Ways to ESKAPE: Identification of potential targets of the cell envelope of *Pseudomonas aeruginosa* for anti-virulence drug development

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Für meine Eltern

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Abbreviations

Ampicillin/ sulbactam	SAM
Aztreonam	ATM
base pair	bp
Cefepime	FEP
Cefotaxime	CTX
Ceftazidime	CAZ
Ciprofloxacin	CIP
Cytoplasm	C
Endopeptidases	EPs
Erythromycin	ERY
Escherichia coli	*Ec*
Extended-spectrum-β-lactams	ESBL
Fosfomycin	FOS
High molecular mass	HMM
Imipenem	IPM
Imipenemase	IMP
Inner membrane	IM
kilo base pairs	kbp
Klebsiella pneumoniae	*Kp*
LB	Lysogeny broth
Levofloxacin	LEV
Low molecular mass	LMM
Lytic transglycosylases	LTs
Mega base pair	Mbp
Meropenem	MEM
Minimal inhibitory concentration	MIC
Multidrug-resistant	MDR
N-acetyl muramic acid	MurNAc
N-acetylglucosamine	GlcNAc
Nanometer	nm
New Dehli metallo-β-lactamase	NDM
Outer mebrane	OM
Penicillin binding protein	PBP
Peptidoglycan	PG

Phospholipid	PL
Piperacillin	PIP
Piperacillin/Tazobactam	TZP
Pseudomonas aeruginosa	*Pa*
Ticaricillin/clavulanate	TIM
Transposon-Directed Insertion Sequencing	TraDIS
Vancomycin	VAN
Verona integron-encoded metallo-β-lactamase	VIM
Whole genome sequencing	WGS
Yersinia enterocolitica	*Ye*
β-barrel assembly machinery complex	BAM
β-barrel outer membrane proteins	OMPs

Abstract

Pseudomonas aeruginosa (*Pa*) is one of the main causative agents of nosocomial infections and the spread of multidrug-resistant *Pa* strains is rising. Therefore, new strategies for therapy are urgently needed. Hence, in this study two different strategies were used to identify possible targets for the development of anti-infective drugs.

One strategy was to investigate proteins involved in the outer membrane assembly in order to disturb the outer membrane. The assembly of outer membrane proteins is managed by the BAM complex and periplasmic chaperones like SurA. Hence, in the first part of this study, deletion mutants of the BAM complex components BamB and BamC and of chaperones HlpA and SurA were generated and investigated. The constructed deletion mutants were analysed regarding their outer membrane permeability, outer membrane composition, morphology and their virulence in the *Galleria mellonella* infection model. The most profound effects were found upon depletion of SurA, including increased membrane permeability, enhanced sensitivity to antibiotics and reduced virulence in the *Galleria mellonella* infection model. More importantly, the SurA depletion in a multi-drug resistant *Pa* strain resensitized the strain to treatment with clinically relevant antibiotics. Depletion of BamB showed less changes in the outer membrane composition and hence less sensitivity against antibiotics and no relevant phenotypical effects. In addition, deletion of *bamC* or *hlpA* did not present any notable effects and none of these deletion mutants could prolong the survival of the *Galleria mellonella* larvae compared to infection with the wildtype strain. Thus, the data underline the importance of SurA in outer membrane biogenesis in *Pa*.

The other strategy was to target non-essential genes that are not directly responsible for resistance mechanisms but essential under exposure to therapeutic antibiotic concentrations. Therefore, a high- density transposon (Tn) library in a multidrug-resistant *Pa* isolate was constructed and exposed to cefepime and meropenem. The depletion of Tn insertions was measured and revealed several interesting genes involved in peptidoglycan metabolism. Validation of in frame deletion mutants by measuring *ampC* expression, AmpC activity and antibiotic susceptibility confirmed their relevance regarding β-lactam reistance and their role as promising targets for anti-infective drugs.

To sum up, by using two different strategies, proteins of the cell envelope or proteins involved in the cell envelope assembly of *Pa* were identified, which may serve as potential targets to develop anti-infective drugs in order to combat the rise of multi-drug resistant pathogens.

Zusammenfassung

Pseudomonas aeruginosa (*Pa*) ist eine der häufigsten Ursachen von nosokomialen Infektionen und die Ausbreitung von multiresistenten *Pa* Stämmen nimmt zu. Aus diesem Grund sind dringend Alternativen zur Behandlung von multiresistenten Erregern erforderlich. In dieser Studie wurden daher zwei verschiedene Strategien angewandt, um potentielle Zielproteine für die Entwicklung von Antiinfektiva zu finden.

Eine Strategie bestand darin, Proteine zu identifizieren, die am Aufbau der bakteriellen Außenmembran beteiligt sind, um diese wieder durchlässig zu machen. Der Einbau von Außenmembranproteinen wird durch den BAM- Komplex und periplasmatischen Chaperonen wie SurA gesteuert. Im ersten Teil dieser Studie wurden daher von den BAM-Komplex Komponenten BamB und BamC und von den Chaperonen HlpA und SurA Mutanten hergestellt und in Bezug auf Außenmembran Durchlässigkeit und -Komposition, ihrer Morphologie und ihrer Pathogenität im *Galleria mellonella* Infektionsmodell untersucht. Den größten Effekt zeigte die Inaktivierung von SurA, mit einer erhöhten Membranpermeabilität, Antibiotikawirksamkeit und einer verringerten Pathogenität im *Galleria mellonella*-Infektionsmodell. Noch bedeutender ist die Tatsache, dass durch die Inaktivierung von SurA in einem multiresistenten *Pa* Stamm, dieser wieder mit klinisch relevanten Antibiotika behandelbar wurde. Die Depletion von BamB weißte geringere Veränderungen in der Außenmembrankomposition auf und zeigte dementsprechend eine geringere Sensitivität gegenüber Antibiotika und keine relevanten phänotypischen Merkmale. Die Deletion von *bamC* oder *hlpA* zeigte hingegen keine nennenswerten Effekte und keiner der Deletionsmutanten konnte das Überleben der *Galleria mellonella* Larven im Vergleich zum Wildtyp Stamm verlängern. Die generierten Daten konnten damit die Relevanz von SurA für die Außenmembranbiogenese in *Pa* aufzeigen.

Eine weitere Strategie bestand darin, nicht essentielle Gene zu finden, die nicht direkt für die Resistenzmechanismen verantwortlich, aber unter therapeutischen Antibiotikakonzentrationen relevant sind. Zu diesem Zweck wurde in einem multiresistenten *Pa* Isolat eine Transposon (Tn) -bibliothek konstruiert und unter Zusatz von Cefepim und Meropenem kultiviert. Die Depletion von Tn-Insertionen wurde anschließend gemessen und zeigte verschiedene interessante Gene auf, die am Peptidoglykanmetabolismus beteiligt sind. Die Validierung von Deletionsmutanten durch Messung von *ampC*-Expression, AmpC-Aktivität und Antibiotika Suszeptibilität konnte ihre Relevanz für β-Laktam Antibiotikaresistenz und als vielversprechende Kandidaten für Antiinfektiva bestätigen.

Zusammenfassend ist festzuhalten, dass unter Anwendung zweier unterschiedlicher Methoden, Zellhüllenproteine bzw. Proteine die am Aufbau der Zellhülle beteiligt sind in *Pa* identifiziert werden konnten, welche als potenzielle Kandidaten für die Entwicklung von Antiinfektiva dienen können, um die weitere Ausbreitung von multiresistenten Krankheitserregern zu verhindern.

List of puplications

Liam Whiteley, Maria Haug, Kristina Klein, Matthias Willmann, Erwin Bohn, Salvatore Chiantia, Sandra Schwarz **(2017)**

Cholesterol and host cell surface proteins contribute to cell-cell fusion induced by the *Burkholderia* type VI secretion system 5

PLOS ONE 12(10): e0185715. https://doi.org/10.1371/journal.pone.0185715

Kristina Klein, Michael S. Sonnabend, Lisa Frank, Karolin Leibiger, Mirita Franz-Wachtel, Boris Macek, Thomas Trunk, Jack C Leo, Ingo B. Autenrieth, Monika Schütz, Erwin Bohn **(2019)**

Deprivation of the Periplasmic Chaperone SurA Reduces Virulence and Restores Antibiotic Susceptibility of Multidrug-Resistant *Pseudomonas aeruginosa*.

Front. Microbiol. 10:100. doi: 10.3389/fmicb.2019.00100, * equal contribution

Erwin Bohn, Michael Sonnabend, Kristina Klein, Ingo B. Autenrieth **(2019)**

Bacterial adhesion and host cell factors leading to effector protein injection by type III secretion system

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M. S. Sonnabend*, K. Klein*, S. Beier, A. Angelov, R. Kluj, C. Mayer, C. Groß, K. Hofmeister, A. Beuttner, M. Willmann, S. Peter, P. Oberhettinger, A. Schmidt, I. B. Autenrieth, M. Schütz and E. Bohn **(2019)**

Identification of drug-resistance determinants in a clinical isolate of *Pseudomonas aeruginosa* by high-density transposon mutagenesis

Antimicrobial Agents and Chemotherapy, accepted Manuscript posted online 09.12.2019, doi:10.1128/AAC.01771-19, * equal contribution

Contributions to publications

Liam Whiteley, Maria Haug, Kristina Klein, Matthias Willmann, Erwin Bohn, Salvatore Chiantia, Sandra Schwarz

Cholesterol and host cell surface proteins contribute to cell-cell fusion induced by the *Burkholderia* type VI secretion system 5

I performed the flow cytometry analysis of RAW264.7 macrophages, which were treated with 0.05% trypsin or 0.05% EDTA and created the corresponding figure and wrote the related part for the Methods section.

Kristina Klein, Michael S. Sonnabend, Lisa Frank, Karolin Leibiger, Mirita Franz-Wachtel, Boris Macek, Thomas Trunk, Jack C Leo, Ingo B. Autenrieth, Monika Schütz, Erwin Bohn (**2019**)

Deprivation of the Periplasmic Chaperone SurA Reduces Virulence and Restores Antibiotic Susceptibility of Multidrug-Resistant *Pseudomonas aeruginosa*.

I generated the deletion and conditional mutants of *surA* in PA14 and ID72 and the strain for the overexpression of *bamB*. Furthermore, I performed the bile salts assays, prepared the samples for Electron Microscopy, performed the growth curves in LB and iron-depleted medium. Moreover, I performed the overexpression and together with Karolin Leibiger the purification of BamB. In addition, Erwin Bohn and I performed Serum Killing assays. Furthermore, I mainly performed the RNA isolation with partial assistance by Michael Sonnabend. On the other hand, Michael Sonnabend mainly performed the qRT-PCR of the RNA samples with partial assistance from me. In addition, I partly conducted Western Blot analysis. *Galleria mellonella* experiments and antibiotic susceptibility testings were performed together with Michael Sonnabend in equal contribution. The manuscript was written by Erwin Bohn, Michael Sonnabend and me. All figures for the publication were designed by me.

Erwin Bohn, Michael Sonnabend, Kristina Klein, Ingo B. Autenrieth **(2019)**

Bacterial adhesion and host cell factors leading to effector protein injection by type III secretion system

The review was mainly written by Erwin Bohn and supported by Michael Sonnabend and me. In addition, the included figure was generated by Erwin Bohn and me.

M. S. Sonnabend*, K. Klein*, S. Beier, A. Angelov, R. Kluj, C. Mayer, C. Groß, K. Hofmeister, A. Beuttner, M. Willmann, S. Peter, P. Oberhettinger, A. Schmidt, I. B. Autenrieth, M. Schütz and E. Bohn **(accepted and posted online 09.12.2019)**

Identification of drug-resistance determinants in a clinical isolate of *Pseudomonas aeruginosa* by high-density transposon mutagenesis

In this study, I assisted Michael Sonnabend by generation of the ID 40 transposon library and partly performed the library preparation for TraDIS and partly conducted the statistical validation of the the experiments. Furthermore, I generated the deletion mutants of *ctpA* and *ctpA*/*mepM1* and the complementation constructs of *amgK*, *ctpA*, *mepM1 mltG* and *ygfB*. In addition, I peformed the RNA isolation and mainly performed the antibiotic susceptibility testings. The manuscript was written in equal contribution by Erwin Bohn, Michael Sonnabend and me. In addition, I generated all figures for the publication.

1 Introduction

1.1 The concept of anti-virulence drugs

In the 21st century the rapid development of antimicrobial resistance has become one of the major challenges to mankind. According to the World Health Organisation (WHO) the spread of resistant pathogens increased in such a way that the possibility of scenarios of a pre-antibiotic era becomes more realistic and that by 2050 more deaths are caused by drug-resistant pathogens (10 million) than from cancer (8.2 million) (Calvert *et al.,* 2018; O'Neill, 2014; O'Neill, 2016). It remains to be seen if this prediction will become a reality, but so far the trend is running only in one direction (O'Neill, 2016). To circumvent the current antimicrobial crisis the development of anti-resistance adjuvants, which promote the effect of known conventional antibiotics, will be a promising tool to combat especially multidrug-resistant (MDR) pathogens. It is well established that compounds such as β-lactamase inhibitors, as adjuvants supressing enzymatic resistance, in combination with β-lactam antibiotics increase the efficacy of the treatment. Successful combinations which have been used are penicillins with the β-lactamase inhibitors clavulanic acid, sulbactam or tazobactam. In addition, efflux pump inhibitors as capsaicin or outer membrane permeabilizers such as cationic peptides or EDTA were also used for treatment of several infections caused by MDR pathogens (Gill *et al.,* 2015).

Another promising strategy to circumvent the rise of MDR pathogens is the development of anti-virulence drugs. The aim of anti-virulence drugs is to disarm the pathogens virulence mechanism, but not growth or viability, and thereby maintaining the bacteria in a state in which they become susceptible to immune clearance or again susceptible to antibiotics (Johnson & Abramovitch, 2017). Targeting the virulence mechanisms instead of growth or viability leads to a reduction of selective pressure, which otherwise promotes the evolution of resistance mechanisms, and at best preserves the host's gut microbiota (Maura *et al.,* 2016; Heras *et al.,* 2015). Furthermore, it would be advantageous if the targeted virulence factor is conserved between different pathogens to allow treatments against polymicrobial infections, whenever it is required (Fleitas Martínez *et al.,* 2019). So far, several anti-virulence strategies have been developed, such as targeting functional membrane microdomains (FMMs), blocking adhesion or biofilm formation, interfering with quorum sensing (QS) or the neutralization of toxins (Calvert *et al.,* 2018; Fleitas Martínez *et al.,* 2019). Most of the anti-virulence strategies developed against *Pseudomonas aeruginosa* (*Pa*) target the biofilm

formation and the QS (Fleitas Martínez *et al.,* 2019). For instance, the furanone derivate C-30 targets the LasR receptor in *Pa* or terrein, isolated from *Aspergillus terreus*, antagonizes QS receptors without affecting the cell growth (Hentzer *et al.,* 2002; Kim *et al.,* 2018). However, resistance against anti-virulence drugs has already been observed (Allen *et al.,* 2014; García-Contreras *et al.,* 2015; Maeda *et al.,* 2012; Imperi *et al.,* 2019) and further examination is needed to find out whether the use of anti-virulence drugs alone is sufficiently effective or if a combinatorial therapy with existing antibiotics is preferable (Calvert *et al.,* 2018). The success of such a co-therapy could be demonstrated by MICHAUD et al. combining a LecB-specific antibiofilm dendrimer together with the antibiotic tobramycin to prevent dispersal and biofilm formation of *Pa* (Michaud *et al.,* 2016).

In sum, anti-virulence strategies offer promising and innovative opportunities to combat infectious diseases, but also face challenges such as development of resistance mechanisms (Maura *et al.,* 2016).

1.2 *Pseudomonas aeruginosa* as a model organism for target identification in Gram- negative bacteria within the ESKAPE group

Pa is an aerobic Gram-negative bacterium, which is ubiquitously distributed in the environment and living sources including soil, plants, animals and humans. The rod shaped bacterium was first isolated by Gessard in 1882 and named *Bacillus pyocyaneus*, following the observation of its green coloration (Morrison & Wenzel, 1984; Lyczak *et al.,* 2000). Besides its minimal growth requirements and ability to tolerate a vast number of physical conditions, *Pa* forms biofilms, harbours many defence mechanisms as well as intrinsic resistance mechanisms against antibiotics and disinfectants (Stover *et al.,* 2000). These characteristics allow the organism to persist in both community and nosocomial settings and made it to one of the top priority pathogens for which new and effective antibiotic treatments are urgently needed as listed by the WHO (Tacconelli *et al.,* 2017). *Pa* is largely associated with hospital acquired infections, particularly in patients with compromised immune defense and is the main cause of morbidity and mortality in cystic fibrosis patients (Moradali *et al.,* 2017; Giwercman *et al.,* 1990). It is a common causative agent of pneumonia (including ventilator-associated pneumonia), blood stream infections and urinary tract, skin, and soft tissue infections (Moradali *et al.,* 2017). Furthermore, reports on *Pa* strains resistant to almost all classes of commonly used antibiotics including aminoglycosides, cephalosporins, carbapenems and fluoroquinones (Poole, 2011; European Centre for Disease

Prevention and Control, 2017; European Centre for Disease Prevention and Control, 2015), underline that *Pa* more and more escapes from the lethal action of antibiotics. *Pa* is not the only pathogens capable of escaping the action of antibiotics, so that the Infectious Diseases Society of America highlighted a group of antibiotic resistant and most challenging nosocomial pathogens as members of the ESKAPE group (***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa* and ***E****nterobacter* spp.) (Rice, 2008). The ESKAPE group is not only important due to its insistent presence in hospital settings. If we could understand the pathogenesis, transmission and resistance of at least one of the most tenacious and pathogenic bacteria, we may be able to apply our knowledge to other species.

In this regard, *Pseudomonas aeruginosa* was used in the present study as a representative of the ESKAPE group to identify potential antiinfective drug targets in order to devise new strategies to treat especially infections caused by MDR *Pa* and to better understand its resistance mechanisms.

The clinical isolate PA14 is a well-established laboratory strain, which is easy to manipulate and for which several infection models exist to study the bacterial characteristics. Furthermore, the AUSUBEL lab created a transposon insertion mutant library of PA14 to enable genome-scale screens (Liberati *et al.,* 2005). In comparison to the other frequently used laboratory strain PAO1, the PA14 genome comprises a few more bases (6.5 Mb: 6.3 Mb) and encodes 5973 open reading frames including pathogenicity islands which contribute to the enhanced virulence of PA14 (Lee *et al.,* 2006).

The chromosome of the MDR bloodstream isolate ID40 (Willmann *et al.,* 2018) is 6.86 Mb in size and encodes 6409 open reading frames. Moreover, ID40 carries a plasmid of 57446 bp comprising 59 open reading frames and in comparison, to PA14 and PAO1, is resistant to several β-lactam antibiotics (Sonnabend et al., accepted 25.11.2019).

1.3 Virulence factors of *Pseudomonas aeruginosa*

Pa harbours an array of virulence factors to counteract the host defence mechanisms, damage the host tissues or to remain competitive with other bacteria (Gellatly & Hancock, 2013).

For the motility and the contact with the epithelium, flagella and pili are used. The appendages adhere to the epithelial surface glycolipid asialo-GM1 to facilitate the contact (Kipnis *et al.,* 2006). In addition, flagella interact with Toll-like receptors TLR5 and TLR2 and thereby activate an NFκB dependent inflammatory response. Pili enable *Pa* to spread

along hydrated surfaces. This special kind of movement is called twitching. The twitching motility is an advantage for colonization of the airway-tissue (Kipnis *et al.,* 2006).

Another player mediating adherence is lipopolysaccharide (LPS) which forms the outer leaflet of the outer membrane and also interacts with asialo-GM1 to facilitate adherence to the host cell. Upon binding to host cell receptors (MD2 and CD14), LPS leads to activation of NFκB and subsequently to the production of pro-inflammatory cytokines, inflammation and sometimes even to endotoxic shock (Gellatly & Hancock, 2013). After the irreversible adhesion by LPS, the type 3 secretion system (T3SS) is activated to secrete the effector proteins ExoY, ExoS, ExoT and ExoU , which promote cell injury e.g by activation of neutrophils or directly causing necrosis (Gellatly & Hancock, 2013). The expression of the four effector proteins varies between different *Pa* strains and isolates. ExoY and ExoT are present together in most strains, while the strains harbour either ExoU or ExoS, but not both (Hauser, 2009). Cytotoxic strains like PA14 express *exoU* and *exoT*, and sometimes *exoY*, whereas invasive strains express *exoS*, *exoT* and often *exoY* (e.g. PAO1, PAK, ID40) (Bleves *et al.,* 2010). However, a group of *Pa* strains lacking the T3SS and the T3SS toxins has recently been reported (Roy *et al.,* 2010; Basso *et al.,* 2017). In contrast to other *Pa* strains, the PA7 group secretes exolysin A (ExlA), which forms a two-partner secretion system with the porin TpsB encoded by *exlB*. Furthermore, the pore-forming toxin ExlA requires type IV pili to get in contact with host cells and induce cell lysis (Basso *et al.,* 2017).

Other virulence factors, such as elastase B (LasB), endotoxin A (ExoA) or phospholipase C, are secreted by the type 2 secretion system (T2SS). These exoproteins also participate in cytotoxicity as they destruct tight-junctions and degrade flagellin (LasB), inhibit protein synthesis (ExoA), or break down phospholipids (phospholipase C) (Gellatly & Hancock, 2013; Hauser, 2009; Kipnis *et al.,* 2006; Casilag *et al.,* 2016).

Furthermore, *Pa* synthesizes the mucoid exopolysaccharide alginate, which is assumed to be involved in biofilm formation in CF lungs (Gellatly & Hancock, 2013; Kipnis *et al.,* 2006)
and pyocyanin, a blue redox-active metabolite, which impedes the host response and induces apoptosis in neutrophils (Managò *et al.,* 2015).

For its metabolism, *Pa* needs to chelate iron from the environment. The siderophore pyoverdine sequesters iron molecules and additionally acts as signalling molecule. The pyoverdine-iron complex interacts subsequently with the receptor FpvR which in turn activates a signalling cascade causing the production of ExoA, endoproteases and also pyoverdine (Lamont *et al.,* 2002; Jimenez *et al.,* 2012).

Moreover, the upregulation of the virulence genes in the whole population is coordinated by quorum sensing (QS) via acyl homoserine lactones, called autoinducers. *Pa* harbours at least four QS systems, including *las*, *rhl*, *iqs and pqs*, which are organized in a hierarchy with the *las* system at the top of the cascade (Lee & Zhang, 2015).

In conclusion, *Pa* produces a great quantity of virulence factors modifying the immune system by adhesion, evading and destroying the tissue for progression of the disease and forming a barrier to antibiotics. Most of the virulence factors can cause acute and chronic infections, which renders *Pa* together with its diverse antibiotic resistance mechanisms (§ 1.4) a serious therapeutic problem for treatment of hospital- and community acquired infections.

1.4 Antibiotic resistance mechanisms of *Pseudomonas aeruginosa*

The treatment of *Pa* infections becomes increasingly more difficult because *Pa* is naturally resistant to a lot of antibiotics and has an extraordinary capacity to develop and acquire new resistance mechanisms (Moradali *et al.,* 2017).

Intrinsically resistance of *Pa* to certain antibiotics is enabled due to the low permeability of the outer membrane (OM), the activity of efflux pumps and the inducible expression of the chromosomally encoded β-lactamase, AmpC (Gellatly & Hancock, 2013).

The lowered permeability of the OM is the result of a reduced number of large porin proteins like OprF and an increased number of more specific porin channels like OprD (Gellatly & Hancock, 2013; Moradali *et al.,* 2017). This adaptation makes the OM of *Pa* 10- to 100-fold less permeable than that of *Escherichia coli* (*Ec*) (Hancock & Speert, 2000) and creates resistance to commonly used antibiotics such as carbapenems and cephalosporins. Resistance to the carbapenems imipenem (IPM) and meropenem (MEM) for instance is usually mediated through mutations, the loss or a reduced copy number of OprD (Trias & Nikaido, 1990; Kao *et al.,* 2016). The high intrinsic resistance of the pathogen is further dependent on the rapid efflux by efflux pumps such as MexAB-OprM or MexDC-OprJ. So far, 12 resistance-nodulation-division (RND) family efflux pumps have been genetically identified in *Pa*, but only 6 have been described (Schweizer, 2003; Poole, 2014). The expression of the efflux systems is regulated by several factors and varies under different conditions. The most clinically important efflux sets are MexAB-OprM and MexXY-OprM, as they have the broadest substrate profile for β-lactams and in addition facilitate resistance to chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, trimethoprim and aminoglycosides (Livermore, 2002; Avarain *et al.,* 2013). Another

important player of intrinsic resistance is AmpC, an inducible chromosomal β-lactamase. It is typically expressed at basal levels, but subinhibitory concentrations of certain β-lactam antibiotics can lead to its induction (Gellatly & Hancock, 2013).

Pa can acquire further resistance to antibiotics through horizontal gene transfer from other bacteria (plasmids) or through mutational changes in intrinsic genes.

AmpC overproduction can be induced through mutational inactivation of *ampD* (repressor of *ampC*) or spontaneous mutations in the regulatory gene *ampR* leading to elevation of *ampC* expression and consequently to an increase of antibiotic resistance (Moradali *et al.,* 2017).

Another common mutation related to *ampC* derepression has been recognized in the gene *dacB*, encoding the nonessential low-molecular-weight penicillin binding protein 4 (PBP4) (Moya *et al.,* 2009). The mutation of *dacB* has been shown to activate the CreBC two-component regulator, which further increases the resistance levels. MOYA and colleagues further demonstrated that laboratory mutants and clinical isolates with mutated PBP4 exhibited a significantly increased *creD* expression. CreD is regulated by the global regulator CreBC, but its role in *ampC* regulation has not been further characterized (Moya *et al.,* 2009; Lister *et al.,* 2009). Interestingly, the knockout of *creBC* decreased the resistance against penicillins, cephalosporins and aztreonam (ATM) in *dacB* mutants and displayed no changes in *ampC* levels, which led MOYA and colleagues to the suggestion that the CreBC system enhances resistance through another pathway as AmpC (Moya *et al.,* 2009). Furthermore, it was recently evidenced that the CreBC system has an important impact on bacterial fitness and biofilm formation even in the absence of β-lactam antibiotics (Zamorano *et al.,* 2014).

Moreover, there exist mutations in a number of other genes encoding for other amidases (AmpDh2 and AmpDh3), other PBPs (PBP5 and PBP7) or e.g. lytic transglycosylases (SltB1 and MltB) that have been shown to boost *ampC* expression (López-Causapé *et al.,* 2018).

In addition, *ampC* expression is tightly connected to the rebuilding of the peptidoglycan cell wall including many enzymes like AmpG permease, amidase AmpD or the N-acetylglucosaminidase NagZ (Ropy *et al.,* 2015), which will be further addressed in section 1.5.4.

Besides the described mutations leading to *ampC* overexpression, other antibiotic resistance mechanisms are also generated by mutational inactivation of intrinsic genes. As already mentioned above, reduction or loss of OprD results in greater resistance to carbapenems. In addition, loss-of-function mutations in the *mexAB-oprM* operon leads to the overproduction of MexAB-OprM and also enhances the resistance to carbapenems (Kao *et al.,* 2016; Lister *et al.,* 2009). Resistance to fluoroquinolones can also be mediated by overproduction of efflux

pumps (Poole, 2011; Morita *et al.,* 2015) or by mutations in the fluoroquinolone target genes, DNA gyrases *gyrA* and *gyrB* or in the subunits ParC and ParE of topoisomerase IV (Lee *et al.,* 2005).

Resistance mechanisms acquired through plasmids or chromosomally integrated transposons confer resistance to several antibiotic classes such as extended-spectrum-β-lactams (ESBL) and carbapenems, fluoroquinolones and aminoglycosides (Moradali *et al.,* 2017).

Genes encoding ESBLs and carbapenemases have largely variable prevalence in each individual country but specific metallo-β-lactamase (MBL) as IMP, VIM and NDM have been found worldwide (Hong *et al.,* 2015). In addition to ESBL and carbapenemase producing isolates, *Pa* also evolved a mechanism to survive treatment with colistin (polymyxin E), one of the last therapeutical options to clear carbapenem resistant *Pa*. The resistance mechanism is primarily caused by post-translational modifications of LPS molecules in the OM. In *Pa* the substitute 4-amino-4-deoxy-L-arabinose (L-Ara4N) is added to lipid A or the core polysaccharide leading to a negative charge of the phosphate residues preventing the binding of polymyxin molecules to the bacterial envelope. The LPS modifying genes are under the control of two-component systems such as PhoPQ, PmrAB, ParRS, CprRS and ColRS. Mutations in genes of the two-component systems cause a constitutive upregulation of the LPS modification and consequently to polymyxin resistance (Jeannot *et al.,* 2017). However, recent findings demonstrated plasmid-mediated colistin resistance through the *mcr*-1 gene, which was first discovered in an *Ec* strain from agricultural products and mobilized to *Klebsiella pneumoniae* (*Kp*) and *Pa* (Liu *et al.,* 2016). Thus, colistin resistance is mediated by a complex regulatory network of chromosomal genes or through the mobilisation of the plasmid-encoded gene mcr-1. Especially, the plasmid-mediated distribution of colistin resistance raises concern emergence of multi- or even pan-drug resistant Gram-negative bacteria and worsen the situation worldwide (Jeannot *et al.,* 2017; Moradali *et al.,* 2017).

1.5 The cell envelope of Gram-negative bacteria

The cell envelope of Gram-negative bacteria consists of an outer membrane (OM), a thin peptidoglycan (PG) layer within the periplasm, the inner membrane (IM) and the cytoplasm. The complex and dynamic cell envelope protects the bacteria from the environment, allows the entrance of nutrients and ensures the excretion of waste products from the inside (Silhavy *et al.,* 2010). The main focus of this work is on outer membrane β-barrel protein (OMP)

biogenesis, in particular the periplasmic chaperone network and the β-barrel assembly machinery (BAM) complex, and additionally covers the PG synthesis and recycling. Consequently, transport across the IM or transport of lipoproteins or phospholipids to the OM will not be discussed in much detail.

1.5.1 Composition and biogenesis of the outer membrane

The OM is an asymmetric lipid bilayer, which serves as a platform for outer membrane β-barrel proteins (OMPs) to perform their cellular functions and as frontline from which the bacteria interact with the environment or host. It is composed of phospholipids in the inner leaflet, lipopolysaccharide (LPS) in the outer leaflet, bilayer-anchored lipoproteins and integral OMPs (May & Silhavy, 2017).

In *E. coli*, three mayor phospholipids (PLs) (phophatidylethanolamine, phosphatidylglycerol and cardiolipin) assemble in the cell envelope. How PLs are transported to the OM still needs to be clarified. So far, three systems have been identified in *E. coli* to maintain OM asymmetry by removal of mislocalized PLs. The phospholipase PdlA degrades mislocalized PLs, the palmitoyltransferase PagP uses mislocalized PLs as a substrate and transfers an acyl chain to lipid A, which contributes to OM stability, and the Mla pathway, composed of MlaABCDEF, that facilitates the retrograde PL trafficking (Dowhan, 2013; Malinverni & Silhavy, 2009; Narita & Tokuda, 2006).

