Novel insights into the connection between peptidoglycan recycling and multidrug resistance in *Pseudomonas aeruginosa*

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Abbreviations

ADP	adenosine diphosphate
1,6-anhMurNAc	1,6-anhydro-N-acetyl muramic acid
BAM	β-barrel assembly machinery
bp	base pairs
Ec	*Escherichia coli*
EP	endopeptidase
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GlcNAc	N-acetyl glucosamine
IM	inner membrane
LB	lysogeny broth
LC-MS/MS	liquid chromatography tandem-mass spectrometry
LFQ	label-free quantification
LMM	low-molecular-mass
LPS	lipopolysaccharide
LT	lytic transglycosylase
MDR	multidrug-resistant
MIC	minimal inhibitory concentration
MurNAc	N-acetyl muramic acid
NPN	1-N-phenylnaphthylamine
OM	outer membrane
OMP	outer membrane protein
Pa	*Pseudomonas aeruginosa*
PBP	penicillin-binding protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PG	peptidoglycan
PPIase	peptidyl-prolyl *cis*/*trans* isomerase
qRT-PCR	real-time quantitative polymerase chain reaction

Skp	seventeen kilodalton protein
SurA	survival protein A
T3SS	type III secretion system
Tn	transposon
TraDIS	Transposon-Directed Insertion Sequencing
UDP	uridine diphosphate
WHO	World Health Organisation
WT	wild type
Ye	*Yersinia enterocolitica*

Zusammenfassung

Pseudomonas aeruginosa (*Pa*) ist ein Gram-negatives, fakultativ pathogenes Bakterium und einer der häufigsten Erreger bei nosokomialen Infektionen mit multiresistenten Bakterien. *Pa* wurde von der World Health Organisation (WHO) als einer der drei bakteriellen Erreger eingestuft, gegen die die Entwicklung neuer Antibiotika am dringendsten benötigt wird. Das Ziel dieser Arbeit war, neue Zielstrukturen in *Pa* für die Entwicklung von Adjuvantien zu finden. Im Gegensatz zu Antibiotika sind Adjuvantien nicht gegen überlebenswichtige Strukturen gerichtet mit dem Ziel, das Bakterium zu eliminieren, sondern gegen Resistenz-vermittelnde Mechanismen. Dadurch wäre es mit Adjuvantien möglich, die Resistenz gegen eine oder sogar mehrere Antibiotikaklassen aufzuheben und damit mehrere Therapieoptionen wieder verfügbar zu machen.

Für das hohe Resistenzlevel von *Pa* sind hauptsächlich drei Mechanismen verantwortlich: die Undurchlässigkeit der Außenmembran, die Inaktivierung von Antibiotika durch Enzyme wie die β-Laktamase AmpC und die Expression von Efflux-Pumpen. In den beiden Studien dieser Arbeit sollten Kandidaten für die Entwicklung von Adjuvantien identifiziert werden, die zum ersten und zweiten Mechanismus beitragen.

In der ersten Studie wurde eine Transposonbank des multiresistenten Isolats ID40 hergestellt, das durch eine Überproduktion von AmpC hochresistent ist gegenüber β-Laktam-Antibiotika. Die Transposonbank wurde in Gegenwart von Cefepim oder Meropenem kultiviert und dann wurden mittels Transposon-Directed Insertion Sequencing (TraDIS) diejenigen Mutanten identifiziert, deren Sensitivität wieder hergestellt war. Neben vielen bekannten Resistenzgenen wurden 3 Gene, die beim Recycling von Peptidoglycan eine Rolle spielen, sowie ein uncharakterisiertes Gen als aussichtsreichste Kandidaten identifiziert, da sie für das Wachstum sowohl in Anwesenheit von Cefepim als auch von Meropenem notwendig waren. Die Deletion dieser Gene hatte eine stark reduzierte *ampC*-Expression und β-Laktamase-Aktivität zur Folge und dadurch eine wiederhergestellte Sensitivität gegenüber mehreren β-Laktam-Antibiotika. Alle vier Gene sind vielversprechende Kandidaten für die Entwicklung von Adjuvantien für die Kombinationstherapie mit β-Laktam-Antibiotika von multiresistenten *Pa*-Stämmen.

In der zweiten Studie wurde der Einfluss von Proteinen untersucht, die den Einbau von Außenmembranproteinen in die Außenmembran fördern. Ein Mangel des periplasmatischen Chaperons SurA führte zu einer starken Veränderung in der Zusammensetzung der Außenmembranproteine, verminderter Virulenz und erhöhter Sensibilität gegenüber verschiedensten Antibiotika. Daher könnte SurA eine gute Möglichkeit sein, die Virulenz von *Pa* zu reduzieren und die Sensitivität multiresistenter Stämme wiederherzustellen.

Abstract

Pseudomonas aeruginosa (*Pa*) is a Gram-negative opportunistic pathogen and a frequent cause of nosocomial infection with multidrug-resistant (MDR) bacteria. *Pa* was classified as one of the three pathogens with the highest priority for the development of novel antibiotics by the World Health Organisation (WHO). The aim of this thesis was to identify novel targets in *Pa* for the development of antibiotic adjuvants. In contrast to antibiotics, adjuvants do not target structures essential for viability to directly eliminate a pathogen but the mechanism which confers resistance. Therefore, adjuvants could restore the sensitivity to one or even several classes of antibiotics and thereby restore several therapy options.

The high resistance of *Pa* is mainly caused by three mechanisms: Low permeability of the outer membrane (OM), inactivation of antibiotics by enzymes like the β-lactamase AmpC and the expression of efflux pumps. In two studies, the aim was to identify targets contributing to the first and the second mechanism.

In the first study, a transposon (Tn) library was generated in the MDR isolate ID40, which is highly resistant to β-lactam antibiotics due to an overproduction of AmpC. The Tn library was grown in presence of cefepime or meropenem at the breakpoint concentration and then mutants with restored sensitivity were identified by transposon-directed insertion sequencing (TraDIS). Besides a lot of known resistance genes, we identified three genes involved in peptidoglycan (PG) recycling as well as a gene with unknown function as most promising candidates, since they were found to be necessary for growth in both the presence of cefepime or meropenem. Deletion of these genes led to strongly reduced *ampC* expression, β-lactamase activity and consequently to restored sensitivity against several β-antibiotics. All four candidates are promising targets for adjuvants for therapy in combination with β-lactam antibiotics in MDR *Pa* strains.

In the second study, we investigated the impact of proteins promoting the insertion of outer membrane proteins (OMP) into the OM. Deprivation of the periplasmic chaperone SurA resulted in a drastically altered OMP composition, impaired virulence and enhanced sensitivity to various antibiotics. SurA could therefore serve as a target to reduce virulence of *Pa* and to restore antibiotic sensitivity in MDR strains.

List of publications

Michael S. Sonnabend*, Kristina Klein*, Sina Beier, Angel Angelov, Robert Kluj, Christoph Mayer, Caspar Groß, Kathrin Hofmeister, Antonia Beuttner, Matthias Willmann, Silke Peter, Philipp Oberhettinger, Annika Schmidt, Ingo B. Autenrieth, Monika Schütz and Erwin Bohn (**2019**)

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

Antimicrob Agents Chemother, Epub 09.12.2019

*equal contribution

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

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

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*equal contribution

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

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

Int J Med Microbiol 2019 Jul;309(5): 344-350. doi: 10.1016/j.ijmm.2019.05.008.

Personal contribution

A more detailed statement about the contributions of the different authors in the two main publications of this thesis can be found in the separate table “Erklärungen zum Eigen- und Fremdanteil”.

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

For this study, I isolated the DNA for Nanopore sequencing, annotated the ID40 genome and mainly generated the Tn library. Moreover, I conducted the growth in antibiotics, the main part of the library preparation, the sequencing for the TraDIS experiment and most of the TraDIS data analysis, the β-lactamase activity assay and the qRT-PCR experiments.

In addition, I generated the deletion mutants Δ*amgK*, Δ*mepM1*, Δ*mltG*, Δ*tuaC* and Δ*ygfB* and participated partly in the antibiotic susceptibility testing experiments by microbroth dilution.

The manuscript was written by Kristina Klein, Erwin Bohn and me in equal contribution.

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

For this study, I performed the NPN assay, generated the overexpression construct for the purification of SurA and conducted the protein purification and size-exclusion chromatography of SurA for the production of antibodies. I did the preparation of OM fractions for mass spectrometry as well as data analysis of the mass spectrometry data and most of the western blots. Moreover, I generated the Δ*bamB* deletion mutant. The Δ*bamC* and Δ*hlpA* mutants were generated by Lisa Frank (MD student) under my supervision.

I participated partly in the RNA isolation and conducted the main part of the qRT-PCR experiments. The experiments in *Galleria mellonella* and the antibiotic susceptibility testing by E-tests were conducted by Kristina Klein and me in equal contribution.

The manuscript was written by Kristina Klein, Erwin Bohn and me in equal contribution.

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

This review was mainly written by Erwin Bohn with contribution of Kristina Klein and me.

Introduction

Pseudomonas aeruginosa

Pa is a Gram-negative, rod-shaped bacterium and one of the most frequent causes for nosocomial infections with MDR bacteria. It was first described by Carle Gessard in 1882 as an organism producing the pigment pyocyanin, which is responsible for the characteristic colour of *Pa* cultures (Gessard, 1984).

Pa occurs ubiquitously and can be isolated from various environments like plants, animals, sinks, contact lens solutions and even from antiseptic solutions (Pollack, 1995; Harris et al., 1984; Pitt, 1998). This ability allows *Pa* to colonize also medical environments and equipment like mechanical ventilation devices and catheters, which is one of the most important infection routes in nosocomial infections with *Pa* (Park et al., 2011; Willmann et al., 2014; Percival et al., 2015). *Pa* can be part of the human microbiome with colonization rates between 2.6 and 24 % of the intestinal tract (Morrison and Wenzel, 1984), which can exceed 50 % during hospitalization (Pollack, 1995).

Colonization with *Pa* usually does not lead to infection in immunocompetent people. However, impaired immunity, cystic fibrosis or disruption of the intestinal microbiota by antibiotic treatment are risk factors for *Pa* infection (Morrison and Wenzel, 1984; Pollack, 1995; Bonten et al., 1999; Takesue et al., 2002; Williams et al., 2010). Therefore, *Pa* is a problem mainly in intensive care units (Richards et al., 1999; Spencer, 1996) causing bacteremia, pneumonia, wound or urinary tract infections (Page and Heim, 2009). The mortality rates can be very high, especially in sepsis and ventilator-associated pneumonia, where mortality rates of 30 % (Williams et al., 2010) and up to 60 % (Page and Heim, 2009) have been observed.

Pa displays a wide variety of virulence factors which contribute to infection. It is able to move in solution with its single polar flagellum as well as on solid surfaces using type IV pili. Type IV pili are in addition the most important adhesins of *Pa*, promoting adhesion to abiotic surfaces as well as to host cells and thereby play an important role in the course of infection (Kipnis et al., 2006; Kohler et al., 2000; Yeung et al., 2009).

After adhesion, *Pa* employs a lot of different effector proteins which are mainly secreted by one of the secretion systems. Effectors of the type I and type II secretion systems are secreted into the extracellular environment and mainly involved in the

degradation of extracellular matrix and complement proteins like the alkaline protease AprA (Laarman et al., 2012) and the elastases LasA and LasB (Mariencheck et al., 2003). But also the AB toxin exotoxin A is secreted by the type II secretion system which causes the adenosine diphosphate (ADP)-ribosylation of elongation factor 2 of the host cell leading to apoptosis (Jenkins et al., 2004). The most important effectors for virulence of *Pa* are secreted by the type III secretion system (T3SS), which has been shown to be crucial for virulence (Schulert et al., 2003; Shaver and Hauser, 2004; Roy-Burman et al., 2001). The effectors ExoY, ExoT, ExoS and ExoU of the T3SS are directly injected into the host cell. *Pa* strains produce either ExoS or ExoU, whereas ExoU is estimated to be 100 times more potent than ExoS (Kipnis et al., 2006; Hauser, 2009; Gellatly and Hancock, 2013). The induction of cell death by these different effectors probably aims to cause breaches in the epithelial barrier and allow *Pa* to reach deeper tissue (Hauser, 2009). Additional important virulence factors are pyocyanin, which causes oxidative stress to the host by disrupting the host catalase (Gellatly and Hancock, 2013; Lau et al., 2004), iron chelators like pyoverdine and lipopolysaccharide (LPS), which plays an important role in sepsis (Ramachandran, 2014). Moreover, *Pa* is able to produce biofilm providing protection against antibiotics (Hall-Stoodley and Stoodley, 2009; Lieleg et al., 2011). Biofilm formation is particularly problematic in patients suffering from cystic fibrosis, where biofilm together with thickened mucus in the lung and multidrug resistance of *Pa* make treatment extremely difficult (Donlan and Costerton, 2002).

Antibiotic resistance mechanisms

The prevalence of MDR *Pa* strains is rising despite the use of combination therapies (Lister et al., 2009; Moore and Flaws, 2011) and poses a serious threat for immunocompromised and hospitalized people. In more and more cases colistin serves as antibiotic of last resort despite its heavy side effects (Livermore, 2002; Biswas et al., 2012). *Pa* belongs to the group of ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) and carbapenem-resistant *Pa* was rated by the WHO as the species for which novel antibiotics are most urgently needed besides carbapenem-resistant *Acinetobacter baumannii* and extended-spectrum β-lactamase producing *Enterobacteriaceae* (Tacconelli et al., 2018). Beside

acquired resistance, high levels and prevalence of antibiotic resistance in *Pa* are mainly due to three resistance mechanisms: The low permeability of the OM, constitutive expression of efflux pumps and enzymes like AmpC which inactivate antibiotics (Strateva and Yordanov, 2009).

The integrity of the OM in Gram-negative bacteria functions as an important barrier against antibiotics. The permeability of the OM of *Pa* has been estimated to be 12-100 fold lower than in *Escherichia coli* (*Ec*) (Nikaido, 1986). Therefore, the permeation of antibiotics is slow or completely prevented and together with the export by efflux pumps, the amount of antibiotic molecules in the bacterial cell is reduced, allowing resistance-conferring enzymes to efficiently inactivate them (Hancock and Speert, 2000).

