

Interfakultäres Institut für Mikrobiologie und Infektionsmedizin
AG Schütz/ Bohn

**Advanced target validation and development of an activity
assay for inhibitor evaluation of MepM murein
endopeptidases as anti-virulence and resistance-breaking
targets**

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Vöhringer, Kathrin

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Dekan: Professor Dr. B. Pichler

1. Berichterstatter: PD Dr. E. Bohn

2. Berichterstatter: Prof. Dr. M. Schindler

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Abbreviations

<i>A. baumannii</i>	<i>Acinetobacter baumannii</i>
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
CDC	Centers for Disease Control and Prevention
d-ala	D-alanine
d-glu	γ -D-glutamate
<i>E. coli</i>	<i>Escherichia coli</i>
ECDC	European Centre for Disease Prevention and Control
FITC	Fluorescein isothiocyanate
GlcNAc	N-Acetylglucosamin
IM/ OM	Inner/ Outer membrane
<i>K. pneumoniae</i>	<i>Klebsiella pneumoniae</i>
l-ana	L-analine
LB	Lysogen broth
LPS	Lipopolysaccharide
mDAP	Meso-diaminopimelic acid
MDR	Multidrug resistant
Mep	Murein endopeptidase
MIC	Minimal inhibitory concentration
MurNac	N-acetylmuramic acid
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PG	Peptidoglycan
RND	Resistance-nodulation-division
RT	Room temperature
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. pneumoniae</i>	<i>Streptococcus pneumoniae</i>
SP	Signal peptide
TM	Transmembrane helix
UC	Ultracentrifuge
<i>V. cholerae</i>	<i>Vibrio cholerae</i>
WCL	Whole cell lysate

1. Introduction

1.1 Emerging antibiotic resistance is an urgent global health threat

The discovery of penicillin by Alexander Fleming back in 1928 and the introduction of the clinical use of antimicrobial drugs was a fundamental step in medical history. The ability to treat infectious diseases rapidly reduced mortality in the 20th century (Armstrong et al., 1999) and led to a strong increase in life expectancy worldwide (Centers for Disease and Prevention, 1999). Until today, antibiotic treatment continues to form the basis for modern medicine, specifically for carrying out invasive surgery, chemotherapy or organ transplantation without patients subsequently dying from infectious diseases (Young and Khadaroo, 2014). The widespread use of antibiotics not only in medicine has since created a selective pressure on antibiotic-resistant bacteria (Levy and Marshall, 2004) and has started a race between the emergence and spread of resistance and the development of new antibiotic therapies. As the discovery of new antibiotics is becoming increasingly difficult (Silver, 2011) and the number of newly discovered antibiotic substances has been very low since the 20th century (Stennett et al., 2022), the emerge of antibiotic resistance is a major threat to global health (Lewis, 2020, Brown and Wright, 2016).

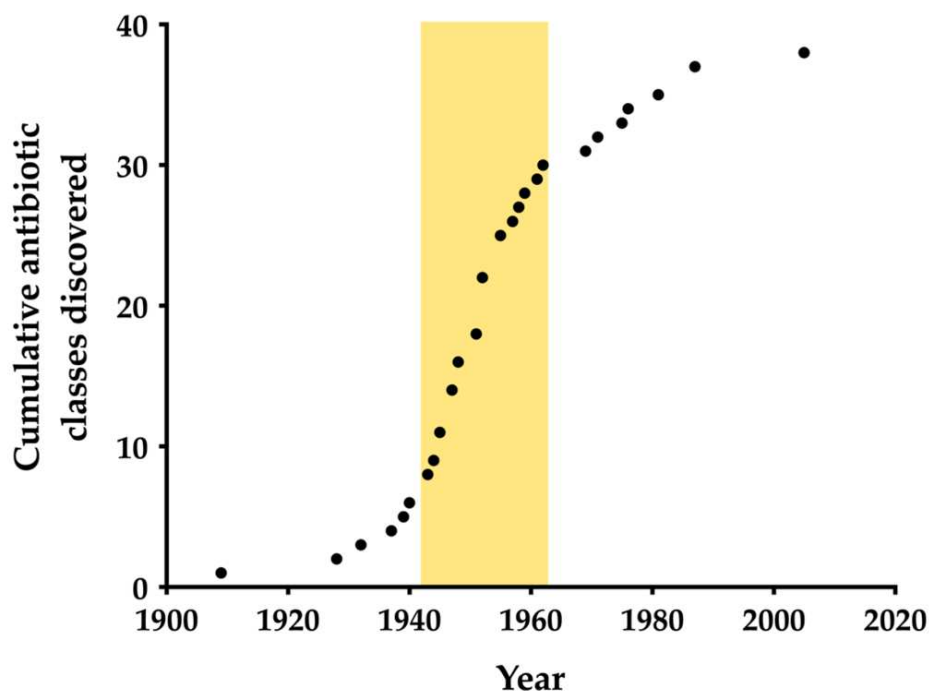


Figure 1: Cumulative discovery of antibiotics

The cumulative discovery of the 38 classes of clinically used antibiotics. The Golden Age of discovery is highlighted in yellow. (Stennett et al., 2022)

Worldwide, the number of deaths caused by antibiotic-resistant bacteria is rising. (European Antimicrobial Resistance, 2022, Antimicrobial Resistance, 2022). The greatest threat comes from the so called ESKAPE pathogens *E. coli*, *S. aureus*, *K. pneumoniae*, *S. pneumoniae*, *A. baumannii* and *P. aeruginosa*, against which new therapeutic strategies are urgently needed (Tacconelli et al., 2018). According to their resistance profile, the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) categorize these pathogens in multidrug resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) (Magiorakos et al., 2012). In eastern Europe, resistance rates of *P. aeruginosa* and other Gram-negative pathogens are already disturbingly high. In Germany 5-10 % of invasive *P. aeruginosa* isolates are resistant to carbapenems (data from 2021). In order to continue to control these infections, broad alternative therapeutical approaches are currently being researched including the inhibition of quorum sensing and bacterial lectins, the use of iron chelates or phage therapy (Chatterjee et al., 2016, Pang et al., 2019). This work contributes to a better understanding of the mechanisms of resistance and presents another potential new strategy for treating antibiotic-resistant *P. aeruginosa*.

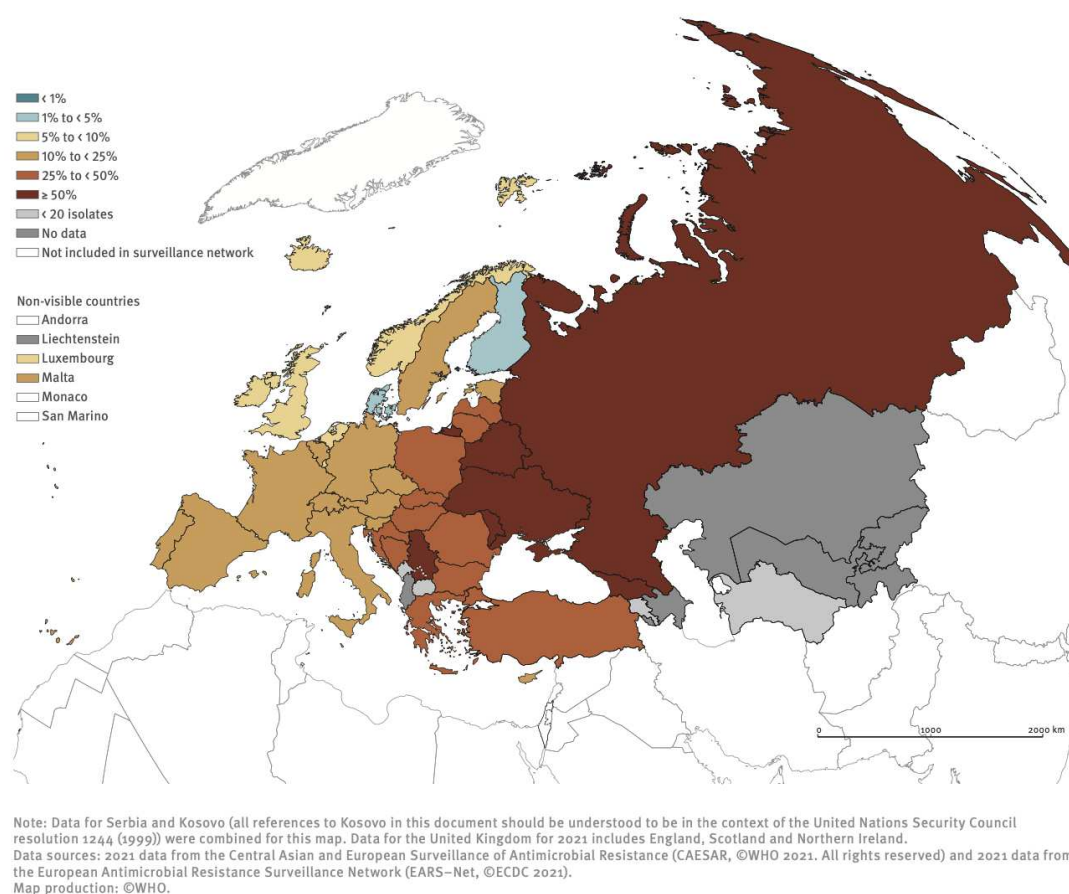


Figure 2: Percentage of invasive *Pseudomonas aeruginosa* isolates with resistance to carbapenems (imipenem/ meropenem), by country, WHO European Region, 2021
source: doi: 10.2900/63495

1.2 *P. aeruginosa* infections

The Gram-negative *P. aeruginosa*, one of the ESKAPE pathogens, is a rod-shaped facultative anaerobic bacterium that occurs ubiquitously in the environment and as an opportunistic pathogen mainly infects immunocompromised patients (Lyczak et al., 2000). It causes 7.1 – 7.3% of all healthcare-associated infections (Weiner-Lastinger et al., 2020, Magill et al., 2014) and even 16.2% of patient infections on intensive care units (Vincent et al., 2020).

Infections caused by *P. aeruginosa* are mainly nosocomial infections like pneumonia (Furtado et al., 2007), surgical site infections (Raouf et al., 2018), urinary tract infections (Rosenthal et al., 2016, Mittal et al., 2009) and infections of wounds (Norbury et al., 2016, Serra et al., 2015). Particularly device-related infections such as ventilator-associated pneumonia or catheter-associated urinary tract infections are frequently caused by *P. aeruginosa* (Weiner-Lastinger et al., 2020). In patients with cystic fibrosis, chronic infection with *P. aeruginosa* is a major concern as it deteriorates lung function and increases morbidity (Mogayzel et al., 2014, Frederiksen et al., 1997).

The most dangerous *P. aeruginosa* infection is a blood stream infection with an estimated mortality rate of 43.2%–58.8% (Kang et al., 2005, Micek et al., 2011, Thaden et al., 2017). This is especially dangerous for immunocompromised patients, in whom 23% of bloodstream infections are caused by *P. aeruginosa* (Tofas et al., 2017). Overall, *P. aeruginosa* poses a serious risk, especially for immunocompromised and hospitalized patients.

Several classes of antibiotics are used to treat *P. aeruginosa* infections, including β -lactams, quinolones and aminoglycosides. The polymyxin colistin is applied as a last resort in multidrug resistant strains because of its side effects like nephrotoxicity and neurotoxicity (Pina-Sánchez et al., 2023, Spapen et al., 2011). Aminoglycosides like gentamicin or amikacin inhibit bacterial protein synthesis through binding to ribosomal subunits (Mingeot-Leclercq et al., 1999). Quinolones like ciprofloxacin and levofloxacin are gyrase and topoisomerase IV inhibitors that interfere with DNA replication (Aldred et al., 2014). The β -lactam antibiotics include ureidopenicillins, cephalosporins, carbapenems and monobactams. They bind to so-called penicillin-binding proteins and thus inhibit cell wall biosynthesis (Poole, 2004). The increasing emerge of resistance to these classes of antibiotics requires the development of new therapeutical strategies. How *P. aeruginosa* manages to become resistant to these antibiotics is described in detail in the following with a focus on β -lactam resistance.

1.3 β -lactam resistome of *P. aeruginosa*

1.3.1 Antibiotic inactivation

P. aeruginosa is intrinsically resistant to early generation cephalosporins and aminopenicillins like ampicillin or amoxicillin, also in combination with β -lactamase inhibitors, due to the chromosomal expression of an inducible β -lactamase AmpC, which inactivates those antibiotics. As AmpC is only produced at a low level in wildtype strains, *P. aeruginosa* is generally sensitive to ureidopenicillins such as piperacillin, carbapenems, monobactams and cephalosporins of generation 3b and higher (Sanders and Sanders, 1986).

P. aeruginosa can achieve acquired resistance through chromosomal mutations, which it is particularly capable of, that lead to *ampC* overexpression (Lopez-Causape et al., 2018). Most of these mutations involve genes belonging to the peptidoglycan recycling regulatory pathway, for example the mutational inactivation of *dacB* or *ampD* (Juan et al., 2005, Moya et al., 2009) or the mutational induction of the transcriptional regulator *ampR* (Juan et al., 2017). In the end all these mutations cause *ampC* overexpression and thus cause resistance to broad-spectrum β -lactam antibiotics. The clinical isolate ID40 used in this work carries an inactive *dacB* gene that causes *ampC* overexpression (Sonnabend et al., 2019).

Mutations in the active site of *ampC* or the active site of narrow-spectrum β -lactamase *oxa-2* are less frequent but can mediate resistance to β -lactam antibiotics also in combination with β -lactamase inhibitors like ceftolozane/tazobactam (Cabot et al., 2014, Fraile-Ribot et al., 2017). Besides chromosomal mutations, *P. aeruginosa* can acquire resistance through horizontal gene transfer of plasmids, encoding carbapenemases or extended-spectrum β -lactamases (ESBLs) (Tenover et al., 2022, Lopez-Causape et al., 2018). These mediate resistance against classical anti-pseudomonal penicillins, cephalosporins and carbapenems. Global distribution of these carbapenemases varies greatly from region to region (Reyes et al., 2023).

1.3.2 Decreased uptake due to low cell permeability

P. aeruginosa has an extremely low permeable outer membrane for hydrophobic molecules compared to other Gram-negative bacteria like *E. coli* and it incorporates only few porin channels (Hancock and Brinkman, 2002). This gives it a high intrinsic resistance to antibiotics, that need to penetrate the outer membrane to reach the periplasm and their site of action. While aminoglycosides and polymyxins achieve this by interacting with bacterial LPS, β -lactam antibiotics are assumed to use porin channels for their uptake (Lambert, 2002). However, it has not been conclusively clarified how β -lactam antibiotics pass the outer membrane and there are

also indications of porin-independent permeation (Ude et al., 2021). For carbapenems it is known that they penetrate the membrane through the porin OprD and downregulation of *oprD* is one of the most common causes for carbapenem resistance in *P. aeruginosa* (Castanheira et al., 2014, Li et al., 2012). Previously, it was investigated whether the deletion of *mepM* in *P. aeruginosa* ID40 influences the expression level of *oprD*, but no correlation was found (Kemper, 2024).

1.3.3 Increased efflux

P. aeruginosa not only prevents the uptake of antibiotics, but also expresses resistance-nodulation-division (RND) efflux pumps that actively excrete toxic compounds including antibiotics (Li and Nikaido, 2009). Each of them contains an outer membrane channel and a cytoplasmatic membrane transporter, which are connected to each other by periplasmic linker proteins (Daury et al., 2016). Four efflux pumps are capable for the efflux of antibiotics in *P. aeruginosa*, namely MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM. Among these, MexAB-OprM, MexCD-OprJ and MexXY-OprM contribute to β -lactam resistance, while MexEF-OprN does not. It pumps out quinolones and tetracyclines (Masuda et al., 2000, Llanes et al., 2011, Maseda et al., 2000). Overexpression of efflux pumps is one of the most common resistance mechanisms in clinically isolated *P. aeruginosa* strains, alongside overexpression of *ampC* and inactivation of *oprD* (Cabot et al., 2011).

1.3.4 Target modification

β -lactam antibiotics inhibit cell wall synthesis by covalently binding to and inhibiting essential transpeptidases, so-called penicillin-binding proteins PBP1a, PBP1b, PBP2 and PBP3. Some studies indicate that mutational modifications or altered expression levels of these targets may reduce affinity for β -lactam antibiotics and promote β -lactam resistance (Zapun et al., 2008, Moya et al., 2012).

1.4 Murein endopeptidases as putative resistance breaking targets in *P. aeruginosa*

Recently it could be demonstrated that besides essential genes, additional chromosomally encoded non-essential genes become essential in the presence of therapeutic concentrations of meropenem or cefepim and thus contribute to β -lactam resistance (Sonnabend et al., 2019). As the search for new antibiotic agents is becoming increasingly difficult, an alternative therapeutic approach is to target these genes with adjuvants that applied together with antibiotics, make resistant bacteria sensitive to these antibiotics again. In *P. aeruginosa* murein endopeptidases (Meps) have been identified as potential targets for such adjuvants as their deletion reduces MIC values of β -lactam antibiotics and the triple deletion of *mepM1*, *mepM2* and *mepM3* breaks resistance against many β -lactam antibiotics (Kemper, 2024, Sonnabend et al., 2019).

The essentiality of crosslinked peptidoglycan for the viability of bacteria has long been recognized and forms the basis of β -lactam therapy, as β -lactam antibiotics inhibit transpeptidases that build up these crosslinks. However, the function and regulation of endopeptidases that cut these crosslinks and their role in mediating β -lactam resistance have only been investigated in the recent years. In the following it will be described what is known so far.

1.4.1 Cell wall of Gram-negative bacteria: structure and function

The cell envelope of Gram-negative bacteria is composed of an outer and inner membrane. The bacterial cell wall, a thin murein layer, is located in between these two lipid layers in the so-called periplasm (Silhavy et al., 2010). This murein layer consists of crosslinked peptidoglycan (PG) that forms a solid mesh-like sacculus that surrounds the entire bacterial cell. This maintains bacterial stability and shape and protects bacteria from environmental stress like osmotic pressure or turgor (Holtje, 1998, Vollmer et al., 2008a).

Fig. 3 (Rajguru et al., 2024) illustrates the structure and composition of the PG of Gram-negative bacteria. The basic structure consists of long glycan strands of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc), that are crosslinked through short stem peptides. These are composed of two to five mostly non-proteinogenic amino acids: L-Alanine (l-ala), γ -D-glutamate (d-glu), meso-diaminopimelic acid (mDAP) and D-alanine (d-ala). Crosslinks between m-DAP³ of one peptide chain and d-ala⁴ hold different glycan strands together and build the mesh-like structure (Schleifer and Kandler, 1972). The amount of crosslinks varies from species to species and depends on different growth conditions (Turner et

al., 2014). In addition, *P. aeruginosa* acquires huge variability in PG composition through modification of the stem peptides with at least 448 unique mucopeptides identified so far. In adaption to changing environmental conditions like nutrient supply *P. aeruginosa* alters the composition of its PG by O-methylation or mDAP deamination (Anderson et al., 2022).

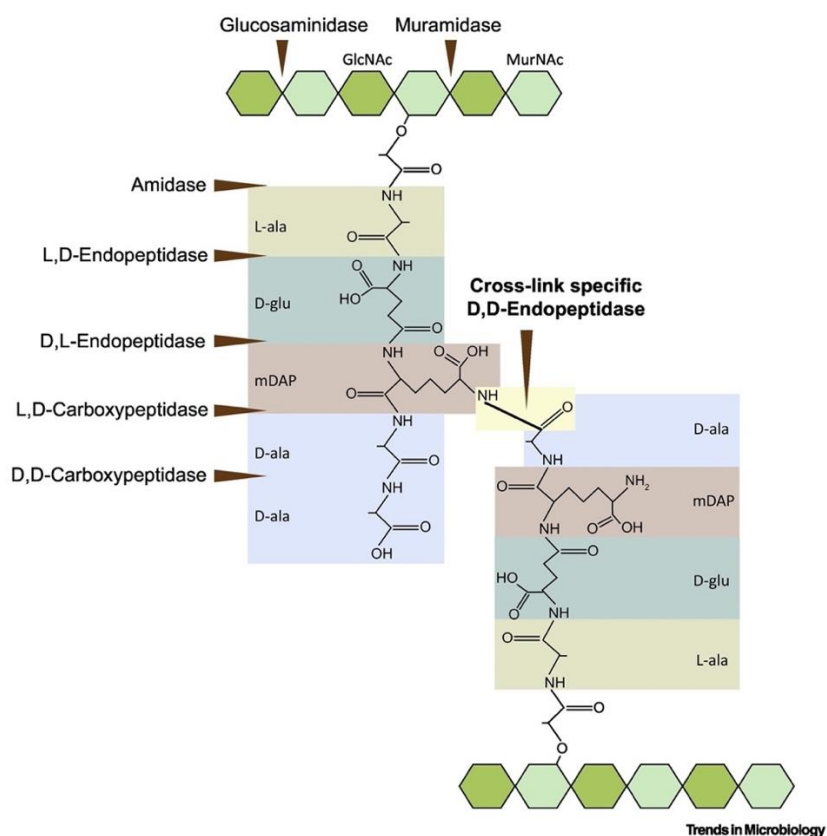


Figure 3: Peptidoglycan (PG) structure and composition

The basic unit of PG comprises a disaccharide of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) linked through a 1,4-glycosidic bond with a pentapeptide stem attached to C3 of MurNAc via lactate moiety. The stem peptide canonically consists of L-alanine (L-ala1), d-glutamic acid (d-glu2), meso-diaminopimelic acid (mDAP3), d-alanine (d-ala4), and d-ala5. The terminal d-ala5 is removed in the mature PG. Two glycan strands are interconnected via crosslinking of the stem peptide either directly or indirectly: a direct 4'-3' crosslink between d-ala4 and mDAP3 residues is depicted here. Cleavage specificities (brown triangles) of various PG hydrolases are shown. (Rajguru et al., 2024)

A rigid cell wall buildup of covalent peptide bonds would not allow the cells to expand. To maintain cell growth and division or to allow the incorporation of transmembrane complexes like efflux pumps or flagella the murein layer must retain its plasticity and adaptability. This is realized by various glycosidases and peptidases, that cleave the covalent bonds and support PG remodeling and elongation through the insertion of new PG (Vollmer et al., 2008b). This includes cleavage of crosslinks between stem peptides by DD-endopeptidases (Voedts et al., 2021). For the synthesis of new PG, disaccharide-peptide precursors are formed in the

cytoplasm and are transported across the inner membrane to the periplasm. There, transglucosylases join the glycans into long chains and transpeptidases build the peptide crosslinks (Garde et al., 2021).

The cleavage sites of different PG hydrolases are illustrated in Fig. 3 (Rajguru et al., 2024). Peptidases include amidases, carboxypeptidases and endopeptidases. Endopeptidases, such as MepMs investigated in this work, cleave specific peptide bonds according to the chirality of amino acids. They can have DD-, LD- or DL- endopeptidase activity (van Heijenoort, 2011). Cell wall remodeling enzymes not only allow cell growth and division but also contribute to antibiotic resistance, for example through the *ampC* transcriptional regulator *ampR* (Dhar et al., 2018, Jacobs et al., 1994).

1.4.2 Murein endopeptidases hydrolyzing peptidoglycan crosslinks in Gram-negative bacteria

Crosslink specific endopeptidases have first been described in *E. coli* in 2012. Spr/MepS, YebA/MepM and YdhO/MepH were identified as three redundantly essential DD-endopeptidases with similar catalytic specificity. As illustrated by Rajguru et al. (2024) in Fig. 4 (A), they are thought to hydrolyze crosslinks and make space for the insertion of new PG into the cell wall, thus support growth and viability. The codeletion of *mepS*, *mepM* and *mepH* in turn leads to rapid cell lysis probably through autolysis (Singh et al., 2012). It has been shown that overproduction of these crosslinking endopeptidases increases antibiotic resistance by inducing PG synthesis (Lai et al., 2017).

Synthesis and hydrolysis of PG crosslinks need to be finely balanced. In *E. coli*, MepS and MepH activity is regulated by a NlpI-Prc multienzyme complex (Banzhaf et al., 2020). How the regulation of MepS depends on PG expansion is illustrated in Fig. 4 (B) (Rajguru et al., 2024). NlpI is an outer membrane lipoprotein adapter and Prc is a periplasmic protease that degrades MepS. MepS is active during the expansion of PG, remaining stable when NlpI-Prc is not degrading it. In the absence of signals indicating PG expansion, NlpI-Prc degrades MepS, thereby inhibiting the cleavage of crosslinks. (Singh et al., 2015, Jeon and Cho, 2022). There is evidence that the NlpI-Prc complex adapts MepS levels to environmental conditions such as nutrient supply or phospholipid moieties and thus controls PG expansion depending on surrounding conditions (Som and Reddy, 2023).

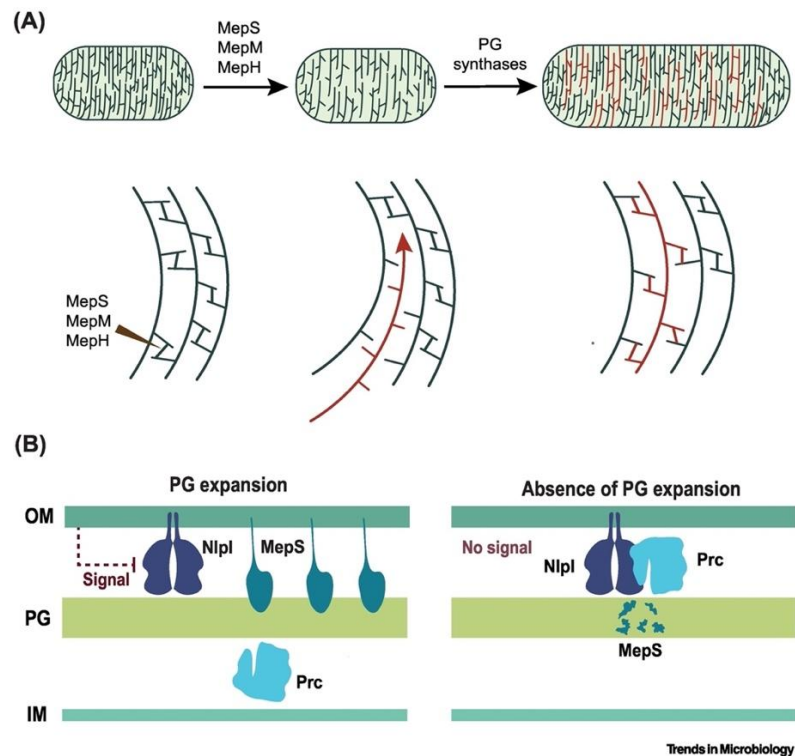


Figure 4: Role of crosslink-specific endopeptidases in peptidoglycan (PG) expansion and their regulation

(A) The elongation-specific endopeptidases MepS, -M, and -H in *Escherichia coli* cleave 4'-3' crosslinks during PG expansion. Hydrolysis (brown triangle) of existing PG (dark green) allows incorporation of nascent PG material (dark orange), followed by resynthesis of crosslinks, leading to cell wall elongation. (B) The endopeptidase activity of MepS (teal) is regulated by the adapter-protease duo Nlpl (blue) and Prc (aqua). During active PG expansion, an outer membrane (OM)-derived signal (dashed red line) modulates the proteolytic activity of Nlpl-Prc, leading to stabilization of MepS. Conversely, in the absence of the signal, the Nlpl-Prc system degrades MepS, preventing crosslink hydrolysis. Abbreviation: IM, inner membrane. (Rajguru et al., 2024)

V. cholerae also carries three 4'-3' crosslink specific endopeptidases ShyA (homologue of *E. coli* MepM), ShyB and ShyC. Analysis on substrate specificity of ShyA revealed another possible regulatory mechanism of how endopeptidases support PG elongation. ShyA specifically hydrolyzes old PG, which has a low pentapeptide content and avoids the hydrolysis of nascent PG dipeptides with high pentapeptide content (Dörr et al., 2013). ShyA and ShyC have also been found to confer β -lactam resistance. *V. cholerae* responds to β -lactam exposure with sphere formation, which makes it resistant, and DD-endopeptidases are essential for this phenotype (Dörr et al., 2015).

P. aeruginosa carries five murein DD-endopeptidases (Meps) that are listed in Table 1. MepM1, MepM3, MepH1 and MepH2 have been discovered as substrates of the periplasmic protease CtpA and its binding partner LbcA. CtpA inactivates Meps by cleavage and deletion of *ctpA* leads to less PG crosslinking most likely due to accumulation of Meps. Phenotypically deletion of *ctpA* is associated with increased biofilm formation, reduced type III secretion and reduced

virulence. Comparisons to endopeptidases in *E. coli* indicate that *paMepM1* is 35% identical to *ecMepM*/YebA. *PaMepH1* and *paMepH2* are predicted to be orthologues of *ecMepS* (Srivastava et al., 2018). Phylogenetic analysis on LytM proteins in *P. aeruginosa* and *E. coli* support that *paMepM1* is related to *ecMepM* (Yakhnina et al., 2015).

In a high-density transposon mutagenesis screening assay *P. aeruginosa* murein DD-endopeptidases have been identified as drug resistance determinants (Sonnabend et al., 2019). Indeed, it could be verified that the deletion of *mepM1*, *mepM2*, *mepM3*, *mepH1* and *mepH2* reduces MIC values of the β -lactam antibiotics meropenem, imipenem, cefepime, ceftazidime, piperacillin/ tazobactam and aztreonam. The codeletion of *mepM1*, *mepM2* and *mepM3* reduces resistance synergistically and lowers the MIC values below the breakpoints according to the current EUCAST Clinical Breakpoint Tables for all the antibiotics mentioned except for imipenem. This can partially be explained by a reduction of *ampC* expression (Kemper, 2024, Sonnabend et al., 2019).

Table 1: Murein DD-endopeptidases in *P. aeruginosa*

Name	Protein ID	Family	Regulated by	Relation to
MepM1	PA0067	LytM/M23	CtpA	<i>E. coli</i> MepM
MepM2	PA3787	LytM/M23		
MepM3	PA4404	LytM/M23	CtpA	
MepH1	PA1199	NlpC/P60	CtpA	<i>E. coli</i> MepS, MepH
MepH2	PA1198	NlpC/P60	CtpA	<i>E. coli</i> MepS, MepH

1.4.3 Structure and domains of murein DD-endopeptidases in *P. aeruginosa*

Analysis of the protein sequences with InterProScan (Jones et al., 2014) from InterPro (Mitchell et al., 2019) by Fabian Renschler assign MepM1, MepM2 and MepM3 to the family of LytM/M23 peptidases. MepH1 and MepH2 are NlpC/P60 peptidases. Comparably in *E. coli* MepM is a LytM/M23 peptidase and MepS and MepH are NlpC/P60 peptidases (Singh et al., 2012). Members of the LytM/M23 family are zinc metalloendopeptidases (Mistry et al., 2021). These enzymes cleave peptidoglycan cross-links in various bacteria and are crucial in processes like cell division or maintaining cell shape (Bonis et al., 2010, Singh et al., 2012).

Additionally, MepM1 contains an OapA domain, which has been demonstrated to interact with peptidoglycan in the *E. coli* protein YtfB (Burke et al., 2013). OapA domains are linked to other domains that are involved in the cleavage of peptidoglycan cross-links (Jorgenson and Young, 2018). MepM2 carries a signal peptide for the Sec translocon for its transportation across the cytoplasmic membrane and MepM3 carries a transmembrane helix.

The available X-ray crystal structure of M23 from *P. aeruginosa* (PDB ID: 2HSI at resolution of 1.90 Å (Ragumani et al., 2008)) and the use of a homology model enabled the prediction of potential quaternary structures of MepM1 and MepM2 that are illustrated in Fig. 5 (A). Computational druggability studies by Thales Kronenberger, University Hospital Tübingen, surprisingly predicted high similar active sites of MepM1 and MepM2 that form druggable pockets, Fig. 5 (B and C). Subsequent *in silico* docking with Gly₄Phosphinate, a known inhibitor of *S. aureus* LytM (Grabowska et al., 2015) and molecular dynamics simulation demonstrate a stable interaction between the phosphinate group and the zinc ion of the available crystal structure of MepM2 during the 500 ns simulation period.

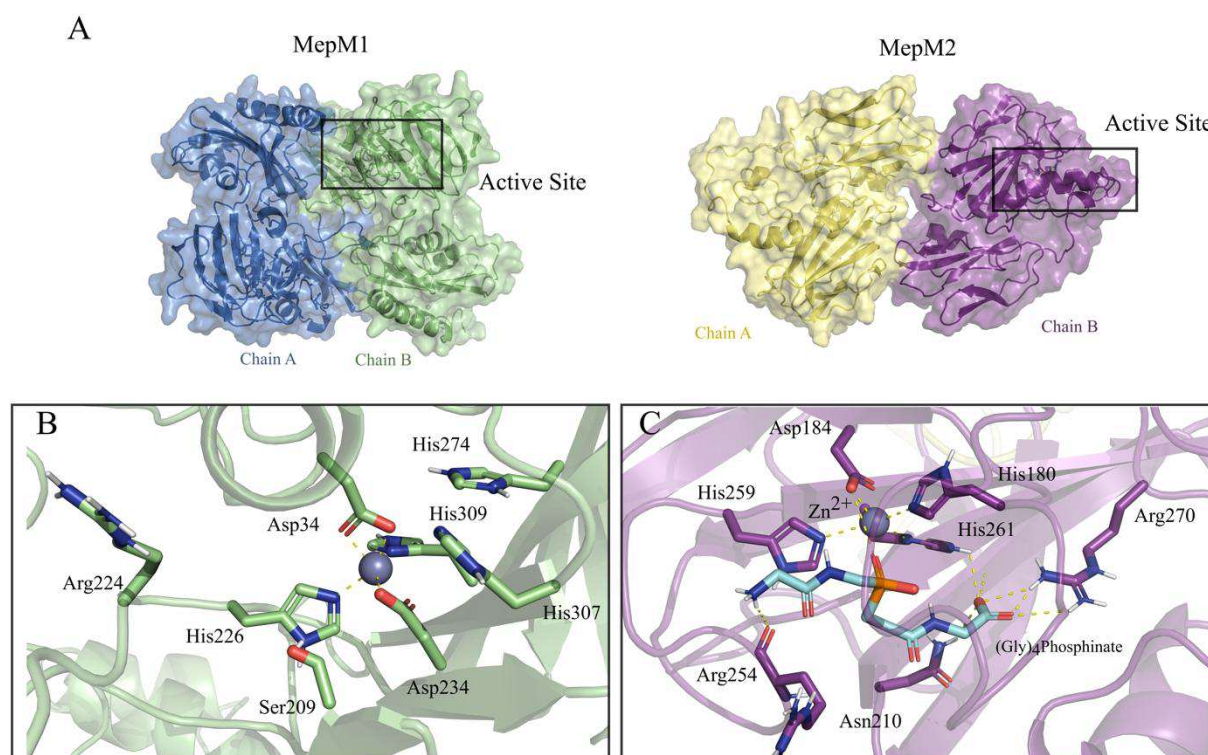


Figure 5: Quaternary structure of *P. aeruginosa* MepM1 and paMepM2

(A) Quaternary structure of MepM1, result from a homology model, and MepM2 (PDB ID: 2HSI), both structures are proposed to work as dimers with MepM1 relying on the flexible loop to integrate the dimerization interface and MepM2 showing a rather smaller interface. Despite the overall dissimilar structure, both active sites are highly conserved among them, with a catalytic Zinc ion coordinated by histidine and aspartate amino acids (structural water molecules are omitted). Snapshots from the molecular dynamics simulation show stable pockets for both MepM1 (B) and for the MepM2 complexed with the potential inhibitor Gly₄Phosphinate (C). Figure and description provided by Fabian Renschler

1.5 Aim of this work

P. aeruginosa is a harmful pathogen especially for immunocompromised and hospitalized patients. Its increasingly developing resistance and the lack of new antibiotic substances makes MDR *P. aeruginosa* a serious health threat. Given these challenges, innovative therapeutic approaches are of great importance. By targeting non-essential enzymes, that become essential in the presence of β -lactam antibiotics, these well-known and well-tolerated β -lactam antibiotics may be used effectively again for treating *P. aeruginosa* infections.

Promising targets are the murein DD-endopeptidases MepM1, MepM2 and MepM3, as their deletion synergistically breaks resistance against many β -lactam antibiotics partly due to a decrease in *ampC* expression. Additionally, MepM1 and MepM2 share the same active site making it possible to target both enzymes at the same time with one inhibitor. First potential MepM inhibitors were identified in virtual screening studies by Thales Kronenberger using commercially available compound libraries and docking followed by molecular dynamics simulation.