LPS is a negatively charged glycolipid which is composed of lipid A, a core oligosaccharide and an O-antigen polysaccharide chain (Sperandeo *et al.,* 2017). Lipid A anchors the LPS into the OM and is well known to activate the innate immune system (Needham & Trent, 2013). Furthermore, lipid A of *Pa* differs from other species like *Ec* or *Nm*, in length and number of acyl chains (King *et al.,* 2009). The core oligosaccharide of *Pa* is the most phosphorylated core among Gram-negative bacteria and has been associated to its pathogenesis and intrinsic resistance to antibiotics, such as novobiocin (King *et al.,* 2009).

The O-specific antigens are exposed on the OM, are highly immunogenic and differ from strain to strain. Most *Pa* strains produce two forms of O-antigens, known as common polysaccharide antigen and O-specific antigen. The differences in the O-units of the O-specific antigen are the basis of antigenic serotyping in *Pa* and so far revealed twenty major serotypes, including PAO1 that belongs to serotype O5 and PA14 that belongs to serotype O10 (Lam *et al.,* 2011; King *et al.,* 2009).

The transport of LPS across the IM starts with the ABC transporter MsbA, and is continued by the Lpt (lipopolysaccharide transport) system, which transfers LPS to the OM. The Lpt

system in *Ec* is composed of seven proteins (LptABCDEFG) that pushes LPS molecules continuously from the IM across the periplasm to the outer leaflet of the OM like a "PEZ candy dispenser" (May & Silhavy, 2017; Sperandeo *et al.,* 2017). Studies in *Neisseria meningitidis* (*Nm*) showed that all proteins of the Lpt apparatus are essential for the LPS transport to the OM, except LptE (Bos *et al.,* 2004; Bos & Tommassen, 2011; Tefsen *et al.,* 2005). Thus far, studies in *Pa* could confirm the essential role of LptD in LPS transport (Srinivas *et al.,* 2010; Werneburg *et al.,* 2012) and that LptH, the homologue of *Ec* LptA, is important for cell envelope biogenesis, growth and pathogenicity in various animal models (Fernández-Piñar *et al.,* 2015). In addition, LptE plays a crucial role as a chaperone and plug for LptD and consequently affects the cell envelope stability, antibiotic resistance and virulence of *Pa* in *Galleria mellonella* infection model (Lo Sciuto *et al.,* 2018).

Lipoproteins and OMPs or proteins destined for the the periplasm are first targeted to the Sec translocon in the IM by an N-terminal signal sequence (Du Plessis *et al.,* 2011). Upon entry into the periplasm, the transport of lipoproteins and OMPs diverge. Most of the mature lipoproteins are transported to the OM by the so called Lol (localization of lipoprotein) pathway, containing an ABC transporter (LolCDE) in the inner membrane, a periplasmic carrier protein LolA and a lipoprotein LolB. LolCDE recognizes and releases the lipoprotein in an ATP-dependent manner to LolA, forming a complex in the periplasm. The LolA-lipoprotein complex then moves across the periplasm to interact with the OM anchored lipoprotein receptor LolB. Finally, LolB incorporates the lipoprotein into the inner leaflet of the OM (Narita & Tokuda, 2006). In contrast, OMPs are guided by periplasmic chaperones, such as SurA, Skp and DegP, to the BAM complex in the OM, that facilitates OM insertion. This is described in more detail in the following sections.

1.5.2 Quality control for the biogenesis of OMPs by periplasmic chaperones

Upon entry into the periplasm, unfolded OMPs are subjected to quality control by periplasmic chaperones and proteases to prevent their aggregation or misfolding, or to degrade misfolded OMPs, which dropped out of the folding pathway, respectively.

Periplasmic chaperones bind to unfolded OMPs and guide them through the periplasm and peptidoglycan layer to the OM. There are three main classes of periplasmic chaperones that are known (Goemans *et al.,* 2014; Rollauer *et al.,* 2015):

(1) Peptidyl-prolyl cis-trans isomerases (PPIases), which interconvert the *cis* and *trans* isomers of peptidyl-prolyl bonds involving a proline residue, such as PpiA, FkpA and PpiD.
(2) Disulfide bond catalysing enzymes, which ensure fast and correct folding in the oxidizing environment of the periplasm, such as DsbA, -B, -C and -D.
(3) Proteins with general chaperone activity and impact on OMP biogenesis, such as SurA, Skp and DegP.

Most peptide bonds are present in *trans*, due to their steric hindrance, but proline residues stabilize the *cis* configuration of peptide bonds. To accelerate the rate-limiting step in protein folding, PPIases catalyse the inter-conversion of peptidyl-prolyl imide bonds in protein substrates (Goemans *et al.,* 2014). So far, a superfamily comprising three families of PPIases has been detected in *Ec*. The cyclophilin group was identified at first, including PpiA. The second group, named FKBP, includes FkpA and the third group harbours PpiD and SurA and is called parvulin group.

Interestingly, experiments on a quadruple mutant of *ppiA*, *ppiD*, *surA* and *fkpA* in *Ec* showed no severe changes to the phenotype, indicating no important role of the PPIases in viability and that SurA and FkpA primarily act as periplasmic chaperones (Justice *et al.,* 2005). The chaperone activity of FkpA was further demonstrated by improved expression of single-chain antibody fragments by co-expression of *fkpA* in a *skp* mutant (Bothmann & Plückthun, 2000) and by structural investigations showing that the N-terminus is required for chaperone activity (Saul *et al.,* 2004). In addition, a *fkpA skp* double mutant revealed their role in folding of the OMPs LptD and FhuA, whereby FkpA can replace Skp to fold LptD (Schwalm *et al.,* 2013).

The exact role of PpiA in cell envelope protein quality control is still unknown and the regulation of the *ppiA* gene by the Cpx system (Pogliano 1997), which activates proteases and folding catalyst in response to damage of the cell envelope, is the only indication for its contribution to the quality control of cell envelope proteins (Goemans *et al.,* 2014). PpiD is associated with the IM and is thought to interact with unfolded polypeptides exiting the Sec translocon. Thereby it influences the OMP translocation across the IM (Antonoaea *et al.,* 2008). The protein harbours three soluble domains, of which the first and third have chaperone activity, and the second is very similar to the parvulin domain of SurA. Despite its parvulin domain, PpiD has no mayor role in OMP biogenesis in *Ec*, cannot compensate loss of SurA, and is not necessary for growth (Justice *et al.,* 2005; Antonoaea *et al.,* 2008; Matern

et al., 2010). Thus, the role of PpiD remains unclear, besides its assistance in OMP translocation.

The second rate-limiting step in protein folding is the disulfide bond formation. This modification is mainly performed on proteins that are exported to the cell envelope or beyond, including proteins involved in virulence like toxins, flagella, pili, adhesins or type I & III secretion systems (Landeta *et al.,* 2015). Two mayor pathways are formed by the Dsb enzymes; an oxidative pathway which catalyzes the formation of disulfide bonds into the folding proteins, and an isomerase pathway which repairs incorrectly tied disulfide bonds. In *Ec*, DsbA and DsbB are part of the oxidative pathway, where DsbA reacts with the unfolded OMPs to oxidize them and introduce disulfide bonds, and DsbB re-oxidizes DsbA (Smith *et al.,* 2016). The isomerisation pathway consists of DsbC and DsbD, and results in a process in which DsbC corrects the disulfide bonds and DsbD reduces its periplasmic substrate DsbC (Goemans *et al.,* 2014). Due to the involvement in disulfide formation in many virulence factors, loss of *dsbA* shows a pleiotropic phenotype, reduced fitness in animal models and attenuated virulence. In particular, without DsbA, toxin subunits cannot fold into their native structures, pili subunit assembly or flagella mediated motility is impaired or T3SS effectors cannot be secreted, because intramolecular disulfide bonds in OM secretins within the T3SS in e.g. *Yersinia pestis*, are not folded correctly (Smith *et al.,* 2016).

In *Pa*, the disulfide bond machinery consists of multiple Dsb proteins, including two DsbA proteins (DsbA1 & DsbA2) and two DsbB (DsbB1 & DsbB2) proteins. DsbA1 is the major disulfide bond catalysing enzyme in *Pa* and is re-oxidized by both, DsbB1 and DsbB2. Hence, only the simultaneous deletion of both DsbB proteins leads to misfolding of several virulence factors and consequently to reduced pathogenicity of *Pa* (Arts *et al.,* 2013). Furthermore, ARTS et al. identified 22 new potential substrates of DsbA1, including periplasmic binding proteins, putative periplasmic enzymes involved in peptidoglycan remodelling and potential virulence factors (Arts *et al.,* 2013).

Periplasmic chaperones with general chaperone activity, including Skp, DegP and SurA, are thought to play important roles as chaperones in the OMP biogenesis as indicated by the fact that the gene expression of the chaperones is regulated by the σ^E envelope stress response (Sklar, Wu & Kahne *et al.,* 2007). Two folding pathways are assumed to prevent the aggregation of OMPs and enable a safe passage across the periplasm to the OM. In *Ec*, SurA has been shown to transport the bulk mass of OMPs to the OM, whereas in a parallel pathway Skp and DegP rescue OMPs that have fallen of the normal assembly pathway or even

compensate the absence of SurA (Hagan *et al.,* 2011). The role of Skp and DegP under normal growth conditions is therefore negligible (Sklar, Wu & Kahne *et al.,* 2007).
The periplasmic chaperone SurA was originally identified as a protein essential for survival in stationary phase (Tormo *et al.,* 1990) and is now known to have both, chaperone activity to assist in OMP assembly (Rouvière & Gross, 1996; Lazar & Kolter, 1996) and PPIase activity to catalyse cis-trans isomerization of peptide bonds (Behrens *et al.,* 2001). SurA displays a four domains architecture with a N-terminal domain, two PPIase domains of the parvulin family (P1 and P2) and a C-terminal domain. In the crystal structure, the first PPIase domain is surrounded by the N- and C-terminal domain, forming a core module, so that the second PPIase domain sticks out from the core, extended by two polypeptide linkers (Rollauer *et al.,* 2015; Goemans *et al.,* 2014). The peptide binding activity of SurA was located in the first PPIase domain P1 (Xu *et al.,* 2007), whereas the PPIase activity resides exclusively in P2 (Behrens *et al.,* 2001). Surprisingly, deletion of both parvulin domains has no impact on the chaperone function of SurA *in vitro* and was shown to almost completely complement the *in vivo* function of native SurA (Behrens *et al.,* 2001). Thus, the parvulin domains promote the efficiency of OMP assembly rather than contribute to the chaperone activity of SurA.
Deletion of *surA* in *Ec* leads to reduced OMP levels and consequently to a highly defective cell envelope which is sensitive to antibiotics, bile salts and detergents and additionally leads to impaired virulence (Lazar & Kolter, 1996; Rouvière & Gross, 1996; Sydenham *et al.,* 2000; Weirich *et al.,* 2017). Several studies in *Ec* have shown that in the absence of SurA, porins like LamB, OmpA, OmpC, OmpF, OmpX and other OMPs like FadL, FecA, FhuA and LptD are not correct folded (Rouvière & Gross, 1996; Lazar & Kolter, 1996; Sklar, Wu & Kahne *et al.,* 2007; Vertommen *et al.,* 2009). Interestingly, only FhuA (ferrichrome receptor) and LptD are true substrates of SurA and do not correlate with the decreased mRNA levels caused by σ^E envelope stress response (Goemans *et al.,* 2014). LptD is an essential β-barrel protein, which associates with the lipoprotein LptE to initiate the transport of LPS to the OM (§1.5.1). The phenotype which is observed upon loss of LptD resembles that of a *surA* mutant, which led to the suggestion that the impaired OM in a *surA* mutant is mainly caused by the reduced LptD levels. Furthermore, it is interesting to mention that *lptD* and *surA* are organized together in an operon (Vertommen *et al.,* 2009). However, further studies have demonstrated the requirement of SurA also for other OMPs like the autotransporters EspP (*Enterobacteriacae*), Hbp (*Ec*) and IcsA (*Shigella flexneri*), as well as for the adhesin intimin (enteropathogenic *Ec*) (Purdy *et al.,* 2007; Bodelón *et al.,* 2009; Ruiz-Perez *et al.,* 2009; Sauri *et al.,* 2009), and additionally for pilus - (Justice *et al.,* 2005) and fimbrial assembly (*Ec*)

(Palomino *et al.,* 2011). Moreover, SurA has been demonstrated to be essential for pathogenesis in *Yesinia pestis* (Southern *et al.,* 2016), *Yersinia pseudotuberculosis* (Obi *et al.,* 2011), *Yersinia enterocolitica* (*Ye*) (Weirich *et al.,* 2017), *Shigella flexneri* (Purdy *et al.,* 2007), uropathogenic *Ec* (Justice *et al.,* 2006; Watts & Hunstad, 2008) and *Salmonella enterica* (Sydenham *et al.,* 2000).

Skp (Seventeen kilodalton protein) shows a jellyfish-like architecture with three α-helical tentacles protruding from a β-barrel core domain. Its relatively small substrate-binding site is located in an inner cavity which is defined by the three tentacles (Walton & Sousa, 2004). The small cavity inside Skp is rather suitable for the binding of precursor OMPs containing aromatic and hydrophobic residues than for OMPs with periplasmic or extracellular domains such as OmpA (Rollauer *et al.,* 2015). Nevertheless, it was shown that Skp binds the β-barrel domain of OmpA in an unfolded state, whereas the periplasmic domain of OmpA resides outside the cavity in a completely folded state (Walton *et al.,* 2009; Qu *et al.,* 2009). However, deletion of Skp in *Ec* does not affect the presence of OMPs significantly, although Skp has an array of substrates including porins, autotransporters and also intimin (Ruiz-Perez *et al.,* 2009; Bodelón *et al.,* 2009). In contrast, depletion of Skp in an *Ec surA* mutant results in a bulk of unfolded OMPs and finally leads to cell death emphasizing that Skp and SurA have many OMP substrates in common (Ricci & Silhavy, 2012). In contrast, deletion of *skp* in *Nm* caused reduced levels of the OMPs PorA and PorB, which was not observed in a *surA* mutant, indicating a less relevant role of SurA in *Nm* (Volokhina *et al.,* 2011). Furthermore, *skp* mutation attenuates *Salmonella enterica in vivo* during mice infection (Rowley *et al.,* 2011) and indirectly leads to decreased cell spread in *Shigella flexneri* (Wagner *et al.,* 2009).

The periplasmic serine protease DegP functions in the same pathway as Skp and its synthesis is activated by the σ^E or Cpx system in response to heat shock or other envelope stresses (Ricci & Silhavy, 2012). DegP recognizes unfolded, misfolded or aggregated OMPs to refold or degrade them (Hagan *et al.,* 2011). The protein consists of a N-terminal chymotrypsin-like protease domain with a His-Asp-Ser catalytic site and of two C-terminal PDZ domains (PDZ1 and PDZ2), which are protein interaction modules found in diverse signalling proteins in prokaryotes, eukaryotes and viruses. PDZ1 is essential for the protease activity by recognizing and offering the substrates to the protease domain at the N-terminus (Iwanczyk *et al.,* 2007). Besides its protease activity, DegP seems to displays also chaperone activity, because its deletion in an *Ec surA* mutant is not viable (Rizzitello *et al.,* 2001). It is thought that the chaperone activity of DegP primarily functions at lower temperatures (below 28°C) and that the protease activity dominates at increasing temperatures (Ricci & Silhavy, 2012). The

substrate spectrum of DegP includes PhoA, PapA, Mals (Kadokura *et al.,* 2001; Jones *et al.,* 2002; Skorko-Glonek *et al.,* 2007) or OMPs like OmpA, OmpC, OmpF and LamB (Krojer *et al.,* 2008). The folding of the α-amylase MalS, for instance, is promoted by DegP under low temperatures, but at higher temperatures MalS is degraded by DegP. Moreover, it could be demonstrated that DegP acts as a protector of OMP monomers to prevent their degradation by other proteases (Krojer *et al.,* 2008). In addition, DegP was shown to be important for survival at elevated temperature and for virulence of *Salmonella enterica* (Mo *et al.,* 2006), as well as of *Brucella abortus* (Elzer & Roop, 1996), *Brucella melitensis* (Phillips *et al.,* 1995), *Ye* (Li *et al.,* 1996), *Kp* (Cortés *et al.,* 2002) and *Pa* (Yorgey *et al.,* 2001). Despite that, how DegP exactly acts as chaperone in OMP biogenesis is not well understood and remains to be determined.

To sum up, three classes of periplasmic chaperones, including SurA, Skp and DegP, build a network with two parallel pathways to control the assembly of OMPs. In this network, SurA plays the pivotal role for OMP assembly and Skp and DegP rescue the OMPs that fall off the SurA pathway. Nevertheless, there can be species specific differences in importance of the chaperones for OM integrity, bacterial fitness and virulence.

1.5.3 The BAM complex and integration of proteins into the outer membrane

After transport of newly synthesized nonlipidated OMPs across the IM by the Sec translocon and through the periplasm by chaperones SurA, Skp and DegP, OMPs are assembled into the OM by the so-called BAM complex.

The BAM complex consist of BamA, an OMP itself, and the four lipoproteins BamB, BamC, BamD and BamE, that bind to the N-terminal periplasmic domain of BamA (Sklar, Wu & Kahne *et al.,* 2007; Wu *et al.,* 2005; Voulhoux *et al.,* 2003).

BamA was original identified in *Nm* and displays the central and essential component of the BAM machinery (Voulhoux *et al.,* 2003). BamA belongs to the Omp85 protein superfamily and forms a 16 stranded β-barrel, spanning the OM. The protein is conserved across all Gram-negative bacteria and orthologs of the protein can be found in mitochondria and chloroplasts (Hagan *et al.,* 2011). Gram-negative BamA proteins contain five polypeptide transport-associated (POTRA) domains, whereas in chloroplasts and mitochondria only three or one POTRA domains can be found, respectively (Hagan *et al.,* 2011). These periplasmic POTRA domains are the binding sites for the four lipoproteins of the BAM complex. BamC, -D and -E

bind to POTRA domain 5 (P5) and BamB interacts with P2-P5, indicating a separate assembly of BamA and BamB (Kim *et al.,* 2007). However, BENNION and colleagues could demonstrate that the periplasmic chaperone SurA of *Ec* binds to the P1 domain (Bennion *et al.,* 2010). Moreover, some of the POTRA domains are essential for viability, as shown for *Ec*, where deletion of P1 and/or P2 can be tolerated, but single deletion of P3, P4 or P5 leads to a defect in OMP assembly and increased mortality of the bacteria (Kim *et al.,* 2007). In contrast, deletion of P1-P4 in BamA of *Nm* does not affect the viability and may be explained by the fact that LptD, which usually requires the POTRA domains P1-P4 for its assembly, is also dispensable in *Nm* (Ricci & Silhavy, 2012).

BamB displays an eight-bladed β-propeller structure in which each propeller is formed by four antiparallel β-sheets forming together a ring-like architecture (Noinaj *et al.,* 2011; Kim & Paetzel, 2011). This nonessential component of the BAM complex is bound via its N-terminal lipid anchor to the periplasmic side of the OM (Charlson *et al.,* 2006) and crystallization experiments in *Ec* indicated that BamB may bind unfolded OMPs by β-augmentation (Wu *et al.,* 2005; Kim *et al.,* 2007). Despite its irrelevant role for viability, deletion of BamB results in significantly aberrant OM biogenesis and consequently to increased sensitivity to lysozyme, detergents and antibiotics or to decreased adherence and reduced virulence in different species (Weirich *et al.,* 2017; Lee *et al.,* 2017; Hsieh *et al.,* 2016). Thus, BamB plays an important role in OMP biogenesis although it is not essential.

Analysis of BamC revealed a modular structure containing a N- and C-terminal domain and additionally a disordered region of 75 amino acids at the N-terminus (Knowles & McClelland *et al.,* 2009; Albrecht & Zeth, 2010; Kim & Paetzel, 2011; Kim, K. H. *et al.,* 2011). Furthermore, the C-terminal domain of BamC is exposed to the cell surface (Webb *et al.,* 2012). Deletion of BamC has no striking impact on the OM composition and assembly of OMPs is still possible (Fardini *et al.,* 2009; Onufryk *et al.,* 2005). Interestingly, BamC is not ubiquitous throughout Gram-negative bacteria and does not show sequence similarity to any known protein (Knowles & Scott-Tucker *et al.,* 2009).

In contrast to the other lipoproteins BamD is the only essential lipoprotein of the BAM machinery, leading to cell death when absent. It is composed of five tetratricopeptide repeat (TPR) domains and presents an overall α-helical structure (Kim, Kelly H. *et al.,* 2011; Albrecht & Zeth, 2010). BamD is highly conserved and according to the current knowledge is thought to initially recognize the C-terminal targeting sequence of OMP precursors (Albrecht & Zeth, 2010). Within the complex, BamD mediates the interaction of BamC and -E with BamA, demonstrated by failed interactions of the lipoproteins caused by C-terminal

truncations of BamD (Sklar, Wu & Gronenberg *et al.,* 2007). In more detail, the disordered N-terminus of BamC is required for the interaction with BamD, by binding in that region of BamD that is believed to target OMP precursors. So, BamC might have a regulatory role, blocking or exposing the targeting sequence-binding site of BamD (Kim, Kelly H. *et al.,* 2011).

BamE interacts with the TPR domains 4 and 5 of BamD and additionally was shown to interact with P5 of BamA, supporting the interplay of BamD and BamA (Sklar, Wu & Gronenberg *et al.,* 2007; Sklar, Wu & Gronenberg *et al.,* 2007; Fardini *et al.,* 2009). Furthermore, BamD binds to the P1 and P2 domain of BamA, further stabilizing the complex structure (Bakelar *et al.,* 2016). Moreover, a recent study showed, that the absence of BamE leads to the accumulation of the surface exposed lipoprotein RcsF (regulator of capsule synthesis protein F) on BamA causing the inactivation of the BAM complex (Tata & Konovalova, 2019). Thus, BamE is responsible for a correct interaction of RcsF-bound BamA together with BamD to enable the assembly of OMP/RcsF complexes (Tata & Konovalova, 2019). Maybe this explains the more severe changes in OM integrity and OMP level upon *bamE* deletion compared to the minor changes upon *bamC* deletion (Sklar, Wu & Gronenberg *et al.,* 2007; Fardini *et al.,* 2009).

Hence, a lot of structural and biochemical data have led to a good knowledge of the interactions between the single components of the BAM complex, but how the folding and insertion of the OMPs into the OM takes place is still not fully understood. At the moment two models have been suggested, both based on BamA features between the β1 (N-terminus) and β16 (C-terminus) strands of the β-barrel (Leo & Linke, 2018; Zhang *et al.,* 2019).

The first, named budding model, implies that BamA opens a lateral gate and interacts with the growing OMP, forming a β-sheet hybrid. Once the lateral gate of BamA is open, β-hairpins bind to the N-terminus of BamA via β-augmentation. This means that β-strands of the newly formed OMP align with the β-sheet of BamA and that the OMP is directly integrated into the barrel of BamA while hydrophobic residues face the outside and hydrophilic residues the inside (Navarro-Garcia, 2019). Finally, the newly formed OMP “buds” away and leaves behind the BAM complex, once more ready for use (Zhang *et al.,* 2019; Navarro-Garcia, 2019). The second, called assisted model, claims a self-insertion of the OMPs caused by decreased hydrophobicity near the lateral gate of BamA. More precisely, the hydrophobic region between BamA and the OM seems to disorder the lipid bilayer and thereby generates a region for a more efficient insertion of folded OMPs into the OM. For its insertion, the OMP forms β-hairpins invading the OM step by step until the full β-barrel is formed. So, the role of

the BAM complex in this model is to catalyse the process and destabilize the OM bilayer and guide the unfolded OMPs to the intended region (Navarro-Garcia, 2019).
However, recent investigations of HÖHR *et al.* on the BamA homolog Sam50 in *Saccharomyces cerevisiae* revealed a mechanism combining both models: formation of a β-sheet hybrid and insertion of β-hairpins into the lateral gate, and subsequent insertion into the thinned membrane (Höhr *et al.,* 2018).
Furthermore, the BAM complex is also crucial for the assembly of autotransporter proteins, consisting of a C-terminal translocator domain (β-domain) that supports the transport of the N-terminal passenger domain through the OM to the cell surface (van Ulsen *et al.,* 2018). The passenger domain harbours the biological function and can interact as protease, toxin, adhesin or shows other functions that cause virulence (Henderson & Nataro, 2001). In *Pa*, several autotransporters have been identified like PlpD, EstA, AaaA and EprS (da Mata Madeira *et al.,* 2016; Wilhelm *et al.,* 2007; Kida *et al.,* 2013; Luckett *et al.,* 2012). Both, the translocator and passenger domain of autotransporters interact with chaperones to fold the translocator domain in the periplasm and fuse it to the BamA barrel. Moreover, interactions with BamB and BamD could also be demonstrated (Ieva *et al.,* 2011). Subsequently, the C-terminus of the passenger domain forms a hairpin like conformation and is pulled through the pore of BamA (Leo *et al.,* 2012). Eventually, the passenger domain is cleaved off and finally the translocator domain is released from the BAM complex (Leo *et al.,* 2012).

To sum up, transport and insertion of OMPs, including autotransporters, occurs by a group of periplasmic chaperones (§ 1.5.2) and five OM BAM proteins (§4 Figure 1). The mechanism of OMP insertion is still controversially discussed and needs to be further analysed.

1.5.4 Architecture, biosynthesis and recycling of the peptidoglycan cell wall

1.5.4.1 Architecture of the peptidoglycan cell wall

The Gram-negative peptidoglycan (PG), also called murein or sacculus, was first detected in 1964 by Wolfhard Weidel and his group in Tübingen (Braun, 2015). It is composed of repeating disaccharides of N-acetyl muramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) that are linked by β 1-4 glycosidic bonds. Attached to the muramyl moiety of the disaccharide chain protrude peptide chains containing up to five amino acids. The peptide chains typically consist of L-alanine, D-glutamic acid, meso-diaminopimelic acid (mDAP) or lysine and two D-alanine and enable a cross-linking between the glycan strands (Koraimann, 2003). Normally, the cross-linking takes place between the mDAP of one peptide chain and

the fourth D-Alanine of the other peptide chain, but cross links are also formed between the mDAPs of both peptide chains (Irazoki *et al.,* 2019; Dhar *et al.,* 2018; Vollmer *et al.,* 2008). Thus, a multi-layered meshwork of glycan strands and peptides is formed and embedded in the periplasmic space. In contrast to the PG of Gram-positive bacteria (up to 80 nm), the PG of Gram-negative bacteria has a thickness of approximately 6 nm (*Ec*, 6.35 ± 0.53 nm; *Pa* 2.41 ± 0.54 nm (Matias *et al.,* 2003)) and withstands the osmotic pressure to maintain cell integrity, keeps the regular bacterial shape and provides to a certain degree the escape of antibiotic action (Dhar *et al.,* 2018; Juan *et al.,* 2018; Zeng & Lin, 2013). The stability of the Gram-negative cell wall is further improved by a covalent linkage to the OM via Braun's lipoproteins (Hantke & Braun, 1973) or other murein lipoproteins such as the PG-associated lipoprotein (Pal) (Juan *et al.,* 2018). Consequently, the architecture and the correct synthesis and maintenance of the PG is essential for a proper protection against environmental threats, viability and virulence of the bacteria. Any interruption in PG biosynthesis or even degradation during cell growth results in cell lysis (Vollmer *et al.,* 2008). During cell growth and cell division the PG is subjected to comprehensive transformation and the synthesis and recycling of the cell wall components is well coordinated to maintain cell integrity (Dhar *et al.,* 2018). Up to 50 % of the murein components are recycled for murein biosynthesis during active bacterial growth and it was demonstrated that an existing glycan strand, first serving as a kind of docking station, is replaced by three new cross-linked glycan strands, giving a "three-for-one model" (Höltje, 1998).

1.5.4.2 Biosynthesis of the peptidoglycan cell wall

The biosynthesis of new glycan strands requires several enzymatic reactions which can be separated into three main steps: (I.) the synthesis of the uridine-diphophate (UDP)-MurNAc pentapeptide precursor, (II.) the attachment of the precursor to a lipid (lipid I) in the cytoplasmic leaflet of the IM, generating PG precursor lipid II, and (III.) the transfer of lipid II into the periplasm by a flippase and subsequent incorporation into the existing glycan strand and cross-linking by high molecular mass penicillin-binding proteins (HMM PBPs) (Zeng & Lin, 2013; Vermassen *et al.,* 2019) (see also Sonnabend et al., Figure 3 for an simplified overview of PG synthesis and recycling).

More precisely, the UDP-MurNAc pentapeptide synthesis starts in the cytoplasm with the conversion of fructose-6-phosphate to UDP-GlcNAc by the enzymes GlmS, GlmU and GlmM (Dhar *et al.,* 2018). Homologs of *Ec* glucosamine synthetase GlmS, N-acetylglucosamine-1-phosphate uridyltransferase GlmU and phosphoglucosamine mutase GlmM are also present in

Pa (PA5549, PA4749 and PA5552, respectively). Subsequently, UDP-GlcNAc is enzymatically transformed by the Mur enzymes A-F to UDP-MurNAc pentapeptide, which is further modified by MraY, adding the aliphatic moiety undecaprenyl pyrophosphate (UndP) to enable the binding to the cytoplasmic leaflet of the IM, and forming lipid I (Dhar *et al.,* 2018). The generation of lipid I by MraY is reversible, but proceeds immediately with the addition of GlcNAc to the MurNAc moiety of lipid I by glycosyltransferase MurG, generating lipid II (Bouhss *et al.,* 2004). Because MraY and MurG both are essential enzymes in PG synthesis and conserved in Gram-negative and Gram-positive bacteria they are promising targets for new antibacterial agents (Zawadzke *et al.,* 2003; Fer *et al.,* 2018).

The third step of PG synthesis starts with the transfer of lipid II into the periplasm, catalysed by a flippase enzyme. The flipping of lipid II has been discussed controversially and in *Ec* RodA, FtsW and MurJ have been reported to perform this reaction (Ruiz, 2015; Taguchi *et al.,* 2018; Bolla *et al.,* 2018; Meeske *et al.,* 2016; Cho *et al.,* 2016; Sham *et al.,* 2014; Mohammadi *et al.,* 2014). Recent analysis now suggests a model for lipid II flipping in which MurJ is recruited by its substrate lipid II and depends on FtsW/PBP3 (Liu *et al.,* 2018). Finally, GlcNAc-MurNAc pentapeptides of lipid II are incorporated into the growing PG sacculus by HMM PBPs with transglycosylase (TG) and/or transpeptidase (TP) activities. In *Pa*, there exist PBP1a, PBP1b, PBP2, PBP3 and PBP3b/3x, whereas PBP3b/3x was demonstrated to be the only essential HMM PBP in *Pa* (Chen *et al.,* 2017).

