Reinforcing Lipid A Acylation on the Cell Surface of *Acinetobacter baumannii* Promotes Cationic Antimicrobial Peptide Resistance and Desiccation Survival

Joseph M. Boll,[a] Ashley T. Tucker,[a] Dustin R. Klein,[c] Alexander M. Beltran,[a] Jennifer S. Brodbelt,[c] Bryan W. Davies,[a,b] M. Stephen Trent[d]

Departments of Molecular Biosciences,[a] Institute for Cellular and Molecular Biology,[b] Department of Chemistry,[c] University of Texas at Austin, Austin, Texas, USA; Department of Infectious Diseases, University of Georgia, College of Veterinary Medicine, Athens, Georgia, USA[d]

ABSTRACT *Acinetobacter baumannii* is an emerging Gram-negative pathogen found in hospitals and intensive care units. In order to persist in hospital environments, *A. baumannii* withstands desiccative conditions and can rapidly develop multidrug resistance to conventional antibiotics. Cationic antimicrobial peptides (CAMPs) have served as therapeutic alternatives because they target the conserved lipid A component of the Gram-negative outer membrane to lyse the bacterial cell. However, many Gram-negative pathogenic bacteria, including *A. baumannii*, fortify their outer membrane with hepta-acylated lipid A to protect the cell from CAMP-dependent cell lysis. Whereas in *Escherichia coli* and *Salmonella*, increased production of the outer membrane acyltransferase PagP results in formation of protective hepta-acylated lipid A, which reinforces the lipopolysaccharide portion of the outer membrane barrier, *A. baumannii* does not carry a gene that encodes a PagP homolog. Instead, *A. baumannii* has evolved a PagP-independent mechanism to synthesize protective hepta-acylated lipid A. Taking advantage of a recently adapted *A. baumannii* genetic recombineering system, we characterized two putative acyltransferases in *A. baumannii* designated $LpxL_{Ab}$ (*A. baumannii* LpxL) and $LpxM_{Ab}$ (*A. baumannii* LpxM), which transfer one and two lauroyl ($C_{12:0}$) acyl chains, respectively, during lipid A biosynthesis. Hepta-acylation of *A. baumannii* lipid A promoted resistance to vertebrate and polymyxin CAMPs, which are prescribed as last-resort treatment options. Intriguingly, our analysis also showed that $LpxM_{Ab}$-dependent acylation of lipid A is essential for *A. baumannii* desiccation survival, a key resistance mechanism for survival in hospital environments. Compounds that inhibit $LpxM_{Ab}$-dependent hepta-acylation of lipid A could act synergistically with CAMPs to provide innovative transmission prevention strategies and treat multidrug-resistant infections.

IMPORTANCE *Acinetobacter baumannii* infections can be life threatening, and disease can progress in a variety of host tissues. Current antibiotic regimen and disinfectant strategies have failed to limit nosocomial *A. baumannii* infections. Instead, the rate of *A. baumannii* infection among health care communities has skyrocketed due to the bacterium's adaptability. Its aptitude for survival over extended periods on inanimate objects, such as catheters, respirators, and surfaces in intensive care units, or on the hands of health care workers and its ability to rapidly develop antibiotic resistance make *A. baumannii* a threat to health care communities. Emergence of multidrug- and extremely drug-resistant *A. baumannii* illustrates the ineffectiveness of current prevention and treatment options. Our analysis to understand how *A. baumannii* resists cationic antimicrobial peptide (CAMP)-mediated and desiccative killing revealed two lipid A acyltransferases that produce protective hepta-acylated lipid A. Our work suggests that inhibiting lipid A biosynthesis by targeting the acyltransferase $LpxM_{Ab}$ (*A. baumannii* LpxM) could provide a novel target to combat this pathogen.

The outer membrane of Gram-negative bacteria is a highly conserved barrier consisting of an inner monolayer of glycerophospholipids and a surface-exposed monolayer of lipopolysaccharide (LPS). The amphipathic properties of LPS and phospholipids allow spontaneous formation of a membrane bilayer where the hydrophobic lipid moieties are sandwiched between the hydrophilic groups. The biophysical membrane properties restrict diffusion of toxic molecules (e.g., antibiotics) across the membrane into the cell. LPS is a biologically distinct glycolipid that contains three domains: the bioactive membrane anchor called lipid A, core sugars that extend from lipid A, and a core-ligated O-antigen carbohydrate repeat (1). Many mucosal pathogens, including *Acinetobacter baumannii* synthesize lipooligosaccharide (LOS), which includes only core and lipid A. Whereas the O-antigen domain is dispensable, lipid A and core are required for bacterial survival in a host. In fact, inhibitors that target essential enzymatic steps in lipid A biosynthesis have provided promising antimicrobial chemotherapeutics (2–4).
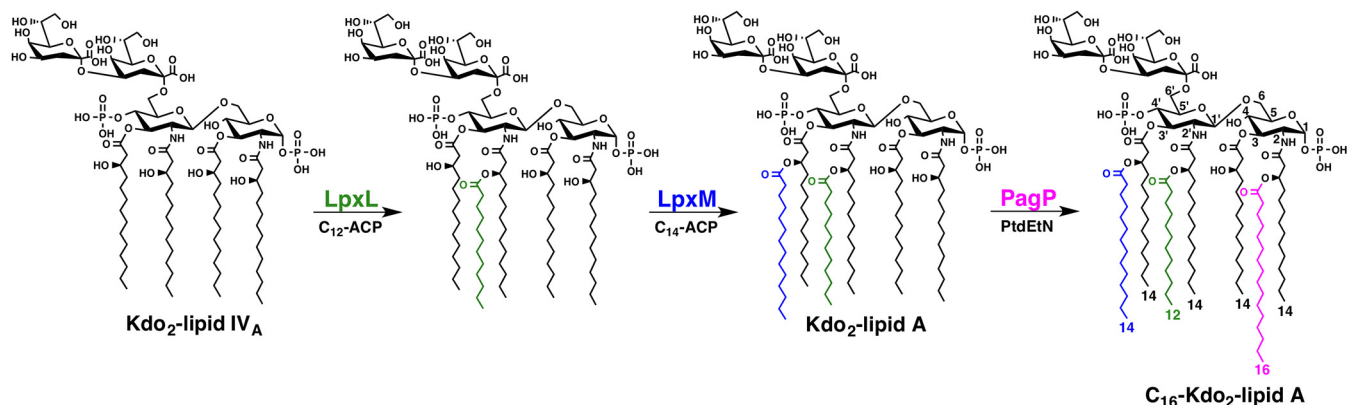
FIG 1 Synthesis of hexa- and hepta-acylated lipid A in *E. coli* K-12. In *E. coli*, LpxL catalyzes transfer of a laurate ($C_{12:0}$) group from an acyl carrier protein (ACP) onto the *R*-2′-hydroxymyristate acyl chain of Kdo_2-lipid IV_A. Subsequently, LpxM-dependent addition of myristate ($C_{14:0}$) onto the *R*-3′-hydroxymysristate residue completes synthesis of hexa-acylated Kdo_2-lipid A. Upon sensing specific environmental cues, PagP activation results in palmitoylation ($C_{16:0}$) of the *R*-2-hydroxymyristate primary acyl chain, altering membrane fluidity and increasing resistance to antimicrobial peptides. PtdEtN, phosphatidylethanolamine.

In the well-defined Gram-negative bacterium *Escherichia coli* K-12, LPS/LOS biosynthesis initiates with formation of Kdo_2-lipid A (Kdo stands for 3-deoxy-D-manno-octulosonic acid) or endotoxin. Nine conserved enzymes, termed the Raetz pathway (1), coordinately synthesize hexa-acylated lipid A. While the first seven enzymes assemble the precursor Kdo_2-lipid IV_A, the last two biosynthetic steps are completed by LpxL and LpxM. LpxL first catalyzes transfer of laurate ($C_{12:0}$) followed by LpxM-dependent myristate ($C_{14:0}$) addition in a stepwise manner to complete synthesis of the hexa-acylated and *bis*-phosphorylated Kdo_2-lipid A molecule (Fig. 1) (5–7).