Pa employs 12 RND-type efflux pumps (Lister et al., 2009) to efflux a wide variety of substances. The main efflux pumps MexAB-OprM and MexXY-OprM are able to export β-lactam antibiotics, fluoroquinolones, chloramphenicol, tetracycline, macrolides and trimethoprim (Livermore, 2002; Schweizer, 2003). Mutations in the regulators (MexR or MexZ, respectively) can furthermore cause overexpression of efflux pumps leading to increased resistance (Islam et al., 2004; Vogne et al., 2004; Evans et al., 2001).

Resistance to β-lactam antibiotics is mainly caused by the expression of β-lactamases, especially *ampC*. The expression level of *ampC* is low in wild type (WT) strains (Sanders and Sanders, 1986) but can be induced by β-lactam antibiotics and β-lactamase inhibitors (Lindberg et al., 1988; Lister et al., 1999; Stobberingh, 1988; Weber and Sanders, 1990). Overproduction of AmpC can also be due to chromosomal mutations like in the *dacB* gene encoding penicillin-binding protein 4 (PBP4) (Moya et al., 2009). Resistance to carbapenems can be achieved by either expressing metallo-β-lactamases like GIM, IMP, SPM and VIM (Castanheira et al., 2004; Gales et al., 2003; Nordmann and Poirel, 2002) or by inactivation of the porin OprD, which is exploited by carbapenems to cross the OM (Margaret et al., 1989; Sakyo et al., 2006; Satake et al., 1991; Trias and Nikaido, 1990). OprD inactivation is mostly achieved by changes in the promoter sequence, premature interruption of transcription (Wolter et al., 2008; Yoneyama and Nakae, 1993; El Amin et al., 2005) or mutations in the *oprD* gene causing frame-shift or premature stop codons (Pirnay et al., 2002).

Peptidoglycan turnover and *ampC* expression

Overproduction of the β-lactamase AmpC plays an important role in the resistance of *Pa* against β-lactam antibiotics and is intimately connected with peptidoglycan (PG) turnover. The *ampC* gene is regulated by AmpR, which can either serve as an activator when bound to 1,6-anhydro-N-acetyl muramic acid (1,6-anhMurNAc)-peptides or as a repressor when bound to uridine diphosphate N-acetyl muramic acid (UDP-MurNAc)-pentapeptide (Jacobs et al., 1997). These molecules are part of the PG recycling pathway and therefore, changes in PG turnover by chromosomal mutations or the action of β-lactam antibiotics can have a strong impact on *ampC* expression and thereby on the level of resistance against β-lactam antibiotics.

The cell wall composed of PG is an essential structure providing shape and protection against cell lysis by osmotic pressure. It consists of a heteropolysaccharide of MurNAc and N-acetyl glucosamine (GlcNAc) linked by glycosidic bonds with short peptide chains attached, which are up to 5 amino acids long (Dhar et al., 2018). The cross-links mainly between the third residue of the one and the fourth residue of the other peptide chain form a mesh-like structure conferring high stability (Dhar et al., 2018).

Peptidoglycan turnover

For better comprehension, a graphic representation of the PG turnover processes can be found in Figure 3 of the publication “Identification of drug-resistance determinants in a clinical isolate of *Pseudomonas aeruginosa* by high-density transposon mutagenesis” in the appendix of this thesis.

The PG layer is constituted by incorporating the precursor molecule UDP-MurNAc-pentapeptide. This precursor is either synthesized *de novo* or by recycling of PG degradation products in the cytoplasm. The *de novo* biosynthesis starts with fructose-6-phosphate, which is converted to UDP-MurNAc-pentapeptide by the addition of UDP and peptides by the Glm and Mur enzyme groups (Mengin-Lecreulx and van Heijenoort, 1994, 1996; Barreteau et al., 2008). After transfer to an undecaprenol carrier, this PG precursor is transported across the inner membrane (IM) into the periplasm by so far undetermined flippase enzymes, presumably FtsW (PA4413) and MviN (PA4562) (Azzolina et al., 2001; Mohammadi et al., 2011; Dhar et al., 2018). In the periplasm the precursor is incorporated into the existing PG layer. This process is facilitated by high molecular mass penicillin-binding proteins (PBPs), which catalyse

the transglycosylation between MurNAc and GlcNAc moieties as well as the transpeptidation between the peptide chains (Ishino et al., 1980; Handfield et al., 1997; Legaree et al., 2007; Chen et al., 2017).

On the catabolic side, degradation of PG is mainly mediated by low molecular mass (LMM) PBPs, lytic transglycosylases (LTs) and amidases (Park and Uehara, 2008). *Pa* harbours three LMM PBPs (PBP4/DacB, PBP5/DacC and PBP7/PbpG), which act as carboxypeptidases and/or endopeptidases (EP) and cleave the crosslinks between the peptide chains (Ropy et al., 2015). Members of the LT family release PG degradation products like GlcNAc-1,6-anhMurNAc from the PG layer (Höltje et al., 1975). In *Pa*, 11 LTs have been identified, exhibiting exolytic as well as endolytic activity (Lee et al., 2017b). Finally, the periplasmic amidases cleave between the peptide chain and the muramyl moieties, either in the PG layer or in the already released degradation products like GlcNAc-1,6-anhMurNAc-peptides (Zhang et al., 2013). For PG recycling, the two periplasmic amidases AmpDh2 and AmpDh3 are important, which are homologues of *Ec* AmiD (Juan et al., 2006) and related to the cytoplasmic amidase AmpD (Moya et al., 2008; Zhang et al., 2013). Since AmpDh2 carries a signal sequence of OM lipoproteins like AmiD from *Ec*, AmpDh2 is thought to be the AmiD homologue in *Pa*, while AmpDh3 is an extra amidase of *Pa* (Moya et al., 2008).

GlcNAc-1,6-anhMurNAc-peptides generated by the different periplasmic enzymes are then transported into the cytoplasm by the permease AmpG and probably its homologue AmpP (Perley-Robertson et al., 2016; Kong et al., 2010). In the cytoplasm, the GlcNAc moiety is cleaved by NagZ (Stubbs et al., 2008) and the peptide chain is removed by the cytoplasmic amidase AmpD, resulting in free peptides and 1,6-anhMurNAc (Zhang et al., 2013). In addition, the L,D-carboxypeptidase LdcA removes the terminal D-alanine from the peptide part (Korza and Bochtler, 2005).

1,6-anhMurNAc and free peptides can then be recycled and reused for the synthesis of the PG precursor UDP-MurNAc-pentapeptide which saves energy compared to *de novo* biosynthesis. In *Pa*, 1,6-anhMurNAc is converted to UDP-MurNAc by the so-called salvage pathway including the sequential action of the enzymes AnmK, MupP, AmgK and MurU (Borisova et al., 2014; Gisin et al., 2013). The free peptides can then be ligated again to UDP-MurNAc by Mpl (Mengin-Lecreulx et al., 1996). Subsequently, MurF adds D-alanine-D-alanine to the peptide chain resulting in UDP-

MurNac-pentapeptide (Duncan et al., 1990) which can then again be transferred into the cytoplasm and integrated into the PG layer. The function of Mpl and MurF has only been demonstrated in *Ec* so far, but homologues are also found in *Pa* (Dhar et al., 2018).

ampC *expression and resistance against β-lactam antibiotics*

Since resistance to β-lactam antibiotics strongly depends on the expression level of the inducible *ampC* gene, which is regulated positively or negatively by AmpR when bound to 1,6-anhMurNAc-peptides or UDP-MurNAc-pentapeptide, respectively, changes in PG recycling can have an impact on β-lactam resistance in *Pa*.

Mutations in the *dacB* gene encoding the LMM-PBP PBP4 are common in clinical isolates resistant against β-lactam antibiotics, probably because the loss of PBP4 results in a higher amount of PG catabolites that finally lead to *ampC* expression (Lee et al., 2015a; Moya et al., 2009; Dhar et al., 2018). The other group of enzymes beside LMM PBPs whose products can end as *ampC*-inducing 1,6-anhMurNAc-peptides are LTs. In *Pa*, the loss of Slt and MltF has been shown to decrease resistance against β-lactam antibiotics (Cavallari et al., 2013). Additionally, the inhibition of Slt, MltD and MltG by bulgecin results in an enhanced sensitivity of *Pa* PAO1 against ceftazidime and meropenem (Dik et al., 2019). Loss of SltB1 and MltB1 on the other hand lead to increased resistance against β-lactam antibiotics, however, this effect was independent from *ampC* expression (Cavallari et al., 2013; Lamers et al., 2015).

After generation in the periplasm, muropeptides are transported into the cytoplasm. If the main permease AmpG is inactivated, the PG catabolites cannot be processed to 1,6-anhMurNAc-peptides in the cytoplasm and therefore *ampC* expression is abrogated (Korfmann and Sanders, 1989). Loss of AmpG can even restore sensitivity of pan-resistant clinical *Pa* isolates against β-lactam antibiotics (Zamorano et al., 2011; Dhar et al., 2018).

In the cytoplasm, NagZ is important for the generation of 1,6-anhMurNAc-peptides by removing GlcNAc from the muropeptides. Therefore, loss of NagZ leads to decreased *ampC* expression and β-lactam resistance (Asgarali et al., 2009; Zamorano et al., 2010). In contrast, AmpD reduces the 1,6-anhMurNAc-peptide pool by cleaving the peptide chain. Therefore, loss of AmpD leads to enrichment of 1,6-anhMurNAc-peptides in the cytoplasm and consequently strong *ampC* induction and

high levels of resistance against β-lactam antibiotics (Jacobs et al., 1995). Mutations in *ampD* are the most common cause for β-lactam resistance by *ampC* overexpression in clinical isolates (Juan et al., 2005).

The role of AnmK, MupP, AmgK and MurU in antibiotic resistance has not been clearly elucidated so far. In one study, it has been shown that deletion of each of these genes led to increased resistance against β-lactam antibiotics in PAO1 (Fumeaux and Bernhardt, 2017). This could be explained by a reduced UDP-MurNAc-pentapeptide pool without a functional recycling pathway and thereby reduced repression of *ampC*. However, another study could not see a change in the minimal inhibitory concentration (MIC) against β-lactam antibiotics of a Δ*amgK* deletion in the same strain (Borisova et al., 2014).

Finally, also loss of the regulator AmpR obviously enhances sensitivity against β-lactam antibiotics since *ampC* expression is reduced without activation (Kong et al., 2005; Kumari et al., 2014).

Outer membrane protein biogenesis

Composition of the outer membrane

Beside expression of β-lactamases and efflux pumps, the integrity of the OM of Gram-negative bacteria is an important property conferring resistance by already preventing many molecules to reach their targets inside the bacterial cell. Most of our current knowledge about OMP biogenesis was derived from other Gram-negative species, especially *Ec* and *Yersinia enterocolitica* (*Ye*) (Sklar et al., 2007; Weirich et al., 2017), but also *Neisseria meningitidis* (Volokhina et al., 2011). The OM is composed of an asymmetric bilayer consisting of an inner phospholipid and an outer LPS leaflet (Patel et al., 2017). Due to its amphiphatic character conferred by the hydrophobic lipid A core and the hydrophilic O-antigen, LPS provides an efficient barrier (Nikaido, 2003, 2005). Additionally, the OM harbours a lot of lipoproteins which are anchored in the inner leaflet (Narita and Tokuda, 2017) and OMPs that span the OM and connect extra- and intracellular space (Choi and Lee, 2019).

LPS is transported to the OM by the Lpt pathway after the formation in the cytoplasm and transfer to the periplasm by the $LptB_2CFG$ complex (Narita and Tokuda, 2009; Sperandeo et al., 2017). LPS molecules are extracted by the $LptB_2CFG$ complex and transferred to LptA which functions as a shuttle protein across the periplasm (Okuda

et al., 2012). The LptDE complex in the OM then inserts LPS into the OM (Gu et al., 2015; Li et al., 2015; Sperandeo et al., 2017).

The exact mechanism of phospholipid transport to the OM still remains to be resolved. Recent data suggest that phospholipids are translocated across the IM by a MlaFEDB complex and then transferred to the periplasmic shuttle MlaC (Hughes et al., 2019). Maintenance of the asymmetry of the OM seems to be important since there are different mechanisms involved in this process: Proteins of the Mla pathway mediate retrograde transport of phospholipids, that were mislocalized into the outer leaflet (Choi and Lee, 2019). PagP is responsible for the transfer of the acyl-chain of surface-exposed phospholipids to lipid A of LPS (Bishop et al., 2000) and the phospholipase PldA degrades phospholipids in the outer leaflet of the OM (Dekker, 2000).

Lipoproteins are transported and inserted into the OM by the Lol pathway. After production in the cytoplasm, lipoproteins are translocated across the IM by the Sec or the Tat system (Konovalova and Silhavy, 2015; Narita and Tokuda, 2017), then extracted by the $LolCD_2E$ complex and transferred to the shuttle protein LolA (Narita and Tokuda, 2017; Yakushi et al., 2000). LolA transports the lipoprotein across the periplasm to LolB in the OM, which inserts the lipoprotein (Grabowicz, 2018).

Outer membrane protein biogenesis

OMPs are transported to the OM in a partially similar fashion like lipoproteins. OMPs contain usually 8 to 24 antiparallel β-strands, forming a β-barrel in the OM by connecting the first and last β-strand by hydrogen bonds (Jacob-Dubuisson et al., 2009). The protein is synthesized in the cytoplasm, held in an unfolded confirmation by SecB (Xu et al., 2000) and translocated into the periplasm by the SecYEG translocon in the IM (Crane and Randall, 2017). Subsequently, the unfolded OMPs are transferred to periplasmic chaperones, which serve as shuttle proteins and transport the OMP to the β-barrel assembly machinery (BAM) complex in the OM (Ruiz et al., 2006).