1.5.4.3 Recycling of the peptidoglycan cell wall

The recycling process of the PG cell wall involves three types of lytic enzymes, including low molecular mass (LMM) PBPs, lytic transglycosylases (LTs) and amidases (Dhar *et al.,* 2018). LMM PBPs can act as carboxypeptidases or endopeptidases, whereas the latter activity facilitates the hydrolysis of cross-links to ensure the continuing PG maturation and recycling.

Ec harbours five LMM PBPs, called PBP4/DacB, PBP5/DacA, PBP6/DacC, PBP6b/DacD and PBP7/8/PbpG (Dhar *et al.,* 2018; Sauvage *et al.,* 2008). PBP5, attached to the IM, mainly fullfils carboxypeptidase activity and its loss provokes abnormalities in the PG layer and increases the presence of free pentapeptides (Nelson & Young, 2000). *Pa* only harbours three LMM PBPs, PBP4/DacB, PBP5/DacC and PBP7/Pbp, and these share the same carboxypeptidase- and/ or endopeptidase activity as their homologs in *Ec*. Moreover, PBP5 of *Pa* is very similar to PBP5 and PBP6 of *Ec* and deletion of PBP5 in *Pa* also evidenced its role as major carboxypeptidase as shown by increased pentapeptide levels (Ropy *et al.,* 2015). Furthermore, loss or mutation of the *dacB* encoded PBP4 is the mayor cause of *ampC*

overexpression in *Pa* resulting in high level β-lactam resistance and similarly to increased expression of *creBC* and *creD* (Moya *et al.,* 2009) (see also § 1.4). Other enzymes, cleaving the cross-bridges, are DD-endopeptidases like MepA, MepH (YdhO), MepM (YebA), MepS (Spr) and the recently identified MepK (Singh *et al.,* 2012; Chodisetti & Reddy, 2019). Loss of the three DD-endopeptidases *mepH*, *mepM* and *mepS* have been reported to cause a severe defect in PG assembly, leading to cell lysis (Singh *et al.,* 2012). The regulation of these PG hydrolases is not known well, but recently it could be shown that *Pa* harbours a homolog of the *Ec* Carboxy-terminal processing protease (CTP) Prc, (Reiling *et al.,* 2005) and a second CTP, named CtpA, which could be shown to regulate four proteins involved in PG hydrolysis, namely MepM1 (PA0667), MepS/MepH2 (PA1198), MepH1 (PA1199) and MepM3 (PA4404) (Srivastava *et al.,* 2018). Thus, *Pa* seems to have two CTPs that help to constrain the PG hydrolysis.

LTs catalyse the non-hydrolytic cleavage of the β-1,4-glycoside bonds between MurNAc and GlcNAc, forming 1,6-anhydroMurNAc, which is the inducer of *ampC* expression (Domínguez-Gil *et al.,* 2016). *Ec* harbours eight LTs encoded by *mltA, mltB, mltC*, *mltD*, *mltE*, *mltF*, *mltG* and *slt70* (Scheurwater & Clarke, 2008; Scheurwater *et al.,* 2008; Yunck *et al.,* 2016). *Pa* owns eleven LTs, including MltA, MltB, MltD, MltF, MltF2, MltG, Slt, SltB1, SltB2, SltH/SltB3 and RlpA. Deletion of LTs are known to reduce the levels of *ampC* inducing 1,6-anhydroMurNAc, leading to decreased minimal inhibitory concentrations (MICs) for β-lactam antibiotics. However, loss of MltA, SltG or MltF in *Pa* had no effect on the resistance phenotype or even worse double deletion of *sltB1* and *mltB* caused an increase of resistance, although no increase of *ampC* expression could be detected (Cavallari *et al.,* 2013; Lamers *et al.,* 2015). Thus, the loss of LTs can also contribute to a further increase of AmpC production (Lamers *et al.,* 2015), making it difficult to use an unspecific LT inhibitor to treat antibiotic resistance.

Periplasmic amidases hydrolyse the amide bond between MurNAc and L-alanine, separating the glycan strand from the stem peptide, as well as between recycling by-products like GlcNAc-1,6-anhydro-MurNAc peptides (Vermassen *et al.,* 2019; Dhar *et al.,* 2018). Loss of amidases in Gram-negative bacteria has been shown to increase the sensitivity to antibiotics, detergents and defensins (Heidrich *et al.,* 2002; Yakhnina *et al.,* 2015). *Ec* harbours the three periplasmic amidases AmiA, AmiB and AmiC and the OM bound amidase AmiD. The periplasmic amidases in *Pa* are AmiA and AmiB and two additional periplasmic amidases, AmpDh2 and AmpDh3, which share homology with *Ec* AmiD. However, this unique feature of *Pa* allows the PG turnover already in the periplasm and is advantageous since loss the

cytoplasmic amidase AmpD does not result in decreased fitness or virulence due to the presence of the extra amidases (Moya *et al.,* 2008). Furthermore, it could be demonstrated that AmpDh3 contributes more than AmpDh2 to *ampC* repression in an *ampD* mutant (Juan *et al.,* 2006) and additionally, AmpDh3 was very recently identified to be delivered to the periplasm of prey bacteria (*Ec* or *Yersinia pseudotuberculosis*) by the type 6 secretion system locus 2 (H2-T6SS) of *Pa*, and to kill it by hydrolysing the PG (Wang *et al.,* 2019, prereleased, but not yet peer-reviewed manuscript).

In the next step the generated muropeptides in the periplasm are transported across the IM by the permease AmpG or less frequently by the permease system OppBCDF or by NagE and MurP (MurP not in *Pa*) (Park, 1993). *Pa* harbours the two permeases AmpG and AmpP (Kong *et al.,* 2010). Depletion of AmpP in PAO1 did not alter its resistance profile (Kong *et al.,* 2010), but lack of AmpG leads to a reduced transfer of the AmpC inducer 1,6-anhydroMurNAc into the cytoplasm and consequently restored the susceptibility to β-lactam antibiotics even in highly resistant clinical isolates (Kong *et al.,* 2010; Zamorano *et al.,* 2011). In connection with the AmpG mediated transport of GlcNAc-1,6-anhydro-MurNAc into the cytoplasm, the β-N-acetylglucosaminidase NagZ, amidase AmpD and LD-carboxypeptidase LdcA divide the muropeptides into the components GlcNAc, anhydro-MurNAc, and L-alanine-D-glutamic acid-mDAP and D-Ala (Dhar *et al.,* 2018). Deletion of NagZ does not affect the growth, but prevents the production of 1,6-anhydro-MurNAc and consequently leads to low AmpC levels (Asgarali *et al.,* 2009; Zamorano *et al.,* 2010). Loss of AmpD causes an increase of 1,6-anhydro-MurNAc-tripeptides and loss of LdcA leads to an increase in 1,6-anhydro-MurNAc-tetrapeptides (Höltje *et al.,* 1994; Templin *et al.,* 1999).

Consequently, the muropeptides are further processed through different pathways in *Ec* and *Pa*. In *Ec*, 1,6-anhydro-MurNAc is processed by the AnmK kinase, the etherase MurQ and by NagA to GlcN-6-P and subsequently is catalysed to fructose-6-P by NagB, the starting product of PG biosynthesis, or is further synthesized to UDP-GlcNAc by GlmM and GlmU (Gisin *et al.,* 2013; Uehara *et al.,* 2005). In *Pa*, a homolog of AnmK, but not of MurQ can be found. So, 1,6-anhydro-MurNAc is also processed by AnmK, but subsequently the phosphatase MupP converts MurNAc-6P to MurNAc (Borisova *et al.,* 2014; Borisova *et al.,* 2017). This salvage pathway, which bypasses the *de novo* biosynthesis of UDP-MurNAc, proceeds with the phosphorylation of MurNAc to MurNac-α-1P by the anomeric MurNAc- and GlcNAc kinase AmgK. Finally, the uridyltransferase MurU modulates the latter to UDP-MurNAc (Borisova *et al.,* 2017). Thus, the presence of AmgK and MurU compensates the absence of MurQ and related synthesis steps for UDP-GlcNAc by NagA, NagB, GlmM and

GlmS (Dhar *et al.,* 2018). Furthermore, the salvage pathway bypasses the need for MurA and MurB and thereby confers intrinsic resistance to fosfomycin, which normally inhibits the UDP-MurNAc synthesis by covalent binding of MurA (Borisova *et al.,* 2017). In addition, distribution analysis indicated that *amgK* and *murU* homologs are mainly present in α-, β-, and γ-proteobacteria but not in enterobacteria like *Ec* or *Kp* (Gisin *et al.,* 2013).

1.5.5 Peptidoglycan metabolism and the regulation of intrinsic β-lactamases

One of the most striking characteristics of *Pa* is its β-lactam resistance mechanism mediated by the chromosomal β-lactamase AmpC. Regulation of *ampC* expression depends on the PG recycling (Torrens *et al.,* 2017). β-lactam antibiotics are the most common administered antibiotics and by mimicking the last two D-Alanine residues of the peptide side chains they prevent binding of HMM PBPs and subsequently prevent the cross-linking of the PG, hence leading to cell lysis (Dhar *et al.,* 2018). In many Gram-negative bacteria, including *Pa*, *Neisseria gonorrhoeae*, *Enterococcus cloacae* and *Citrobacter freundii*, the AmpC levels can be induced by the presence of β-lactam antibiotics (Juan *et al.,* 2018). So far, it is assumed that under normal growth UDP-MurNAc-pentapeptide binds to the LysR-type transcriptional regulator AmpR and acts as corepressor to repress *ampC* expression. The presence of β-lactam antibiotics increases the amount of PG derived fragments, so that AmpD may get saturated, the level of 1,6-anhydro-MurNAc-tripeptide increases and competes for the binding of AmpR. Subsequently, AmpR performs a conformational change and activates *ampC* expression (Jacobs *et al.,* 1994; Jacobs *et al.,* 1997; Domínguez-Gil *et al.,* 2016).

In addition, the AmpR regulated induction of *ampC* expression requires the products of AmpG, AmpD, NagZ, AmpDh2 and AmpDh3, since they play important roles in the PG cell wall synthesis and recycling as described in the previous section.

The inactivation of AmpD is one of the main drivers of β-lactam resistance in *Pa* clinical strains as well as in Enterobacteriaceae and leads to *ampC* overexpression, even in the absence of β-lactam antibiotics (Juan *et al.,* 2006). Deletion of all three genes *ampD*, *ampDh2* and *ampDh3* renders the situation even worse due to a constant hyperexpression of *ampC* (Moya *et al.,* 2008). AmpG and NagZ are further key players in *ampC* mediated β-lactam resistance, since they allow the entrance of muropeptides into the cytoplasm and their modulation to *ampC* inducing molecules (Juan *et al.,* 2018). Interestingly, NagZ inactivation attenuates β-lactam resistance in *ampD* and *dacB* mutants due to the inability to produce 1,6-

anhydro-muropeptides or blocking the overexpression of *creD*, respectively (Zamorano *et al.,* 2010).

The second main driver of AmpC hyperproduction is the mutational inactivation of *dacB*, encoding PBP4. If *dacB* is disrupted, the CreBC two-component system, including the IM effector protein CreD, is activated and indirectly triggers the AmpR-dependent overproduction of AmpC. The CreBC system is another essential player in the interplay of intrinsic β-lactamase induction and PG metabolism and acts as a global regulator, especially under stress conditions such as exposure to β-lactams. Nevertheless, the association of this system and β-lactam resistance has been documented only in *dacB* mutants and not in other AmpC hyperproducing strains carrying mutations in *ampD*, *ampDh2* or *ampDh3* (Moya *et al.,* 2009). Interestingly, inactivation of *ampC* in the *dacB* mutant affects the *creD* activation, but *nagZ* or *ampG* deletions showed no impact, indicating that *ampC* expression itself drives the *creD* inducibility and that blocking PG cell wall metabolites attenuates *creD* expression (Zamorano *et al.,* 2014). However, the hyperproduction of AmpC always depends on the regulator AmpR, since it directly controls the *ampC* expression through the formation of an AmpR-DNA complex. Loss of AmpR causes a significant increase in β-lactam resistance and additionally leads to the activation of other virulence genes (Kong *et al.,* 2005). AmpR was shown to be involved in the regulation of the MexEF-OprN efflux pump (efflux of fluoroquinolones, chloramphenicol and trimethoprim), biofilm formation, QS-regulated virulence factors and to control the transcription of PoxB β-lactamase, underlining its role as global regulator in *Pa* (Kong *et al.,* 2005; Balasubramanian *et al.,* 2012). For instance, inactivation of AmpR reduced virulence as shown by *Caenorhabditis elegans* paralytic assays (Balasubramanian *et al.,* 2012), or caused a decreased aminoglycoside resistance (Balasubramanian *et al.,* 2014).

To sum up, the interplay between the PG cell wall recycling and the induction of β-lactamase AmpC production is very complex and requires the global transcriptional regulator AmpR, the three AmpD amidases, AmpG and NagZ. In addition, AmpR also regulates resistance to non-β-lactam antibiotics, biofilm formation and QS-regulated virulence factors. Moreover, the CreBC two-component system plays a decisive role in β-lactam resistance in *dacB* mutants and additionally acts as a global regulator.

2 Aims of the thesis

Pa is one of the main causative agents of nosocomial infections such as pneumonia, urinary tract infection, wound infections and potentially life-threatening blood stream infection. *Pa* can be intrinsically resistant to certain antibiotics due to a low permeability of the OM, the activity of efflux pumps and the inducible expression of the chromosomal β-lactamase AmpC. Furthermore, the spread of MDR *Pa* strains is rising and therefore new strategies for therapy are urgently needed.

The OM serves as a platform for OMPs, including virulence associated proteins, to perform their cellular functions and as frontline from which the bacteria interact with the environment or host. Therefore, one strategy is to define targets for anti-virulence adjuvants that are associated with the OM assembly and its maintenance. In this respect, the main aim of the current work was to investigate the suitability of several proteins involved in the OM assembly as potential targets in order to possibly develop new strategies to treat *Pa* infections. Thus, single gene deletion mutants of the non-essential BAM complex components BamB and BamC, of the periplasmic chaperones HlpA as well as a conditional mutant of the periplasmic chaperone SurA were generated and investigated with regards to membrane integrity, antibiotic susceptibility, sensitivity to bile salts, serum resistance and virulence in a *Galleria mellonella* infection model.

A second strategy to define targets for anti-virulence adjuvants is to target chromosomal non-essential genes that are not directly responsible for resistance mechanism but essential under therapeutic antibiotic concentrations, summarized herein as the "secondary resistome". Therefore, a high- density transposon (Tn) insertion mutant library in a MDR *Pa* bloodstream isolate (ID40) was generated and subjected to cefepime (FEP) and MEM. Subsequently, Transposon-Directed Insertion Sequencing (TraDIS) was performed and revealed several known and unknown non-essential candidate genes as possible targets. Moreover, some candidates were further validated by analysing their respective deletion mutants for antibiotic susceptibility, β-lactamase activity and *ampC* expression.

The gained knowledge may help to develop new anti-virulence adjuvants to circumvent antimicrobial resistance and to further highlight the species-specific differences, which render the identification of a potential anti-virulence drug target, which is present in several Gram-negative species, more difficult, but at the same time might facilitate high-precision infection therapy.

3 Results and Discussion

3.1 Deprivation of the periplasmic chaperones SurA reduces virulence and restores antibiotic susceptibility of a multidrug-resistant *Pseudomonas aeruginosa*

Pa is one of the main causative agents of nosocomial infections and belongs to the so called ESKAPE group of multidrug-resistant pathogens for which new strategies for therapy are urgently needed (Rice, 2008). *Pa* harbours several intrinsic and acquired resistance mechanisms against antibiotics including enzymatic and mutational resistance mechanisms like the production of β-lactamases, overexpression of efflux-pumps, and the OM, that limits the entrance of antibiotic molecules through its low permeability (Gellatly & Hancock, 2013; Klein *et al.,* 2019). Especially, the passage of drugs across the OM of *Pa* and other Gram-negative bacteria is the major challenge and needs to be resolved (Klein *et al.,* 2019).
The OM is an asymmetric lipid bilayer, composed of phospholipids in the inner leaflet, LPS in the outer leaflet and bilayer-anchored lipoproteins and OMPs (May & Silhavy, 2017). Most of the OMPs are porins and autotransporter proteins that comprise a β-barrel domain and allow the passage of solutes and contribute to the interaction with the environment or host (Silhavy *et al.,* 2010; Henderson & Nataro, 2001). The insertion of the OMPs into the OM is enabled by a machinery composed of periplasmic proteins like Skp (HlpA), SurA and DegP (MucD) that serve as quality control and guidance for the proteins during the transport across the periplasm, and the BAM complex, consisting of BamA-E, which assembles the OMPs into the intended region in the OM. The important role of periplasmic chaperones and the BAM complex in membrane integrity and antibiotic resistance has been investigated already in other species such as *Ec*, *Ye* and *Nm* and thereby revealed species-specific differences. Investigations in *Ec* and *Ye* demonstrated a major role for SurA and a minor role for Skp in folding and OMP assembly (García-Contreras *et al.,* 2015; Sklar, Wu & Kahne *et al.,* 2007; Weirich *et al.,* 2017), but in *Nm* Skp plays the prominent role for OMP assembly (Volokhina *et al.,* 2011). Furthermore, deletion of non-essential lipoproteins of the BAM complex or periplasmic chaperones leads to altered OMP composition resulting in disturbed OM integrity that renders the bacteria more susceptible to antibiotics, human serum, bile salts or detergents (Lee *et al.,* 2017; Weirich *et al.,* 2017; Navarro-Garcia, 2019; Klein *et al.,* 2019).
Thus, the aim of this study was to investigate the role of the non-essential BAM components BamB and BamC as well as the periplasmic chaperones SurA and the Skp homolog HlpA in

Pa PA14 and additionally the depletion of SurA in the MDR bloodstream isolate ID72, for OM integrity and composition, antibiotic resistance and virulence.

The OM integrity of the mutants was tested on the one hand by using N-phenylnaphthylamine (NPN) and on the other hand by growth in bile salts. NPN fluoresces only in hydrophobic environments like the phospholipid bilayer of the inner leaflet of the OM, so higher fluorescence values indicate a reduced OM integrity. The outcome of the experiments revealed a significant entrance of NPN into the conditional *surA* mutant, but not into the *bamB*, *bamC* or *hlpA* mutant compared to the PA14 WT. The complementation of the *surA* mutant (*surA* SurA+) by the addition of 0.2 % arabinose could restore the NPN fluorescence signal to WT levels (Klein *et al.,* 2019). Moreover, the growth studies in LB containing 0.3 % bile salts revealed a significantly reduced growth of the conditional *surA* mutant, as well as of the *bamB* and *bamC* mutant. In contrast, the growth of the *hlpA* mutant and *surA* SurA+ was not affected. For complementation of the *bamB* deletion mutant, *bamB* was expressed from a plasmid containing an arabinose inducible promoter (*bamB* BamB+). The addition of 0.2 % arabinose caused a decrease in growth reduction almost to WT level, but not significantly (Klein *et al.,* 2019). Hence, the depletion of SurA and BamB showed the most impact on OM integrity.

To see if the altered OM integrity results in morphological changes, transmission electron microscopy pictures were taken from the conditional *surA* mutant in the presence and absence of arabinose as well as from the *bamB* mutant and PA14 WT. Here, the *bamB* mutant revealed numerous vesicles attached to the cell surface and in the absence of arabinose the conditional *surA* mutant showed also vesicles and additionally the cells looked slightly bloated. Comparable results in sensitivity to bile salts and morphological changes were obtained for *surA* and *bamB* deletion mutants in *Ye* (Weirich *et al.,* 2017).

To further analyse the changes in the OM of the different mutants, OM fractions were prepared and analysed by LC-MS/MS. The data displayed only minor changes in the OM composition of the *bamB*, *bamC* and *hlpA* mutants, whereas the depletion of SurA showed major alterations in the OM composition. In the *hlpA* mutant only HlpA itself was not detectable. The deletion of *bamC* only reduced the level of porin OmpH, whereas the deletion of *bamB* caused the reduction of more proteins like FecA, OprB and PlpD, but without significance (Klein *et al.,* 2019). However, the depletion of SurA altered the levels of 42 proteins, including TonB dependent receptors, siderophore receptors, autotransporters, BAM complex components and porins (Klein *et al.,* 2019). Many porins, important for the nutrient uptake, especially members of the OprD family (OpdO, OpdN, OpdP and OprD), were

reduced in the absence of SurA. Additionally, autotransporter proteins such as PlpD, AaaA, EprS or EstA showed reduced amounts or were absent upon SurA depletion. Thus, the decreased levels of OMPs in the conditional *surA* mutant can explain the higher permeability of the OM, demonstrated by the stronger fluorescence signal in the NPN assay and indicate that SurA in *Pa* seems to play the major role for OMP biogenesis like in *Ec* and *Ye* (Sklar, Wu & Kahne *et al.,* 2007; Weirich *et al.,* 2017; Klein *et al.,* 2019). Moreover, the induced changes of siderophore receptors (FpvA, FecA, FiuA) in the conditional *surA* mutant influenced the fitness of the bacteria, which could be demonstrated by monitoring the growth of the conditional *surA* mutant and the WT in the presence of the iron chelator 2,2'-Bipyridyl (BiP). The loss of SurA resulted in a significant BiP dose-dependent growth reduction compared to WT (Klein *et al.,* 2019) . This result completes previous findings on the siderophore receptor FiuA, which showed upon loss of the receptor significant pleiotropic effects like reduced virulence and decreased biofilm formation (Lee *et al.,* 2016). Additionally, the proteomics data revealed significantly reduced amounts of the LptD/E complex, which is important for the LPS assembly. Together with other proteins like OprD and PlpD, LptD could be confirmed as a true substrate of SurA (qRT-PCR) as already shown in *Ye* and *Ec* (Vertommen *et al.,* 2009; Weirich *et al.,* 2017). In addition, LO SCIUTO et al. could show that loss of LptE affects the functionality of LptD resulting in impaired cell envelope stability, reduced virulence and decreased antibiotic resistance (Lo Sciuto *et al.,* 2018). Thus, the inhibition of SurA affects several biological functions/pathways such as iron uptake and LPS transport and generally leads to reduced abundance of OMPs in the OM, probably caused by enhanced degradation of the proteins in the periplasm. Moreover, HlpA could not be identified as a substrate of SurA, assuming that HlpA might compensate to a certain level the loss of SurA (Klein *et al.,* 2019).

To further validate the proteomics data, SurA, OprD, and PlpD were additionally detected in PA14 WT and the mutant strains by Western Blot analysis of whole cell lysates (Klein *et al.,* 2019). In the absence of arabinose, no SurA could be detected indicating a functional depletion of SurA. In addition, the presence of arabinose partially recovered the SurA level to 64 % compared to WT. Moreover, the decreased amounts of OprD (15 %) and PlpD (24 %) in the absence of arabinose further confirmed the proteomics data of the conditional *surA* mutant (Klein *et al.,* 2019).

Next, all mutant strains were exposed to human serum to investigate if the OM composition influences the serum resistance. The treatment with 10 % human serum revealed that only the depletion of SurA had an impact on the serum resistance, since the conditional *surA* mutant

was immediately killed after exposure to human serum. More importantly, the mutant strains were investigated for their virulence using the *Galleria mellonella* infection model. The analysis demonstrated that only the depletion of SurA led to a significantly delay in time to death compared to the WT (Klein *et al.,* 2019). However, application of arabinose was not practicable in this experiment, so that the conditional *surA* mutant was grown in the presence (SurA +) or absence (SurA-) of arabinose just prior to infection. No significant difference between the survival curves were observed, indicating that without continuous addition of arabinose, the SurA production drops rather fast. Hence, it could not be shown if the conditional *surA* mutant regains its full virulence in the presence of arabinose. Despite that, it could be shown that SurA of *Pa* is crucial for virulence in *G. mellonellla* (Klein *et al.,* 2019). Alltogether, it can be assumed that the inhibition of SurA and the accompanying affected LPS transport and the reduced level of siderophore receptors are the main drivers causing the reduced resistance to complement mediated killing (Hancock 1983, Larmann 2012), reduced fitness under iron depleted conditions and the prolonged survival of the *G. mellonella* larvae (Lo Sciuto *et al.,* 2018).

Since the conditional *surA* mutant and the *bamB* mutant showed the most promising effects on OM integrity, it was analysed if the depletion of SurA or BamB affects the susceptibility to antibiotics. Additionally, a conditional *surA* mutant of a clinical MDR *Pa* bloodstream isolate ID72 was tested. The mutant strains and the PA14 and ID72 WT strains were subjected to E-test of penicillins, cephalosporins, carbapenems, fluoroquinolones and antibiotics which are not used against Gram-negative bacteria such as vancomycin (VAN). In the *bamB* deletion mutant the MIC values for ampicillin/sulbactam (SAM), ceftazidime (CAZ), fosfomycin (FOS) and VAN were at least 4-fold reduced and could be restored in the presence of arabinose, except for FOS (Klein *et al.,* 2019). However, the depletion of SurA in PA14 as well as in ID72 reduced the MIC at least 4-fold for ticaricillin/clavulanate (TIM), CAZ, levofloxacin (LEV), ciprofloxacin (CIP) and VAN. Depletion of SurA in PA14 additionally reduced the MIC for SAM and in ID72 additionally for FEP but not vice versa. More importantly, the SurA depletion in the MDR *Pa* strain ID72 resensitized the strain to treatment with the clinically relevant antibiotics FEP, CAZ and LEV, highlighting the crucial role of SurA for OM integrity (Klein *et al.,* 2019). Thus, the higher permeability of the OM might again be the reason for these effects.

Furthermore, due to the obtained effects and the fact that we did not succeed in generating an in frame deletion of *surA* and that transposon (Tn) libraries of different *Pa* strains did not detect a viable *surA* Tn mutant (Skurnik *et al.,* 2013; Lee *et al.,* 2015; Turner *et al.,* 2015), we

suggest that *surA* might be essential in *Pa* (Klein *et al.,* 2019). However, the Tn library of PA14 by LIBERATI et al. includes three Tn mutants of *surA* (Liberati *et al.,* 2006). The Tn insertion of mutant ID38436 starts already after 17 bps and the Tn mutant displayed similar characteristics as the conditional *surA* mutant and in addition SurA could not be detected by Western blot analysis (data not shown). We speculate that in this Tn mutant other mutations occurred which on the one hand compensated the loss of SurA to enable viable cells, but on the other hand still possessed defects in the OM, showing a similar phenotype (Klein *et al.,* 2019).

The less dramatic phenotype of the *bamB* mutant (reduced sensitivity to bile salts and higher susceptibility to antibiotics) matches to the minor changes in OMP composition given by the proteomics data (Klein *et al.,* 2019). Minor changes in the OMP composition were also detected in *bamB* mutants of *Ec*, *Ye and Kp* (Charlson *et al.,* 2006; Weirich *et al.,* 2017; Hsieh *et al.,* 2016). In all species, especially the number of porins and autotransporter proteins with large numbers of β-strands was affected by the deletion of *bamB*. In *Pa*, porins OpdO and OprB, as well as autotransporters like AaaA and PlpD were less abundant in the OM upon *bamB* deletion (Klein *et al.,* 2019). This might be reasoned in the observation that BamB is more important for the biogenesis of OMPs that have a large number of β-strands (Palomino *et al.,* 2011; Weirich *et al.,* 2017). However, the consequences on the phenotype strongly differ between the species. A recent study in *Kp* showed that the deletion of *bamB* caused a 15-fold reduction in adherence to and invasion to host cells and additionally was attenuated for virulence in a *Kp* model of bacteremia. Moreover, the *bamB* mutant in *Kp* displayed significantly decreased resistance to VAN and erythromycin (ERY) (Hsieh *et al.,* 2016). In *Ye*, loss of *bamB* also lead to attenuated virulence and reduced MICs for several antibiotics such as VAN. In addition, the *Ye bamB* mutant was sensitive against bile salts and complement activity (Weirich *et al.,* 2017). The latter and the attenuation of virulence could not be observed for the *bamB* mutant in *Pa* (Klein *et al.,* 2019). In contrast, another study could show that deletion of *bamB* in *Pa* PAO1 leads to sensitization against lysozyme and against VAN and cefotaxime (CTX) (Lee *et al.,* 2017). The sensitivity against the antibiotics could be confirmed by the constructed PA14 *bamB* mutant. However, the effects of the *bamB* deletion in *Pa* PA14 are not that promising and do not justify to consider BamB as a potential drug target, since the deletion has no impact on the virulence or serum resistance (Klein *et al.,* 2019). Deletion of *bamC* and the *skp* homolog *hlpA* showed neither distinct phenotypes nor attenuation of virulence in the *G. mellonella* infection model, so that they are not suitable as drug targets (Klein *et al.,* 2019).

In sum, the most profound changes in the OM composition were detected upon depletion of SurA, collectively causing a reduced fitness and consequently leading to reduced virulence in the *G. mellonella* infection model. This underlines the importance of SurA in OMP biogenesis and its suitable role as anti-infective drug target in order to reduce the spread of MDR *Pa*.

3.2 Identification of drug-resistance determinants in a clinical isolate of *Pseudomonas aeruginosa* by high-density transposon mutagenesis

The spread of antimicrobial resistance in Gram-negative pathogens, particularly for β-lactams, is rising and causes thousands of deaths due to nosocomial infections by pathogens such as *Pa* (Torrens *et al.,* 2017). Among its intrinsic and acquired resistance mechanism, the β-lactam resistance mediated by chromosomal β-lactamase AmpC is notable. The regulation of *ampC* is tightly linked to the PG recycling and therefore mutations of different PG recycling components such as the transcriptional regulator *ampR*, amidase *ampD* or *dacB*, encoding PBP4, can increase the expression levels of *ampC* and lead to clinical resistance to β-lactam antibiotics (Juan *et al.,* 2006; Moya *et al.,* 2009; Balasubramanian *et al.,* 2012). Moreover, the upregulation of β-lactamase AmpC can be triggered by the application of β-lactam antibiotics and β-lactamase inhibitors (Sanders & Sanders Jr, 1986).

However, the use of β-lactam-β-lactamase inhibitor combinations is one way to combat MDR *Pa* strains, but resistance against β-lactamase inhibitors have already been demonstrated and are increasing (Drawz & Bonomo, 2010). Consequently, new therapeutic solutions are needed to at least render the infections less harmful for the patient without blocking or reverse pathways leading to β-lactam resistance (Juan *et al.,* 2018). Hence, one strategy is to use a different class of antibiotic adjuvants, which do not act on primary resistance mechanisms, but on genes essential under low antibiotic concentrations as previously peformed by JANA et al. by subjecting *Kp* to colistin, CIP and IPM (Jana *et al.,* 2017). Therefore, Transposon-Directed Insertion Sequencing (TraDIS) of the clinical bloodstream isolate ID40 (Willmann *et al.,* 2018) was performed, previously subjected to subinhibitory concentrations of FEP and MEM, to identify the secondary resistome of *Pa* (Sonnabend *et al.,* accepted).

