In vivo synthesis of monolysocardiolipin and cardiolipin by Acinetobacter baumannii phospholipase D and effect on cationic antimicrobial peptide resistance

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Summary
Acinetobacter baumannii is an opportunistic pathogen, which has become a rising threat in healthcare facilities worldwide due to increasing antibiotic resistances and optimal adaptation to clinical environments and the human host. We reported in a former publication on the identification of three phospholipases of the phospholipase D (PLD) superfamily in A. baumannii ATCC 19606T acting in concerted manner as virulence factors in Galleria mellonella infection and lung epithelial cell invasion. This study focussed on the function of the three PLDs. A Δpld1-3 mutant was defect in biosynthesis of the phospholipids cardiolipin (CL) and monolysocardiolipin (MLCL), whereas the deletion of pld2 and pld3 abolished the production of MLCL. Complementation of the Δpld1-3 mutant with pld1 restored CL biosynthesis demonstrating that the PLD1 is implicated in CL biosynthesis. Complementation of the Δpld1-3 mutant with either pld2 or pld3 restored MLCL and CL production leading to the conclusion that PLD2 and PLD3 are implicated in CL and MLCL production. Mutant studies revealed that two catalytic motifs are essential for the PLD3-mediated biosynthesis of CL and MLCL. The Δpld1-3 mutant exhibited a decreased colistin and polymyxin B resistance indicating a role of CL in cationic antimicrobial peptides (CAMPs) resistance.

Introduction
Multidrug resistant and extensive drug resistant Acinetobacter baumannii strains have become a major healthcare threat worldwide and are considered by the World Health Organization as critical pathogens, which pose the greatest threat to human health and for which new antibiotics are urgently needed (World Health Organization, 2017). A. baumannii induces pneumonia, skin and urinary tract infections and bacteraemia, which can lead to high morbidity and mortality especially in immunocompromised individuals (Fournier and Richet, 2006; Falagas and Rafailidis, 2007; Peleg et al., 2008). Several factors contribute to the success of A. baumannii as major hospital-acquired pathogen such as persistence in clinical settings under desiccation, nutrient starvation and resistance against high concentrations of different antibiotics (Peleg et al., 2008; Antunes et al., 2011; Antunes et al., 2014; Harding et al., 2018). While there have been many insights into the epidemiology and antibiotic resistance of A. baumannii, information with respect to the molecular basis of virulence and pathogenicity is limited. However, it has become clear that the pathogenicity of A. baumannii implies several determinants such as the outer membrane protein OmpA, iron-uptake systems, pili, secretion systems, porins, capsular polysaccharides, outer membrane vesicles, and phospholipases (PLs) (Peleg et al., 2008; Kwon et al., 2009; Antunes et al., 2011; Moubareck and Halat, 2020). The latter are key enzymes for the metabolism of phospholipids, which are abundant in the membranes of host cells, the first barriers encountered by pathogens upon colonization of the human host. Many PLs produced by pathogenic bacteria are involved in virulence by supporting the colonization of different host tissues, the establishment of infections or the escape of the immune response of the host (Songer, 1997; van der Meer-Janssen et al., 2010).

PLs are assigned to three different classes depending on the cleavage site. Phospholipases A (PLAs) hydrolyze the fatty acids from glycerol, phospholipases C (PLCs) release the phosphorylated headgroup from the...
phospholipid and phospholipases D (PLDs) hydrolyze the phosphodiester bond between the phosphate and the headgroup of the phospholipid thereby generating phosphatidic acid and an alcohol moiety (Songer, 1997; Ramrakhiani and Chand, 2011; Damnjanovic and Iwasaki, 2013). In addition, PLDs are able to catalyse transphosphatidylation reactions between two phospholipids (Dawson, 1967; Yang et al., 1967; Stncev and Stuhec-Sealec, 1970). Most members of the PLD superfamily share a common core structure and two PLDc_2 PFAM domains each encompassing a conserved H,K,D,x,G,S,N catalytic motif (HKD motif), which is present in two copies per enzyme (Hammond et al., 1995; Koonin, 1996; Ponting and Kerr, 1996; Stuckey and Dixon, 1999; Selvy et al., 2011). Only rarely, PLDs with one copy of the HKD motif were found (Nishimasu et al., 2012; Celma et al., 2017).