Therefore, specific objectives of this work are

- (i) To uncover further resistance mechanisms and gain a better understanding of the general function of MepMs in *P. aeruginosa*. This will be achieved through investigations of AmpC activity and possible changes in the membrane proteome due to *mepM* deletion
- (ii) To purify recombinant MepM proteins for (iii) and to produce antibodies, enabling MepM detection *in vivo* and thereby supporting the first objective
- (iii) To establish a high-throughput MepM activity assay allowing for potential MepM inhibitor screening using the purified proteins (ii)
- (iv) To test whether first potential commercially available MepM inhibitors can bind to recombinant MepMs and inhibit their enzymatic activity

2. Material and Methods

2.1 Materials

2.1.1 Bacteria

Table 2: *P. aeruginosa* strains used in this work

Bacterium	Description	Source
<i>P. aeruginosa</i>		
ID40	multi-resistant clinical isolate from a blood stream infection	(Willmann et al., 2018)
ID40 Δ mepM1	in-frame deletion mutants of ID40	(Sonnabend et al., 2020)
ID40 Δ mepM2		(Kemper, 2024)
ID40 Δ mepM3		
ID40 Δ mepMH1/2		
ID40 Δ mepM1 Δ mepM2		
ID40 Δ mepM1 Δ mepM3		
ID40 Δ mepM1 Δ mepM2 Δ mepM3		
ID40 Δ ctpA		
ID40 Δ ctpA Δ mepM1 Δ		
ID40 Δ ctpA Δ mepM1 Δ mepM3		
ID40 Δ mepM1:: <i>rha</i> -mepM1 Δ mepH1/2	in-frame deletion mutants of ID40 with <i>mepM1</i> complementation	generated in this work
ID40 Δ mepM1:: <i>rha</i> -mepM1 Δ mepM3 Δ mepH1/2		
ID40:: <i>mexE</i> -HiBiT	ID40 and deletion mutants of ID40 with HiBiT-tagged <i>mexE</i> or <i>mexY</i>	generated by my working group
ID40:: <i>mexY</i> -HiBiT		
ID40 Δ mepM1 Δ mepM2 Δ mepM3:: <i>mexE</i> -HiBiT		
ID40 Δ mepM1 Δ mepM2 Δ mepM3:: <i>mexY</i> -HiBiT		
ID40 Δ mexE/ <i>mexF</i> / <i>oprN</i>		
ID40 Δ mepM1 Δ mepM2 Δ mepM3 Δ mexE/ <i>mexF</i> / <i>oprN</i>	In-frame deletion mutants of ID40	

Table 3: *E. coli* strains used in this work

Bacterium	Description	Source
<i>E. coli</i>		
SM10 λ pir	<i>E. coli</i> donor strain for horizontal gene transfer	(Hmelo et al., 2015)
BL21 DE3	Competent cells for protein expression	New England BioLabs (NEB)

2.1.2 Plasmids

Table 4: Plasmids used in this work

Plasmid	Use	Selection features	Source
pEXG2_Δ <i>mepM3</i>	in-frame deletion of <i>mepM3</i> (flanking regions)	gentamicin resistance, <i>sacB</i> gene for sucrose counterselection	(Kemper, 2024)
pColi_His-3C- <i>paMepM1</i>	Low copy number plasmid for protein overexpression under a lactose/ IPTG promotor	ampicillin resistance	created by Fabian Renschler using pColi-His-3C plasmid backbone (Weir Lab, MPI for Development Biology)
pColi_His-3C- <i>paMepM2</i>			
pColi_His-3C- <i>paMepM3</i>			

2.1.3 Primer

Table 5: Primer used in this work

Primer	Use	Sequence (5' to 3')
MepM3_seq_f	verification of <i>mepM3</i> deletion	CATTTCGGCGATCTTGCC
MepM3_seq_r		CAACTGGGTCGATGCCTG
MepM3_inside_r		GGATGGCCGGGTAATGAATC

2.1.4 Antibodies

Table 6: Antibodies used in this work

Primary antibody	Concentration in Western Blots	Origin
rabbit α-PlpD (PAO1)	1:10000	(Klein et al., 2019)
rabbit α-SurA (PA14)	1:200	(Klein et al., 2019)
rabbit α-RpoB	1:2000	Abcam
rabbit α-MepM1	1:1000	Eurogentec (generated in this work)
rabbit α-MepM2	1:1000	Eurogentec (generated in this work)
Secondary antibody		
POX goat-F(ab') ₂ α-rabbit IgG (H+L), HRP	1:5000	Thermo Fisher Scientific

2.1.5 Enzymes, substrates and compounds

Table 7: Enzymes, substrates and compounds used in this work

Enzyme/ compound	Manufacturer/ Supplier	Description
rMepM1	Purified in this work	<i>P. aeruginosa</i> enzyme overexpressed in <i>E. coli</i>
rMepM2	Purified in this work	<i>P. aeruginosa</i> enzyme overexpressed in <i>E. coli</i>
Quenched fluorescence substrate IIIId	Intavis Peptide Services GmbH, Tübingen	Quenched fluorescence substrate for high throughput rMepM activity assay, designed by Fabian Renschler
MepMi_1 – 5	Enamine, Kyiv	Potential inhibitors of rMepM1 and rMepM2, selected through in silico analysis by Thales Kronenberger
Lysozyme from white chicken egg	Merck, Darmstadt	

2.1.6 Culture media

Table 8: Culture media used in this work

Culture medium	Components	Used for
Lysogeny Broth medium (LB), Miller's modification	10 g tryptone 5 g yeast extract 10 g NaCl ddH ₂ O ad 1 L	General liquid culture medium
LB glycerol 20% (v/v)	100 mL glycerol 400 mL LB	Cryopreservation of bacterial cultures
Lysogeny Broth (LB) agar, Miller's modification	10 g tryptone 5 g yeast extract 10 g NaCl 15 g agar ddH ₂ O ad 1 L	General solid culture medium
Selective LB agar (75 µg/mL gentamicin, 25 µg/mL irgasan)	3.75 mL gentamicin (10 µg/µL) 500 µL irgasan solution (25 µg/µL) LB agar ad 500 mL	Solid culture medium for allelic exchange: positive selection of mutants that have integrated the plasmid genome (first crossover)
Non-selective-lysogen-broth (NSLB) sucrose agar	10 g tryptone 5 g yeast extract 5 g NaCl 15 g agar 150 g sucrose ddH ₂ O ad 1 L	Solid culture medium for allelic exchange: positive selection of mutants that fulfilled second crossover

Terrific Broth (TB) auto induction medium, Carl Roth, pH 7.0	12 g Tryptone 24 g Yeast Extract 0.15 g MgSO ₄ - 7.1 g (NH ₄) ₂ SO ₄ - 6.5 g KH ₂ PO ₄ - 7.1 g Na ₂ HPO ₄ - 0.5 g glucose 2 g lactose ddH ₂ O ad 1 L	Autoinduction medium for protein overexpression in <i>E. coli</i>
Mueller Hinton II (MH) Broth Micronaut	BioTrading Benelux B.V.	Liquid culture medium for Microbroth Dilution Assay

2.1.7 Buffers

pH values (unless otherwise described) were adjusted with HCl or NaOH.

Table 9: Buffers used in this work

Buffer	Components	Source/ reference
5 x TRIS-Borat-EDTA (TBE) electrophoresis buffer	54 g Trizma base 27.5 g boric acid 20 mL EDTA (0,5 M, pH = 8) ddH ₂ O ad 2 L	
0.5 x TBE electrophoresis buffer		1:10 dilution of 5 x TBE
Buffer K, 4 °C	50 mM triethanolamine 250 mM sucrose 1 mM EDTA pH = 7,5 at 4 °C adjusted with acetic acid (HAc) ddH ₂ O ad 500 mL	(Zilkenat et al., 2017)
Buffer 2xM	100 mM triethanolamine 2 mM EDTA pH = 7,5 adjusted with acetic acid (HAc) ddH ₂ O ad 250 mL	(Zilkenat et al., 2017)
Buffer 1xM	50 mM triethanolamine 1 mM EDTA	1:1 dilution of Buffer 2xM
5 x SDS running buffer	60.4 g Trizma base 288 g glycine 20 g SDS ddH ₂ O ad 2 L	
1 x SDS running buffer	200 mL 5 x SDS running buffer ddH ₂ O ad 1 L	
10 x Western Blot transfer buffer	60.4 g Trizma base 288 g glycine ddH ₂ O ad 2 L	

1 x Western Blot transfer buffer	100 mL of 10 x Western blot transfer buffer 200 mL methanol ddH ₂ O ad 1 L	
10 x TBS buffer	24.2 g Trizma base 180 g NaCl pH = 7,4 DI-H ₂ O ad 2 L	
TBS-T1%	100 mL 10 x TBS buffer 1 mL Tween-20 ddH ₂ O ad 1 L	
Lysis buffer (rMepM1, rMepM2, rMepM3), 4°C	20 mM Tris 300 mM NaCl 10% Glycerol 20 mM Imidazole pH = 8	
Elution/ Enzyme buffer (rMepM1), 4°C	20 mM Tris 300 mM NaCl 10% Glycerol pH = 8	
Elution/ Enzyme buffer (rMepM2, rMepM3), 4°C	1 x PBS pH = 8	
Immunization buffer (rMepM1), 4°C	20 mM Tris 300 mM NaCl 3% Glycerol pH = 8	
Immunization buffer (rMepM2), 4°C	1 x PBS, pH = 8	
FITC PG labeling buffer	0.5 M NaHCO ₃ pH = 9 – 9,3	
MepM activity assay reaction buffer	50mM Tris pH = 8	
Laemmli-buffer 4x with β-mercaptoethanol	900 μL 4x Laemmli buffer 100 μL β-mercaptoethanol	
Blueblock PF (10x)		SERVA Electrophoresis GmbH, Heidelberg

2.1.8 Chemicals

Table 10: Chemicals used in this work

Chemical	Manufacturer
Acetic acid CH ₃ COOH	Merck, Darmstadt
acetone	Merck, Darmstadt
Agar	Roth, Karlsruhe
Agarose powder	Lonza, Rockland (USA)
Ampicillin Sodium Salt BioChemica	AppliChem, Darmstadt

Boric acid	PanReac AppliChem, Darmstadt
Bradford reagent	SERVA Electrophoresis GmbH, Heidelberg
Coomassie Brilliant Blue G-250	Serva Electrophoresis, Heidelberg
Dimethyl sulfoxid (DMSO)	Sigma-Aldrich, Taufkirchen
Dithiothreitol (DTT)	PanReac AppliChem, Darmstadt
DNase I recombinant, RNase-free	Roche, Rotkreuz (CH)
DPBS (Gibco)	Thermo Fisher, Schwerte
Ethanol absolut ≥99.8%, AnalaR NORMAPUR	VWR, Radnor (US)
Ethylenediaminetetraacetic acid (EDTA)	Merck, Darmstadt
Fluorescein isothiocyanate (FITC)	Sigma-Aldrich, St. Louis (US)
Gene ruler 1 kb Plus DNA Ladder	Thermo Fisher Scientific, Schwerte
Gentamicin sulfate	PanReac AppliChem, Darmstadt
Glucose	Merck, Darmstadt
Glycerol, SOLVAGREEN ≥98 %	Carl Roth, Karlsruhe
Glycin, PUFFERAN ≥99 %, p.a.	Carl Roth, Karlsruhe
Hydrochloric acid (HCl)	Merck, Darmstadt
Imidazole, ReagentPlus, 99%	Sigma-Aldrich, St. Louis (US)
Irgasan	Sigma-Aldrich, St. Louis (US)
L-Rhamnose Monohydrat, suitable for microbiology, ≥99.0%	Sigma-Aldrich, St. Louis (US)
4x Laemmli sample buffer	Bio-Rad, Hercules (USA)
Magnesium chloride (MgCl ₂)	Applichem, Darmstadt
MangoMix PCR reaction mix	Bioline, London (UK)
Sodium chloride (NaCl), 99.5-100.5%, AnalaR NORMAPUR	VWR, Radnor (US)
Sodium hydroxide (NaOH)	Merck, Darmstadt
PageRuler™ Prestained Protein	Thermo Fisher Scientific, Schwerte
Peptidoglycan from <i>B. subtilis</i>	Marina Borisova-Mayer
Peptidoglycan from <i>E. coli</i> , FITC-labeled	Manuel Banzhaf
Protease inhibitor cocktail complete	Sigma-Aldrich, Taufkirchen
Sodium dodecyl sulfate (SDS)	Carl Roth, Karlsruhe
Sodium bicarbonate (NaHCO ₃)	Sigma-Aldrich, St. Louis (US)
β-mercaptoethanol	AppliChem, Darmstadt
Sucrose	Merck, Darmstadt
SYBR Safe DNA gel stain (0,01%)	Thermo Fisher Scientific, Schwerte
Triethanolamine	Sigma-Aldrich, St. Louis (US)
Tris(hydroxymethyl)aminomethan (Tris-base), Trizma Base, Primary Standard and Buffer, ≥99.9% (titration), crystalline	Sigma-Aldrich, St. Louis (US)
Triton X-100 for molecular biology	Sigma-Aldrich, St. Louis (US)
α-lactose	Sigma-Aldrich, St. Louis (US)
Water deionized (ddH ₂ O)	In-house water line
Tween 20	Sigma-Aldrich, St. Louis (US)

2.1.9 Commercial Kits

Table 11: Commercial Kits used in this work

Kit	Manufacturer
Beta-Lactamase Activity Kit	BioVision, Milpitas (USA)
Clarity Western ECL Substrate	BIO-RAD
DNeasy® UltraClean®	Qiagen, Hilden
Nano-Glo® HiBiT Blotting System	Promega
Nano-Glo® HiBiT Lytic Detection System	Promega
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, Schwerte

2.1.10 Consumables

Table 12: Consumables used in this work

Consumable	Manufacturer
Acrodisc 25 mm w/0.2 µm Supor STRL	Pall Corporation, New York (US)
Amicon Ultra 15 ml Centrifugal Filters (cutoff: 10 kDa or 30 kDa)	Merck Millipore, Darmstadt
BD Plastipak, Syringe with Luer-Lok adapter	Becton Dickinson, Franklin Lakes (US)
Blotting paper	Bio-Rad, München
Combitips plus (10 mL)	Eppendorf, Hamburg
Cryotubes	Thermo Fisher Scientific, Schwerte
Cuvettes, UV-Vis	Sarstedt, Nümbrecht
Dialysis membrane ZelluTrans, MWCO 3500	Carl Roth, Karlsruhe
Falcon tubes conical 15 mL	Greiner Bio-One, Frickenhausen
Falcon tubes conical 50 mL	Corning Life Sciences, Corning (USA)
Glass bottles (Duran glass, 50 – 2000 mL)	DWK Life Sciences, Mainz
Glass spatula	WU, Mainz
Gloves (Peha-soft nitrile)	Hartmann Heidenheim
Inoculation loop	Greiner Bio-One, Frickenhausen
Injekt, Luer Solo (2 – 20 mL)	B. Braun, Melsungen
Kimtech Precision wipes	Kimberly-Clark, Dallas (USA)
Microtiter 384 well plate, F-bottom, black	Greiner Bio-One, Frickenhausen
Microtiter 96 well plates, F-bottom, clear/ black/ white	Greiner Bio-One, Frickenhausen
Ni ²⁺ -NTA-agarose beads	Qiagen, Hilden
Parafilm	Bemis, Neenah (US)
PCR reaction tubes (0.2 ml), 8-tube strip	Biozym Scientific GmbH, Oldendorf
Petri dishes	Greiner Bio-One, Frickenhausen
Pipette tips (10 µL)	Brand, Wertheim
Pipette tips (1000 µL)	Greiner Bio-One, Frickenhausen
Pipette tips (200 µL)	Sarstedt, Nümbrecht
Reaction tubes Safe-Lock (1.5 ml, 2 ml)	Eppendorf, Hamburg

Round bottom tubes 14 ml	Corning Life Sciences, Corning (US)
Scalpel	B. Braun, Melsungen
Scalpels (surgical, disposable)	Braun, Melsungen
Screw caps for cryotubes	Sarstedt, Nümbrecht
SDS gels (gradient, 4-20%)	Bio-Rad, München
Sealing foil, gas-permeable	Thermo Fisher Scientific, Waltham (US)
Sensititre Gram Negative EUX2NF AST Plate	Thermo Fisher Scientific, Waltham (US)
Sensititre Gram Negative GN2F AST Plate	Thermo Fisher Scientific, Waltham (US)
Steritop 45 mm Neck Size, Millipore Express PLUS 0.22 µm	Merck Millipore, Darmstadt
Steritop™ Filter Units (0.22 µm pore size)	Merck, Darmstadt
Western blotting membranes Amersham Protran 0.45 NC nitrocellulose	Cytivia, Marlborough (US)
Whatman Gel Blotting Paper	GE Healthcare, Chicago (US)
ZelluTrans dialysis tube 3,4 kDa cutoff	Roth

2.1.11 Instruments

Table 13: Instruments used in this work

Instrument	Manufacturer	Used for
ÄKTAprime Plus	Cytivia, Marlborough (US)	Protein purification
Analytic Balance Competence CPA225D-0CE	Sartorius, Göttingen	Weighing
Balance Precision EG 4200-2NM	Kern, Balingen	Weighing
BioPhotometer	Eppendorf, Hamburg	Measurement of OD _{600nm}
Centrifuge 5417 R	Eppendorf, Hamburg	Centrifugation
Centrifuge 5810 R	Eppendorf, Hamburg	Centrifugation
Centrifuge Avanti J-26 XP (rotor: JLA 8.1000)	Beckman Coulter, Brea (US)	Harvest of bacteria
Centrifuge Optima XPN-80 (rotor: 45 Ti or SW 41 Ti)	Beckman Coulter, Brea (US)	Centrifugation
Columns Econo Chromatography	Bio-Rad, Hercules (US)	Protein purification
DensiChek plus	Biomerieux, Marcy-l'Étoile (FR)	Microbroth Dilution Assay
Freezer G 5216 Index 21B/001	Liebherr, Bulle (CH)	Storage of samples
Freezer Hera (-20°C)	Thermo Fisher Scientific, Waltham (US)	Storage
Freezer Hera HFU T Series (-80°C)	Thermo Fisher Scientific, Waltham (US)	Storage
French Press Cell Disrupter	Thermo Fisher Scientific, Waltham (US)	Membrane isolation

Fridge KT 1840 Index 24A/001	Liebherr, Bulle (CH)	Storage of samples
Gradient station	Biocomp, Fredericton NB (Canada)	Membrane isolation
Heating block Thermomixer comfort	Eppendorf, Hamburg	Heating and mixing
HiLoad 16/600 Superdex 200 pg	Cytivia, Marlborough (US)	Protein purification
HiLoad 26/600 Superdex 75 pg	Cytivia, Marlborough (US)	Protein purification
Ice machine AF 156 Xsafe	Scotsman, Vernon Hills (US)	Production of ice
Imaging chemiluminescence Fusion Solo S system	Vilma Lourbat, Eberhardzell	Visualization of SDS-PAGE gels
Imaging System FastGene FAS-V	Nippon Genetics, Düren	Visualization of agarose gels
Incubator Function Line	Heraeus, Hanau	Incubation of bacteria on agar plates
Laminar flow cabinet BDK-S 1200, 1300	Weiss, Sonnenbühl	Sterile working conditions
Magnetic stirrer MR3001 K	Heidolph, Schwabach	Stirring
Microwave MW7775	Severin Elektrogeräte GmbH, Sundern	Heating, boiling
Mini Trans-Blot Cell	Bio-Rad, Hercules (US)	SDS-PAGE
Mixing block MB-102	BIOER, Hangzhou (China)	Mixing
Nanodrop One	Thermo Fisher Scientific, Waltham (US)	Photometric quantification of proteins/ nucleid acids
Orbital shaker SU1000	Sunlab Instruments, Mannheim	Mixing
pH electrode LE438	Mettler Toledo GmbH, Gießen	pH adjustment
pH meter FiveEasy F20	Mettler Toledo GmbH, Gießen	pH adjustment
Pipette Multipipette plus	Eppendorf, Hamburg	Pipetting, FITC assay
Pipette VIAFLO 12 Channel 5-125 µl	Integra Biosciences, Zizers (CH)	Pipetting, Ild Assay
Pipettes Research plus	Eppendorf, Hamburg	Pipetting
Pipetus	Hirschmann Laborgeräte, Eberstadt	Pipetting, protein purification
PowerPac 300	Bio-Rad, Hercules (US)	SDS-PAGE
Rotor 45 Ti	Beckman Coulter, Brea (US)	Membrane isolation
Rotor Avanti JLA 8.1000	Beckman Coulter, Brea (US)	Harvest of bacteria
Rotor SW 41 Ti	Beckman Coulter, Brea (US)	Membrane isolation
Shaking incubators Ecotron, Multitron and Minitron	Infors HT, Bottmingen	Incubation of bacteria in liquid culture
Sonifier 250	Branson Ultrasonics, Brookfield (US)	Cell lysis
Sub Cell and Mini-Sub Cell GT System	Bio-Rad, Hercules (US)	Agarose gel electrophoresis

Tecan Infinite M Plex	Tecan, Männedorf (CH)	Microplate reader
Tecan Infinite® 200 PRO plate reader	Tecan, Männedorf (CH)	Microplate reader
Thermocycler C1000 Touch	Bio-Rad, Hercules (US)	PCR
Vacuum pump CVC 2000	Vacuumbrand, Wertheim	Protein purification
Vortex Genie 2	Thermo Fisher Scientific, Waltham (US)	Vortexing
Waterbath WB10	Memmert, Schwabach	Heating
Wheel of fortune neoLab Line Rotator	NeoLab Migge, Heidelberg	Mixing

2.1.12 Software

Table 14: Software used in this work

Software	Manufacturer
Adobe Acrobat Illustrator	Adobe Systems Inc., San José (USA)
Adobe Photoshop	Adobe Systems Inc., San José (USA)
EndNote	Clarivate, Philadelphia (USA)
GraphPad Prism	GraphPad Software, Inc., San Diego (USA)
I-control for Tecan infinite reader	Tecan, Crailsheim
Microsoft Office Word, Excel and Powerpoint	Microsoft Corporation, Redmond (USA)
SnapGene	GSL Biotech, San Diego (USA)
DeepL	DeepL SE, Cologne, Germany

2.2 MepM target validation methods

2.2.1 Cultivation of bacteria

Unless otherwise described, bacteria in liquid culture were incubated at 37 °C and with shaking at 200 rpm (liquid medium).

2.2.1.1 *Production of bacterial overnight cultures in liquid medium*

5 mL of liquid lysogeny broth (LB) in 14 mL round bottom tubes were inoculated with bacteria (*P. aeruginosa*, *E. coli*) from frozen cryocultures or bacteria growing on plates (mutagenesis) and were incubated overnight. The LB was supplemented with appropriate antibiotics if the bacteria carried a plasmid with an antibiotic resistance cassette, or with rhamnose 0.1% to activate the expression of genes under a rhamnose promotor.

2.2.1.2 *Production of bacterial subcultures in liquid medium*

Subcultures were prepared by diluting overnight cultures 1:20 in LB and incubation for two to four hours. The LB was supplemented with selective antibiotics or rhamnose if needed.

2.2.1.3 *Cultivation of bacteria on plates*

Bacteria were spread on LB plates or supplemented selective plates and plates were incubated overnight.

2.2.1.4 *Production of bacterial cryocultures*

5 mL of overnight culture was centrifuged at 4995 g for 5 min. The pellet was resuspended in 3 mL LB + 20% glycerol, which prevents ice crystallization in the cytoplasm of the cells during freezing. Aliquots of 1 mL were immediately stored at -80 °C.

2.2.1.5 *Photometric detection of total cell biomass*

The bacterial density of a culture was determined by photometric measurement of the absorbance at 600 nm (OD_{600nm}). For this purpose, the culture samples were diluted in cuvettes to obtain measured values between 0.1 and 1.

2.2.2 Mutagenesis and molecular genetic methods

2.2.2.1 Isolation of genomic DNA (gDNA)

gDNA was isolated from overnight cultures using DNeasy® UltraClean® Microbial Kit (QIAGEN) according to the manufacturer's protocol.

2.2.2.2 Quantification of DNA concentration

The DNA concentration in a sample was quantified photometrically using a NanoDrop Microvolume Spectrophotometer (Thermo Fisher).

2.2.2.3 Deletion of *mepM3* in *P. aeruginosa* ID40 by allelic exchange

The *mepM3* suicide plasmid pEXG2 (Fig. 6) generated by Kemper (2024) carries a truncated and non-functional version of *mepM3* designed for in-frame deletion of *mepM3* in the ID40 strain by allelic exchange. The gentamicin resistance cassette and a *sacB* site on the plasmid allow positive and negative selection of bacteria at different points of mutagenesis.

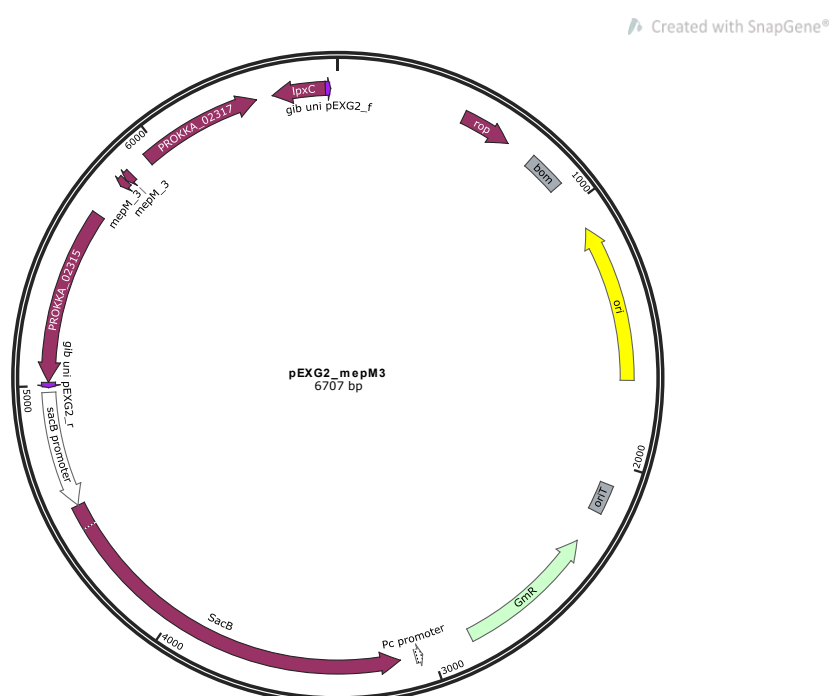


Figure 6: pEXG2 *mepM3* suicide plasmid

The plasmid carries a truncated and non-functional version of *mepM3*, which allows allelic exchange by crossover at homologous sequences. The gentamicin resistance cassette (GmR) and the *sacB* gene enable positive and negative selection of plasmid genome carrying bacteria.

Fig. 7 depicts the steps of the gene deletion. The underlying principle is shown in Fig. 8. The knock-out plasmid was transformed from *E. coli* SM10λpir (donor) into *P. aeruginosa* (acceptor) by conjugation. For this purpose, overnight cultures of plasmid carrying *E. coli* SM10λpir (200 µL) and *P. aeruginosa* ID40Δ*mepM1::rha-mepM1ΔmepH1/2* (400 µL) were combined and centrifuged for 1 min at 10000 g. The pellet was resuspended in 100 µL LB, placed as a droplet on a LB plate and incubated overnight. Bacteria were collected using an inoculation loop and resuspended in 2 mL LB. 100 µL and 20 µL were plated out on selective LB plates containing irgasan 20 µg/mL and gentamicin 75 µg/mL and were incubated overnight. This selected gentamicin resistant *P. aeruginosa* after first crossover from *P. aeruginosa* that have not integrated the plasmid genome and at the same time prevented growth of irgasan sensitive *E. coli*. Four single colonies were picked, each spread on non-selective LB plates and incubated overnight to allow a second crossover. A single colony was picked from each plate and grown overnight in 5 mL LB containing 22.5 % sucrose. The cultures were spread on non-selective-lysogen-broth plates (NSLB) containing 15% sucrose and incubated overnight. Levansucrase, encoded by *sacB*, hydrolyses sucrose and produces the toxic metabolite levan, which kills *P. aeruginosa* that have not performed the second crossover. To select potential deletion mutants, single colonies were picked and spread first on a LB plate containing 75 µg/mL gentamicin and second on a non-selective LB plate. Sucrose-resistant merodiploid *P. aeruginosa* grow on both plates. Growth on only the LB plate indicates a fulfilled second crossover. To distinguish wildtypes from mutants, colony PCR (2.2.2.4) of potential mutants was performed.

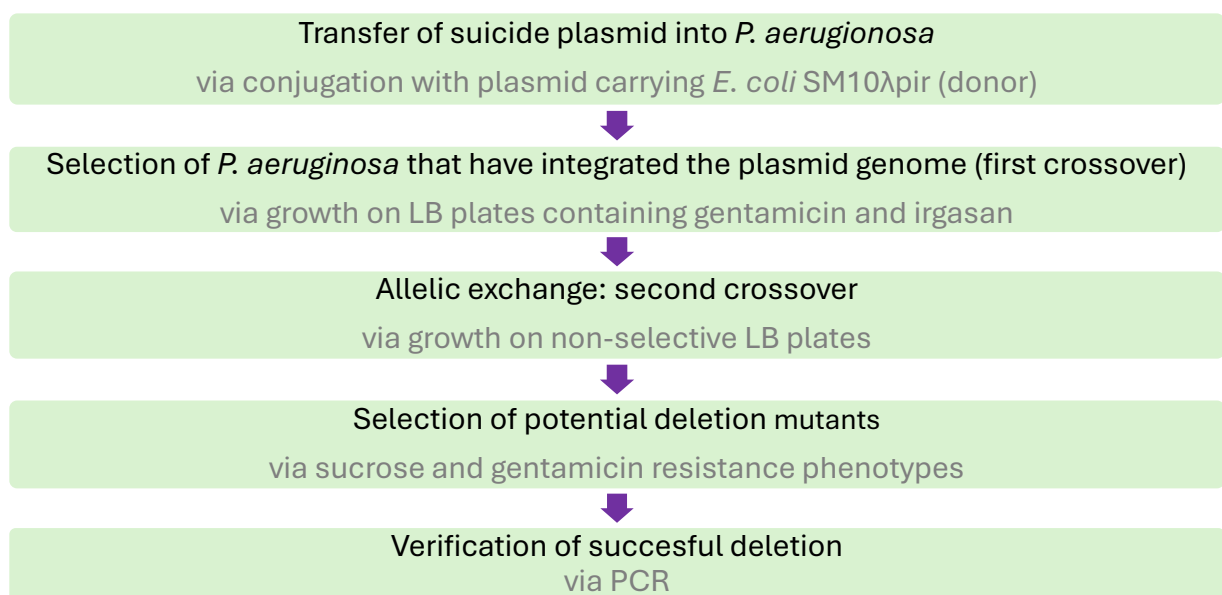


Figure 7: Steps of in-frame *mepM3* deletion by allelic exchange in *P. aeruginosa*

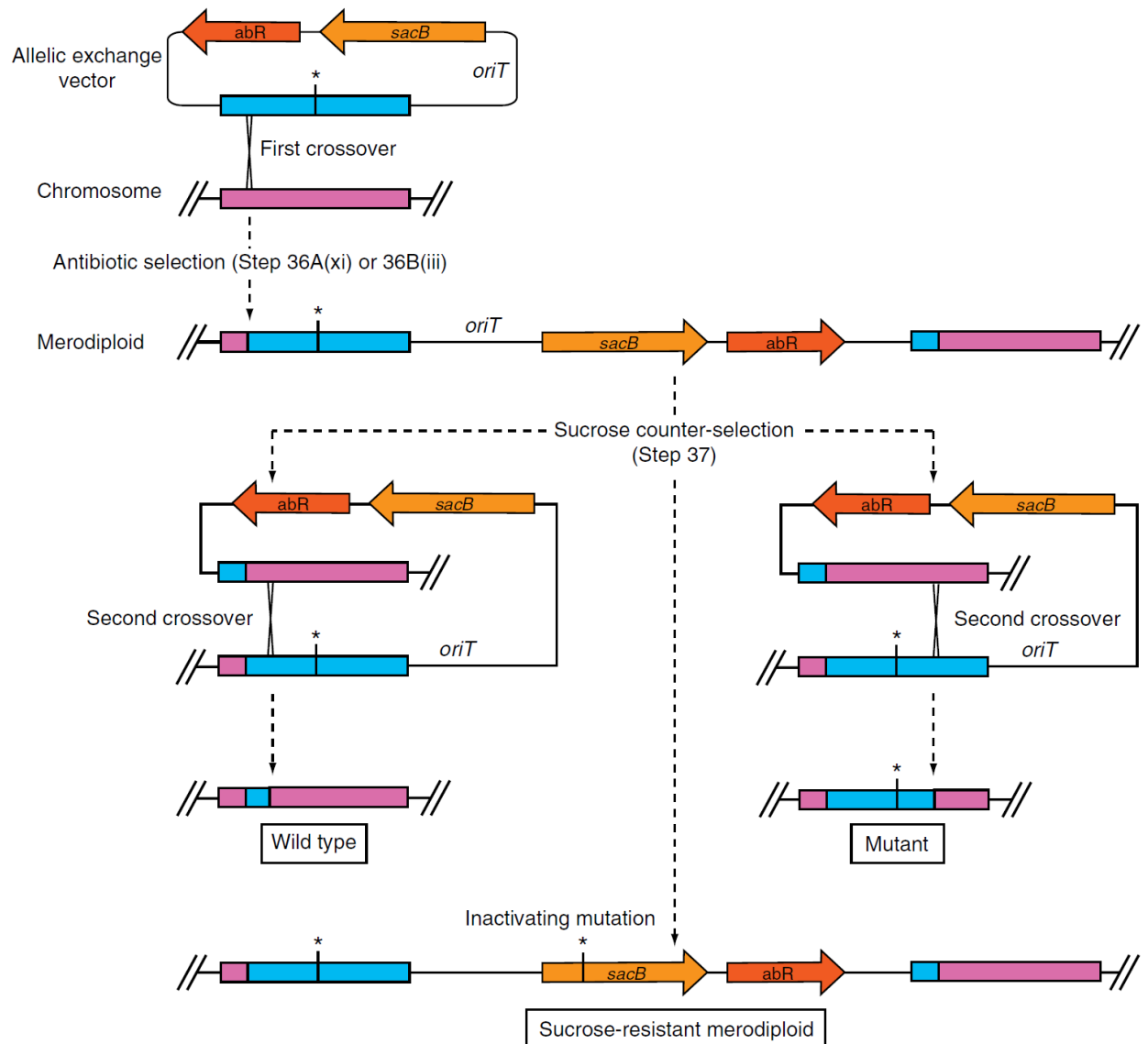


Figure 8: Allelic exchange principle (Hmelo et al., 2015)

The plasmid genome is integrated through a first crossover at homologous DNA sequences on the plasmid and the target gene. The antibiotic resistance cassette (*abR*) and the *sacB* gene allow the selection of bacteria at different points of allelic exchange.

2.2.2.4 Colony PCR of potential deletion mutants (results not shown)

To obtain genetic material, bacteria were picked from LB plates (2.2.2.3) and resuspended in 50 µL sterile ddH₂O. Bacteria were lysed by incubation at 95 °C for 5 min and centrifuged for 5 min at 5000 g. The supernatants were used as template DNA. Table 15 describes the composition of the reaction mixture per tube. Table 16 lists the applied thermocycler settings. The primers binding upstream and downstream of *mepM3* (outside PCR) amplified a 621 bp fragment from $\Delta mepM3$ mutant DNA, whereas PCR with ID40 wildtype DNA amplified a 1485 bp fragment. (Table 17)

Table 15: Composition of the PCR reaction mixture per tube

compound	volume per tube
MangoMix	7.5 µL
forward primer	0.5 µL (10 pmol)
reverse primer	0.5 µL (10 pmol)
template supernatant of lysed bacteria or gDNA (2.2.2.5)	3 µL or 1 µL (20 - 40 ng)
sterile ddH₂O	adjust to 15 µL

Table 16: Thermocycler settings for the PCR reaction

step	temperature	time	cycles
Initial denaturation	95 °C	5 min	
Denaturation	95 °C	1 min	30 x
Annealing	see table 17	30 s	
Extension	72 °C	(30 s/ kb fragment)	
Final extension	72 °C	5 min	
Hold	4 °C	∞	

2.2.2.5 PCR of potential deletion mutants with isolated gDNA

To verify a successful deletion of *mepM3*, PCR was repeated with isolated gDNA from potential mutants.

gDNA was diluted in ddH₂O adjusting a concentration of 20 – 40 ng in 1 µL. The PCR with outside primers (described in 2.2.2.4) was supplemented by a second PCR with primers that bind inside and outside the target gene (inside PCR). Table 17 summarizes the primers used, annealing temperatures, and expected lengths of the amplified fragments. The PCRs were performed as described in 2.2.2.4 with 1 µL gDNA as template instead of 3 µL supernatant.

Table 17: Primers and amplified fragments in PCR verifying *mepM3* deletion

Two primer pairs were used that bind inside or outside the *mepM3* gene locus.

PCR reaction	Forward Primer	Reverse Primer	Annealing temperature	Fragment from wildtype gDNA	Fragment from mutant gDNA
outside PCR	MepM3_seq_f 975	MepM3_seq_r 976	54°C	1485 bp	621 bp
inside PCR	MepM3_seq_f 975	MepM3_inside_r 977	54°C	497 bp	no fragment

2.2.2.6 Agarose gel electrophoresis

The length of the fragments amplified in the PCR was determined by agarose gel electrophoresis.

For gel preparation 1 mg agarose powder was dissolved in 100 mL 0.5% TBE buffer while heating in the microwave. The addition of 0.05 µL SYBR Safe per mL buffer stained the DNA and allowed visual detection of DNA fragments in the gel. 5 µL of DNA marker and 10 µL of PCR product were added per pocket. Negatively charged DNA fragments were separated by applying a constant voltage of 110 mV for 50 min in 0.5% TBE buffer. Stained DNA fragments were visualized and captured with FAS-V documentation system.

2.2.3 Microbroth dilution assay generating antibiograms

Minimum inhibitory concentrations (MIC values) of ID40 and the deletion mutants were determined by microbroth dilution in microtiter plates (Sensititre™ EUX2NF and Sensititre™ GN2F (Thermo Fisher)).

The bacteria were grown overnight on LB plates. Colonies were homogenised in about 1 mL NaCl 0.9% adjusting a McFarland value of 0.5. 62.5 µL of the suspension was mixed with 15 mL Müller Hinten broth (MH broth). In the + rhamnose approaches, 0.1% rhamnose was added. The microtiter plates were filled with 50 µL per well, incubated at 37 °C for 18h and the OD_{600nm} was measured in a Tecan Infinite® 200 PRO plate reader. After subtracting the blank values (OD_{600nm} of a plate filled with sterile MH broth) from the measured OD_{600nm} values, wells with OD_{600nm} ≥ 0,05 were considered cultured. The lowest antibiotic concentration at which bacterial growth no longer occurred corresponded to the MIC value. According to the EUCAST MIC breakpoints, the strains were classified as sensitive or resistant to the respective antibiotic.

2.2.4 β-lactamase AmpC activity assay

The β-lactamase AmpC activity of ID40 and the deletion mutants was determined using the commercially available Beta-Lactamase Activity Assay Kit (BioVision) following the manufacturer's protocol.

The principle of the assay is based on the hydrolysis of the yellow substrate nitrocefin by beta-lactamase, producing a red product which can be detected photometrically at 490 nm.

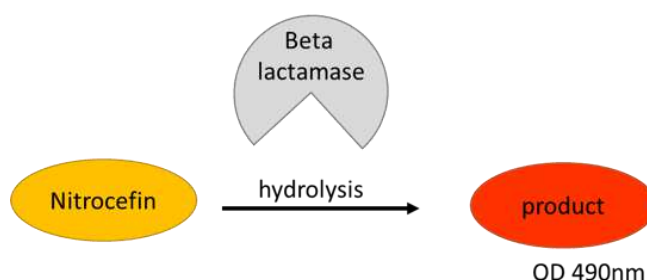


Figure 9: β-lactamase AmpC activity assay principle

AmpC hydrolyses the yellow substrate nitrocefin forming a red product which can be detected photometrically at 490 nm.