For the transport between IM and OM, two possible pathways have been described in Gram-negative bacteria: Either survival protein A (SurA) or the serine EP DegP together with the seventeen kilodalton protein (Skp) can transfer the OMP to the BAM complex. The SurA pathway is the preferred one and the DegP/Skp pathway is thought to be a substitute in case the SurA pathway is disturbed (Sklar et al., 2007).

SurA exerts peptidyl-prolyl-*cis/trans*-isomerase (PPIase) as well as chaperone activity (Behrens et al., 2001) and was originally identified as a protein necessary for survival in stationary phase (Tormo et al., 1990; Sklar et al., 2007). It is composed of a big N-terminal, two parvulin-like and a small C-terminal domain (Rahfeld et al., 1994; Behrens et al., 2001; Bitto and McKay, 2002). As shuttle from the SecYEG translocon to the BAM complex, it functions as a holdase (Malinverni and Silhavy, 2011) which holds OMPs in an unfolded confirmation to prevent misfolding in the periplasm. SurA binds preferentially to unfolded OMPs (Bitto and McKay, 2004) and porins (Behrens et al., 2001). At the OM, SurA interacts with BamA to transfer its substrate to the BAM complex (Sklar et al., 2007). Loss of SurA leads to increased sensitivity against hydrophobic antibiotics, SDS and bile salts (Lazar and Kolter, 1996; Rouviere and Gross, 1996; Weirich et al., 2017), reduced OMP levels and OM density (Sklar et al., 2007) and appearance of aberrant OMPs in the periplasm (Behrens et al., 2001; Lazar and Kolter, 1996; Onufryk et al., 2005; Rouviere and Gross, 1996).

DegP was described as an EP with temperature-dependent chaperone activity (Strauch et al., 1989; Lipinska et al., 1990). It serves as a holdase at low temperature and as protease at high temperature (Spiess et al., 1999). DegP was shown to be responsible for the degradation of unfolded OMPs in a *surA*/*skp* double mutant depletion strain (Sklar et al., 2007). Skp forms homotrimers and binds to denatured OMPs. Loss of Skp leads to reduced levels of various OMPs (Chen and Henning, 1996). DegP and Skp can together compensate for the loss of SurA in *Ec* (Rizzitello et al., 2001; Sklar et al., 2007; Malinverni and Silhavy, 2011). Absence of DegP or Skp results in the accumulation of unfolded OMPs in the periplasm and in reduction of OM integrity (Chen and Henning, 1996; Dartigalongue et al., 2001; Missiakas et al., 1996).

After delivery by the periplasmic chaperones, OMPs are inserted into the OM by the BAM complex. The BAM complex consists of the integral β-barrel protein BamA and four lipoproteins (BamB-E) (Konovalova et al., 2017). The exact mechanism of insertion is still not clear and two models are discussed: Either the β-barrel is formed in the BamA pore and then escapes into the OM through lateral opening of BamA or the OMPs are inserted by homooligomers formed by several BAM complexes which form a protected environment, in which β-barrels can be formed (Malinverni and Silhavy, 2011).

Of the BAM complex components, BamA and BamD are essential. Depletion of one of them results in accumulation of misfolded OMPs and reduced OM density (Wu et al., 2005; Malinverni et al., 2006; Doerrler and Raetz, 2005). Loss of the non-essential components was shown to lead to lower OMP levels and reduced OM integrity in *Ec* and *Ye* (Malinverni et al., 2006; Onufryk et al., 2005; Sklar et al., 2007; Weirich et al., 2017).

The periplasmic chaperones and BAM complex components are regulated by the σ^E stress response (Rhodius et al., 2006; Dartigalongue et al., 2001; Onufryk et al., 2005), which is activated by the presence of unfolded OMPs (Mecsas et al., 1993; Walsh et al., 2003; Sklar et al., 2007), and by Cpx, which in contrast to σ^E only regulates DegP and Skp (Danese and Silhavy, 1997; Dartigalongue et al., 2001). σ^E enhances DegP and Skp levels and decreases OMP synthesis (Erickson and Gross, 1989; Vogel and Papenfort, 2006; Guisbert et al., 2007) by inhibiting the translation of important OMPs with small RNAs (Johansen et al., 2008; Johansen et al., 2006; Udekwu and Wagner, 2007).

While much is known about OMP biogenesis in *Ec* and *Ye*, in *Pa* only parts of this process have been described. Like in *Ec* and *Ye*, BamA is lethal also in *Pa* (Hoang et al., 2011). Depletion of BamA leads to strongly reduced OprF levels in the OM and increased expression of *mucD* that encodes a DegP-homologue (Hoang et al., 2011; Tashiro et al., 2009). Inhibition of the interaction between BamA and BamD was shown to result in a higher sensitivity against antibiotics, reduced production of OMPs and reduced OM integrity (Mori et al., 2012). Deletion of *bamB* leads to sensitivity against lysozyme, slightly impaired OM integrity, enhanced sensitivity against cell wall targeting antibiotics and reduced virulence (Lee et al., 2017a). Moreover, the deletion of a BamE homologue leads to enhanced sensitivity against SDS, deoxycholate and antibiotics (Ochsner et al., 1999). From the periplasmic chaperones only MucD was shown to be important for resistance against oxidative stress and virulence (Yorgey et al., 2001).

The concept of adjuvants

The development of resistance against antibiotics is known since the first antibiotics have been discovered (Abraham and Chain, 1988). Beside the search for novel antibiotics, the most promising approach to nevertheless be able to control bacterial infection is the development of adjuvants. The aim of this concept is not to target

essential structures in bacteria but to target the resistance mechanism and thereby restore the sensitivity against existing antibiotics when administered in combination with the respective adjuvant. Targeting a global resistance mechanism has the potential to restore the sensitivity against multiple antibiotics and broaden the available repertoire for treatment of infections with MDR strains. The best-known examples for adjuvants are β-lactamase inhibitors, which are widely used for therapy in combination with β-lactam antibiotics such as piperacillin/tazobactam, ampicillin/sulbactam or ceftazidime/avibactam.

The main aim of this thesis was to find novel targets involved in either induction of *ampC* expression or OM integrity with the long-term aim being the development of adjuvants, which could restore the sensitivity of MDR *Pa* against antibiotics.

Objectives of the thesis

Since the frequency of infections with MDR *Pa* strains is increasing and treatment becomes more and more difficult, novel options to control infections with MDR *Pa* strains are urgently required. The major aim of this thesis was to find novel targets for adjuvants that could restore the sensitivity of MDR strains against antibiotics and thereby restore the possibility to use these antibiotics for therapy.

For this purpose, we addressed two central resistance mechanisms that enhance the resistance level of *Pa* against multiple classes of antibiotics: The expression of the β-lactamase *ampC* and the permeability of the OM. To address the first mechanism, we used a screening approach called TraDIS to identify all genes that contribute to resistance against β-lactam antibiotics, which is mainly mediated by AmpC. Deletion mutants of the most interesting candidates were then tested for sensitivity against β-lactam antibiotics, β-lactamase activity and *ampC* expression. To address the second mechanism on the other hand, we used a hypothesis-driven approach and analysed the potential of targeting four proteins involved in OM biogenesis to decrease the OM integrity and to thereby enable antibiotics to cross the OM more efficiently.

Beside the major aim to find novel targets for antibiotic adjuvants, we wanted to further elucidate the relation between changes in PG turnover and *ampC* expression in a MDR isolate and the players contributing to an enhanced level of AmpC. This knowledge contributes to a better understanding of the mechanisms involved in the regulation of *ampC* and could therefore help to generate novel strategies to combat resistance against β-lactam antibiotics.

Moreover, in the second study we were interested in the importance of the four investigated proteins for OM biogenesis in comparison to other species since the participating proteins in OM biogenesis are conserved in the most Gram-negative bacteria but their contribution to OM composition differs. Especially for SurA in *Pa*, we wanted to analyse in detail which OMPs are inserted SurA-dependently and which changes in OMP composition a deprivation of SurA implicates. These data can contribute to a broader understanding of the biogenesis and insertion of OMPs into the OM in general.

Results and discussion

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

Aim of this study was to identify potential targets in *Pa* to restore the sensitivity of MDR strains to treatment with existing antibiotics. Therefore, we generated a Tn library in a clinical MDR bloodstream isolate called ID40. Biparental mating was carried out with an *Ec* SM10 λpir strain containing the plasmid pBT20 which encodes the transposon sequence as well as a transposase. The generated Tn library of ID40 was then grown in presence of meropenem or cefepime, which are commonly used antibiotics for treatment of *Pa* infection and against which ID40 is resistant (Sonnabend et al., 2019). Therefore, only Tn mutants with restored sensitivity against these antibiotics were killed by the antibiotics. By TraDIS we then identified these Tn mutants. The respective genes were considered to be promising candidates for novel targets for antibiotic adjuvants. For the most interesting candidates the restored sensitivity was verified by MIC determination of a broader panel of β-lactam antibiotics in respective clean deletion mutants. For the verified candidates in addition the reason for the restored sensitivity was investigated by analysing β-lactamase activity and expression of *ampC*.

Before conducting the experiment and TraDIS, the ID40 strain was sequenced by long-read sequencing (Nanopore). Together with short-read sequencing (Illumina) data provided by Willmann et al. (Willmann et al., 2018) the reference genome was generated by hybrid assembly and then annotated with Prokka (version 1.11) (Bankevich et al., 2012; Seemann, 2014). ID40 harbours a chromosome of 6.86 mega base pairs (Mbp) and a plasmid of 57446 bp encoding 6468 genes in total and belongs to the sequence type ST-252 as determined by multi-locus sequence typing (MLST 2.0, Center for Genomic Epidemiology, DTU, Denmark (Larsen et al., 2012)) (Sonnabend et al., 2019). MIC analysis using microbroth dilution was performed to analyse the resistance profile. According to the breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), ID40 is resistant against piperacillin, piperacillin/tazobactam, cefepime, ceftazidime, aztreonam, imipenem, levofloxacin and ciprofloxacin, intermediate for meropenem and sensitive against amikacin, gentamicin, tobramycin and colistin (Sonnabend et al., 2019). The sensitivity to aminoglycosides was a necessary property because gentamicin was

used as a resistance marker integrated into the transposon sequence. Beside common resistance genes like the β-lactamase genes *ampC* (PDC-3) and *bla*/*poxB* as well as *crpP* and *fosA* associated with resistance to fluoroquinolone and fosfomycin, respectively, a point mutation in *dacB* encoding PBP4 was found (G-A nt1310, G437D) (Sonnabend et al., 2019). This point mutation has been described to cause resistance of *Pa* against ceftazidime with an increase in MIC from 1 µg/ml to 32 µg/ml in PAO1 (Moya et al., 2009) and is a likely explanation for the resistance of ID40 against all β-lactam antibiotics.

Analysis of the Tn library grown in lysogeny broth (LB) as a control showed a homogeneous distribution of Tn insertions across the whole genome with approximately 100000 unique insertion sites. 697 genes were identified to be essential for viability in ID40, 9 further genes were ambiguous (Sonnabend et al., 2019).

For the identification of promising candidates to restore antibiotic sensitivity, the Tn library was grown in cefepime or meropenem at the respective breakpoint concentration according to the EUCAST (cefepime: 8 µg/ml, meropenem: 2 µg/ml), defined as the maximum MIC at which a strain is still sensitive. Afterwards, the DNA was isolated and Tn-containing fragments were enriched and sequenced. The read counts of the samples grown in cefepime or meropenem were compared to those grown in LB by DeSeq2 (Love et al., 2014). Comparison to the control grown in LB showed significant changes in read counts (adjusted p value < 0.05) in 102 genes for cefepime and in 140 genes for meropenem (Sonnabend et al., 2019). Genes with an at least 5-fold reduction in read counts in comparison to the WT were considered to be the most interesting candidates. These criteria were fulfilled by 19 genes for cefepime and by 18 genes for meropenem. Out of these, 13 genes were found in the analysis for both antibiotics (Sonnabend et al., 2019). The identification of many genes known to confer resistance confirmed that the TraDIS screening approach was suitable for the aim to identify genes involved in antibiotic resistance. Among these genes were *mexA* and *mexB* from one of the most important efflux pumps, the main β-lactamase *ampC* as well as *ampG* and *nagZ* (Sonnabend et al., 2019).

Most of the identified genes contribute to PG recycling: besides *ampC*, *ampG*, *nagZ* and *slt*, which have already been described to be crucial for resistance against β-lactam antibiotics, we identified the LT *mltG*, the EPs *mepM1* and *mepM2* and all four members of the salvage pathway which recycles 1,6-anhMurNAc to UDP-MurNAc in

the cytoplasm (*anmK*, *mupP*, *amgK* and *murU*). From the PG biosynthesis pathway the putative PG glycosyltransferase *ftsW*, *mrcA* encoding PBP1 and the PBP-activator *lpoA* were identified (Sonnabend et al., 2019). We decided to further investigate the genes that were found to be important for resistance against both cefepime and meropenem since they have the biggest potential as targets for antibiotic adjuvants. Besides *mltG* and *mepM1*, we chose *amgK* as representative of the salvage pathway and the two unknown genes *ygfB* and *tuaC*. We generated deletion mutants of each gene and analysed the MICs of the most common β-lactam antibiotics by microbroth dilution, their β-lactamase activity by nitrocefin turnover as well as their expression of the *ampC* β-lactamase by real-time quantitative PCR (qRT-PCR) in order to find out whether the restored sensitivity is a consequence of altered β-lactamase regulation.