In a first step, to identify the secondary resistome of ID40, the WGS of ID40 was annotated and revealed a chromosome of 6.86 Mbp encoding 6409 open reading frames and the presence of a plasmid of 57446 bp containing 59 putative genes (https://www.ebi.ac.uk/ena; accession number PRJEB32702) (Sonnabend *et al.,* accepted). Analysis by the ResFinder (Zankari *et al.,* 2012) identified resistance genes against aminoglycosides (aph(3')-IIb (*neo*)), β-lactam antibiotics (blaOXA-486 (*bla*/*poxB*) and OXAPAO1 (*ampC*)), fluoroquinolones (crP (*crp*)) and fosfomycin (fosA (*fosA*_1)). Furthermore, a point mutation in *dacB* could be detected, which is known to be responsible for upregulating AmpC levels as mentioned above. Other gene mutations which could explain resistance mechanisms like mutations in *oprD* or genes encoding efflux pumps were not specifically taken into account, but sequence analysis of OprD did not point to a dysfunctional protein.

Thus, the different resistance phenotypes of ID40 and PA14, which shares the same resistance genes, but is sensitive to all β-lactams, may be explained by the mutation of *dacB* (Sonnabend *et al.,* accepted).

The constructed TraDIS library showed with 100000 Tn insertions across the genome a homogenous distribution and with on average 18 Tn insertions per 1 kbp of coding sequences a homogenous coverage of the whole genome. Furthermore, the Tn library of ID40 grown in LB, showed that 697 of 6468 genes were determined to be essential and only nine genes were determined to be ambiguous (Sonnabend *et al.,* accepted).

The exposure to subinhibitory concentrations of FEP and MEM, showed that under the defined criteria the read counts of 140 genes upon MEM treatment and 102 genes upon FEP treatment were significantly changed. In total, 24 genes were at least five-fold reduced and of these 13 genes upon both MEM and FEP treatment, 5 only upon MEM and 6 only upon FEP treatment (Sonnabend *et al.,* accepted). Most of the identified genes are involved in PG recycling such as *ampG* and *nagZ*, which contribute to β-lactam resistance (Asgarali *et al.,* 2009; Korfmann & Sanders, 1989; Zamorano *et al.,* 2011). The identified genes can be divided into three categories based on their localization or pathway, namely players in the periplasm, cytoplasm or players of the salvage pathway. Additionally, a category of uncharacterized players is presented. To validate the findings, in frame deletion mutants of selected genes were generated (*mltG*, *mepM1*, *amgK*, *ygfB*, *tuaC*, *ctpA* and *ctpA*/*mepM1*) and their susceptibility to β-lactam antibiotics using microbroth dilution assays was measured and their *ampC* activity was determined using a nitrocefin based assay as well as by measuring the mRNA quantity of *ampC* (Sonnabend *et al.,* accepted).

The cell wall recycling starts in the periplasm with three types of lytic enzymes that generate the PG muropeptides, which contribute to the transcriptional regulation of *ampC*. These are LTs such as MltG and Slt, LMM PBPs, EPs such as MepM1 and amidases such as AmpDh2 and AmpDh3. They altogether cleave the PG layer to facilitate the insertion of new murein chains and simultaneously release soluble PG fragments into the cytoplasm (Dhar *et al.,* 2018).

The antibiotic treatment of the ID40 Tn library caused the most profound effects on the LTs MltG and Slt. Slt was already shown to display decreased resistance against piperacillin (PIP) and cephalosporin antibiotics if inactivated (Cavallari *et al.,* 2013), and MltG was recently shown to be inhibited by the sulfonated glycopeptide bulgecin, causing decreased MICs against CAZ and MEM (Dik *et al.,* 2019). Additionally, the LTs MltF and MltD were identified to maintain resistance, but to a lesser extent than MltG and Slt. In contrast, SltB and

SltH were found to counteract the resistance. The findings of MltF and MltD confirmed previous studies which addressed theses LTs as possible drug targets to circumvent resistance (Lamers *et al.,* 2015; Cavallari *et al.,* 2013; Dik *et al.,* 2019; Sonnabend *et al.,* accepted). Furthermore, the generated *mltG* mutant showed reduced MIC values of all tested antibiotics and especially the MICs of FEP, PIP and PIP/tazobactam (TZP), IPM, CAZ and ATM were reduced below the breakpoint, rendering it to the best candidate for drug development. Moreover, its significantly reduced *ampC* expression and accompanying lower β-lactamase activity could underline the important role of MltG in *ampC* expression and PG recycling, since MltG acts as terminase to control PG chain length and thereby produces *ampC* inducing muropeptides (Yunck *et al.,* 2016). In sum, it can be concluded that LTs represent one of the most promising targets to resensitize MDR *Pa* strains to clinically relevant β-lactam antibiotics, as previously demonstrated by DIK and colleagues using the LT inhibitor bulgecin (Dik *et al.,* 2019; Sonnabend *et al.,* accepted).

The read counts of EP MepM1 (YebA, PA0667) were also reduced. MepM1 putatively modulates PG crosslinking and was found to be a substrate of the protease CtpA (Srivastava *et al.,* 2018). Other substrates of the cross-links regulating protease are MepM3 (PA4404/TUEID40_02316), MepH1 (PA1199/TUEID40_01414) and MepS/MepH2 (PA1198/TUEID40_01415). The read counts of the latter were also reduced upon FEP and MEM treatment, but not like MepM1. Furthermore, the EP MepM2/TUEID40_04881, which was not evidenced as CtpA substrate in PAK (Srivastava *et al.,* 2018), showed also reduced read counts in the screen, but only upon MEM treatment. Despite that, it might be involved in antibiotic resistance. However, EPs also take part in *ampC* induction. This study revealed for *mepM1* the highest reduction in Tn insertion read counts, while Tn insertions in MepS/MepH2 showed only a slightly impact on the growth in the presence of MEM. Thus, deletion mutants of *mepM1* and *ctpA* were generated to further analyse their role in *ampC* induction and antibiotic susceptibility. The microbroth dilution assays showed reduced MIC values of all tested β-lactam antibiotics in Δ*mepM1*, except for IPM and MEM. The MIC of ATM and FEP were additionally reduced below the breakpoint. In contrast, the MIC values of ATM, FEP, PIP and MEM were increased in Δ*ctpA*, causing hyperresistance. Likewise, loss of CtpA lead to elevated *ampC* expression and a higher β-lactamase activity. Thus, it is very likely that the increased activity of MepM1 or other CtpA substrates leads to increased resistance (Sonnabend *et al.,* accepted). Moreover, the deletion of *mepM1* in the *ctpA* deletion mutant showed reduced MIC values for ATM and PIP compared to Δ*ctpA*, but still higher MIC values in comparison to Δ*mepM1*. This indicates the contribution of other EPs in *ampC*

upregulation and may indicate that elevated MepM1 levels lead to stronger *ampC* expression. According to this, the inhibition of these EPs could be a possible strategy to break resistance against β-lactam antibiotics. Furthermore, the important role of CtpA in regulating EPs is emphasized by the hyperresistance of the mutant in ID40 (Sonnabend *et al.,* accepted) and its impact could further be supported by SANZ-GARCÍA et al., who demonstrated the occurrence of mutations in *ctpA* under ceftazidime-avibactam challenge and consequently increased resistance (Sanz-García *et al.,* 2018).

Upon transport into the cytoplasm by the permease AmpG and partly AmpP (Perley-Robertson *et al.,* 2016), the muropeptides are catalysed by several enzymes to form the PG precursor UDP-GlcNAcMurNAc-pentapeptide (Dhar *et al.,* 2018). In the first steps 1,6-anhydro-MurNAc-peptides, which bind to the transcriptional regulator AmpR to induce *ampC* expression, are degraded by LdcA, NagZ and AmpD. The amidase AmpD cleaves the peptide chains of the muropeptides generating 1,6-anhydro-MurNAc, which is subsequently directed to the salvage pathway to catalyse the formation of UDP-MurNAc by the enzymes AnmK, MupP, AmgK and MurU (Gisin *et al.,* 2013; Borisova *et al.,* 2014). After that, the Mur enzymes transform UDP-MurNAc to UDP-MurNAc-pentapeptide, which binds AmpR to repress *ampC* expression (Dhar *et al.,* 2018). The TraDIS experiment revealed increased susceptibility towards β-lactam antibiotics upon loss of *ampG* and *nagZ*, which has already been demonstrated to be reasoned in decreased amounts of 1,6-anhydroMurNAc-peptides (Asgarali *et al.,* 2009; Zamorano *et al.,* 2010). On the other hand, increased resistance is caused by the accumulation of 1,6-anhydroMurNAc-peptides upon loss of *ampD*, one of the most occurring mutations in MDR clinical *Pa* isolates (Jacobs *et al.,* 1995; Langaee *et al.,* 2000; Juan *et al.,* 2005; Sonnabend *et al.,* accepted).

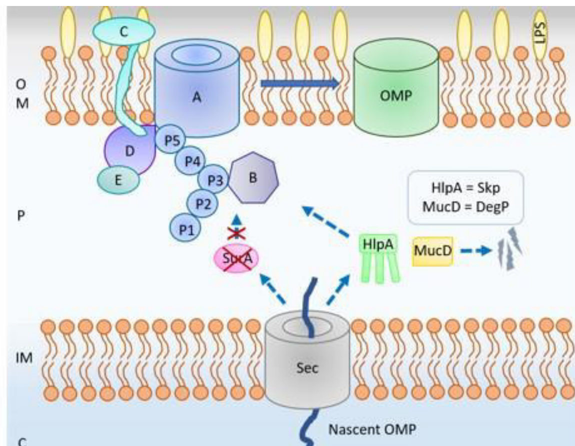
The four genes *anmK*, *amgK* , *mupP* and *murU* of the salvage pathway, which mediates intrinsic resistance to FOS (Gisin *et al.,* 2013), were identified in the TraDIS screen too.

All genes of the pathway have been shown to lead to increased resistance to CAZ and CTX upon inactivation (Fumeaux & Bernhardt, 2017). This effect could not be explained so far, but it is suggested that the reduction of the steady state level of *ampC* repressor UDP-MurNAc-pentapeptide creates this situation, so that 1,6-anhydro-MurNAc-peptides predominate and derepress *ampC* (Fumeaux & Bernhardt, 2017). In contrast, a study by BORISOVA et al. in *Pa* PAO1 (Borisova *et al.,* 2014), as well as the present results showed no increased resistance to CAZ and IPM in PAO1 Δ*amgK*. However, reduced MIC values for all β-lactam antibiotics except for MEM and MICs below the breakpoint for ATM, FEP and CAZ could be obtained in ID40 Δ*amgK*. Moreover, the role of *amgK* in FOS resistance could be confirmed and the

ID40 *amgK* mutant showed reduced *ampC* expression levels and reduced β-lactamase activity. At the moment this outcome seems to be counterintuitive and more detailed studies have to be done (Sonnabend *et al.,* accepted). However, it is possible that the anabolic recycling pathway somehow counteracts the derepression of *ampC* expression in the *dacB* background of ID40. This finding leads to the assumption that the salvage pathway may also play an important role in the maintenance of β-lactam resistance (Sonnabend *et al.,* accepted).
The TraDIS screening additionally identified uncharacterized genes. Namely, a member of the glycosyltransferase 1 family TUEID40_05543/*tuaC*, and TUEID40_03245 encoding an YgfB-like protein, which has not been characterized so far (Sonnabend *et al.,* accepted).
The deletion of *tuaC* displayed only minimal reduced MIC for TZP. Because of that we focused on TUEID40_03245, which shares similarity with the homologous gene *ygfB* in *Ec*. Hence, the unknown gene was termed *ygfB* and an in-frame deletion mutant was generated. The deletion of *ygfB* caused downregulated *ampC* mRNA levels and reduced β-lactamase activity resulting in reduced MICs for all tested β-lactam antibiotics and for ATM and FEP, below the respective breakpoint concentrations. To our knowledge, *ygfB* has not been mentioned so far in the context of antibiotic resistance, rendering it to an interesting candidate of drug development (Sonnabend *et al.,* accepted). However, *ygfB* is located in an operon consisting of *pepP*; *ubiH,* PA14_68970 orthologue and *ubiI*. *ubiH* and *ubiI* are important for ubiquinone biosynthesis and *pepP* encodes an aminopeptidase. Such operons can be found in the same order in *Ec*, *Acinetobacter baumannii* (*Ab*) and *Legionella pneumophila* (*Lp*), whereas YgfB of *Pa* shares 33 % identity with *Ec* and *Ab* YgfB and 32 % with YgfB of *Lp* (Sonnabend *et al.,* accepted). Interestingly, studies of Tn mutants in *Pa* PAO1 indicated a contributing role of YgfB for virulence in *C. elegans* (Feinbaum *et al.,* 2012) and additionally TraDIS experiments in *Pa* PA14 by SKURNIK et al. revealed a potential role of *ygfB* orthologue PA14_69010 in effective colonization in the caecum of mice (Skurnik *et al.,* 2013). Thus, YgfB possibly effects virulence and is able to modulate antibiotic resistance, rendering it to a promising target of antibiotic adjuvants which might reduce antibiotic resistance and virulence at the same time (Sonnabend *et al.,* accepted). Noteworthy, *pepP* was also identified in this approach, but lead to the suggestion that the inactivation of *pepP* might provoke hyperresistance (Sonnabend *et al.,* accepted).
To sum up, the performed TraDIS approach revealed a set of non-essential genes which are highly relevant for the induction of AmpC production and consequently β-lactam resistance. To our point of view, these genes are promising targets for anti-infective drugs to reverse multidrug resistance in *Pa* strains with high AmpC activity. Especially, the function of YgfB

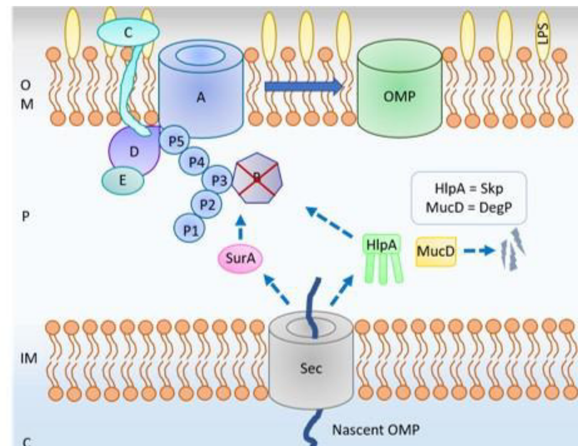
and its specific role in antibiotic resistance and virulence will be further investigated (Sonnabend *et al.,* accepted).

4 Graphic Summary



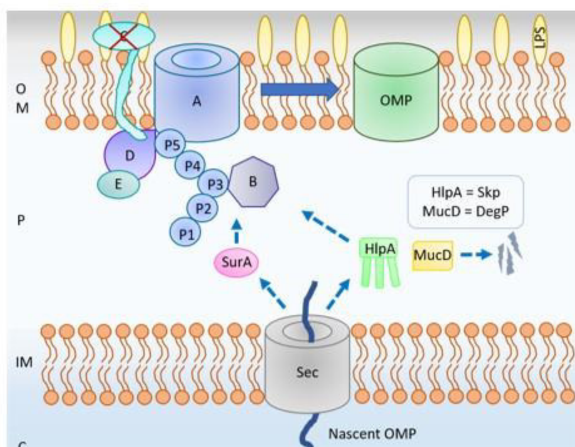
(A) Upon depletion of SurA:

- Drastically altered OM composition
- Increased membrane permeability and morphological changes of the OM
- Increased susceptibility to killing by complement system
- Enhanced sensitivity to antibiotic treatment
- Attenuated virulence in a *G. mellonella* infection model



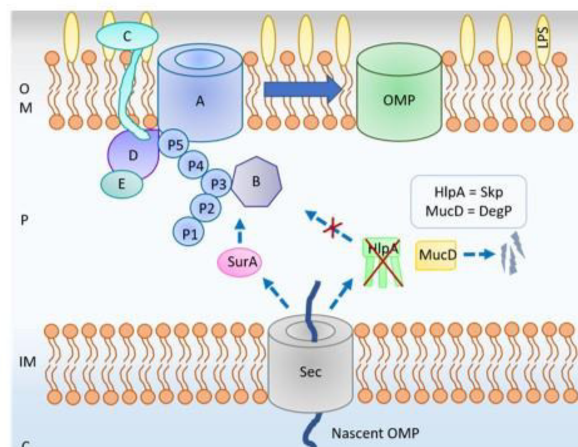
(B) Upon depletion of BamB:

- Altered OM composition
- Increased membrane permeability and morphological changes of the OM
- Enhanced sensitivity to antibiotic treatment



(C) Upon depletion of BamC:

- Only minor changes in OM composition
- No other obvious phenotypes



(D) Upon depletion of HlpA:

- No changes in OM composition
- No other obvious phenotypes

Figure 1. **Biogenesis of β-barrel outer membrane proteins in Gram-negative bacteria and effects upon loss of SurA, BamB, BamC or HlpA**. Nascent β-barrel outer membrane proteins (OMPs) are synthesized in the cytoplasm (C) and transported across the inner membrane (IM) into the periplasm by the Sec translocon. Subsequently, chaperones SurA or

HlpA (Skp in *Ec*) bind the OMPs and guide them through the periplasm (P) to the BAM complex, comprising BamA with POTRA domains P1-P5 and BamB-D, located in the outer membrane (OM). The BAM machinery then folds and inserts the OMPs into the OM. Misfolded OMPs or OMPs that fall off the SurA pathway are degraded by MucD (DegP in *Ec*). **(A)** Drastic effects upon SurA depletion; **(B)** Effects upon BamB depletion; **(C)** Minor effects upon BamC depletion; **(D)** No effects upon HlpA depletion.

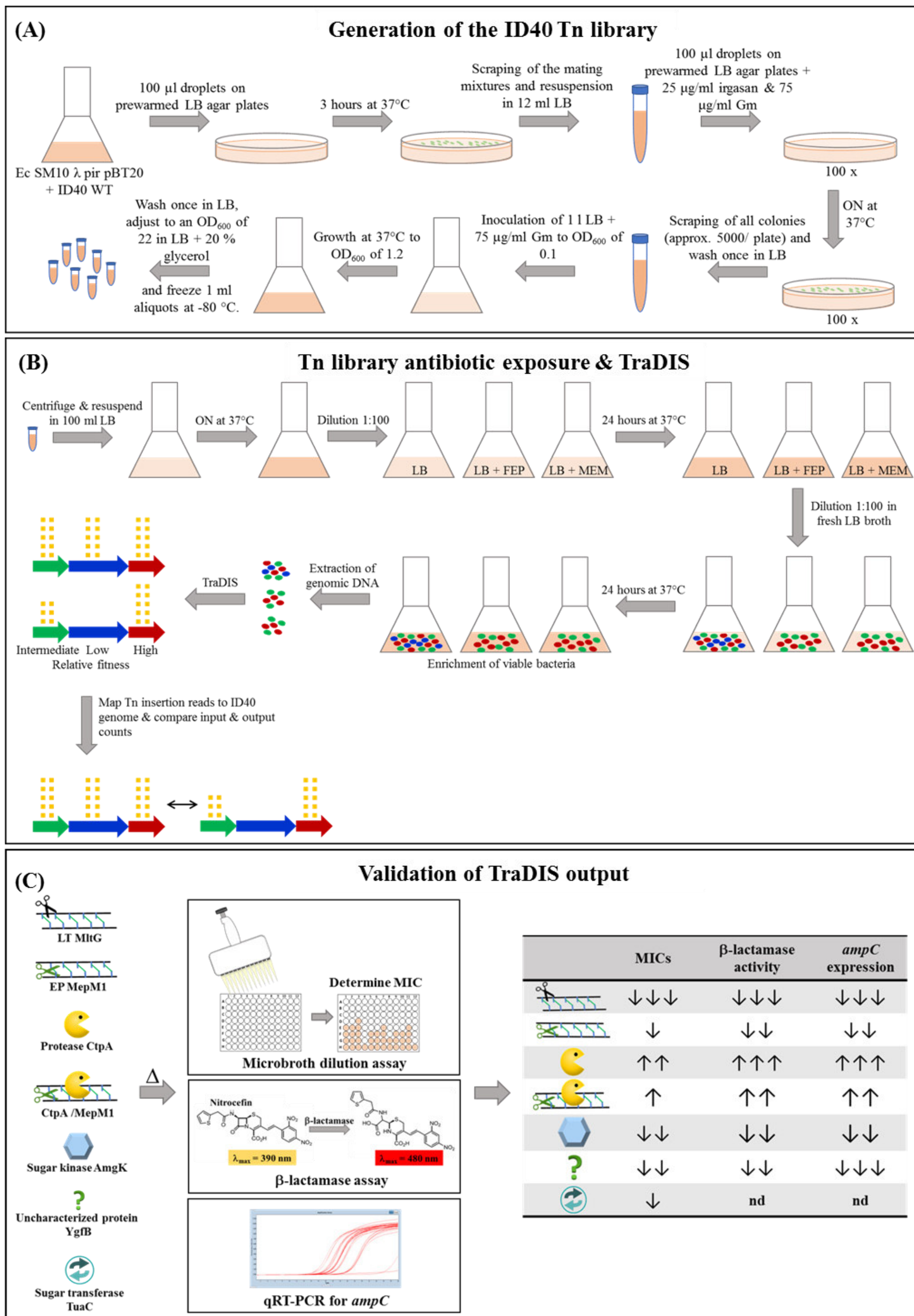


Figure 2. **TraDIS approach and validation**. (**A**) Diagram of the experimental procedure to generate a Tn library in *Pa* ID40. (**B**) Growth of the ID40 Tn library under different

conditions (LB/ LB + FEP or LB + MEM) following extraction of genomic DNA and TraDIS. (**C**) Validation of TraDIS output by generating deletion mutants (Δ*mltG*, Δ*mepM1*, Δ*ctpA*, Δ*ctpA*Δ*mepM1*, Δ*amgK*, Δ*ygfB* and Δ*tuaC*) and analysis by microbroth dilution assays and measurement of β-lactamase activity (Nitrocefin- based assay) and *ampC* expression (qRT-PCR). ↓ indicates reduction and ↑ indicates increase; the number of arrows illustrates the strength of the reduction or increase; nd, not detected.

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6 Appendix

Accepted manuscripts

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Deprivation of the Periplasmic Chaperone SurA Reduces Virulence and Restores Antibiotic Susceptibility of Multidrug-Resistant *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is one of the main causative agents of nosocomial infections and the spread of multidrug-resistant strains is rising. Therefore, novel strategies for therapy are urgently required. The outer membrane composition of Gram-negative pathogens and especially of *Pa* restricts the efficacy of antibiotic entry into the cell and determines virulence. For efficient outer membrane protein biogenesis, the β-barrel assembly machinery (BAM) complex in the outer membrane and periplasmic chaperones like Skp and SurA are crucial. Previous studies indicated that the importance of individual proteins involved in outer membrane protein biogenesis may vary between different Gram-negative species. In addition, since multidrug-resistant *Pa* strains pose a serious global threat, the interference with both virulence and antibiotic resistance by disturbing outer membrane protein biogenesis might be a new strategy to cope with this challenge. Therefore, deletion mutants of the non-essential BAM complex components *bamB* and *bamC*, of the *skp* homolog *hlpA* as well as a conditional mutant of *surA* were investigated. The most profound effects for both traits were associated with reduced levels of SurA, characterized by increased membrane permeability, enhanced sensitivity to antibiotic treatment and attenuation of virulence in a *Galleria mellonella* infection model. Strikingly, the depletion of SurA in a multidrug-resistant clinical bloodstream isolate re-sensitized the strain to antibiotic treatment. From our data we conclude that SurA of *Pa* serves as a promising target for developing a drug that shows antiinfective activity and re-sensitizes multidrug-resistant strains to antibiotics.

Keywords: SurA, *Pseudomonas aeruginosa*, virulence, multidrug resistance, antibiotics, outer membrane protein biogenesis

INTRODUCTION

The widespread use of antibiotics is causative for the rapid development of multidrug-resistant strains. Particularly, the emergence of carbapenem-resistant bacteria poses a significant threat to public health (Pendleton et al., 2013). The Gram-negative, opportunistic pathogen *Pseudomonas aeruginosa* (*Pa)* belongs to the so-called ESKAPE group, comprising a group of the most common and multidrug-resistant bacteria (Rice, 2008). *Pa* can cause infections in a wide range of animal and plant hosts and is a leading cause of nosocomial infections, which are almost exclusively found in immunocompromised hosts (Lyczak et al., 2000; Lister et al., 2009). *Pa* displays numerous intrinsic and acquired resistance mechanism against antibiotics: (i) enzymatic and mutational resistance mechanisms like the production of β-lactamases, (ii) overexpression of efflux systems, and (iii) the low permeability of the outer membrane (OM) that limits the penetration of antibiotic molecules (Yoshimura and Nikaido, 1982).

The major challenge for drugs against *Pa* and Gram-negative bacteria in general is to pass the bacterial OM. The OM provides a highly effective barrier against foreign and harmful molecules, allows import and export of essential substances such as nutrients and iron, is necessary for communication and harbors many virulence factors. The outer leaflet of the OM is constituted mainly by lipopolysaccharides (LPS), whereas the inner leaflet consists of phospholipids. This bilayer houses a great variety of outer membrane proteins (OMPs) that facilitate transport and other essential functions, and act as virulence factors (Nikaido, 2003). Many OMPs are porins and autotransporters. Both comprise a β-barrel domain and either facilitate transport of molecules across the OM (Chevalier et al., 2017) or can form cell surface exposed moieties that shape the interaction with the host and the extracellular environment (Leyton et al., 2012). For the insertion of these β-barrel proteins, Gram-negative bacteria employ a conserved transport system consisting of the periplasmic chaperones SurA, Skp, and DegP, which protect and guide newly synthesized proteins from the Sec translocon in the inner membrane to the OM and the β-barrel assembly machinery (BAM) complex (Sklar et al., 2007; Tashiro et al., 2009; Goemans et al., 2014; Li et al., 2018). Both SurA and Skp act as chaperones and are thought to form a partially redundant network. The importance of SurA and Skp for the OMP biogenesis is controversially discussed. At least in *Escherichia coli (Ec)* and *Yersinia enterocolitica (Ye)*, SurA plays the major and Skp a less prominent role in folding and assembly of OMPs (Sklar et al., 2007; Volokhina et al., 2011; Weirich et al., 2017). However, in *Neisseria mengitidis*, Skp is more important for shaping the OMP composition than SurA, indicating species-specific differences (Tamae et al., 2008).

The BAM complex, which inserts the β-barrel proteins into the OM, consists of the central component BamA and the four lipoproteins BamB, BamC, BamD, and BamE (Noinaj et al., 2017). Of these subunits, only BamA and BamD are essential in most of the so far investigated Gram-negative bacteria, except *Borrelia burgdorferi* and *Salmonella enterica* (Wu et al., 2005; Malinverni et al., 2006; Fardini et al., 2009; Dunn et al., 2015). BamA is a β-barrel protein itself (Noinaj et al., 2017). Its C-terminal β-barrel domain is connected to an N-terminal periplasmic domain which consists of five polypeptide transport-associated (POTRA) domains. The POTRA domains form several interactions with the other Bam subunits, building up the BAM complex and interact with both substrates and periplasmic chaperones such as SurA (Gu et al., 2016). BamB directly binds to the POTRA domains 2-5 of BamA and supports the stabilization of nascent OMPs by binding and delivering OMP β-strands to BamA (Heuck et al., 2011).

In *Ec*, the deletion of one of the non-essential BAM complex components or the related periplasmic shuttle protein SurA may lead to an altered protein composition in the OM and/or disturbed OM integrity and therefore to a higher susceptibility to various antibiotics (Behrens et al., 2001; Onufryk et al., 2005). Using *Ye* we have previously shown that the deletion of *surA* and *bamB* implies a significantly decreased virulence and more efficient clearance of *Ye* infection by the host *in vivo* (Weirich et al., 2017).

In *Pa,* BamA, and the BamE-homolog OmlA have already been recognized to play a role in the stability of the OM and susceptibility to environmental stress (Ochsner et al., 1999; Yorgey et al., 2001; Hoang et al., 2011). For BamB of *Pa*, an enhanced susceptibility against lysozyme and cell wall targeting antibiotics as well as a decreased growth *in vivo* have been demonstrated recently (Lee et al., 2017).

Thus, it is well recognized that the BAM complex itself as well as chaperones in delivering proteins to the outer membrane are critical for membrane integrity as well as antibiotic resistance and could therefore be targets for drug development (Tamae et al., 2008; Weirich et al., 2017; Storek et al., 2018; Vij et al., 2018). Nevertheless, previous studies revealed species-specific differences in the importance of individual components in OMP biogenesis such as Skp and SurA (Sklar et al., 2007; Volokhina et al., 2011; Weirich et al., 2017). In addition, for considering such proteins as targets for *Pa* it would be mandatory to affect multidrug-resistant strains and break resistance against commonly used antibiotics.

To identify potential targets in order to possibly develop new strategies to treat especially infections caused by multidrug-resistant *Pa*, we investigated the role of components involved in the assembly of proteins into the OM by deletion of the non-essential BAM complex components BamB and a BamC homolog as well as the periplasmic shuttle proteins SurA and HlpA (a Skp-like protein) in *Pa* PA14. Depletion of SurA had the greatest impact on OM integrity and caused profound changes in the protein composition of the OM. These changes broadened the spectrum of antibiotics that could be used for treatment of *Pa* infection, and they lowered the minimum inhibitory concentration of clinically important antibiotics. Additionally, depletion of SurA enhanced clearance of *Pa* infection by the host. Taken together, our findings indicate that specifically SurA could serve as a novel antivirulence and/or resistance-breaking target even in multidrug-resistant strains of *Pa*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bacterial strains and plasmids used in this study are listed in **Table S1**. Bacteria were cultivated overnight at 37°C with shaking at 200 rpm in lysogeny broth (LB) containing suitable antibiotics but without any additives such as arabinose, if not otherwise stated. Antibiotics were added at the following concentrations: Tetracycline (Tet; AppliChem #A2228) 15 μg/ml, ampicillin (Amp; AppliChem #A0839) 100 μg/ml and gentamicin (Gm; AppliChem #A1492) 15 μg/ml (*Ec* strains) or 75 μg/ml for Gm and 50 μg/ml for Tet (*Pa* strains). If not stated otherwise, overnight cultures were diluted 1:20 into fresh LB medium containing suitable antibiotics (and/or additives like 0.2% arabinose (Sigma Aldrich #A3256) for the conditional *surA* mutant) and grown for 3 h at 37°C and 200 rpm to obtain subcultures in exponential phase ($OD_{600} = 0.5$). The growth of bacteria in LB at 37°C in a 24-well-plate was measured using Tecan Infinite® 200 PRO at 37°C. To investigate the growth under iron-restricted conditions, indicated concentrations of 2,2′-Bipyridyl (Sigma Aldrich #D216305) were added.

Generation of In-frame Deletion Mutants

In-frame deletion mutants were generated using the suicide plasmid pEXG2 (Rietsch et al., 2005). The primers used in this study are listed in **Table S2**. First, the flanking regions (consisting of 30 bp at the 3′ end and 30 bp at the 5′ end of the gene of interest plus ∼ 800 bp for each flanking region) and a pEXG2 fragment were amplified by PCR and ligated using Gibson assembly (Gibson, 2009). In general, constructed plasmids were verified by DNA sequencing, transformed into *Ec* SM10 λ *pir* and subsequently mobilized by conjugation into PA14. Merodiploids were selected on LB agar plates containing irgasan (25 μg/ml; Sigma Aldrich #72779) and Gm (75 μg/ml). To achieve the second cross-over, counter selection on no-salt lysogeny broth (NSLB) agar containing 15% sucrose was performed (Sigma Aldrich #S7903). Finally, the loss of the plasmid was tested by streaking colonies on LB agar plates containing Gm (75 μg/ml) and in parallel on LB agar plates without antibiotics. In-frame deletion mutants were confirmed by PCR using (i) a primer pair flanking the target gene and (ii) a primer pair where one primer binds to the coding region of the target gene.