The manufacturers protocol was implemented as follows: 20 mL subculture was grown for 3 hours. The OD_{600nm} at harvest was measured. Bacteria were collected by centrifugation for 5 min at 4950 g and completely transferred to pre-weighted 1.5 mL Eppendorf caps. The bacteria were dried by three centrifugation steps (in total 13 min, 10000 g) and removal of the

supernatant. Dry pellets were weighed on a fine balance and resuspended in 20 µL assay buffer per mg pellet. Cells were lysed by sonication on ice for 5 min (1-2 min intervals) at 10% duty cycle and output control 1.3. To set the OD_{490nm} readings within the linear range of the nitrocefin standard curve, samples were prediluted 1:25 with assay buffer. 1 µL sample and 49 µL of assay buffer were mixed in a 96-well plate. The OD_{490nm} was measured every minute for 1 h at room temperature with a Tecan Infinite® 200 PRO plate reader.

For the analysis, an equation describing the linear course of the standard curve was created by simple linear regression analysis with GraphPad. Time intervals with linear enzyme activity ΔT (min) were defined for each strain. The absolute nitrocefin turnover B (nmol) in this time range was calculated by substituting the ΔOD_{490nm} in this time range into the equation of the standard curve. The β -lactamase activity was explained as nitrocefin turnover per min per mL sample volume in a time interval with constant substrate turnover per time (complete enzyme saturation).

$$\beta - \text{lactamase AmpC activity} = \frac{B}{\Delta T \times V} \times D$$

B	Nitrocefin turnover (nmol)
ΔT	time (min)
V	sample volume added to the reaction well (mL)
D	sample dilution factor

2.2.5 Membrane proteome analysis

2.2.5.1 Membrane protein isolation

For the first membrane protein purification, per strain 1 L LB medium was inoculated with bacteria from overnight cultures to adjust $OD_{600nm} = 0.05$ and incubated until $OD_{600nm} = 1$. Bacteria were harvested by centrifugation at 6000 g for 15 min with rotor JLA 8.1. The pellet was resuspended in 35 mL 1xPBS and centrifuged for 10 min at 8000 g and 4 °C. The pellet was resuspended in 15 mL buffer K supplemented with 10 µg/mL DNase, 1 mM EDTA, 20 µg/mL lysozyme, 1 mM $MgCl_2$ and complete protease inhibitor cocktail. Bacteria were mechanically lysed in the French pressure cell press (three circles at 1100 psi) and centrifuged for 20 min at 8000 g and 4 °C. From the supernatant, 1 mL sample (input ultracentrifuge = input UZ) was taken and stored at -80 °C for later SDS-PAGE analysis. It contains the entire membrane proteome. The remaining supernatant was centrifuged at 42000 rpm (rotor 45 Ti) and 4 °C for 45 min. From the supernatant, 1 mL sample (supernatant ultracentrifuge = supernatant UZ) was taken and stored at -80 °C for later SDS-PAGE analysis. It contains the soluble cytoplasmatic and periplasmatic proteins. The pellet containing the membrane proteins was homogenised in 500 µL buffer 1xM. A 30 – 53% sucrose gradient was prepared in Gradient station (Biocomp Instruments). The homogenised pellet (about 550 µL) was added completely on top of the sucrose gradient. The tube was centrifuged for 15 h at 41000 rpm and 4 °C (Rotor SW41 Ti, slow/slow). The separated membrane proteome was finally fractionated in the Gradient station (Biocomp Instruments).

The second membrane protein purification was performed likewise omitting the subsequent separation of the membrane proteins by the sucrose gradient and fractionation. Instead, triplicates of ID40 and ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ were produced. The samples from the first and the second membrane protein purification were further processed in the same way.

2.2.5.2 BCA Assay for determination of total protein concentration in the proteome samples

Total protein concentrations of the purified proteome samples were determined using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit according to the manufacturer's protocol. The assay principle is based on the reduction of Cu^{2+} to Cu^{1+} by proteins in alkaline medium. Bicinchoninic acid (BCA) chelates these copper cations (Cu^{1+}). The resulting BCA-copper complex can be detected photometrically at 562 nm.

2.2.5.3 SDS-PAGE of membrane proteins

To visualize the isolated membrane proteins, SDS-PAGE was performed.

60 µL of protein sample was mixed with 20 µL 4x Lämmli + β-ME and heated to 95 °C for 10 min. UZ-samples, frozen away during membrane isolation (2.2.5.1) were also processed in this way.

Depending on the total protein concentration (2.2.5.2), different sample volumes were loaded on a pre-cast 4-20% SDS gel adjusting the same amount of total protein per pocket. 10 µL of precast protein ladder was loaded as a marker for protein size. The proteins were separated at a constant voltage of 120 V for 60 min in 1x SDS buffer. The gel was washed in H₂O and stained in Coomassie blue. The stained proteins were visualized with Imaging chemiluminescence Fusion Solo S system.

2.2.5.4 Western Blot analysis (anti-RpoB, anti-PlpD and anti-SurA)

To verify successful purification and separation of inner and outer membrane proteins, Western Blots were performed with antibodies against periplasmatic (SurA), cytoplasmatic (RpoB) and outer membrane proteins (PlpD). For this, a pre-cast 4-20% SDS gel was loaded with the membrane proteome samples and SDS-PAGE was performed as described in 2.2.5.3, omitting the washing and staining of the gel. For electroblotting, a transfer sandwich was assembled in a gel holder cassette with the following components (from cathode to anode): sponge, transfer paper, gel, nitrocellulose membrane, transfer paper, sponge. The proteins were transferred to the nitrocellulose membrane applying a constant current of 0.35 A for 1 h at 4 °C with stirring at 100 rpm in transfer buffer.

The following steps were performed at room temperature (RT) on a seesaw. The antibody concentrations used are described in Table 18. The membrane was blocked in 10 mL 1x BlueBlock for 1 h, then the primary antibody was added and incubated for 1 h. The membrane was washed three times with 30 mL TBS-T1%. The secondary antibody was added in 10 mL 1x BlueBlock and was incubated for 1 hour. The membrane was washed three times with 30 mL TBS-T1% and finally with 30 mL 1x PBS. Blotting or antibody incubation was partially performed at 4°C overnight instead of for 1 h at RT. Bound antibodies were visualized using Clarity Western ECL Substrate (BIO-RAD) according to the manufacturer's protocol and Imaging chemiluminescence Fusion Solo S system.

Table 18: antibody concentrations for anti-RpoB, anti-SurA and anti-PlpD Western Blot analysis

primary antibody (AB)	primary AB concentration	secondary AB	secondary AB concentration
rabbit anti-RpoB	1:2000	POX goat anti-rabbit IgG	1:5000
rabbit anti-SurA	1:200		
rabbit anti-PlpD	1:10000		

2.2.5.5 Comparative mass spectrometric analysis of the membrane proteome of ID40 and ID40 Δ mepM1 Δ mepM2 Δ mepM3

Mass spectrometric analysis of the purified membrane samples was performed by the Proteome Center Tübingen. In the first approach, samples with inner membrane proteins (combined fractions 3 and 4) and samples with outer membrane proteins (combined fractions 7 and 8) from ID40 and ID40 Δ mepM1 Δ mepM2 Δ mepM3 were analyzed using dimethylation labeling (light: CH₂O; medium: CD₂O). LC-MS/MS analysis was performed on Proxeon Easy-nLC coupled to an Orbitrap Exploris 480 mass spectrometer (methods: 130 min, Top20, Fast). MaxQuant software (vs 1.6.14.0) was used to process of data and spectra were searched against a Uniprot *P. aeruginosa* database (5565 entries, downloaded 7th October 2020). Statistical downstream analysis was done using Perseus software (vs 1.6.14.0)

In the second approach the separation into inner and outer membrane proteins as well as dimethylation labeling was omitted. Instead, whole membrane proteome samples of ID40 and ID40 Δ mepM1 Δ mepM2 Δ mepM3 were analyzed in three independent replicates. Statistical analysis of the second approach was done by Ulrich Schoppmeier, Institute for Medical Microbiology and Hygiene, Tübingen. Gene and protein names, PA01 orthologs and predicted localization of the proteins were searched on <https://pseudomonas.com> and <https://www.uniprot.org>. Proteins that showed at least a 2-fold change in the normalized ratios and had an adjusted P value of at most 0.05 were considered altered.

2.2.6 Verification of mass spectrometry results

2.2.6.1 MexE- and MexY-HiBiT Western Blot

Whole cell lysates of ID40-HiBiT strains were prepared from subcultures: After measuring the OD_{600nm} (2.2.1.5), 1 mL subculture was centrifuged at 5000 g for 5 min. The cell pellet were resuspended in ddH₂O according to the following formula:

$$volume\ (\mu L) = \frac{OD_{600nm} \times 100}{2}$$

4x Lämmli + β -ME was added in a ratio of 1:1 and the sample was heated up to 95 °C for 10 min. A precast 4-20 % SDS gel was loaded with 10 μ L of whole cell lysate per pocket and 10 μ L precast protein ladder. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane as described in 2.2.5.3 and 2.2.5.4. The membrane was blocked in 10 mL 1x BlueBlock for 1 h at RT on a seesaw. Tagged proteins were detected with the Nano-Glo® HiBiT Blotting System (Promega) following the manufacturer's protocol.

2.2.6.2 *MexE- and MexY-HiBiT luciferase assay*

The Nano-Glo® HiBiT Lytic Detection System (Promega) allowed quantification of HiBiT-tagged protein levels (MexE and MexY) in whole cell lysates. It was implemented as follows: Overnight cultures of ID40-HiBiT strains were diluted adjusting OD_{600nm} of 0.05 in 6 mL LB and cultures were grown for 4 h. Bacteria were collected by centrifugation at 4995 g for 10 min and washed by resuspending in 1 mL PBS and centrifugation for 1 min at 10000 g. The pellet was resuspended in 1 mL PBS and OD_{600nm} was measured. The same number of bacteria per strain was transferred to a 1.5 mL Eppendorf cup according to the following formula:

$$volume (\mu L) = \frac{1000 \mu L}{OD_{600nm}}$$

After centrifugation (10000 g, 1 min) the supernatant was removed with a pipet tip. This step was repeated once. For cell lysis, the pellet was resuspended in 500 μ L buffer K supplemented with 10 μ g/mL DNase, 20 μ g/mL lysozyme, 1 mM MgCl₂, 1 mM EDTA, 0.5 % Triton-X 100 and complete protease inhibitor cocktail and was incubated on ice for 30 min. 50 μ L of cell lysate were filled in a white, flat-bottom 96-well plate in triplicates. Following the manufacturer's protocol, 50 μ L of lytic buffer supplemented with LgBit 1:100 and substrate 1:50 was added per well. The plate was shaken for 2 min and luminescence was detected in a Tecan Infinite® 200 PRO plate reader.

2.3 rMepM/ Biochemical methods

The protocol for purification of recombinant *paMepM1*, *paMepM2* and *paMepM3* was derived from the protocol of Singh et al. (2012) for *ecMepM*.

2.3.1 Protein overexpression and purification of His-tagged rMepM

His-tagged rMepM1, rMepM2 and rMepM3 were overexpressed in *E. coli* BL21 carrying His-*pamepM* under an inducible lactose/IPTG promotor on a plasmid (results, Fig. 30). Overexpressed proteins were purified by Nickel affinity purification and size exclusion chromatography.

2.3.1.1 Overexpression of rMepM1, rMepM2 and rMepM3

E. coli BL21 cryocultures carrying the pColi-His-3C-MepM overexpression plasmids (results, Fig. 30) were kindly generated by Fabian Renschler before the start of my work.

50 mL LB supplemented with 100 µg/mL ampicillin was inoculated with *E. coli* BL21 cryoculture and incubated overnight at 37 °C and 200 rpm shaking. For rMepM2, reducing agent DTT (1 mM) was added to the buffer during purification to prevent disulfide bond formation.

The preculture was centrifuged at 4995 g for 5 min and the pellet was resuspended in 50 mL Terrific Broth (TB) auto-induction medium supplemented with 0.5 g/L glucose and 2 g/L α -lactose. 1 L of supplemented TB was inoculated with the preculture adjusting an OD_{600nm} of 0.1 – 0.2. The overexpression culture was incubated at 37 °C and shaking at 180 rpm until reaching OD_{600nm} of 0.8. The temperature was lowered to 20 °C and a 0.5 mL sample “induction” was taken. The culture was incubated overnight. The next day a 0.5 mL sample “harvest” was taken. Bacteria were harvested by centrifugation at 6000 rpm (rotor JA 8.1000) and 6 °C for 10 min, transferred to a 50 mL Falcon tube and either processed directly (2.3.1.2) or stored at -20 °C for later purification.

The samples taken during overexpression and purification were immediately processed by centrifugation at 5000 g for 5 minutes. The supernatant was removed and the pellet was resuspended in 180 µL ddH₂O for cell lysis. 70 µL 4x Lämmli + β -ME were added and the sample was stored at -20 °C for later SDS-PAGE analysis.

2.3.1.2 Nickel affinity purification of rMepM1 and rMepM2

Overexpressed His-tagged protein was purified in two steps by nickel affinity purification and size exclusion chromatography. His-tagged proteins bind to the nickel beads and are thus separated from unbound proteins, which are washed off the column.

A gravity flow column was loaded with Ni^{2+} -NTA-agarose beads (Qiagen), washed with 4 column volumes (CV) ddH₂O and equilibrated with 4 CV lysis buffer.

The bacterial pellet (2.3.1.1) was resuspended in 35 mL with lysis buffer to which an eyelet of Triton X-100, lysozyme powder, DNase powder and complete protease inhibitor cocktail tablet were added. 10 μL of sample “total” was taken and mixed with 20 μL 4x Lämmli + β -ME for later SDS-PAGE analysis. This applies to all samples taken during nickel affinity purification. Sonication of the bacterial resuspension for 3 x 1 min (duty cycle 50%, output control 2.5) induced cell lysis. The sample “lysis” was taken. Insoluble cell components were removed by centrifugation for 30 min at 35000 g (rotor JA 14.5) and 6 °C. The supernatant containing the soluble proteins was filtered through a 0.22 μm filter and the sample “supernatant” was taken. The supernatant was incubated with the equilibrated beads for 15 min at 4 °C.

Subsequently, the fractions were collected under the column in individual Falcon tubes after each step and kept on ice. Samples were taken from collected fractions after each step for later SDS-PAGE analysis.

The column was opened and the sample “flowthrough” was taken. Unbound proteins were washed from the column with 1 CV lysis buffer (“wash 1”) and washing was continued with 5 CV lysis buffer (“wash 2”). The bound proteins were eluted with elution buffer (mixture of 15 ml 1 M Imidazole with 30 ml lysis buffer). The concentration of eluted protein was monitored by a colour change when mixing 10 μL collected from a droplet under the column with 100 μL Bradford reagent. When the protein concentration dropped, the subsequent flowthrough was collected in a new Falcon tube. Samples “elution 1” and “elution 2” were taken.

The collected samples were analyzed by SDS-PAGE (2.3.1.5).

2.3.1.3 Buffer change of rMepM1 and rMepM2 via dialysis

For further purification of the eluted proteins by size exclusion chromatography, the imidazole was removed by buffer change. Buffers also had to be changed to meet the required conditions for immunisation at Eurogentec to produce anti-MepM1 and anti-MepM2 antibodies.

The elutions (2.3.1.2) or protein samples for immunisation were dialyzed overnight using a ZelluTrans (Roth) dialysis tube with 3.4 kDa cutoff in 1 L dialysis buffer at 4 °C with stirring at 100 rpm.

2.3.1.4 Size exclusion chromatography of rMepM1 and rMepM2

In size exclusion chromatography, proteins are separated according to their molecular weight. The concentration of the eluted proteins is determined by UV detection.

Dialyzed elutions (2.3.1.3) were concentrated by various centrifugation steps (each 5 min, 4995 g, 6 °C) over a centrifugal concentration unit (30 kDa cut off for rMepM1, 10 kDa cut off for rMepM2). Concentration was stopped when the volume above the filter fell below 5 mL or protein precipitation occurred (rMepM1). Precipitated insoluble protein was pelleted by centrifugation for 10 min at 16000 g and 6 °C. From the supernatant, 20 µL sample “input” was taken and mixed with 10 µL 4x Lämmli + β-ME. The remaining supernatant was passed over the equilibrated size exclusion column (rMepM1: S200_16_600, rMepM2: S75_26_600).

From every second peak fraction, 20 µL sample was taken and mixed with 10 µL 4x Lämmli + β-ME. The samples were analysed by SDS-PAGE (2.3.1.5).

2.3.1.5 SDS-PAGE verifying rMepM overexpression and purification

To verify the successful overexpression and purification of the proteins, SDS-PAGE was performed.

The samples in 4x Lämmli + β-ME were put on 95 °C for 10 min. A precast 4-20% SDS gel was loaded with 5 µL of Lämmli sample per pocket and 10 µL of precast protein ladder as a marker for protein size. The proteins were separated at a constant voltage of 120 mV for 60 min in 1x SDS buffer. The gel was washed in H₂O and stained in Coomassie blue. The stained proteins were visualized with Imaging chemiluminescence Fusion Solo S system.

2.3.1.6 Photometric determination of protein concentration

To determine the protein concentration in pure rMepM samples, OD_{260nm} was measured with a NanodropTM spectrophotometer and the protein concentration was calculated from the extinction coefficient ϵ of rMepM1 ($\epsilon = 27850 \frac{\text{L}}{\text{mol} * \text{cm}}$) and rMepM2 ($\epsilon = 16960 \frac{\text{L}}{\text{mol} * \text{cm}}$) according to the following formula:

$$c (M) = \frac{E}{\epsilon * d}$$

E = extinction at 260 nm

ϵ = extinction coefficient in $\frac{\text{L}}{\text{mol} * \text{cm}}$

d = layer thickness (= 1 cm)

2.3.1.7 rMepM1 and rMepM2 concentration on filters

The fractions from size exclusion chromatography (2.3.1.4) containing pure and concentrated rMepM were combined, mixed, and concentrated via a centrifugal concentration unit as described in 2.3.1.4 to achieve higher protein concentrations in the storage samples.

2.3.1.8 rMepM1 and rMepM2 storage

Protein aliquots were frozen in liquid nitrogen N₂ and were stored at -80 °C.

2.3.2 Production of anti-MepM1 and anti-MepM2 antibodies

Polyclonal affinity purified antibodies against rMepM1 and rMepM2 were produced by Eurogentec. Two rabbits each were immunised with purified rMepM1 and rMepM2 according to their 28 days speedy program (Fig. 36, results).

2.3.2.1 Western Blot analysis detecting MepM in whole cell lysates of ID40 strains

Whole cell lysates of ID40 strains were prepared as described in 2.2.6.1. A precast 4-20% SDS gel was loaded with 10 µL of whole cell lysate and 10 µL of precast protein ladder as a marker for protein size. Proteins were separated via SDS-PAGE and blotted against MepM1 and MepM2 (Table 19) as describe in 2.2.5.4.

To select suitable rabbits for immunisation, whole cell lysates of ID40, ID40 Δ mepM1 Δ mepM2 and ID40 Δ ctpA strains were blotted against preimmune sera from ten rabbits as described above (results not shown). Rabbits whose pre-immune serum did not detect *P. aeruginosa* proteins of the size of MepM1 and MepM2, meaning in which no bands appeared at the expected MepM1 and MepM2 band positions in the Western blot analysis, were selected for immunization.

Table 19: Antibody concentrations for Western Blots analysis of whole cell lysates with preimmune sera and affinity purified anti-MepM antibodies

		antibody	concentration
preimmune sera	primary AB	preimmune serum	1:200
	secondary AB	POX goat anti-rabbit AB	1:2000
affinity purified anti-MepM antibodies	primary AB	purified final bleeds	1:1000
	secondary AB	POX goat anti-rabbit AB	1:10000

2.3.3 Western Blot analysis detecting MepM in membrane proteome samples

Western Blots were performed as described in 2.2.5.4. Table 20 describes the applied antibody concentrations.

Table 20: Antibody concentrations in anti-MepM1 and anti-MepM2 Western Blots for detection of MepM in membrane proteome samples

Isolated membrane fractions of ID40 strains were blotted against MepM1 and MepM2 to identify the proteins as inner/outer membrane proteins or soluble periplasmatic or cytoplasmatic proteins.

Primary AB	Primary AB concentration	Secondary AB	Secondary AB concentration
Anti-MepM1	1:2000	POX goat anti-rabbit AB	1:10000
Anti-MepM2	1:2000		

2.4 MepM activity assays methods

2.4.1 FITC-PG assay

2.4.1.1 FITC- labeling of purified peptidoglycan

Purified *B. subtilis* PG (kindly provided by Marina Borisova-Mayer) was mixed with Fluorescein isothiocyanate (FITC) powder to a ratio of 2:1 in a preweighted Eppendorf cup in the dark. 4 mL of 0.5 M sodium bicarbonate (NaHCO₃) buffer (pH 9 - 9.3) was added to the PG-FITC and incubated for 4 hours at 37 °C h in the dark under constant inverting on a rotary wheel incubator. FITC-PG was pelleted by centrifugation (15 min, 20000 g). The pellet was washed 3 times by resuspending in 4 mL NaHCO₃ buffer (pH 9 – 9.3) and subsequent centrifugation. FITC-PG was washed with 4 mL ddH₂O in various centrifugation steps. The fluorescence in the supernatant was measured after each centrifugation step by detection in the Tecan Infinite® 200 PRO. Washing was stopped when the fluorescence signal of the supernatant remained constant (after 10 centrifugation steps). FITC-PG was last washed with ethanol and acetone in two centrifugation steps each. Acetone was discarded and FITC-PG was dried by centrifugation for 15 min at 20000 g. The FITC-PG was weighed and resuspended in ddH₂O adjusting a concentration of 10 mg/mL and stored at 4 °C.

2.4.1.2 FITC-PG degradation assay

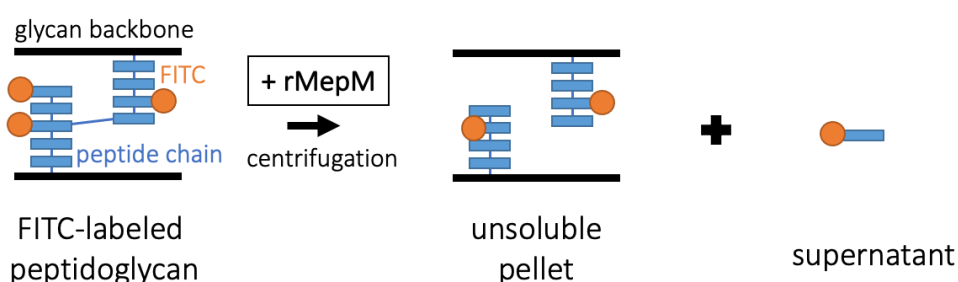


Figure 10: FITC-PG degradation assay principle

FITC-labelled PG is incubated with rMepM and incubation is stopped by centrifugation. Since the FITC-PG fraction is insoluble in water, the release of short soluble FITC-PG fragments into the supernatant indicates PG cleavage mediated by rMepM.

The *in vivo* MepM substrate peptidoglycan was labeled with FITC and used as a substrate for a first MepM activity assay. Degradation of FITC-PG by rMepM releases short soluble FITC-PG fragments that are found in the supernatant after centrifugation. In the following, the optimised assay protocol is described. The volumes, concentrations and incubation times described may differ from earlier approaches during assay optimization described in the results.

Stored purified rMepM (-80 °C) was thawed on ice, mixed and protein concentration determined photometrically (2.3.1.6). rMepM concentration was adjusted to 12-14 μM and 30 μL were transferred to a new Eppendorf tube. FITC-PG master mix was prepared by mixing 5 μL of FITC-PG + 65 μL reaction buffer per sample in a brown 1.5 mL Eppendorf tube. One extra unit of FITC-PG and reaction buffer were used to obtain sufficient master mix. 30 μL rMepM or reaction buffer (blank) was filled into brown 1.5 mL Eppendorf tube (Table 21). The reaction was started by adding 70 μL master mix per Eppendorf tube and resuspending three times. The Eppendorf tubes were incubated at 37 °C for 20 min in a heating block with shaking at 1400 rpm. The reaction was stopped by centrifugation at 20000 g for 10 min and 4 °C. The supernatant was transferred to new brown 1.5 mL Eppendorf tube and kept on ice. A black, flat-bottom 96-well plate was filled with 70 μL supernatant per well. Emission at 519 nm was measured with the Tecan Infinite® 200 PRO plate reader after excitation at 480 nm.

Table 21: Composition per reaction tube in FITC-PG degradation assay

rMepM and reaction buffer were filled into brown 1.5 mL Eppendorf caps and addition of FITC-PG master mix started the reaction.

Sample		rMepM	Blank
rMepM		30 μL (final conc. 12-14 μM)	-
reaction buffer			30 μL
FITC-PG master mix	FITC-PG (10 mg/mL)	5 μL	5 μL
	reaction buffer	65 μL	65 μL
Final volume		100 μL	100 μL

2.4.2 Quenched fluorescence substrate IIId assay

2.4.2.1 High-throughput MepM activity IIId assay

The cleavage of substrate IIId by rMepM separates the quencher on the one side from the fluorophore on the other generating a fluorescence signal that can be detected in the Tecan Infinite® 200 PRO.

The stored purified rMepM was thawed on ice and the concentration determined photometrically (2.3.1.6). A 4 µM rMepM predilution was prepared in enzyme buffer. Master mixes were prepared according to Table 22. A black flat-bottom 384-well-plate was filled with 22.5 µL of master mix per well in triplicates. A 500 µM substrate IIId predilution was prepared in DMSO in a brown 1.5 mL Eppendorf tube. The reaction was started by adding 2.5 µL substrate predilution per well (or 2.5 µL DMSO in the “buffer only” wells) and resuspending three times with a multichannel pipet. The plate was placed in the Tecan Infinite® 200 PRO plate reader and fluorescence was measured every 5 min (rMepM1) or 30 min (rMepM2) with the following settings: excitation at 320 nm, emission at 420 nm, 37 °C.

Table 22: Components of master mixes for rMepM activity IIId assay

22.5 µL of master mix were filled in a 384-well plate in triplicates and reaction was started by adding 2.5 µL of substrate IIId (final conc.: 50 µM) per well.

	rMepM + IIId substrate (1:50)	negative control	IIId substrate only	buffer only
rMepM1 or rMepM2 4 µM predilution → final conc.: 1 µM	25 µL	25 µL	-	-
enzyme buffer rMepM1: 20 mM Tris, 300 mM NaCl, 10% Glycerol, pH = 8 at 4 °C rMepM2: 1 x PBS, pH = 8	-	-	25 µL	25 µL
EDTA 100 mM stock in H ₂ O → final conc.: 1 mM	-	1 µL	-	-
H₂O	1 µL	-	1 µL	1 µL
reaction buffer	64 µL	64 µL	64 µL	64 µL
final volume	90 µL	90 µL	90 µL	90 µL

2.4.2.2 MepM inhibitor screening with Ild assay

To screen for possible MepM inhibitors, the assay was performed as described in 2.4.2.1. Possible inhibitors were added to the master mixes adjusting a final assay concentration of 300 μ M (Table 23). To reduce the number of components, the volume of assay buffer in the master mixes that did not contain EDTA was increased by 1 μ L instead of substituting 1 μ L H₂O. The master mixes were preincubated with inhibitors for 15 min at RT before filling the 384-well plate.

Table 23: Components of master mixes for inhibitor screening with quenched fluorescence Ild assay

22.5 μ L of master mix were filled in a 384-well plate in triplicates and reaction was started by adding 2.5 μ L of substrate Ild (final conc.: 50 μ M) per well.

	rMepM + Ild substrate (1:50)	negative control	Ild substrate only	buffer only	rMepM + Ild substrate + inhibitor	inhibitor + Ild substrate
rMepM1 or rMepM2 4 μ M predilution → final conc.: 1 μ M	25 μ L	25 μ L	-	-	25 μ L	-
enzyme buffer rMepM1: 20 mM Tris, 300 mM NaCl, 10% Glycerol, pH = 8 at 4 °C rMepM2: 1 x PBS, pH = 8	-	-	25 μ L	25 μ L	-	25 μ L
EDTA 100 mM stock in H ₂ O → final conc.: 1 mM	-	1 μ L	-	-	-	-
inhibitor 6 mM stock in DMSO → final conc.: 300 μ M	-	-	-	-	5 μ L	5 μ L
DMSO	5 μ L	5 μ L	5 μ L	5 μ L	-	-
reaction Buffer	60 μ L	59 μ L	60 μ L	60 μ L	60 μ L	60 μ L
Final volume	90 μ L	90 μ L	90 μ L	90 μ L	90 μ L	90 μ L

3. Results

3.1 MepM target validation

3.1.1 Generation of a conditional knockout mutant ID40 Δ mepM1::rha-mepM1 Δ mepM3 Δ mepH1 Δ mepH2 lacking all CtpA regulated meps

The generation of an ID40 mutant lacking all CtpA regulated Meps (MepM1, MepM3, MepH1 and MepH2) has failed (Kemper, 2024). This led to the hypothesis that deletion of all CtpA regulated *meps* results in a lethal phenotype.

To investigate this, a conditional knockout mutant ID40 Δ mepM1::rha-mepM1 Δ mepM3 Δ mepH1 Δ mepH2 carrying *mepM1* under a rhamnose promotor was generated. To achieve this, *mepM3* was knocked out by allelic exchange in the pre-existing conditional mutant ID40 Δ mepM1::rha-mepM1 Δ mepH1 Δ mepH2. Addition of rhamnose 0.1% to the medium supplemented MepM1 during all steps of mutagenesis hence preventing the presumably lethal phenotype.

3.1.2 Verification of *mepM3* deletion in ID40 Δ mepM1::rha-mepM1 Δ mepH1 Δ mepH2

To verify a successful deletion of *mepM3*, genomic DNA was isolated from potential mutants after mutagenesis. Fig. 11 and 12 describe the principle of identification of deletion mutants by PCR. DNA fragments of a specific length were amplified using primers that bind either inside (Fig. 11) or outside (Fig. 12) the *mepM3* gene locus. The lengths of the synthesized fragments were identified by agarose gel electrophoresis.

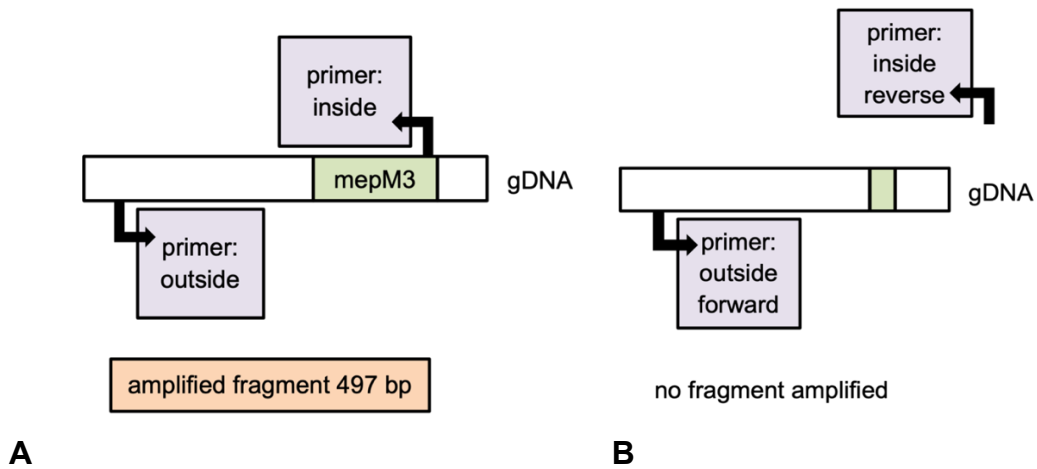


Figure 11: Identification of *mepM3* deletion mutants by inside PCR

(A) Inside PCR with ID40 gDNA carrying the intact *mepM3* locus amplifies a 497 bp fragment. (B) Inside PCR with Δ *mepM3* mutant gDNA does not amplify any fragment as there is no binding site for the reverse primer on the gDNA.

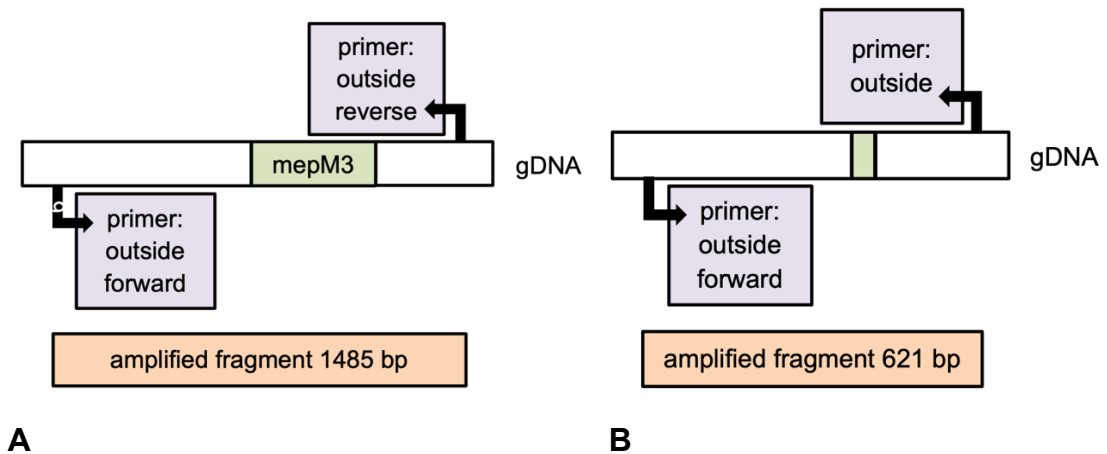


Figure 12: Identification of *mepM3* deletion mutants by outside PCR

(A) Outside PCR with ID40 gDNA carrying the intact *mepM3* locus amplifies a 1485 bp fragment. (B) Outside PCR with $\Delta mepM3$ mutant gDNA amplifies a 621 bp fragment.

ID40 $\Delta mepM1::rha-mepM1\Delta mepM3\Delta mepH1\Delta mepH2$ lacks a binding site for the primer within the *mepM3* gene locus. Therefore, no gene fragment was amplified using the inside primer pair whereas inside PCR with ID40 gDNA amplified a 497 bp fragment. (Fig. 11 and 13) Primers binding upstream and downstream of *mepM3* amplified a 621 bp fragment from $\Delta mepM3$ mutant gDNA, whereas PCR with ID40 gDNA amplified a 1485 bp fragment. (Fig. 12 and 14)

Generation of a conditional knock out mutant ID40 $\Delta mepM1::rhamepM1\Delta mepM3\Delta mepH1\Delta mepH2$ was successful in four clones (Fig. 13 and 14). The first clone was stored in cryocultures and used for subsequent experiments.

The mutant was viable even without adding rhamnose to the medium. Thus, ID40 could either be viable in the absence of all CtpA regulated *meps*, or very low *mepM1* expression due to leakiness of the rhamnose promotor is sufficient for bacterial survival.

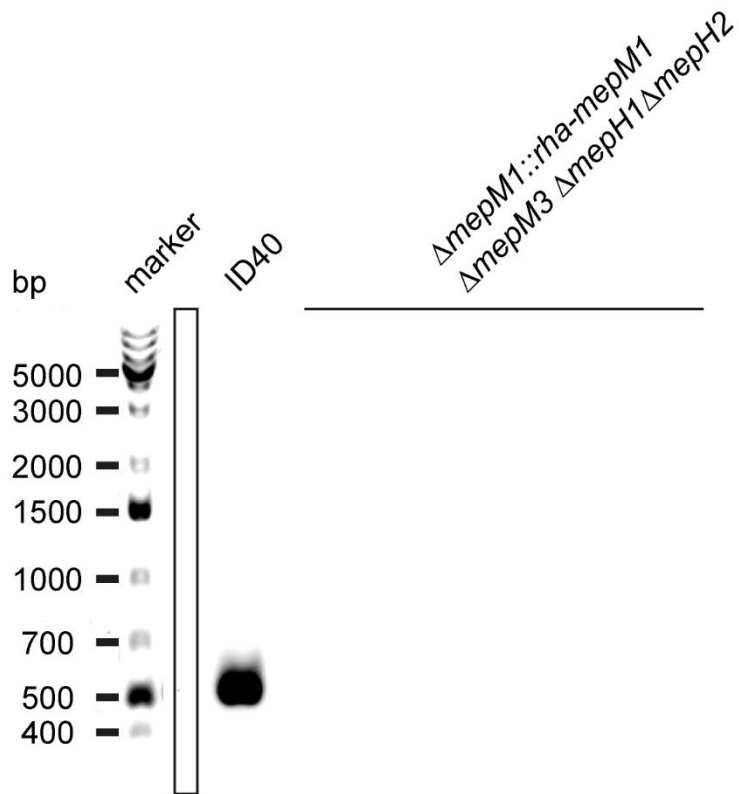


Figure 13: Agarose gel electrophoresis of DNA fragments from inside PCR

No gene fragment is amplified from $\Delta mepM3$ mutants gDNA whereas inside PCR with ID40 gDNA amplified a 497 bp fragment.

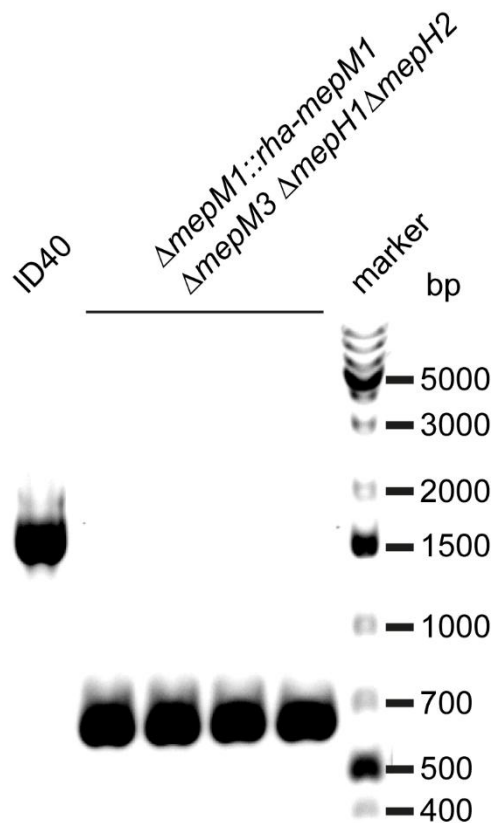


Figure 14: Agarose gel electrophoresis of DNA fragments from outside PCR

PCR with outside primers amplified a 621 bp fragment from $\Delta mepM3$ mutants gDNA whereas PCR with ID40 gDNA amplified a 1485 bp fragment.