MltG is one of eleven LTs in *Pa*. It was described to exert endolytic LT activity and may act as terminase of PG chain length (Yunck et al., 2016). In contrast to Slt, MltB, MltD, MltF, SltB1 and SltH (Cavallari et al., 2013; Lamers et al., 2015), its role in antibiotic resistance has not been investigated in detail so far. The deletion of *slt* caused a decreased MIC of piperacillin, cefotaxime and ceftazidime. On the other hand, deletion mutants of *sltB1*, *sltH*, *mltB*, *mltD* and *mltF2* were slightly less sensitive against piperacillin and cefotaxime (Cavallari et al., 2013; Lamers et al., 2015). MltG was shown so far to be one of several LT substrates for the LT inhibitor bulgecin A (Dik et al., 2019; Sonnabend et al., 2019). Bulgecin A inhibits Slt, MltD and MltG and thereby potentiates the effect of ceftazidime and meropenem. A Tn mutant of *mltG* in the laboratory strain PAO1 showed reduced growth in sub-MIC of meropenem but not ceftazidime (Dik et al., 2019). In our experiments in ID40, the Δ*mltG* mutant showed strikingly reduced MICs and sensitivity was restored against all β-lactam antibiotics except for meropenem according to the EUCAST breakpoints. This directly corresponds to a strongly reduced β-lactamase activity and *ampC* expression, which were almost as low as those of the control laboratory strain PA14 indicating that deletion of *mltG* almost compensates for the effect of the *dacB* mutation (Sonnabend et al., 2019). MltG is therefore a promising target with the ability to restore sensitivity against all β-lactam antibiotics at least in *dacB* mutant strains like ID40. The inhibitor bulgecin A showed only a minor reduction in MICs against ceftazidime and meropenem (Dik et al., 2019). However, the strains tested in Dik et al. were all sensitive to meropenem and 8 out of 10 strains were sensitive to

ceftazidime. It would be therefore very interesting to examine whether bulgecin A shows a bigger effect in strains resistant to β-lactam antibiotics, especially in *dacB* mutants.

As a second candidate involved in PG degradation we investigated MepM1. MepM1 is a metallo-EP cleaving the crosslinks between the PG strands with specificity for D-Ala−mDAP cross-links (Singh et al., 2012). MepM1 is non-essential, but a double deletion mutant of *mepM1* and *spr,* another D,D-EP with similar function, is synthetically lethal in *Ec* indicating the importance of D,D-EPs for viability (Singh et al., 2012). MepM1 together with three other EPs (TUEID40_02316 (homologue in PAO1: PA4404), TUEID40_01415 (PA1198) and TUEID40_01414 (PA1199)) is inactivated by the carboxy-terminal processing protease CtpA (Srivastava et al., 2018; Sonnabend et al., 2019). CtpA requires the lipoprotein LbcA to exert its protease activity on the EP substrates. The deletion of *ctpA* has already been characterised to lead to a defective T3SS, enhanced surface attachment and low salt sensitivity (Srivastava et al., 2018). Except the low salt sensitivity, the phenotype could be reverted by the deletion of *mepM1* in the Δ*ctpA* mutant (Srivastava et al., 2018). To investigate whether a Δ*ctpA* mutant shows the opposite phenotype than Δ*mepM1* also regarding antibiotic resistance, we generated a Δ*ctpA* as well as a Δ*ctpA*Δ*mepM1* double mutant. From the other EPs inactivated by CtpA, additionally TUEID40_01415 was identified in the TraDIS experiment but the reduction in read counts was much less than for *mepM1*. On the other hand, for *mepM2* from the same protein family a significant and strong reduction in read counts was found after treatment with meropenem, but MepM2 is not inactivated by CtpA (Srivastava et al., 2018; Sonnabend et al., 2019).

The Δ*mepM1* mutant showed reduced MIC values for all investigated β-lactam antibiotics except for meropenem and imipenem and its sensitivity was restored for cefepime and aztreonam. Correspondingly, the β-lactamase activity as well as the expression of *ampC* were significantly reduced, but to a clearly lesser extent than in the Δ*mltG* mutant. In the Δ*ctpA* mutant the MICs of meropenem, cefepime, piperacillin and aztreonam were even increased in comparison to the WT. Consistently, the β-lactamase activity as well as the expression of *ampC* were increased indicating that loss of inactivation of the four EPs (including MepM1) by CtpA leads to hyperresistance due to derepression of *ampC* (Sonnabend et al., 2019). This finding is consistent with the observation by Sanz-Garcia et al. who

reported the occurrence of mutations in *ctpA* in strains that developed resistance against ceftazidime/avibactam (Sanz-Garcia et al., 2018). The MICs of piperacillin and aztreonam, the β-lactamase activity and the *ampC* expression of the Δ*ctpA*Δ*mepM1* double mutant were comparable to those of the WT but not reverted to those of the Δ*mepM1* mutant (Sonnabend et al., 2019). Therefore, the three other EP substrates of CtpA also play a role for the level of resistance since their deregulation in absence of CtpA can compensate for the loss of MepM1. An inhibitor targeting multiple of the structurally similar EPs like MepM1 and MepM2 or MepM1 and TUEID40_01415 could be a good option to further decrease β-lactamase expression and resistance against β-lactam antibiotics.

After the degradation of the PG by LTs, EPs and amidases in the periplasm, the PG catabolites are transported into the cytoplasm by the permease AmpG. In the periplasm, the catabolites are further processed by NagZ, LdcA and AmpD. AmpG and NagZ contribute to the pool of 1,6-anhMurNAc-peptides and were consistently identified in the TraDIS results since their deletion leads to a reduced amount of 1,6-anhMurNAc-peptides in the cytoplasm and therefore a lower expression level of *ampC*. AmpD on the other hand degrades 1,6-anhMurNAc-peptides to free peptides and 1,6-AnhMurNAc. Loss of AmpD therefore increases the pool of 1,6-anhMurNAc-peptides and is a frequent cause of resistance against β-lactam antibiotics (Jacobs et al., 1995; Sonnabend et al., 2019). After cleavage by AmpD, the released 1,6-anhMurNAc can then be recycled to UDP-MurNAc by the salvage pathway and subsequently reused for PG biosynthesis.

From the four players of the salvage pathway *amgK*, *anmK* and *murU* were identified for both cefepime and meropenem in the TraDIS screen and *mupP* was identified for cefepime (Sonnabend et al., 2019). The salvage pathway was discovered in *Pa* and bypasses the fosfomycin-sensitive *de novo* PG biosynthesis (Gisin et al., 2013) by recycling of 1,6-anhMurNAc to UDP-MurNAc. Therefore, it is responsible for resistance against fosfomycin. A connection to resistance against β-lactam antibiotics has been suggested as the deletion of *amgK* leads to a slight increase of MICs against ceftazidime and cefotaxime (Fumeaux and Bernhardt, 2017). However, another study did not see increased MIC values in PAO1 (Borisova et al., 2014), which could also be confirmed in our study for all investigated β-lactam antibiotics (Sonnabend et al., 2019). Surprisingly, the Δ*amgK* mutant in ID40 showed a strong reduction in MICs for all investigated β-lactam antibiotics except for meropenem as

well as reduced β-lactamase activity and *ampC* expression in addition to a reduction of the MIC of fosfomycin (Sonnabend et al., 2019). The higher sensitivity to fosfomycin was expected because without a functional salvage pathway, PG synthesis can only be conducted by the *de novo* biosynthesis pathway which comprises MurA, the target of fosfomycin. Reduced MICs against fosfomycin with an impaired salvage pathway have been previously reported (Borisova et al., 2014). In addition to the reduced MIC of fosfomycin, the sensitivity of ID40 Δ*amgK* to cefepime, ceftazidime and aztreonam was restored (Sonnabend et al., 2019). Therefore, at least AmgK and presumably the whole salvage pathway are crucial for resistance against β-lactam antibiotics at least in ID40. This finding underlines the necessity to investigate antibiotic resistance in MDR strains instead of laboratory strains and shows that depending on the genetic background of a strain the same players can have different roles.

In addition to genes involved in PG recycling, also two genes with unknown function were identified by the TraDIS experiment. *tuaC* is a gene with unknown function belonging to the glycosyltransferase 1 family. However, the restored sensitivity of the Tn mutant in the TraDIS experiment could not be confirmed with the Δ*tuaC* deletion mutant and therefore, the Δ*tuaC* mutant was not further analysed (Sonnabend et al., 2019). The reason for our finding that the deletion mutant did not show the same phenotype like the Tn mutant could be that the Tn insertions did not affect the functionality of the protein due to an insertion at the very 3'-end of the gene. Another explanation could be that not the inactivation of *tuaC* itself caused the restored sensitivity but an additional effect of the Tn insertion on another gene downstream of *tuaC*. This result confirms the need of verification of results obtained by TraDIS by the generation of clean deletion mutants.

The Δ*ygfB* mutant on the other hand showed reduced MICs very similar to the Δ*amgK* mutant with restored sensitivity against cefepime and aztreonam. Accordingly, also β-lactamase activity and expression of *ampC* were strongly reduced and had the lowest expression level except for the Δ*mltG* mutant (Sonnabend et al., 2019). The function of YgfB was not characterized so far. It is located in an operon comprising the aminopeptidase *pepP*, the ubiquinone biosynthesis genes *ubiH* and *ubiI* and another unknown gene. Similar operons with genes homologous to *ygfB* can be found in *Ec*, *Acinetobacter baumannii* (each 33 % identical amino acids) and *Legionella pneumophila* (32 % identical amino acids), but

also in these species the function of YgfB is unknown (Sonnabend et al., 2019). Data from experiments with Tn mutants in PAO1 and PA14, however, suggested that YgfB could be important for the colonization of the gastrointestinal tract in mice (Skurnik et al., 2013) as well as for virulence in *Caenorhabditis elegans*, respectively (Feinbaum et al., 2012). These findings emphasize that YgfB is an interesting target since an inhibitor would not only restore sensitivity against β-lactam antibiotics but maybe also reduce colonization and virulence of *Pa*.

Mass spectrometry analysis of whole cell lysates showed a strikingly higher amount of AmpDh3 in the Δ*ygfB* mutant in comparison to the WT (unpublished data). While in the WT, the amount of AmpDh3 was below the detection limit, in the Δ*ygfB* mutant a 226-fold higher amount of AmpDh3 compared to the detection limit was found. AmpDh3 is a periplasmic homologue of AmpD. Presumably, higher AmpDh3 levels in the periplasm lead to enhanced cleavage of the peptides from 1,6-anhMurNAc-peptides and related precursors and subsequently result in lower levels of 1,6-anhMurNAc-peptides in the cytoplasm. Therefore, it is quite plausible that the reduction in *ampC* expression and the restored sensitivity of the Δ*ygfB* mutant is based on the strong upregulation of *ampDh3*. This hypothesis is supported by the finding that deletion of *ampDh3* leads to a strong increase in *ampC* expression and consequently in increased MICs of β-lactam antibiotics (Juan et al., 2006). While the molecular function of YgfB still has to be determined in *Pa* and also in other Gram-negative species, it seems to either directly or indirectly suppress the expression of *ampDh3* and thereby to indirectly contribute to the amount of 1,6-anhMurNAc-peptides and *ampC* expression.

In conclusion, we identified several genes which have not yet or only partly been described to be important for resistance against β-lactam antibiotics so far. They serve as promising targets since a potent inhibitor could reduce β-lactamase expression and activity and therefore allow to reconsider various classes of β-lactam antibiotics for treatment of MDR *Pa* strains at least with a *dacB* mutant background.

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

Besides expression of resistance-conferring enzymes like AmpC and efflux pumps, the low permeability of the OM is the most important intrinsic resistance mechanism in Gram-negative bacteria, especially in *Pa*. Therefore, in this study we addressed the OMP biogenesis pathway, whose function is crucial for OM integrity, to identify targets for adjuvants that could restore antibiotic resistance by facilitating the entry of antibiotics into the bacterial cell.

In different Gram-negative species the importance of various players in OM biogenesis can be different: SurA is the major periplasmic chaperone in *Ec* and *Ye* and Skp and DegP are thought to comprise the rescue pathway when SurA is lacking (Sklar et al., 2007), while in *Neisseria meningitidis,* Skp seems to be more important compared to SurA (Volokhina et al., 2011). The non-essential BAM complex components BamB and BamC have been shown to have an impact on OM integrity and composition in *Ec* and *Ye* (Malinverni et al., 2006; Onufryk et al., 2005; Sklar et al., 2007; Weirich et al., 2017).

To assess the importance and the potential as targets for antibiotic adjuvants in *Pa*, we generated mutants of BamB and BamC as well as the periplasmic chaperones SurA and the Skp-homologue HlpA in PA14. Since it was not possible to generate a Δ*surA* deletion mutant, we constructed a conditional mutant by introducing a copy of *surA* under the control of an arabinose-inducible promotor and subsequently deleting the intrinsic *surA* gene (Klein et al., 2019). Deletion of the genes was verified by polymerase chain reaction (PCR) and by the absence of the respective proteins in the mass spectrometry experiment. Depletion of SurA without addition of arabinose and complementation by the addition of 0.2 % arabinose was verified by qRT-PCR and western blot. qRT-PCR revealed a reduced *surA* expression by 92 %, which can be explained by a residual expression because of the leaky *araC*-P_{BAD} promoter (Meisner and Goldberg, 2016; Klein et al., 2019). In absence of arabinose, SurA was not detectable by western blot but the protein level was restored after addition of arabinose (Klein et al., 2019). The impossibility to generate a *surA* deletion mutant was surprising since in other Gram-negative bacteria, *surA* is not essential. This seems to be different in *Pa* supported by the fact that in the most Tn libraries of *Pa* no *surA* Tn mutant is available (Skurnik et al., 2013; Lee et al., 2015b; Turner et al.,

2015; Sonnabend et al., 2019). Only in one Tn library of PA14, three different mutants with Tn insertions at the very beginning or the very end of the *surA* gene are viable. Possible explanations for this could be that the biggest part of the gene is intact and a slightly truncated protein with residual functionality is still formed or that compensatory mutations have been acquired in these strains.

Growth kinetics at 37 °C in LB medium were assessed for all mutants and revealed a slightly but significantly reduced growth of the conditional *surA* mutant while all other mutants showed a growth comparable to the WT. Using electron microscopy, morphological changes were observed for the Δ*bamB* and the conditional *surA* mutant: in the Δ*bamB* mutant numerous vesicles were formed on the cell surface and the conditional *surA* mutant looked slightly bloated and also showed vesicle formation. This is probably a sign of cell envelope stress in both mutants (Klein et al., 2019).