Despite *E. coli* K-12 providing a basic model to understand lipid A biosynthesis in Gram-negative bacteria, many pathogens remodel the hexa-acylated molecule into diverse lipid A-based structures. Lipid A modifications directly affect the ability of a pathogen to survive in its host by altering outer membrane permeability, by camouflaging the pathogen from host immune detection, and by promoting resistance to antimicrobial peptides (1, 8). While pathogens exploit diverse strategies to survive in a host, a detailed understanding of the molecular mechanisms that mediate bacterial survival is key for the development of new and more-effective antimicrobial treatments.

Gram-negative bacteria can alter their lipid A structure by incorporating additional chemical moieties or by altering the lipid A phosphate or acyl chain groups (8, 9). Well-defined transcriptional and posttranscriptional regulatory systems tightly control the lipid A enzymatic modification machinery, illustrating the importance of lipid A on bacterial survival (8, 10, 11). One well-characterized lipid A modification involves palmitate addition to form hepta-acylated lipid A, which promotes bacterial resistance to cationic antimicrobial peptides (CAMPs), fortifies the outer membrane, and reduces host immune recognition (). In a number of pathogens, hepta-acylation is a protective response to a specific environmental stressor. In the presence of the signal, the bacterium initiates a regulatory phosphorylation cascade that culminates in increased expression of the outer membrane protein PagP (12). In the outer membrane, PagP transfers a palmitoyl ($C_{16:0}$) group to lipid A with phosphatidylethanolamine serving as the acyl donor (13).

Unlike most Gram-negative pathogens, which synthesize hexa-acylated lipid A under standard growth conditions, the nosocomial pathogen *Acinetobacter baumannii* produces a predominant hepta-acylated lipid A molecule in a PagP-independent fashion (15, 16). In this study, we investigate the biosynthetic mechanism utilized for production of hepta-acylated lipid A in *A. baumannii* and characterize how it impacts bacterial fitness. *A. baumannii* is a resilient Gram-negative pathogen that is well adapted to survive in hospital or intensive care unit environments. Over the past decade, the rate of *A. baumannii* transmission and infection in health care communities has significantly increased patient morbidity and mortality (17). *A. baumannii* infections are diverse, and treatment options have become extremely limited due to a propensity for *A. baumannii* to develop resistance to conventional antibiotics. The bacterium develops antibiotic resistance through acquisition of resistance genes, upregulation of efflux pumps, and through modification of outer membrane structures (18). Oftentimes acquisition of resistance mechanisms results in reduced bacterial fitness (19–21). Emergence of multidrug-resistant (MDR) and extensively drug-resistant strains has required increased prescription of last-resort antibiotics (17, 22–25). Currently, last-line treatments include a CAMP called colistin which targets lipid A to perturb the membrane and lyse the bacterium (26). Despite success of last-resort CAMPs like colistin, recent studies have shown that *A. baumannii* can develop resistance under selective pressure (25). Therefore, a detailed understanding of the bacterial factors essential for CAMP resistance in *A. baumannii* is necessary to direct innovative chemotherapeutic strategies.

Another important factor contributing to the spread of *A. baumannii* is its propensity to withstand desiccation. Uniquely, *A. baumannii* survives on inanimate objects without nutrients or water for extended periods, which promotes transmission throughout health care environments (27). The molecular factors that mediate desiccation survival are not well understood, but identification of desiccation fitness determinants could result in more effective eradication strategies.

In this study, we characterized two genes designated *lpxL*$_{Ab}$ (*A. baumannii* lpxL) and *lpxM*$_{Ab}$ (*A. baumannii* lpxM) that are required for biosynthesis of hepta-acylated lipid A in *A. baumannii*. Unlike lipid A biosynthesis in most other Gram-negative

pathogens, *A. baumannii* encodes a dual acyltransferase designated LpxM$_{Ab}$ (*A. baumannii* LpxM), which transfers two lauroyl groups onto lipid A to synthesize hepta-acylated lipid A via a PagP-independent mechanism. Our analysis demonstrated that hepta-acylated lipid A is the dominant glycolipid on the surface of *A. baumannii* and activates human Toll-like receptor 4 (TLR-4). In contrast, LOS isolated from *lpxL*$_{Ab}$ and *lpxM*$_{Ab}$ mutants failed to stimulate the human TLR-4 innate immune pathway. Similar to studies performed in *Salmonella*, hepta-acylated lipid A promoted bacterial resistance to vertebrate CAMPs (10); however, hepta-acylated lipid A in *A. baumannii* also conferred resistance to polymyxin CAMPs such as the last-resort antimicrobial agent colistin. In addition, we determined that hepta-acylated lipid A is important for bacterial desiccation survival, indicating that loss of outer membrane fatty acids is detrimental to *A. baumannii* persistence on inanimate objects in the hospital.

RESULTS

A. baumannii encodes LpxL and LpxM lipid A acyltransferase homologs.

Previous studies to characterize the biosynthesis of lipid A, the bioactive moiety that anchors LPS/LOS to the outer membranes of Gram-negative bacteria, established that two secondary acyltransferases catalyze the final two enzymatic steps. In *E. coli*, LpxL functions first by transferring a laurate group onto Kdo$_2$-lipid IV$_A$ followed by myristate addition via LpxM to complete biosynthesis of hexa-acylated Kdo$_2$-lipid A (Fig. 1) (5–7). The two secondary acylation steps in lipid A biosynthesis are generally conserved among Gram-negative bacteria. However, lipid A secondary acyltransferases that recognize noncanonical substrates in an epsilonproteobacterium have been reported (28).

Instead of synthesizing hexa-acylated lipid A, like many Gram-negative bacterial pathogens, *A. baumannii* synthesizes a predominant hepta-acylated lipid A species under normal growth conditions (15, 16). While the mechanism that leads to *A. baumannii* lipid A hepta-acylation has not been characterized, analysis of *E. coli* and *Salmonella* determined that lipid A hepta-acylation is dependent on the outer membrane protein PagP (Fig. 1) (10, 13, 29). PagP expression increases in response to specific stimuli that perturb the outer membrane, leading to migration of phospholipids in the outer leaflet (30). Mechanistically, PagP transfers a palmitate acyl chain (C$_{16:0}$) from phosphatidylethanolamine to the lipid A *R*-2-hydroxymyristate to generate hepta-acylated lipid A (Fig. 1) (13). In *E. coli* and *Salmonella*, palmitate addition protects the cell from vertebrate CAMPs by reducing the permeability of the outer membrane barrier (10). However, the biological role of hepta-acylated lipid A in *A. baumannii* has not been examined. Intriguingly, *A. baumannii* does not carry a gene that encodes a PagP homolog, suggesting that it has evolved a PagP-independent acylation mechanism.

In order to identify putative lipid A acyltransferases in *A. baumannii*, *E. coli* LpxL and LpxM amino acid sequences were analyzed by Basic Local Alignment Search Tool (BLAST). Bioinformatics analysis identified two *A. baumannii* genes that encode putative lipid A acyltransferases, designated in strain ATCC 17978 as *A1S_0431* and *A1S_2609*. *E. coli* LpxL shared 39% identity with the *A1S_0431* gene product, designated LpxL$_{Ab}$, while LpxM shared 23% identity to the *A1S_2609* gene product, which we designated LpxM$_{Ab}$. LpxL$_{Ab}$ and LpxM$_{Ab}$ shared 29% identity to each other.

Targeted removal of each putative acyltransferase gene was performed by genetic recombineering, which was recently adapted for use in *A. baumannii* (31). Strains harboring nonpolar markerless deletions of *lpxL*$_{Ab}$ and *lpxM*$_{Ab}$ were isolated. While *lpxM*$_{Ab}$ is not transcribed in an operon, *lpxL*$_{Ab}$ could potentially be cotranscribed with *A1S_0430*, encoding LpsB, and *A1S_0432*, which encodes a putative transport protein. LpsB is a glycosyltransferase required for core oligosaccharide attachment to lipid A (32), which is important for *A. baumannii* virulence, CAMP resistance, and polymyxin resistance (33). For complementation of each acyltransferase, each respective coding sequence was expressed from an isopropyl-β-ᴅ-thiogalactopyranoside (IPTG)-inducible promoter on a replicon in the mutant strain (see Table S1 in the supplemental material). Growth curve analysis of the mutants and complemented strains did not reveal any major growth defect compared with the wild-type strain (see Fig. S1 in the supplemental material).