Generation of Conditional Depletion Mutants

As stated also in the results section, we were not able to create an in-frame *surA* deletion mutant. Therefore, a conditional mutant was generated, starting from a merodiploid PA14::pEXG2-*surA* clone. For the integration of exogenous *surA*, the plasmid mini-CTX1-*araC*P_{BAD}-*surA* (PA14) was constructed. The mini-CTX1 (Hoang et al., 2000) is an optimized self-proficient integration vector for *Pa* containing a φCTX attachment site for integration of foreign genes into the chromosome. The coding sequence (cds) of *tolB* of the vector mini-CTX1-*araC*P_{BAD}-*tolB* (Lo Sciuto et al., 2014) was replaced by the cds of *surA* using PCR amplification and Gibson assembly. The mini-CTX1-*araC*P_{BAD}-*surA* construct was integrated into the *attB* neutral site of the chromosome of PA14::pEXG2-*surA* as described recently (Hoang et al., 2000; Lo Sciuto et al., 2014) in the presence of Tet (50 μg/ml), Gm (100 μg/ml) and arabinose (0.2%). Afterwards, the endogenous copy of the *surA* gene was deleted in-frame under SurA-inducing conditions and confirmed as described above. Excision of the mini-CTX1 backbone containing the Tet resistance cassette was performed using Flp recombinase as described (Hoang et al., 2000) and verified by PCR. Likewise, a conditional *surA* mutant of the clinical *Pa* isolate ID72 was generated, using mini-CTX1-*araC*P_{BAD}-*surA* and the mutator plasmid pEXG2-*surA* ID72. For the complementation of *bamB*, the mini-CTX1 vector was used to introduce an arabinose-inducible copy of *bamB* into the genome of PA14 *bam*B as described for *surA*.

Electron Microscopy

A total of 5 x 10^9 bacteria were harvested and fixed in Karnovsky's fixative, embedded in agarose, cut in small blocks and fixed again in Karnovsky's fixative. After post-fixation and embedding in glycid ether, blocks were cut using an ultramicrotome. Sections (30 nm) were mounted on copper grids and analyzed using a Zeiss LIBRA transmission electron microscope.

Generation of Overexpression Plasmids for Protein Purification

The cds of PA14 *surA* was subcloned into the vector pTXB1, resulting in pTXB1-*surA*-Intein. pET28a-*bamB*-His_6 was generated by Genscript Inc. Both plasmids were transformed into *Ec* BL21 (DE3) (Invitrogen #C600003). The sequence encoding full-length *plpD* from *Pa* PAO1 was synthesized with *Ec* codon optimization (ThermoFisher Scientific). The region coding for the passenger and the POTRA domain (residues 18-406) were subcloned into the expression vector pET28a+ (Novagen #69864) using Gibson assembly with mutations leading to an inactive lipase and encoding a C-terminal hexa-histidine tag resulting in pET28a-*plpD* S60A/D207N-His (Liu and Naismith, 2008).

Protein Purification and Generation of Polyclonal Antibodies

For purification of SurA, *Ec* BL21 (DE3) harboring pTXB1-*surA*-Intein was grown to an OD_{600} of 0.4, induced by the addition of 100 μM IPTG (Peqlab #37-2020) and grown for another 4 h at 37°C. Protein purification was performed using the IMPACT™ kit (New England Biolab #E6901S) according to the manufacturer's instructions with subsequent size-exclusion chromatography on a HiLoad™ 16/600 Superdex™ 200 pg column (GE Lifesciences). Fractions containing purified SurA were pooled, concentrated and validated by SDS-PAGE. For purification of BamB, *Ec* BL21 (DE3) harboring pTXB1-*bamB*-His_6 were grown to an OD_{600} of 0.6, induced by the addition of 100 μM IPTG and grown overnight at 37°C. Bacteria were pelleted and resuspended in buffer A [40 mM HEPES (Carl Roth #9105.4), pH 7.4; 150 mM NaCl (VWR Chemicals #27810.295)] following an incubation under stirring for 20 min at 4°C with 10 mM $MgSO_4$ (AppliChem #A6414), 20 mg/ml lysozyme (Sigma Aldrich #6876), protease inhibitor tablets

(Sigma Aldrich #S8830) and a pinch of DNase (Sigma Aldrich #DN25). Subsequently, bacteria were lysed using a French pressure cell, followed by sequential centrifugation steps at 4°C (4,500 × *g*, 15 min; 20,000 × *g*, 20 min; 40,000 × *g*, 1 h). Finally, the sterile-filtered (0.2 μm filter, Sarstedt) His_6-tagged protein was subjected to metal affinity chromatography (HisTrap^{TM} HP, 5 ml, GE Life Sciences) and concentrated. Antibodies were raised in 2 rabbits each for SurA or BamB-His_6 and subsequently affinity-purified against purified SurA or BamB protein, respectively (Eurogentec).

For purification of PlpD lipase + POTRA domains, *Ec* BL21 Gold (DE3) cells (Agilent Technologies #230132) harboring pET28a-*plpD* S60A/D207N-His were grown in autoinducing ZYP-5052 medium (Studier, 2005) at 30°C, harvested 24 h post-inoculation by centrifugation and resuspended in running buffer containing 40 mM sodium phosphate (Carl Roth #K300.1), 400 mM NaCl and 20 mM imidazole, pH 8.0 (AppliChem #A1073). For lysis, additional EDTA-free protease inhibitor, 1 mM $MgCl_2$ (Sigma Aldrich #M8266), 1 mM $MnCl_2$ (Merck #8059300100), 0.1 mg/ml lysozyme and a pinch of DNase were added to the buffer before application to a French pressure cell. After centrifugation at 20,000 × *g* and 4°C for 35 min, the sterile-filtered supernatant containing the His_6-tagged protein was applied to a HisTrap^{Tm} FF column (GE Healthcare) and purified on an NGC Chromatography System (Bio-Rad). The protein was eluted from the column using a gradient of imidazole (to 0.5 M) and further purified on a HiPrep 26/60 Sephacryl S200 HR size exclusion column (GE Healthcare, USA) using 20 mM Tris and 300 mM NaCl at pH 7.5. The production of antibodies was performed at the Section for Experimental Biomedicine (University of Life Sciences, Oslo, Norway) with license of the Norwegian Animal Research Authority (NARA) (http://www.mattilsynet.no/dyr_og_dyrehold/dyrevelferd/forsoksdyr/).

NPN Assay

To determine changes in the OM permeability of the generated mutants, the fluorescent, hydrophobic 1-N-phenylnaphthylamine (NPN) (Acros organics #90-30-2) was used as described (Konovalova et al., 2016). Subcultured bacteria were washed and adjusted to an OD_{600} of 0.5 in 5 mM HEPES buffer (pH 7.2). NPN was added to the bacteria to a final concentration of 10 μM. 200 μl of the bacterial suspension were transferred to 96-well F-bottom, black, non-binding plates (Greiner Bio-one #89089-582). Subsequently, fluorescence (excitation and emission wavelengths 350 and 420 nm, respectively) was measured using the Tecan Infinite® 200 PRO. Polymyxin B (PMB, Merck #A 231-40) served as a positive control and was added to a final concentration of 8 μg/ml. Values obtained for a buffer-only control were subtracted from all values.

Bile Salt Assay

To analyze the sensitivity to bile salts, 10^7 bacteria per well were inoculated in duplicates into a 24 well microtiter plate containing either 1 ml LB or 1 ml LB + 0.3 % bile salts (Sigma Aldrich #B8756). The conditional *surA* mutant was additionally supplemented with 0.2 % arabinose. The plate was incubated at 37°C and shaking at 160 rpm for 8 h and OD_{600} was determined using the Tecan Infinite® 200 PRO.

Western Blot Analysis

5×10^8 bacteria per ml of subcultures grown for 3 h were boiled in 2.5 × Laemmli buffer (Bio-Rad #161-0747) containing 50 mM DTT (Thermo Fisher Scientific #R0861) at 95°C for 10 min. SDS-PAGE was performed with 5×10^6 bacteria per lane using a 10 % Mini-PROTEAN® TGX^{TM} Precast Protein gel (Bio-Rad). Subsequently, proteins were transferred to a nitrocellulose membrane. After blocking in 5% skim milk in TBS (10 mM Tris-HCL (Sigma #T1503), 150 mM NaCl; pH 7.6), the membrane was incubated with the primary antibody [rabbit anti-SurA, 1:200; rabbit anti-BamB-His_6, 1:200; rabbit anti-OprD (kindly provided by Thilo Köhler, University of Geneva; Epp et al., 2001), 1:2,000; rabbit anti-PlpD serum 1:10,000; rabbit anti-RpoB (*Ec*), 1:2,000 (Abcam #mAb EPR18704)] and afterwards with the secondary antibody (horseradish-peroxidase-conjugated goat anti-rabbit antibody 1:5,000, Thermo Fisher Scientific #31460). Clarity^{TM} Western ECL Substrate (Bio-Rad #170-5061) was added and signals were detected using a Fusion Solo S imager (Vilber). Protein bands were quantified via ImageJ. In contrast to SurA and OprD, where RpoB was used as a loading control for quantification, for PlpD the unspecific band of ∼75 kDa served as a loading control.

Enrichment of OM Fractions

Preparation of the OM was conducted as described (Thein et al., 2010; Oberhettinger et al., 2015; Weirich et al., 2017). In short, PA14 strains including the conditional *surA* mutant were grown overnight in LB. Subcultures (1:20 dilution) were then grown in LB to an OD_{600} of 0.5–0.7. For complementation of the conditional *surA* mutant 0.2% arabinose was added in the subculture. After centrifugation, 2.5×10^{10} bacteria were resuspended in 0.5 ml of resuspension buffer (0.2 M Tris, 1 M sucrose, 1 mM EDTA (Applichem #A5097), pH 8.0), then 5,000 U lysozyme were added and incubated for 5 min at room temperature. Subsequently, 3.2 ml H_2O were added and incubated for 20 min at room temperature until spheroplasts were formed. Then, 5 ml of extraction buffer (2% Triton X-100 (AppliChem #A4975), 50 mM Tris, 10 mM $MgCl_2$, pH 8.0) together with 5 μl DNase I (Roche Applied Science #03539121103) were added and incubated on a rotator for 20 min at room temperature to solubilize the inner membrane fraction with Triton X-100 (Schnaitman, 1971; Page and Taylor, 1988). The lysate was centrifuged at 85,000 × *g* for 1 h at 4°C and the pellet containing the OM fraction was washed three times in 2.5 ml H_2O by centrifugation at 292,000 × *g* for 15 min at 4°C. The pellet containing the OM fraction was resuspended in 300 μl H_2O.

NanoLC-MS/MS Analysis and Data Processing

The protein concentration of the OM samples was measured using the Pierce^{TM} BCA Protein Assay Kit (Thermo Fisher Scientific #23225). 10 μg of each sample was subjected to SDS-PAGE and stained with Roti®-Blue Colloidal Coomassie Staining

Solution. OM fractions were analyzed as described previously (Weirich et al., 2017) with slight modification: Coomassie-stained gel pieces were digested in-gel with trypsin (Borchert et al., 2010), and desalted peptide mixtures (Rappsilber et al., 2007) were separated on an Easy-nLC 1200 (Thermo Scientific) system coupled to an LTQ Orbitrap Elite mass spectrometer (Thermo Scientific). The peptide mixtures were injected onto the column in HPLC solvent A (0.1% formic acid) at a flow rate of 500 nl/min and subsequently eluted with an 127 min segmented gradient of 5-33-50-90% of HPLC solvent B (80% acetonitrile in 0.1% formic acid) at a flow rate of 200 nl/min. The mass spectrometer was operated in positive ion mode, and spectra were recorded in a mass range from m/z 300 to 2000 with a resolution of 120,000. The 15 most intense ions were sequentially isolated and fragmented in the linear ion trap using collision-induced dissociation (CID) and default CID settings. The target values for MS scans and MS/ MS fragmentation were 10^6 and 5,000 charges, respectively. Sequenced precursor masses were excluded from further selection for 60 s.

Acquired MS spectra were processed with MaxQuant software package version 1.5.2.8 (Cox and Mann, 2008) with integrated Andromeda search engine (Elias and Gygi, 2007). Database search was performed against a target-decoy *Pa* UCBPP-PA14 database obtained from Uniprot, containing 5886 protein entries, and 285 commonly observed contaminants. Endoprotease trypsin was defined as protease with a maximum of two missed cleavages. Oxidation of methionine and N-terminal acetylation were specified as variable modifications, and carbamidomethylation on cysteine was set as fixed modification. Initial maximum allowed mass tolerance was set to 4.5 ppm (for the survey scan) and 0.5 Da for CID fragment ions. Peptide, protein and modification site identifications were reported at a false discovery rate (FDR) of 0.01, estimated by the target/decoy approach (Elias and Gygi, 2007). The label-free algorithm was enabled, as was the "match between runs" option (Luber et al., 2010). The detection limit was calculated as the mean of the lowest label-free quantification (LFQ) values of each sample. Multiple *t*-tests were performed and FDR of differences in the $\log_2$ protein amount between mutant and wild type (WT) were assessed using the two-stage step-up method (Benjamini et al., 2006) with GraphPad Prism 7.04 software. Differences in protein amount with a FDR < 0.1 were considered significant.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD011849 (Username: reviewer54276@ebi.ac.uk, Password: i3rXLDrr).

RNA Isolation and qRT-PCR

5×10^9 bacteria grown as described for the mass spectrometry analyses were resuspended in 1 ml TRIzol™ Reagent (Thermo Fisher Scientific #15596018). RNA isolation and DNase digestion were conducted as described previously (Goerke et al., 2000; Münzenmayer et al., 2016). The RNA (0.1 µg/µl in RNA storage solution, Invitrogen #AM7000) was diluted 1:10 with RNase-free water (Ambion #AM9937). To exclude samples with detectable DNA contamination, a quantitative PCR using the QuantiFast SYBR Green PCR Kit (Qiagen # 204054) for the house keeping gene *gyrB* was performed. mRNA expression was assessed by quantitative RT-PCR using the QuantiFast SYBR Green qRT-PCR Kit (Qiagen # 204154) according to the manufacturer. A standard curve was generated by a serial dilution of one sample. Efficiency of the PCR and Cp values were calculated with the help of LightCycler480 software (Roche). Relative quantification was conducted as described by Pfaffl (Pfaffl, 2001). The used primers are listed in **Table S2**.

Serum Killing Assay

A serum killing assay was performed using the BacTiter-Glo™ Microbial Cell Viability Assay (Promega) as described (Necchi et al., 2017) with slight modifications. Normal human serum (NHS) from healthy donors (Transfusion medicine, University hospital Tübingen) was stored in aliquots at −80°C. Heat inactivated serum (HIS) was generated by incubating the serum at 56°C for 30 min immediately before use. 5×10^6 bacteria were incubated at 37°C in 100 µl 10% HIS- or 10% NHS-PBS in a 96 well V-bottom microtiter plate (Greiner bio-one #651101) in triplicates for various time periods. After that, plates were centrifuged at 3,500 × *g* for 5 min and the pelleted bacteria were resuspended in 100 µl PBS (Gibco™ #14040-091). To determine the number of viable bacterial cells, 50 µl bacterial suspension and 50 µl BacTiter-Glo™ reagent (Promega #G8321) were transferred to a white lumitrac 96 well F-bottom microtiter plate (Greiner bio-one #655075) and the ATP levels inside the bacteria were quantified with a Tecan Infinite® 200 PRO.

Galleria mellonella Infection Model

Galleria mellonella (TruLarv™) larvae were purchased from Biosystems Technology. Subcultured bacteria were serially diluted to 10^3/ml in PBS. Each *G. mellonella* larva was injected with 10 µl of 10^3/ml bacterial dilution using a 30 gauge syringe (BD Biosciences). The larvae were then incubated at 37°C and monitored for 3 days after infection. Larvae were considered dead when no movement could be triggered by touching the larvae with a forceps. Ten microliter aliquots of the bacterial dilutions injected into the larvae were plated in triplicates on LB agar plates and the CFU was determined. The mean administered bacterial dose for all experiments was 12 ± 2 bacteria.

Antibiotic Susceptibility Testing

For determination of antibiotic susceptibility, bacterial strains were grown at 37°C overnight. Physiological sodium chloride solution was inoculated to a McFarland standard of 0.5. From this solution, bacteria were streaked with cotton swabs onto Mueller-Hinton agar plates with or without 0.2 % arabinose. E-tests (Liofilchem) were conducted according to CLSI standard protocols to test the sensitivity of the different strains for the following antibiotics: ampicillin/sulbactam (#92070); piperacillin/ tazobactam (#92108); ticarcillin/ clavulanic acid (#921171); doripenem (#92040); meropenem (#920840); cefotaxime (#920061); cefepime (#921271); ceftazidime (#921380); levofloxacin (#92081); ciprofloxacin (#920450); fosfomycin (#920790); vancomycin (#920570); erythromycin (#92051); trimethoprim/ sulfamethoxazole (#921231).

Statistics

Statistics were performed using GraphPad Prism 7.04 software as described for each experiment in the table or figure legends.

RESULTS

Generation of *Pa* Strains Carrying Deletions for BAM Complex Components and Periplasmic Chaperones

The BAM complex and associated chaperones may be interesting targets for developing novel drugs against Gram-negative bacteria. Their inhibition could possibly re-sensitize Gram-negative pathogens to antibiotics to which they are resistant or enable the use of antibiotics typically not being able to cross the OM barrier and thus not applicable for treatment of infection with Gram-negative pathogens (e.g., vancomycin) (Sydenham et al., 2000; Rolhion et al., 2005; Fardini et al., 2009; Weirich et al., 2017). Because of the clinical importance and increasing numbers of multidrug-resistant strains we addressed the role of *Pa* BamB (PA14_14910), BamC (PA14_51260), the Skp-like protein HlpA (PA14_17170), and SurA (PA14_07760) for fitness and virulence of *Pa* in order to determine which factors might be the best targets for drug development. For this purpose we generated single gene deletions, which were verified by PCR using genomic DNA as template. Mass spectrometry analyses of OM fractions (typically highly contaminated with cytoplasmic proteins) of the *bamB*, *bamC,* and *hlpA* deletion strains compared to wild type (WT) revealed the absence of the corresponding proteins (highlighted in boldface in **Table S3B**).

Although we initiated numerous attempts, we were not able to generate a *surA* deletion mutant. As an alternative, we created a stable and unmarked PA14 *surA* conditional mutant harboring an arabinose-inducible copy of the *surA* coding sequence, resulting in the conditional *surA* mutant Δ*surA araC*-P_{BAD}-*surA* (**Figure 1A**), for convenience termed *surA*. Complementation of *surA* was achieved by the addition of 0.2 % arabinose to the culture media where appropriate (termed *surA* SurA+). To check for expression of *surA*, mRNA levels were determined by quantitative RT-PCR, using *gyrB* as a housekeeping gene (**Table S4**). The relative number of mRNA transcripts of the conditional *surA* mutant grown in the absence of arabinose was reduced by 92 % compared to bacteria harvested after growth in the presence of arabinose (*surA* SurA+). Therefore, in the absence of arabinose *surA* is still expressed in a low amount because the *araC*-P_{BAD} promoter is leaky and cannot be repressed by catabolite repression (Meisner and Goldberg, 2016). In addition, we assessed the presence of SurA protein in whole cell lysates by Western blot analysis (**Figure 1B**). Using the conditional *surA* mutant, SurA protein could not be detected after growth in the absence of arabinose indicating a SurA protein level below the detection limit of the Western blot analysis, while production of SurA was restored in the presence of arabinose. Growth of the (conditional) mutants was investigated at 37°C in LB medium (**Figure 1C**). Only a slight but significant reduction in growth ($p < 0.01$) was observed between 6 h and 12 h after start of the experiment for the conditional *surA* mutant, while all other mutants grew comparably to the PA14 WT strain.

SurA and BamB Are Important for OM Integrity

Integrity of the OM is a pivotal feature of Gram-negative bacteria mediating protection against drugs and harsh environments including mucosal surfaces with antimicrobial peptide production. Since SurA delivers OMPs to the OM, where they are inserted by the BAM complex, an inhibition of parts of this pathway should result in an altered OM composition and possibly a reduced OM integrity. To evaluate changes in OM integrity induced by SurA depletion, or *bamB*, *bamC* or *hlpA* deletion, we first performed a 1-N-phenylnaphthylamine (NPN) assay. NPN fluoresces only in hydrophobic environments. Thus, if the integrity of the OM is compromised in one of the mutant strains, NPN can reach the phospholipid bilayer of the inner OM leaflet more efficiently (Konovalova et al., 2016). Higher fluorescence values therefore indicate a reduced OM integrity. It was shown previously that disturbance of the OM by polymyxin B (PMB) leads to a strong and significant increase of NPN fluorescence. Therefore, PMB was used as a positive control in our assay (**Figure 2A**). We found that the depletion of SurA, but not the deletion of *bamB*, *bamC* or *hlpA* led to a significant increase of fluorescence, compared to the wildtype strain (WT). This means that only the depletion of SurA significantly enhances the entry of NPN. The complementation of *surA* by growing the strain in the presence of arabinose (*surA* SurA+) resulted in a NPN fluorescence signal comparable to that of PA14 WT, indicating that the phenotype can be fully restored by the complementation.

Next we investigated the susceptibility to bile salts, which act as physiological detergents in the intestinal tract (Merritt and Donaldson, 2009). Treatment with 0.3% bile salts significantly reduced the growth of the (conditional) *surA*, *bamB*, and *bamC* mutants, but not of the *hlpA* mutant or *surA* SurA+ (**Figure 2B**). For complementation of the *bamB* deletion mutant, a mini-CTX1 plasmid expressing *bamB* under the control of an arabinose-inducible promoter was introduced and induced with 0.2% arabinose (*bamB* BamB+).

Depletion of SurA and BamB Induces Morphological Changes of *Pa*

Since we had observed that both SurA and to a lesser extent BamB have an impact on OM integrity of *Pa*, we were interested if these changes result in obvious morphological changes. For this purpose, PA14 WT, the *bamB* and the conditional *surA* mutant strains grown in the presence or absence of arabinose were harvested, fixed in Karnovsky's fixative and visualized by transmission electron microscopy (**Figure S1**). The morphology of the PA14 WT strain was characterized by regular-shaped cells with a continuous, plain surface without any vesicles or protrusions attached. The BamB-deficient strain very much resembled the phenotype of a corresponding *Ye* mutant strain (Weirich et al., 2017). It was characterized by numerous vesicles attached to the cell surface, probably a sign

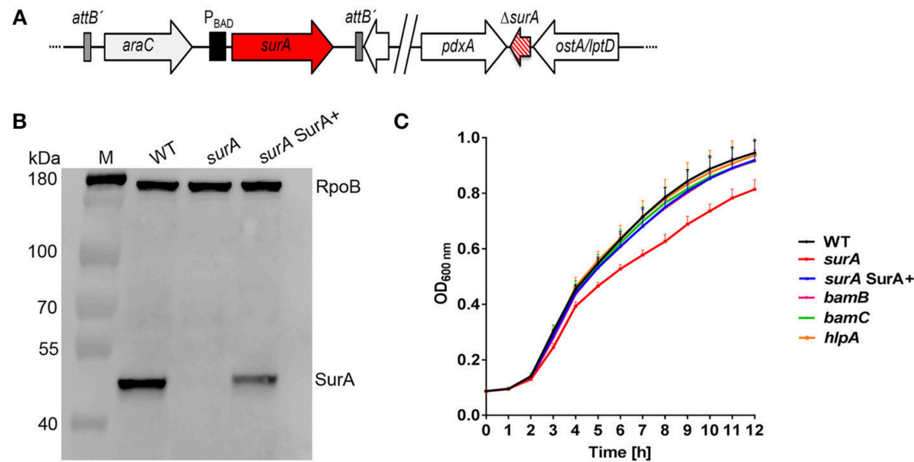

FIGURE 1 | Scheme of the conditional *surA* mutant, verification and impact of SurA, BamB, BamC and HlpA on *Pa* growth. **(A)** Schematic view of the genomic organization of the conditional *surA* mutant. **(B)** Western blot analysis of SurA and RpoB of PA14 WT and the conditional *surA* mutant in the absence (*surA*) and presence of 0.2% arabinose (*surA* SurA+). **(C)** Growth curves of indicated strains. Data depict the mean and SD of at least 3 experiments. Growth curve of the conditional *surA* mutant is highlighted in red. ANOVA analyses revealed significant differences ($p < 0.01$) for both WT vs *surA* and *surA* SurA+ vs *surA* in the time range between 6 and 12 h.

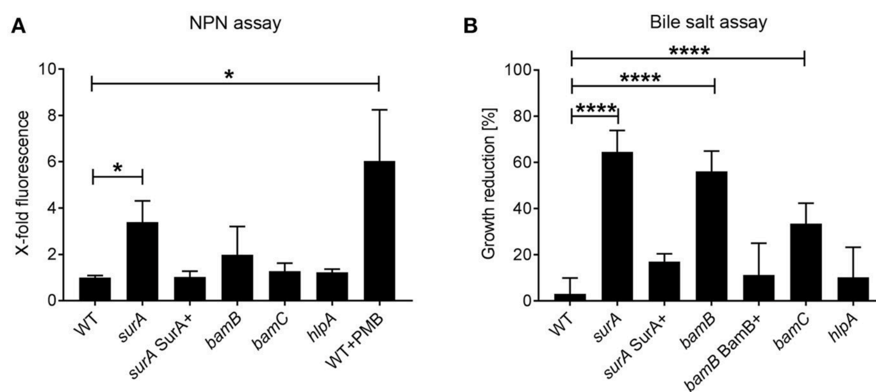

FIGURE 2 | Role of SurA, BamB, BamC, and HlpA for membrane integrity and sensitivity against bile salts. **(A)** NPN Assay. A conditional *surA* and *bamB*, *bamC,* and *hlpA* deletion mutants were treated with NPN. Data depict the mean and SD of 3–5 independent experiments with triplicates. The fluorescence signal derived from matched numbers of bacteria was compared to that of WT. Polymyxin B (PMB) was used as a positive control. Asterisks indicate significant differences ($p < 0.05$) compared to WT using ANOVA analysis. **(B)** Bile Salt Assay. Growth of the indicated *Pa* strains was measured in the absence or presence of 0.3 % bile salts after 8 h. Data depict the mean and SD of the growth reduction in 0.3 % bile salts in LB compared to LB alone of at least 3 independent experiments with duplicates. Asterisks indicate significant differences (****$p < 0.0001$ or *$p < 0.05$) as analyzed by ANOVA analysis.

for envelope stress (Kulp and Kuehn, 2010). Cells of the SurA depletion strain grown in the absence of arabinose also appeared rather regular-shaped, however, they looked slightly bloated and had some vesicles attached to their surface. Taken together, both a *bamB* and a conditional *surA* mutant of *Pa* showed visible changes in cell morphology, which corroborates previous findings obtained with *Ye*.

Depletion of SurA Results in a Drastically Altered Composition of OMPs

To analyze the OMP composition, OM fractions of WT and mutants were prepared and semi-quantitative proteomic analysis was performed using tryptic in-gel digestion and LC-MS/MS analysis. The ratio of label-free quantification (LFQ) intensities between the mutants and the WT was calculated. All differences in $\log_2$ LFQ intensities with a false discovery rate (FDR) <0.1 were considered significant. A list of all significant alterations is found in **Table S3** (S3A: OMPs, S3B: all proteins). For the raw data please refer to http://proteomecentral.proteomexchange.org/cgi/GetDataset with the dataset identifier PXD011849.

The deletion mutant strains for *hlpA*, *bamC* and *bamB* exhibited just minor changes. In the *hlpA* deletion mutant, only HlpA was reduced in abundance, as it was no longer detectable in the OM fraction. The *bamC* deletion led to a significant reduction only of OmpH. Deletion of *bamB* led to a reduction of quite

a number of proteins (e.g. FecA, OprB, PlpD) also found to be reduced in the SurA-depleted strain, however these changes were not significant according to our selection criteria. The relatively mild alterations in the OM composition may explain the comparably weak phenotypes of the *hlpA*, *bamC* and *bamB* deletion mutants with regards to OM integrity.

More interesting were the effects observed for SurA: depletion of SurA significantly altered the level of 42 proteins predicted to be localized in the OM (**Table 1**). Essentially, three groups could be differentiated: (i) proteins highly abundant in the OM of the WT but not detectable in the OM fraction of the conditional *surA* mutant (ratio *surA*/WT < 0.01). This group included TonB-dependent receptors and the siderophore receptors FpvA, FiuA and FecA, and Type V secretion systems (autotransporters). (ii) Proteins highly abundant in the OM fraction of WT and significantly reduced more than 3-fold in the OM fraction of *surA*. This group included proteins of the BAM complex and porins (e.g., OprD, OprF, OprH). Finally (iii) a small group of proteins that showed higher protein levels in the OM fraction of the conditional *surA* mutant (e.g., OprM, OpmG, OpmB) compared to the WT.

In order to find out if the changes in protein abundance were caused on the transcriptional level, we assessed the relative mRNA levels of selected genes from the different functional groups of OMPs of the SurA depletion strain (grown exactly as for the mass spectrometry analyses) by quantitative RT-PCR and compared to the WT (**Figure S2**). From the genes tested, elevated amounts of mRNA transcripts were only found for *hlpA* (2.4-fold), which might be a regulatory effect to compensate the reduced level of SurA. The transcriptional level of all other investigated genes was comparable for all WT, the conditional *surA* mutant and *surA* SurA+. These results indicate that the genes including the type Vd autotransporter PlpD (Salacha et al., 2010) and porins such as OprD seem to be true substrates of SurA and that their reduced abundance in the OM is probably the result of degradation within the periplasm.

Validation of MS/MS Findings: Verification of Selected OMP Levels by Western Blot Analyses

To further validate the proteomics data, the protein levels of SurA, OprD, and PlpD of the WT and the mutants were determined in whole cell lysates by Western blot analysis (**Figures 3A,B**). Comparable RpoB levels in all samples demonstrate equal loading of the lanes. Under depleting conditions (*surA*), no SurA was detectable by Western blot analysis demonstrating that the depletion worked well. Production of SurA in the *surA* SurA+ sample shows at least a partial recovery (64%) compared to the PA14 WT strain. In accordance with the proteomics data (**Table 1** and **Figure 3C**), we found a decreased amount of OprD (15%) and PlpD (24%) in the whole cell lysate of the conditional *surA* mutant. As the PlpD antibody resulted in several bands in Western blot, a *plpD* deletion strain was employed to identify the band corresponding to PlpD.