To investigate the impact of the different deletions on OM integrity, we analysed the influx of the fluorescent dye 1-N-phenylnaphthylamine (NPN) as well as the susceptibility of the strains to bile salts. NPN is not able to cross the OM of *Pa* WT strains. It fluoresces only when it reaches the hydrophobic environment of the inner phospholipid layer of the OM (Konovalova et al., 2016). Therefore, NPN fluorescence indicates that the dye is able to cross the OM due to a reduced OM integrity. The conditional *surA* mutant showed a significantly higher NPN signal than the WT which was complemented upon addition of arabinose. The other mutants did not show a significant difference to WT indicating that only loss of SurA results in a reduced OM integrity to an extent that NPN is able to cross the OM (Klein et al., 2019).

Bile salts are physiological detergents occurring in the intestinal tract. Treatment with a concentration of bile salts that does not harm the WT (0.3 %) resulted in significantly reduced growth of the conditional *surA* mutant as well as of the Δ*bamB* and Δ*bamC* mutant. Only the Δ*hlpA* mutant did not show a higher susceptibility to bile salts (Klein et al., 2019). Therefore, also the OM integrity in the Δ*bamB* and Δ*bamC* mutant is slightly disturbed while for Δ*hlpA* no change in OM integrity could be observed.

OM integrity is determined by its composition and changes in the amount of OMPs or LPS or can lead to reduced OM integrity. Therefore, we prepared OM fractions of all strains and analysed their OMP composition by liquid chromatography tandem-mass spectrometry (LC-MS/MS). Differences in the $\log_2$ of intensities of label-free

quantification (LFQ) values with a false discovery rate of < 0.1 were considered to be significant. The ratio between the LFQ value of a mutant and the LFQ value of the WT was calculated.

The most prominent change in OMP composition in the OM fraction was found in the SurA-depleted strain: 42 proteins predicted to be localized in the OM were significantly lower or higher abundant in the conditional *surA* mutant compared to the WT. Mainly the amount of members of the type V secretion system (T5SS) family, TonB-dependent receptors, porins, BAM complex components and LptD and LptE was strongly reduced in the OM of the conditional *surA* mutant (Klein et al., 2019).

From other species it is known that SurA is crucial for the insertion of autotransporter proteins, which belong to the T5SS, into the OM (Sklar et al., 2007; Wu et al., 2005; Oberhettinger et al., 2012). The autotransporter proteins PlpD and AaaA as well as a two-partner secretion system consisting of the membrane transporter PA14_32790 and its partner PA14_32780 were not detectable in the OM of the conditional *surA* mutant. Similarly, the amount of the autotransporter protein EprS was strongly reduced in comparison to the WT (Klein et al., 2019). Therefore, SurA is crucial for the insertion of auotransporter proteins into the OM also in *Pa*.

In a comparable manner, siderophore receptors and other TonB-dependent receptors were absent or at least strongly reduced in the OM of the conditional *surA* mutant (Klein et al., 2019). In addition to three uncharacterized TonB-dependent receptors, three major iron uptake systems were completely or almost absent: The pyoverdine receptor FpvA, the ferric citrate receptor FecA and the ferrichrome receptor FiuA (Klein et al., 2019). Since iron uptake is very important for bacterial growth, the reduction in iron uptake systems could contribute to the reduced fitness of the conditional *surA* mutant. Loss of FiuA has been shown to have a detrimental impact on elastase level and reduced virulence in an airway infection model (Lee et al., 2016). To further investigate the consequences of the strong reduction in iron uptake system, we analysed the growth under iron-depleted conditions. The growth of the conditional *surA* mutant in presence of the iron chelator 2,2'-Bipyridyl was significantly and dose-dependently reduced compared to the WT.

The biggest group of OMPs affected by SurA deprivation were porins: 13 porins were less abundant or not detectable at all (OpdO, OpdN) in the conditional *surA* mutant, while 3 porins of the OprM family (OprM, OpmB, OpmG) were significantly more abundant than in the OM fraction of the WT. Among the reduced porins, members of

the OprD family (OpdO, OpdN, OpdP and OprD) were the strongest reduced. The porins that were found are mainly responsible for the uptake of different nutrients like pyroglutamate (OpdO), glycine-glutamate (OpdP), arginine (OprD and OprQ) and glucose (OprB) (Chevalier et al., 2017; Klein et al., 2019). A reduction of these porins in the OM could therefore also contribute to reduced fitness and attenuation. Moreover, the major OMP of *Pa*, OprF, was reduced in abundance by over 50 % in the conditional *surA* mutant. OprF has been shown to fulfil a lot of functions like nonspecific diffusion of ions and low-molecular-mass sugars (Nestorovich et al., 2006), OM permeability and adhesion to eukaryotic cells (Azghani et al., 2002). Loss of OprF leads to an impaired production of a number of virulence factors (Fito-Boncompte et al., 2011) and increased sensitivity to a range of antibiotics (Dötsch et al., 2009) and therefore its reduced level in the OM contributes to all observed phenotypes of the conditional *surA* mutant.

Surprisingly, the insertion of OprM family members seems to be independent from SurA in contrast to the other porins since their level was even enhanced in the conditional *surA* mutant. It was previously observed in other species that a particular subset of OMPs was affected only weakly by loss of periplasmic chaperones and non-essential BAM complex components (Mahoney et al., 2016; Weirich et al., 2017). Possibly, this also accounts for the different dependence on SurA of OprM-like proteins. Members of the OprM family are the OM component of the two most important efflux pumps of *Pa*, MexAB and MexXY (Poole, 2000). They are involved in resistance against β-lactams, chloramphenicol, macrolides, quinolones, tetracycline (Li et al., 1995; Masuda et al., 2000) and aminoglycosides (Mao et al., 2001; Klein et al., 2019). Consequently, also the associated efflux pump proteins were found in a higher amount in the OM of the conditional *surA* mutant (Klein et al., 2019). Nevertheless, the higher abundance of these two major efflux pumps did not compensate for the reduced OM integrity and thereby higher sensitivity to antibiotics.

Furthermore, the amount of LptD and LptE, which form a complex in the OM and are responsible for the insertion of LPS into the OM (Chimalakonda et al., 2011), was clearly reduced in the conditional *surA* mutant. It has previously been shown that a depletion of LptE and LptD results in impaired OM integrity, reduced virulence and reduced antibiotic resistance (Lo Sciuto et al., 2018; Klein et al., 2019). Therefore, the reduction of LptD and LptE in the OM is likely to be at least partly responsible for the observed phenotypes in the conditional *surA* mutant.

In the Δ*bamB* mutant, a similar tendency in the changes of the OMP composition was observed like in the conditional *surA* mutant. However, the effect was less pronounced and not significant except for the loss of BamB itself. In the Δ*bamC* mutant besides the loss of BamC only OmpH was significantly reduced and in the Δ*hlpA* mutant, no significant changes in OMP composition could be observed (Klein et al., 2019). Therefore, HlpA seems to play an even minor role for OMP composition compared to other Gram-negative species, where the loss of Skp had a slight impact on OM integrity and composition (Chen and Henning, 1996; Weirich et al., 2017).

Changes in OMP composition can either be caused by a reduced insertion of OMPs by lack of SurA or by transcriptional downregulation of OMP expression by the σ^E stress response triggered by the accumulation of misfolded β-barrel proteins in the periplasm (Mecsas et al., 1993; Walsh et al., 2003; Guisbert et al., 2007). We analysed the expression level of selected genes in the conditional *surA* mutant. The level of all investigated genes encoding OMPs was comparable to the WT, indicating that the reduced OMP levels in the OM are due to reduced insertion because of the lack of SurA, but not due to an indirect downregulation of expression by the σ^E stress response. The only gene for which a significantly altered expression level was observed was *hlpA*. A 2.4-fold increase in transcription suggests a compensatory upregulation of *hlpA* to rescue the effects of the SurA deprivation (Klein et al., 2019).

To confirm the proteomics data we determined the protein levels of SurA, OprD and PlpD in whole bacterial lysates by western blot. As expected, SurA was not detectable in the conditional *surA* mutant without induction but restored to 64 % in the presence of arabinose according to the quantification of the western blot signals. In the deletion strains, no difference in SurA protein level was detected. Consistent with the result of the mass spectrometry analyses, the protein levels of OprD (15 %) and PlpD (24 %) were clearly reduced in the conditional *surA* mutant. In addition, the level of OprD was slightly reduced in the Δ*bamB* mutant and the level of PlpD was reduced in the Δ*bamB* and the Δ*hlpA* mutant, which also fits to the proteomics data albeit the effects are not significant (Klein et al., 2019).

Several proteins that are important for full virulence like FpvA and OprF were found to be less abundant or absent in the OM of the conditional *surA* mutant. Therefore, we were interested in the ability of the conditional *surA* mutant to cause infection and analysed its ability to survive in human serum as well as its virulence in an *in vivo* infection model in *Galleria mellonella*. The serum complement is an important

defense mechanism of the innate immune system to eliminate pathogens in the bloodstream. The strains were grown in either 10 % human serum in phosphate-buffered saline (PBS) or in 10 % heat-inactivated serum. While the WT and the Δ*bamB*, Δ*bamC* and Δ*hlpA* deletion mutants were able to survive, the conditional *surA* mutant was rapidly killed by the active human serum (Klein et al., 2019). Therefore, the changes in OM composition in the conditional *surA* mutant lead to sensitivity against the complement system and thereby withdraw an important property of *Pa* to cause sepsis which is associated with high mortality rates (Suarez et al., 2009).

To assess the general virulence of the mutants, we used the *Galleria mellonella* infection model which has been shown to be a valuable tool in bacterial infection research (Jander et al., 2000; Junqueira, 2012). In total, 60 larvae per group were injected with 12 ± 2 bacteria and survival of the larvae was monitored at physiological temperature. The larvae injected with the WT, the Δ*bamB*, Δ*bamC* or the Δ*hlpA* mutant were rapidly killed within 24 hours. In contrast, larvae injected with the conditional *surA* mutant survived significantly longer, some up to 72 hours (Klein et al., 2019). Deprivation of SurA and consequent reduction of the amount of several virulence factors in the OM therefore lead to reduced virulence *in vivo*. *Galleria mellonella* larvae injected with the conditional *surA* mutant grown in presence of arabinose prior to infection showed very similar survival curves compared to larvae injected with the conditional *surA* mutant grown without arabinose. This indicates that the level of SurA after injection and thereby removal of the inductor quickly declines in the complementation strain. For *in vivo* complementation, arabinose would have to be continuously administered to the larvae, which was not feasible in our experimental setting (Klein et al., 2019). In conclusion, SurA is important for virulence while the changes induced by the deletion of *bamB*, *bamC* or *hlpA* are not sufficient to reduce virulence.

The OM integrity is especially important for high intrinsic antibiotic resistance in *Pa* since many antibiotics are not able to cross the OM barrier. To investigate whether the reduced OM integrity leads to a higher sensitivity to antibiotics, we analysed the MICs of various antibiotics for the Δ*bamB* and the conditional *surA* mutant using E-tests. Since we aimed to restore antibiotic susceptibility in MDR *Pa* strains, we additionally tested a conditional *surA* mutant in a MDR clinical isolate called ID72. In PA14, the MICs of all tested antibiotics except carbapenems and erythromycin (in the

conditional *surA* mutant), namely for ampicillin/sulbactam, piperacillin/tazobactam, ticarcillin/clavulanate, cefotaxime, cefepime, ceftazidime, levofloxacin, ciprofloxacin, fosfomycin, vancomycin and trimethoprim/sulfamethoxazole were reduced for the Δ*bamB* and the conditional *surA* mutant (Klein et al., 2019). Vancomycin served as a marker for OM integrity defects as shown for corresponding mutants in different species (Ruiz et al., 2005; Weirich et al., 2017). Complementation by the addition of arabinose revealed similar MICs like the WT. For doripenem and meropenem in contrast a slightly higher MIC was observed (Klein et al., 2019). This is consistent with the reduced amount of OprD found in the OM of the mutants, since OprD serves as an entry site for carbapenems into the bacterial cell and carbapenem resistance is often caused by mutation or downregulation of OprD (Wolter et al., 2008; Yoneyama and Nakae, 1993; El Amin et al., 2005; Pirnay et al., 2002). The ID72 strain was highly resistant against all tested antibiotics except ciprofloxacin. In the conditional *surA* mutant, the MICs of ticarcillin/clavulanate, cefepime, ceftazidime, ciprofloxacin, levofloxacin, fosfomycin and vancomycin were reduced. In case of cefepime, ceftazidime and levofloxacin, the sensitivity of ID72 could be restored (Klein et al., 2019). Therefore, the global effect on OM integrity by deprivation of SurA allows a wide variety of antibiotics to better cross the OM. This resulted in reduced MICs of almost all tested antibiotics. The data obtained from the conditional *surA* mutant in a MDR *Pa* isolate demonstrate that deprivation of SurA is a possibility to restore sensitivity against various clinically important antibiotics.

In conclusion, SurA is an important protein for proper OM composition and integrity. The non-essential BAM complex components BamB and BamC as well as the Skp-homologue HlpA on the other hand are not suitable as targets since the effects in the Δ*bamB* and the Δ*bamC* strain are very small. For the Δ*hlpA* mutant, no change in OM composition and integrity could be observed indicating that Skp plays an even minor role in *Pa* than in other Gram-negative species. This is supported by our finding that SurA seems to be essential in *Pa* and Skp and DegP are obviously not able to rescue a complete loss of SurA. Deprivation of SurA however causes global effects like reduced OM integrity, reduced fitness in human serum due to high susceptibility to the complement system, impaired virulence *in vivo* and enhanced sensitivity to various antibiotics. Therefore, SurA is a promising target for the development of an antibiotic adjuvant which could restore sensitivity of MDR strains and in addition reduce *in vivo* fitness and virulence.