To analyze the effect on lipid A produced by each *A. baumannii* strain, ^{32}P-radiolabeled lipid A was isolated and chromatographically separated based on hydrophobicity. Quantitative thin-layer chromatography (TLC) showed that wild-type *A. baumannii* produced a predominant hepta-acylated lipid A species with relatively minor amounts of a hexa-acylated species (Fig. 2A, lane 1). Strains with deletions in the LpxL$_{Ab}$ and LpxM$_{Ab}$ genetic coding sequences exhibited reduced hydrophobic migration patterns indicative of decreased acylation relative to the wild-type strain (Fig. 2A, lanes 2 and 4). Complementation restored production of wild-type hepta-acylated lipid A in each mutant strain (Fig. 2A, lanes 3 and 5). Unlike the well-defined *E. coli* lipid A biosynthetic pathway, these initial results also indicate that secondary acylation in *A. baumannii* is not strictly ordered, since both *lpxL*$_{Ab}$ and *lpxM*$_{Ab}$ mutants retain secondary acyl chains.

To determine the mutant acylation pattern, matrix-assisted laser desorption ionization−time of flight (MALDI-TOF) mass spectrometry (MS) was performed on isolated lipid A. Spectra collected from wild-type and complemented *A. baumannii* strains indicated major molecular ions at *m/z* 1,728.1 and 1,911.2 (Fig. 2B; see Fig. S2 in the supplemental material), while the Δ*lpxL*$_{Ab}$ strain revealed one major ion at *m/z* 1,713.1 (Fig. 2C) and the Δ*lpxM*$_{Ab}$ strain revealed one major ion species at *m/z* 1,546.0 (Fig. 2D). On the basis of our observed *m/z* ratios, we proposed chemical structures to fit the exact theoretical mass where wild-type *A. baumannii* synthesizes two lipid A species, including a minor hexa-acylated lipid A and a predominant hepta-acylated lipid A (Fig. 2B). Based on our chemical structures in the mutant strains, the Δ*lpxL*$_{Ab}$ strain lacked a hydroxylauric acid, while the Δ*lpxM*$_{Ab}$ strain resulted in production of penta-acylated lipid A lacking two lauroyl groups (Fig. 2C and D).

To verify the *A. baumannii* lipid A secondary acyl groups, higher-energy collisional dissociation (HCD) and UV photodissociation (UVPD) fragmentation spectra were collected for the previously indicated molecular ions. HCD and UVPD are high-energy ion activation methods that produce rich fragmentation patterns and thus enable a detailed structural analysis (34, 35). Unique fragments in the HCD and UVPD spectra at *m/z* 1,911.2, corresponding to wild-type hepta-acylated lipid A, confirmed the presence and length of all seven acyl chains (see Fig. S3 in the supplemental material). Specifically, our analysis revealed four acyl chains attached to the distal side and three on the proximal side of the lipid A molecule as shown in Fig. 3C.
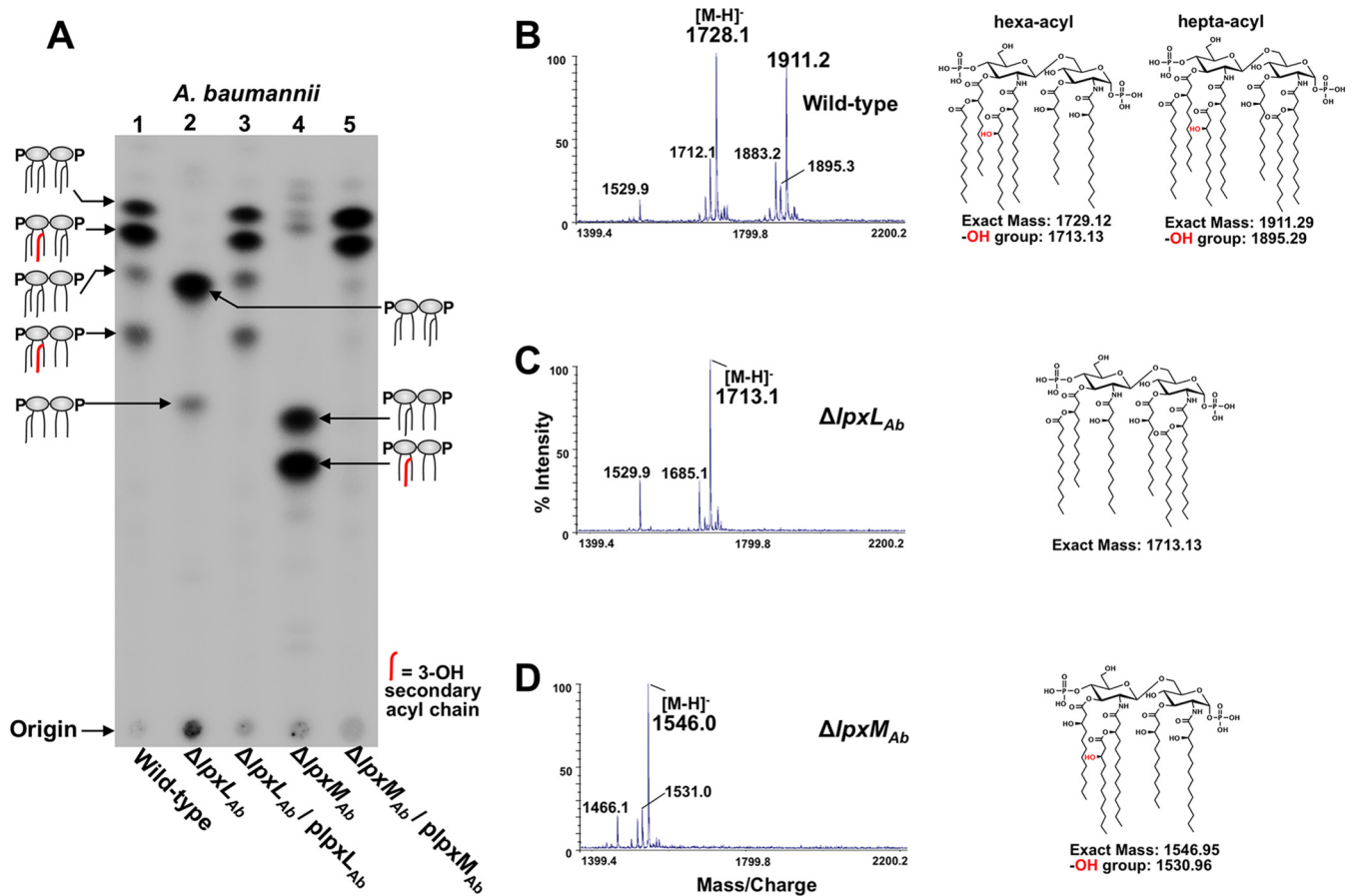
FIG 2 Structural characterization of the *A. baumannii* secondary lipid A acyltransferases. Characterization of lipid A isolated from *A. baumannii* strains. (A) ^{32}P-radiolabeled lipid A from *A. baumannii* was isolated and separated based on hydrophobicity using TLC. Fatty acyl chains bearing a hydroxyl group at position 3 are indicated in red. (B) MALDI-TOF MS and structures of wild-type *A. baumannii* lipid A with *m/z* ratios of 1,729.12 and 1,911.29, corresponding to the hexa- and hepta-acylated species, respectively. Addition of a hydroxyl group (red) corresponds to the *m/z* ratios 1,713.13 and 1,895.16. (C) MALDI-TOF MS of the $\Delta lpxL_{Ab}$ mutant indicating a major species at *m/z* 1,713.1. (D) MALDI-TOF MS of the $\Delta lpxM_{Ab}$ mutant indicating a major species at *m/z* 1,546.0. Addition of the hydroxyl group to lipid A (red) results in a lipid A at *m/z* 1,530.96.