Validation of MS/MS Findings: Impact of Reduced Siderophore Receptor Abundance

As a consequence of the highly reduced levels of siderophore receptors (FpvA, FiuA, and FecA) under SurA-depleted conditions we assumed that the strain might suffer from a defective uptake of siderophore-iron complexes. Under iron-restricted conditions this should consequently lead to a growth reduction. Therefore, we assessed the growth characteristics of PA14 and the *surA* mutant under iron limitation. This was achieved by the addition of various amounts of the iron chelator 2,2′-Bipyridyl (BiP) to the growth medium (**Figure S3**). As assumed, under iron limitation (+BiP), the SurA-depleted strain exhibited a significantly stronger BiP dose-dependent growth defect compared to the WT.

Depletion of SurA Increases the Susceptibility for Killing by the Complement System

An important first line host defense against invading bacteria specifically in bloodstream infection is the serum complement system. Therefore, we investigated whether serum resistance of *Pa* is altered in the (conditional) *surA*, *bamB*, *bamC,* and *hlpA* mutants. To this end, serum killing tests using human serum were performed. The strains were incubated in 10% heat inactivated serum (HIS) or 10 % normal human serum (NHS). Survival of bacteria was then quantified at indicated time points over a maximum period of 4 h (**Figure 4A**). While deletion of *bamB*, *bamC* or *hlpA* had no impact on survival in active serum, the conditional *surA* mutant was killed rapidly when grown in the absence of arabinose (**Figure 4B**), indicating that the depletion of SurA alters the OM in a way that renders *Pa* highly susceptible to killing by the serum complement system.

SurA Is Important for Virulence of *Pa* in the *Galleria mellonella* Infection Model

To address the importance of the investigated genes for virulence, the *Galleria mellonella* infection model was used. For this purpose, 12 ± 2 cells of PA14 WT or the (conditional) *surA*, *bamB, bamC* or *hlpA* mutant were injected into the hemolymph of *G. mellonella* larvae. Thereafter, the survival of the larvae was monitored (**Figure 5**). Neither deletion of *bamB*, *bamC,* nor *hlpA* altered the survival compared to infection with the WT. However, infection with the conditional *surA* mutant led to a significant delay in the time to death. The conditional *surA* mutant was grown under two growth conditions prior to infection: (i) arabinose induced–SurA present prior to infection (SurA+) or (ii) uninduced–SurA absent prior to infection (SurA–). However, no significant difference was found between the survival curves of SurA+ and SurA–. This indicates that SurA production may decline rather quickly under *in vivo* conditions without continuous application of arabinose, which was not applicable in our experimental setting. Therefore, we could not test whether a complementation would fully rescue virulence. Nevertheless, our data demonstrate that SurA is critical for virulence of *Pa* in *G. mellonella*.

TABLE 1 | Outer membrane proteins affected by SurA depletion.

Function	Gene name	Ratio *surA*/WT	β-strands	PDB ID**
Type V secretion	PA14_32780	**<0.01**	16*	
	PA14_32790	**<0.01**	–	
	PA14_61190	**0.23**	16*	
	PlpD	**<0.01**	16	5F4A, 5FQU
	AaaA (PA14_04290)	**<0.01**	12*	
	EprS (PA14_18630)	**0.04**	12*	
	EstA	**0.20**	12	3KVN
Siderophore receptors and other TonB-dependent receptors	FpvA	**<0.01**	22	2W75, 2W16
	FecA	**<0.01**	22	1PO0, 1PO3
	FiuA	**0.04**	22*	
	PA14_34990	**<0.01**	22*	
	PA14_54180	**<0.01**	22*	
	PA14_26420	**0.02**	22*	
BAM-complex	BamD/ComL	**0.30**	–	
	BamA	**0.31**	16	4C4V
	BamE/OmlA	**0.31**	–	
	BamB	**0.35**	–	
	BamC (PA14_51260)	**0.84**	–	
Porins	OpdO	**<0.01**	18	2Y0K, 2Y06
	OpdN	**<0.01**	18	4FSO
	OprG	**0.07**	8	2X27
	OprE	**0.11**	18*	
Porins	OpdP	**0.13**	18	3SYB
	OprD	**0.14**	18	3SY7
	OprB	**0.22**	16	4GY, 4GF
	OprQ	**0.25**	22*	
	OprC	**0.28**	22*	
	OprH	**0.32**	8	2LHF
	OpdC (PA14_02020)	**0.35**	18	3SY9
	OprF	**0.47**	8	4RLC
	PA14_31680	**0.55**	–	
	OprM	**1.52**	4	3D5K
	OpmB (PA14_31920)	**1.88**	4*	
	OpmG	**7.37**	4*	
LPS bio-synthesis	LptD	**0.32**	26	5IVA
	LptE	**0.38**	–	
T3SS	ExsB (PA14 42400)	**<0.01**	–	
Others	Gbt	**<0.01**	4*	
	FadL (PA14_60730)	**<0.01**	14	3DWO
	PA14_13130	**0.03**	–	
	PA14_24360	**0.04**	–	
	PA14_36020	**7.28**	–	
	FusA (PA14_13520)	**>20.40**	4*	

*OM fractions of PA14 WT and the conditional surA mutant derived from three independent experiments were analyzed by mass spectrometry. Table depicts proteins which are described to be located in the OM and are significantly reduced or increased due to SurA depletion. Multiple t-testing was performed. Significant differences (FDR < 0.1) are shown in bold face. Number of β-strands of β-barrel proteins is indicated. *Predicted with Boctopus (Hayat and Elofsson, 2012); **Accession number of protein data bank (www.rcsb.org) of indicated proteins or orthologs.*

Susceptibility to Antibiotics

The impermeability of the OM is the main reason that many antibiotics are not effective against Gram-negative bacteria, since they cannot pass the OM to reach their target. To investigate whether the depletion of SurA or BamB influences antibiotic susceptibility, we performed a comprehensive analysis with E-tests using the *bamB* deletion mutant, the conditional *surA* strains of PA14 and the clinical multidrug-resistant *Pa* bloodstream isolate ID72 (Willmann et al., 2018) [resistant against 3 classes out of the following: (I) 3rd and 4th generation cephalosporines (e.g., cefotaxim, ceftazidim), (II) acylureidopenicillins (e.g., piperacillin), (III)

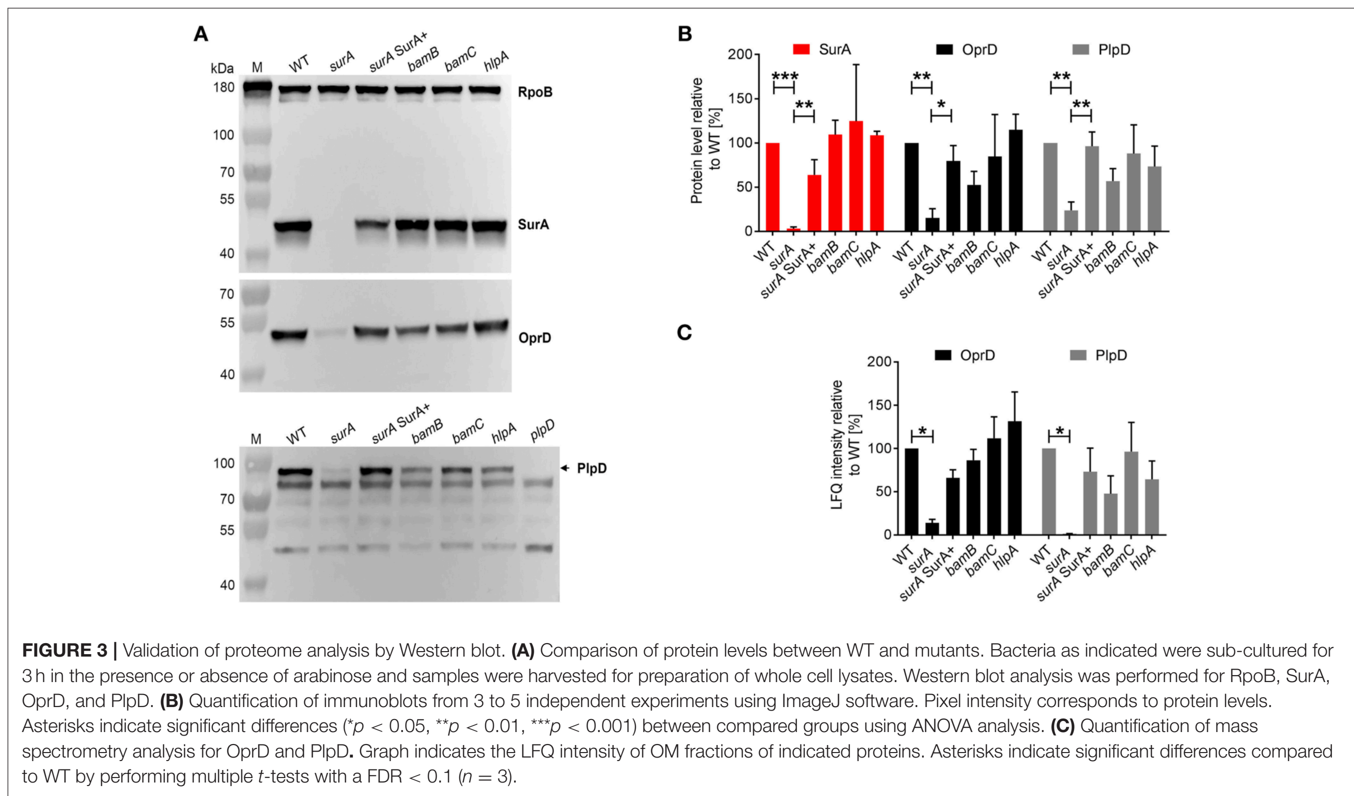

FIGURE 3 | Validation of proteome analysis by Western blot. **(A)** Comparison of protein levels between WT and mutants. Bacteria as indicated were sub-cultured for 3 h in the presence or absence of arabinose and samples were harvested for preparation of whole cell lysates. Western blot analysis was performed for RpoB, SurA, OprD, and PlpD. **(B)** Quantification of immunoblots from 3 to 5 independent experiments using ImageJ software. Pixel intensity corresponds to protein levels. Asterisks indicate significant differences (**p* < 0.05, ***p* < 0.01, ****p* < 0.001) between compared groups using ANOVA analysis. **(C)** Quantification of mass spectrometry analysis for OprD and PlpD**.** Graph indicates the LFQ intensity of OM fractions of indicated proteins. Asterisks indicate significant differences compared to WT by performing multiple *t*-tests with a FDR < 0.1 (*n* = 3).

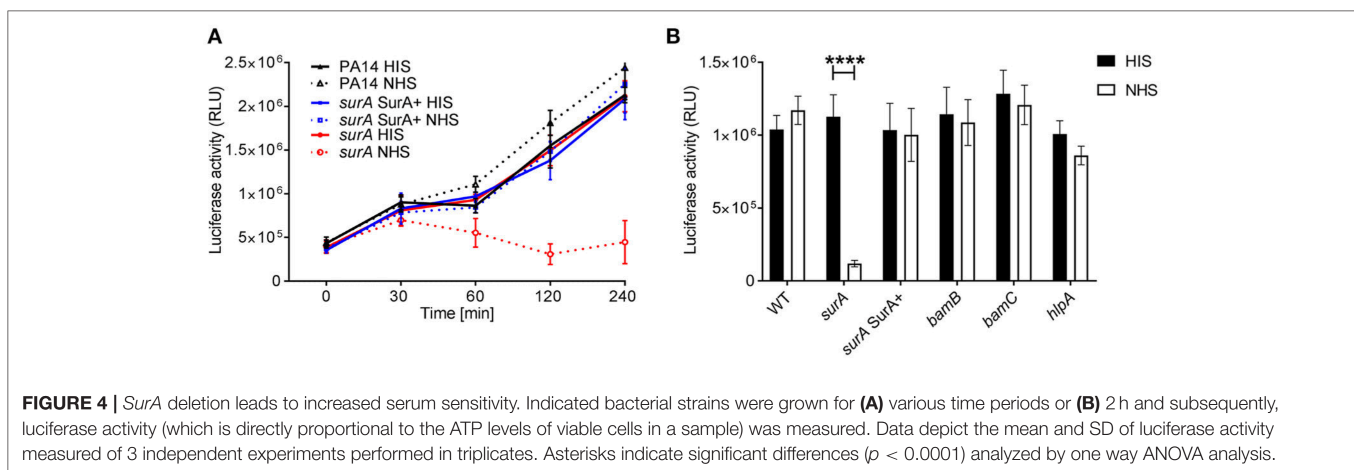

FIGURE 4 | *SurA* deletion leads to increased serum sensitivity. Indicated bacterial strains were grown for **(A)** various time periods or **(B)** 2 h and subsequently, luciferase activity (which is directly proportional to the ATP levels of viable cells in a sample) was measured. Data depict the mean and SD of luciferase activity measured of 3 independent experiments performed in triplicates. Asterisks indicate significant differences (*p* < 0.0001) analyzed by one way ANOVA analysis.

fluorchinolones (e.g., ciprofloxacin), and (IV) carbapenems (e.g., imipenem, meropenem)] (**Figure S4**) and the corresponding complemented strains compared to the WT control strains. Our test set additionally included several antibiotics not applicable for treatment of Gram-negative pathogens. However, these substances (vancomycin, erythromycin) can be used to detect OM defects in Gram-negatives (Wu et al., 2005). The deletion of *bamB* reduced the MIC values at least 4-fold for ampicillin/sulbactam, ceftazidime, fosfomycin and vancomycin (**Table 2**). The complementation with arabinose-induced BamB (*bamB* BamB+) restored the resistance against these antibiotics with the exception of fosfomycin. In summary, our data demonstrate that *bamB* deletion leads to a moderate increase in antibiotic susceptibility against several antibiotics.

Interestingly, for some of the tested antibiotics, we could observe at least a 4-fold reduction of the MIC for both the PA14 and the ID72 conditional *surA* mutant. This was the case for ticarcillin/clavulanate (PA14 32→6 mg/l; ID72 >256→64 mg/l), ceftazidime (PA14 2→0.5 mg/l; ID72 >256→8 mg/l), levofloxacin (PA14 0.38→0.094 mg/l; ID72 1.5→0.064 mg/l), ciprofloxacin (PA14 0.19→0.038 mg/l; ID72 0.38→0.064 mg/l) and vancomycin (PA14 >256→12 mg/l; ID72 >256→64 mg/l). For the SurA-depleted strain in the PA14 background, we additionally observed a reduced MIC for ampicillin/sulbactam (PA14 >256→24 mg/l). Moreover, the mutant in the ID72

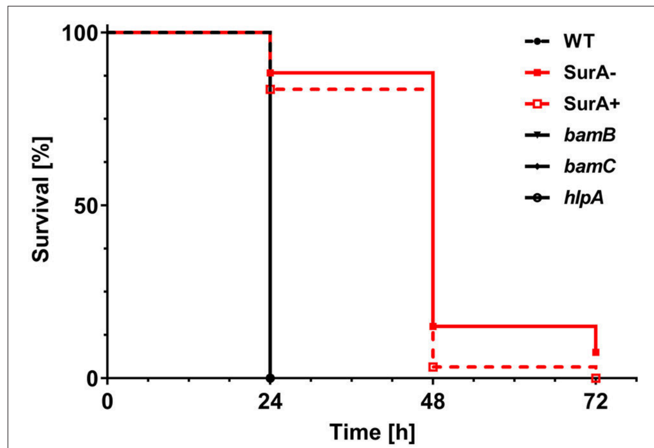

FIGURE 5 | *SurA* deletion leads to attenuated virulence in the *Galleria mellonella* infection model. In total, 60 *G. mellonella* larvae per group were infected in 3 independent experiments with a CFU of 12 ± 2 for the indicated time period and survival of larvae was monitored by touching with a forceps. The conditional *surA* mutant strain was tested both when expressing SurA (SurA+) and after depletion of SurA (SurA–) at the time point of infection. Please note that the survival curves of WT, *bamB*, *bamC,* and *hlpA* are identical. Statistical analysis was performed using a log rank test (Mantel-Cox test). A significant difference between WT and the conditional *surA* mutant was observed ($p < 0.0001$).

background displayed a reduced MIC for cefepime (>32→3 mg/l). Strain-specific differences mediated by SurA depletion were found for ampicillin/sulbactam (increased sensitivity of PA14 *surA* but not ID72 *surA*) and cefepime (increased sensitivity of ID72 *surA* but not PA14 *surA*). Strikingly, in the SurA-depleted multidrug-resistant clinical bloodstream isolate ID72, the MIC values for cefepime, ceftazidime and levofloxacin were reduced to such an extent that according to the current EUCAST Clinical Breakpoint Tables (v. 8.1.), ID72 was re-sensitized to treatment with these antibiotics. In the case of ticarcillin/clavulanate, the MIC value was reduced. However, it did not drop below the critical breakpoint. Taken together, our data demonstrate that SurA depletion leads to an increased susceptibility against some representatives of clinically relevant antibiotics, even in the case of a multidrug-resistant *Pa* strain. Thus, SurA could possibly be used as a drug target to re-sensitize resistant strains to antibiotic therapy.

DISCUSSION

Pa is a difficult-to-treat pathogen and, compared to other Gram-negative bacteria, associated with a higher mortality that cannot be attributed to resistance only (Aloush et al., 2006; Willmann et al., 2014; Thaden et al., 2017). Often colistin is considered as a last resort antibiotic to defeat infections caused by *Pa*, however, it has severe side effects and is rather nephrotoxic (Jeannot et al., 2017). Therefore, novel drugs and drug targets are required to control *Pa* infections (Perez et al., 2016).

The BAM complex and associated chaperones are responsible for the transport and insertion of the great majority of OMPs into the Gram-negative OM. Previous studies already highlighted

TABLE 2 | Sensitivity of *Pa* strains against selected antibiotics measured by *E*-tests.

			MIC Breakpoint (mg/L)		PA14 SurA			ID72 SurA			PA14 BamB		
			S ≤	R >	PA14 WT	PA14 *surA*	PA14 *surA* SurA+	ID72	ID72 *surA*	ID72 *surA* SurA+	PA14 WT	PA14 *bamB*	PA14 *bamB* BamB+
Penicillins	AMS	Ampicillin-sulbactam	–	–	>256	24	>256	>256	>256	>256	>256	32	>256
	PIT	Piperacillin-tazobactam	16	16	6	3	6	>256	<256	>256	6	2	6
	TIL	Ticarcillin-clavulanate	16	16	32	**6**	24	>256	64	>256	32	**12**	192
Carbapenems	DOR	Doripenem	1	2	0.25	0.38	0.38	>32	>32	>32	0.25	0.25	0.5
	MER	Meropenem	2	8	0.38	0.75	0.5	>32	>32	>32	0.38	0.5	1.5
Cephalosporins	CTA	Cefotaxime	–	–	16	8	16	>256	>256	>256	16	8	32
	CEP	Cefepime	8	8	0.75	0.25	0.75	>32	**3**	> 32	0.75	0.38	0.5
	CTZ	Ceftazidime	8	8	2	0.5	1	>256	**8**	64	2	0.38	1.5
Fluoroquinolones	LEV	Levofloxacin	1	1	0.38	0.094	0.38	1.5	**0.064**	0.75	0.38	0.25	0.38
	CIP	Ciprofloxacin	0.5	0.5	0.19	0.038	0.094	0.38	0.064	0.125	0.19	0.064	0.19
	FOS	Fosfomycin	–	–	64	24	64	64	16	64	64	12	8
	VAN	Vancomycin	–	–	>256	12	>256	>256	64	>256	>256	48	<256
	ERY	Erythromycin	–	–	>256	>256	>256	>256	>256	>256	>256	96	<256
	TRS	Trimethoprim-sulfamethoxazole	–	–	4	1.5	3	>32	>32	>32	4	2	8

The following strains were investigated for antibiotic sensitivity: PA14 WT, a conditional surA mutant grown in the absence (surA) and the presence of 0.2% arabinose (surA SurA+), the bamB deletion mutant (bamB) and a conditional bamB deletion mutant grown in the presence of 0.2% arabinose (bamB BamB+). Reduction of MIC values compared to WT is marked in red. Bold face indicates reduction of MIC values below the breakpoint.

the importance of the BAM complex as a putative drug target for several Gram-negative bacteria (Vertommen et al., 2009; Namdari et al., 2012; Hagan et al., 2015; Krachler, 2016; Weirich et al., 2017; Storek et al., 2018). The delivery of OMPs to the BAM complex is performed by the well-known chaperones SurA and Skp. Interestingly, according to the literature there are striking differences in the importance of these chaperones for OMP biogenesis. In *Ec* and *Ye*, SurA seems to play a major and Skp only a minor role for OMP biogenesis (Sklar et al., 2007). In contrast, in *Neisseria meningitidis* Skp but not SurA seems to play the major role for OMP biogenesis (Volokhina et al., 2011). According to the importance of *Pa* in clinical settings, we wanted to know which of the components of the BAM complex might be more useful as a target.

Therefore, we analyzed the role of distinct components of the BAM complex and the periplasmic chaperones HlpA/Skp and SurA for OM integrity and composition, virulence and antibiotic resistance. The main findings of this study are that depletion of SurA severely alters *Pa* OMP composition, which in consequence strongly influences OM integrity as well as resistance to bile salts, complement activity and antibiotics, which altogether leads to attenuated virulence and enhanced susceptibility to several antibiotics even in a multidrug-resistant bloodstream isolate of *Pa*.

A comparably lower impact of the *bamB* deletion on *Pa* sensitivity against antimicrobial substances is perfectly in line with the milder phenotypes and minor changes in OMP composition of the *bamB* mutant. Similar findings have been made with *Ec* and *Ye* (Charlson et al., 2006; Weirich et al., 2017). Deletion of the *skp* homolog *hlpA* and the BAM complex component *bamC* did not result in obvious phenotypes in our hands. In addition, none of these deletion mutants showed attenuation of virulence in the *G. mellonella* infection model. This is in line with previous studies on Skp in *Ec* where it was shown that Skp/HlpA may play only a minor role as chaperone to deliver OMPs to the BAM complex (Sklar et al., 2007).

Recently, it was asked whether BamB might be the achilles' heel for targeting *Klebsiella pneumoniae (Kp)* infection (Krachler, 2016). It was found that deletion of *bamB* led to a 15-fold decrease in *Kp* adherence to retinal, intestinal and lung epithelial cells and consequently decreased invasion. *bamB* deletion had a pleiotropic effect on the profile of OMPs including a decrease of some porins as well as of type I fimbriae. Moreover, *bamB* deletion led to a significant attenuation of virulence in mice challenged intraperitoneally with *Kp* (Hsieh et al., 2016). Attenuation of virulence of a *bamB* deletion mutant was also found during *Ye* infection (Behrens et al., 2001). *In vitro* assays showed increased sensitivity against antimicrobial components such as bile salts and complement activity. In addition, *bamB* deletion mutants of *Ye* were sensitized to various antibiotics (typically not active against Gram-negative bacteria), such as vancomycin (Weirich et al., 2017). Like in *Kp*, several porins as well as the autotransporter invasin were significantly decreased in *Ye*. Another study addressing the role of BamB in *Pa* PAO1 already showed that *bamB* deletion also leads to sensitization against lysozyme, vancomycin and cefotaxime (Lee et al., 2017), which could be confirmed in our study. However, in contrast to *Ye* or *Kp,* neither increased sensitivity against human serum nor attenuation of virulence was observed. A common impact of *bamB* deletion in various species seems to be the reduction of the abundance of some porins (Malinverni et al., 2006; Hagan et al., 2010). In line with this, in the *Pa bamB* deletion mutant, porins such as OpdO (>93% reduced) and OprB (45% reduced) were found in lower levels in the OM. Some autotransporters like AaaA (67% reduced) and PlpD (52% reduced) were also found in lower levels in the OM. This is in agreement with previous studies, where it was observed that BamB-dependency of autotransporter proteins seemed to be correlated with the number of β-strands contained. Especially those proteins possessing a large number of β-strands were negatively affected by the absence of BamB, whereas others were not (Rossiter et al., 2011; Weirich et al., 2017). However, these effects were rather moderate. Thus, BamB may contribute to the assembly of porins and autotransporters in *Pa*, but in contrast to the function of BamB in *Kp* or *Ye*, the rather mild phenotypes we found upon deletion of *bamB* in *Pa* PA14 do not justify considering it as a promising target for drug development from our point of view. Nonetheless, given the results that have been obtained with e.g., *Pa* PAO1 and *Salmonella* (Namdari et al., 2012; Lee et al., 2017), it cannot be ruled out that the importance of BamB for OM composition and consequently the resulting phenotypes might vary significantly between strains and species.

The most interesting candidate as a putative drug target addressed in this study was found to be SurA. We recognized quite early during our studies that SurA might play an important role in *Pa* PA14, because it was not feasible to generate an in-frame deletion mutant of *surA*. This indicated that *surA* might be essential in PA14, which would be in line with the findings of various other groups since there was no viable *surA* transposon mutant detected in their transposon libraries of different *Pa* strains (Skurnik et al., 2013; Lee et al., 2015; Turner et al., 2015) and also with own unpublished observations. Nevertheless, there is one transposon library in PA14 that contains three different mutants with transposons inserted into *surA* (Liberati et al., 2006). The transposon mutant with the ID38436 included in the available PA14NR set showed a similar phenotype like the conditional *surA* mutant in various assays and no SurA was detectable by Western blot analysis (data not shown). The insertion site of this mutant is located at the very beginning of the gene (at base pair 17), indicating inactivation of the gene. One possible explanation that this mutant is viable might be that compensatory mutations occurred in this transposon mutant. Altogether, we assume that SurA in *Pa* is essential in contrast to other Gram-negative bacteria. Nevertheless, the phenotypes observed in the SurA depletion strain of *Pa* are very similar to those of the deletion mutant in *Ye* (Weirich et al., 2017).

While *bamB* deletion only leads to mild alteration in the OM composition, the depletion of SurA disturbed the insertion of a wide variety of OMPs of different functions, resulting in a drastically altered OM composition. Since the proper composition of the Gram-negative OM is important for its function as an impermeable barrier for many substances, it is reasonable that the reduced amount of several OMPs resulted in a higher permeability to the fluorescent dye NPN.

The permeability barrier of the OM and the export of substances by efflux pumps are the main reasons for the high intrinsic resistance of *Pa* against many antibiotics (Nikaido, 1989; Poole, 2001). The reduced integrity of the OM could be an important reason, why the conditional *surA* mutants of PA14 and ID72 were better accessible to antibiotics such as vancomycin that are usually not able to cross the OM of *Pa* and reach their target inside the bacterial cell. Nevertheless, it cannot be excluded that other effects such as alteration in OMP composition or stress response may contribute to the increased antibiotic sensitivity. Thus, an inhibition of SurA could possibly permit a re-purposing of approved antimicrobials, currently active only against Gram-positive pathogens, for use in Gram-negative bacteria. Of course this could work only if (i) the current limitation of use is a result of the inefficient entry and if (ii) the antimicrobial target is conserved and also present in the Gram-negative species. These data are in line with previous data found for the commensal *Ec* K12 as well as *Ye* (Tamae et al., 2008; Weirich et al., 2017).

However, a critical precondition to consider SurA as a target specifically in species like *Pa* would be to break the resistance against therapeutically used antibiotics of multidrug-resistant strains. By using a conditional ID72 *surA* mutant this could indeed be demonstrated for various antibiotics such as cephalosporins and fluoroquinolones.

In summary, from all the investigated factors, SurA was identified as the best target candidate to restore the sensitivity against some antibiotics by distortion of the OM specifically in multidrug-resistant strains. In the *surA* conditional mutant we found that the OM contained a higher amount of some single proteins like the OprM family porins OprM, OpmB and OpmG that are associated with the MexAB and MexXY efflux pumps (Poole, 2000). They are involved in mediating resistance against β-lactams, chloramphenicol, macrolides, quinolones and tetracycline (Li et al., 1995; Masuda et al., 2000), and aminoglycosides (Mao et al., 2001), respectively. Their increased abundance indicates that these porins are no dedicated substrates of SurA and their insertion into the OM may be facilitated in a different way, independent of SurA. OprM actually assembles into a trimer (Akama et al., 2004). It has been previously observed that a distinct subset of OMPs belonging to the TolC-like BAM substrates (i.e., multimeric with each monomer having only few β-strands) were affected only weakly by the absence of the non-essential Bam proteins and periplasmic chaperones. However, they were highly dependent on the essential Bam proteins BamA and BamD (Mahoney et al., 2016; Weirich et al., 2017). This might also apply to OprM family porins. Also the associated efflux pumps were found in a relatively higher amount in the OM of the conditional *surA* mutant, but this does not seem to influence its antibiotic sensitivity (**Table 2**).

With the exception of OprM, OpmG, and OpmB, many porins were detected in a significantly lower amount in the OM of the conditional *surA* mutant, including the most striking reduction observed for members of the OprD family (OpdO, OpdN, OpdP, and OprD). This may lead to a deprivation of nutrients, since most of these porins are specific transporters for different nutrients like pyroglutamate (OpdO), glycine-glutamate (OpdP), arginine (OprD and OprQ) and glucose (OprB) (Chevalier et al., 2017) and could also contribute to attenuation.

Besides the porins, also other groups of OMPs were strikingly affected by the depletion of SurA. We found that especially siderophore receptors and other TonB-dependent receptors (e.g., FpvA or FecA) (Pederick et al., 2015; Luscher et al., 2018) as well as different autotransporter proteins (e.g., PlpD or AaaA) were absent or less abundant in the OM upon depletion of SurA. The mRNA expression analysis suggested that the autotransporter protein PlpD is also a true substrate of SurA, similar to the autotransporter Inv of *Ye* (Weirich et al., 2017). This means that these proteins are reduced in abundance because they cannot use any alternative insertion pathway when SurA is depleted. Thus they presumably are degraded by periplasmic proteases such as DegP (Sklar et al., 2007).

The finding that so many proteins involved in iron acquisition and transport were completely or almost completely absent in the conditional *surA* mutant, including the pyoverdine receptor FpvA, the ferric citrate transporter FecA and the ferrichrome receptor FiuA, suggests a reduced fitness of the conditional *surA* mutant under iron-limited conditions. This is in line with our findings that SurA depletion strongly affects growth in LB medium under iron-restricted conditions. In addition, it was previously shown that deletion of *fiuA*, besides its involvement in iron acquisition, leads to pleiotropic effects such as reduction of elastase levels and reduced virulence in an airway infection model (Lee et al., 2016). Therefore, the reduced abundance of siderophore receptors and the associated downstream effects could also contribute to attenuation of the SurA-depleted PA14 in the *G. mellonella* infection model.

Furthermore, the significantly reduced amount of the LptD/E complex (Chimalakonda et al., 2011) in the conditional *surA* mutant might result in an altered level of LptD in the OM (Lo Sciuto et al., 2018). The stable LptD/E complex is present at the OM and functions in the final stages of LPS assembly. The lipopolysaccharide transport (Lpt) is responsible for transporting LPS from the periplasmic side of the OM to the cell surface (Balibar and Grabowicz, 2016; Andolina et al., 2018). In line with previous studies (Vertommen et al., 2009; Weirich et al., 2017), LptD was shown to be a true substrate of SurA. Furthermore, it was shown that LptE depletion leads to reduced functionality of LptD resulting in impaired cell envelope integrity, reduced virulence and decreased antibiotic resistance (Lo Sciuto et al., 2018), which identifies LptD as a promising target for drug development. Actually, LptD is already addressed as a drug target by the macrocyle inhibitor Murepavadin (Polyphor POL7080), which is currently tested in a phase III clinical trial (Martin-Loeches et al., 2018). This fact renders the concept of a SurA inhibitor -which is able to significantly reduce the cellular LptD protein levels- even more attractive.