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Appendix

Publications and accepted manuscripts

Michael S. Sonnabend*, Kristina Klein*, Sina Beier, Angel Angelov, Robert Kluj, Christoph Mayer, Caspar Groß, Kathrin Hofmeister, Antonia Beuttner, Matthias Willmann, Silke Peter, Philipp Oberhettinger, Annika Schmidt, Ingo B. Autenrieth, Monika Schütz and Erwin Bohn (**2019**)

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*equal contribution

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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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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

52 targets for developing drugs that could be used to restore the sensitivity towards
53 existing antibiotics specifically in MDR strains of *Pa*.
54
55 **Introduction**
56 *Pa* is one of the most important pathogens involved in nosocomial infections, such as
57 pneumonia, urinary tract infection, wound infections and potentially life threating
58 blood stream infection. In particular, intensive care and immunocompromised
59 patients are at risk to develop severe infections. MDR strains are emerging which
60 makes treatment of *Pa* infection even more difficult. For this reason, the WHO ranked
61 carbapenem-resistant *Pa* into the top class of its list of priority pathogens for which
62 new antibiotics are urgently needed (1). For an increasing number of cases colistin is
63 the last treatment option despite its neuro-and nephrotoxic side effects.
64 *Pa* employs various intrinsic and acquired antibiotic resistance mechanisms. The
65 high intrinsic resistance is mainly caused by a very low permeability of the outer
66 membrane (2) and the inducible expression of efflux pumps and enzymes mediating
67 resistance like AmpC (3). *ampC* is expressed at a low level in wildtype strains but can
68 be strongly increased in strains in which *ampC* is derepressed. Derepression of
69 *ampC* is often caused by mutations in the transcriptional regulator AmpR, in AmpD
70 (4, 5) or in the *dacB* gene encoding muropeptide amidase and penicillin-binding
71 protein 4 (PBP4), respectively (6), leading to an increased pool of 1,6-
72 anhydromuropeptides originating from the peptidoglycan (PG) recycling pathway (7).
73 Moreover, *ampC* expression can be induced by β-lactam antibiotics and β-lactamase
74 inhibitors leading to resistance against most β-lactam antibiotics (8).
75 One strategy to reconsider antibiotics that have become ineffective caused by the
76 development of resistance is the inactivation of the primary resistance mechanism.
77 Thus, the combination of β-lactam antibiotics with β-lactamase inhibitors such as

78 tazobactam, which block the activity of β-lactamases, makes it possible to reconsider
79 antibiotics such as piperacillin. However, often such combinations fail again to kill
80 microbial pathogens because of β-lactamases which are resistant against the β-
81 lactamase inhibitors (9-11). One upcoming strategy is to use a different class of
82 antibiotic adjuvants. Such adjuvants would not inactivate a primary resistance
83 mechanism but would rather act on a secondary resistance gene. Several examples
84 for such a strategy have been described (12-16). In this study, we wanted to find out
85 which proteins could serve as targets to resensitize MDR *Pa* strains to treatment with
86 β-lactam antibiotics.
87 To answer this question we performed Transposon-Directed Insertion Sequencing
88 (TraDIS) using the clinical bloodstream isolate ID40, which is resistant against many
89 β-lactam antibiotics, to assess the resistome of *Pa* in a similar approach described by
90 Jana et al. (17). TraDIS has been shown to be a valuable tool under particular
91 conditions and in various approaches to find genes responsible for growth (18-21).
92 We constructed a Tn mutant library in the MDR ID40 strain and subjected it to
93 cefepime (FEP) or meropenem (MEM). TraDIS revealed non-essential candidate
94 genes including well-known as well as so far unknown genes whose inactivation
95 breaks resistance against these antibiotics. Some candidates were verified by testing
96 respective deletion mutants for their antibiotic sensitivity, β-lactamase activity and
97 *ampC* expression. The presence of these genes seems to be crucial to achieve or
98 maintain antibiotic resistance. These genes may comprise the most promising non-
99 essential target genes for the development of novel antibiotic adjuvants to reconsider
100 β-lactam antibiotics in resistant strains of *Pa*.
101
102 **Results**
103 **ID40 sequence and resistance profile**

104 To determine the resistome of a MDR *Pa* strain against β-lactam antibiotics, we used
105 the bloodstream isolate ID40 (22). ID40 belongs to the sequence type ST-252
106 (determined by MLST 2.0, Center for Genomic Epidemiology, DTU, Denmark (23))
107 and is resistant against piperacillin (PIP), piperacillin/tazobactam (TZP), cefepime
108 (FEP), ceftazidime (CAZ), aztreonam (ATM), levofloxacin (LEV), ciprofloxacin (CIP)
109 and imipenem (IMP). Moreover, ID40 is intermediate for meropenem (MEM) and
110 sensitive against amikacin (AMI), gentamicin (GEN), tobramycin (TOB) and colistin
111 (COL) (Table S1). The whole genome and the plasmid sequence were annotated
112 and submitted to the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena;
113 accession number PRJEB32702).
114 The ID40 chromosome is 6.86 Mbp in size and encodes 6409 open reading frames
115 and carries a plasmid of 57446 bp comprising 59 putative genes. Resistance genes
116 were searched using ResFinder (24) revealing the following resistance genes:
117 aph(3')-IIb (*neo*) for aminoglycoside resistance, blaOXA-486 (*bla*) and OxaPAO1
118 (*ampC*, PDC-3) for β-lactam resistance, crpP (*crpP*) for fluoroquinolone resistance
119 and fosA (*fosA_1*) for fosfomycin resistance. Additionally, we found a point mutation
120 in the *dacB* gene (PBP4; G-A nt1310, G437D), which is known to be responsible for
121 resistance against β-lactam antibiotics as shown by an increased MIC for CAZ from 1
122 µg/ml to 32 µg/ml in *Pa* PAO1 (6). Therefore, most likely the mutation in *dacB*
123 rationalizes the different resistance level of ID40 in comparison to strain PA14, which
124 comprises the same resistance genes but is sensitive to all β-lactam antibiotics.
125 Other resistance mechanisms like reduced expression of *oprD* and overexpression of
126 efflux pumps were not specifically addressed, but their contribution to resistance
127 cannot be finally excluded. Analysis of the OprD sequence and comparison to the
128 literature did not provide any clear evidence that OprD of ID40 is dysfunctional (25-
129 28).

130

131 **Construction of a high-density mutant library and TraDIS sequencing**

132 Growth of the Tn library in LB revealed approximately 100000 unique Tn insertions
133 distributed across the genome with an average of 18 Tn insertion sites per 1 kbp of
134 coding sequences. Homogenous distribution of Tn insertions and homogenous
135 coverage of the whole genome are shown in **Figure S1**.
136 Analysis of the unchallenged Tn library showed that from 6468 genes 697 genes
137 were determined to be essential for viability (10.8 %) (**Data set S1**) and 9 were
138 determined to be ambiguous (0.14 %) (**Data set S2**). Among these, many genes
139 were previously described to be essential, for example *dnaA*, *gyrB* or *lolA* (29, 30).

140

141 **Identification of genes important for resistance against meropenem and**
142 **cefepime**

143 The contribution of non-essential genes to antimicrobial resistance was measured by
144 quantifying the depletion of Tn insertion mutants upon exposure with FEP and MEM
145 at the respective breakpoint concentration defining a *Pa* strain as sensitive according
146 to EUCAST (FEP: 8 µg/ml, MEM: 2 µg/ml). For analysis of the TraDIS results we
147 chose only genes in which the read number in LB control was > 10 in all three
148 independent experiments and additionally showed a significant change in read
149 counts upon treatment and had an adjusted p value < 0.05 (**Data set S3**). Genes that
150 showed a significant change in read counts in comparison to the untreated sample
151 are visualized in **Figure 1**. In total, 140 genes fulfilled these criteria upon MEM
152 treatment and 102 genes upon FEP treatment.
153 Non-essential genes in which the read counts for Tn insertion were at least 5-fold
154 reduced with a high level of significance (adjusted p value < 0.05) are listed in
155 **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

208 the breakpoint for for PIP, TZP and IMP. These data confirm the validity of the
209 TraDIS screen and demonstrate the contribution of these genes to resistance against
210 β-lactam antibiotics in the ID40 strain.
211 Deletion of *ctpA* increased MIC values for MEM, FEP, PIP and ATM. Thus, we
212 hypothesize that increased activity of MepM1 and other CtpA substrates leads to
213 increased resistance. The MIC values of the double mutant Δ*ctpA*Δ*mepM1* for PIP
214 and ATM were lower compared to those of Δ*ctpA* but higher compared to those of
215 the Δ*mepM1* deletion mutant, indicating that the other substrates of CtpA might also
216 contribute to resistance against β-lactam antibiotics and compensate for the loss of
217 MepM1 without the inactivation of CtpA. According to the TraDIS data the most
218 promising CtpA-regulated substrates which may, in combination with MepM1,
219 contribute to β-lactam resistance are TUEID40_02316 and TUEID40_01415 (**Data**
220 **set S3**). Furthermore, it could be confirmed that deletion of *amgK* results in reduced
221 resistance against fosfomycin as previously described (**Table 2** and **S1**) (39).
222 For complementation, conditional mutants (Δ*mltG::mltG*, Δ*mepM1::mepM1*,
223 Δ*ctpA::ctpA,* Δ*ygfB::ygfB)* under control of a rhamnose-inducible promoter were
224 generated. In the presence of 0.1% rhamnose complementation could be achieved
225 (**Table S1**).
226
227 **MltG, MepM1, AmgK and YgfB contribute to β-lactam resistance in ID40 by**
228 **promoting *ampC* expression**
229 To assess in more detail the reason why the mutants show restored susceptibility to
230 β-lactam antibiotics, we measured the β-lactamase activity of ID40, the different
231 deletion mutants as well as of the laboratory strain PA14, which is sensitive to all
232 tested antibiotics (**Table 2**). As determined by a nitrocefin-based assay, β-lactamase
233 activity was strongly reduced in Δ*mltG,* Δ*mepM1,* Δ*ygfB*, and Δ*amgK* with the most

234 profound reduction in Δ*mltG* showing a β-lactamase activity almost as low as the
235 PA14 strain (**Figure 2A**), and being sensitive to all tested β-lactam antibiotics (**Table**
236 **S1**). The β-lactamase activity corresponds directly to the MIC values of the different
237 mutants. Similarly, a higher β-lactamase activity was found in the hyperresistant
238 Δ*ctpA* mutant. Therefore, the changes in MICs are presumably caused by an altered
239 β-lactamase activity in the mutants compared to ID40 wildtype. No significant change
240 in β-lactamase activity was found in Δ*ctpA*Δ*mepM1* compared to Δ*ctpA*, indicating
241 that the uncontrolled levels of other CtpA substrates can compensate the lack of
242 MepM1.
243 In the ID40 genome, two β-lactamases are encoded (*ampC* and OXA-486/*bla*/*poxB*).
244 For PoxB it has been shown that it does not contribute to β-lactam resistance (44).
245 We quantified the expression level of *ampC* to investigate whether the lower β-
246 lactamase activity is due to reduced *ampC* expression. Semi-quantitative RT-PCR
247 revealed that deletion of *mltG*, *mepM1*, *amgK* or *ygfB* significantly decreased *ampC*
248 mRNA expression **(Figure 2B)**. Deletion of *ctpA*, presumably resulting in a higher
249 level of MepM1 and its other substrates, caused an increase in *ampC* expression.
250 The expression level of *ampC* in the different mutants is in agreement with the levels
251 of β-lactamase activity and the MICs of β-lactam antibiotics that we have measured.
252 These results indicate that the different levels of resistance of the ID40 mutants are
253 due to different levels of *ampC* expression.
254

255 **4 Discussion**

256 Here, we report the - to our knowledge - first application of TraDIS in a MDR
257 *Pseudomonas aeruginosa* strain and the evaluation of its non-essential resistome
258 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

311 cleaves the peptide chain attached to 1,6-anhMurNAc so that the generated 1,6-
312 anhMurNAc can subsequently be recycled to UDP-MurNAc by the so-called cell wall
313 salvage pathway via AnmK, MupP, AmgK and MurU which bypasses *de novo*
314 biosynthesis of UDP-MurNAc (38, 39). Finally, UDP-MurNAc is modified by the Mur
315 enzymes to form UDP-MurNAc-pentapeptide (45). Both 1,6-anhMurNAc-peptides
316 and UDP-MurNAc-pentapeptide can bind to the *ampC* regulator AmpR. Thereby, 1,6-
317 anhMurNAc-peptides induce *ampC* expression, while UDP-MurNAc-pentapeptide
318 bound to AmpR represses *ampC* expression.
319 As observed in our TraDIS data and also shown previously loss of AmpG or NagZ
320 results in decreased amounts of 1,6-anhMurNAc peptides and hence results in
321 increased susceptibility towards β-lactam antibiotics (32, 47). On the other hand, loss
322 of AmpD leads to accumulation of 1,6-anhMurNAc-peptides and therefore an
323 increased *ampC* expression (51) and is a frequent cause of high *ampC* expression in
324 clinical isolates of *Pa* (52, 53).
325
326 ***Players of the cell wall salvage pathway***
327 The individual deletion of each of the 4 (*anmK*, *mupP*, *amgK* and *murU*) genes of the
328 cell wall salvage pathway in PAO1 has been shown to lead to increased β-lactamase
329 activity and a subtle increase of resistance against cefotaxime and CAZ (41).
330 Although this effect could not be explained so far, it was proposed that it might be
331 due to the reduction of the steady state level of the *ampC* repressor UDP-MurNAc-
332 pentapeptide. Consequently, 1,6-anhMurNAc-peptides would be more likely to bind
333 to AmpR and thereby induce *ampC* expression (41). In contrast, another study
334 showed that the deletion of *amgK* also in *Pa* PAO1 had no impact on CAZ and IMP
335 resistance (39), which could be confirmed in our study for all tested β-lactam
336 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

363 interest as a target to develop antibiotic adjuvants which might additionally reduce
364 virulence. In further studies we will address the function of YgfB and its specific role
365 in mediating antibiotic resistance.
366 In conclusion, using TraDIS we identified a set of nonessential genes which are
367 crucial for the induction of *ampC* expression and β-lactam resistance. As shown in a
368 recent study, overexpression of *ampC* is the most frequent cause for the
369 development of resistance in strains capable of expressing *ampC* as shown by the
370 acquisition of mutations in *dacB*, *ampD* and *mpl* after exposure of *Pa* PAO1 WT to
371 increasing concentrations of ceftazidime (56). However, there are additional
372 mechanisms to develop resistance against β-lactam antibiotics which gain more
373 importance when *ampC* expression is hindered. Mutations in *ftsI* leading to
374 modification of PBP3, the target of β-lactam antibiotics, mutations or overexpression
375 of the efflux pump MexAB-OprM as well as large chromosomal deletions led to
376 resistance against ceftazidime albeit to a lower level compared to β-lactamase-
377 dependent resistance (56). This aspect will have to be considered for the
378 development of adjuvants leading to decreased expression of *ampC*.
379 Nevertheless, the genes identified in our study provide promising candidates as
380 targets to develop novel adjuvants to restore the function of β-lactam antibiotics in
381 MDR *Pa* strains with high AmpC activity.
382

383 **Material and Methods**

384 **Bacterial strains and culture conditions**

385 Bacterial strains and plasmids used in this study are listed in **Table S2**. Bacteria were
386 cultivated overnight at 37 °C with shaking at 200 rpm in lysogeny broth (LB)
387 containing suitable antibiotics if necessary. Overnight cultures were diluted 1:20 into
388 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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865 **Figure legends**

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

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

874

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

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

882

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

885 The bacterial murein matrix is formed by chains of the two alternating amino sugars
886 MurNAc (M) and GlcNAc (G), which are linked by β(1→4) glycosidic bonds. Attached
887 to the MurNAc residues is a pentapeptide side chain which typically is composed of
888 L-alanine-ɣ-D-glutamate *meso*-diaminopimelic acid-D-alanyl-D-alanine (L-Ala-ɣ-
889 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.