3.1.3 Effect of $\Delta mepM$ and $\Delta mepH$ codeletion on antibiotic resistance

As ID40 lacking all CtpA regulated *meps* was viable, the effect of this codeletion on antibiotic resistance was investigated. MIC values of the newly generated mutant ID40 $\Delta mepM1::rha-mepM1\Delta mepM3\Delta mepH1\Delta mepH2$ with active and inactive rhamnose promotor (i.e. presence or absence of 0.1% rhamnose in the medium) were determined by microbroth dilution assays. Each strain was tested on two different antimicrobial susceptibility testing plates, EUX2NF and GN2F. The results were compared to the MIC values of ID40 and the Δmep mutants determined by Kemper (2024).

Table 24 shows my results (ID40 $\Delta mepM1::rha-mepM1\Delta mepM3\Delta mepH1\Delta mepH2$) as well as Kemper's results (all other strains). MIC values higher than ID40 are written in red. MIC values lower than ID40 are written in green. MIC values that classify bacteria as sensitive to the tested antibiotic according to EUCAST MIC breakpoints are highlighted in grey.

The codeletion of *mepM1* and any tested murein endopeptidase gene synergistically reduces antibiotic resistance.

In ID40 $\Delta mepM1::rha-mepM1\Delta mepM3\Delta mepH1\Delta mepH2$ the absence of *mepM1* due to inactivation of the rhamnose promotor further reduces antibiotic resistance to PIP and FEP. As known before, MepM1 has the clearly strongest effect on antibiotic resistance. ID40 lacking all CtpA regulated *meps* is only slightly more sensitive than ID40 $\Delta mepM1$ single deletion mutant. The most sensitive mutant remains ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$. Therefore, this work will focus on MepM1, MepM2 and MepM3 in the following rather than MepH1 and MepH2.

Table 24: MICs of ID40 and $\Delta mepM$ deletion mutants

MIC values of ID40 and $\Delta mepM$ deletion mutants were determined by microbroth dilution assays. In red: MIC values higher than ID40, green: MIC values lower than ID40, grey background: MIC values that classify bacteria as sensitive to the tested antibiotic according to EUCAST MIC breakpoints. Combined results of Vöhringer and Kemper (2024)

	MIC Breakpoint (mg/L)		ID40	$\Delta mepM1$	$\Delta mepM3$	$\Delta mepM1$ $\Delta mepM3$	$\Delta mepH1$ $\Delta mepH2$	$\Delta mepM1$::rha-mepM1 $\Delta mepM3$ $\Delta mepH1$ $\Delta mepH2$ +rha	$\Delta mepM1$::rha-mepM1 $\Delta mepM3$ $\Delta mepH1$ $\Delta mepH2$ -rha	$\Delta mepM1$ $\Delta mepM2$ $\Delta mepM3$
	S ≤	R >								
Meropenem	2	8	8	2-8	4 - 8	2 - 4	2-4	2	2	≤1 - 1
Imipenem	4	4	32	16	32 - 64	16	32	16	16	4 - 8
Cefepim	8	8	16 - 32	8	16	8	8 - >8	16	8	4
Ceftazidim	8	8	32 - >32	16 - 32	32 - >32	16 - 32	16 - 32	16	16	4 - 8
Piperacillin	16	16	128 - >128	16 - 32	>128	32	32 - 64	64	32	8
Piperacillin-Tazobactam	16	16	128 - >128	32 - 64	64 - 128	32	32	32	32	8 - 16
Aztreonam	16	16	16 - 32	8 - 16	16 - 32	8 - 16	8	8	8	4

3.1.4 MepM mediated resistance mechanisms

Deletion of *mepM1*, *mepM2* and *mepM3* synergistically breaks antibiotic resistance. This effect can only be partially explained by a reduction in *ampC* expression (Kemper, 2024). To further understand MepMs contribution to resistance, possible posttranscriptional regulation of AmpC leading to reduced enzyme activity and changes in the membrane proteome were investigated.

3.1.4.1 *β*-lactamase AmpC activity

The commercially available Colorimetric Nitrocefin Assay (Biovision) determines *β*-lactamase AmpC activity by measuring AmpC mediated Nitrocefin hydrolysis. Both transcriptional as well as posttranscriptional effects of *mepM* deletion on AmpC activity are detected by this assay.

Absolute Nitrocefin turnover was calculated by photometric detection of the colorful product and application of the measured OD_{600nm} values to a standard curve. The *β*-lactamase activity is explained as nitrocefin turnover per min per ml sample volume in a time interval with constant substrate turnover per time (complete enzyme saturation).

$$\beta - \text{lactamase AmpC activity} = \frac{B}{\Delta T \times V} \times D$$

B	Nitrocefin turnover (nmol)
ΔT	time (min)
V	sample volume added to the reaction well (ml)
D	sample dilution factor

As described in the kit's protocol, dry bacterial pellets were resuspended in different volumes of dilution buffer according to the measured dry pellet weight, resulting in equal bacterial concentration per well. Comparison of the measured dry pellet weight with the OD_{600nm} of the subcultures at harvest confirmed a linear relationship between pellet weight and bacterial count (Fig. 15).

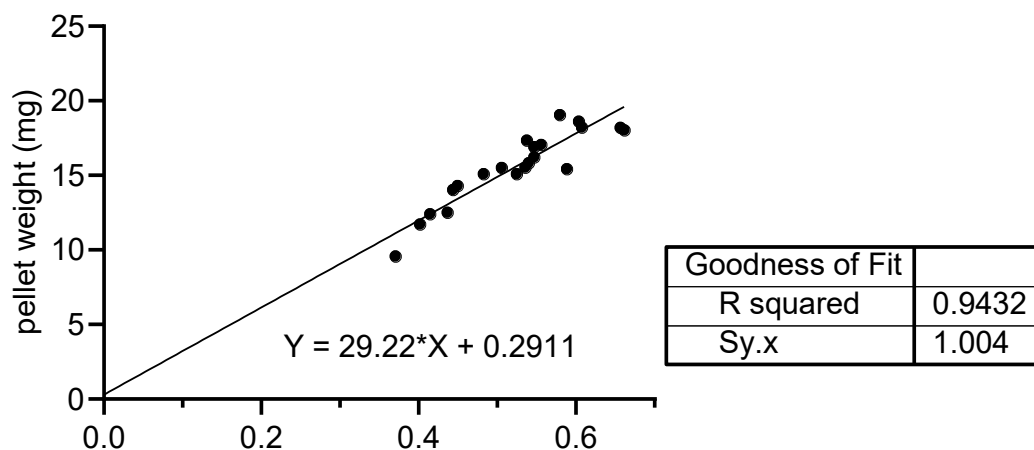


Figure 15: Linear correlation between optical density and pellet weight

The graph shows all values from the three experiments analyzed in Fig. 16. Comparing the measured dry pellet weights to the OD_{600nm} of the subcultures at harvest confirms a linear relationship between pellet weight and bacterial count. Each point refers to one subculture.

For analysis, AmpC activity rates were normalized to the ID40 activity rate of each experiment (Fig. 16, A). Three experiments were analyzed, with each experiment comprising two technical replicates. Significance levels were determined by statistical analysis using an ordinary one-way ANOVA. To analyze the effect of codeletions, AmpC activity rates were normalized to the $\Delta mepM1$ activity rate of each experiment. (Fig. 16, B)

Single deletion of *mepM1* and *mepM2* reduced AmpC activity by 66% and 31%, respectively. Codeletion of *mepM1* and *mepM2* does not further reduce AmpC activity below the single *mepM1* deletion. Single deletion of *mepM3* has no effect on AmpC activity. In ID40 $\Delta mepM1\Delta mepM3$ and ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ the additional deletion of *mepM3* even seems to prevent the $\Delta mepM1$ induced decrease in AmpC activity at least in case of the ID40 $\Delta mepM1\Delta mepM3$ deletion strain.

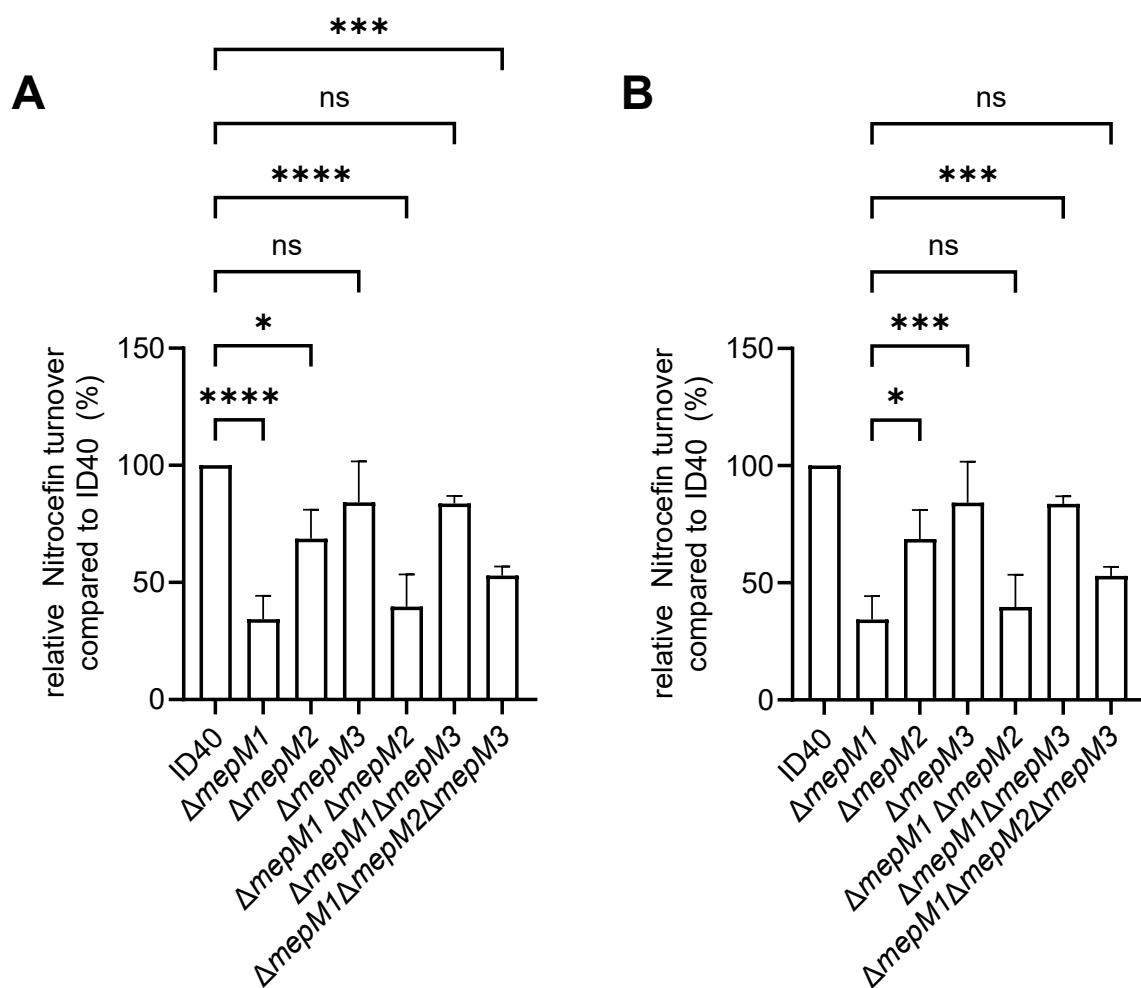


Figure 16: β -lactamase AmpC activity of $\Delta mepM$ deletion mutants normalized to ID40

The graph depicts the AmpC activity rates of the $\Delta mepM$ deletion mutants from the same three experiments. **(A)** AmpC activity rates were normalized to the ID40 activity rate of each experiment. Deletion of *mepM1* and *mepM2* reduces AmpC activity by 66% and 31%, respectively. **(B)** AmpC activity rates were normalized to the $\Delta mepM1$ activity rate of each experiment. Codeletion of *mepM1* and *mepM2* or *mepM3* does not further reduce AmpC activity below the single *mepM1* deletion.

The results are consistent with the findings on *ampC* expression by Kemper (2024). The deletion of *mepM1* and *mepM2* reduces AmpC activity by decreasing *ampC* expression. There is no evidence for further MepM mediated posttranscriptional regulation of AmpC activity.

3.1.4.2 Membrane proteome analysis

The $\Delta mepM1\Delta mepM2\Delta mepM3$ mediated break of resistance can only be partially explained by a reduction in β -lactamase AmpC activity. Other resistance mechanisms might be related to changes in the membrane proteome due to an altered peptidoglycan structure of $\Delta mepM$ deletion mutants.

To determine the effects of $\Delta mepM1\Delta mepM2\Delta mepM3$ triple deletion on membrane proteome composition, membrane proteins from ID40 and ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ were isolated and identified by mass spectrometry. This was done twice, and the results of both membrane isolations were compared to find up- or downregulated membrane proteins.

3.1.4.3 Membrane isolations

The membrane proteins from the first isolation were very finely separated into inner and outer membrane samples and analyzed by dimethyl labeling mass spectrometry.

In the second membrane isolation, the separation into inner and outer membrane proteins was omitted. The final samples from each strain contained the entire membrane proteome. Triplicates of each strain were analyzed by ordinary mass spectrometry.

3.1.4.3.1 First membrane isolation

Bacteria were grown in 1 L LB medium until the cultures reached an OD_{600nm} of 1. The ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ culture formed foam, while ID40 did not.

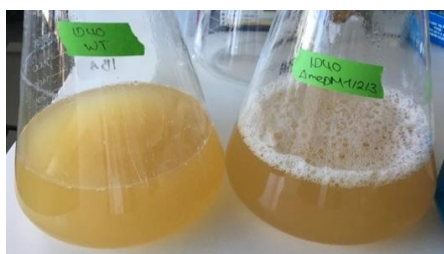


Figure 17: ID40 and ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ cultures at harvest

Cultures were grown in 1 L LB medium until reaching an OD_{600nm} of 1. The ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ culture formed foam, while ID40 did not.

The bacterial cultures were harvested by centrifugation. The ID40 pellet (right) showed dark discoloration, while the ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ pellet (left) was not discolored.



Figure 18: ID40 and ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ pellets after harvest

Cells were harvested by centrifugation. The ID40 pellet (right) showed dark discoloration.

After cell lysis and membrane purification, inner and outer membrane proteins were separated by a sucrose gradient. Samples were fractionated into 12 fractions, and total protein concentrations were measured by the BCA protein assay. The same amount of total protein per fractions was loaded onto a SDS gel. Proteins were separated according to their molecular size applying constant voltage and stained in Coomassie Blue.

The individual banding patterns of the separated fractions differed. The respective fractions of ID40 (Fig. 19) and ID40 Δ mepM1 Δ mepM2 Δ mepM3 (Fig. 20) each showed similar banding patterns. The isolation of the membrane proteins and separation into specific fractions was successful.

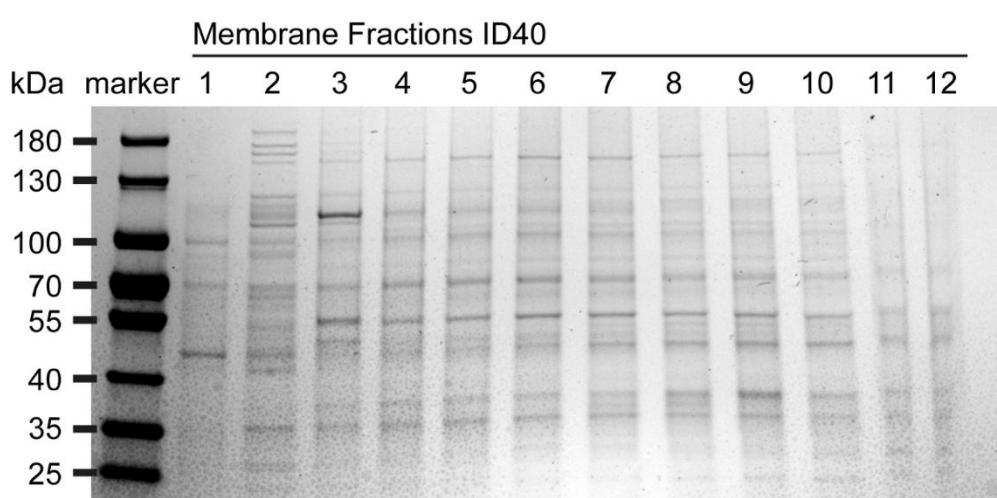


Figure 19: Banding patterns of the ID40 fractions containing the membrane proteome
Fractions were separated by a sucrose gradient. Same amount of total protein was loaded per line. Proteins were separated by SDS-PAGE and stained with Coomassie blue.

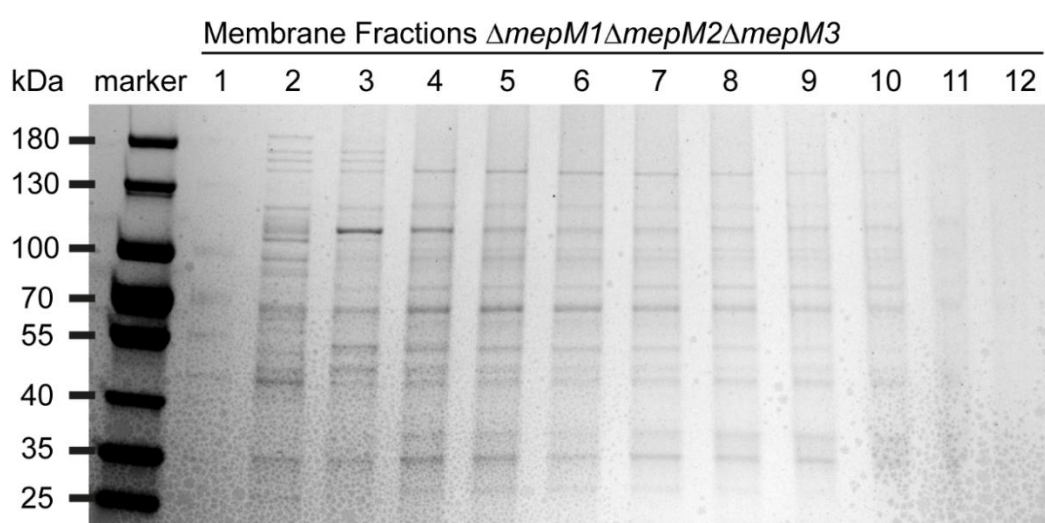


Figure 20: Banding patterns of the Δ mepM1 Δ mepM2 Δ mepM3 fractions containing the membrane proteome
Fractions were separated by a sucrose gradient. Same amount of total protein was loaded per line. Proteins were separated by SDS-PAGE and stained with Coomassie blue.

To verify which fractions contained the membrane proteins and which contained cytoplasmic and periplasmic contaminants, Western Blots against periplasmic (SurA), cytoplasmic (RpoB) and outer membrane (PlpD) proteins were performed. Unfortunately, no antibody against an inner membrane protein was available. Fraction samples as well as two samples taken during membrane purification (input and supernatant UC) were blotted against SurA, RpoB and PlpD. The sample taken before ultracentrifugation (input UC) contains all bacterial proteins. The supernatant after ultracentrifugation (supernatant UC) is supposed to contain separated soluble cytoplasmic proteins. Same amount of total protein was loaded per lane.

The outer membrane protein PlpD (80.9 kDA) was concentrated mainly in fractions 7, 8 and 9.

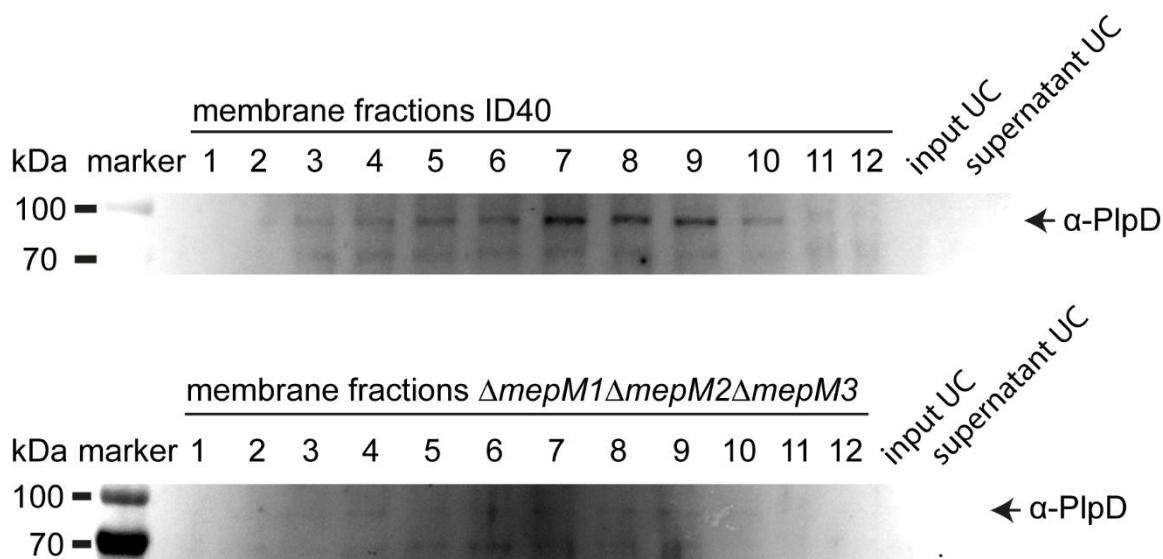


Figure 21: PlpD detection in membrane proteome fractions

Western blots were performed using membrane fractions of (A) ID40 and (B) ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ to detect the outer membrane protein PlpD. Fractions were incubated with anti-PlpD antibody, HRP-conjugated secondary antibody and finally clarity Western ECL solution to measure chemiluminescence. PlpD was mainly detectable in fractions 7-9.

Cytoplasmatic (RpoB, 150.8 kDA) and periplasmatic (SurA, 48.4 kDA) proteins were mainly separated by ultracentrifugation and are mainly detectable in the supernatant UC. Remaining contamination by those proteins is found in fractions 1, 2 and 3 (Fig. 21 and 22). This can be explained by the inner membrane being located in between the cytosol and the periplasm.

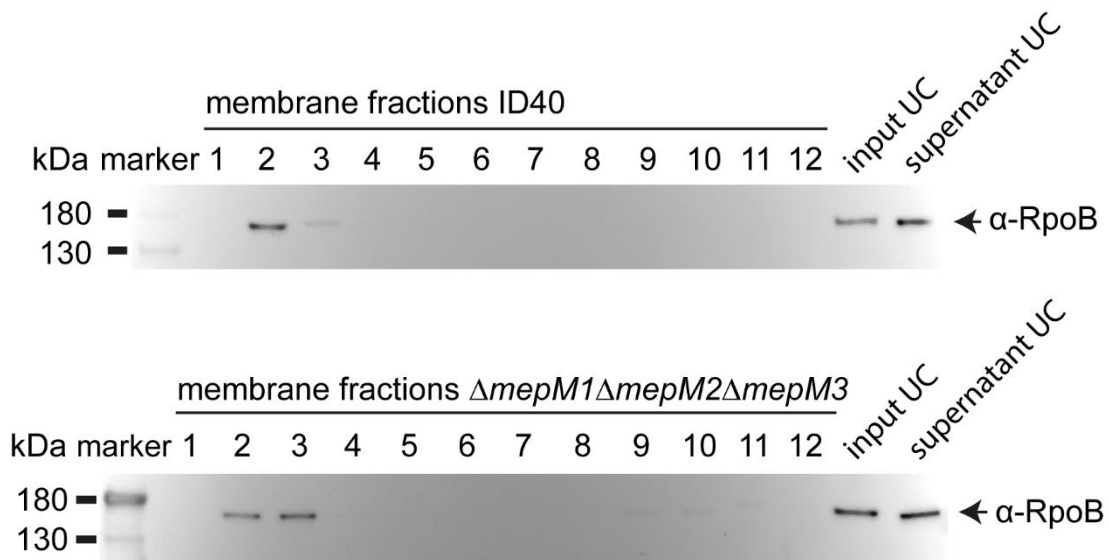


Figure 22: RpoB detection in membrane proteome fractions

Western blots were performed using membrane fractions of (A) ID40 and (B) ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ to detect the cytoplasmatic protein RpoB. Fractions were incubated with anti-RpoB antibody, HRP-conjugated secondary antibody and finally clarity Western ECL solution to measure chemiluminescence. RpoB was mainly detectable in the input before and the supernatant after ultracentrifugation (UC) as well as in fractions 2-3 of the membrane isolation.

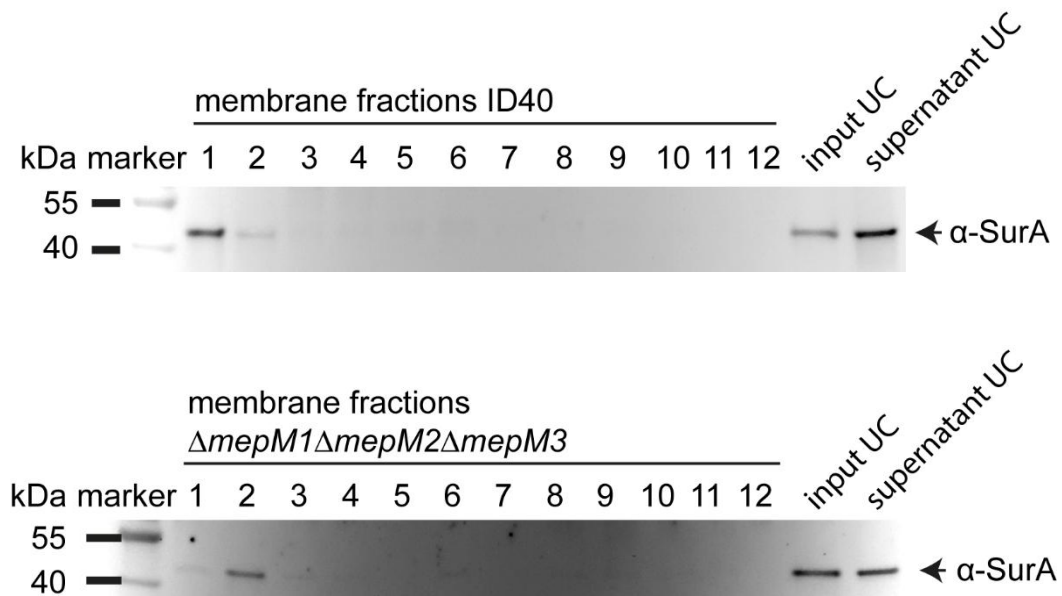


Figure 23: SurA detection in membrane proteome fractions

Western blots were performed using membrane fractions of (A) ID40 and (B) ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ to detect the periplasmatic protein SurA. Fractions were incubated with anti-SurA antibody, HRP-conjugated secondary antibody and finally clarity Western ECL solution to measure chemiluminescence. SurA was mainly detectable in the input before and the supernatant after ultracentrifugation (UC) as well as in fractions 1-2 of the membrane isolation.

According to the described findings (banding patterns in SDS-PAGE and Western blot results), fractions 2 and 3 were combined for the mass spectrometry analysis of inner membrane proteins and fractions 7 and 8 for the outer membrane proteins.

3.1.4.3.2 Second membrane isolation

The second membrane purification was performed in the same way as the first one, omitting the separation into inner and outer membrane proteins. The final samples of each strain contained the entire membrane proteome.

The SDS gel (Fig. 24) shows the banding patterns of membrane and cytosolic protein samples in triplicates as results of the second membrane isolation. The same amount of total protein was loaded per lane. There is a visible difference in the membrane proteome banding pattern of ID40 and ID40 Δ mepM1 Δ mepM2 Δ mepM3 at around 35 kDa, 55 kDa and 70 kDa.

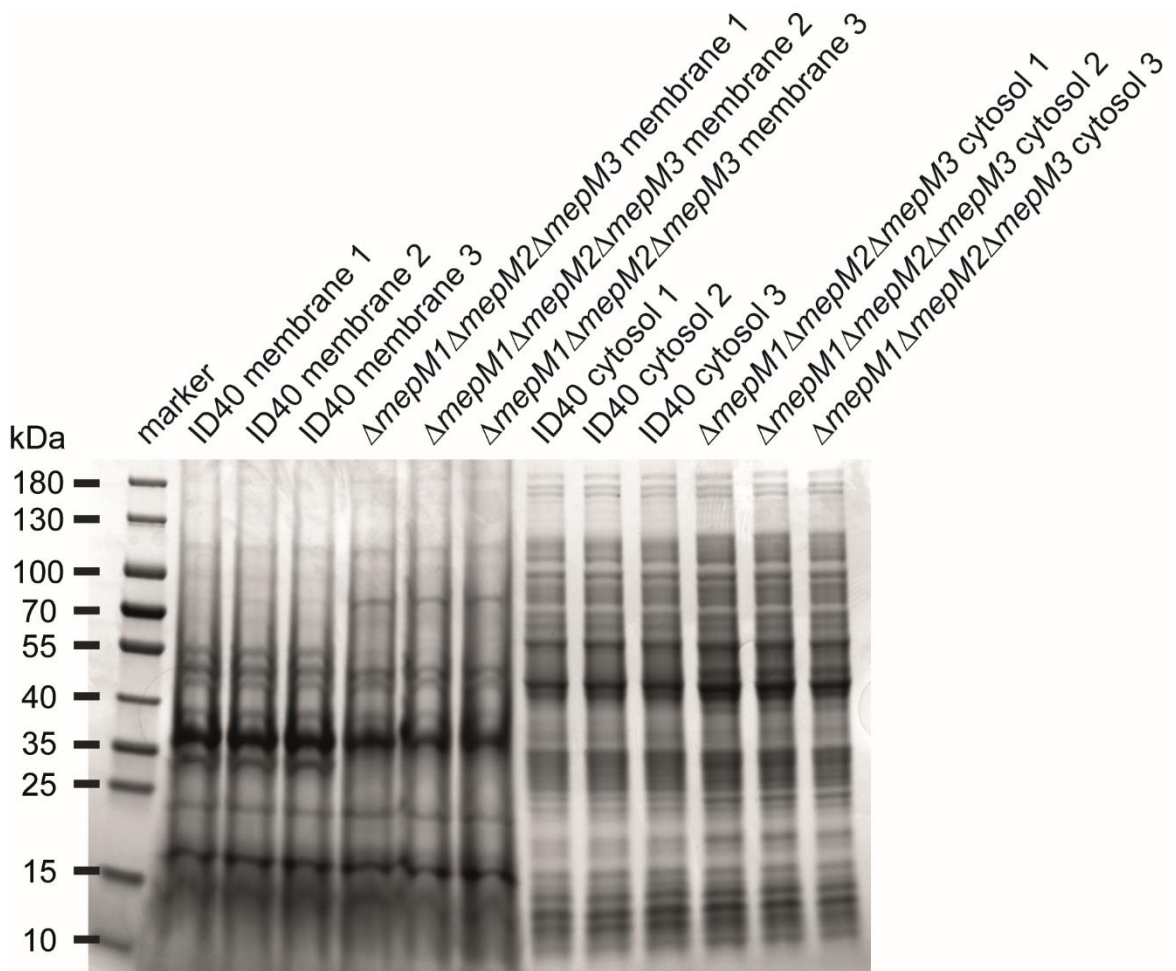


Figure 24: SDS-PAGE of the membrane and cytosolic proteome of ID40 and ID40 Δ mepM1 Δ mepM2 Δ mepM3

Loaded samples each result from membrane isolation of 1 L bacterial culture at OD_{600nm} = 1. Same amount of total protein was loaded per line. Proteins were separated by SDS-PAGE and stained with Coomassie blue.

3.1.4.4 Comparative mass spectrometric analysis of the membrane proteome of ID40 and ID40 Δ mepM1 Δ mepM2 Δ mepM3

In the first approach, samples with inner membrane proteins (set 1, combined fractions 3 and 4) and samples with outer membrane proteins (set 2, combined fractions 7 and 8) from ID40 and ID40 Δ mepM1 Δ mepM2 Δ mepM3 were analyzed using dimethylation labeling. In the second approach the separation into inner and outer membrane proteins as well as dimethylation labeling was omitted. Instead, whole membrane proteome samples of ID40 and ID40 Δ mepM1 Δ mepM2 Δ mepM3 were analyzed in three independent replicates.

Proteins that showed at least a 2-fold change in the normalized ratios and had an adjusted P value of at most 0.05 were considered altered. In the first analysis 94 proteins with altered protein levels were detected. 14 of those are outer membrane proteins, 28 inner membrane proteins, 1 flagella protein, 3 located in the membrane unknown if inner or outer membrane, 34 cytoplasmatic proteins, 3 periplasmatic proteins and 10 of unknown localization. In the second experiment 59 proteins with altered protein levels were detected. 9 were outer membrane proteins, 27 inner membrane proteins, 2 flagella proteins, 3 membrane proteins unknown if inner or outer membrane, 12 cytoplasmatic proteins, 2 periplasmatic proteins and 4 of unknown localization. Proteins not localized in the membrane are interpreted as impurities and were excluded from further analysis.

The tables 25 and 26 list all detected altered proteins of known membrane localization and the corresponding log2 ratios mutant/ wildtype as well as the statistically adjusted P values. Table 25 lists all membrane proteins with significantly higher and Table 26 with lower levels in ID40 Δ mepM1 Δ mepM2 Δ mepM3 compared to ID40. The proteins are sorted according to the level of the log2 ratio mutant/ wildtype (WT) and those were illustrated with colored data bars. Protein and gene names, PA01 orthologs and predicted localization of the proteins were retrieved from Pseudomonas.com (Winsor et al., 2016) and UniProt (Consortium, 2024).

Only few membrane proteins showed higher or lower levels in both experiments. Some of those proteins belong to efflux pump operons. Components of MexX/MexY/OprM and MexE/MexF/OprN were detected with higher levels in ID40 Δ mepM1 Δ mepM2 Δ mepM3 and are highlighted in bold.

Table 25: Membrane proteins with higher levels in ID40Δ*mepM1*Δ*mepM2*Δ*mepM3* compared to ID40

The table presents results from comparative mass spectrometry of the purified membrane proteome of ID40Δ*mepM1*Δ*mepM2*Δ*mepM3* versus ID40. It includes membrane proteins with at least a 2-fold increase in the normalized ratio and an adjusted P value ≤ 0.05. In the first experiment, inner (set 1) and outer (set 2) membrane proteins were separated during purification and analyzed individually using dimethylation labeling. In the second experiment whole membrane proteome samples were analyzed in three independent replicates. Proteins are sorted by the log2 ratio mutant/ wildtype (WT). Protein and gene names, PA01 orthologs and predicted localization were retrieved from Pseudomonas.com and UniProt. Efflux pump proteins are highlighted in bold.

Protein ID	ID40 gene	PA01 ortholog	Name	Localisation	log2 Ratio mutant/ WT			Significance		
					1st exp. set 1	1st exp. set 2	2nd exp.	adj. P value 1st exp., set 1	adj. P value 1st exp., set 2	adj. P value 2nd exp.
Q9HXN4	nagE	PA3761	N-Acetyl-D-Glucosamine phosphotransferase system transporter	CM			7,83			0,0000044
Q9HVV3	mltB1	PA4444	Soluble and membrane-bound lytic transglycosylase	CM			6,18			0,000839
Q9IOJ3	nuoJ	PA2645	NADH-quinone oxidoreductase subunit	CM			5,82			0,00313
Q9IOY7	oprN	PA2495	Multidrug efflux outer membrane protein	OM		1,89	9,44		2,15E-05	7,44E-14
Q9HWG6	TUEID40_04360	PA4223	Probable ATP-binding component of ABC transporter	CM			5,41			0,00152
Q9I4S6	yiaD	PA1048	Probable outer membrane protein	OM	2,93	2,20	8,19	1,39E-10	7,92E-07	7,44E-14
G3XCW2; REV_G3XCW2	mexY	PA2018	Efflux pump membrane transporter	CM	2,84	1,36	7,05	5,16E-10	0,002038261	0,000136
P42512	fpIA	PA4221	Fe(3+)-pyochelin receptor	OM	1,53	1,77	6,99	0,00072467	6,70E-05	0,00000398
Q9X6V6	rlpA	PA4000	Endolytic peptidoglycan transglycosylase RlpA	membrane	1,04	1,96	1,98	0,070575042	1,11E-05	0,0168
Q9HTR6	glnK	PA5288	Nitrogen regulatory protein P-II 2	CM	2,90			4,55E-07		
Q9IOY8	mexF	PA2494	Efflux pump membrane transporter	CM	2,84			4,91E-10		
G3XD21	mexX	PA2019	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein	CM	3,38	1,56		1,43E-13	0,000415534	
Q9HWG6	TUEID40_04361	PA4223	Probable ATP-binding component of ABC transporter	CM	2,78	2,11		1,31E-06	0,000121666	
Q9I3C1	nicB_2	PA1600	Probable cytochrome c	CM	2,41			2,62E-05		
Q9HWG8	ampP	PA4218	Probable transporter	CM		2,34			2,42E-05	
Q9I4S3	dsdX	PA1051	Probable transporter	CM	2,33			5,02E-05		
Q9IOY9	mexE	PA2493	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein	CM	2,14			2,61E-06		
Q9I202	opdO	PA2113	Pyroglutamate porin	OM	2,00	2,01		0,000506227	0,00023781	
Q9HTV2	oatA_1	PA5238	Probable O-antigen acetylase	CM	1,95			0,000687578		
G3XD89	oprC	PA3790	Putative copper transport outer membrane porin	OM	2,00	1,84		1,15E-05	3,69E-05	
Q9HWG7	irb	PA4222	Probable ATP-binding component of ABC transporter	CM	1,89	1,91		0,000970328	0,000425809	
P11221	oprI	PA2853	Major outer membrane lipoprotein	OM	1,71			0,000159338		
Q9HTW9	ubil	PA5221	Probable FAD-dependent monooxygenase	CM	1,53			0,007527014		
Q9HVM8	pilY1	PA4554	Type IV pilus biogenesis factor	OM	1,45	1,58		0,001370086	0,000370305	
Q9HVM9	pilX	PA4553	type 4 fimbrial biogenesis protein PilX	membrane	1,40	1,44		0,002027646	0,001127522	
Q9I6D5	TUEID40_03985	PA0359	Probable multidrug transporter	CM		1,50			0,004403382	
G3XD43	pilE	PA4556	Type 4 fimbrial biogenesis protein PilE	CM			1,5			0,0404
Q9HYX0	pleC	PA3271	Histidine kinase	CM	1,49			0,001038461		
Q9I653	creD	PA0465	Inner membrane protein	CM	1,38			0,00221576		
Q9I5Z1	mmoC	PA0545	FAD-binding FR-type domain-containing protein	CM	1,25	1,29		0,005447399	0,003378302	
Q9I3G0	ccoN2	PA1557	Cytochrome-c oxidase	CM	1,27			0,005031422		
Q9I3B3	mcpS_2	PA1608	Probable chemotaxis transducer	OM		1,27			0,004029729	
P34750	pilQ	PA5040	Fimbrial assembly protein	OM		1,26			0,004157805	
Q9HVD1	pagL	PA4661	Lipid A 3-O-deacylase	OM	1,18			0,009116467		

Table 26: Membrane proteins with lower levels in ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ compared to ID40

The table presents results from comparative mass spectrometry of the purified membrane proteome of ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ versus ID40. It includes membrane proteins with at least a 2-fold decrease in the normalized ratio and an adjusted P value ≤ 0.05 . In the first experiment, inner (set 1) and outer (set 2) membrane proteins were separated during purification and analyzed individually using dimethylation labeling. In the second experiment whole membrane proteome samples were analyzed in three independent replicates. Proteins are sorted by the log2 ratio mutant/ wildtype (WT). Protein and gene names, PA01 orthologs and predicted localization were retrieved from Pseudomonas.com and UniProt.