Heterologous expression of LpxM$_{Ab}$ in *E. coli* revealed dual transferase activity. Previously characterized LpxL and LpxM acyltransferases exclusively add one fatty acid to lipid A (5). However, our data indicated that *A. baumannii* $\Delta lpxM_{Ab}$ strains lacked two lauroyl groups. To determine whether LpxM$_{Ab}$ is sufficient to catalyze transfer of two laurate groups onto *A. baumannii* lipid A, we expressed the *lpxL$_{Ab}$* and *lpxM$_{Ab}$* genetic coding sequences in BN2, an *E. coli* strain with deletions in both *lpxM* and *pagP*. *E. coli* strain BN2 synthesizes penta-acyl lipid A with one laurate group attached at position 2′ (Fig. 3A, leftmost lane) (9). Expression of LpxL$_{Ab}$ in strain BN2 did not alter the lipid A structure, suggesting that it is not active in *E. coli* or that it functions like *E. coli* LpxL and only adds a secondary acyl chain at position 2′ (Fig. 3A, middle lane, and Fig. 1) (36). Importantly, expression of LpxM$_{Ab}$ in strain BN2 resulted in formation of both hexa- and hepta-acylated lipid species (Fig. 3A, rightmost lane). Thus, LpxM$_{Ab}$ is sufficient to attach lauroyl groups at two distinct locations of the lipid A molecule, a characteristic unique to LpxM$_{Ab}$. We confirmed attachment of the acyl chains by mass spectrometry where spectra showed two major species at *m/z* 1,768.2 and 1,951.2, which indicate two separate acylation sites (Fig. 3B and C).

Determining LpxM$_{Ab}$ acyl chain placement and hydroxylation. Importantly, the palmitate acyl chain added by PagP at the *R*-2-hydroxymyristate position of lipid A in *E. coli* and *Salmonella* was shown to be important for resistance against host CAMPs (Fig. 1) (10). Therefore, we sought to determine whether placement of the secondary acyl chain in *A. baumannii* lipid A occurs at an equivalent position.

Formation of hepta-acylated lipid A through addition of an acyl chain onto *A. baumannii* lipid A at the same site that PagP functions in *E. coli* and *Salmonella* could be important for survival during host infection. To investigate LpxM$_{Ab}$-dependent acyl chain placement, we used a genetic approach to confirm attachment of a lauroyl group to the *R*-2-hydroxymyristate in *A. baumannii*, which is the equivalent position that PagP-dependent myristate addition occurs in *E. coli* (37). To do this, LpxM$_{Ab}$ was coexpressed with the lipid A deacylase PagL in an *E. coli* strain. PagL efficiently removes the 3-*O*-linked acyl chain of lipid A following transport of LPS/LOS to the outer membrane (Fig. 4A) (38). Lipid A isolated from cells coexpressing LpxM$_{Ab}$ and PagL was analyzed by MALDI-TOF mass spectrometry, and major species at *m/z* 1,725.2 and 1,753.2 were present in the spectra
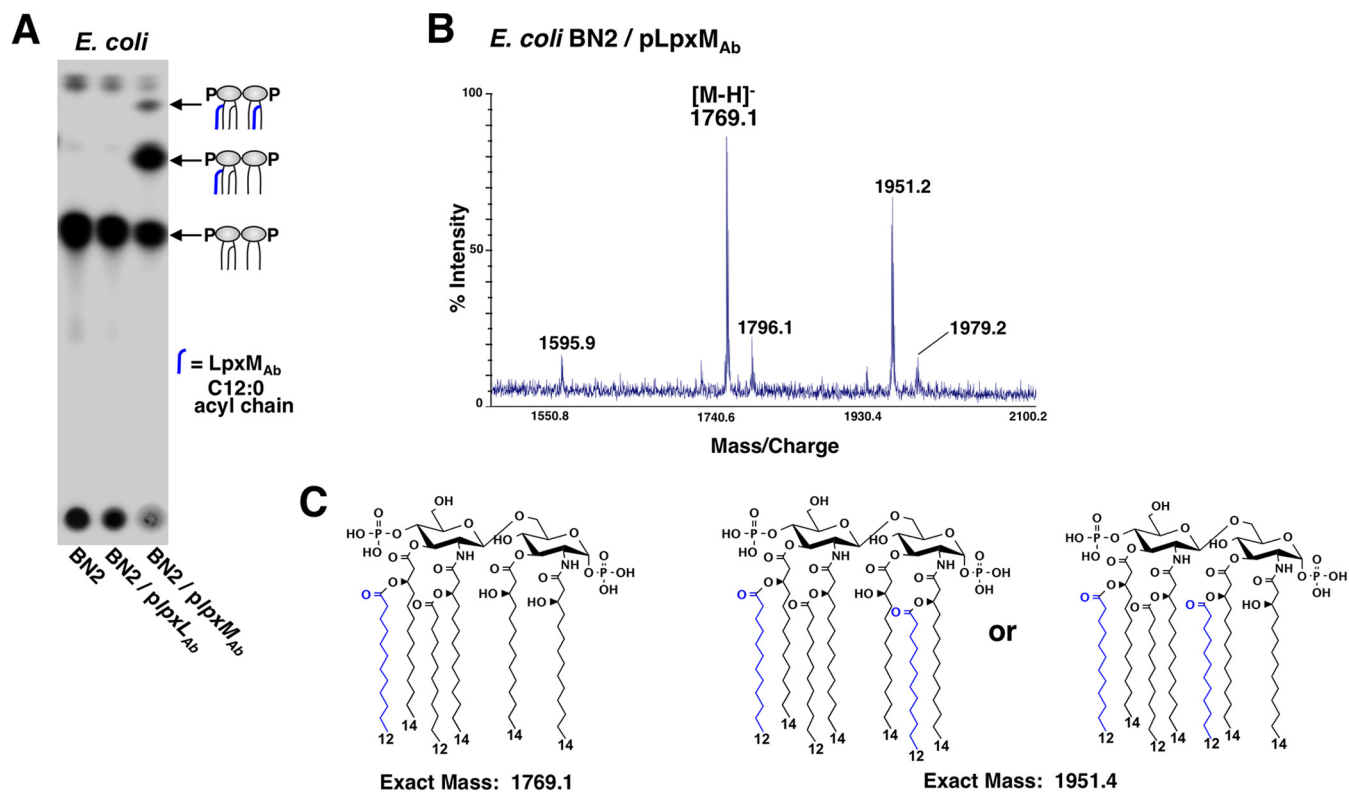
FIG 3 LpxM$_{Ab}$ is a dual acyltransferase. Characterization of LpxM$_{Ab}$ revealed a unique dual acyltransferase activity. (A) ^{32}P-radiolabeled lipid A from *E. coli* BN2 was isolated and separated based on hydrophobicity using TLC. The blue acyl chains in all lipid A structures correspond to lauroyl groups derived from LpxM$_{Ab}$. (B) MALDI-TOF MS analysis of lipid A isolated from a PagP- and LpxM-deficient strain, BN2, expressing LpxM$_{Ab}$. The *m/z* at 1,769.1 and 1,951.2 correspond to LpxM$_{Ab}$-dependent acylation. (C) Proposed chemical structures of LpxM$_{Ab}$-dependent acylation in *E. coli*.

(Fig. 4B). These data indicate that deacylation at position 3 by PagL, which takes place in the outer membrane, is not inhibited by inner membrane-associated LpxM$_{Ab}$-dependent acylation (Fig. 4B). Furthermore, HCD and UVPD fragmentation spectra of *m/z* 1,712.12, corresponding to the Δ*lpxL$_{Ab}$* mutant, also confirmed that the secondary laurate group is attached at position 2 (see Fig. S4 in the supplemental material). On the basis of our genetic data and high-resolution HCD and UVPD data, we confirmed that LpxM$_{Ab}$ and PagP add a lauroyl group and a palmitoyl group, respectively, at the *R*-2-hydroxymyristate position of lipid A (Fig. 1 and 4C). In summary, LpxM$_{Ab}$-dependent lauroyl group addition occurs at the *R*-3′- and *R*-2-hydroxymyrsitate positions of lipid A (Fig. 4C).