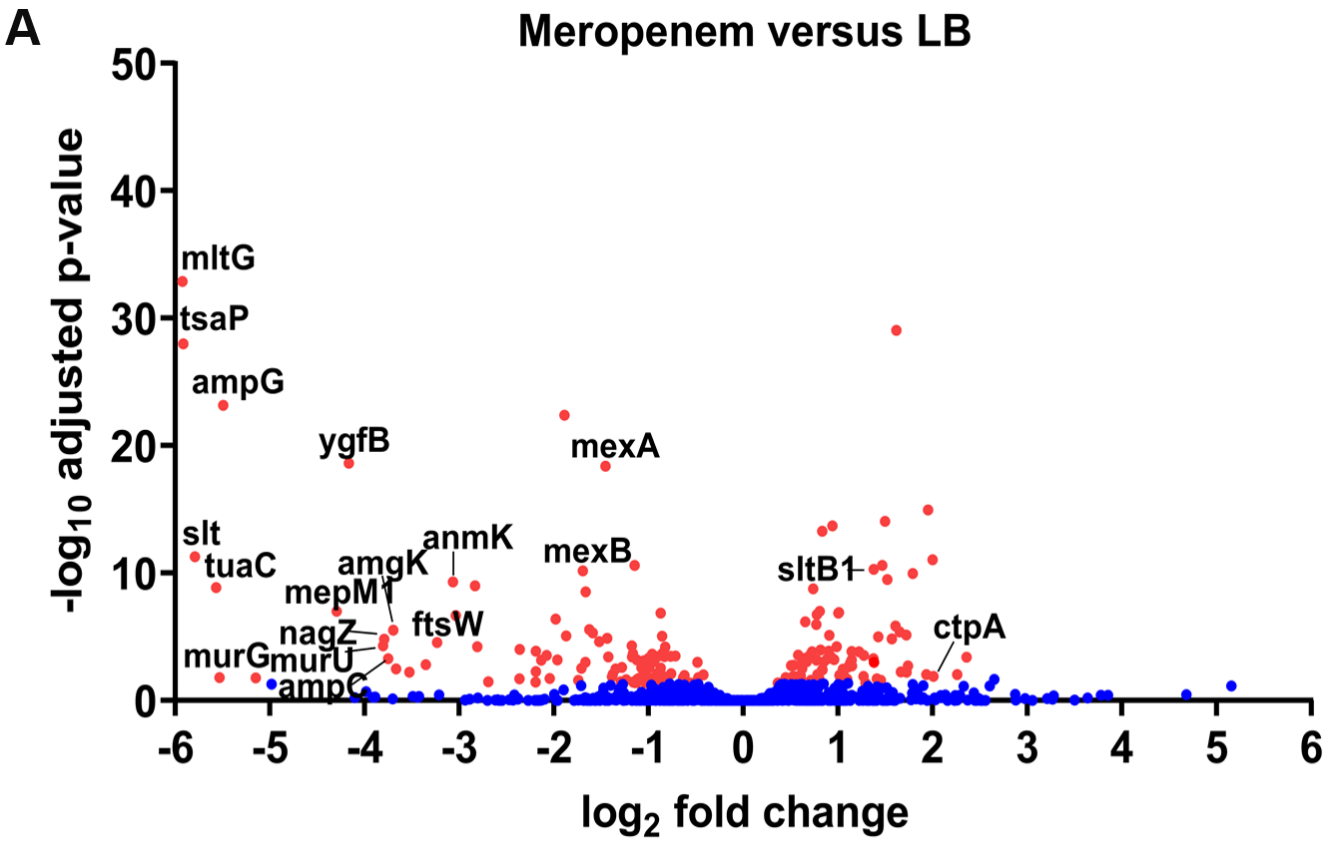
Category				MEM *vs* LB		FEP *vs* LB		Orthologues
	ID	Gene	Name/Function	Ratio	p value	Ratio	p value	
Genes with an adjusted p value < 0.05 and ≥ 5-fold reduction for MEM and FEP								
Resistance	TUEID40_04486	*ampC*	β-lactamase	0.07	0.00052	0.05	3.87E-5	PA14_10790; PA4110
PG synthesis/ recycling	TUEID40_05675	*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-	0.10	1.58E-03	0.135	6.43E-03	PA3155

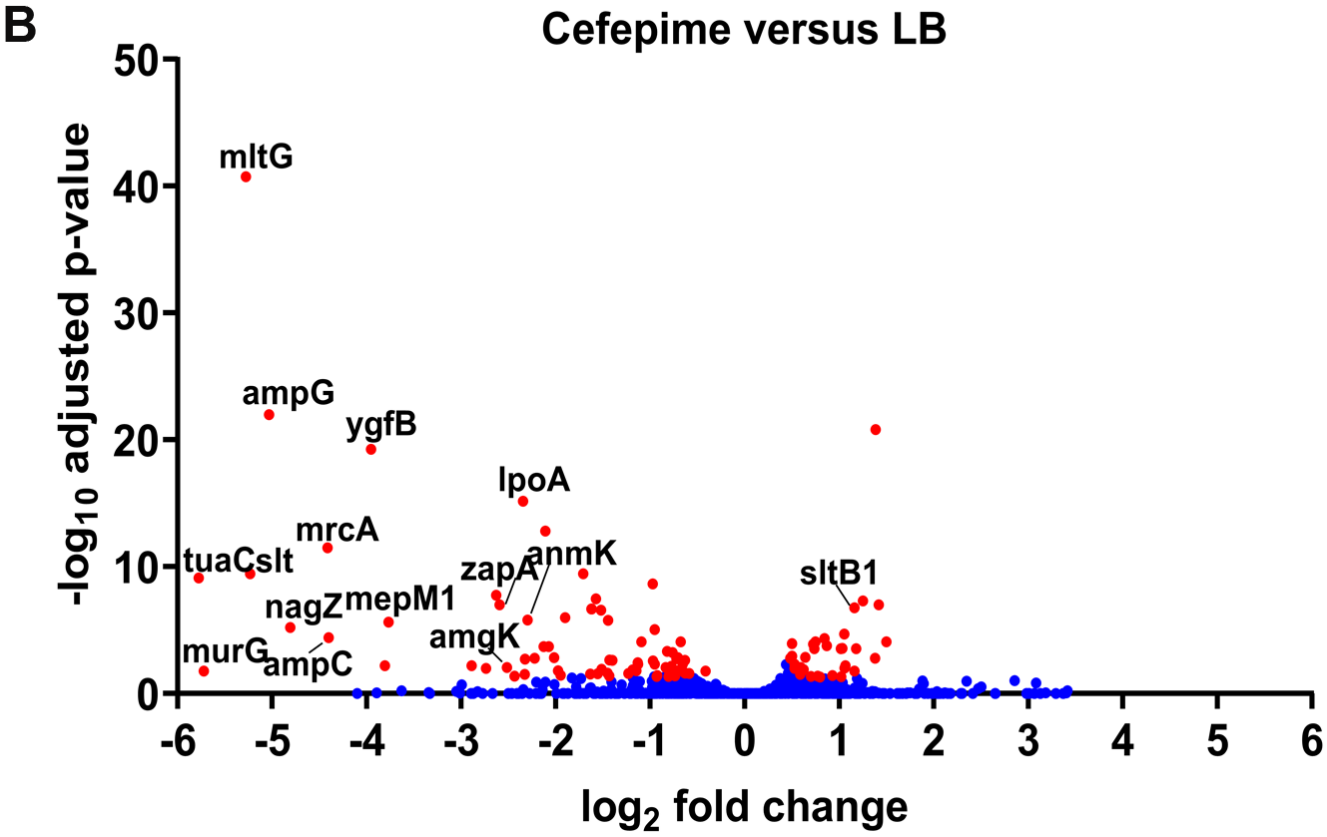
			oxo-D-glucuronate aminotransferase					
Unknown	TUEID40_03245	*ygfB*	ygfB-like proteins, unknown	0.06	2.35E-19	0.06	5.56E˙20	PA14_69010; PA5225
	TUEID40_05543	*tuaC*	Glycosyltransferase family 1	0.02	1.35E-09	0.02	7.79E-10	-
Genes with an adjusted p value < 0.05 and ≥ 5-fold reduction only for MEM								
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

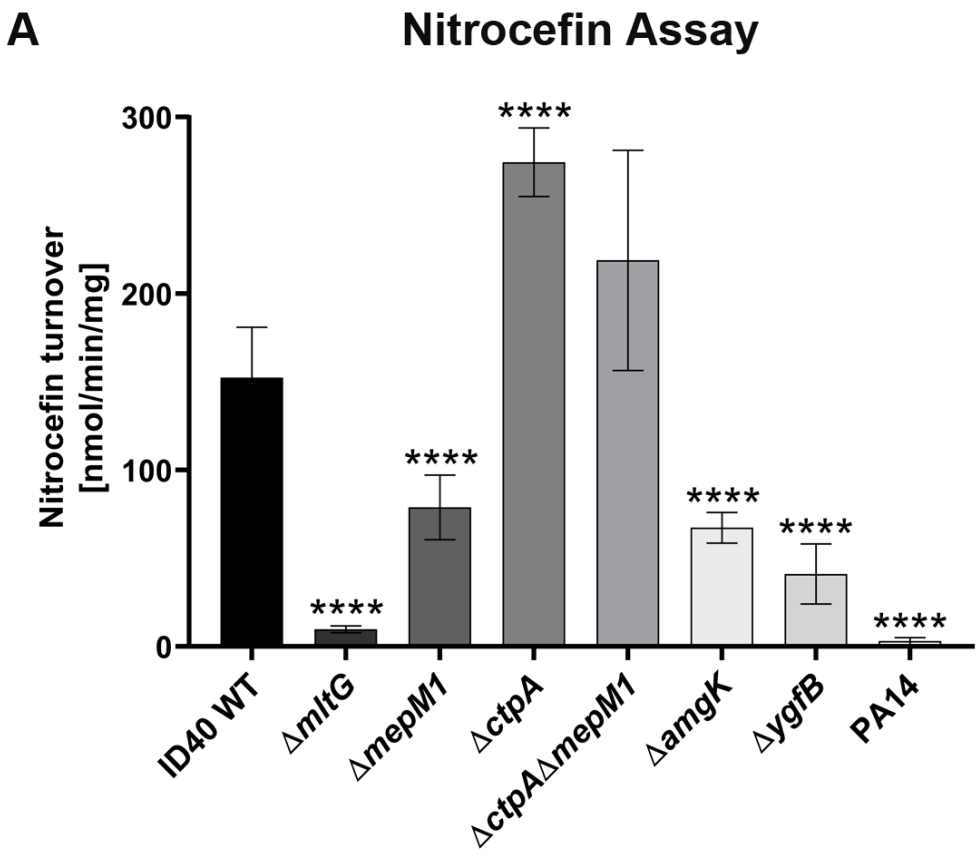
Table 2. Susceptibility of ID40 WT and deletion mutants against β-lactam antibiotics. Minimal inhibitory concentrations (MICs) of ID40 WT and deletion mutant strains were determined by microbroth dilution or by E-Test for fosfomycin. MIC values of the deletion mutants lower than that of ID40 WT are highlighted 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.