Protein ID	ID40 gene	PA01 ortholog	Name	Localisation	log2 Ratio mutant/ WT			Significance		
					1st exp. set 1	1st exp. set 2	2nd exp.	adj. P value 1st exp., set 1	adj. P value 1st exp., set 2	adj. P value 2nd exp.
P32722	oprD	PA0958	Porin D	OM	-2,84	-3,98	-10,90	8,21E-05	2,50E-06	7,44E-14
Q9HWQ0	TUEID40_04464	PA4131	Probable iron-sulfur protein	CM	-3,51	-3,48	-7,27	1,08E-06	3,96E-05	7,44E-14
Q9K3C5	fliD	PA1094	B-type flagellar hook-associated protein	flagellum	-4,86	-3,27	-6,17	1,59E-11	0,00166417	0,000155
Q9HY75	TUEID40_05161	PA3535	Probable serine protease	OM			-4,92			0,00000179
Q9HWP8; Q9I2N9	TUEID40_04462	PA4133	Cytochrome-c oxidase	CM	-3,07	-4,58		2,05E-05	5,95E-08	
Q9HXU8	lptF	PA3692	Lipotoxon F	OM	-3,06	-3,76	-4,30	2,14E-05	8,72E-06	2,93E-12
Q9HXW3	mexJ	PA3677	Probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein	CM			-4,13			0,000193
Q9I6E1	xanP_2	PA0352	Probable transporter	CM			-4,10			0,00107
Q9I3T6	PA1408	PA1408	DUF3772 domain-containing protein	CM			-4,02			0,00108
P72151	fliC	PA1092	B-type flagellin	flagellum			-3,77			1,93E-08
Q9I4F7	PA1181	PA1181	Uncharacterized protein	CM			-3,53			0,0123
Q9HUT7	osmE	PA4876	Osmotically inducible lipoprotein OsmE	OM	-1,97	-2,63	-3,37	0,006619857	0,002007835	0,0000869
Q9HXZ9	TUEID40_05056	PA3631	Uncharacterized protein	CM			-3,37			0,00534
Q9HU67	typA	PA5117	Regulatory protein TypA	CM	-1,60	-3,33		0,026580487	8,69E-05	
Q9HXB2	fecA	PA3901	Fe(III) dicitrate transport protein	OM	-1,48	-3,15	-2,50	0,041282549	0,000206766	0,00159
Q9I6W7	TUEID40_03784	PA0165	Uncharacterized protein	OM			-3,15			0,00497
Q9I6X4	triC	PA0158	Resistance-Nodulation-Cell Division (RND) efflux transporter	CM			-3,03			0,000677
Q9I0J0	nuoM	PA2648	NADH-quinone oxidoreductase subunit M	CM		-2,99			0,000425129	
Q9HXC3	opuCB	PA3890	Probable permease of ABC transporter	CM	-2,03			0,004923237		
Q9HUN9	mcsS_2	PA4925	Uncharacterized protein	CM	-2,01	-1,42		0,005449782	0,21987647	
Q9HZ51	ugpC	PA3187	Probable ATP-binding component of ABC transporter	CM	-1,98			0,006206685		
Q03024	aprD	PA1246	Alkaline protease secretion ATP-binding protein	CM	-1,93			0,007602442		
Q9I028	fadE	PA2815	Acyl-coenzyme A dehydrogenase	CM	-1,91		-2,15	0,008448308		0,0000637
Q9I1N8	pslA	PA2231	cell-cell and/or cell-surface interaction in biofilm formation	CM			-1,98			0,00413
Q9I6X0	opdC	PA0162	Histidine porin	OM		-2,28	-2,61		0,007391047	0,00000404
Q9HXC2	opuCA	PA3891	Probable ATP-binding component of ABC transporter	CM			-2,54			8,65E-09
Q9HW12	mcsS_1	PA4394	Uncharacterized protein	CM			-2,47			0,00282
Q9HZG7	TUEID40_05652	PA3041	Uncharacterized protein	CM			-2,29			0,0127
Q9HZG6	PROKKA_05651	PA3042	DUF883 domain-containing protein	membrane	-2,14		-2,23	0,003107612		5,3E-09
Q9I089	PROKKA_05975	PA2754	conserved hypothetical protein	CM	-2,02			0,005279851		
Q9HUA6	opgH	PA5077	Glucans biosynthesis glucosyltransferase H	CM			-1,96			0,00864
Q9HZG8	PROKKA_05653	PA3040	DUF883 domain-containing protein	CM			-1,94			0,015
Q9HVR7	dppC	PA4504	Probable permease of ABC transporter	CM			-1,79			0,000433
Q9HTR3	betT2	PA5291	Probable choline transporter	CM			-1,74			0,00192
Q9I6X6	triA	PA0156	Probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein	membrane			-1,65			0,00516
Q00513	xcpS	PA3102	Type II secretion system protein F	CM			-1,56			0,0128
Q9HUT4	PROKKA_02832	PA4897	AsmA domain-containing protein	CM			-1,49			0,0287
Q9HY19	potA2	PA3607	Spermidine/putrescine import ATP-binding protein	CM			-1,41			0,0365
Q9I5F5	putP	PA0783	Sodium/proline symporter	CM			-1,26			0,00846

3.1.4.5 Verification of altered protein levels found in mass spectrometry

As upregulation of efflux pumps is a known resistance mechanism, finding elevated levels of MexX/MexY and MexE/MexF/OprN in the sensitive ID40 Δ mepM1 Δ mepM2 Δ mepM3 was surprising. However, in a previous screening experiment for resistance related genes, Sonnabend et al. (2020) found that the deletion of *mexE* and *mexF* appeared to increase resistance in ID40 against Meropenem and Cefepim. These findings are consistent with my results on membrane proteome changes. To verify the higher levels of MexE/MexF/OprN and MexX/MexY in ID40 Δ mepM1 Δ mepM2 Δ mepM3, four knock-in strains were kindly generated by my working group:

- ID40::*mexE*-HiBiT
- ID40::*mexY*-HiBiT
- ID40 Δ mepM1 Δ mepM2 Δ mepM3::*mexE*-HiBiT
- ID40 Δ mepM1 Δ mepM2 Δ mepM3::*mexY*-HiBiT

The HiBiT-tag is a luminescent reporter system that allows detection of tagged proteins in Western Blots as well as quantification of protein levels in luciferase assays.

3.1.4.5.1 Detection of MexE-HiBiT and MexY-HiBiT by Western Blotting

Three independent whole cell lysates of ID40::*mexE*-HiBiT and ID40 Δ *mepM1* Δ *mepM2* Δ *mepM3*::*mexE*-HiBiT were blotted against HiBiT (Fig. 25). Blotting against RpoB served as loading control.

Western Blots verified higher levels of MexE in ID40 Δ *mepM1* Δ *mepM2* Δ *mepM3*.

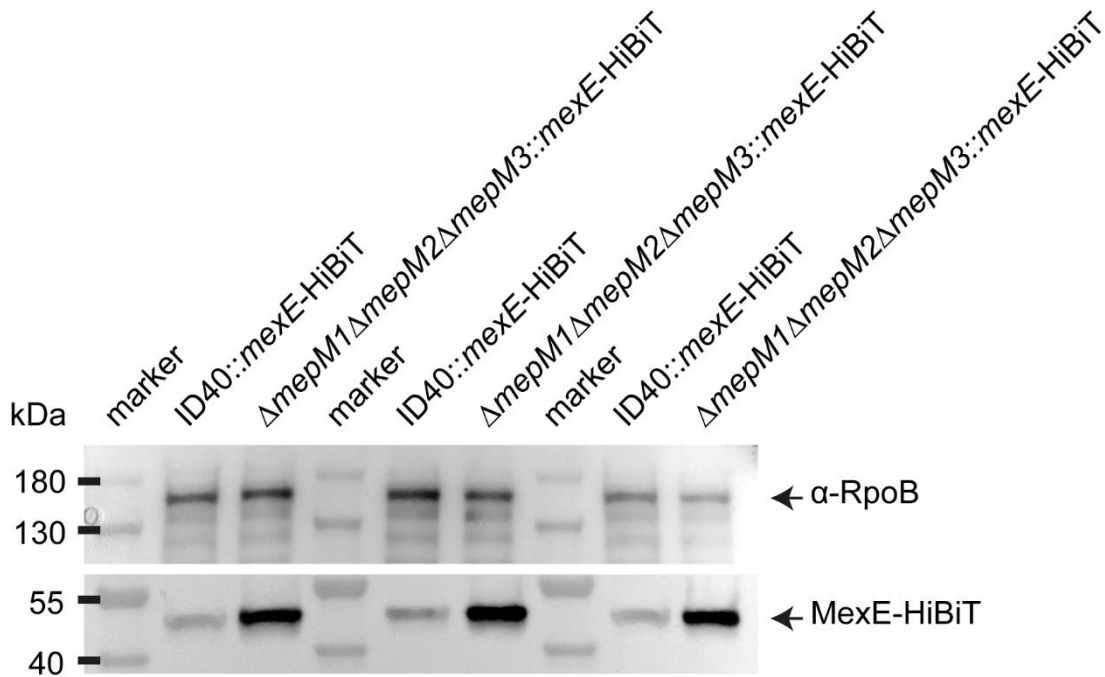


Figure 25: Increased MexE levels in ID40 Δ *mepM1* Δ *mepM2* Δ *mepM3* compared to ID40

Western blot analysis of whole cell lysates (WCL) of MexE-HiBiT tagged strains are shown. WCL were incubated with LgBit which binds to HiBiT resulting in a luciferase which converts the substrate furimazine leading to detectable chemoluminescence. RpoB as a loading control was detected using anti-RpoB antibody, a HRP conjugated secondary antibody and ECL as the substrate.

Two independent whole cell lysates of ID40::*mexY*-HiBiT and ID40 Δ *mepM1* Δ *mepM2* Δ *mepM3*::*mexY*-HiBiT were blotted against HiBiT (Fig. 26). Blotting against RpoB served as loading control.

Western Blots did not verify higher levels of MexY in ID40 Δ *mepM1* Δ *mepM2* Δ *mepM3*.

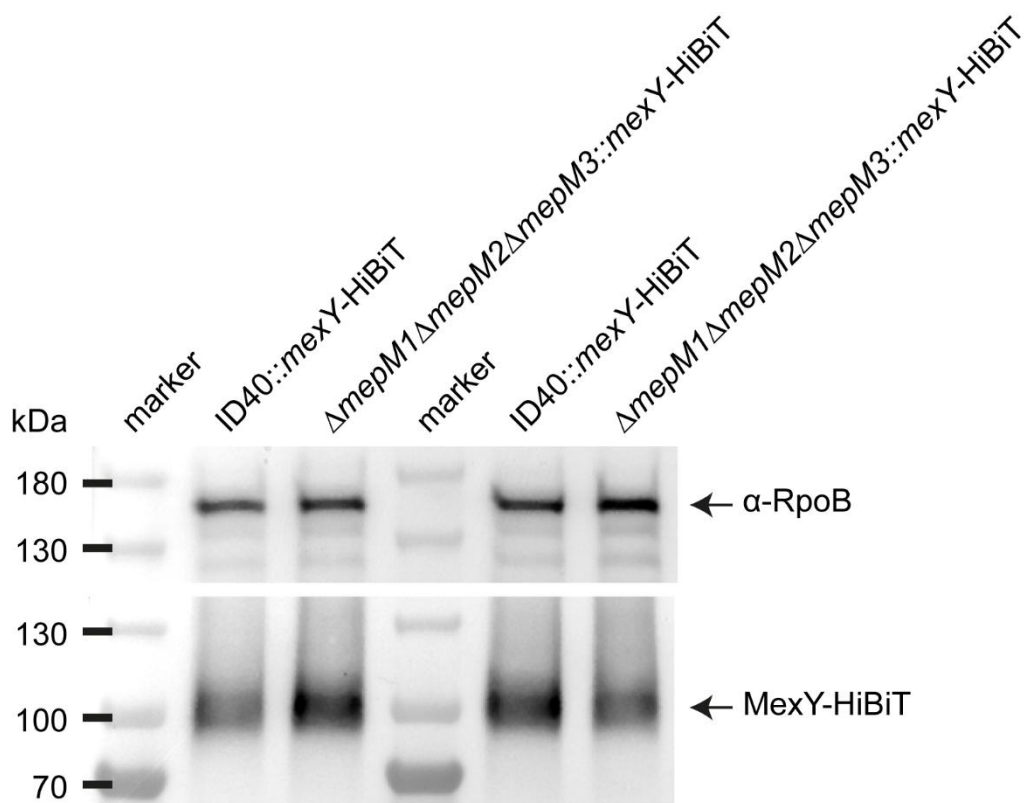


Figure 26: Equal MexY levels in ID40 Δ *mepM1* Δ *mepM2* Δ *mepM3* and ID40

Western blot analysis of whole cell lysates (WCL) of MexY-HiBiT tagged strains are shown. WCL were incubated with LgBit which binds to HiBiT resulting in a luciferase which converts the substrate furimazine leading to detectable chemoluminescence. RpoB as a loading control was detected using anti-RpoB antibody, a HRP conjugated secondary antibody and ECL as the substrate.

3.1.4.5.2 Quantification of MexE-HiBiT and MexY-HiBiT expression by luciferase assays

To quantify the Western Blot results, semiquantitative luciferase assays with HiBiT-labeled strains were performed. Addition of LgBiT to HiBiT results in the formation of an active HiBiT-LgBiT luciferase which converts a substrate and thereby generates luminescence.

Fig. 27 shows the results of three individual experiments, each experiment comprising three technical replicates. Significance levels were determined by statistical analysis using an unpaired t-test. MexE levels were 3.7 times higher in ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ compared to ID40.

Fig. 28 shows the results of one experiment comprising three technical replicates. MexY levels do not differ between those two strands.

These results agree with the Western Blot results.

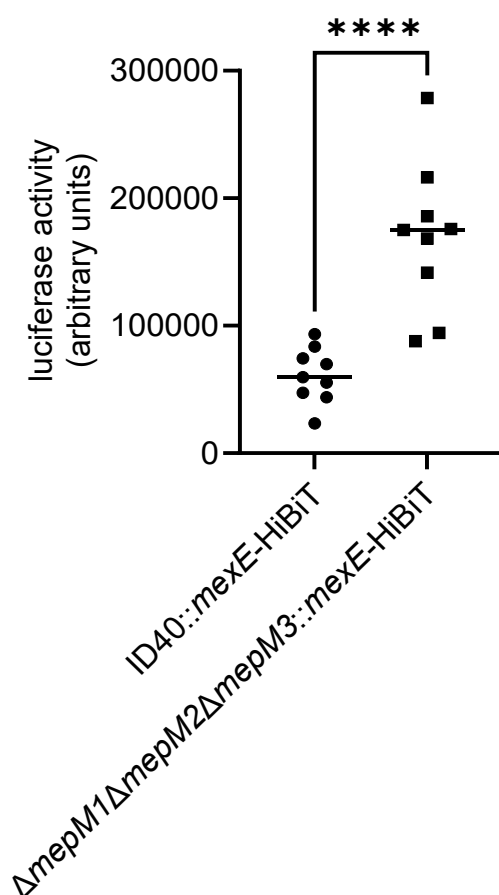


Figure 27: MexE-HiBiT expression in ID40 and ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$

Graph depicts results from three luciferase assays each comprising three technical replicates.

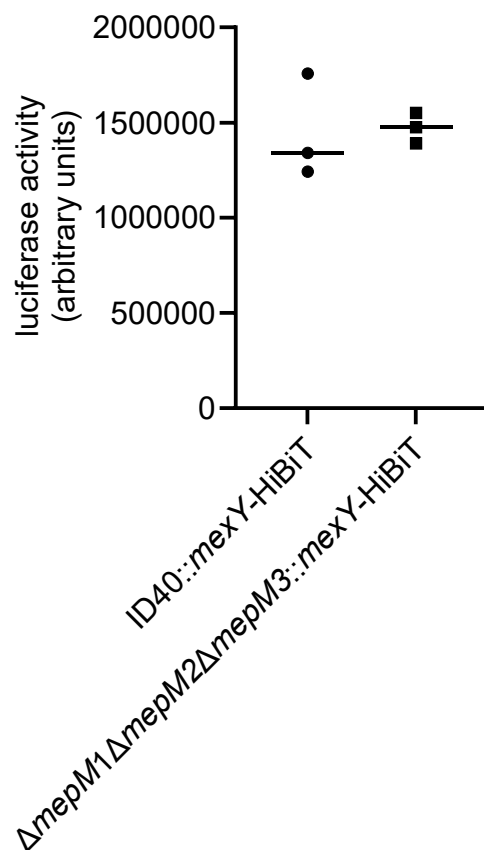


Figure 28: MexY-HiBiT expression in ID40 and ID40 Δ mepM1 Δ mepM2 Δ mepM3
Graph depicts results from one luciferase assay comprising three technical replicates.

3.1.4.6 Antibigrams verifying Δ mepM1 Δ mepM2 Δ mepM3 mediated reduction in resistance by upregulation of *mexE/mexF/oprN*

The described results led to the hypothesis that increased expression of the *mexE/mexF/oprN* operon in the absence of MepMs are crucial to achieve reduced β -lactam resistance. To verify this, the *mexE/mexF/oprN* operon was deleted in ID40 and ID40 Δ mepM1 Δ mepM2 Δ mepM3 by my working group.

Microbroth dilution assays on EUX2NF plates were used to determine MIC values of the four strains ID40, Δ mepM1 Δ mepM2 Δ mepM3, Δ mexE/mexF/oprN and Δ mepM1 Δ mepM2 Δ mepM3 Δ mexE/mexF/oprN. The detected MIC values of ID40 and Δ mepM1 Δ mepM2 Δ mepM3 are consistent with those measured by Kemper (see Table 24).

Table 27 summarizes the MIC values determined in two individual tests. MIC values higher than ID40 are marked in red. MIC values lower than ID40 are marked in green. Deletion of *mexE/mexF/oprN* in ID40 reduces resistance to ciprofloxacin, imipenem, levofloxacin,

trimethoprim sulfamethoxazole and polymixin B. It increases resistance in ID40 to gentamicin, tobramycin and colistin and slightly increases resistance to cefepim, meropenem and amikacin. Additional deletion of the *mexE/mexF/oprN* operon in the sensitive ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ partially restores resistance against meropenem, imipenem, ceftazidime, piperacillin-tazobactam and colistin (underlaid in violet). This effect is also observed, albeit weaker, for cefepim, aztreonam, doripenem and amikacin.

Table 27: MIC values of $\Delta mepM1\Delta mepM2\Delta mepM3$ and $\Delta mexE/mexF/oprN$ deletion mutants
MIC values were determined in two individual tests. In red: MIC values higher than ID40, in green: MIC values lower than ID40, underlaid in violet: additional deletion of *mexE/mexF/oprN* partially restores resistance to those antibiotics in the sensitive ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$.

	MIC Breakpoint (mg/L)		ID40	$\Delta mepM1$ $\Delta mepM2$ $\Delta mepM3$	$\Delta mepM1$ $\Delta mepM2$ $\Delta mepM3$ $\Delta mexE/mexF/oprN$	$\Delta mexE/mexF/oprN$
	S ≤	R >				
Piperacillin-Tazobactam	0.001	16	>64	16	32	>64
Meropenem	2	8	8	1	2	8-16
Imipenem	4	4	64->64	16	32	64
Doripenem			4-8	0.5-1	1	8
Aztreonam	0.001	16	32	4-8	8	32->32
Ceftazidime	0.001	8	>32	8	16	>32
Cefepime	8	8	32	4-8	8	32->32
Gentamicin	IE	IE	2	2-4	2-4	4
Tobramycin	2	2	0.5	0.5	0.5	1
Amikacin			8	4	4-8	8-16
Ciprofloxacin	0.001	0.5	>2	>2	>2	2
Levofloxacin	0.001	1	>4	>4	>4	4
Tigecycline			>2	>2	>2	>2
Trimethoprim sulfamethoxazole			> 8/125	> 8/125	8/125	8/125
Polymixin B			4-8	1	1	4
Colistin	2	2	0.5-1	0.5-1	1-2	1-2

In summary of this part, it can be shown that deletion of *mepM1*, *mepM2* and *mepM3* leads to higher production of MexE indicating higher production of the complete efflux consisting of MexE, MexF and OprN. Moreover, evidence could be provided that this upregulation contributes to the reduced resistance found for ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$.

3.2 Protein purification, Antibodies and Activity Assays

Purified rMepMs were required for the establishment of an *in vitro* MepM activity assay to screen possible MepM inhibitors and for the generation of anti-MepM1 and anti-MepM2 antibodies for the detection of MepM1 and MepM2 *in vivo*.

3.2.1 Protein purification of His-tagged rMepM1, rMepM2 and rMepM3

3.2.1.1 Protein overexpression of His-tagged rMepMs in *E. coli* BL21

Fig. 29 describes the structure and domains of paMepM1, paMepM2 and paMepM3 *in vivo*. rMepM1 was overexpressed in full length (aa 1-447). rMepM2 was overexpressed lacking the N-terminal signal peptide (SP) as it is truncated *in vivo* during translocation (aa 28-282). rMepM3 was overexpressed lacking the transmembrane helix (TM) to make the protein water soluble and allow protein purification (aa 47-307).

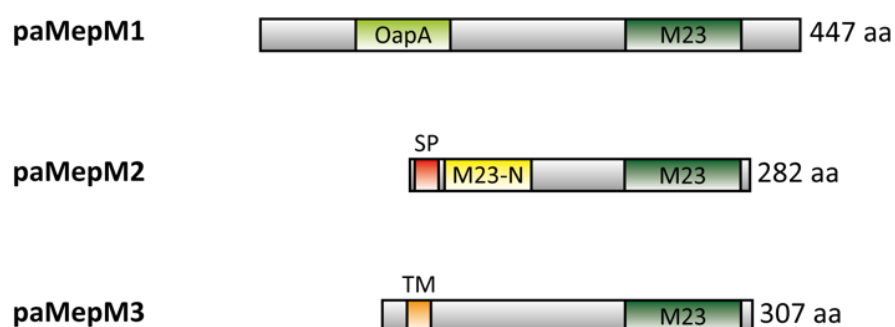


Figure 29: Domain organization of paMepM1, paMepM2 and paMepM3

rMepM1 was expressed in full length, rMepM2 and rMepM3 were expressed lacking the signal peptide (SP) or the transmembrane helix (TM), respectively. aa = amino acid

His-tagged rMepM1, rMepM2 and rMepM3 were overexpressed in *E. coli* BL21 carrying His-*pamepM* under an inducible lactose/IPTG promotor on a plasmid (Fig. 30). *E. coli* cryocultures carrying the overexpression plasmids were already generated by Fabian Renschler before the start of my work.

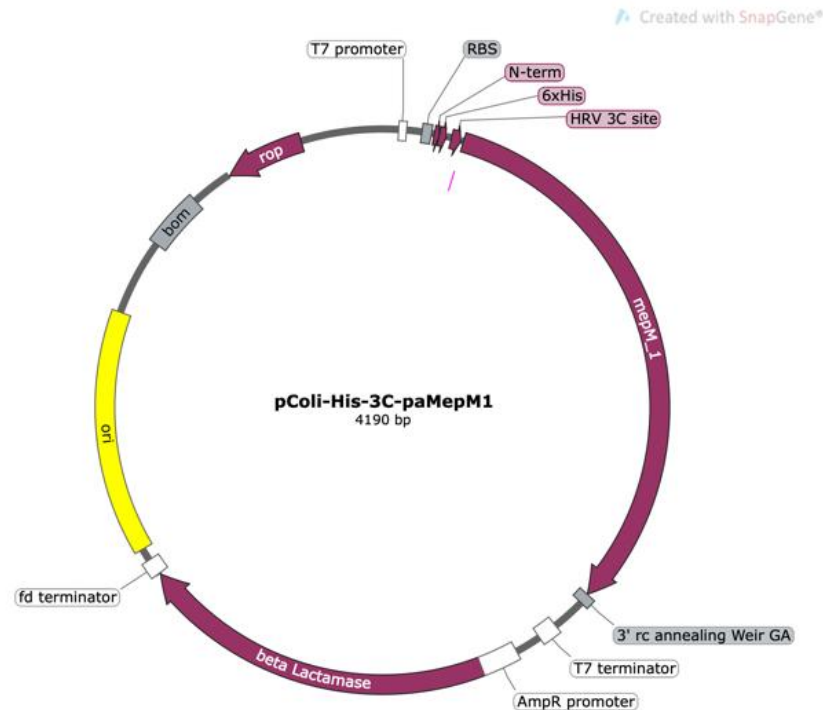


Figure 30: Overexpression plasmid pColi-His-3C-paMepM1

The plasmid carries His-tagged *pamepM1* under control of a T7 promoter inducible by IPTG or lactose. The β -lactamase enables antibiotic selection with Ampicillin of plasmid-carrying *E. coli*. HRV-3C-site allows removal of the His-tag after protein overexpression.

The structure of the plasmid is considered an exemplary one for all three expression plasmids for rMepM1, rMepM2 and rMepM3.

Fig. 31 depicts SDS-PAGE of induction and harvest samples after Coomassie staining. Loaded samples were obtained from 500 μ L culture just before (induction) and after overexpression (harvest). The band strength of the lines varies due to the different bacterial density in the samples.

The increased band intensities for (A) rMepM1 (52 kDa), (B) rMepM2 (30 kDa) and (C) rMepM3 (30 kDa) in the harvest samples indicate successful overexpression of rMepMs. Due to a low basic promotor activity, slight overexpression is already seen in the preculture sample of rMepM3. rMepM1 was overexpressed in two times 1 L bacterial culture in parallel (Fig. 31 A, cultures A and B) and the harvest of both cultures was then purified together.

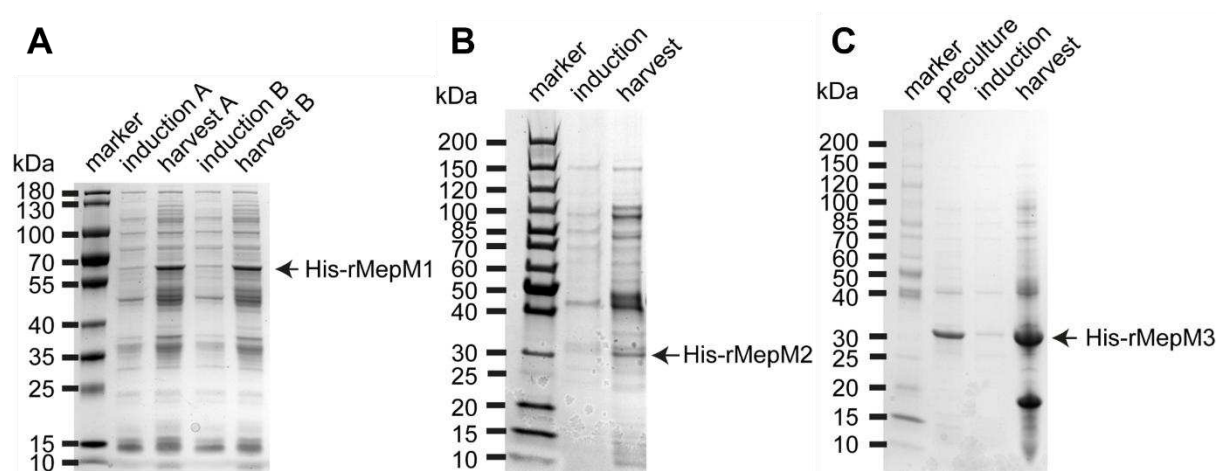


Figure 31: SDS-PAGE confirming rMepM overexpression in *E. coli* BL21

Coomassie stained SDS-PAGEs are shown. Loaded samples were obtained from 500 μ L culture just before (induction) and after (harvest) lactose promoter-induced overexpression. The increased band intensities for (A) rMepM1 (52 kDa), (B) rMepM2 (30 kDa) and (C) rMepM3 (30 kDa) in the harvest samples indicate successful overexpression of rMepMs.

3.2.1.2 Nickel affinity chromatography of His-tagged rMepM1, rMepM2 and rMepM3

Overexpressed rMepMs were purified in two steps. First, His-tagged rMepMs were separated by nickel affinity purification. In a second step rMepMs were further purified by size exclusion chromatography.

Fig. 32 depicts SDS-PAGE of samples taken at different steps of nickel affinity purification. After cell lysis and centrifugation, soluble proteins such as rMepM1 and rMepM2 were found in the supernatant. rMepM3 appeared to be an insoluble protein that remained in the pellet and therefore could not be purified.

His-tagged rMepMs bound to the Nickel beads were separated from unbound proteins in flowthrough and wash samples. rMepM1 and rMepM2 appeared in the elution samples.

For rMepM1 purification, supernatant A and B were combined and both loaded onto the same column. Starting with 70 mL of supernatant, rMepM1 was eluted in 19 mL (elution 2). rMepM2 was eluted in 20 mL (elution 1) starting with 35 mL of supernatant. Hence, His-tagged rMepM1 and rMepM2 could be purified by Nickel affinity purification, but not rMepM3 as it was not water soluble.

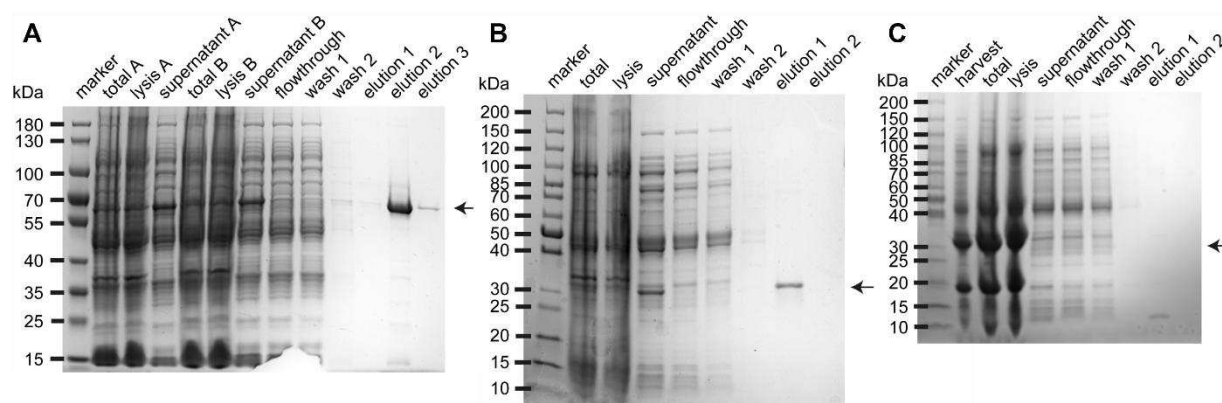


Figure 32: SDS-PAGE of samples taken at different steps during nickel affinity purification of His-tagged rMepMs

Coomassie stained SDS-PAGEs of various steps of the purification process are shown. After cell lysis and centrifugation, His-tagged rMepM1 (A) and rMepM2 (B) appeared in the supernatant. Insoluble rMepM3 (C) appeared in the pellet. Nickel affinity purification separated the eluted His-tagged proteins from the non-binding proteins collected in the flowthrough and wash samples. The same amount of sample was loaded per line. Arrows indicate the positions of rMepM1 (52 kDa), rMepM2 (30 kDa), and rMepM3 (30 kDa), respectively.

3.2.1.3 Size exclusion chromatography of His-tagged rMepM1 and rMepM2

In a second purification step, rMepMs were purified by size exclusion chromatography.

Chromatograms in Fig. 33 (A) and Fig. 34 (A) illustrate the total protein concentrations measured by UV absorbance (y-axis) of the separated fractions (x-axis). Fractions under the peak contain the largest amount of protein.

Fig. 33 shows the chromatogram of size exclusion chromatography (S200 16/600 column) of rMepM1 (A) and SDS-PAGE of the corresponding peak fractions 15 – 39 after Coomassie staining (B). Fractions 15 – 37 were combined, concentrated, flash frozen in liquid N₂ and stored at -80°C.

Fig. 34 shows the chromatogram of size exclusion chromatography (S75 26/600 column) of rMepM2 (A) and SDS-PAGE of the corresponding peak fractions 35 – 47 after Coomassie staining (B). Fractions 38 – 43 were combined, concentrated, flash frozen in liquid N₂ and stored at -80°C.

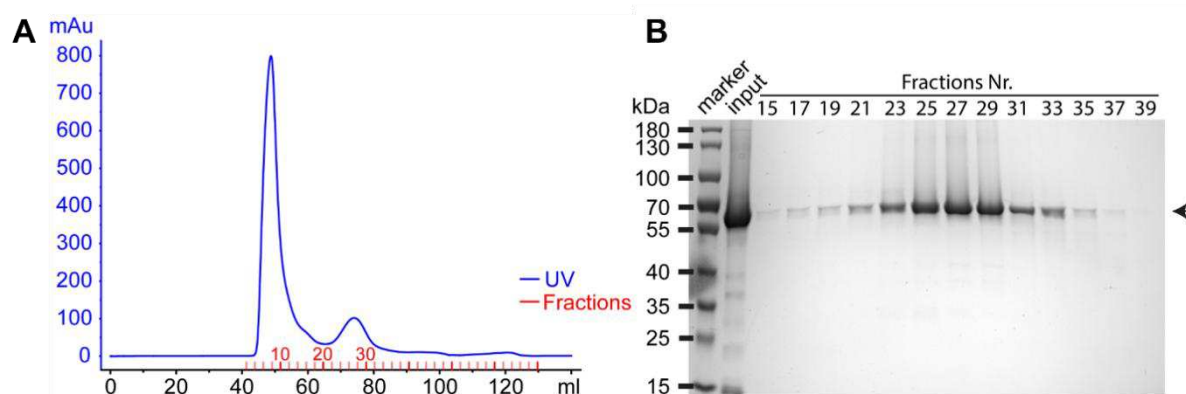


Figure 33: Size exclusion chromatography of rMepM1 on S200_16_600

(A) Chromatogram illustrates the protein concentrations measured by UV absorbance (y-axis) of the separated fractions (x-axis). Fractions under the peak contain the largest amount of protein. (B) SDS-PAGE of the corresponding peak fractions 15 – 39 after Coomassie staining. The arrow indicates the positions of rMepM1 (52 kDa).

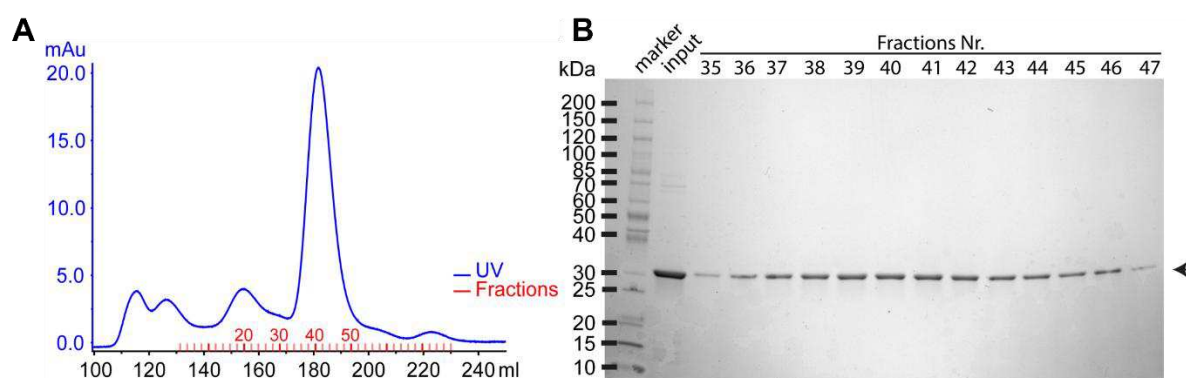


Figure 34: Size exclusion chromatography of rMepM2 on S75_26_600

(A) Chromatogram illustrates the protein concentrations measured by UV absorbance (y-axis) of the separated fractions (x-axis). Fractions under the peak contain the largest amount of protein. (B) SDS-PAGE of the corresponding peak fractions 15 – 39 after Coomassie staining. The arrows indicates the positions of rMepM2 (30 kDa).

The purification methods for rMepM1 and rMepM2 were successfully established. A yield of 11 mg rMepM1 and 12.2 mg rMepM2 was obtained from one liter *E. coli* BL21 culture. Fig. 35 depicts SDS-PAGE after Coomassie staining of purified His-tagged rMepM1 and rMepM2.

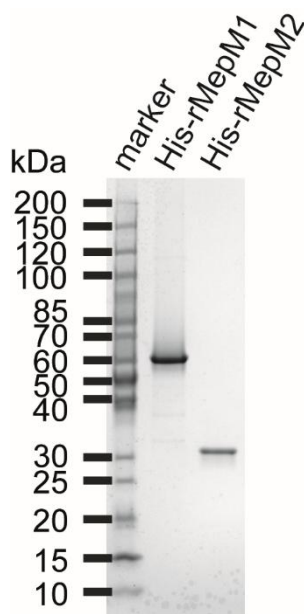


Figure 35: Purified His-tagged rMepM1 and rMepM2

SDS-PAGE after Coomassie staining. Proteins were purified by nickel affinity chromatography followed by size exclusion chromatography.