In addition, lipid A from *A. baumannii* strains was analyzed by TLC; lipid A from the wild-type strain, *lpxM$_{Ab}$* mutant, and all complemented *A. baumannii* strains migrated as doublets, indicating the presence of a modified fatty acid in all strains except the *lpxL$_{Ab}$* mutant (Fig. 2A). Mass spectrometry indicated a mass difference of 16 in lipid A, which is indicative of hydroxylation modification (Fig. 2B and D). Therefore, we concluded that the *lpxL$_{Ab}$*-dependent acyl chain is hydroxylated. Hydroxylation of lipid A fatty acids is common in Gram-negative bacteria where LpxO hydroxylates fatty acids. Furthermore, BLAST analysis revealed that gene *A1S_0308* encodes a putative *A. baumannii* LpxO homolog with 59% identity to *Salmonella enterica* serovar Typhimurium LT-2 LpxO.

A. baumannii lipid A differentially stimulates human TLR-4/MD-2. Microbes continuously challenge the host immune sys-

tem, but mammals have evolved receptors like TLR-4/MD-2 to detect invading bacterial cells via pathogen-associated molecular patterns (39). Specifically, the lipid A portion of Gram-negative bacterial LPS/LOS is a pathogen-associated molecular pattern that is bound by the TLR-4/MD-2 complex with high affinity (40). The interaction between lipid A and TLR-4/MD-2 strongly activates MyD88- and TRIF-dependent innate immune pathways to result in inflammation and clearance of the infection via innate immune recruitment (41). Coevolution of the TLR-4/MD-2 receptor and lipid A ligand has resulted in strong immune activation when hexa-acylated lipid A is detected. However, many pathogens modify the lipid A chemical structure to avoid TLR-4-dependent immune detection (8). Therefore, we analyzed human TLR-4/MD-2 activation using LOS isolated from the *A. baumannii* acyltransferase mutants.

To analyze immune activation by *A. baumannii* LOS, we used a human embryonic kidney reporter cell line (HEK-blue; InvivoGen) that expressed the human TLR-4, MD-2, and CD-14 receptor complex along with the NF-κB and activator protein-1-dependent reporter secreted embryonic alkaline phosphatase (SEAP) (9). LOS was isolated and purified from wild-type, Δ*lpxL$_{Ab}$*, Δ*lpxM$_{Ab}$*, and complemented strains (see Fig. S5 in the supplemental material). Increasing concentrations of LOS were incubated with HEK-blue cells to activate potential signaling pathways. The wild type and the complemented strains activated reporter expression in a dose-dependent manner indicating that *A. baumannii* hepta-acylated lipid A activates the human TLR-4/MD-2 innate immune pathway (Fig. 5). In contrast, LOS stimu-
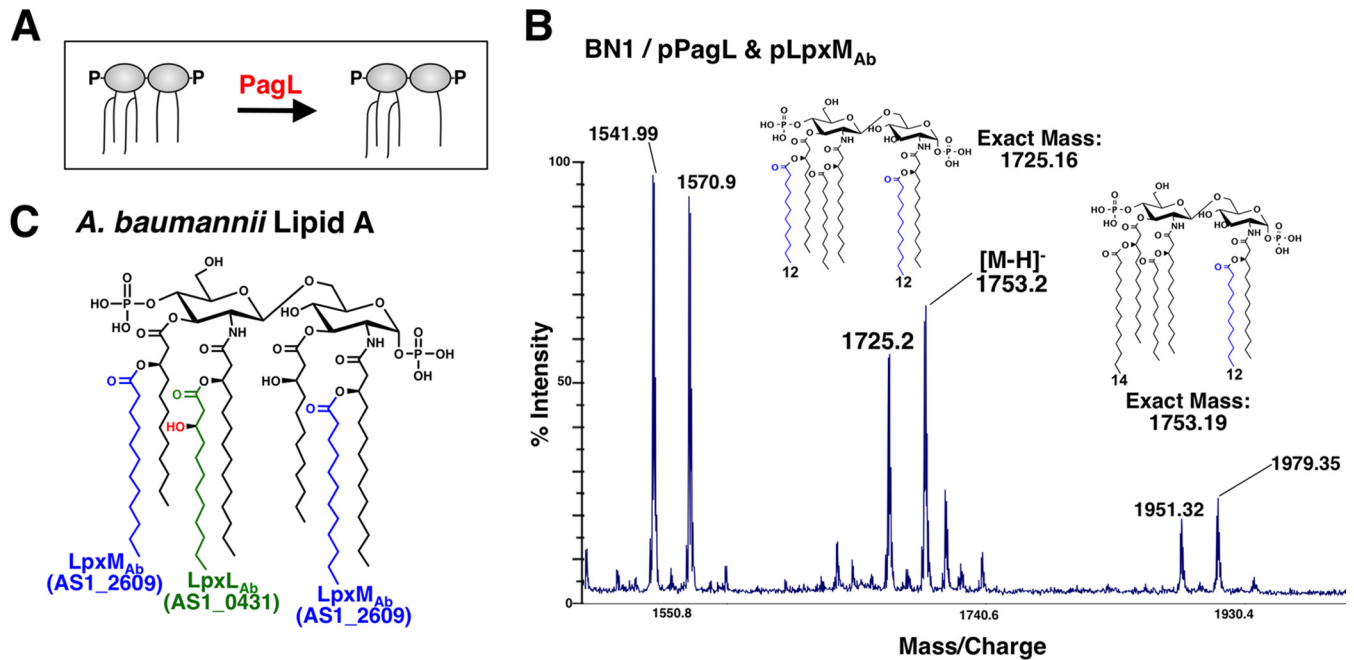
FIG 4 Determining LpxM_Ab-dependent acyl chain placement. (A) PagL removes the 3-O-linked acyl chain of lipid A. (B) MALDI-TOF MS analysis of lipid A from the PagP-deficient *E. coli* strain BN1 expressing PagL and LpxM_Ab. The *m/z* at 1,725.16 and 1,753.19 correspond to lipid A structures that have a secondary laurate group (blue) at positions 3′ and 2 and only at position 2, respectively. (C) Chemical structure of wild-type *A. baumannii* lipid A that is based on both genetic studies and chemical analysis. LpxL_Ab catalyzes addition of a secondary lauroyl group to position 2′ (green), which can be hydroxylated (red). LpxM_Ab catalyzes transfer of secondary acyl chain at both positions 2 and 3′ (blue).

lation by *lpxL_Ab* and *lpxM_Ab* was dramatically reduced (Fig. 5). Similar to findings in *E. coli*, the pattern of TLR-4/MD-2 reporter activation indicates that acylation of *A. baumannii* lipid A is important for immune recognition by the host immune system (8, 37).

LpxM_Ab protects *A. baumannii* from CAMPs. The innate immune system produces CAMPs that bind to and lyse Gram-negative bacteria through disruption of the outer membrane. CAMPs are directed to LPS/LOS on the bacterial surface by electrostatic interactions (42). Presumably PagP-dependent lipid A palmitoylation increases the hydrophobic van der Waals forces of the lipid bilayer to prevent CAMP insertion and membrane dis-

FIG 5 Stimulation of the human TLR-4 immune signaling pathway by *A. baumannii* LOS. Stimulation of human TLR-4 following incubation with increasing concentrations (0, 0.1, 1.0, 10, 50, and 100 ng/ml) of isolated LOS with HEK-blue cells expressing TLR-4, MD-2, and CD-14 is depicted. Detection of a secreted reporter indicates differential activation of TLR-4 in response to LOS.

ruption. In *Salmonella*, hepta-acylation of lipid A protected bacterial cells from vertebrate CAMPs such as C18G, an α-helical peptide found at the C terminus of the secreted cytokine human platelet factor IV (10, 43).

Based on quantitative TLC analysis of wild-type *A. baumannii* lipid A, hepta-acylated lipid A dominated the cell surface (Fig. 2A, leftmost lane). Therefore, we first determined whether hepta-acylated lipid A in *A. baumannii* conferred resistance to vertebrate CAMPs. Increasing concentrations of the C18G CAMP facilitated concentration-dependent killing in all *A. baumannii* strains. Importantly, the *lpxM_Ab* mutant displayed increased sensitivity to C18G, where a 1,000-fold decrease in the number of CFU per milliliter was recovered at a concentration of 10 μg/ml compared to the wild-type strain. Complementation fully restored resistance of the *lpxM_Ab* mutation back to wild-type levels, indicating that LpxM_Ab-mediated hepta-acylation confers protection against vertebrate CAMPs in *A. baumannii* (Fig. 6A).