In previous studies, we identified three PLDs in A. baumannii ATCC 19606T (Stahl et al., 2015). Two of the PLDs, PLD1 and PLD2 each harbour two PLDc_2 domains. Each domain contains a highly conserved 28 amino acid long PLD phosphodiesterase active site motif, which spans a H,K,D,x,G,S,N pattern. Interestingly, the third PLD (PLD3) harbours only one PLDc_2 domain and only one conserved HKD motif. A second HKD-like motif was found to overlap with the C-terminal PLDc_2 domain, but here the highly conserved aspartic acid (D) is replaced by a tyrosine. Galleria mellonella (greater wax moth) larvae is an accepted model organism for bacterial infection studies. Its short generation time and its innate immune response which shows significant similarities to the immune response of vertebrates makes it an ideal alternative to the mouse model (Tsai et al., 2016). Moreover, all three PLDs were found to act together in A549 human lung epithelial cell invasion.

In the present study, we analysed the possible function of the three PLDs in modulation of the A. baumannii lipid profile. We provide clear evidence that the three PLDs are implicated in CL biosynthesis with an additional role of PLD2 and PLD3 in MLCL biosynthesis. Our recent finding that the Δpld2 + 3 mutant, which does not produce any MLCL, is significantly affected in lung epithelial infection suggests that MLCL contributes to host cell infection. Here, we found CL to play a role in resistance against cationic antimicrobial peptides (CAMPs) such as colistin and polymyxin.

Results

Lipid composition of single, double and triple pld deletion mutants

To analyse the role of the three PLDs in phospholipid biosynthesis, total lipids of the A. baumannii ATCC 19606T wild type, single, double and triple pld deletion mutants were prepared and analysed by thin layer chromatography (TLC) (Fig. 1A). In A. baumannii ATCC 19606T wild type cells, the phospholipids CL, phosphatidylethanolamine (PE), lyso-PE, phosphatidylglycerol (PG) and acyl-PG, as well as the uncommon phospholipid MLCL were detected. This corresponds to our previous finding (Lopalco et al., 2017). CL, PE and MLCL were the most abundant lipids followed by PG. Acyl-PG and lyso-PE were detected in minor amounts. Δpld1, Δpld2, Δpld3 mutants as well as Δpld1 + 2 and Δpld1 + 3 mutants exhibited lipid profiles, which were identical to those of the wild type cells. Deletion of pld2 + 3 resulted in the absence of MLCL in the total lipids, whereas the deletion of all three plds led to the absence of MLCL and a significant decrease in the amount of CL (Fig. 1A). The absence of MLCL in the Δpld2 + 3 mutant and the Δpld1-3 mutant was accompanied by an increase in the amount of PG (Fig. 1A). Complementation of the Δpld1-3 mutant with pld1 restored CL production but not MLCL production. Complementation of the Δpld1-3 mutant with either pld2 or pld3 restored CL production, as well as MLCL production (Fig. 1B).

To verify the results obtained by the TLC analyses, the total lipid extracts of all A. baumannii pld mutants were analysed by MALDI-TOF/MS in the negative ion mode (Fig. 2). The peaks in the MALDI-TOF/MS lipid profile can be grouped in three main m/z ranges: the interval m/z 650–800 where the peaks of main phospholipids are present; the m/z interval 1100–1200 containing the MLCLs peaks and the m/z range 1350–1450 containing the CLs. The major phospholipid signals are at m/z 716.4.
and 747.8, attributable to PE (34:1) and PG (34:1) respectively; in addition, minor peaks attributable to other species of PE and PG are also present. In the m/z ranges of MLCLs and CLs, the main peaks are at m/z 1165.1 attributable to MLCL (52:2) and m/z 1403.3 attributable to CL (68:2). Major and minor peaks in the lipid profiles, correspond to the lipid species previously described (Lopalco et al., 2017). As in the wild type cells, the abundant phospholipids, CL and MLCL were detected in all single pld deletion mutants as well as in Δpld1 + 2 and Δpld1 + 3 mutants. In consistency with our TLC results, the total lipids of the Δpld2 + 3 mutant did not contain MLCLs and neither CLs nor MLCLs could be detected in the Δpld1-3 mutant. Taken together, the lipid profiles of the different pld mutants together with those of the complemented Δpld1-3 mutant lead to the conclusion that the PLD1 plays a major role in MLCL biosynthesis, whereas PLD2 and PLD3 are essential for both, the synthesis of CL and MLCL, respectively.

