionally, the often described SNP T345A in the ORF of *mprF* leading to increased resistance to DAP appeared to cause no change in the amount of cell-bound DAP. This suggests that – besides the known basic resistance against CAMPs and CAMP-like antibiotics via charge repulsion – there is another resistance mechanism which is conferred by T345A, specifically against DAP without cross-resistance, and is supposedly mediating alterations of how DAP and the cell surface with less PG interact.

For another thing, assumed interactions between *dlt* and *mprF* were looked into. It could be shown that MprF and both its sub-domains interact with other proteins of the lipoteichoic acid (LTA) synthesis machinery and the D-alanylation pathway, namely YpfP, LtaA, LtaS and DltD. Interestingly, when *dlt* was knocked out and therefore the DltABCD system was not at hand, which is the synthesis apparatus for LTAs as membrane lipids, MprF alone was not

able to confer resistance to polymyxin B. This implies some kind of dependence between the function of MprF and the presence of LTAs.

Thirdly, the topology and function of MprF has been investigated in more detail. At a defined amino acid position within a predicted loop of MprF, cysteine residues were inserted. By detecting these cysteine residues, the localisation of the loop in the membrane could be deduced. The extended cytoplasmic loop (aa 245-298) of the flippase domain could be detected on the outer side of the membrane. But when disabling the synthase domain via SNP D731A, a signal on the outer side of the layer could not be detected any more. This implies, firstly, that the aforementioned loop (aa 245-298) flips from the inner to the outer side of the membrane and, secondly, that this flipping occurs only as long as the synthase domain of MprF is intact, suggesting a dependence between the flipping of the extended loop and the presence of the substrate LysPG. Therefore, this loop possibly depicts the active center of the MprF flippase.

Zusammenfassung

Ein wichtiger Virulenzfaktor für bakterielle Pathogenität ist der Multiple Peptide Resistance Factor (MprF). Dieses Membranprotein stellt das positiv geladene Phospholipid Lysyl-Phosphatidylglycerol (LysPG) her, das bakterielle Resistenz gegen kationische antimikrobielle Peptide (KAMPs), KAMP-ähnliche Antibiotika und das humane Immunsystem verleiht.

Diese Arbeit diente zur besseren Einordnung der Rolle MprFs in der Interaktion mit anderen Proteinen der Synthesemaschinerie von Membranlipiden. Zudem soll die Funktion von MprF näher untersucht werden, um eventuell neue potenzielle Ziele für antivirulente Therapeutika zu finden. Solche Inhibitoren könnten, im Gegensatz zu Antibiotika, die bekannt dafür sind, durch Selektionsdruck einen Vorteil zu erhalten, bakterielle Pathogene angreifbarer für unser Immunsystem machen und dadurch alternative Therapieoptionen liefern.

Drei Aspekte von MprF wurden untersucht.

Erstens wurde die Resistenz verglichen, welche durch *mprF* und *mprFT345A* gewährt wird. Es konnte gezeigt werden, dass die gesteigerte Duldung von DAP durch *S. aureus* mittels MprFs nicht auf einer geringeren Bindung von DAP basiert, was impliziert, dass es nicht auf verstärkter Ladungsabstoßung beruht. Zusätzlich schien eine oft beschriebene SNP (T345A) im ORF von *mprF*, welche zu erhöhter Resistenz gegen DAP führt, ebenso keine Änderung der Menge an zellgebundenem DAP zu zeigen. Dies deutet darauf hin, dass es – neben der bekannten Basisresistenz gegen KAMPs und KAMP-artige Antibiotika durch Ladungsabstoßung – einen weiteren Resistenzmechanismus gibt, der auf T345A beruht, spezifisch gegen Daptomycin und ohne Kreuzresistenz wirkt, und dass er vermutlich Änderungen vermittelt, wie DAP und die Zelloberfläche, die weniger PG enthält, interagieren.

Zweitens wurden vermutete Interaktionen zwischen *dlt* und *mprF* untersucht. Es konnte gezeigt werden, dass MprF und seine beiden Sub-Domänen mit anderen Proteinen der LTA-Synthesemaschinerie und der D-Alanylierungsbahn interagieren, nämlich YpfP, LtaA, LtaS und DltD. Wenn *dlt* abgeschalten war und daher kein DltABCD-System bestand, welches der Syntheseapparat für

LTAs und Membranlipide ist, war interessanterweise MprF eigenständig nicht in der Lage, eine Resistenz gegen Polymyxin B zu gewähren. Dies impliziert eine Art von Abhängigkeit zwischen der Funktion von MprF und der Gegenwart von LTAs.

Drittens wurden die Topologie und die Funktion von MprF detaillierter untersucht. indem es mit Cysteinresten markiert und selbige detektiert worden sind. An einer definierten Aminosäureposition innerhalb einer vorhergesagten Schleife von MprF wurden Cystein-Reste eingefügt. Indem diese Cystein-Reste detektiert wurden, konnte die Lokalisation der Schleife in der Membran abgeleitet werden. Die erweiterte zytoplasmatische Schleife (AS 245-298) der Flippasedomäne konnte an der äußeren Seite der Membran nachgewiesen werden. Jedoch, als die Synthasedomäne durch die SNP D731A deaktiviert wurde, konnte an der äußeren Seite der Membranschicht kein Signal mehr detektiert werden. Dies impliziert erstens, dass die oben genannte Schleife (AS 245-298) von der inneren an die äußere Seite der Membran flippt, und zweitens, dass dieses Flipping sich nur dann ereignet, wenn die Synthasedomäne von MprF intakt ist, was eine Abhägigkeit nahelegt zwischen dem Flipping der erweiterten Schleife und der Gegenwart des Substrates LysPG. Daher stellt diese Schleife möglicherweise das aktive Zentrum der MprF-Flippase dar.

6 Literature

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7 Erklärung zum Eigenanteil

Herr Prof. Dr. A. Peschel und Herrn MSc C. Slavetinsky haben die Studie konzipiert, sie haben die Arbeit betreut und haben das Manuskript korrigiert.
Herr A. Geyer hat, zunächst unter Anleitung und Mitwirkung durch Herrn MSc S. Kuhn und vor allem Herrn MSc C. Slavetinsky, die Kultivierung der Bakterien, die molekulargenetischen Versuche (Plasmidisolation, PCR, SDM, Restriktionsverdau, Gelelectrophorese, Gelextraktion, Purifikation), Transformationen (Hitzeschock, Elektroperforation) sowie Phagentransduktion bzw. Auswertungen durchgeführt. Herr Geyer hat die Bestimmung der MICs durchgeführt. Herr Geyer hat, zunächst unter Anleitung durch Herrn D. Kühner, die Bestimmungen mittels UPLC durchgeführt. Herr Geyer hat, zunächst unter Anleitung durch Frau Dr. S. Zilkenat, den Cystein Assay durchgeführt.
Herr A. Geyer hat, zunächst unter Anleitung von Herrn MSc C. Slavetinsky, die Datenrecherche durchgeführt.
Herr MSc S. Kuhn hat Daten zur Interaktion zwischen MprF und anderen Proteinen der Membranlipidsynthese zur Verfügung gestellt.
Herr MSc S. Kuhn hat anfangs, Herr MSc C. Slavetinsky hauptsächlich die Betreuung übernommen.
Herr Prof. Dr. A. Peschel und Herr MSc C. Slavetinsky haben das Manuskript korrigiert.

Winnenden, den 13.04.2020

André Geyer

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Óma, csillagod tényleg kis doctor.