Another class of CAMPs called polymyxins includes polymyxin B and E, which are lipopeptides synthesized by Gram-positive soil bacilli. These cyclic peptides contain a hydrophobic tail that inserts into the membrane to kill by forming pores in the Gram-negative outer membrane (44). Polymyxin B is currently used in topical solutions such as neosporin, while polymyxin E (colistin) is a last-line antimicrobial used to treat Gram-negative multidrug-resistant bacterial infections. We observed increased killing by polymyxin B (Fig. 6B) and colistin (Fig. 6C), indicating that these drugs could kill *A. baumannii* more effectively when LpxM_Ab-dependent acylation was inhibited. The enhanced killing that we observed was fully complemented when each gene was expressed in *trans*. Therefore, LpxM_Ab-dependent acylation in
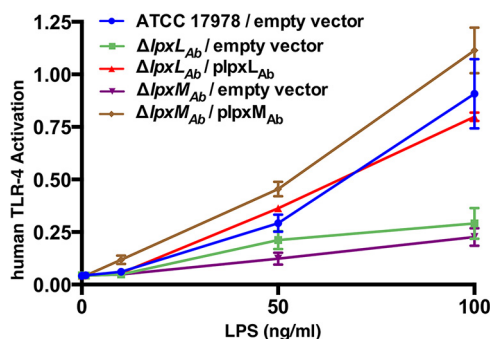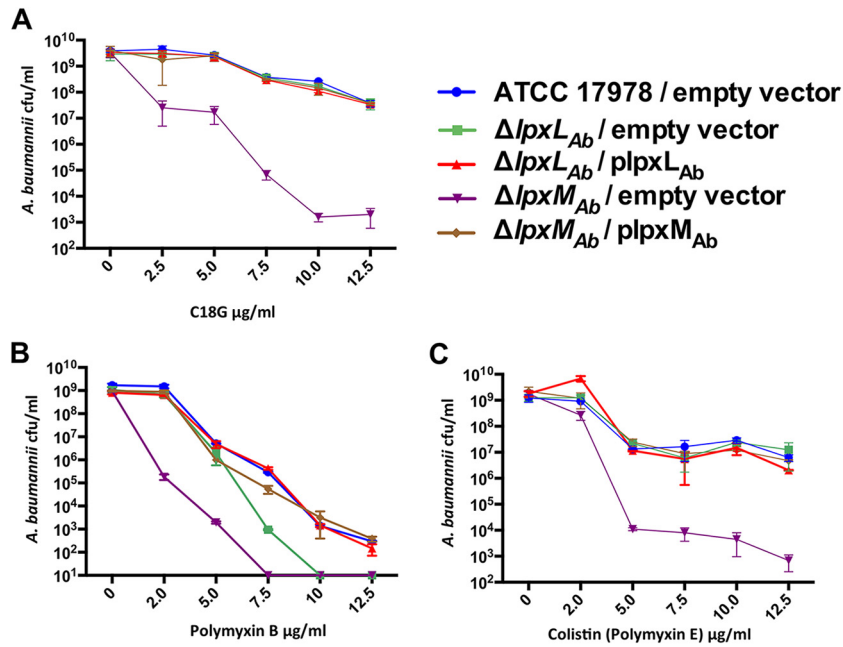
FIG 6 LpxM$_{Ab}$ contributes to vertebrate and polymyxin CAMP resistance in *A. baumannii*. *A. baumannii* CAMP survival assays. *A. baumannii* strains were incubated with the indicated concentrations of the mammalian CAMP, C18G (A), polymyxin A (B), or polymyxin E (C) for 2 h. Bacterial cells were serially diluted and plated to recover viable bacteria. Colony counts, reported as CFU per milliliter, determined cell survival.

A. baumannii promotes resistance not only to vertebrate CAMPs but also to medically relevant polymyxin antibiotics.

LpxM$_{Ab}$ is required for virulence in *Galleria mellonella*. A well-established model to determine virulence of *A. baumannii* strains is the *Galleria mellonella* infection model (45–47). Importantly, a strong correlation between the virulence of several bacteria, including *A. baumannii*, in *G. mellonella* and mammalian virulence models has been established (47–49). Furthermore, *G. mellonella* carries a gene that encodes a humoral immune response, which is activated when large bacterial loads are injected. While invertebrates do not mount an antibody response, within 3 h of challenge, antimicrobial peptides are synthesized and released into the hemolymph to neutralize the bacterial infection (50, 51).

To determine the susceptibility of killing in the *A. baumannii* acyltransferase mutants, we injected equivalent numbers of CFU from wild-type, mutant, and complemented strains into *G. mellonella* and monitored bacterial killing. After 72 h, all wax moth larvae injected with the wild-type and complemented *A. bauman-*

nii strains died, whereas all worms that were challenged with phosphate-buffered saline (PBS) and the *A. baumannii* Δ*lpxM$_{Ab}$* strain survived. In contrast, only one of the larvae injected with the *A. baumannii* LpxL$_{Ab}$ mutant survived (Fig. 7). These data suggest that LpxM$_{Ab}$-dependent lipid A acylation is important for *A. baumannii* survival and virulence in invertebrates.

LpxM$_{Ab}$-dependent acylation of *A. baumannii* lipid A contributes to desiccation survival. *A. baumannii* is a nosocomial pathogen that persists on inanimate objects in hospitals for months and even years (52). The organism's unique ability to survive under desiccative conditions results in increased infections and transmission rates. The molecular mechanisms that contribute to desiccation tolerance in *A. baumannii* have not been characterized and are not well understood. In other Gram-negative bacteria, proteins involved in exopolysaccharide biosynthesis, in maintaining protein stability, and in detoxification of reactive oxygen species were identified as important to desiccation resistance (53, 54). However, the protective role of the outer membrane barrier has not been explored.

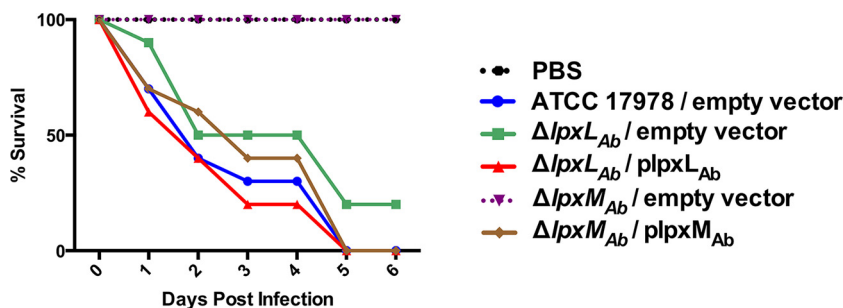
FIG 7 *Galleria mellonella* virulence model. *G. mellonella* survival is dependent on LpxM$_{Ab}$. *G. mellonella* larvae were injected with approximately 1×10^6 CFU and incubated at 37°C. Killing was monitored every 24 h for 6 days.
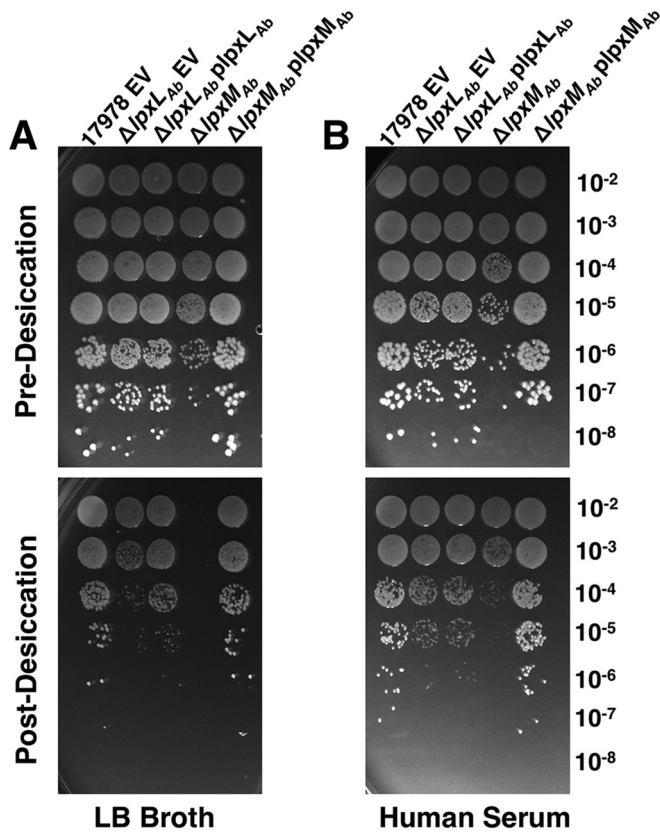
FIG 8 LpxM$_{Ab}$ contributes to *A. baumannii* desiccative survival. An *A. baumannii* desiccation survival assay was performed. *A. baumannii* strains, including ATCC 17978 carrying an empty vector (EV), at an OD$_{600}$ of 0.1 serially diluted and spotted onto LB plates before and after desiccation on polystyrene. Cells were washed in LB broth (A) or human serum (B) before desiccation.