Growth of A. baumannii ATCC 19606T wild type and Δpld1-3 mutant

We reported in a former publication that the Δpld1-3 mutant was unaffected in growth in mineral medium (MM) with 20 mM acetate at 37°C (Stahl et al., 2015) exhibiting a growth rate of 0.75–0.8 h⁻¹ and a maximal optical density of 1.3. However, this does not exclude a role of CL and MLCL during growth under stress conditions. To analyse the effect of the absence of CL and MLCL on growth under stress conditions, wild type and Δpld1-3 mutant cells were grown in MM with 20 mM acetate at 22°C, 42°C or at 37°C in the presence of 0.5 M NaCl (Fig. 3A–C). Growth at 22°C resulted in a prolonged lag phase of 2 h, a growth rate of 0.44 h⁻¹ and a final OD₆₀₀ of 1.09. Cells that were grown at 42°C were severely impaired in growth and exhibited a growth rate of 0.16 h⁻¹ and a final OD₆₀₀ of 0.3. In addition, growth of wild type and the Δpld1-3 mutant cells at 37°C in the presence of 0.5 M NaCl led to a prolonged lag phase of 4 h and a decrease in growth rate to 0.15–0.19 h⁻¹. Also, the final OD₆₀₀ of 0.49 was decreased in comparison to the growth without osmotic stress. However, no difference in growth of the wild type and the Δpld1-3 mutant was observed. This leads to the conclusion, that CL and MLCL are not required for growth of A. baumannii under these stress conditions.

Fig. 2. MALDI-TOF/MS analysis of the total lipid extract of A. baumannii mutants. Total lipids of A. baumannii wild type, single, double and triple pld deletion mutants were separated by TLC and mass spectra of the samples were acquired in negative ion mode. Mass spectrum represents the MALDI-TOF/MS lipid profile in the full m/z range 600–1500. [Color figure can be viewed at wileyonlinelibrary.com]
growth of the Δpld2 + 3 mutant and Δpld1-3 mutant was monitored in the presence of colistin or polymyxin B. Therefore, serial dilutions of the Δpld2 + 3 mutant, the Δpld1-3 mutant and the wild type strain were spotted on LB agar plates containing the respective drug. As shown in Fig. 4, growth of the Δpld2 + 3 mutant was not affected by 0.35 μg ml⁻¹ colistin, whereas the Δpld1-3 mutant was affected in growth. In the 10⁻⁵ and 10⁻⁶ dilutions of the Δpld1-3 mutant significantly less colonies were detected than in the analogous dilutions of the wild type strain. The same was found with respect to the polymyxin B resistance. Taken together, these results lead to the conclusion that the absence of MLCL and CL in the Δpld1-3 mutant leads to increased CAMP sensitivities.

Role of the two catalytic motifs of PLD3 in CL and MLCL biosynthesis

Our previous finding that the second catalytic motif of the PLD3 differs from the conserved HKD motifs raised the question whether both HKD motifs are important for CL and MLCL synthesis. To address this question, the catalytically relevant histidines of the two motifs were replaced either by aspartate or by alanine (Fig. 5) by site-directed mutagenesis. Total lipids of the wild type and Δpld1-3 mutant producing PLD3 variants were analysed by TLC (Fig. 6). Complementation of the Δpld1-3 mutant with a PLD3 variant where the catalytic histidine 222 (His222) was changed to aspartate or alanine led to the absence of CL or MLCL. Also, the complementation of the Δpld1-3 mutant with a PLD3 variant where the histidine of the second modified HKD motif was exchanged against aspartate (His393Asp, His393Ala) did not restore MLCL and CL production. These findings lead to the conclusion that both HKD motifs are essential for the function of the PLD3 in CL and MLCL production.