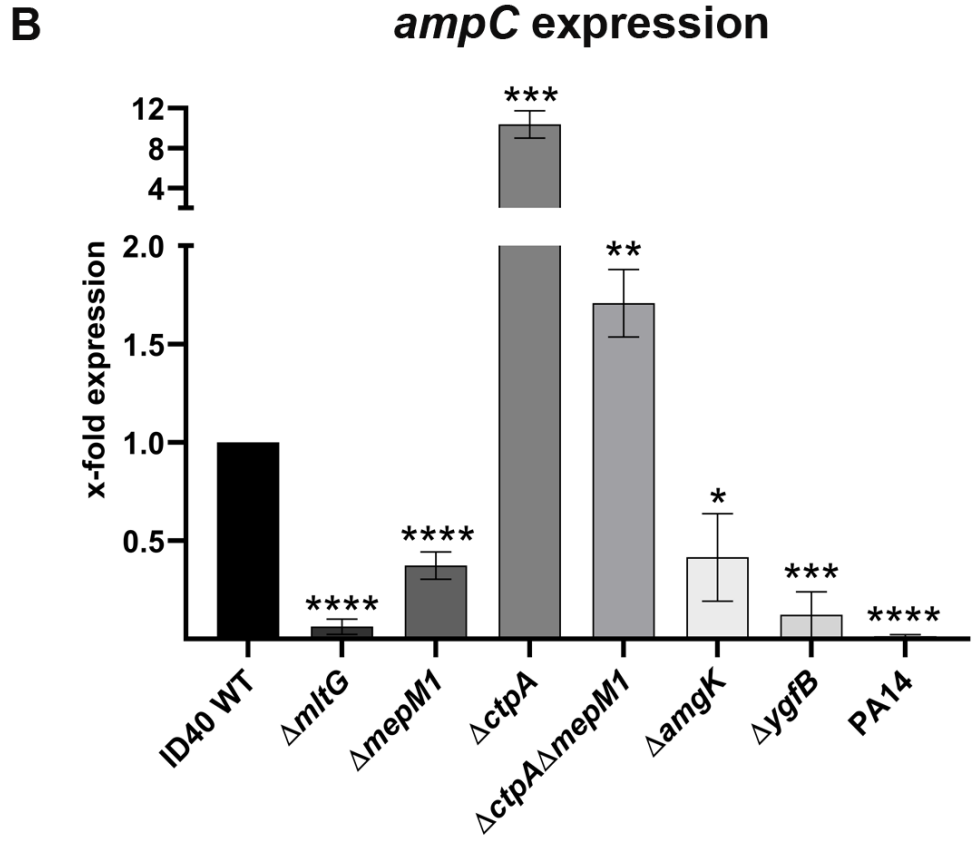
	MIC Breakpoint (mg/L)		ID40 WT	Δ*mltG*	Δ*mepM1*	Δ*ctpA*	Δ*mepM1* Δ*ctpA*	Δ*ygfB*	Δ*amgK*	Δ*tuaC*	PA14
	S ≤	R >									
MEM	2	8	8	4	8	16	16	4	8	8	<0.125
IMP	4	4	32	**4**	32	32	32	8	8	32	<1
FEP	8	8	16	**4**	**4**	32	32	**8**	**8**	16	<1
CAZ	8	8	32	**2**	16	32	32	16	**8**	32	<1
PIP	16	16	128	**<4**	64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

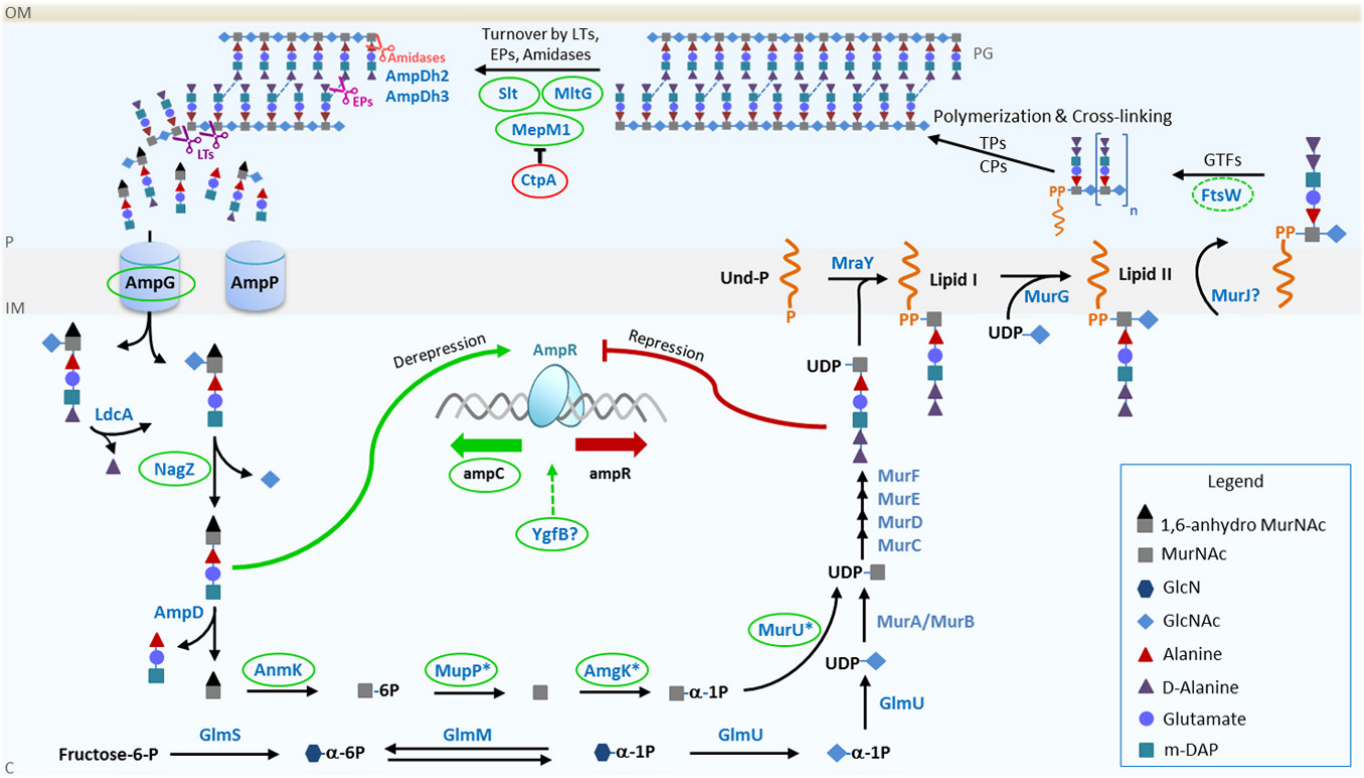
MEM, meropenem; IMP, imipenem; FEP, cefepime; CAZ, ceftazidime; PIP, piperacillin; TZP, piperacillin/tazobactam; ATM, aztreonam; FOS, fosfomycin; *E-test











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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

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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-*ara*CP_{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-*ara*CP_{BAD}-*tolB* (Lo Sciuto et al., 2014) was replaced by the cds of *surA* using PCR amplification and Gibson assembly. The mini-CTX1-*ara*CP_{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-*ara*CP_{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™ 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™ 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™ 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™ 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™ 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

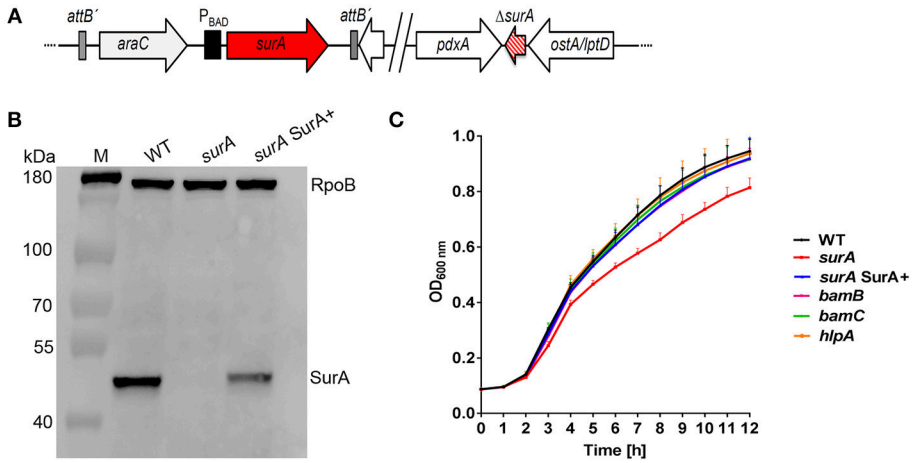

FIGURE 1 | Scheme of the conditional *surA* mutant, verification and impact of SurA, BamB, BamC and HlpA on *Pa* growth. **(A)** Schematic view of the genomic organization of the conditional *surA* mutant. **(B)** Western blot analysis of SurA and RpoB of PA14 WT and the conditional *surA* mutant in 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.

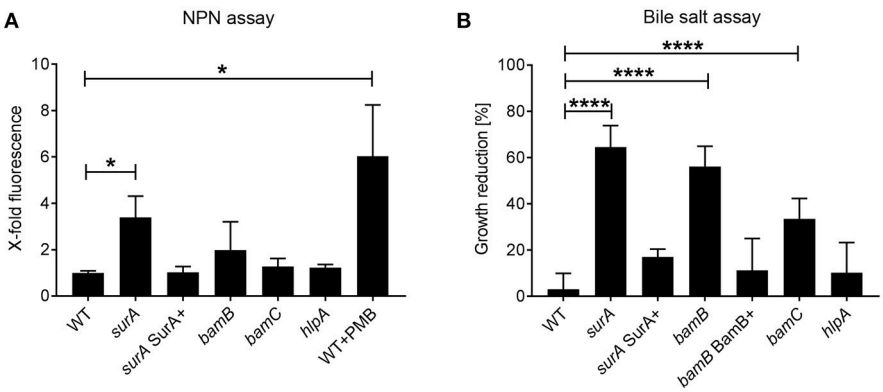

FIGURE 2 | Role of SurA, BamB, BamC, and HlpA for membrane integrity and sensitivity against bile salts. **(A)** NPN Assay. A conditional *surA* and *bamB*, *bamC,* and *hlpA* deletion mutants were treated with NPN. Data depict the mean and SD of 3–5 independent experiments with triplicates. The fluorescence 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)

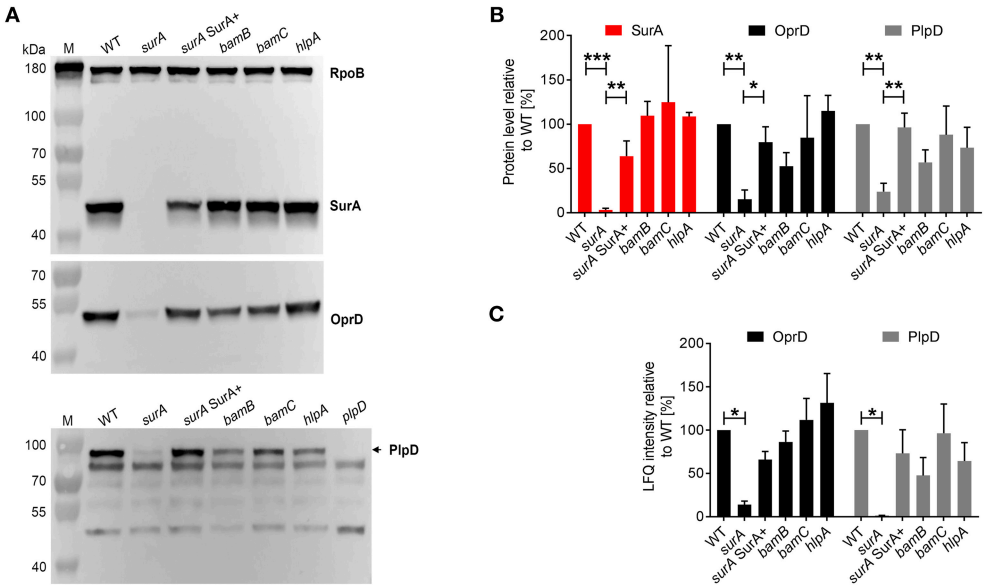

FIGURE 3 | Validation of proteome analysis by Western blot. **(A)** Comparison of protein levels between WT and mutants. Bacteria as indicated were sub-cultured for 3 h in the presence or absence of arabinose and samples were harvested for preparation of whole cell lysates. Western blot analysis was 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 ($n = 3$).

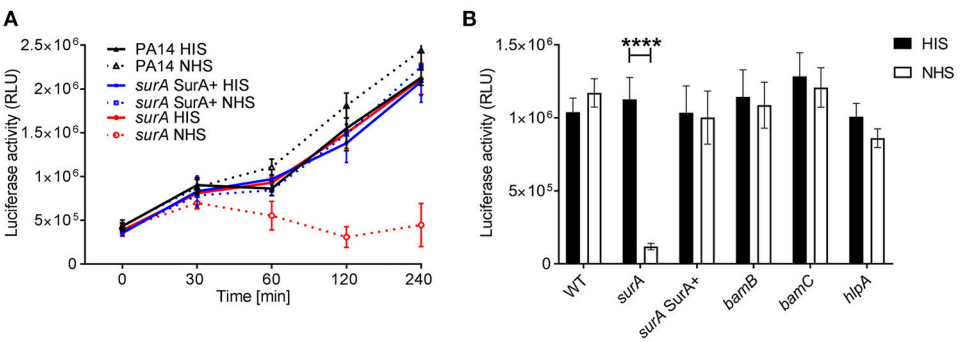

FIGURE 4 | *SurA* deletion leads to increased serum sensitivity. Indicated bacterial strains were grown for **(A)** various time periods or **(B)** 2 h and subsequently, luciferase activity (which is directly proportional to the ATP levels of viable cells in a sample) was measured. Data depict the mean 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

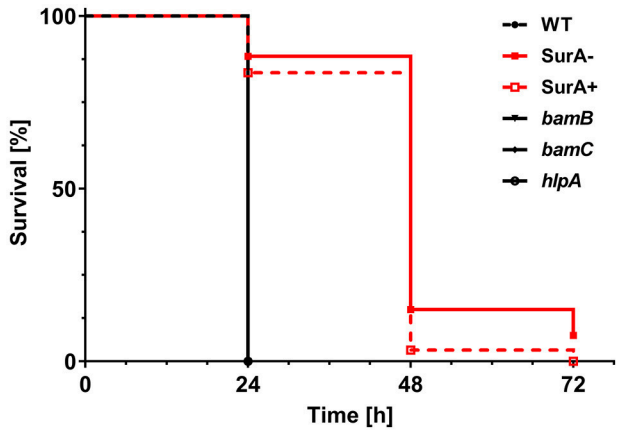

FIGURE 5 | *SurA* deletion leads to attenuated virulence in the *Galleria mellonella* infection model. In total, 60 *G. mellonella* larvae per group were infected in 3 independent experiments with a CFU of 12 ± 2 for the indicated time period and survival of larvae was monitored by touching with a forceps. 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	Fostomycin	–	–	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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Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich die zur Promotion eingereichte Arbeit mit dem Titel:

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Tübingen, den 10.01.2020