In order to further characterize our lipid A acyltransferase mutants, we subjected the wild type, Δ*lpxL$_{Ab}$*, Δ*lpxM$_{Ab}$*, and the complemented strains to a desiccation survival assay. Strains were spotted on agar in a 10-fold dilution series before and after desiccation on polystyrene (Fig. 8; see Fig. S6 in the supplemental material). Following desiccation, bacterial CFUs were recovered for all strains except for the *A. baumannii* Δ*lpxM$_{Ab}$* strain, for which no viable cells were recovered at either cell density tested, indicating a >1,000-fold decrease in CFU (Fig. 8A and Fig. S6A). In addition, we performed desiccation assays where we resuspended *A. baumannii* cells in human serum before desiccation. Following desiccation, cells were recovered in all groups and spotted (Fig. 8B and Fig. S6B). However, we recovered 100-fold less of the *lpxM$_{Ab}$* mutant strain following desiccation (Fig. 8B and Fig. S6B). Under each condition, in *trans* expression of *lpxM$_{Ab}$* fully complemented the mutant phenotype and restored bacterial survival to the wild-type level. These results suggest that the lipid components in the outer membrane contribute to desiccation survival in *A. baumannii*.

DISCUSSION

The lipid A domain of LPS/LOS provides Gram-negative bacteria with a formidable barrier to protect the cell from its environment. The work described in this study suggests that hepta-acylation of lipid A fortifies the outer membrane to protect *A. baumannii* from CAMPs and desiccation. While many pathogens synthesize hexa-acylated lipid A under standard growth conditions, PagP-dependent hepta-acylation of lipid A occurs upon exposure to stressful factors (10). Hepta-acylation reinforces the outer membrane by packing the hydrophobic membrane with palmitate added at the *R*-2-hydroxymyristate position of lipid A (37). PagP-dependent acylation of lipid A protects Gram-negative bacteria from antimicrobial peptides in vertebrate mucosal secretions (e.g., CAMPs) (10). Whereas most pathogens regulate synthesis of hepta-acylated lipid A by regulating PagP expression, *A. baumannii* does not carry a gene that encodes a PagP homolog and constitutively synthesizes hepta-acylated lipid A under standard growth conditions. The PagP-independent mechanism utilized by *A. baumannii* illustrates an additional strategy for the addition of a secondary acyl chain at position 2 on lipid A. Intriguingly, *A. baumannii* could have evolved its PagP-independent mechanism to continuously produce hepta-acylated lipid A on its surface due to the selective pressures encountered in its environmental niche. Since *A. baumannii* is primarily found in health care settings, assault by hospital-like environmental factors may suggest why *A. baumannii* encodes a LpxM dual acyltransferase homolog to continually reinforce its outer membrane.

The previously characterized LpxL and LpxM acyltransferases of *E. coli* add acyl chains to a specific site of the lipid A molecule in an ordered fashion (5–7). In contrast, our results suggest that the *A. baumannii* homologs LpxL$_{Ab}$ and LpxM$_{Ab}$ catalyze lipid A acylation independently of the other. Furthermore, the dual acyltransferase activity of LpxM$_{Ab}$, illustrated in a heterologous *E. coli* system, involved a unique transferase activity where two lauroyl groups are attached to lipid A. While beyond the scope of this study, a more detailed examination of the LpxM$_{Ab}$ molecular structure could reveal the requirements that mediate the relaxed acyltransferase fidelity. Intriguingly, *Moraxella catarrhalis*, which is responsible for otitis media in infants and is closely related to *Acinetobacter*, also carries a gene that encodes an enzyme with potential dual acyltransferase activity. While *A. baumannii* adds two C$_{12:0}$ groups, *M. catarrhalis* adds two C$_{10:0}$ groups to lipid A, both of which are essential for its survival in the mouse lung and human serum (55).

While the host immune system exploits a number of immune mechanisms to clear bacterial infections, a common lipid A structure, including the number, length, and arrangement of fatty acyl chains and exposed phosphates, is key (40). The human TLR-4/MD-2 receptor complex has evolved to bind hexa-acylated *bis*-phosphorylated lipid A with high affinity to result in strong immune activation. TLR-4-dependent pathways include MyD88- and TRIF-dependent signaling cascades, which trigger an immune response at the site of infection (56). While we cannot directly compare TLR-4/MD-2 activation by *A. baumannii* LOS to hepta-acylated *E. coli* LPS because of the different lipid A acyl chain lengths, hepta-acylated LOS extracted from wild-type *A. baumannii* activated a human TLR-4 reporter cell line (Fig. 5). In contrast, hexa-acylated *lpxL$_{Ab}$* and penta-acylated *lpxM$_{Ab}$* mutants failed to stimulate our reporter cell line, suggesting that the acyl chains added via these enzymes are important for human TLR-4/MD-2 recognition of *Acinetobacter* LOS. The inability of the *lpxM$_{Ab}$* and *lpxM$_{Ab}$* mutants to stimulate human TLR-4 illustrates the importance of acyl chain placement in human immune recognition of LPS/LOS.

In addition to immune surveillance systems, the mammalian innate immune system secretes CAMPs to deter bacterial invasion into host tissues. In *Salmonella*, PagP-dependent hepta-acylation of lipid A increased resistance to vertebrate CAMPs, such as C18G CAMP, which is a cytokine secreted by host cells to inhibit bacterial growth and prevent infection (57). However, hepta-acylation did not result in increased resistance to polymyxin CAMPs (10). Similar to *Salmonella pagP* mutants, *A. baumannii lpxM$_{Ab}$* mutants exhibited sensitivity to C18G (Fig. 6A). However, removal of *lpxM$_{Ab}$* also sensitized *A. baumannii* to the bactericidal effects of polymyxin CAMPs (Fig. 6B and C). Whereas *pagP* mutants produce hexa-acylated lipid A, our analysis showed that the *A. baumannii lpxM$_{Ab}$* mutant synthesizes penta-acylated lipid A (Fig. 2D). Therefore, we suspect that the observed polymyxin killing sensitivity results from the loss of two laurate acyl chains from *A. baumannii* lipid A. Importantly, LpxM$_{Ab}$ inhibition effectively sensitizes *A. baumannii* cells to a wide range of host and polymyxin CAMPs, suggesting potential for enhanced drug delivery as a synergistic antimicrobial agent.

To demonstrate the essentiality of LpxM$_{Ab}$-dependent survival in a host, we used an invertebrate model to assess CAMP-dependent killing. *Galleria mellonella* provides an optimal model, because it carries a gene(s) encoding a humoral immune response that synthesizes and releases antimicrobial peptides into the hemolymph upon sensing bacterial assault (50). Our results demonstrate that deletion of *lpxM$_{Ab}$* resulted in attenuated virulence (Fig. 7). Production of antimicrobial peptides likely plays a large role in neutralizing *A. baumannii* infections (58). The inability of the *lpxM$_{Ab}$* mutant *A. baumannii* to kill wax moth larvae is likely because the humoral antimicrobial peptide response can rapidly lyse the penta-acylated lipid A *lpxM$_{Ab}$* mutant, but not cells that synthesize wild-type lipid A.