3.2.2 Production of antibodies against MepM1 and MepM2

Purified proteins were shipped to Eurogentec for the production of antibodies against MepM1 and MepM2. Rabbits were selected for immunization based on their Pre-immune bleeds. Preimmune sera were checked by Western Blot for their interaction with ID40 whole cell lysates (i.e. *P. aeruginosa* proteins) to identify animals with previous exposures to *P. aeruginosa* or with cross-reactive antibodies which might hamper our attempts to raise antibodies against MepM1 and MepM2. Two animals each, which did not show signals at the corresponding molecular weights of the MepM proteins, were chosen for immunization. Rabbits 12474 and 27475 were immunized against rMepM1 and rabbits 12467 and 12471 against rMepM2.

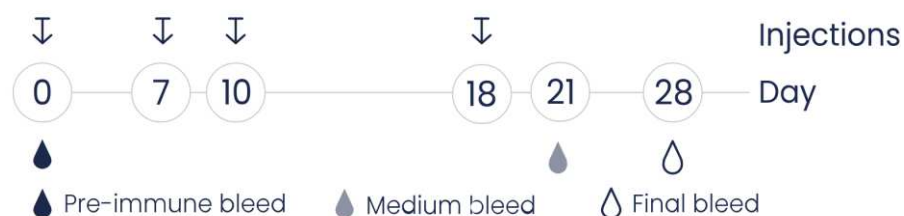


Figure 36: Eurogentec © Speedy 28-Day immunization protocol for the generation of anti-MepM1 and anti-MepM2 antibodies

rMepM1 and rMepM2 were each injected into two rabbits on days 0, 7, 10 and 18. The final bleeds were collected on day 28, combined, affinity purified and contained the final antibodies.

Antibodies (purified final bleeds) were tested in Western Blots against whole cell lysates (WCL) of ID40, ID40 $\Delta mepM1\Delta mepM2$ and ID40 $\Delta ctpA$. Fig. 37 shows the results of Western blots with rabbit sera after immunization and affinity purification. The newly produced anti-MepM1 and anti-MepM2 antibodies detect MepM1 and MepM2 in WCL of ID40 strains. Anti-MepM2 binds specifically to MepM2. Anti-MepM1 detects MepM1 but also binds to some non-specific proteins.

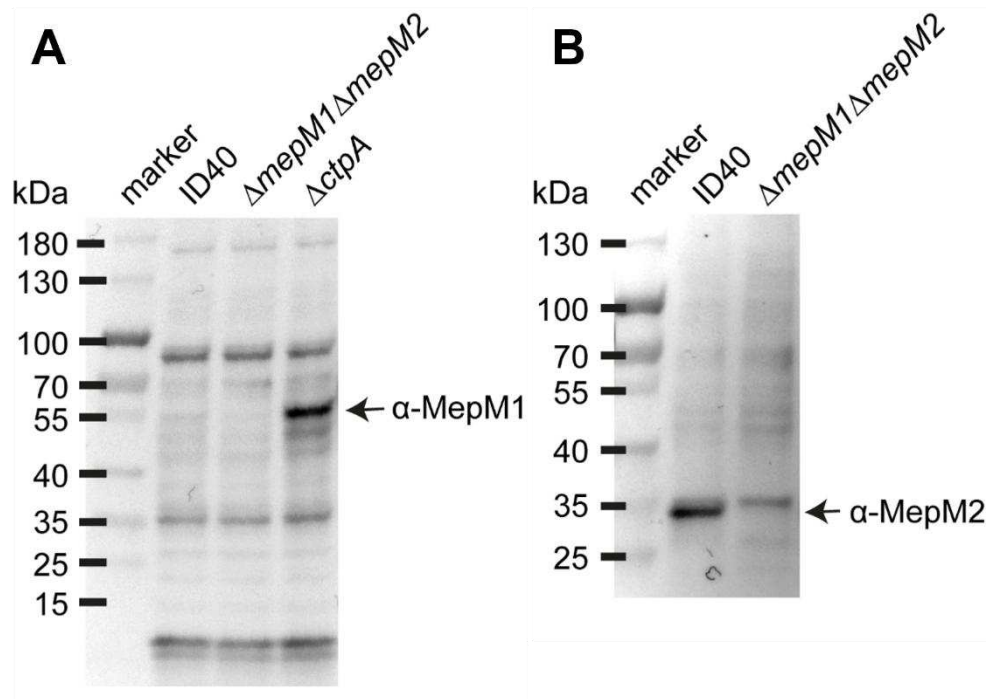


Figure 37: Newly produced antibodies detect MepM1 and MepM2 in Western Blots with whole cell lysates (WCL)

Western blot analysis of whole cell lysates (WCL) of ID40 and deletion mutants are shown. Chemiluminescence was detected after incubation with primary (A) anti-MepM1 or (B) anti-MepM2 antibody, HRP conjugated secondary antibody and clarity Western ECL. The higher MepM1 levels in $\Delta ctpA$ allow for a better visualization of the MepM1 band, which is only weakly visualizable in ID40.

For optimization different primary antibody concentrations were tested (Fig. 38). At an antibody concentration of 1:1000, MepM1 is still detected by the antibody in ID40 with less non-specific bands. The anti-MepM2 antibody shows very clear results at a concentration of 1:1000, but still detects MepM2 at 1:2000.

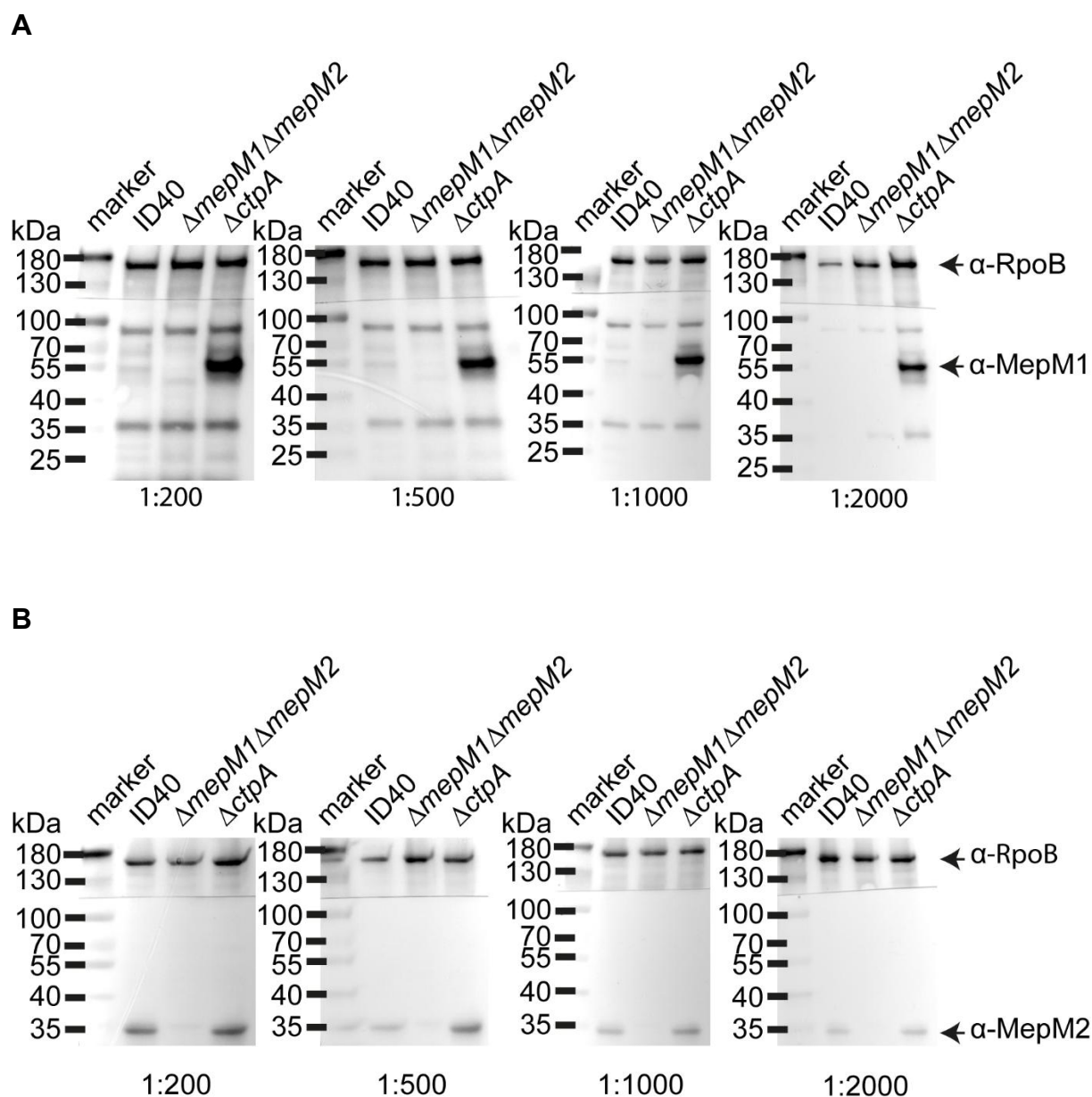


Figure 38: Concentrations of anti-MepM1 and anti-MepM2 primary antibodies in Western Blots
Western blot analysis of whole cell lysates (WCL) of ID40 and deletion mutants are shown. Chemiluminescence was detected after incubation with various concentrations of primary (A) anti-MepM1 or (B) anti-MepM2 antibody, HRP conjugated secondary antibody and clarity Western ECL solution. The best results are obtained at a primary antibody concentration of 1:1000 for anti-MepM1 and anti-MepM2 antibodies.

3.2.2.1 MepM1 and MepM2 protein levels in ID40 and the deletion mutants

The newly generated antibodies allowed detection of MepM1 and MepM2 levels in ID40 and the deletion mutants.

Fig. 39 shows the results of Western Blot analysis of WCL of ID40 strains with anti-MepM1 and anti-MepM2 antibodies. Blotting against the ubiquitous RpoB served as a loading control. Deletion of *mepM1* and *mepM2* was demonstrated in all $\Delta mepM1$ and $\Delta mepM2$ deletion mutants. High MepM1 levels were detected in the whole cell lysate of the $\Delta ctpA$ deletion mutant, indicating that in principle the anti-MepM1 antibody can detect MepM1 well. In contrast, MepM1 levels in ID40 are barely but clearly detectable, whereas in the $\Delta mepM1$ deletion mutants MepM1 is undetectable. It can be concluded that MepM1 levels in ID40 are kept low due to CtpA. The anti-MepM2 antibody allows to detect MepM2 very clearly. Since the strength of the band in the $\Delta ctpA$ deletion mutant and ID40 is comparable, this data also shows that MepM2, in contrast to MepM1, is not regulated by CtpA.

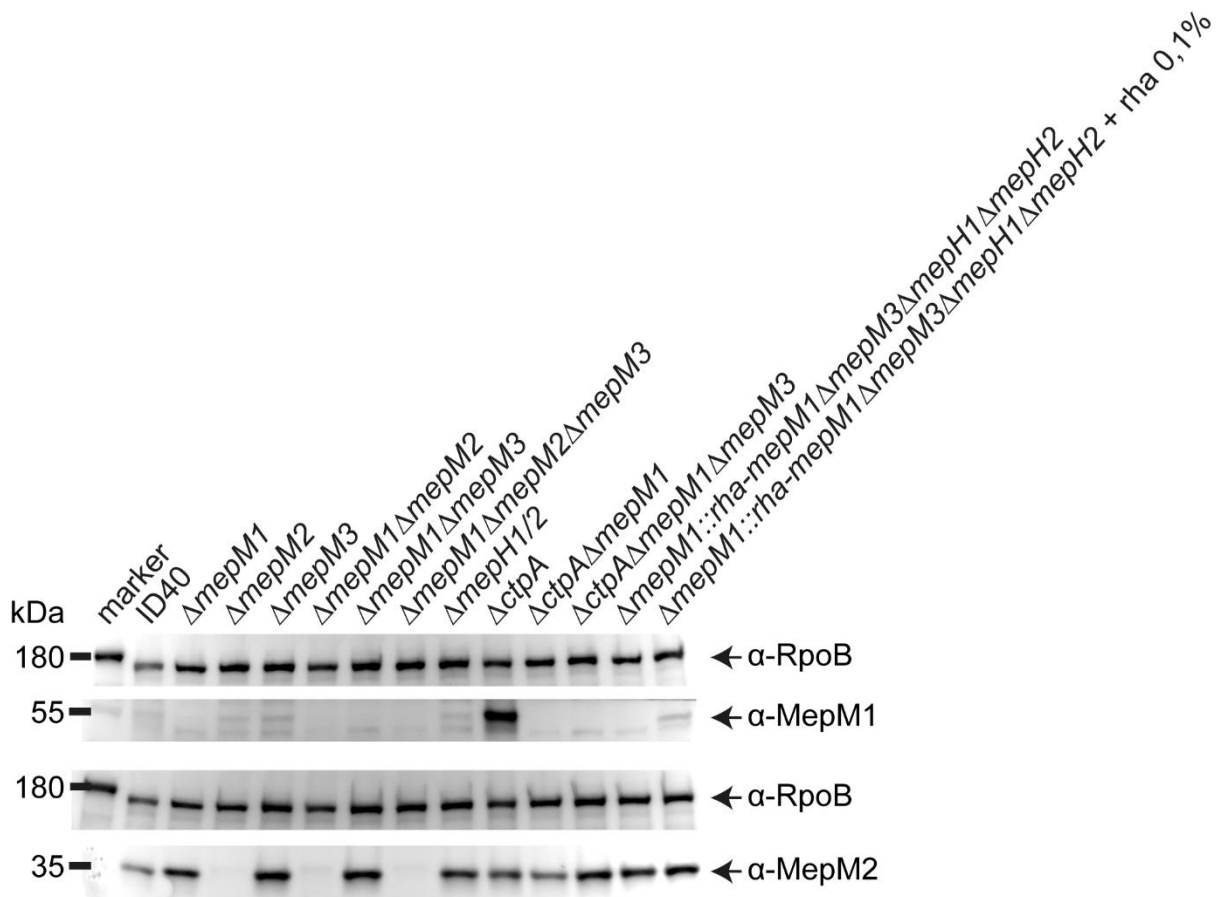


Figure 39: MepM1 and MepM2 levels in ID40 and $\Delta mepM$ deletion mutants

Western blot analyses were performed using whole cell lysates of indicated strains. Chemiluminescence was detected after incubation with indicated primary antibody, HRP conjugated secondary antibody and clarity Western ECL solution.

3.2.2.2 MepM localization

In 3.1.4.3.1 the ID40 membrane proteome was isolated and separated into inner and outer membrane proteins or soluble cytoplasmic and periplasmic proteins. Blotting of the resulting fractions against MepM1 and MepM2 allowed localization of MepM1 and MepM2. Previously performed Western Blots against periplasmic (SurA), cytoplasmic (RpoB) and outer membrane (PlpD) proteins were used for comparison.

MepM2 is concentrated in the same fractions as SurA and is not present in the outer membrane fractions as the OM marker protein PlpD. Since periplasm and cytoplasm were not separated during membrane purification and no blot against an inner membrane protein was available for comparison, this results only indicate periplasmic or cytoplasmic localization of MepM2.

Since MepM1 is expressed at low levels, it could not be detected in the Western Blot. A very weak band may indicate localization of MepM1 in the outer membrane (results not shown).

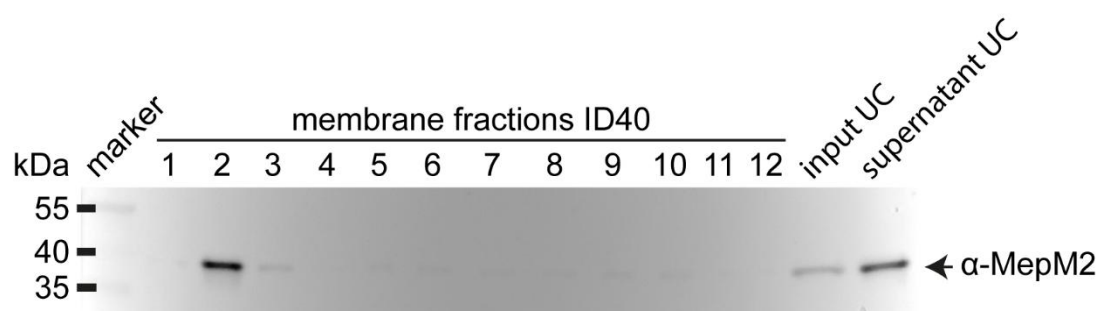


Figure 40: MepM2 is detected in membrane proteome fractions of ID40 using anti-MepM2 antibody

Western blots were performed to detect MepM2 in membrane fractions of purified ID40 membrane. Chemiluminescence was detected after incubation with anti-MepM2 antibody, HRP-conjugated secondary antibody and finally clarity Western ECL solution. MepM2 was mainly detectable in fraction 2 indicating periplasmic or cytoplasmic localization of MepM2 after comparison with results in 3.1.4.3.1.

3.2.3 rMepM1 and rMepM2 Activity Assays

To enable screening for MepM inhibitors, a major goal of this work was to develop a high-throughput *in vitro* activity assay for MepM1 and MepM2.

3.2.3.1 FITC-peptidoglycan (FITC-PG) Assay

To investigate whether purified rMepM1 and rMepM2 are in principle suitable in enzyme activity assays, the *in vivo* MepM substrate peptidoglycan was used as substrate for a first MepM activity assay. As described in detail in Materials and Methods, the peptidoglycan was labeled with FITC. The FITC-labeled PG was incubated with rMepM and the incubation was stopped by centrifugation (Fig. 41). Since the FITC-PG is insoluble in water, the release of short soluble FITC-PG fragments into the supernatant indicates PG cleavage mediated by rMepM. These fragments were detected by fluorescence measurement in the Tecan reader.

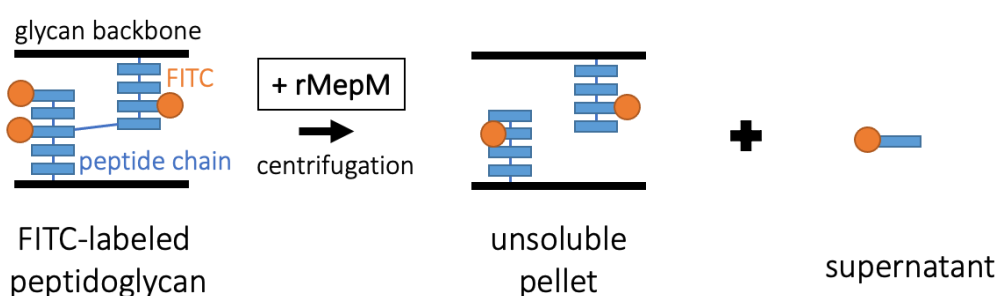


Figure 41: FITC-PG Assay principle

FITC-labeled PG is incubated with rMepM and incubation is stopped by centrifugation. Since the FITC-PG fraction is insoluble in water, the release of short soluble FITC-PG fragments into the supernatant indicates PG cleavage mediated by rMepM.

3.2.3.1.1 rMepM1 and rMepM2 are active enzymes that cleave *E. coli* PG

In a first step, it was evaluated whether the purified proteins rMepM1 and rMepM2 are active enzymes. For this purpose, *E. coli* FITC-PG, kindly provided by Manuel Banzhaf, was incubated with rMepM1 and rMepM2.

Fig. 42 illustrates the fluorescence emissions detected in the supernatants of FITC-PG after incubation with rMepM1 and rMepM2. Uncleaved FITC-PG (no enzyme) corresponds to the background. The assay confirmed that rMepM1 and rMepM2 are active enzymes that can cleave FITC-labeled *E. coli* PG. The signal to background ratio was 2.9 for rMepM1 and 2.2 for rMepM2.

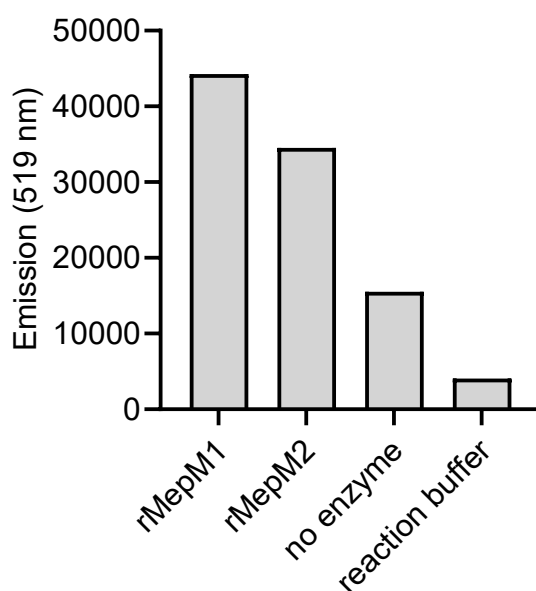


Figure 42: rMepM1 and rMepM2 cleave *E. coli* PG

1 mg/mL *E. coli* FITC-PG was incubated with 1 μ M rMepM for 90 min in 150 mM NaCl buffer. Emission was measured with gain 106 (optimal) in the Tecan reader.

3.2.3.1.2 rMepM2 cleaves *Bacillus subtilis* PG

For further experiments, available Gram-positive *Bacillus subtilis* (*B. subtilis*) PG, kindly provided by Marina Borisova-Mayer, was labeled with FITC. To save resources, the optimization of the assay conditions was initially performed with rMepM2 only. Since the emissions were measured with optimal gain, the absolute values differ between the individual experiments and must be considered in comparison to the background of each experiment.

Fig. 43 illustrates the emissions detected in the supernatants of FITC-labeled *B. subtilis* PG after incubation with rMepM2 for 60 min. Supernatant of uncleaved FITC-PG (no enzyme) corresponds to the background.

FITC-labeling of *B. subtilis* PG was successful. rMepM2-mediated cleavage of FITC-labeled *B. subtilis* PG can be measured using this assay setup. However, the signal to background ratio with *B. subtilis* PG was only 2.0 for rMepM2 and needed to be increased.

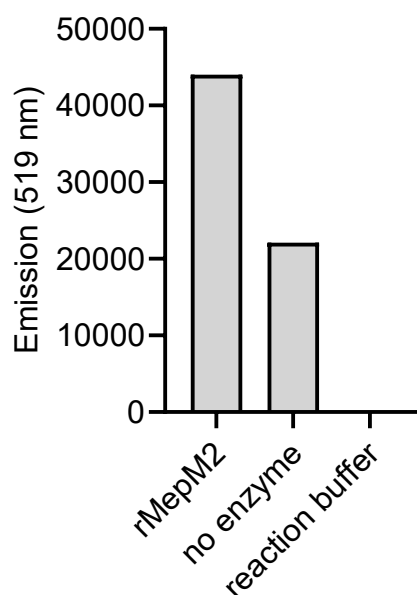


Figure 43: rMepM2 cleaves FITC-labeled *B. subtilis* PG

1 mg/mL FITC-labeled *B. subtilis* PG was incubated with 4.6 μ M rMepM2 for 60 min in 150 mM NaCl buffer. Emissions were measured with gain 112 (optimal) in the Tecan reader.

3.2.3.1.3 Optimization of FITC-PG Assay conditions

In a second step, the assay conditions were optimized to increase the signal to background ratio. Signal to background ratios were calculated following this formula:

$$\text{signal to background ratio} = \frac{rMepM2}{\text{no enzyme}}$$

3.2.3.1.3.1 Incubation time

The initial incubation time of 1 h was extended to 2 h and 3 h. The extended incubation time did not result in further FITC-PG cleavage but increased the background. After 1 h either all PG-crosslinks have already been cleaved or rMepM2 has become inactive due to enzyme instability.

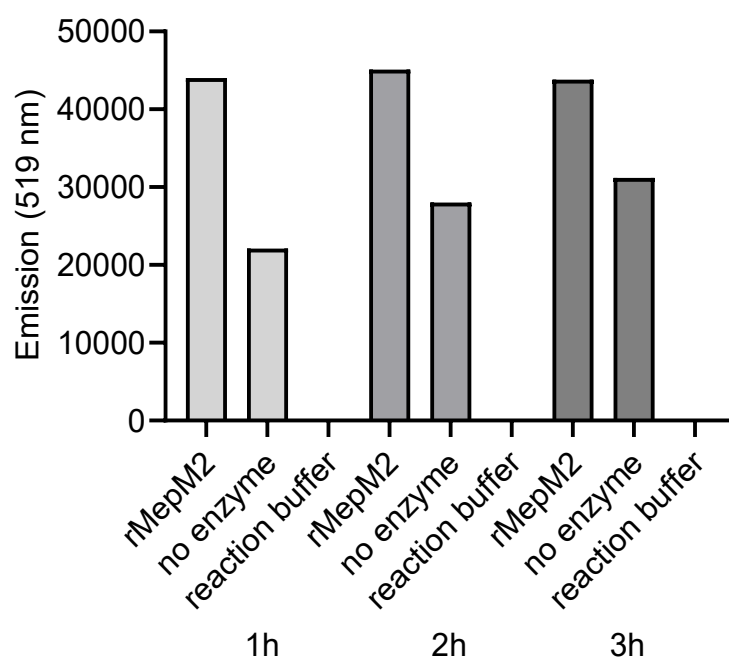


Figure 44: FITC-PG assay optimization: extended incubation time

1 mg/mL FITC-labeled *B. subtilis* PG was incubated with 4.6 μ M rMepM2 for 1 h, 2 h or 3 h in 150 mM NaCl buffer. Emissions were measured with optimal gain 112 (1 h), 111 (2 h) and 108 (3 h) in the Tecan reader.

3.2.3.1.3.2 rMepM2 enzyme concentration

rMepM2 concentration was increased by factor two to three (Fig. 45). The emission signal raised proportionally to the amount of enzyme used. The ratio of emission to rMepM2 concentration was analysed using a simple linear regression in GraphPad and confirmed a linear correlation (Fig. 46). Signal to background ratio was raised to 2.4 by using a three times higher concentration of rMepM2.

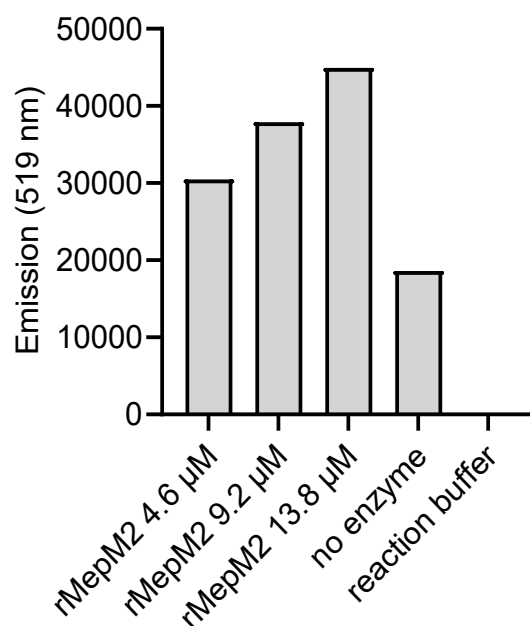


Figure 45: FITC-PG assay optimization: increase of rMepM2 concentration

1 mg/mL FITC-labeled *B. subtilis* PG was incubated with different concentrations of rMepM2 for 60 min in 150 mM NaCl buffer. Emissions were measured with gain 110 (optimal) in the Tecan reader.

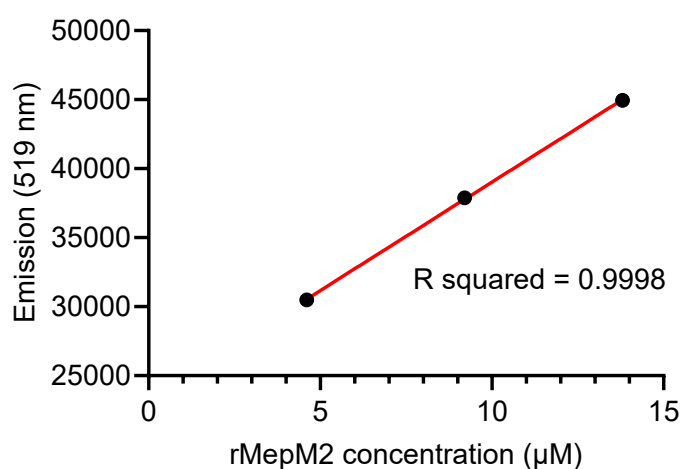


Figure 46: Signal increases proportionally to rMepM2 concentration

The ratio of emission signal to rMepM2 concentration was analysed using a simple linear regression and confirmed a linear correlation.

3.2.3.1.3.3 FITC-PG substrate concentration

The substrate concentration was halved to test if we can reduce the background signal. We opted to use the highest protein concentration used so far to obtain the maximum substrate turnover.

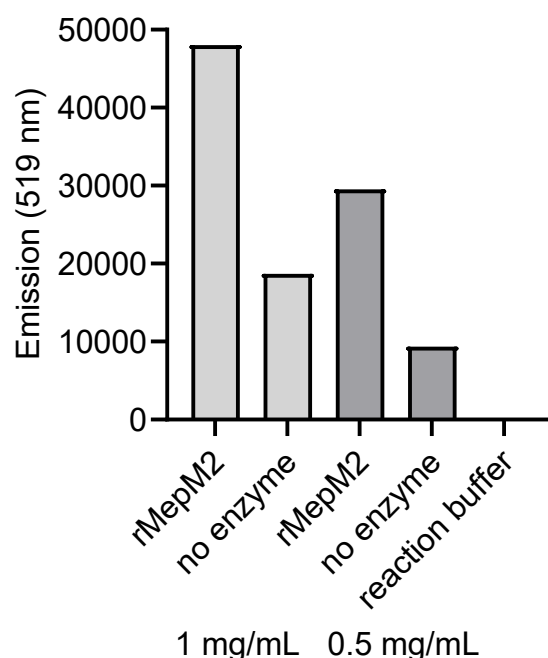


Figure 47: FITC-PG assay optimization: FITC-PG substrate concentration

FITC-labeled *B. subtilis* PG was incubated with 12 μ M rMepM2 for 60 min in no salt buffer. Emissions were measured with gain 100 in the Tecan reader.

Table 28: Signal to background ratios depending on FITC-PG concentration

Halving the amount of FITC-PG increases the signal to background ratio.

FITC-PG substrate concentration	Signal to background ratio
1 mg/mL	2.6
0.5 mg/mL	3.2

Signal to background ratio was raised to 3.2 (Table 28) by reducing the substrate concentration to 0.5 mg/mL FITC-PG.

3.2.3.1.3.4 Buffer conditions

3.2.3.1.3.4.1 pH value

The assay was performed at different pH values between 7 and 9 to evaluate at which pH value rMepM2 is most active (Fig. 48). Very high or low pH values of the buffer caused a non-specific decay of FITC-PG and thus increased the background. Since FITC-PG will not be used as a substrate for the high-throughput assay, this effect was secondary. After subtracting the background signals from the respective rMepM2 signals (Table 29), rMepM2 is most active at pH 8.

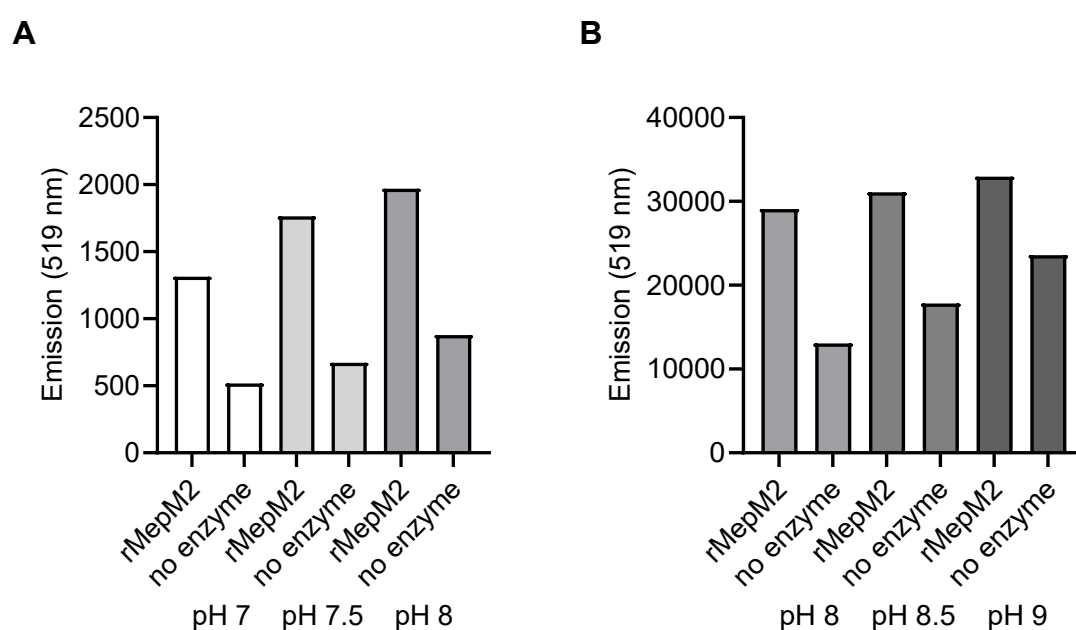


Figure 48: FITC-PG assay optimization: pH value of assay buffer

1 mg/mL FITC-labeled *B. subtilis* PG was incubated with 11.4 μ M rMepM2 for 60 min in 150 mM NaCl buffer. Emissions were measured with gain 100 in the Tecan reader. (A) experiment with low pH buffers (B) experiment with high pH buffers

Table 29: rMepM2 mediated PG cleavage at different pH values

The table illustrates data generated in two assays (low and high pH). In both assays, rMepM2 was most active at pH 8.

	pH value	Signal minus background
Low pH assay	7	9713
	7.5	13137
	8	13388
High pH assay	8	16035
	8.5	13257
	9	9393

3.2.3.1.3.4.2 Salt concentration

The assay was performed using assay buffers with different salt concentrations (Fig. 49). rMepM2 was most active at no salt conditions.

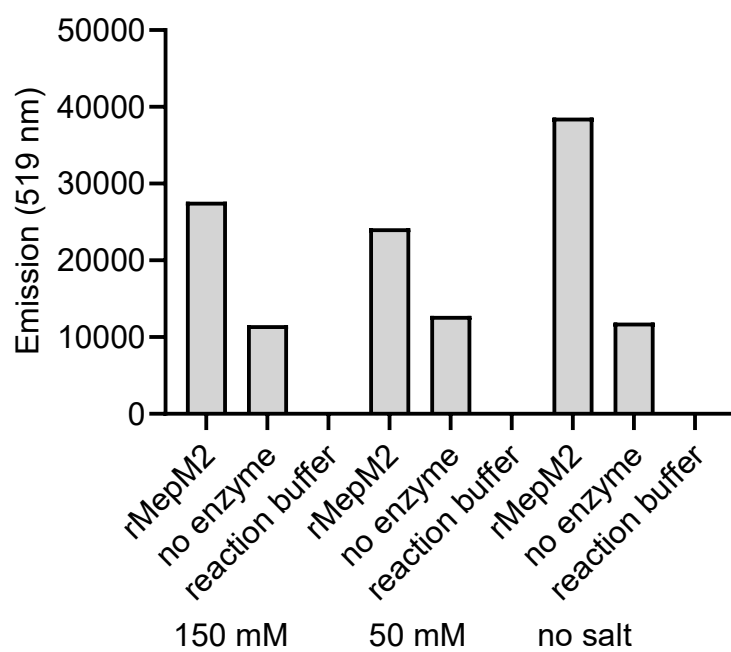


Figure 49: FITC-PG assay optimization: salt concentration of assay buffer

1 mg/mL FITC-labeled *B. subtilis* PG was incubated with 12.3 μ M rMepM2 for 60 min in assay buffer with 150 mM NaCl, 50 mM NaCl and under no salt conditions. Emissions were measured with gain 100 in the Tecan reader.

3.2.3.1.3.4.3 Addition of ZnCl_2 and MgCl_2

As some endopeptidases are known to require Zinc or Magnesium as cofactors, a possible effect of Mg^{2+} or Zn^{2+} ions on rMepM2 activity was investigated (Fig. 50). Addition of ZnCl_2 1mM (A) as well as MgCl_2 1 mM (B) to the buffer reduced rMepM2 activity. Unfortunately, the reaction buffer control was not measured in the ZnCl_2 experiment.

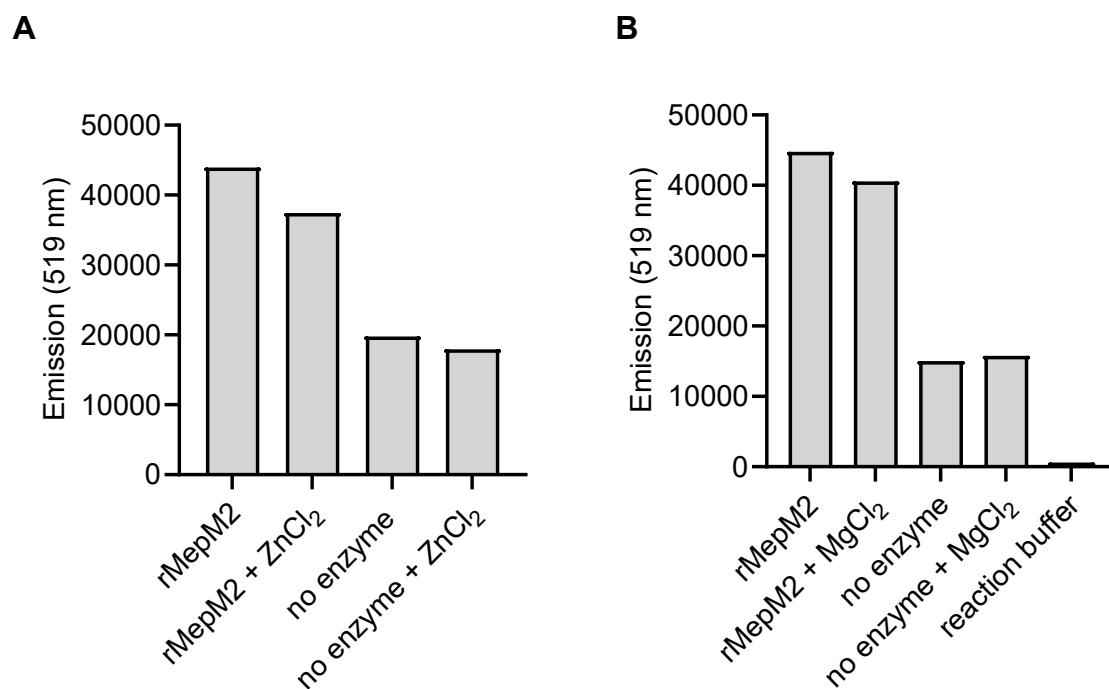


Figure 50: FITC-PG assay optimization: addition of Zn (A) or Mg (B)

0.5 mg/mL FITC-labeled *Bacillus subtilis* PG was incubated with 12.6 μM rMepM2 for 60 min in no salt buffer. Emissions were measured with gain 107 (optimal) in the Tecan reader.