Discussion

Members of the PLD family are widespread in nature and exhibit a broad range of activities using different substrates (Hananhan and Chaikoff, 1947; Ella et al., 1995). Here, we demonstrate that all three PLDs of A. baumannii ATCC 19606T mediate CL synthesis. Moreover, PLD2 and PLD3 were found to be also involved in MLCL biosynthesis. In addition to CL and MLCL A. baumannii ATCC 19606T produces the abundant phospholipids PE, PG, Acyl-PG and LPE (Lopalco et al., 2017). Deletions resulting in a lack of MLCL or MLCL and CL led to an increase in PG indicating that PG serves as a substrate for the PLD mediated CL production. This will be addressed in future biochemical studies of purified PLDs. Indeed, it is known that CL is either synthesized from two molecules of PG or from the reaction of one molecule PG with CDP-DAG (Nishijima et al., 1988; Sandoval-Calderon et al., 2009). MLCL could then be generated from CL by the action of a PLA removing one acylchain.

CL is an abundant lipid in membranes of prokaryotes, eukaryotes and archaea (Schlame, 2008). In aerobic bacteria and in the membranes of mitochondria, CL is important for the function and stability of respiratory chain complexes (Lange et al., 2001; Mileykovskaya et al., 2005; Sediak et al., 2006). For Rhizobium tropici, it is known, that the lipid composition of the membrane has an impact on antibiotic resistance (Sohlenkamp et al., 2007). Our studies revealed that this is also the case for A. baumannii ATCC 19606T as evidenced by the higher susceptibility to the CAMPs colistin and polymyxin B in the absence of CL and MLCL from the total lipids. CLs are known to interact with cationic amphipathic helical protein segments on bilayer surfaces (Schlame, 2008) and might direct CAMPs to specific CL-rich domains in the membrane, thereby increasing
bacterial resistance (Tran et al., 2013; Dalebrout et al., 2014). Resistance against polymyxins can also be mediated by modification of the lipid A anchor of the lipopolysaccharide (LPS) of Gram-negative bacteria (Bader et al., 2005; Hankins and Trent, 2009; Fernández et al., 2010).

Members of the PLD-superfamily share a conserved catalytic motif, which contains the duplicated HxKx4Dx6GS/GGxN sequence (Hammond et al., 1995; Ponting and Kerr, 1996). Although most members of the family contain two catalytic HKD motifs, enzymes with one HKD motif have been described. For those enzymes, activity relies on dimerization (Damnjanovic and Iwasaki, 2013; Celma et al., 2017). It has been shown before that the histidine residues of the HKD motifs are essential for enzyme activity (Sung et al., 1997). A similar observation was made here, such as the loss of histidines led to a loss of CL/MLCL synthesis.

The PLDs of A. baumannii ATCC 19606T have been already identified as virulence factors. Each of them contributes to virulence in the G. mellonella infection model and plays an important role in host cell invasion (Stahl et al., 2015) and in lung epithelial cell invasion (Jacobs et al., 2010). These findings together with the role of the PLDs in MLCL and CL production lead to the conclusion that MLCL and CL play a role in infection and persistence of A. baumannii in the human host. A role of CL in virulence has already been demonstrated for different pathogenic bacteria, such as in the pathogen Shigella flexneri, CL is important for the intercellular spread of this intracellular bacterium (Rossi et al., 2017). Movement of the bacterium is facilitated by IcsA-mediated actin polymerization (Bernardini et al., 1989) and it has been shown that CL is needed in the outer membrane for proper presentation of the protein (Rossi et al., 2017). In addition, in Moraxella catarrhalis, CL is required for the attachment of the organism to human epithelial cells and it is hypothesized that the lipid is required for the proper function or localization of adhesion proteins (Buskirk and Lafontaine, 2014). In contrast to this, the Δpld1-3 mutant of A. baumannii which does not produce MLCL and CL is not impaired in adhesion to human lung epithelial cells (Stahl et al., 2015). However, a general impact of CL stabilizing membrane proteins required for host cell invasion and persistence cannot be excluded.
The large variety of MLCLs detected in A. baumannii ATCC 19606T and other clinical A. baumannii strains in contrast to the low MLCL levels in the nonpathogenic A. baylyi ADP1 (Lopalco et al., 2017) are consistent with the conclusion that MLCL also plays a role in virulence of A. baumannii. MLCL has been detected in only very few bacteria (Torregrossa et al., 1977; Hansen et al., 2015; Lopalco et al., 2017). For Lactobacillus acidophilus La-5, it is assumed that MLCL and CL are used by the organism to regulate its membrane fluidity and bilayer stability by modification of the saturation levels of the lipids (Hansen et al., 2015) and other lysocardiolipins have also been identified in Clostridium inoccum, Streptomyces hygroscopicus and A. radioresistens (Johnston et al., 1994; Hoischen et al., 1997; Luo et al., 2018). The human pathogen Helicobacter pylori is able to produce two distinct morphology states, a HpL-type that does not adhere to or invade epithelial cells and a HpS-type that is capable of adhesion and invasion (Bukholm et al., 1997). Interestingly, the HpS-type produces significantly more lysolipids, such as lysocardiolipin, than the HpL-type, indicating a role of lysolipids in virulence of the organism (Tannaes et al., 2000). However, to our knowledge, nothing is known with respect to the role of MLCL in virulence of bacteria. The role of MLCL in host cell invasion of A. baumannii will be addressed in future studies.