Furthermore, CAMPs that target the outer membrane such as magainin and cecropin are potential alternatives to currently prescribed antibiotics. It was previously demonstrated that magainin disorders the outer membrane acyl chains of Gram-negative bacteria, whereas cecropin disrupts lipid moieties in biological membranes to form pores (59–61). These CAMPs and countless others could act synergistically with an LpxM$_{Ab}$ inhibitor to effectively lyse multidrug-resistant *A. baumannii* cells, providing an innovative antimicrobial strategy.

Unexpectedly, our analysis of lipids in the outer membrane determined that the fatty acid lipid content of lipid A is important for *A. baumannii* survival during desiccation (Fig. 8; see Fig. S6 in the supplemental material). While a detailed understanding of the factors required for desiccation survival in any bacterium is lacking, surface structures such as carbohydrates and oxidative damage have been hypothesized to play a role. We suspect that the penta-acylated lipid A outer membrane synthesized by the *lpxM$_{Ab}$* mutant is more fluid than the wild-type hepta-acylated membrane. Increased membrane fluidity would likely permit leakage of water and hydrophilic nutrients out of the cell. The resultant loss of essential growth factors would potentially be deadly to the bacterial cell. In this study, the penta-acylated *A. baumannii* bacteria could not survive for even 48 h in a desiccative environment, while the wild-type *A. baumannii* cells could persist under desiccative conditions for weeks and even months. Identification of outer membrane lipid species as important factors for desiccation survival may aid in the development of new antiseptics.

MATERIALS AND METHODS

Bacterial strains and growth. All strains and plasmids used in this study are listed in Table S1 in the supplemental material. *A. baumannii* ATCC 17978 strains were initially grown from freezer stocks on Luria-Bertani (LB) agar at 37°C. Isolated colonies were used to inoculate LB broth at 37°C. Carbenicillin was used at 150 μg/ml, and kanamycin was used at 25 or 7.5 μg/ml for selection.

Construction of mutant and complementation *A. baumannii* strains. Primers used in this study are listed in Table S2 in the supplemental material. All *A. baumannii* genetic mutants were engineered as previously described (31). In brief, *A. baumannii* ATCC 17978 carrying plasmid pMMB67EH containing the REC$_{Ab}$ coding sequences was diluted from a culture grown overnight into LB broth at an optical density at 600 nm (OD$_{600}$) of 0.05 and grown for 45 min. Expression of REC$_{Ab}$ (pAT03) was induced by the addition of 2 mM IPTG, and cells were grown at 37°C until they were in mid-log growth phase (OD$_{600}$ of 0.4). After the cells were washed in ice-cold 10% glycerol, 10^{10} cells were electroporated in a 2-mm cuvette at 1.8 mV with 5 μg of a recombineering linear PCR product. The cells were immediately grown for 4 h in 4 ml of LB broth with 2 mM IPTG and plated on LB agar containing 7.5 μg/ml or 25 μg/ml of kanamycin. All mutants were verified by PCR and sequenced.

To cure isolated mutants of the pMMB67EH::REC$_{Ab}$ Tet^r plasmid following mutant isolation, strains were streaked for isolated colonies on 2 mM NiCl$_2$ to select for cells that have lost the tetracycline cassette (62). Cured mutants were electroporated with pMMBR67EH carrying the FLP recombinase (pAT04). Cells were recovered for 1 h in 4 ml of LB broth and plated on LB agar containing 2 mM IPTG to induce expression of the FLP recombinase. Excision of the kanamycin cassette was confirmed by PCR and sequenced.

To complement *A. baumannii* mutants, the coding sequences from *A1S_0431* (*lpxL$_{Ab}$*) and *A1S_2609* (*lpxM$_{Ab}$*) were cloned into the KpnI and SalI sites in pMMB67EH. The plasmids were expressed in the respective mutants, and all strains were grown in 0.05 mM IPTG for expression.

Isolation of lipid A. Isolation of lipid A for TLC analysis involved ^{32}P radiolabeling of whole cells as previously described (63). In brief, 14 ml of *A. baumannii* was grown at 37°C to an OD$_{600}$ of 1.0. Bacteria were harvested by centrifugation at 10,000 × *g* for 10 min. Lipid A extraction was carried out by mild-acid hydrolysis as previously described (64).

Isolation and quantification of LOS. LOS was isolated as previously described (63). In brief, *A. baumannii* (1 liter) was grown at 37°C to an OD$_{600}$ of 1.0. Cells were harvested by centrifugation and lyophilized overnight. The phenol-water method was used to isolate LOS (65). Quantification of LOS was performed by using the 3-deoxy-3-manno-octulosonic acid (Kdo) colorimetric assay as previously described (66).

Mass spectrometry. For mass spectrometry, lipid A was analyzed using a MALDI-TOF/TOF (ABI 4700 proteomics analyzer) mass spectrometer in the negative ion mode as previously described (63). All UVPD and HCD spectra were collected in the negative ion mode on a Thermo Scientific Orbitrap Elite mass spectrometer (Bremen, Germany) modified with a Coherent ExciStar XS ArF excimer laser (Santa Clara, CA), as previously described (34, 35). HCD was performed with the normalized collision energy (NCE) of 55% with a cell pressure of approximately 0.13 × 10^2 Pa. UVPD was performed with the laser emitting 193-nm photons at 4 mJ per laser pulse with four pulses per scan. The laser pulse repetition rate was 500 Hz. The instrument was operated at 120,000 resolving power with a precursor isolation window of 5 *m/z*. All samples were dissolved in 50:50 methanol (MeOH)-CHCl$_3$ and directly infused into the mass spectrometer at a rate of 3 μl/min with a spray voltage of 4 kV. The presented HCD and UVPD spectra are composed of 104 to 330 averages.

TLR-4 signaling assays. The HEK-blue human TLR-4 (hTLR-4) cell line was maintained according to the manufacturer's specifications (InvivoGen). LOS samples were serially diluted for assays as previously described (9, 63). At least two biological replicates were each done in triplicate, and one representative set was shown.

Bactericidal assays. Overnight cultures of *A. baumannii* strains were diluted to an OD_{600} of 0.15, and 100 μl was added to three replicate wells on a 96-well microtiter plate. CAMPs were added to each well at the desired concentration, and the plate was incubated at 37°C for 2 h (10). Serial dilutions were plated on LB agar and incubated overnight at 37°C. Colony counts were reported as CFU per milliliter. Two biological replicates were performed in triplicate for each strain, and all groups were included in the reported data.

Desiccation assays. Wild-type and mutant *A. baumannii* strains were diluted to OD_{600}s of 0.1 and 0.01 in 10-μl and 100-μl volumes, respectively, and diluted in LB broth or human serum. Predesiccated samples were serially diluted in 10-fold increments and spotted. Samples were desiccated on polystyrene at 25°C and 40% humidity for 48 h. Following desiccation, samples were resuspended in LB broth, serially diluted in 10-fold increments, and spotted. Survival of each strain was assessed by the relative loss of CFU compared to the wild type.

***Galleria mellonella* virulence assay.** The *Galleria mellonella* virulence assay was performed as previously described (45). Larvae (Vanderhorst, Inc., St. Marys, OH) were stored in the dark and used within 7 days of receipt. Ten randomly chosen caterpillars of similar weight were used for each group in the experiment. Following bacterial injection, caterpillars were stored at 37°C and monitored every 12 h for death. Caterpillars were considered dead when they became nonresponsive to touch. The experiment was performed in triplicate with data from one representative group reported.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00478-15/-/DCSupplemental.

Figure S1, PDF file, 0.2 MB.
Figure S2, PDF file, 0.3 MB.
Figure S3, PDF file, 0.6 MB.
Figure S4, PDF file, 0.5 MB.
Figure S5, PDF file, 0.5 MB.
Figure S6, PDF file, 1.1 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.05 MB.

ACKNOWLEDGMENTS

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