3.2.3.1.3.5 Kinetics

In a third step, kinetics were recorded. Since FITC-PG cleavage is irreversibly terminated by the centrifugation step, mastermixes were prepared for each batch and divided into five individual reaction tubes. FITC-PG cleavage could thus be stopped at five different time points. Fig. 51 illustrates the fluorescence signals in the supernatants after different incubation times. The greatest rMepM2 substrate turnover occurred within the first 5 min. A finer time resolution could not be resolved with this assay setup, as stopping the reaction by centrifugation takes several minutes. After 30 min, background increases and no further rMepM mediated PG cleavage occurs. Reaction time for further experiments was reduced to 20 min.

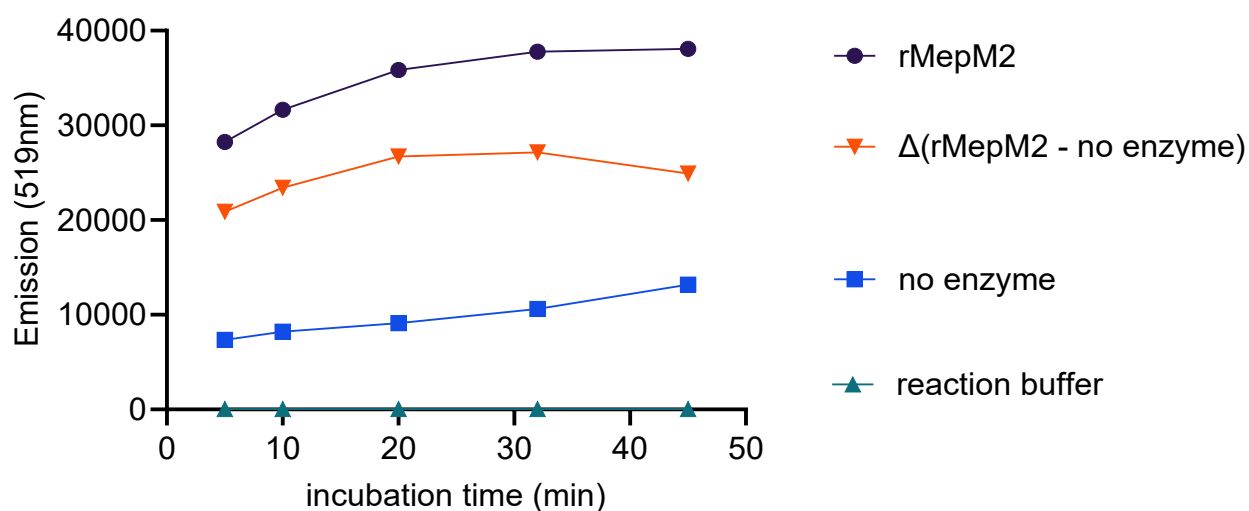


Figure 51: rMepM2 enzyme kinetics recorded with the FITC-PG assay

0.5 mg/mL FITC-labeled *B. subtilis* PG was incubated with 12 μ M rMepM2 in no salt buffer. MasterMix for every approach were divided among 5 reaction tubes and these were incubated for 5 to 45 min. Emissions were measured with gain 107 in the Tecan reader. “ Δ (rMepM2-no enzyme)” (orange) values were generated by subtracting “no enzyme” (blue) from “rMepM2” (violet) values.

Table 30: Signal to background ratio depending on incubation time

The strongest signal to background ratio is obtained after 20 min of incubation. By shortening the incubation time to 20 min, the signal to background ratio was increased to 3.9.

Incubation time (min)	Signal to background ratio
5	3.8
10	3.8
20	3.9
32	3.6
45	2.9

3.2.3.1.3.6 EDTA as negative control

In a final step, a negative control was established for the rMepM2 activity assay. EDTA binds Zinc ions in the active site of rMepM2 and thereby inhibits rMepM2 activity. The results in Fig. 52 demonstrate that rMepM2 activity is completely inhibited by the addition of 1 mM EDTA.

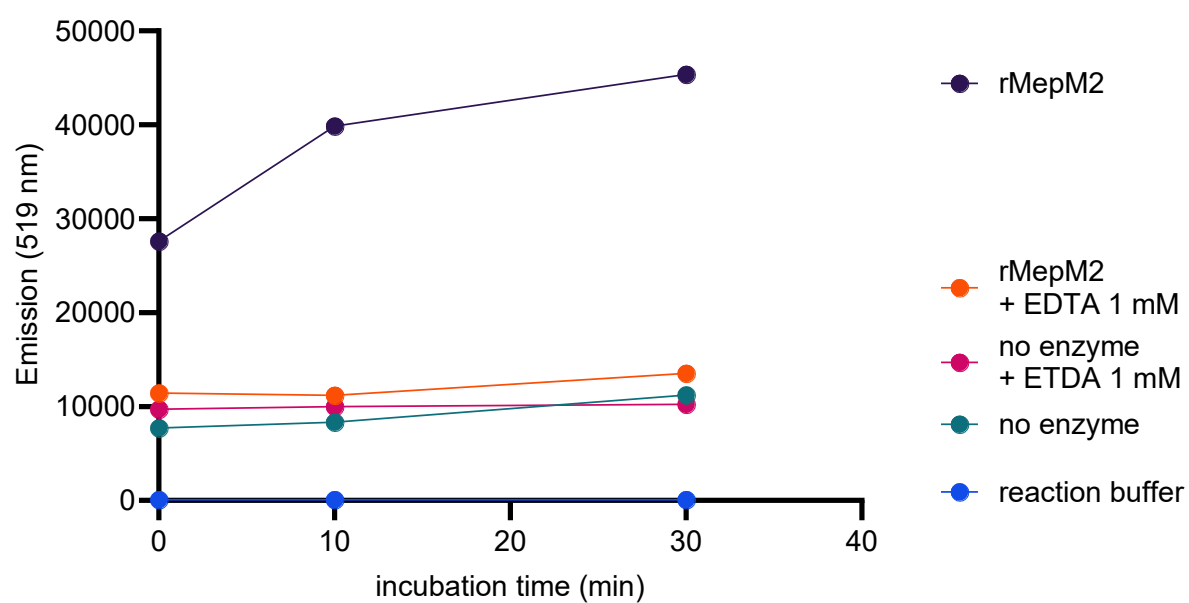


Figure 52: EDTA as negative control for rMepM2 activity assay

0.5 mg/mL FITC-labeled *B. subtilis* PG was incubated with 12 μ M rMepM2 in no salt buffer. MasterMixes for every approach were divided among 3 reaction tubes and these were incubated for 0 to 30 min. Emissions were measured with gain 107 in the Tecan reader.

3.2.3.1.3.7 Final results: optimal assay conditions for rMepM2 FITC-PG Assay

Taken together, using FITC-labeled PG, it could be demonstrated that the purified proteins rMepM1 and rMepM2 show clearly enzymatic activity. Moreover, the FITC-PG assay could be used to find optimal buffer conditions (Table 31) and introduced EDTA as a perfect negative control. By optimizing the assay conditions, the signal to background ratio for the rMepM2 FITC-PG Assay with *B. subtilis* PG was increased from 2.0 to 3.9.

However, since the purification of PG is tedious and time consuming, such FITC-PG assay is not optimal for high-throughput screening for many compounds. Another limitation is that enzyme kinetics cannot be detected at full enzyme saturation in the first few minutes of incubation.

The next goal was to establish an assay which could be used for high-throughput screening of possible MepM inhibitors.

Table 31: Optimal assay conditions for FITC-PG rMepM2 activity assay

The table summarizes the optimal conditions for conducting the FITC-PG assay.

Incubation time		20 min
rMepM2 enzyme concentration		12 – 14 μ M
FITC-PG substrate concentration		0.5 mg/mL
Buffer conditions	pH value (1 x PBS)	pH 8
	salt concentration (NaCl)	no salt
	cofactors (Mg^{2+} , Zn^{2+})	no additional cofactors

3.2.3.2 High-throughput assay with quenched fluorescent substrate IIIId

For the establishment of a high-throughput assay Fabian Renschler designed the fluorescent substrate IIIId following *in silico* analysis of the active sites of MepMs by Thales Kronenberger. Intavis Peptide Services synthesized the tetrapeptide IIIId from commercially available building blocks. It carries a fluorophore (Abz) attached to one side and a quencher (Dnp) on the other side (Fig. 53). IIIId cleavage by rMepM1 or rMepM2 between the D-Ala and D-Lys separates the quencher from the fluorophore generating a fluorescence signal (Fig. 54).

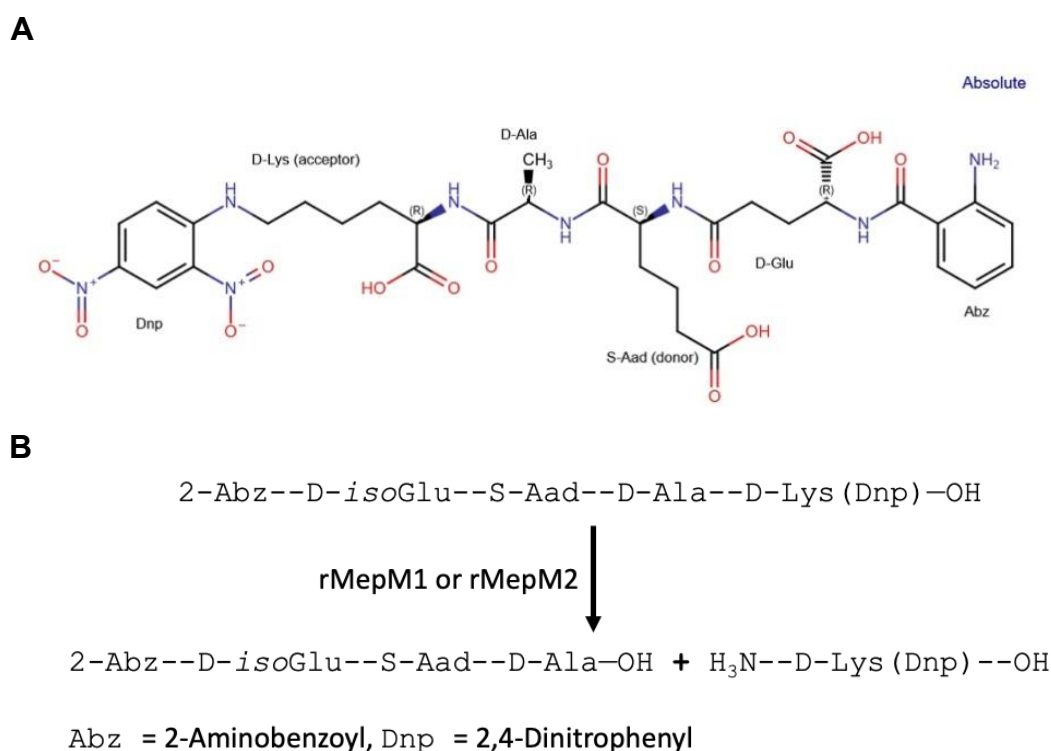


Figure 53: Molecular structure of quenched fluorescent substrate IIIId

(A) The substrate IIIId carries a fluorescent Abz on one side, which is quenched by a Dnp on the other side. (B) IIIId cleavage by rMepM between the D-Ala and D-Lys separates the quencher from the fluorophore generating a fluorescence signal.

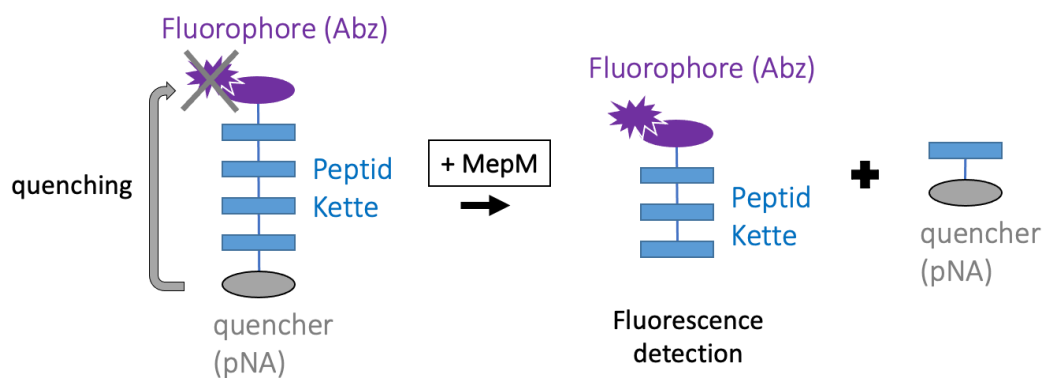


Figure 54: Quenched fluorescent IIIId Assay principle

IIIId cleavage by rMepM separates the quencher from the fluorophore generating a fluorescence signal that can be detected in the Tecan reader.

The IIIId Assay was performed as described in detail in Materials and Methods. Buffer conditions optimized in the FITC-PG assay were adopted. The optimal concentrations for rMepM1, rMepM2 and IIIId were determined and are listed in Table 32.

Table 32: Enzyme and substrate concentrations for IIIId Assay

	rMepM concentration	IIIId concentration	Enzyme to substrate ratio
rMepM1	1 μ M	50 μ M	1:50
rMepM2	1 μ M	50 μ M	1:50

Fluorescence signals show a linear increase of substrate turnover over time by rMepM1 (Fig. 55) and rMepM2 (Fig. 56). The addition of EDTA 1 mM served as a negative control. rMepM1 and rMepM2 activity can be detected with the IIId Assay.

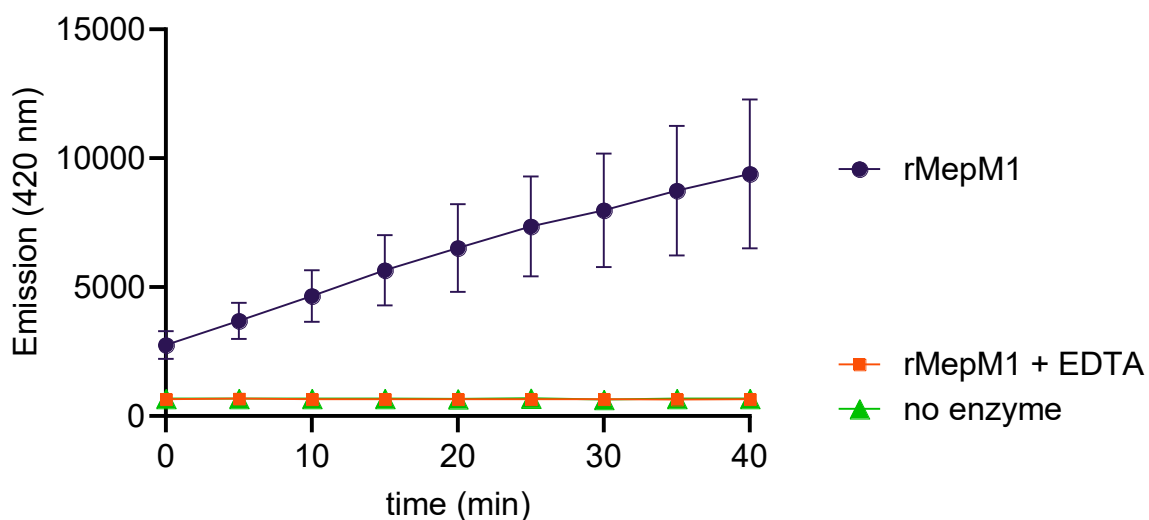


Figure 55: rMepM1 cleaves IIId substrate

IIId 50 μ M was cleaved by rMepM1 1 μ M at 37 °C. Fluorescence was measured in the old Tecan reader at gain 130.

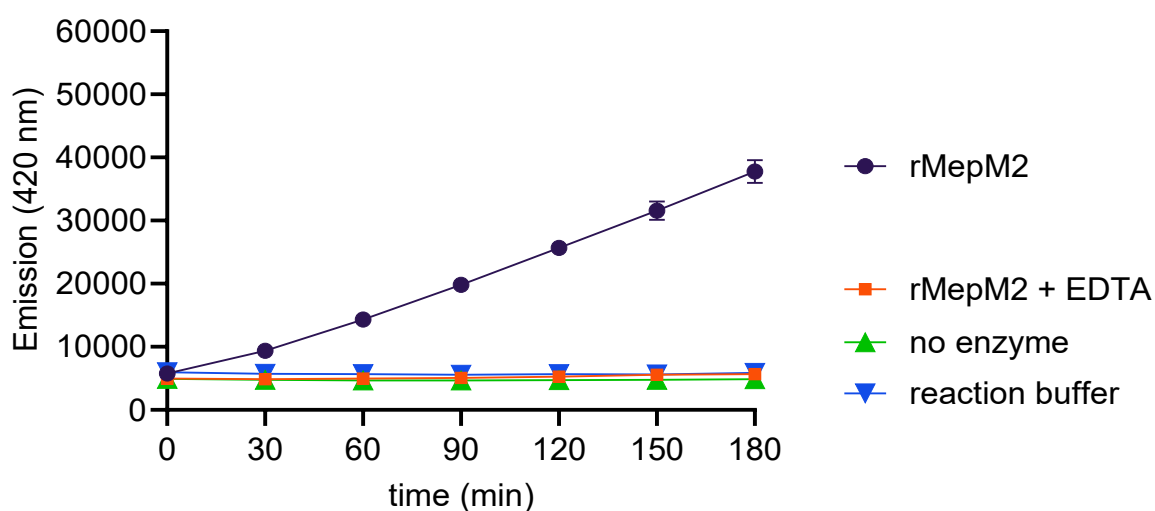


Figure 56: rMepM2 cleaves IIId substrate

IIId 50 μ M was cleaved by rMepM2 1 μ M at 37 °C. Fluorescence was measured in the new Tecan reader at gain 130. Graph depicts raw data from one experiment combining three technical replicates.

3.2.3.3 Inhibitor screening

Since an assay with substrate IIId could be established for high throughput screening in a 384 well format, we tried to indeed find inhibitors for rMepM1 and rMepM2. For this purpose, Thales Kronenberger screened commercially available compounds *in silico* for their ability to bind to the both active sites of MepM1 and MepM2 and hence inhibit their enzymatic activity.

In a first approach five compounds were tested for their ability to inhibit rMepM1 and rMepM2 activity. rMepM1 or rMepM2 were preincubated with these compounds for 15 min before substrate IIId was added and the measurement was started. Figures 57 and 58 show exemplified two of the five compounds tested to inhibit rMepM2. While compound MepMi_001 showed inhibition of enzyme activity (Fig. 57), MepMi_002 (Fig. 58) and all other not shown compounds showed no inhibition of enzyme activity.

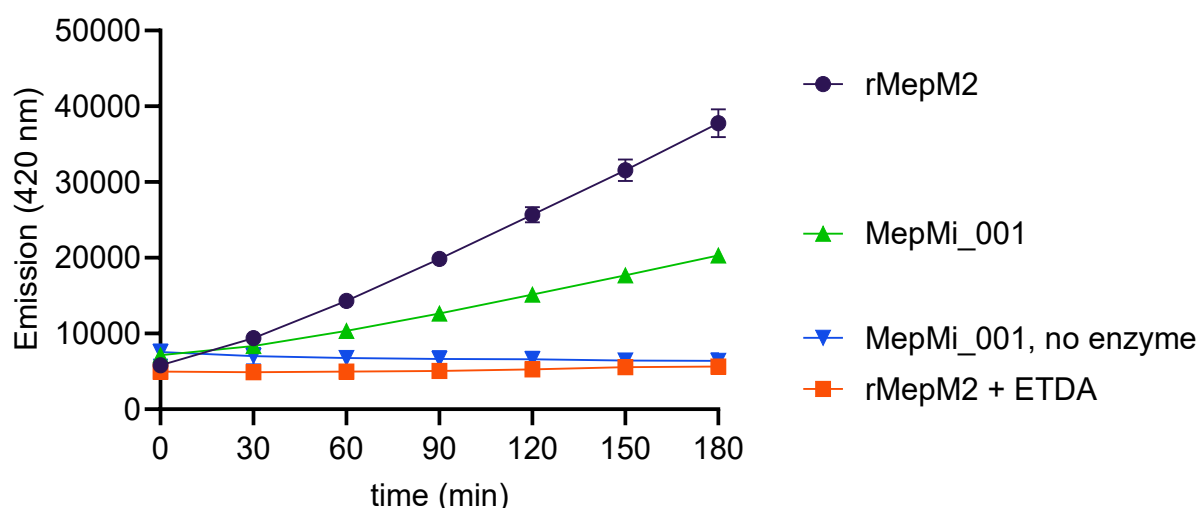


Figure 57: MepMi_001 inhibits rMepM2 activity

IIId 50 μ M is cleaved by rMepM2 1 μ M at 37 °C. MepMi_001 300 μ M inhibits rMepM2 mediated turnover of IIId substrate. Fluorescence was measured in the new Tecan reader at gain 130. Graph depicts raw data from one experiment combining three technical replicates.

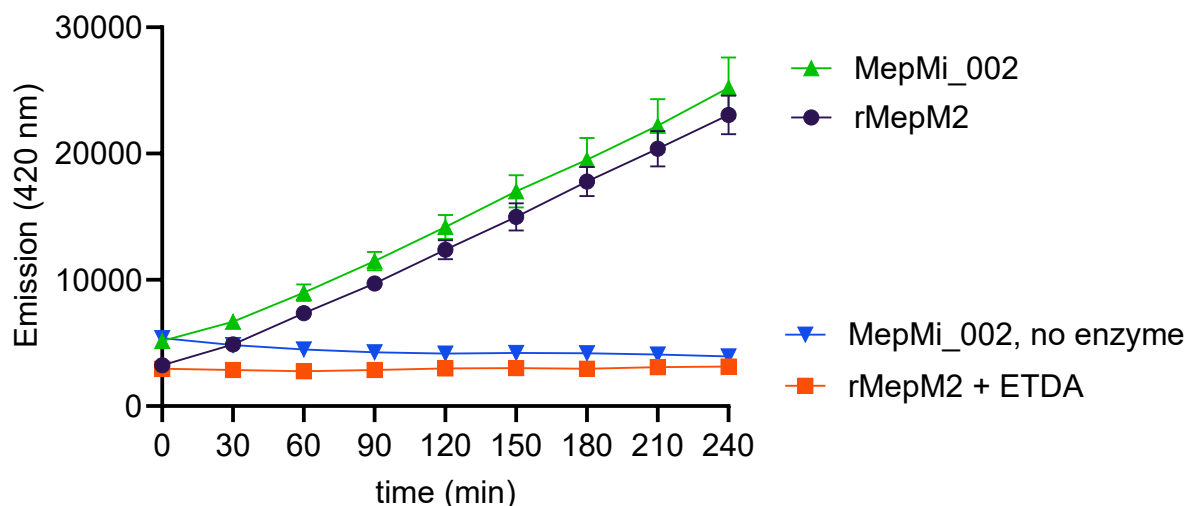


Figure 58: MepMi_002 does not inhibit rMepM2 activity

IIId 50 μ M is cleaved by rMepM2 1 μ M at 37 °C. MepMi_002 300 μ M does not inhibit rMepM2 mediated turnover of IIId substrate. Fluorescence was measured in the old Tecan reader at gain 130. Graph depicts raw data from one experiment combining three technical replicates.

The emission intensities of the individual measurements differ due to fluorescence detection in two different Tecan readers (“old” and “new”). To quantify the inhibitory effect of the compounds and to combine data of several measurements, the respective background was subtracted from the individual readings and the slopes of the linear regressions, corresponding to the enzyme activity, were normalized to the slope of rMepM2 + IIId = 100%. Rates of significance were determined by a nested one-way ANOVA.

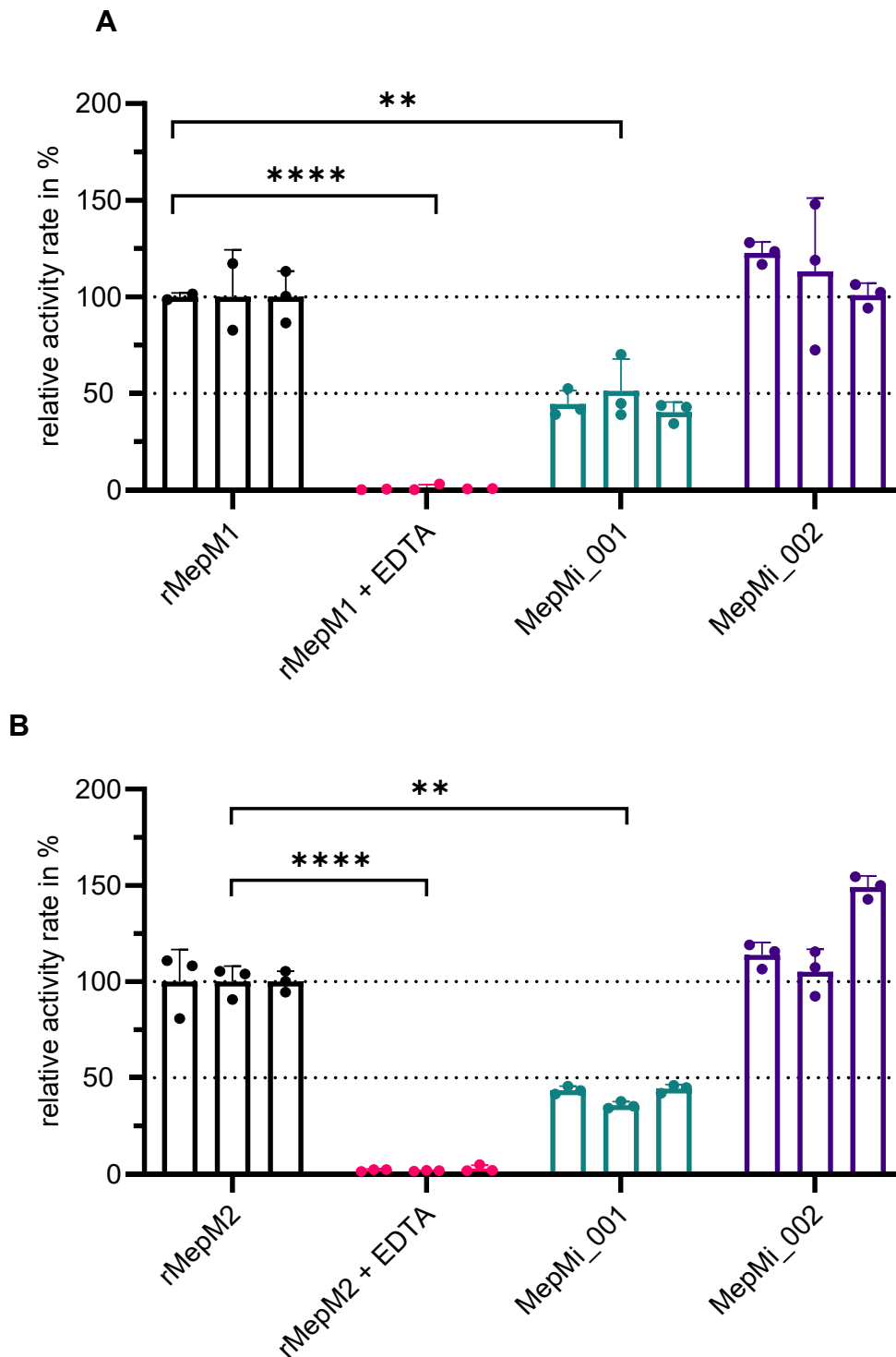


Figure 59: Inhibitory effect of MepMi compounds on rMepM1 (A) and rMepM2 (B) activity

Graph combines data from three experiments comprising three technical replicates each. Activity rates were normalized to rMepM2 = 100% of each experiment. MepMi_001 reduces III δ turnover by rMepM1 and rMepM2 by about 50%. MepMi_002 does not inhibit rMepM1 or rMepM2 activity.

Taken together, a high-throughput assay to test many compounds at once could be established and as proof of principle one first compound showing at least an inhibitory effect on rMepM1 and rMepM2 activity could be identified.

4. Discussion

4.1 CtpA-regulated DD-endoropeptidases (MepM1, MepM3, MepH1, MepH2) are not redundantly essential in *P. aeruginosa*

Initially, co-deletion of all four CtpA-regulated *meps* (*mepM1*, *mepM3*, *mepH1*, *mepH2*) has failed (Kemper, 2024), suggesting that they might be redundantly essential enzymes as described for murein endopeptidases in *E. coli*. There, MepM (homologue of MepM1), MepS and MepH (homologues of MepH1/2) are redundantly essential for bacterial growth and viability and their codeletion leads to rapid lysis (Singh et al., 2012). However, the successful generation of the conditional deletion mutant ID40 Δ *mepM1::rha-mepM1* Δ *mepM3* Δ *mepH1/2* and its growth even in rhamnose-free medium demonstrates that either these genes are not redundantly essential for viability in nutrient rich conditions such as LB-based media, or that very low *mepM1* expression is sufficient for bacterial survival. This could be further explored in growth curves with and without rhamnose-enriched medium inducing *mepM1* expression at different levels and by growing under special conditions, such as limited nutrient supply.

In LB-based media none of the investigated co-deletions of murein endopeptidases in *P. aeruginosa* is lethal but their codeletion synergistically breaks antibiotic resistance.

Trying to understand how different DD-endoropeptidases work together, Banzhaf et al. (2020) discovered that in *E. coli* they are part of multi-enzyme complexes around the outer membrane lipoprotein Nlpl. Those complexes regulate and contribute to both PG synthesis and degradation. Focusing on redundancy of endopeptidases, Jeon and Cho (2022) were able to suppress a growth defect of *E. coli* Δ *mepS* Δ *mepM* by overexpressing *mepH*, *pbpG* or *mepA*, but still observed wider cells, implying that the wildtype phenotype could not be fully restored.

For *P. aeruginosa* so far, a partially differential and partially redundant function of MepMs seems likely. In the following, putative functions of MepMs in *P. aeruginosa* and MepM's role in mediating antibiotic resistance are discussed.

4.2 Deletion of *mepMs* effects AmpC activity in *P. aeruginosa*

Murein endopeptidases have been identified as potential targets for restoring antibiotic susceptibility in *P. aeruginosa* (Sonnabend et al., 2020) and deletion of *mepMs* in MDR *P. aeruginosa* ID40 (Willmann et al., 2018) indeed reduces antibiotic resistance synergistically (Kemper, 2024).

Comparing all ID40 Δmep deletion mutants, the strongest reduction in resistance occurs in the ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ triple deletion mutant, yet stronger than in the newly generated ID40 $\Delta mepM1::rha-mepM1\Delta mepM3\Delta mepH1/2$ mutant, which lacks all CtpA-regulated *meps*. However, due to the leakiness of the rhamnose promoter, low *mepM1* expression in the conditional mutant cannot be excluded and MIC levels may be lower in a non-conditional mutant.

The $\Delta mepM$ mediated break of resistance is partially due to a reduction in *ampC* expression (Kemper, 2024). Results of this work confirm reduced AmpC activity at the level of enzyme activity in the $\Delta mepM1$ and $\Delta mepM2$ single deletion mutants. As previously observed at the level of mRNA expression, no further reduction in AmpC activity occurs in the $\Delta mepM1\Delta mepM2$ and $\Delta mepM1\Delta mepM2\Delta mepM3$ co-deletion mutants. This contrasts with the synergistic reduction in MIC values in these mutants. There must be other, yet unknown MepM-mediated resistance mechanisms. Since *E. coli*, unlike *P. aeruginosa*, constitutively expresses a non-inducible AmpC (Honore et al., 1986), the findings of Lai et al. (2017) on *E. coli* murein endopeptidases mediating resistance to the β -lactam mecillinam support this assumption.

The weaker reduction of AmpC activity in ID40 $\Delta mepM1\Delta mepM3$ compared to the $\Delta mepM1$ single deletion mutant is consistent with the results on *ampC* expression, but difficult to explain. According to the MIC values, additional deletion of *mepM3* leads to a further reduction in resistance. It is possible that the deletion of *mepM3* partially reactivates AmpC activity but at the same time suppresses another resistance mechanism, resulting in overall decreased resistance.

It can be concluded that deletion of at least *mepM1* reduces AmpC activity, but in addition, other mechanisms appear to occur in the $\Delta mepM1\Delta mepM2$ and $\Delta mepM1\Delta mepM2\Delta mepM3$ co-deletion mutants that might explain the further reduction in resistance compared to the single deletion mutants. To generate hypothesis about further *mepM*-mediated resistance mechanisms and to identify putative functional consequences of *mepM* deletions, membrane proteome analysis was performed.

4.3 Membrane proteome analysis: changes in the composition of the membrane proteome by co-deletion of *mepM1*, *mepM2* and *mepM3*

The bacterial membrane is exposed to various environmental conditions. In a wide range of pH values, salt conditions and fluctuating nutrient and metal supply, it must maintain its integrity and adapt the composition of its membrane proteome to the changing conditions. The membrane proteome of *P. aeruginosa* adapted to a cystic fibrosis lung, for example, differs markedly from that of one isolated from a nutrient-deficient burn wound. (Sriramulu et al., 2005). Since MepMs function as peptidoglycan modifiers, it can be hypothesized that they may play a major role in these adaption processes and that their deletion could alter the composition of the membrane proteome.

To identify possible changes in the membrane proteome composition due to the deletion of *mepMs*, purified membrane proteome of ID40 wildtype and ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ was analyzed by mass spectrometry. Comparing the membrane proteome of the most sensitive ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ with that of the ID40 wildtype provides insight into the overall membrane proteome changes due to triple deletion of DD-endopeptidases in *P. aeruginosa*. Considering possible opposing effects of the individual *mepM* deletions, this approach may hide some of the effects of the single *mepM* deletions. Comparing the membrane proteome of the single deletion mutants could facilitate the identification of these individual effects but would also be more time and resource consuming. Our approach was used to investigate the combined effect of concurrent inhibition of at best MepM1, MepM2 and MepM3 with similar active sites, which is the idea for establishing a therapeutic MepM inhibitor.

In the first experiment, the membrane proteins were very finely separated into inner and outer membrane proteins. For the second experiment, the separation into inner and outer membrane proteins was omitted. Protein levels were designated as altered if they showed at least a twofold change. Since only a little number of altered proteins were found in the first approach, the idea was that proteins could be lost through the separation, i.e. the additional purification steps. Thus, more proteins could be identified by analyzing the proteome from the direct purification sample. In fact, however, even fewer altered proteins were identified in the second approach. Of all proteins detected only 5.7% (first membrane isolation) respectively 3.2% (second membrane isolation) have at least a twofold change in abundance.

The low number of proteins altered suggests that at growth in LB MepMs affect specific membrane functions rather than mediating a fundamental change in the membrane proteome composition. The deletion of *mepM* may have an even greater impact under special conditions such as limited nutrient supply. The fact that the deleted MepMs themselves do not appear in the list suggests that they are expressed at very low levels in LB or are not localized in the membrane.

Blotting of isolated membrane fractions against MepM2 (3.2.2.2) indicates its localization in the periplasm (or cytoplasm) and not in the membrane. The activity of purified rMepM2 confirms that the truncated N-terminal signal peptide sequence is not required for its activity and might be responsible for its transport into the periplasm.

Since MepM1 is expressed at low levels, it could not be easily detected in the Western Blot. A very weak band may indicate localization of MepM1 in the outer membrane (results not shown).

Mass spectrometry analysis of both membrane proteome isolations identified a total of 73 membrane associated proteins with altered abundance in ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ compared to ID40. 50 are cytoplasmatic membrane proteins, 17 outer membrane proteins, 2 flagella proteins and 4 membrane proteins not further assigned to the cytoplasmatic or outer membrane. Of all these proteins, 15 were identified in both experiments.

First follow-up studies on the presence of MexE validate the proteome data showing indeed higher levels in ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$. Follow-up studies on the presence of MexY, however, show that not all the proteomic changes found can be verified. This clearly demonstrates that it is important to validate the proteome data by Western Blot analysis or luciferase assays. Nevertheless, the mass spectrometric results described provide important insights into possible proteomic alterations due to deletion of *mepMs*. Since little is currently known about the function of MepMs in *P. aeruginosa*, the list will continue to be useful for reconciliation with new findings in the future.

In the following, the putative role of the detected increased MexE/MexF/OprN abundance is discussed. Subsequently it is hypothesized how further, not yet verified changes in the membrane proteome could provide clues to the presumed functions of MepMs and how MepMs contribute to antibiotic resistance.

4.3.1 Putative role of increased MexE/MexF/OprN in ID40 Δ mepM1 Δ mepM2 Δ mepM3: reduction of β -lactam resistance and virulence and modulation of quorum sensing

Finding higher efflux pump MexE/MexF/OprN abundance in ID40 Δ mepM1 Δ mepM2 Δ mepM3 was surprising, as efflux pump induction is a known resistance mechanism in *P. aeruginosa* (Aeschlimann, 2003). However, consistent with my results, Sonnabend et al. (2020) found that the deletion of *mexE* and *mexF* increases resistance in ID40 against Meropenem and Cefepim. Follow-up studies indeed confirm elevated MexE levels in ID40 Δ mepM1 Δ mepM2 Δ mepM3 compared to the wildtype.

MexE/MexF/OprN is responsible for efflux of fluorquinolones, chloramphenicol and trimethoprim (Köhler, 1997) but not for efflux of β -lactams. In *P. aeruginosa* β -lactams are exported by the efflux pump MexA/MexB/OprM (Li et al., 2000) and MexC/MexD/OprJ (Poole et al., 1996). Between MexA/MexB/OprM and MexE/MexF/OprN an inverse correlation is described (Li et al., 2000).

Results on antibiotic susceptibility testing (results 1.4.3) confirm that co-deletion of *mexE/mexF/oprN* in ID40 Δ mepM1 Δ mepM2 Δ mepM3 indeed partially restores resistance. Although the effect is weak, it is striking that it occurs with all β -lactam antibiotics tested and simultaneously only with β -lactam antibiotics, colistin and amikacin. At the same time, the deletion of Δ *mexE/mexF/oprN* reduces resistance to ciprofloxacin, levofloxacin, imipenem, trimethoprim sulfamethoxazole and polymyxin B in ID40. This fits with MexE/MexF/OprN being responsible for the efflux of quinolones, trimethoprim and chloramphenicol, but not of β -lactams. (Köhler et al., 1997, Maseda et al., 2000).