The global changes in the OM composition of the conditional *surA* mutant including the reduced levels of many porins important for nutrient uptake, iron transport systems and proteins involved in LPS transport may in sum accumulate in reduced fitness. This is in line with the results of the *G. mellonella* infection model, since the larvae showed a prolonged time to death when infected with the conditional PA14 *surA* mutant. For

the *in vivo* experiments, the leakiness of the *araC*-P_{BAD} promoter (Meisner and Goldberg, 2016), still resulting in some mRNA expression, was actually a convenient feature: a partial reduction of SurA simulates the potential inhibition of the protein by a putative SurA inhibitor more realistically than a clean deletion.

Taken together, SurA is an important protein in *Pa* determining proper composition of the OM and seems to be an attractive target for an antiinfective drug. Its inhibition may lead to reduced fitness, may dampen multidrug resistance and could simultaneously render *Pa* accessible to various antibiotics that are usually not effective because of the OM barrier.

DATA AVAILABILITY

The dataset of the LC-MS/MS analysis for determination of OMP composition of the investigated bacterial strains can be found in the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD011849 (http://proteomecentral.proteomexchange.org/cgi/GetDataset, Username: reviewer54276@ebi.ac.uk, Password: i3rXLDrr).

AUTHOR CONTRIBUTIONS

The study was designed and supervised by EB, MS, and IA. Mass spectrometry and data analyses were performed by MF-W and BM. All other experimental data and analyses and generation of tools were performed by KK, MSS, LF, TT, EB, MS, JL, and KL. The manuscript was written by KK, MSS, MS, and EB with contribution of all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00100/full#supplementary-material

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Identification of drug-resistance determinants in a clinical isolate of *Pseudomonas aeruginosa* by high-density transposon mutagenesis

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Running title: The resistome of a MDR *Pseudomonas aeruginosa*

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Abstract

With the aim to identify potential new targets to restore antimicrobial susceptibility of multidrug-resistant (MDR) *Pseudomonas aeruginosa* (*Pa*), we generated a high-density transposon (Tn) insertion mutant library in a MDR *Pa* bloodstream isolate (ID40). The depletion of Tn insertion mutants upon exposure to cefepime or meropenem was measured in order to determine the common resistome for these clinically important antipseudomonal β-lactam antibiotics. The approach was validated by clean deletions of genes involved in peptidoglycan synthesis/recycling such as the lytic transglycosylase MltG, the murein endopeptidase MepM1*,* the MurNAc/GlcNAc-kinase AmgK and the uncharacterized protein YgfB that all were identified in our screen as playing a decisive role for survival of treatment with cefepime or meropenem. We found that the antibiotic resistance of *Pa* can be overcome by targeting usually non-essential genes that turn essential in the presence of therapeutic concentrations of antibiotics. For all validated genes, we demonstrated that their deletion leads to the reduction of *ampC* expression, resulting in a significant decrease of β-lactamase activity and consequently these mutants partly or completely lost resistance against cephalosporins, carbapenems and acylaminopenicillins. In summary, the determined resistome may comprise promising targets for developing drugs that could be used to restore the sensitivity towards existing antibiotics specifically in MDR strains of *Pa*.

Introduction

Pa is one of the most important pathogens involved in nosocomial infections, such as pneumonia, urinary tract infection, wound infections and potentially life threating blood stream infection. In particular, intensive care and immunocompromised patients are at risk to develop severe infections. MDR strains are emerging which makes treatment of *Pa* infection even more difficult. For this reason, the WHO ranked carbapenem-resistant *Pa* into the top class of its list of priority pathogens for which new antibiotics are urgently needed (1). For an increasing number of cases colistin is the last treatment option despite its neuro-and nephrotoxic side effects.

Pa employs various intrinsic and acquired antibiotic resistance mechanisms. The high intrinsic resistance is mainly caused by a very low permeability of the outer membrane (2) and the inducible expression of efflux pumps and enzymes mediating resistance like AmpC (3). *ampC* is expressed at a low level in wildtype strains but can be strongly increased in strains in which *ampC* is derepressed. Derepression of *ampC* is often caused by mutations in the transcriptional regulator AmpR, in AmpD (4, 5) or in the *dacB* gene encoding muropeptide amidase and penicillin-binding protein 4 (PBP4), respectively (6), leading to an increased pool of 1,6-anhydromuropeptides originating from the peptidoglycan (PG) recycling pathway (7). Moreover, *ampC* expression can be induced by β-lactam antibiotics and β-lactamase inhibitors leading to resistance against most β-lactam antibiotics (8).

One strategy to reconsider antibiotics that have become ineffective caused by the development of resistance is the inactivation of the primary resistance mechanism. Thus, the combination of β-lactam antibiotics with β-lactamase inhibitors such as tazobactam, which block the activity of β-lactamases, makes it possible to reconsider antibiotics such as piperacillin. However, often such combinations fail again to kill

microbial pathogens because of β-lactamases which are resistant against the β-lactamase inhibitors (9-11). One upcoming strategy is to use a different class of antibiotic adjuvants. Such adjuvants would not inactivate a primary resistance mechanism but would rather act on a secondary resistance gene. Several examples for such a strategy have been described (12-16). In this study, we wanted to find out which proteins could serve as targets to resensitize MDR *Pa* strains to treatment with β-lactam antibiotics.

To answer this question we performed Transposon-Directed Insertion Sequencing (TraDIS) using the clinical bloodstream isolate ID40, which is resistant against many β-lactam antibiotics, to assess the resistome of *Pa* in a similar approach described by Jana et al. (17). TraDIS has been shown to be a valuable tool under particular conditions and in various approaches to find genes responsible for growth (18-21). We constructed a Tn mutant library in the MDR ID40 strain and subjected it to cefepime (FEP) or meropenem (MEM). TraDIS revealed non-essential candidate genes including well-known as well as so far unknown genes whose inactivation breaks resistance against these antibiotics. Some candidates were verified by testing respective deletion mutants for their antibiotic sensitivity, β-lactamase activity and *ampC* expression. The presence of these genes seems to be crucial to achieve or maintain antibiotic resistance. These genes may comprise the most promising non-essential target genes for the development of novel antibiotic adjuvants to reconsider β-lactam antibiotics in resistant strains of *Pa*.

Results

ID40 sequence and resistance profile

To determine the resistome of a MDR *Pa* strain against β-lactam antibiotics, we used the bloodstream isolate ID40 (22). ID40 belongs to the sequence type ST-252

(determined by MLST 2.0, Center for Genomic Epidemiology, DTU, Denmark (23)) and is resistant against piperacillin (PIP), piperacillin/tazobactam (TZP), cefepime (FEP), ceftazidime (CAZ), aztreonam (ATM), levofloxacin (LEV), ciprofloxacin (CIP) and imipenem (IMP). Moreover, ID40 is intermediate for meropenem (MEM) and sensitive against amikacin (AMI), gentamicin (GEN), tobramycin (TOB) and colistin (COL) (Table S1). The whole genome and the plasmid sequence were annotated and submitted to the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena; accession number PRJEB32702).

The ID40 chromosome is 6.86 Mbp in size and encodes 6409 open reading frames and carries a plasmid of 57446 bp comprising 59 putative genes. Resistance genes were searched using ResFinder (24) revealing the following resistance genes: aph(3')-IIb (*neo*) for aminoglycoside resistance, blaOXA-486 (*bla*) and OxaPAO1 (*ampC*, PDC-3) for β-lactam resistance, crpP (*crpP*) for fluoroquinolone resistance and fosA (*fosA_1*) for fosfomycin resistance. Additionally, we found a point mutation in the *dacB* gene (PBP4; G-A nt1310, G437D), which is known to be responsible for resistance against β-lactam antibiotics as shown by an increased MIC for CAZ from 1 µg/ml to 32 µg/ml in *Pa* PAO1 (6). Therefore, most likely the mutation in *dacB* rationalizes the different resistance level of ID40 in comparison to strain PA14, which comprises the same resistance genes but is sensitive to all β-lactam antibiotics. Other resistance mechanisms like reduced expression of *oprD* and overexpression of efflux pumps were not specifically addressed, but their contribution to resistance cannot be finally excluded. Analysis of the OprD sequence and comparison to the literature did not provide any clear evidence that OprD of ID40 is dysfunctional (25-28).

Construction of a high-density mutant library and TraDIS sequencing

Growth of the Tn library in LB revealed approximately 100000 unique Tn insertions distributed across the genome with an average of 18 Tn insertion sites per 1 kbp of coding sequences. Homogenous distribution of Tn insertions and homogenous coverage of the whole genome are shown in **Figure S1**.

Analysis of the unchallenged Tn library showed that from 6468 genes 697 genes were determined to be essential for viability (10.8 %) (**Data set S1**) and 9 were determined to be ambiguous (0.14 %) (**Data set S2**). Among these, many genes were previously described to be essential, for example *dnaA*, *gyrB* or *lolA* (29, 30).

Identification of genes important for resistance against meropenem and cefepime

The contribution of non-essential genes to antimicrobial resistance was measured by quantifying the depletion of Tn insertion mutants upon exposure with FEP and MEM at the respective breakpoint concentration defining a *Pa* strain as sensitive according to EUCAST (FEP: 8 µg/ml, MEM: 2 µg/ml). For analysis of the TraDIS results we chose only genes in which the read number in LB control was > 10 in all three independent experiments and additionally showed a significant change in read counts upon treatment and had an adjusted p value < 0.05 (**Data set S3**). Genes that showed a significant change in read counts in comparison to the untreated sample are visualized in **Figure 1**. In total, 140 genes fulfilled these criteria upon MEM treatment and 102 genes upon FEP treatment.

Non-essential genes in which the read counts for Tn insertion were at least 5-fold reduced with a high level of significance (adjusted p value < 0.05) are listed in **Table 1**. In total, 24 such genes were identified. 13 of those genes fulfilled these criteria for both MEM and FEP, 5 only for MEM and 6 only for FEP. Most genes were found to be involved in PG synthesis and recycling. The most interesting genes identified in this

screening were those which showed significant reduction in read counts after both MEM and FEP treatment. All TraDIS sequence data were uploaded to ENA (https://www.ebi.ac.uk/ena; accession number PRJEB32702).

We found several genes dedicated to the PG recycling metabolism such as *ampG* and *nagZ*, known to be important for resistance against β-lactam antibiotics (31-36). In addition, the efflux pump genes *mexA* and *mexB* (**Data set S3**) as well as the porin OprF were also identified in our screen and have been described to be involved in antibiotic resistance (37) (**Table 1**). This points out that our approach can identify non-essential genes involved in antibiotic resistance.

A pathway that connects cell wall recycling to PG *de novo* biosynthesis is responsible for the intrinsic resistance of *Pa* to fosfomycin, inhibiting the synthesis of PG by blocking the formation of N-acetylmuramic acid (MurNAc) (38-41). This cell wall salvage pathway comprises anhydro-MurNAc kinase (AnmK), an anomeric cell wall amino sugar kinase (AmgK), MurNAc-6-phosphatase (MupP) and an uridylyl transferase (MurU), together converting 1,6-anhydro-N-acetylmuramic acid (AnhMurNAc) to uridine diphosphate (UDP)-MurNAc, thereby bypassing the fosfomycin-sensitive *de novo* synthesis of UDP-MurNAc. We identified all these four genes **(Table 1)** and conclude that the anabolic recycling pathway may play a critical role to maintain resistance against β-lactam antibiotics at least in strains with high β-lactamase activity.

Moreover, genes encoding the lytic transglycosylases (LTs) Slt and MltG were found to be associated with resistance upon treatment with MEM and FEP **(Table 1)**. Loss of Slt was shown to reduce resistance against β-lactam antibiotics in PAO1 (42). MltG was described as one of several LTs to be inhibited by bulgecin, a sulfonated glycopeptide originally isolated from *P. acidophila* and *P. mesoacidophila*, resulting in a slightly reduced MIC of CAZ and MEM (16).

MepM1 (YebA, PA0667) belongs to a group of murein endopeptidases (EPs) which putatively modulate PG crosslinking (43). A study revealed that the protease CtpA (PA5134) inactivates various EPs, namely PA0667/TUEID40_04290/*mepM1*, PA4404/TUEID40_02316, PA1198/TUEID40_01415, PA1199/TUEID40_01414 and thereby controls the level of PG crosslinking (43). TUEID40_01415 showed also reduced read counts upon treatment with MEM and/or FEP, but to a much lesser extent than MepM1 (**Data set S3**). In addition, the EP MepM2, which is not regulated by CtpA at least in the *Pa* PAK strain (43) seems also to be involved in maintaining antibiotic resistance (**Table 1**).

Furthermore, we identified two so far unknown or uncharacterized candidate genes putatively involved in antibiotic resistance against both MEM and FEP: TUEID40_05543/*tuaC* belongs to the glycosyltransferase 1 family, and TUEID40_03245 encodes an YgfB-like protein with so far unknown function which will be referred here to as YgfB.

Confirmation of selected genes involved in antimicrobial resistance

To validate our TraDIS results, deletion mutants for *mltG, mepM1*, *amgK*, *ygfB*, *tuaC* as well as *ctpA* and a *ctpA*/*mepM1* double mutant were tested for their sensitivity against β-lactam antibiotics. Microbroth dilution assays indicated that deletion of *mltG*, *mepM1, ygfB* and *amgK* reduced the MIC values for all tested β-lactam antibiotics (**Table 2**) except for IMP (Δ*mepM1*) and MEM (Δ*mepM1*, Δ*amgK*), while deletion of *tuaC* showed only a slight reduction in MIC for TZP. The MIC values were reduced below the breakpoint for FEP and ATM in Δ*mltG,* Δ*mepM1*, Δ*ygfB* and Δ*amgK* and for CAZ in Δ*mltG* and Δ*amgK*. Additionally, Δ*mltG* showed MICs below the breakpoint for for PIP, TZP and IMP. These data confirm the validity of the TraDIS screen and

demonstrate the contribution of these genes to resistance against β-lactam antibiotics in the ID40 strain.

Deletion of *ctpA* increased MIC values for MEM, FEP, PIP and ATM. Thus, we hypothesize that increased activity of MepM1 and other CtpA substrates leads to increased resistance. The MIC values of the double mutant Δ*ctpA*Δ*mepM1* for PIP and ATM were lower compared to those of Δ*ctpA* but higher compared to those of the Δ*mepM1* deletion mutant, indicating that the other substrates of CtpA might also contribute to resistance against β-lactam antibiotics and compensate for the loss of MepM1 without the inactivation of CtpA. According to the TraDIS data the most promising CtpA-regulated substrates which may, in combination with MepM1, contribute to β-lactam resistance are TUEID40_02316 and TUEID40_01415 (**Data set S3**). Furthermore, it could be confirmed that deletion of *amgK* results in reduced resistance against fosfomycin as previously described (**Table 2** and **S1**) (39).

For complementation, conditional mutants (Δ*mltG::mltG*, Δ*mepM1::mepM1*, Δ*ctpA::ctpA,* Δ*ygfB::ygfB)* under control of a rhamnose-inducible promoter were generated. In the presence of 0.1% rhamnose complementation could be achieved (**Table S1**).

MltG, MepM1, AmgK and YgfB contribute to β-lactam resistance in ID40 by promoting *ampC* expression

To assess in more detail the reason why the mutants show restored susceptibility to β-lactam antibiotics, we measured the β-lactamase activity of ID40, the different deletion mutants as well as of the laboratory strain PA14, which is sensitive to all tested antibiotics (**Table 2**). As determined by a nitrocefin-based assay, β-lactamase activity was strongly reduced in Δ*mltG,* Δ*mepM1,* Δ*ygfB*, and Δ*amgK* with the most profound reduction in Δ*mltG* showing a β-lactamase activity almost as low as the PA14 strain

(**Figure 2A**), and being sensitive to all tested β-lactam antibiotics (**Table S1**). The β-lactamase activity corresponds directly to the MIC values of the different mutants. Similarly, a higher β-lactamase activity was found in the hyperresistant Δ*ctpA* mutant. Therefore, the changes in MICs are presumably caused by an altered β-lactamase activity in the mutants compared to ID40 wildtype. No significant change in β-lactamase activity was found in Δ*ctpA*Δ*mepM1* compared to Δ*ctpA*, indicating that the uncontrolled levels of other CtpA substrates can compensate the lack of MepM1.

In the ID40 genome, two β-lactamases are encoded (*ampC* and OXA-486/*bla*/*poxB*). For PoxB it has been shown that it does not contribute to β-lactam resistance (44). We quantified the expression level of *ampC* to investigate whether the lower β-lactamase activity is due to reduced *ampC* expression. Semi-quantitative RT-PCR revealed that deletion of *mltG*, *mepM1*, *amgK* or *ygfB* significantly decreased *ampC* mRNA expression **(Figure 2B)**. Deletion of *ctpA*, presumably resulting in a higher level of MepM1 and its other substrates, caused an increase in *ampC* expression. The expression level of *ampC* in the different mutants is in agreement with the levels of β-lactamase activity and the MICs of β-lactam antibiotics that we have measured. These results indicate that the different levels of resistance of the ID40 mutants are due to different levels of *ampC* expression.

4 Discussion

Here, we report the - to our knowledge - first application of TraDIS in a MDR *Pseudomonas aeruginosa* strain and the evaluation of its non-essential resistome upon exposure to two clinically relevant β-lactam antibiotics. The identified genes might represent targets that could be exploited to resensitize resistant strains for treatment with β-lactam antibiotics.

Many of the genes important for high β-lactam resistance found in the TraDIS approach are part of the PG recycling pathway of *Pa* (45) showing its critical role for β-lactam resistance in ID40 (46). A simplified scheme of the PG recycling and synthesis pathway of *Pa* and the genes identified by the TraDIS approach as well as genes described to modulate resistance against β-lactam antibiotics is summarized in **Figure 3**.

Players in the periplasm

The precursors of the PG catabolites contributing to transcriptional regulation of *ampC* are generated in the periplasm. LTs (such as MltG and Slt) together with low molecular mass penicillin-binding proteins, EPs (such as MepM1) and amidases (such as AmpDh2 and 3) cleave the PG layer to facilitate the insertion of new glycan strands and simultaneously release PG degradation products from the matrix into the cytoplasm (45).

Upon treatment with antibiotics, the strongest impact on LTs in the screening was found for *mltG* and *slt*. In addition, and in agreement with previous studies (16, 42, 47), we also found the LTs *mltF* and *mltD* to maintain resistance, but to a lesser extent compared to *slt* and *mltG* (**Data set S3**). On the other hand, *sltB* and *sltH* seem to counteract resistance (**Data set S3**). The recently described MltG may act as a terminase and determine PG chain length (48). Deletion of *mltG* in ID40 significantly reduced *ampC* expression and consequently β-lactamase activity and broke resistance against IMP, FEP, CAZ, PIP, TZP and ATM. These findings confirm the validity of our study and underline the importance of MltG for induction of *ampC* expression in ID40. As previously demonstrated, MltG, Slt and MltD are targets of the LT inhibitor bulgecin reducing the MIC against β-lactam antibiotics (16). According to our data, LTs represent one of the most promising targets for re-sensitization for treatment with β-lactam antibiotics.

EPs may also contribute to the induction of *ampC* expression. As demonstrated, the protease CtpA inactivates and thereby determines the levels of four EPs that control PG crosslinking (43). Of this group, *mepM1* showed the highest reduction of Tn insertion read counts when comparing treatment with antibiotics and control, while Tn insertions in PA1198/TUEID40_01415 had a minor impact on growth in the presence of MEM. In addition, *mepM2*/TUEID40_04881, a further EP which is not regulated by CtpA in PAK (43), also seems to contribute to resistance against β-lactam antibiotics. While deletion of *mepM1* leads to reduced MIC values of β-lactam antibiotics, deletion of *ctpA* leads to hyperresistance probably by deregulating the levels of its substrates. The role of deleted or non-functional CtpA in mediating hyperresistance is further supported by Sanz-García et al. who showed that upon ceftazidime/avibactam treatment, mutations in the *ctpA* gene emerge which leads to resistance (49). Additional deletion of *mepM1* in the *ctpA* mutant reduces MIC values compared to Δ*ctpA* for PIP and ATM, but results in still higher MIC values compared to the *mepM1* deletion mutant, indicating that other CtpA-dependent EPs also contribute to upregulation of *ampC* expression. These data suggest that high activity of MepM1 promotes increased *ampC* expression. Thus, inhibition of several of these EPs could be a possibility to break antibiotic resistance.

Players in the cytoplasm

After PG catabolites have been formed in the periplasm, they are transported into the cytoplasm by the permease AmpG and partly by AmpP (50). In the following the 1,6-anh-MurNAc-peptides are degraded by LdcA, NagZ and AmpD. The amidase AmpD cleaves the peptide chain attached to 1,6-anhMurNAc so that the generated 1,6-anhMurNAc can subsequently be recycled to UDP-MurNAc by the so-called cell wall salvage pathway via AnmK, MupP, AmgK and MurU which bypasses *de novo*

biosynthesis of UDP-MurNAc (38, 39). Finally, UDP-MurNAc is modified by the Mur enzymes to form UDP-MurNAc-pentapeptide (45). Both 1,6-anhMurNAc-peptides and UDP-MurNAc-pentapeptide can bind to the *ampC* regulator AmpR. Thereby, 1,6-anhMurNAc-peptides induce *ampC* expression, while UDP-MurNAc-pentapeptide bound to AmpR represses *ampC* expression.

As observed in our TraDIS data and also shown previously loss of AmpG or NagZ results in decreased amounts of 1,6-anhMurNAc peptides and hence results in increased susceptibility towards β-lactam antibiotics (32, 47). On the other hand, loss of AmpD leads to accumulation of 1,6-anhMurNAc-peptides and therefore an increased *ampC* expression (51) and is a frequent cause of high *ampC* expression in clinical isolates of *Pa* (52, 53).

Players of the cell wall salvage pathway

The individual deletion of each of the 4 (*anmK*, *mupP*, *amgK* and *murU*) genes of the cell wall salvage pathway in PAO1 has been shown to lead to increased β-lactamase activity and a subtle increase of resistance against cefotaxime and CAZ (41). Although this effect could not be explained so far, it was proposed that it might be due to the reduction of the steady state level of the *ampC* repressor UDP-MurNAc-pentapeptide. Consequently, 1,6-anhMurNAc-peptides would be more likely to bind to AmpR and thereby induce *ampC* expression (41). In contrast, another study showed that the deletion of *amgK* also in *Pa* PAO1 had no impact on CAZ and IMP resistance (39), which could be confirmed in our study for all tested β-lactam antibiotics (**Table S1**). Interestingly, in our study we observed that Tn insertions in all genes of the MurU pathway reduce β-lactam resistance. Validation of the screening results using an *amgK* deletion mutant confirmed these results. This finding is indeed counterintuitive and more detailed explorations are necessary to clarify this issue. Presumably, the anabolic

recycling pathway somehow counteracts derepression of *ampC* in the *dacB* background of ID40.

Uncharacterized players

Additionally, we identified several uncharacterized genes in the presented TraDIS screening. Since deletion of the gene *tuaC* showed only a slight reduction in the MICs against some β-lactam antibiotics, we focused on TUEID40_03245, which we termed *ygfB* due to its similarity to the homologous gene in *Ec*. Deletion of *ygfB* resulted in decreased *ampC* expression and β-lactamase activity and broke resistance against FEP and ATM in ID40. To our best knowledge, this gene was so far not described in the context of antibiotic resistance. *ygfB* is located in an operon together with the *pepP*, *ubiH*, *PA14_68970* orthologue and *ubiI*. *ubiI* and *ubiH* are essential genes important for ubiquinone biosynthesis. Similar operon structures are found also in *Ec*, *Acinetobacter baumannii (Ab)* and *Legionella pneumophila (Lp)*. *Pa* YgfB shares 33 % identical amino acids with *Ec* and *Ab* YgfB and 32 % with *Lp* YgfB. Interestingly, the aminopeptidase gene *pepP*, which is encoded adjacent to *ygfB*, was also identified in the TraDIS screening, but Tn insertion read counts indicate that lack of *pepP* might contribute to hyperresistance.

Moreover, experiments with PAO1 Tn mutants suggested that *Pa* YgfB may contribute to virulence in a *C. elegans* infection model (54). In addition, a TraDIS experiment suggested that the *ygfB* orthologue PA14_69010 may play a role for effective colonization in the caecum of mice (55). Thus, the possible role in virulence as well as the ability to modulate antibiotic resistance could mean that this gene is of interest as a target to develop antibiotic adjuvants which might additionally reduce virulence. In further studies we will address the function of YgfB and its specific role in mediating antibiotic resistance.

In conclusion, using TraDIS we identified a set of nonessential genes which are crucial for the induction of *ampC* expression and β-lactam resistance. As shown in a recent study, overexpression of *ampC* is the most frequent cause for the development of resistance in strains capable of expressing *ampC* as shown by the acquisition of mutations in *dacB*, *ampD* and *mpl* after exposure of *Pa* PAO1 WT to increasing concentrations of ceftazidime (56). However, there are additional mechanisms to develop resistance against β-lactam antibiotics which gain more importance when *ampC* expression is hindered. Mutations in *ftsI* leading to modification of PBP3, the target of β-lactam antibiotics, mutations or overexpression of the efflux pump MexAB-OprM as well as large chromosomal deletions led to resistance against ceftazidime albeit to a lower level compared to β-lactamase-dependent resistance (56). This aspect will have to be considered for the development of adjuvants leading to decreased expression of *ampC*.

Nevertheless, the genes identified in our study provide promising candidates as targets to develop novel adjuvants to restore the function of β-lactam antibiotics in MDR *Pa* strains with high AmpC activity.

Material and Methods

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in **Table S2**. Bacteria were cultivated overnight at 37 °C with shaking at 200 rpm in lysogeny broth (LB) containing suitable antibiotics if necessary. Overnight cultures were diluted 1:20 into LB broth containing suitable antibiotics or additives like L-rhamnose and grown for 3 h at 37 °C and 200 rpm. The growth of bacteria in LB at 37 °C in a 24-well-plate was measured using a Tecan Infinite® 200 PRO.

WGS of the ID40 isolate

DNA isolation, library preparation and Illumina sequencing of the ID40 strain are described in Willmann et al. (22).

For Nanopore sequencing, the DNA was isolated using the DNeasy UltraClean Microbial Kit (Qiagen). Library preparation was conducted using the Ligation Sequencing Kit (Oxford Nanopore Technologies). Sequencing was performed on a PromethION sequencer (Oxford Nanopore Technologies) on a FLO-PRO002 flow cell, version R9.

The ID40 genome was assembled using a hybrid assembly approach that combines the Nanopore long reads with exact Illumina short reads. We used the hybrid assembly pipeline pathoLogic (57) with default settings and selected Unicycler (58) as the main assembly algorithm. Further manual scaffolding yielded a single circular plasmid and a circular chromosome. The assembled genome as well as the plasmid sequence was annotated using Prokka (version 1.11) (59, 60).

Generation of the ID40 Tn library

The ID40 Tn mutant library was generated as described previously (55, 61) with some modifications. The donor strain *Ec* SM10 *λ pir* containing pBT20 was grown in LB broth containing 15 µg/ml gentamicin (Gm) and the recipient strain ID40 in LB broth. Cell suspensions of both strains were adjusted to an OD_{600} of 2.0, mixed, and droplets of 100 µl were spotted onto pre-warmed LB agar plates. After incubation at 37 °C for 3 h, mating mixtures were scraped off the plate and resuspended in 12 ml LB broth. 100 µl aliquots were plated onto 100 LB agar plates containing 25 µg/ml irgasan and 75 µg/ml Gm. After overnight growth at 37 °C, all colonies (approximately 5000 per plate) were scraped off the LB agar, resuspended and washed once in LB broth. To eliminate satellite colonies 1 l LB broth containing 75 µg/ml Gm was inoculated with the

suspension to an OD_{600} of 0.1 and grown to an OD_{600} of 1.2. The bacteria were washed once, adjusted to an OD_{600} of 22 in LB broth containing 20 % glycerol and finally aliquots of 1 ml were frozen at -80 °C.

Tn library antibiotic exposure

One aliquot of the Tn library was centrifuged, resuspended in LB broth and grown in 100 ml LB broth overnight. The overnight cultures were diluted 1:100 into 100 ml LB broth with or without 8 µg/ml FEP or 2 µg/ml MEM and grown at 37 °C. After 24 h, the cultures were diluted 1:100 into fresh LB broth and grown for another 24 h at 37°C to enrich viable bacteria.

Library preparation for TraDIS

Genomic DNA of 5 x 10^9 bacteria per sample was isolated using DNeasy® UltraClean® Microbial Kit (Qiagen).

2 µg DNA per sample were sheared into fragments of 300 bp with a M220 Focused-ultrasonicator™ (Covaris) and a clean-up was conducted with a 1.5-fold volume of Agencourt AMPure XP Beads (Beckman Coulter). End repair, A-Tailing and adapter ligation were done using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (NEB). A splinkerette and the P7 indexed primer were used as adapters leading to an enrichment of Tn containing fragments in the PCR (62-64). Fragments were size-selected using Agencourt AMPure Beads and amplified by PCR with one Tn specific and one index primer (Illumina®) in 20 cycles using Kapa HiFi HotStart ReadyMix (Kapa Biosystems). Proper size distribution and quality of the samples were assessed with the Agilent DNA High Sensitivity Kit on a 2100 Bioanalyzer (Agilent Technologies). After a final clean-up, concentration of total fragments and of Tn-containing fragments was measured by qPCR using Kapa SYBR® FAST qPCR Master Mix (2X) Kit (Kapa

Biosystems) with one P5- and one P7-specific or one Tn- and one P7-specific primer, respectively.

Sequencing

Samples were adjusted to 4 nM in resuspension buffer (Illumina®), pooled and denatured with 0.2 N NaOH. Subsequently, the library was diluted to 8 pM in hybridization buffer (Illumina®) and sequenced with the MiSeq Reagent Kit v2 (50 cycles) on a MiSeq™ (Illumina®) with a PhiX (Illumina®) spike-in of 5 % and dark cycles (62).

TraDIS data analysis

Sequencing reads containing the Tn tag were mapped against the ID40 reference genome, using the Bio::TraDIS pipeline (62) in order to determine the locations and numbers of Tn insertions. For each gene, an 'insertion index' was calculated by dividing the number of insertions in a gene by total gene length. The bimodal distribution of insertion indices allows the determination between essential and non-essential genes as recently described (15, 65). Genes that fulfilled the cut-off criteria of an insertion index < 0.0019 for essential or > 0.0026 for nonessential genes were categorized in these groups. All other genes were considered as ambiguous (**Data set S2**).