**Experimental procedures**

**Bacterial strains and culture conditions**

Acinetobacter baumannii ATCC 19606T wild type and markerless pld deletion mutants (Δpld1, Δpld2, Δpld3, Δpld1 + 2, Δpld1 + 3, Δpld2 + 3 and Δpld1-3 (Stahl et al., 2015)) were grown at 37℃ in MM. The MM consists of 50 mM phosphate buffer, pH 6.8, mineral solution (composition per litre: 1 g NH₄Cl, 580 mg MgSO₄ × 7 H₂O, 100 mg KNO₃, 67 mg CaCl₂ × 2 H₂O, 2 mg (NH₄)₂MoO₄ × 4 H₂O, 1 ml SL9 (per litre: 12.8 g Tiritplex, 2 g FeSO₄ × 7 H₂O, 190 mg CoCl₂ × 6 H₂O, 122 mg MnCl₂ × 4 H₂O, 70 mg ZnCl₂, 36 mg MoNa₂O₄ × 2 H₂O, 24 mg NiCl₂ × 6 H₂O, 6 mg H₂BO₃, 2 mg CuCl₂ × H₂O; pH 6.5) (Tschech and Pfennig, 1984)) and 20 mM acetate as carbon source. Kanamycin was added to a final concentration of 50 μg ml⁻¹ when appropriate.

**Extraction of total lipids**

Bacteria were grown in MM and harvested by centrifugation (10 min, 4700 rpm) in the stationary growth phase (OD₆₀₀ 1.3), washed with 0.9% [w/v] NaCl, frozen in liquid nitrogen and stored at −20℃ until used for lipid extraction. Total lipids were extracted from frozen and thawed cells using a method modified from Bligh and Dyer (methanol/chloroform/water; 2:1:0.8, by volume) (Bligh and Dyer, 1959). To optimize the lipid yield, 4 ml of 0.2 M KCl and 2 ml of chloroform were added (Folch et al., 1957). The extracts were dried under N₂ before weighing and then dissolved in chloroform (final concentration 10 mg ml⁻¹).

**Analysis of phospholipid species by thin layer chromatography (TLC)**

Total lipid extracts were analysed by TLC. Silica gel TLC plates were obtained from Machery-Nagel (ALUGRAM® Xtra SILGUR, layer thickness 0.2 mm) or Merck (20 Å/C₂₄/C₁₄/C₈, 10 cm, layer thickness 0.2 mm). The plates were washed twice with chloroform/methanol (1:1, by volume) and activated at 180℃ before use. Lipids were then spotted onto the silica gel TLC plate and polar lipids were eluted with solvent A (chloroform/methanol/acetic acid/water 85:15:10:3.5, by volume). Phospholipid detection was carried out by spraying the plate with molybdnum blue spray reagent (Sigma-Aldrich) (Lopalco et al., 2017).

**Preparation of samples for MALDI-TOF/MS lipid analyses**

A total of 3 μl lipid extract in chloroform solution (10 μg μl⁻¹) was diluted in 27 μl of 2-propanol/acetonitrile (60/40, by volume), then 10 μl of the diluted solution were used.
mixed with 10 μl of matrix solution (9-AA, 10 μg μl⁻¹ in 2-propanol/acetonitrile 60/40, by volume), as previously described (Sun et al., 2008; Lopalco et al., 2013). The resulting lipids-matrix solution was then spotted onto the MALDI target (Micro Scout Plate, MSP 96 ground steel target) in droplets of 0.35 μl and analysed as described below. After the evaporation of the matrix solvent, the samples are ready to be directly analysed with MALDI-TOF/MS.