Besides its role in the efflux of antibiotics, MexE/MexF/OprN fulfills more functions. MexE/MexF/OprN may play a central role in maintaining intracellular redox homeostasis (Juarez et al., 2017) and is induced by metabolic stress, suggesting a function in metabolic supply (Kumar and Schweizer, 2011). In addition, MexE/MexF/OprN has been shown to modulate quorum sensing through secretion of the quorum sensing precursor (PQS) 2-heptyl-4-quinolone. Induction of MexE/MexF/OprN thereby reduced virulence factor expression and swarming motility (Lamarche and Déziel, 2011). A MexE/MexF/OprN overexpression mutant produced less cyanin, elastase and rhamnolipids, likely due to altered intracellular PQS levels (Köhler et al., 2001).

Oshri et al. (2018) demonstrated further that cooperative growth of a quorum-sensing deficient Δ *lasR* mutant was partially resumed by downregulation of MexE/MexF/OprN via its positive

regulator *mexT* (Köhler et al., 1999). Via transcriptomic analysis, Wang et al. (2013) found a linkage between the ParS/ParR two component system, MexE/MexF/OprN and quorum sensing. Deletion of *parS* and *parR* induced expression of *mexE/mexF/oprN* by repressing the negative regulator *mexS*. In contradiction to Lamarche and Deziel, they simultaneously found increased swarming motility, phenazine and rhamnolipid synthesis.

More proteins associated with quorum sensing or virulence were found in the proteome analyses with altered abundance in membrane proteome analysis but have not yet been validated.

The FadE level is potentially reduced in the $\Delta mepM$ triple deletion mutant. This could increase the level of PQS precursors (Wells et al., 2017) and support the assumption of MepMs influencing quorum sensing.

The copper porin OprC (Yoneyama and Nakae, 1996) which is thought to affect motility, quorum sensing, LPS and pyocyanin levels in *P. aeruginosa* (Gao et al., 2020), might be increased in ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$.

Korgaonkar and Whiteley (2011) discovered NagE, which might also be increased in the $\Delta mepM$ triple deletion mutant, being required for growth on and uptake of GlcNAc, a component of the peptidoglycan recycling and synthesis pathway (Sonnabend et al., 2019). Furthermore, they have shown that exogenous peptidoglycan promotes pyocyanin production and proposed that *P. aeruginosa* senses surrounding bacteria by monitoring exogenous peptidoglycan. MepMs might be involved in the recognition and reaction to bacteria in the environment by cleavage of exogenous peptidoglycan.

Looking at $\Delta mepM$ deletion phenotypes in other bacteria, deletion of *bb0761* (*E. coli* MepM homologue) in *B. burgdorferi* resulted in reduced resistance to osmotic and oxidative stress, a growth deficit, an abnormal filamentous phenotype and complete loss of infectivity in a mouse model (Thompson et al., 2022).

Follow up studies are required to assess whether the higher abundance of MexE/MexF/OprN can be assigned to one specific or to several MepMs. Measurement of intracellular and secreted quorum sensing signals could demonstrate MepM's influence on quorum sensing. Research on motility, the secretion of virulence factors and biofilm formation might promise further understanding of the MexE/MexF/OprN mediated functions of MepMs.

4.3.2 Putative changes in the membrane proteome which might explain increased susceptibility to antibiotics

It has been demonstrated that the deletion of *mepM1* reduces AmpC activity, but in addition, other mechanisms appear to occur in the $\Delta mepM1\Delta mepM2$ and $\Delta mepM1\Delta mepM2\Delta mepM3$ co-deletion mutants that may explain the further reduction in resistance compared to the single deletion mutants. The influence of various peptidoglycan modifying enzymes on antibiotic resistance has been studied. Some affect *ampC* expression but also mediate intrinsic resistance independently of AmpC (Ropy et al., 2015). Analysis of the membrane proteome revealed genes known to mediate resistance. Based on this, hypotheses on *mepM*-mediated resistance mechanisms are generated in the following even though altered abundance of those genes remains to be validated.

4.3.2.1 Lytic transglycosylase MltB1

The analysis of the membrane proteome reveals an increased MltB1 abundance in $\Delta mepM1\Delta mepM2\Delta mepM3$, but this has not yet been validated. Considering the results of other studies, MltB1 could possibly be involved in the break of resistance through the deletion of *mepMs*. Lamers et al. (2015) describe that deletion of the two lytic transglycosylases *sltB1* and *mltB* in *P. aeruginosa* leads to increased β -lactam resistance independent of AmpC. They explain this with a slower rate of autolysis, an anti-suicide phenotype. In agreement with this, overexpression of *mltB1* in *E. coli* indeed leads to rapid cell lysis (Ehlert et al., 1995). Putative increased MltB levels in the $\Delta mepM$ triple deletion mutant may reduce resistance by increased autolysis.

In *Stenotrophomas maltophilia*, deletion of *mltB1* increases outer membrane permeability, thereby increasing sensitivity to macrolides and aminoglycosides (Wu et al., 2016a). Indeed, the $\Delta mepM$ triple deletion mutant showed slightly increased resistance to gentamicin (results 1.4.3) possibly due to a slight decrease in outer membrane permeability.

Cavallari et al. (2013) found that deletion of the lytic transglycosylase *mltB* in *P. aeruginosa* leads to high-level AmpC-mediated resistance without changing AmpC levels in the absence of antibiotic exposure. As AmpC activity in our assay was measured in the absence of antibiotics, these mechanisms could explain the lack of further reduction of AmpC activity (results 1.4.1) in the more sensitive $\Delta mepM1\Delta mepM2$ and $\Delta mepM1\Delta mepM2\Delta mepM3$ mutants.

To sum up, the increase in sensitivity by additional co-deletion of *mepM2* and *mepM3* in the ID40 $\Delta mepM1$ mutant could be mediated by an increase in MltB1, thereby inducing autolysis

and possibly additionally reducing AmpC resistance without affecting AmpC levels in the absence of antibiotics.

Interestingly, there is also a publication on MltB1 which, in contrast to the one just mentioned, describes a positive correlation between MltB1 and AmpC activity. In *Stenotrophomonas maltophilia*, the lytic transglycosylase MltB1 was found to increase uninduced AmpC activity depending on the CreBC-two component system and the AmpNG-AmpDI-NagZ-AmpR regulatory circuit. (Huang et al., 2015). It is conceivable that MltB1 affects AmpC activity differently via different signaling pathways. Incorporating my results on AmpC activity, this inconsistency could explain the contradictory results by additional deletion of *mepM3* in ID40 $\Delta mepM1$ and ID40 $\Delta mepM1\Delta mepM2$. Deletion of *mepM3* may be responsible for an increase in *mltB1* and thereby induce AmpC activity via the CreBC system. At the same time, increased *mltB1* could enhance autolysis (Ehlert et al., 1995, Lamers et al., 2015) and thus further reduce overall resistance.

So far, this is only a hypothesis, but I strongly suggest for follow up to confirm that MltB1 is indeed more abundant in the $\Delta mepM$ triple deletion mutant. Additional deletion of *mltB1* in the triple deletion mutant might then also partially restore resistance.

4.3.2.2 Outer membrane lipoprotein YiaD

Membrane proteome analysis brought up increased levels of the outer membrane lipoprotein YiaD in ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$, which are not yet validated.

In *Acinetobacter baumannii* downregulation of *yiaD* caused adaptive resistance to meropenem upon exposure to subinhibitory concentrations (Han et al., 2022). In these studies, deletion of *yiaD* increased the MIC for meropenem 4-fold. The deletion of *yiaD* also led to an enlarged and irregular colony shape and affected biofilm formation. The impact of *yiaD* on meropenem resistance is at least partially related to its interaction with the carbapenemase Oxa-23 (Wu et al., 2016b). Putative elevated levels of YiaD in the $\Delta mepM$ triple deletion mutant might be the cause of the lowered meropenem resistance.

Tachikawa and Kato (2011) identified the lipoprotein YiaD as an accessory protein of BamD, which interacts with PG and *yiaD* overexpression is thought to increase BamD levels in the outer membrane. In changing environmental conditions or during growth, it is supposed to support the incorporation of proteins into the outer membrane of *E. coli*. Suitable for this, YiaD interacts with the SecYEG-complex, that as well is involved the incorporation of outer membrane proteins. (Watkins et al., 2022)

In mutants with artificially expanded periplasm due to mutated elongated Lpp, *yiaD* becomes essential. It is neither essential in wildtype *E. coli* or a Δlpp deletion mutant. (Mandela et al. (2022)). *YiaD* is thus supposed to be relevant for outer membrane proteome biogenesis especially when the periplasm is expanded.

Previously, it was found that β -barrel assembly machinery (BAM) activity in *E. coli* depends on peptidoglycan maturation. While mature tetrapeptide-rich peptidoglycan, conceivably modified by MepMs, binds to and suppresses the BAM, nascent peptidoglycan containing pentapeptides does not interfere with the BAM. This leads to an increased insertion of outer membrane proteins into nascent growing peptidoglycan and a reduced BAM activity in mature peptidoglycan (Mamou et al., 2022). MepMs might be involved in the adaptation of the cell envelope to changing environmental conditions. A change in the peptidoglycan structure due to MepM cleavage could affect YiaD levels and BAM activity, thereby influencing the incorporation of proteins into the outer membrane. The deletion of *mepMs* could reduce adaptability of *P. aeruginosa* to changing environmental conditions.

Although my assumptions are highly speculative, I propose for follow up projects to validate whether indeed MltB and YiaD are more abundant in ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ and whether deletion of either *mltB* or *yiaD* restores partially β -lactam resistance in this mutant. Further I propose to investigate whether the deletion of *mepM* induces autolysis and to test the activity of AmpC after stimulation with subinhibitory concentrations of β -lactam antibiotics. Phenotyping of the *mepM* deletion mutants under altered environmental conditions such as salt concentration, pH values or limited nutrient supply could reveal specific functions of MepMs.

To sum up, deletion of *mepM1* in ID40 reduces resistance by reducing AmpC activity. The additional deletion of *mepM2* and *mepM3* leads to a further reduction in resistance, but not to a further reduction in AmpC activity. This suggests the presence of other MepM-mediated AmpC-independent resistance mechanisms in *P. aeruginosa*. Membrane proteome analysis led to new hypothesis about *mepM* mediated resistance mechanisms, which remain to be verified. These assume that increased MltB1 levels could induce autolysis and further reduce AmpC resistance without changing basic AmpC activity in the absence of β -lactams. Reduced meropenem resistance might be mediated by increased YiaD levels and the deletion of *mepMs* could reduce adaptability of *P. aeruginosa* to surrounding environmental conditions.

4.3.3 Membrane proteome analysis brings up new hypothesis for putative functions of MepM1, MepM2 and MepM3 in *P. aeruginosa*

The low number of proteins altered in ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ compared to the wildtype suggests that at growth in LB MepMs affect specific membrane functions rather than mediating a fundamental change in the membrane proteome composition.

The analysis of those altered membrane proteins and their functions may reveal possible functions of MepMs in *P. aeruginosa*. As none of these altered protein levels has been validated yet (except MexE), these are only hypothesis that would need to be verified by follow-up studies.

4.3.3.1 The deletion of MepMs might induce rhamnolipid production

At harvest of the liquid cultures, foaming was observed in the $\Delta mepM$ triple deletion mutant culture but not in the ID40 culture (results 3.1.4.3.1). Schleheck et al. (2009) discovered foam production in *P. aeruginosa* PAO1 under comparable cultivation conditions and assumed a release of rhamnolipid surfactants. Rhamnolipid production is regulated by the transcriptional factor RhlR (Ochsner et al., 1994), which belongs to a quorum sensing system (Mukherjee et al., 2018) and is associated with growth under limited nutrient and carbon supply (Chayabutra et al., 2001), antimicrobial activity by dispersion of biofilms (Wood et al., 2018), maintenance of own biofilm architecture (Davey et al., 2003), altered cell surface hydrophobicity by the release of lipopolysaccharides (Al-Tahhan et al., 2000) and swarming motility (Kohler et al., 2000). To assess whether MepMs influence these cell functions, it should first be tested if the deletion of *mepMs* indeed increases rhamnolipid production. One way to address changes in rhamnolipid production would be quantification of biosurfactants based on the dye Victoria Pure Blue BO as introduced by Kubicki et al. (2020).

4.3.3.2 MepMs might support adaption to changing nutrient supply

For the uptake of certain nutrients through its low permeable membrane, *P. aeruginosa* expresses different outer membrane porins (Chevalier et al., 2017). Several of those, belonging to the OprD subfamily, showed altered levels in the membrane of ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$, which remain to be validated. The functions of the porins mentioned in the following were described by Tamber et al. (2006). Decreased levels of OprD, an arginine porin, and OpdC, a histidine and arginine porin, were found while OpdO, a pyroglutamate porin, was increased. As downregulation of *oprD*, which also imports imipenem and meropenem into the cell, is a known

resistance mechanism in *P. aeruginosa* (Yoneyama and Nakae, 1993), finding decreased OprD levels in the sensitive mutant was surprising. These porins are required mainly in nutrient-poor environments, but OprD and OpdC are also produced at background levels in glucose-containing medium. OpdO expression is induced by its substrate pyroglutamate (Tamber et al., 2006). Arginine supplemented growth medium promotes biofilm formation and represses swarming over semi-solid surfaces in *P. aeruginosa* (Bernier et al., 2011) and administration of arginine to wound infections reduced the virulence of *P. aeruginosa* in an *in vivo* mouse model (Everett et al., 2017). The deletion of *mepM* could thus influence virulence, biofilm formation and swarming in *P. aeruginosa* via altered levels of arginine-absorbing porins.

Kim et al. (2021) discovered that a growth defect of *E. coli* $\Delta mepS\Delta mepM$ only occurs in amino acid-rich medium, while growth in minimal medium is almost normal. Consistent with this, a strong decrease of MepM and MepS levels was observed in wildtype *E. coli* cultivated in minimal medium. In line with that, MepS was induced by aromatic amino acids and MepM by glutamate. MepM and MepS activity in *E. coli* appears to be more important in nutrient rich environments. How far this applies to *P. aeruginosa* MepMs seems promising to investigate. The expression levels of MepM1 and MepM2 upon growth under nutrient-rich and -poor conditions and after induction with amino acids and glutamate could be measured in Western Blots using the newly generated anti-MepM antibodies. Growth curves of the deletion mutants in amino acid-rich and minimal medium could reveal growth defects under specific conditions.

In addition to the altered incorporation of membrane proteins, MepMs could contribute to adaption to changing environmental conditions by altering the peptidoglycan structure, e.g. by modifying peptidoglycan peptides like mDAP residues. Such endopeptidase-mediated changes have been described for *P. aeruginosa* grown under conditions resembling cystic fibrosis lung infections (Anderson et al., 2022). Detailed examination of the membrane and peptidoglycan components of ID40 wildtype and the $\Delta mepM$ mutants could reveal changes in modified peptidoglycan structures. A comparison of the peptidoglycan of both strains during growth under two different environmental conditions could reveal an adaptation deficit in the *mepM* deletion mutant.

4.3.3.3 *MepMs might reduce the number of flagella*

The porins OprD and OpdC which are according to the proteome data lower abundant in the *mepM123* deletion mutant. belong to the sigma factors regulons FliA and SigX (Schulz et al., 2015), meaning that they are regulated by the same sigma factors.

In addition, proteome data suggested that also the flagellin associated proteins FliC and FliD seem to be decreased in the $\Delta mepM$ triple mutant. FliC subunits form the flagellum and FliD forms the flagellar cap protein. This enables mucin adhesion, which promotes biofilm production, and is required for a functional flagellum and swimming motility. (Landry et al., 2006).

FliA regulates also flagellin biosynthesis (Potvin et al., 2008). This leads to the speculation that the triple deletion of *mepMs* may affect the FliA and SigX regulatory circuits. Since FliC and FliD form the flagella, the question arises as to whether ID40 $\Delta mepM1\Delta mepM2\Delta mepM3$ possesses a reduced number of flagella. Since *Pseudomonas aeruginosa* possesses only an unique flagellum (Bouteiller et al. 2021 doi: 10.3390/ijms22073337) this could mean that the triple deletion might be flagella-deficient. Therefore the preliminary proteome data propose to test whether the *mepM123* deletion shows a non-motile phenotype, which should be tested in motility assays. Since flagella are excreted in high amounts into supernatants and FliC is the dominant protein in such supernatants (Ince et al., 2015), first evidence for changes in flagella could be provided by TCA precipitation of bacterial supernatants and subsequent SDS-PAGE with Coomassie-staining and Western Blotting against FliC.

In summary, very little is known so far about the function of murein endopeptidases in *P. aeruginosa*. The analysis of the membrane proteome brought up new hypothesis about putative functions of MepMs, that remain to be investigated in follow-up studies. These hypotheses assume that MepMs might play an essential role in adaption to changing environmental conditions like limited nutrient supply. This could be due to an altered incorporation of membrane porins, especially for amino acids, or an altered peptidoglycan structure. In this context, they might impair rhamnolipid production or amino acid supply. In addition, the deletion of *mepMs* might reduce swimming motility.

4.4 Successful purification of soluble rMepM1 and rMepM2

For the establishment of an *in vitro* MepM activity assay to screen possible MepM inhibitors and for the generation of anti-MepM1 and anti-MepM2 antibodies to detect MepM1 and MepM2 purified rMepMs were required.

Purification of water soluble rMepM1 and rMepM2 was successful. The purification protocol for rMepM1 and rMepM2 established in this work can be used in the future for the purification of more protein by my research group and possibly also by other groups.

rMepM3 could not be released from the pellet after being overexpressed in *E. coli* probably due to formation of inclusion bodies. Purification of rMepM3 could still succeed under denaturing conditions with subsequent renaturation (Spriestersbach et al., 2015).

4.4.1 Generation of anti-MepM antibodies enables detection of MepM1 and MepM2 in whole cell lysates of ID40 mutants

High MepM1 levels are detected in the whole cell lysate of the $\Delta ctpA$ deletion mutant, indicating that in principle the anti-MepM1 antibody can detect MepM1 well. In contrast, MepM1 levels in ID40 are barely but clearly detectable, whereas in the $\Delta mepM1$ deletion mutants MepM1 is undetectable. It can be concluded that MepM1 levels in ID40 are kept low due to CtpA, as described previously (Srivastava et al., 2018). The anti-MepM2 antibody allows to detect MepM2 very clearly. Since the strength of the band in the $\Delta ctpA$ deletion mutant and ID40 is comparable, this data also shows that MepM2, in contrast to MepM1, is not regulated by CtpA.

Blotting of whole cell lysates against MepMs confirms the successful deletion of *mepM1* and *mepM2* in all deletion mutants.

4.4.2 rMepM1 and rMepM2 are active enzymes that cleave FITC-labeled peptidoglycan *in vitro*

A first assay using the natural MepM substrate peptidoglycan isolated from *E. coli* demonstrates that purified rMepM1 and rMepM2 are active enzymes that cleave peptidoglycan. The linear relationship between rMepM2 concentration and substrate turnover thereby confirmed that the release of FITC-labeled peptidoglycan building blocks was indeed due to rMepM cleavage. This makes it possible for the first time to measure the *in vitro* activity of *P. aeruginosa* MepMs. In Gram-positive *B. subtilis* peptidoglycan the peptide chain is mainly composed of L-Ala, D-Glu, mDAP, D-Ala (Atrih et al., 1999), just as in Gram-negative bacteria like *E. coli* (Mengin-

Lecreulx and Lemaitre, 2005). It could be shown that the purified rMepMs can cleave peptidoglycan from *E. coli* as well as from Gram-positive *B. subtilis*. This makes it easier to repeat the assay, as peptidoglycan from Gram-positive bacteria is little less time- and resource-consuming to purify.

MepM1 carries a domain that is also present in the LysM/M23 family peptidase ShyA in *V. cholerae* which is assumed to be an inhibitory of the peptidase activity. According to the crystal structure of ShyA, this domain is released from the active site by a conformational change from an inactive closed to an open form that enables substrate binding. Mutations in the interface residues of the different domains, which probably favor an open form, caused increased activity of the purified enzymes, while the wildtype ShyA is only active at a low level. To test whether this regulatory mechanism also occurs in *P. aeruginosa*, rMepM1 with mutations in interface residues of this domain must be purified and the activity of the mutated enzymes compared with that of wildtype rMepM1. In any case, rMepM1 carrying the suspected inhibitory domain is an active enzyme that cleaves its substrate peptidoglycan at least at a detectable level.

4.4.3 *In vitro* activity of rMepM2 depends on buffer conditions like pH value, salt concentration or addition of MgCl and ZnCl and is inhibited by EDTA

For the quantification of photometrically measured substrate turnover, calculation of the signal to noise ratio is well established (Bisswanger, 2014). To save peptidoglycan, which is time-consuming to purify, the signal to background ratio was calculated for the FITC-PG MepM activity assay instead. This does not require replicates and was completely sufficient for a first optimization of the buffer conditions. In the following quenched fluorescence substrate assay, the more precise signal to noise ratio from replicates was calculated. It confirms a fluorescence signal resulting from rMepM activity that is significantly higher than the background noise.

Intensive optimization of the FITC-PG MepM activity assay clearly increased the signal to background ratio. To save resources the optimization experiments were only carried out with rMepM2, as this purified enzyme was available in larger quantities. It is shown that *in vitro* rMepM2 activity depends on buffer conditions like pH value (optimum at pH 8) and salt concentration (most active at salt-free conditions), but that *in vitro* rMepM2 is still active in a broad spectrum of conditions from at least pH 7 to 9 and 0 – 150 mM NaCl. Only the addition of 1 mM EDTA completely suppresses rMepM1 and rMepM2 activity *in vitro* and qualifies EDTA supplementation as a perfect negative control. This is probably due to the removal of the

Zn²⁺-ion from the active site via chelation with EDTA. Both activity assays could be further optimized by establishing a negative control with purified rMepMs inactivated by mutation in the active site.

In the following, the dependency of *in vitro* rMepM2 activity on different buffer conditions is correlated with known functions of MepM homologs and their deletion phenotypes in other bacteria. The aim is to derive new hypotheses about possible functions of MepMs in *P. aeruginosa* and about the regulation of their activity *in vivo*, which can then be investigated in follow-up studies.

4.4.4 MepM activity *in vitro* depends on salinity, and MepMs *in vivo* might mediate growth at varying salt concentrations

The putative localization of paMepM1 in the membrane and paMepM2 in the periplasm or cytoplasm, as detected in the respective fractions of membrane purification via Western Blotting, indicates different, localization-specific functions of the individual MepMs. Following their genetic analysis of the domains of peptidoglycan endopeptidases in *E. coli*, Park et al. (2020) also suggest specific functions of the different DD-endopeptidases depending on their domains and *in vivo* localization. They found that MepM (paMepM1 homologue) is required for growth under high salt conditions and MepS (paMepH1/2 homologue) is associated with EDTA sensitivity. Interestingly, the $\Delta mepM$ phenotype could only be restored by *mepM* whereas the $\Delta mepS$ phenotype could be restored by overexpression of several DD-endopeptidases (*mepS*, *mepH*, *pbpG*, *mepM*). My *in vitro* studies on purified rMepM2 show that the activity of rMepM2 is indeed dependent on salinity and is suppressed by addition of 1 mM EDTA. More precisely, rMepM2 activity *in vitro* was higher at salt-free conditions compared to 50 mM and 150 mM NaCl. While wildtype *P. aeruginosa* can adapt to high and low salt conditions, it is described that a *mepM1* overexpressing $\Delta ctpA$ mutant grows poorly in salt-free medium (Srivastava et al., 2018). This could be due to excessive MepM1 activity in the absence of repression by CtpA and concomitant increased activity in salt-free conditions. However, the low-salt sensitivity phenotype could not be fully restored by co-deleting *mepM1*, indicating additional influence of other CtpA-regulated endopeptidases (MepM3, MepH1/2). In contrast, a *E. coli* $\Delta mepM$ mutant is sensitive to high salt (750 mM NaCl) conditions (Park et al., 2020). The *E. coli* $\Delta mepS\Delta mepM$ phenotype was differentially affected in salt-free and LB medium (Jeon and Cho, 2022).

The salinity-dependent rMepM activity combined with the phenotypical descriptions strongly suggest a role for MepMs in adaptation to salt concentration. Regulated rMepM activity appears to be critical for both high and low salt adaptation. The activity of rMepM1 and rMepM2 at no salt to high salt (750 mM NaCl) conditions can be investigated in detail using the established quenched fluorescent substrate assay. A subsequent follow-up study of my working group could indeed demonstrate that MepM1 supports resistance to high salt concentrations (450 mM) *in vivo*.

4.4.5 Zinc dependency and EDTA sensitivity of rMepM1 and rMepM2

It is shown that rMepM2 *in vitro* activity depends on buffer conditions like pH value and salt concentration, but that rMepM2 is still active in a broad spectrum of conditions. Only the addition of 1 mM EDTA completely suppresses rMepM2 activity *in vitro* probably due to the removal of the Zn^{2+} -ion from the active site via complexation with EDTA. rMepM1 and rMepM2 activities *in vitro* are both suppressed by 1mM EDTA. rMepM2 activity *in vitro* is slightly reduced, if at all, by zinc or magnesium excess (1 mM). Compatible with this, *in vitro* inhibition by an excess of zinc occurs in further zinc-dependent metalloproteases (Gomez-Ortiz et al., 1997) likewise in *V. cholerae* ShyA (Dörr et al., 2013). Two of the three M23-metallo-endopeptidases in *V. cholerae*, ShyA and ShyC, are zinc dependent, whereas ShyB is more resistant to zinc limitation. Of note, all three Shy-proteins share homology to paMepM1 in their domain organization. ShyA and ShyC are expressed in rich growth medium, and their activity *in vitro* is inhibited by EDTA. ShyB instead is not expressed under standard laboratory conditions. Its expression is induced by zinc restriction and its putative function is therefore to maintain cell growth in zinc-deficient environments. *In vitro* ShyB is more EDTA resistant than ShyA and ShyC (Murphy et al., 2019).

The suppressibility of rMepM activity *in vitro* by even higher zinc concentrations (>2 mM) and a break of EDTA mediated inhibition *in vitro* by addition of zinc at a higher concentrated than EDTA remain to be tested. Western blots of whole cell lysates with the newly generated anti-MepM antibodies could reveal a possible increased expression of *mepM1* and *mepM2* under zinc restriction. The levels of MepM1 and MepM2 in whole cell lysates grown under zinc limitation would have to be compared with those grown under normal laboratory conditions. After the successful purification of rMepM3, it can further be investigated whether the activity of rMepM3 is dependent on zinc and whether it is also inhibited by EDTA.

4.4.6 High through-put quenched fluorescence substrate assay enables screening for MepM inhibitors

Several enzymes of peptidoglycan remodeling are being investigated as potential targets in treating *P. aeruginosa* and other Gram-negative pathogens. Especially as adjuvants, inhibitors of cell wall enzymes have great potential to restore sensitivity to β -lactam antibiotics. Inhibition of the glycosidase NagZ, for example, has shown promising results *in vitro* and *in vivo* mouse models so far (Mondon et al., 2013, Bouquet et al., 2017) and Bulgecin A, an inhibitor of lytic transglykosylases, potentiates β -lactamase efficacy in *P. aeruginosa* (Dik et al., 2019) and *Neisseria* strains (Williams et al., 2017).

The establishment of a high throughput rMepM activity assay allows screening for potential MepM inhibitors and is a major step in the search for new therapeutic options against *P. aeruginosa* infections, that are urgently needed. The quenched fluorescent substrate IIId, designed was synthesized from available commercial building blocks and can thus be produced easily. Successful cleavage of the IIId substrate confirms that rMepM1 and rMepM2 cleave D-Ala m-Dap peptide peptidoglycan crosslinks as described previously for their *E. coli* homologs (Singh et al., 2012).

Among the first five compounds tested (only selected data are shown), one compound showing at least an inhibitory effect on rMepM1 and rMepM2 activity was identified. This confirms the basic suitability of the assay for screening for MepM inhibitors. Since initial screening was performed with high concentrations of inhibitors (300 μ M inhibitor, 1 μ M rMepM), the potency subsequently needs to be increased by chemical optimization of the inhibitor as a next step towards potential drug development. Then, it needs to be tested whether the combined use of antibiotics and MepM inhibitor lowers the MIC values of resistant bacteria.

4.4.7 Achievements of this work

In search for mechanisms explaining the reduction of resistance to β -lactam antibiotics by *mepM* deletion in *P. aeruginosa*, this work identified reduced β -lactamase activity and increased MexE/MexF/OprN levels as contributing factors. The successful generation of the conditional deletion mutant ID40 $\Delta mepM1::rha-mepM1\Delta mepM3\Delta mepH1$ further revealed that CtpA regulated Meps are not essential for bacterial survival in nutrient rich conditions or that very low *mepM1* expression is sufficient.

Through mass spectrometry analysis, this study provided putative alterations in the membrane proteome upon $\Delta mepM1\Delta mepM2\Delta mepM3$ triple deletion, revealing potential MepM resistance mechanisms including MltB1 induced autolysis, YiaD mediated meropenem susceptibility and impaired environmental adaptability. The findings recommend further research into MepMs role in nutrient adaption, membrane porin regulation, peptidoglycan integrity, and rhamnolipid production, which may also impact *P. aeruginosa* antibiotic resistance.

The establishment of purification methods for recombinant paMepM1 and paMepM2 provides a resource for further studies. The generation of rabbit-anti-paMepM1 und rabbit-anti-paMepM2 antibodies has enabled the validation of $\Delta mepM1$ and $\Delta mepM2$ deletions, while confirming that MepM1 is regulated by CtpA, unlike MepM2.

The establishment of functional assays demonstrated the enzymatic activity of purified recombinant paMepM1 and paMepM2. This enabled, for the first time, the *in vitro* measurement of *P. aeruginosa* murein DD-endopeptidases activity, laying the foundation for further enzymatic characterization. Finally, the development of a high-throughput assay for testing potential MepM inhibitors represents a promising step towards restoring *P. aeruginosa* susceptibility to β -lactam antibiotics. The identification of a first inhibitory compound highlights the potential for discovering therapeutic agents targeting MepMs. Given the urgent need to combat multidrug-resistant *P. aeruginosa* infections, these findings provide valuable insights for antibiotic resistance research and drug development.

5. Summary

The discovery of antibiotics was a milestone in the history of medicine. They led to a significant reduction in mortality rates and enabled invasive medical treatments to be developed by controlling infectious complications. However, their widespread use since then has encouraged the worldwide development of resistance, which today poses a serious threat to global health. Of particular concern are multidrug-resistant Gram-negative ESKAPE pathogens, such as *Pseudomonas aeruginosa*, which is associated with severe nosocomial infections and high mortality rates in immunocompromised patients.

Key mechanisms of *P. aeruginosa* β -lactam resistance include antibiotic inactivation via the overexpression of the chromosomal β -lactamase *ampC*, reduced outer membrane permeability, increased efflux through RND transporters and target modifications in penicillin-binding proteins.

Under antibiotic pressure, non-essential genes can become essential. Targeting the corresponding proteins with adjuvants could restore antibiotic susceptibility in resistant bacteria. The LytM/M23 zinc DD-endopeptidases MepM1, MepM2 and MepM3 have been identified as promising adjuvant targets in *P. aeruginosa*, as deleting them breaks resistance synergistically against various well-known and well-tolerated β -lactam antibiotics. Additionally, MepM1 and MepM2 share the same active site, enabling both enzymes to be targeted simultaneously with a single inhibitor.

This work demonstrated that reduced β -lactamase activity and, surprisingly, increased MexE/MexF/OprN levels are responsible for the reduced resistance to β -lactam antibiotics observed following the deletion of *mepM*. It is hypothesised that elevated MexE/MexF/OprN levels may affect quorum sensing, motility, or biofilm formation, thereby influencing antibiotic resistance.

Analyses using mass spectrometry revealed further potential changes of the membrane proteome following the deletion of *mepM1*, *mepM2* and *mepM3*. This brought up new potential resistance mechanisms and functions of MepMs including adaptation to altered environmental conditions through nutrient uptake, quorum sensing, motility, biofilm formation and remodeling of the cell envelope.

The successful development of purification methods for the recombinant, active enzymes PaMepM1 and PaMepM2, as well as the generation of anti-MepM antibodies, provides important resources for future research. These antibodies were used to validate the $\Delta mepM1$ and $\Delta mepM2$ deletions, confirming that MepM1 is regulated by CtpA, in contrast to MepM2.

The development of functional enzyme activity assays made it possible to measure *P. aeruginosa* murein DD-endopeptidase activity *in vitro* for the first time, paving the way for further enzymatic characterization. Finally, a high-throughput assay for testing potential MepM inhibitors was developed, representing a promising step towards the discovery of therapeutic agents that target MepMs. The identification of the first inhibitory compound proves the functionality of the experimental design.

Considering the urgent need to combat multidrug-resistant *P. aeruginosa* infections, this work makes a valuable contribution to research into antibiotic resistance and the development of new antimicrobial and antivirulence strategies.

6. Deutsche Zusammenfassung

Die Entdeckung der Antibiotika war ein Meilenstein in der Geschichte der Medizin. Sie führten zu einer erheblichen Verringerung der Sterblichkeitsraten und ermöglichten durch die Kontrolle infektiöser Komplikationen die Entwicklung invasiver medizinischer Behandlungen. Ihre weit verbreitete Verwendung hat jedoch auch die weltweite Entwicklung von Resistenzen gefördert, die heute eine ernsthafte Bedrohung für die globale Gesundheit darstellen. Besonders besorgniserregend sind multiresistente Gram-negative ESKAPE-Erreger wie *Pseudomonas aeruginosa*. Dieser verursacht schwere nosokomiale Infektionen mit hohen Sterblichkeitsraten bei immungeschwächten Patienten.

Zu den wichtigsten Mechanismen der β -Lactam-Resistenz von *P. aeruginosa* gehören die Inaktivierung von Antibiotika durch die Überexpression der chromosomalen β -Lactamase AmpC, eine verringerte Permeabilität der äußeren Membran, ein erhöhter Efflux durch RND-Transporter sowie Veränderungen der Zielproteine, also der Penicillin-bindenden Proteinen.

Unter dem Druck von Antibiotika können auch nicht-essenzielle Gene essenziell werden. Die gezielte Hemmung der entsprechenden Proteine durch Adjuvantien könnte die Empfindlichkeit resistenter Bakterien gegenüber Antibiotika wiederherstellen. In *P. aeruginosa* wurden die LytM/M23-Zink-DD-Endopeptidasen MepM1, MepM2 und MepM3 als vielversprechende Wirkziele einer Adjuvantien-Therapie identifiziert, da ihre Deletion die Resistenz gegenüber verschiedener bekannter und gut verträglicher β -Lactam-Antibiotika synergistisch bricht. Darüber hinaus teilen sich MepM1 und MepM2 das gleiche aktive Zentrum, sodass beide Enzyme mit einem einzigen Hemmstoff inhibiert werden könnten.

Diese Arbeit konnte zeigen, dass eine verringerte β -Lactamase-Aktivität sowie überraschenderweise erhöhte MexE/MexF/OprN-Spiegel verantwortlich sind für die Verringerung der Resistenz gegen β -Lactam-Antibiotika nach Deletion von *mepM*. Es wird angenommen, dass erhöhte MexE/MexF/OprN-Spiegel das Quorum Sensing, die Motilität oder die Biofilmbildung beeinflussen und damit die Antibiotikaresistenz reduzieren können.

Massenspektrometrische Analysen ergaben weitere potenzielle Veränderungen des Membranproteoms durch die Deletion von *mepM1*, *mepM2* und *mepM3*. Dies brachte neue potenzielle Resistenzmechanismen und Funktionen von MepMs auf. Hierzu zählt die Anpassung an veränderte Umweltbedingungen durch Nährstoffaufnahme, Quorum Sensing, Motilität, Biofilmbildung und Umbau der Zellhülle.

Die erfolgreiche Entwicklung von Aufreinigungsprotokollen für die rekombinanten, aktiven Enzyme PaMepM1 und PaMepM2 sowie die Herstellung von Anti-MepM-Antikörpern stellen

wichtige Ressourcen für die zukünftige Forschung dar. Die Antikörper wurden zur Validierung der $\Delta mepM1$ - und $\Delta mepM2$ -Deletionsmutanten verwendet und bestätigten, dass MepM1, im Gegensatz zu MepM2, durch CtpA reguliert wird.

Durch die Entwicklung funktioneller Enzymaktivitätstests konnte erstmals die Aktivität der Murein-DD-Endopeptidasen von *P. aeruginosa* *in vitro* gemessen werden, was den Weg für eine weitere enzymatische Charakterisierung ebnete. Schließlich wurde ein Hochdurchsatz-Assay zum Testen potenzieller MepM-Inhibitoren entwickelt. Die Identifizierung der ersten hemmenden Verbindung beweist die Funktionalität des Versuchsaufbaus.

Angesichts der dringenden Notwendigkeit, multiresistente *P. aeruginosa*-Infektionen zu bekämpfen, leistet diese Arbeit einen wertvollen Beitrag zur Erforschung der Antibiotikaresistenz und zur Entwicklung neuer antimikrobieller und antivirulenter Strategien.

7. List of references

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8. Erklärung zum Eigenanteil

Die Arbeit wurde am Interfakultären Institut für Mikrobiologie und Infektionsmedizin unter Betreuung von PD Dr. Erwin Bohn durchgeführt.

Die Konzeption des Projekts erfolgte gemeinsam mit PD Dr. Erwin Bohn, PD Dr. Monika Schütz und Dr. Fabian Renschler.

Sofern nicht explizit anderweitig beschrieben, wurden sämtliche Versuche nach Einarbeitung durch die Labormitglieder PD Dr. Erwin Bohn, PD Dr. Monika Schütz, Dr. Fabian Renschler, Dr. Karolin Birkle und Dr. Silke Malsheimer von mir eigenständig durchgeführt. Die Mutagenese der *MexE*-HiBiT und *MexY*-HiBiT Stämmen sowie der $\Delta mexE/mexF/oprN$ Deletionsmutanten wurden von PD Dr. Erwin Bohn durchgeführt.

Die statistische Auswertung erfolgte sofern nicht explizit anderweitig beschrieben nach Anleitung durch PD Dr. Erwin Bohn durch mich.

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

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