Statistical analysis was performed using DESeq2 (https://bioconductor.org) (66). Differential genes expression analysis was performed for group comparisons MEM vs. control and FEP vs. control. Genes were categorized as differentially enriched or depleted if the adjusted p value was < 0.05.

Generation of in-frame deletion mutants

In-frame deletion mutants were generated using the suicide plasmid pEXG2 (67) as described in Klein et al. (68). Primers used in this study are listed in **Table S3**.

Generation of complementation constructs

For complementation of the *ctpA, mepM1*, *mltG* and *ygfB* mutant strains, the coding sequences were amplified by PCR from genomic DNA of ID40 and were assembled with the plasmid pJM220 (pUC18T-miniTn7T-gm-rhaSR-PrhaBAD) (69) by Gibson cloning. The constructed plasmids were transformed into *Ec* SM10 λ pir and mobilized by conjugation into the mutant strains as described (70) with some modifications. A triparental mating was conducted by combining the recipient strain together with the mini-Tn7T harbouring SM10 λ pir strain and SM10 λ pir pTNS3, harbouring a Tn7 transposase. Insertion of the mini-Tn7T construct into the *att*Tn7 site was monitored by PCR. Excision of the pJM220 backbone containing the Gm resistance cassette was performed by expressing Flp recombinase from a conjugative plasmid, pFLP2. Finally, sucrose resistant, but Gm and Cb sensitive colonies were verified by PCR.

RNA isolation and qRT-PCR

RNA isolation and qRT-PCR were performed as previously described (68).

β-lactamase activity assay

β-lactamase colorimetric activity assay (BioVision) based on nitrocefin turnover was performed according to manufacturers' instructions after dissolving the bacteria in 5 µl/mg β-lactamase assay buffer and diluting the supernatant of sonified bacteria 1:50 in β-lactamase assay buffer.

Antibiotic susceptibility testing

For antibiotic susceptibility testing by microbroth dilution, bacterial strains were grown overnight at 37 °C in LB medium with or without 0.1 % rhamnose. Physiological NaCl solution was inoculated to a McFarland standard of 0.5 and subsequently 62.5 µl of the suspension were transferred into 15 ml MH broth (+ 0.1 % rhamnose for complementation strains) and mixed well. According to the manufacturers instruction 50-100 µl of the suspension was transferred into each well of a microbroth dilution microtiter plate (Micronaut-S MHK Pseudomonas-2 #E1-099-100, Micronaut-S β-Lactamases #E1-111-040 (Merlin Diagnostika); Sensititre™ GN2F, Sensititre™ EUX2NF (Thermo Fisher Scientific)). Microtiter plates were incubated for 18 h at 37°C and OD_{600} was measured using the Tecan Infinite® 200 PRO. Bacterial strains were considered as sensitive to the respective antibiotic concentration if an OD_{600} value below 0.05 was measured.

E-Tests (Liofilchem) were conducted as previously described (68).

Statistics

Statistics were performed using GraphPad Prism 7.04 software as described for each experiment in the table or figure legends.

Data availability

The whole genome and the plasmid sequence were annotated and submitted to the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena; accession number PRJEB32702. In similar all TraDIS sequence data were uploaded to ENA (https://www.ebi.ac.uk/ena; accession number PRJEB32702). A more detailed description of the files is shown in **Table S4**.

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Figure legends

Figure 1. Resistome of the MDR ID40 determined by TraDIS.

The ID40 Tn library was grown in LB broth with or without 2 µg/ml MEM (A) or 8 µg/ml FEP (B) in 3 independent experiments and then the DNA of the surviving bacteria was used for sequencing of the Tn-genome junctions. Fold change and adjusted p value of the samples grown in antibiotics in comparison to the samples grown in LB broth were calculated with DeSeq2 for all annotated genes. All genes with significantly different (adjusted p value < 0.05) read counts in comparison to the LB control are colored in red.

Figure 2. β-lactamase activity and *ampC* expression in selected deletion mutants.

WT and deletion mutant strains were subcultured and β-lactamase activity was measured by nitrocefin turnover (A) or expression of the β-lactamase gene *ampC* was determined by qRT-PCR (B) in at least 3 independent experiments. Graphs depict means and SD. Student's t-test was performed for each mutant strain in comparison to WT (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Figure 3. **Simplified scheme of PG recycling and synthesis pathway of *Pa* and illustration of proteins identified by TraDIS.**

The bacterial murein matrix is formed by chains of the two alternating amino sugars MurNAc (M) and GlcNAc (G), which are linked by β(1→4) glycosidic bonds. Attached to the MurNAc residues is a pentapeptide side chain which typically is composed of L-alanine-ɣ-D-glutamate *meso*-diaminopimelic acid-D-alanyl-D-alanine (L-Ala-ɣ-DGlu-m-DAP-D-Ala-D-Ala). Cross-links between adjacent glycans are mainly built by

connecting m-DAP of one chain with D-Ala of the other chain. PG synthesis starts in the cytoplasm where fructose-6-phosphate is converted in several steps by GlmS, GlmM and GlmU to UDP-GlcNAc. UDP-GlcNAc is further converted to UDP-MurNAc by Mur enzymes A and B, and subsequently a peptide chain is added by Mur ligases C, D, E & F to form UDP-MurNAc-pentapeptide. An alternative route to generate UDP-MurNAc-pentapeptide starts with the transfer of GlcNAc-1,6-anhMurNAc-peptides (muropeptides) along with GlcNAc-anhMurNAc into the cytoplasm by the permase AmpG. Some muropeptides (however not GlcNAc-1,6-anhMurNAc-peptides) or free peptides may also be transported through AmpP, but its function in cell wall recycling has not been elucidated so far. The imported muropeptides are subsequently degraded by NagZ, L,D-carboxypeptidase LdcA and AmpD, producing D-Ala, GlcNAc, L-Ala-iso-D-glutamate-mDAP-tripeptide and 1,6-anhMurNAc. AnmK then catalyzes the phosphorylation of 1,6-anhMurNAc, generating MurNAc-6P, which is further processed by MupP and the sugar kinase AmgK to MurNAc-□-1P. The uridylyltransferase MurU then converts the latter to UDP-MurNAc, following the formation of UDP-MurNAc-pentapeptide. The phospho-MurNAc-pentapeptide moiety is then transferred by the cytosolic translocase MraY to the lipid carrier undecaprenol phosphate (Und-P) to generate lipid I, which is subsequently catalyzed by MurG to lipid II by adding GlcNAc to it. Lipid II is then flipped into the periplasm (likely by the putative flippase MurJ) where GlcNAc-MurNAc peptides are integrated into the growing PG by high molecular mass penicillin-binding proteins, glycosyltransferases (GTFs) such as FtsW and RodA, transpeptidases (TPs) and DD-carboxypeptidases (CPs). Low molecular mass penicillin-binding proteins, endopeptidases (EPs) as MepM1, lytic transglycosylases as MltG and Slt and amidases as AmpDh2 and AmpDh3 finally cleave the existing PG layer to facilitate the insertion of new glycan strands and simultaneously to release the PG degradation products from the matrix into the

cytoplasm. Under normal conditions the PG precursor UDP-MurNAc-pentapeptide binds to AmpR causing repression of *ampC* transcription. In the case of β-lactam treatment, the turnover of the muropeptides is increased (by blockage of PG-crosslinks) resulting in accumulation of 1,6- anhMurNAc-pentapeptide in the cytoplasm. The 1,6-anhMurNAc-muropeptides are able to displace UDP-MurNAc-pentapeptides from AmpR causing derepression and hence activation of *ampC* transcription. YgfB also modulates *ampC* expression contributing finally to β-lactam resistance, but its specific role in mediating antibiotic resistance remains to be investigated. The proteins found via TraDIS are highlighted with a circle in red for proteins mediating repression and in green for proteins mediating derepression of *ampC* expression. The putative FtsW protein (so far not verified in *Pa*) and the unknown mechanism of YgfB are labeled with interrupted lines. OM, outer membrane; P, periplasm; IM, inner membrane; C, cytoplasm; PG, peptidoglycan; CPs, DD-carboxypeptidases; GTFs, glycosyltransferases; EPs, endopeptidases; LTs, lytic transglycosylases. *AmgK, MupP and MurU cell-wall recycling enzymes found in *Pa* but not in enterobacteria such as *Ec* (38, 40, 41).

Table 1. Meropenem and cefepime resistome in *Pa* ID40. Genes for which insertion sequence abundance was significantly (> 5-fold, adjusted p value < 0.05) reduced upon exposure with 2 µg/ml MEM or 8 µg/ml FEP. Differences in insertion sequence abundance are expressed as mean of the ratio of normalized sequence read numbers of antibiotic treated in relation to the normalized sequence read numbers of the LB control culture of the Tn library. In total three independent experiments were performed.

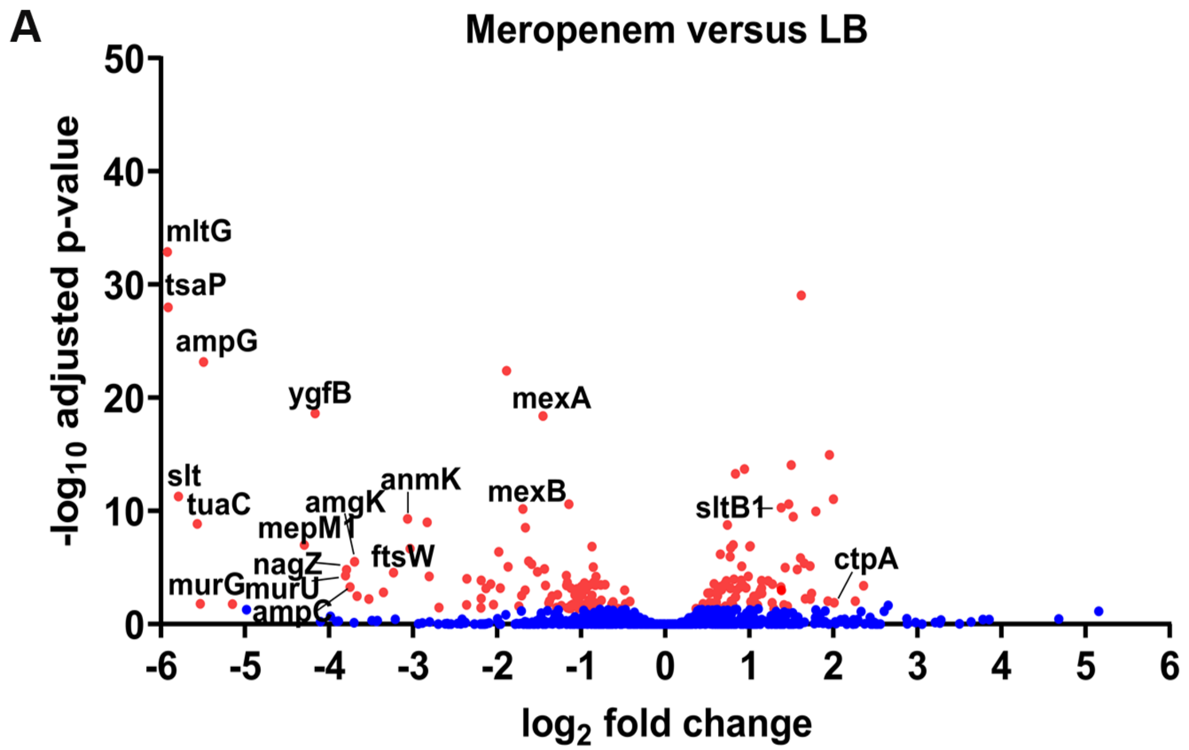
Category				MEM *vs* LB		FEP *vs* LB		Orthologues
	ID	Gene	Name/Function	Ratio	p value	Ratio	p value	
Genes with an adjusted p value < 0.05 and ≥ 5-fold reduction for MEM and FEP								
Resistance	TUEID40_04486	*ampC*	β-lactamase	0.07	0.00052	0.05	3.87E-5	PA14_10790; PA4110
PG synthesis/ recycling	TUEID40_05675	*slt*	Soluble lytic transglycosylase	0.02	5.08E-12	0.03	3.61E-10	PA14_25000; PA3020
	TUEID40_05736	*mltG*	Endolytic murein transglycosylase	0.02	1.32E-33	0.03	1.77E-41	PA14_25730; PA2963
	TUEID40_04290	*mepM1*	Murein-DD endopeptidase	0.05	1.01E-07	0.07	2.29E-06	PA14_08540; PA0667
	TUEID40_02325	*ftsW*	Synthesis of septal peptidoglycan during cell division	0.11	2.76E-05	0.20	0.0019	PA14_57360; PA4413
	TUEID40_02305	*ampG*	Permease	0.02	7.00E-24	0.03	1.01E-22	PA14_57100; PA4393
	TUEID40_05690	*nagZ*	β-N-acetyl-D-glucosaminindase	0.07	1.56E-05	0.04	6.23E-06	PA14_25195; PA3005
	TUEID40_04289	*anmK*	Anhydro-N-acetylmuramic acid kinase	0.12	4.938E-10	0.20	1.62E-06	PA14_08520; PA0666
	TUEID40_04233	*amgK*	N-acetylmuramate/ N-acetylglucosamine kinase	0.08	3.05E-06	0.17	0.0085	PA14_07780; PA0596
	TUEID40_04234	*hddC/ murU*	Similar to N acetyl-muramate alpha-1-phosphate uridylyl-transferase murU of *Pseudomonas putida*	0.07	5.10E-05	0.15	0.0001	PA14_07790; PA0597

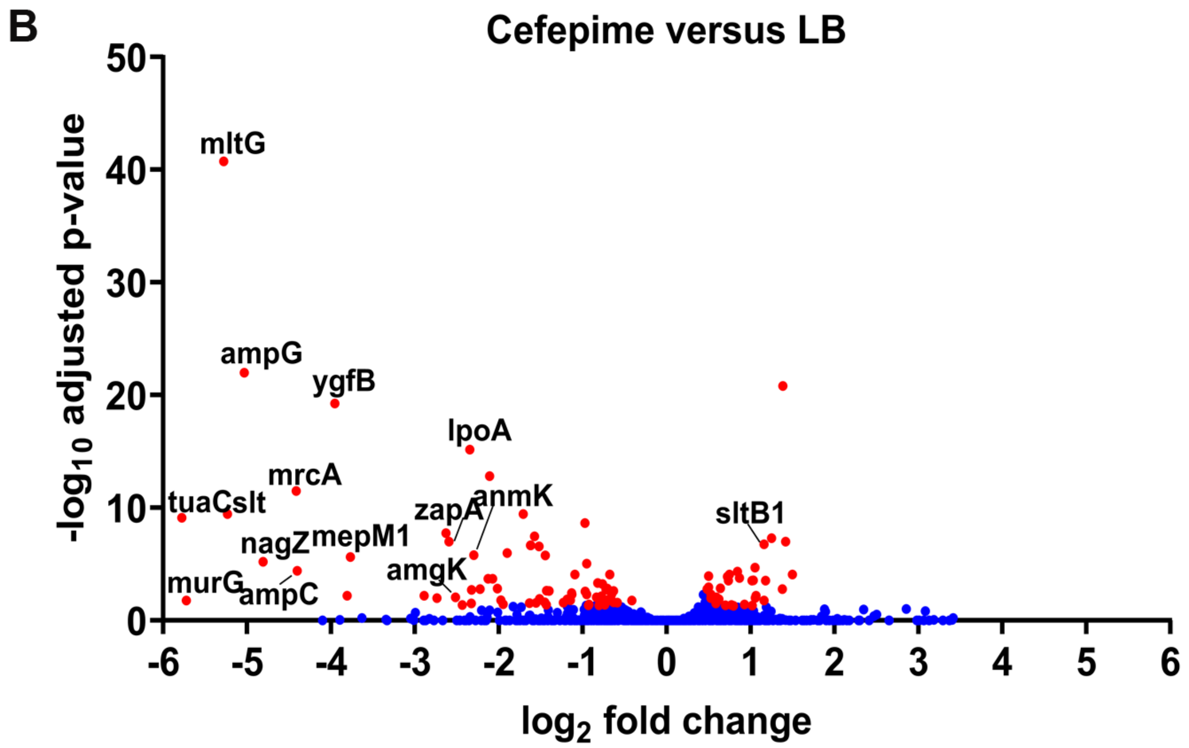
LPS	TUEID40_05537	*wbpE*	UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase	0.10	1.58E-03	0.135	6.43E-03	PA3155
Unknown	TUEID40_03245	*ygfB*	ygfB-like proteins, unknown	0.06	2.35E-19	0.06	5.56E-20	PA14_69010; PA5225
	TUEID40_05543	*tuaC*	Glycosyltransferase family 1	0.02	1.35E-09	0.02	7.79E-10	-
Genes with an adjusted p value < 0.05 and ≥ 5-fold reduction only for MEM								
PG synthesis/ recycling	TUEID40_04881	*mepM2*	Murein DD-endopeptidase MepM, unknown function	0.14	3.60E-12	0.37	0.002	PA14_15100; PA3787
Type IV pili assembly	TUEID40_03621	*tsaP*	Type IV pilus secretin-associated protein; anchors the outer membrane type IV pili secretin complex to the peptidoglycan	0.02	1.02E-28	0.31	3.61E-10	PA14_00210; PA0020
β-barrel assembly	TUEID40_01638	*bepA/ ygfC_1*	β-barrel assembly enhancing protease	0.12	2.17E-07	0.24	1.20E-06	PA14_51320; PA1005
Unknown	TUEID40_03216		putative zinc protease	0.14	6.06E-05	0.21	0.001	PA14_68640; PA5196
	TUEID40_05674	-	Uncharacterized conserved protein YecT. DUF1311 family	0.19	9.84E-05	0.74	1.0	PA14_24990; PA3021
Genes with an adjusted p value < 0.05 and ≥ 5-fold reduction only for FEP								
PG synthesis/ recycling	TUEID40_05519	*gph_2/ mupP*	N-Acetylmuramic Acid 6-Phosphate Phosphatase MupP	0.27	0.14	0.185	4.12E-02	PA14_23210; PA3172
	TUEID40_03006	*mrcA*	Penicillin binding protein 1	0.73	0.40	0.05	3.15E-12	PA14_66670; PA5045
	TUEID40_02335	*lpoA*	Penicillin binding protein activator	1.03	1	0.20	6.87E-16	PA14_57480;PA 4423
Cell division	TUEID40_03247	*zapA*	Cell divison protein zapA	0.39	0.00017	0.17	1.00E-07	PA14_69030; PA5227
Porin	TUEID40_00776	*oprF*	Outer membrane protein F	0,22	0.036	0.20	0.03	PA14_41570; PA1777
Unknown	TUEID40_01298	-	Uncharacterized putative membrane-bound PQQ-dependent dehydrogenase	0.45	0.0018	0.16	1.80E-08	PA14_47350; PA1305

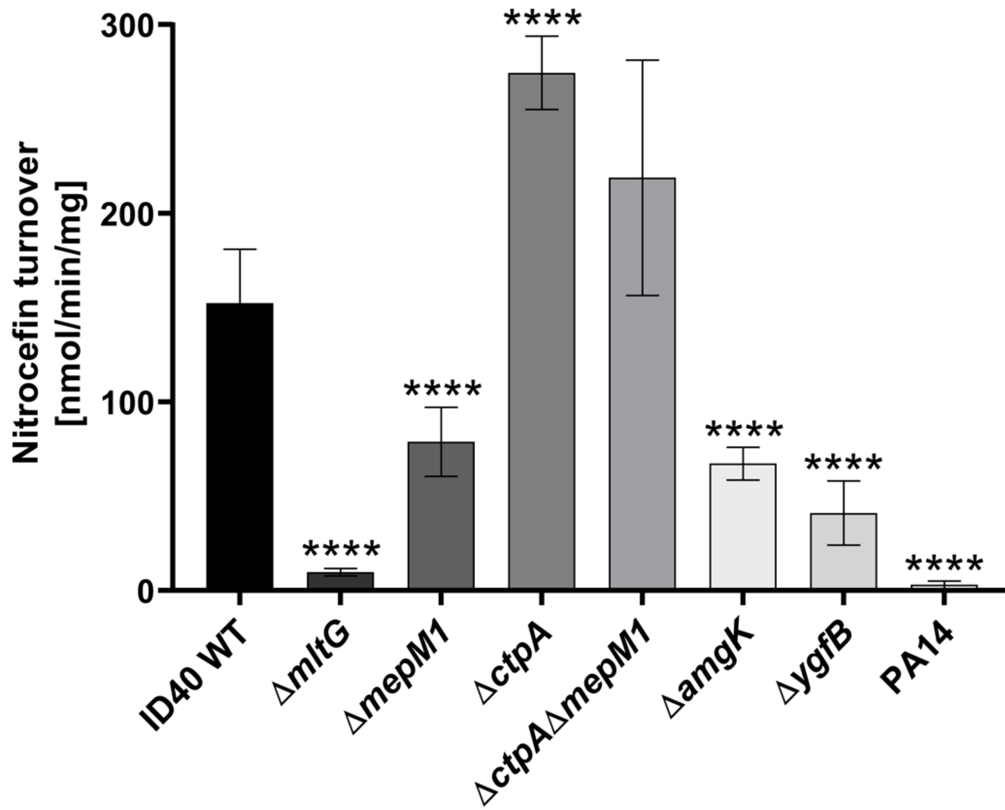
Table 2. Susceptibility of ID40 WT and deletion mutants against β-lactam antibiotics. Minimal inhibitory concentrations (MICs) of ID40 WT and deletion mutant strains were determined by microbroth dilution or by E-Test for fosfomycin. MIC values of the deletion mutants lower than that of ID40 WT are highlighted in green and those below the MIC breakpoint in bold green and light green background. MIC values higher compared to that of ID40 WT are highlighted in red.

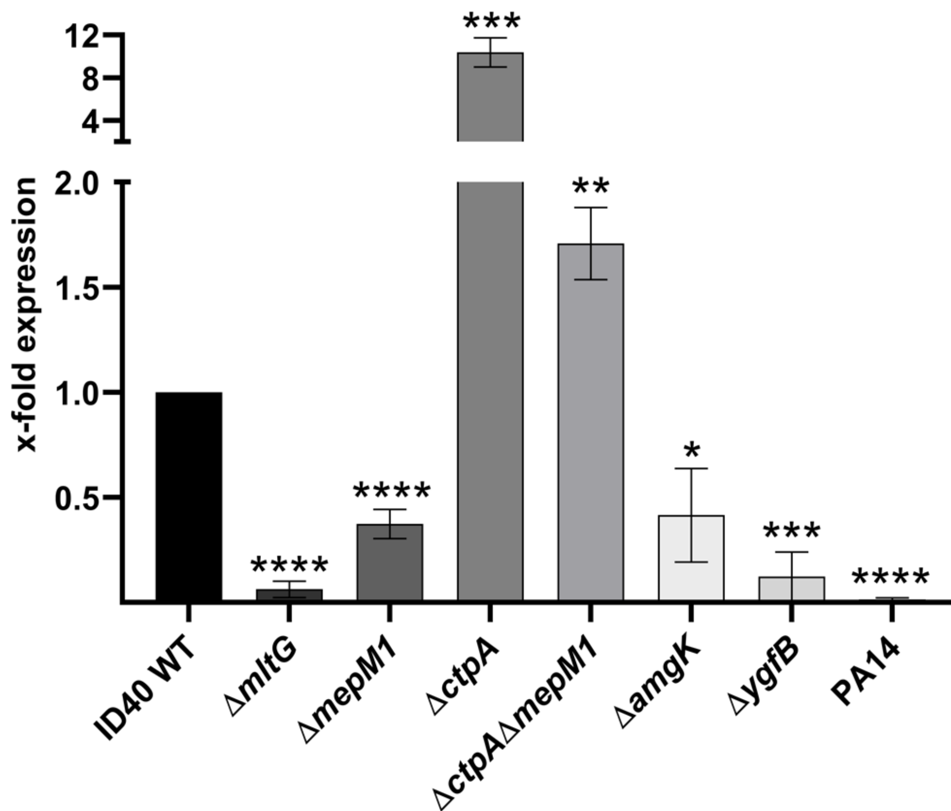
	MIC Breakpoint (mg/L)		ID40 WT	Δ*mltG*	Δ*mepM1*	Δ*ctpA*	Δ*mepM1* Δ*ctpA*	Δ*ygfB*	Δ*amgK*	Δ*tuaC*	PA14
	S ≤	R >									
MEM	2	8	8	4	8	16	16	4	8	8	<0.125
IMP	4	4	32	**4**	32	32	32	8	8	32	<1
FEP	8	8	16	**4**	**4**	32	32	**8**	**8**	16	<1
CAZ	8	8	32	**2**	16	32	32	16	**8**	32	<1
PIP	16	16	128	**<4**	64	>128	128	32	32	128	<4
TZP	16	16	128	**4**	32	128	128	32	32	64	4
ATM	16	16	32	**2**	**16**	>32	32	**16**	**8**	>32	8
FOS *	-	-	96	96	96	96	64	128	48	96	48

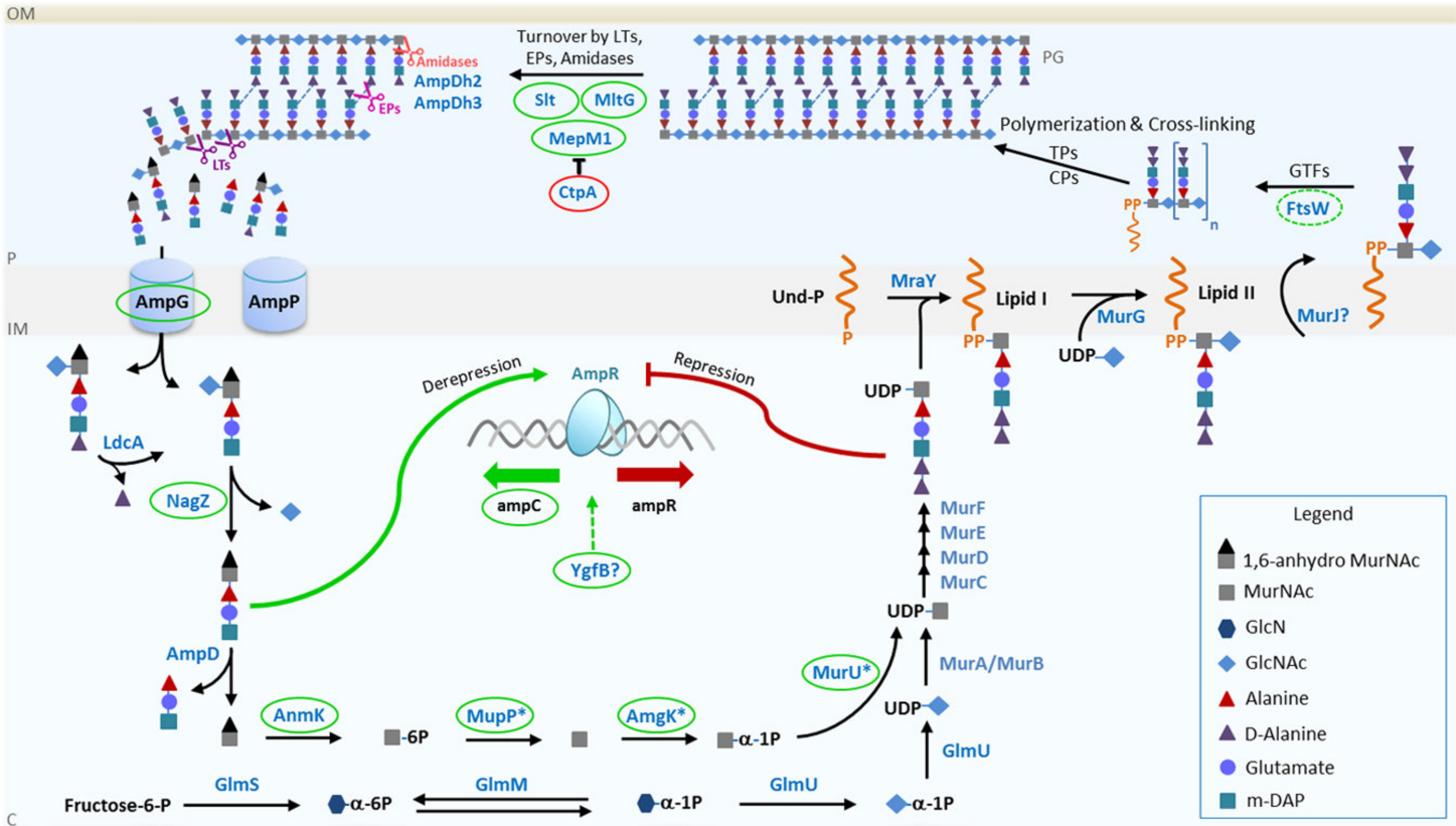
MEM, meropenem; IMP, imipenem; FEP, cefepime; CAZ, ceftazidime; PIP, piperacillin; TZP, piperacillin/tazobactam; ATM, aztreonam; FOS, fosfomycin; *E-test


A
Meropenem versus LB
-log10 adjusted p-value
log2 fold change
mltG
tsaP
ampG
ygfB
mexA
slt
tuaC
amgK
anmK
mexB
sltB1
mepM1
nagZ
ftsW
ctpA
murG
murU
ampC


B
Cefepime versus LB
-log10 adjusted p-value
log2 fold change
mltG
ampG
ygfB
lpoA
mrcA
tuaC
slt
zapA
anmK
sltB1
nagZ
mepM1
amgK
murG
ampC


A
Nitrocefin Assay
Nitrocefin turnover
[nmol/min/mg]
300
200
100
0
****
****
****
****
****
****
ID40 WT
ΔmltG
ΔmepM1
ΔctpA
ΔctpAΔmepM1
ΔamgK
ΔygfB
PA14


B
ampC expression
x-fold expression
12
8
4
2.0
1.5
1.0
0.5
***
**
****
****
*
***
****
ID40 WT
ΔmltG
ΔmepM1
ΔctpA
ΔctpAΔmepM1
ΔamgK
ΔygfB
PA14


OM
PG
P
IM
C
Turnover by LTs,
EPs, Amidases
Amidases
AmpDh2
AmpDh3
EPs
LTs
Slt
MltG
MepM1
CtpA
Polymerization & Cross-linking
TPs
CPs
GTFs
FtsW
PP
n
AmpG
AmpP
Und-P
MraY
Lipid I
MurG
Lipid II
MurJ?
UDP
LdcA
NagZ
Derepression
AmpR
Repression
ampC
ampR
YgfB?
MurF
MurE
MurD
MurC
AmpD
MurU*
MurA/MurB
AnmK
-6P
MupP*
AmgK*
α-1P
GlmU
Fructose-6-P
GlmS
α-6P
GlmM
Legend
1,6-anhydro MurNAc
MurNAc
GlcN
GlcNAc
Alanine
D-Alanine
Glutamate
m-DAP

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides Statt, dass ich die vorliegende Arbeit zum Thema

„Ways to ESKAPE: Identification of potential targets of the cell envelope of *Pseudomonas aeruginosa* for anti-virulence drug development"

eigenständig, ohne unerlaubte Hilfe und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe. Alle sinngemäß und wörtlich übernommenen Textstellen aus Veröffentlichungen oder aus anderwärtigen, fremden Äußerungen habe ich als solche erkenntlich gemacht.

Tübingen, den 12.12.2019