**MALDI-TOF mass spectrometry**

MALDI-TOF mass spectra of lipid extracts were acquired on a Bruker Microflex LRF mass spectrometer (Bruker Daltonics, Bremen, Germany). The system utilizes a pulsed nitrogen laser, emitting at 337 nm, the extraction voltage was 20 kV and gated matrix suppression was applied to prevent detector saturation. For each mass spectrum, 2000 single laser shots (sum of 4 × 500) were averaged. The laser fluence was kept about 5% above threshold to have a good signal-to-noise ratio. The spectra were acquired in reflector mode (detection range: 200–2000 mass/charge, m/z) using the delayed pulsed extraction; spectra were acquired in negative ion modes. Spectral mass resolutions and signal-to-noise ratios were determined by the software for the instrument ‘Flex Analysis 3.3’ (Bruker Daltonics). A mix containing: 1,2-dimyristoyl-snglycero-3-phosphate, 1,2-dimyristoyl-sn-glycero-3-phospho-(1'–rac-glycerol), 1,2-dimyristoyl-sn-glycero-3-phospho-serine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine, 1',3'-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol, 1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol, was always spotted next to the sample as external standard and an external calibration was performed before each measurement; the mass range of the authentic standards is 590–1450 amu.

**Complementation of the Δpld1-3 mutant with wildtype pld1, pld2 and pld3**

To complement the Δpld1-3 mutant with distinct pld genes the three pld genes and their upstream promoter region were amplified from chromosomal DNA of A. baumannii ATCC 19606T using the primers 1–6 (Supporting Information Table S1). The amplified pld genes were integrated into the E. coli-A. baumannii shuttle vector pBAV1K, which was generated from pBAV1K_T5_gfp (Bryksin and Matsumura, 2010) by liberation of the pBAV1K backbone from pBAV1K_T5_gfp (deletion of the T5 promoter and gfp) using NotI followed by religation yielding pBAV1K. For pBAV1K_pld1, the amplified pld1 gene with its corresponding upstream region was cloned into NotI and PstI sites of pBAV1K. pBAV1K_pld2 and pBAV1K_pld3 were generated using Gibson assembly (Gibson et al., 2008). Therefore, pBAV1K was amplified using primers 7 and 8 (Supporting Information Table S1) and assembled with pld2 (primers 3 and 4, Supporting Information Table S1) or using primers 9 and 10 and assembled with pld3 (primers 5 and 6, Supporting Information Table S1). The plasmids were transferred into the A. baumannii Δpld1-3 mutant.

**Antibiotic sensitivity test**

A. baumannii ATCC 19606T was grown over night in LB broth. The OD₆₀₀ was adjusted to 1 and serial dilutions (1:10 in phosphate buffered saline) were prepared. 4 μl of the dilutions were spotted on LB agar plates containing 0.35 μg ml⁻¹ colistin or 0.25 μg ml⁻¹ polymyxin B.

**Site-directed mutagenesis of pld3**

To analyse the individual role of the conserved amino acid residues of the catalytic HKD motifs in the PLD3 the E. coli-A. baumannii shuttle vector pBAV1K carrying the pld3 gene (pBAV1K_pld3) was used as template to exchange the histidine residues against alanine or asparagine resulting in plasmids pBAV1K_pld3*H222A, pBAV1K_pld3*H222D, pBAV1K_pld3*H393A and pBAV1K_pld3*H393D. For pBAV1K_pld3*H222A and pBAV1K_pld3*H222D pBAV1K_pld3 was amplified using the primer pairs 11/12 and 13/14 (Supporting Information Table S1) respectively. The resulting PCR products were circularized using Gibson assembly (Gibson et al., 2008). For pBAV1K_pld3*H393A and pBAV1K_pld3*H393D pBAV1K_pld3 was amplified using the primer pairs 15/16 and 16/17 (Supporting Information Table S1) respectively. PCR products were circularized via blunt end ligation. The resulting plasmids were transferred into the A. baumannii Δpld1-3 mutant.

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