A penicillin-binding protein inhibits selection of colistin-resistant, lipooligosaccharide-deficient *Acinetobacter baumannii*

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The Gram-negative bacterial outer membrane fortifies the cell against environmental toxins including antibiotics. Unique glycolipids called lipopolysaccharide/lipooligosaccharide (LPS/LOS) are enriched in the cell-surface monolayer of the outer membrane and promote antimicrobial resistance. Colistin, which targets the lipid A domain of LPS/LOS to lyse the cell, is the last-line treatment for multidrug-resistant Gram-negative infections. Lipid A is essential for the survival of most Gram-negative bacteria, but colistin-resistant *Acinetobacter baumannii* lacking lipid A were isolated after colistin exposure. Previously, strain ATCC 19606 was the only *A. baumannii* strain demonstrated to subsist without lipid A. Here, we show that other *A. baumannii* strains can also survive without lipid A, but some cannot, affording a unique model to study endotoxin essentiality. We assessed the capacity of 15 clinical *A. baumannii* isolates including 9 recent clinical isolates to develop colistin resistance through inactivation of the lipid A biosynthetic pathway, the products of which assemble the LOS precursor. Our investigation determined that expression of the well-conserved penicillin-binding protein (PBP) 1A, prevented LOS-deficient colony isolation. The glycosyltransferase activity of PBP1A, which aids in the polymerization of the peptidoglycan cell wall, was lethal to LOS-deficient *A. baumannii*. Global transcriptomic analysis of a PBP1A-deficient mutant and four LOS-deficient *A. baumannii* strains showed a concomitant increase in transcription of lipoproteins and their transporters. Examination of the LOS-deficient *A. baumannii* cell surface demonstrated that specific lipoproteins were overexpressed and decorated the cell surface, potentially compensating for LOS removal. This work expands our knowledge of lipid A essentiality and elucidates a drug resistance mechanism.

Acinetobacter | peptidoglycan | colistin | lipoprotein | lipopolysaccharide

The bacterial cell envelope is a multifaceted structure that regulates uptake of essential nutrients and cofactors while protecting the cell from its often-hostile environment. The defining feature of Gram-negative bacteria is an essential second (outer) membrane in the cell envelope that encases the periplasm and peptidoglycan cell wall. The outer membrane forms a unique asymmetrical lipid bilayer barrier that enhances resistance to a variety of antibiotics and host immune mechanisms. Whereas the inner monolayer of the outer membrane is composed of glycerophospholipids, the cell surface-exposed monolayer is enriched in either ***l****ipo****p****oly****s****accharide* (LPS) or ***l****ipo****o****ligo****s****accharide* (LOS), which are amphipathic glycolipids synthesized from the essential precursor, lipid A. The lipid A domain anchors LPS/LOS into the outer membrane, whereas the hydrophilic components including the core polysaccharides and O-polysaccharide(s) (in the case of LPS) extend into the extracellular milieu (1).

Host immune receptors recognize ***p****athogen-****a****ssociated* ***m****olecular* ***p****atterns* (PAMPs) in the bacterial cell envelope during infection. The highly conserved lipid A (via TLR4/MD2 receptors) and peptidoglycan (via Nod receptors) chemical structures are PAMPs that stimulate immune pathways to clear the bacterial infection (2, 3). To circumvent detection by the host immune system, many Gram-negative pathogens alter structural motifs on the cell surface by adding chemical moieties to the lipid A domain of LPS/LOS. Not only does camouflaging PAMPs offer protection from host innate immune detection, but also chemical modification of lipid A enhances resistance to many antimicrobial agents (4). Consequently, bacterial remodeling of outer-membrane lipid A limits the effective antimicrobial arsenal to counteract Gram-negative bacterial infections.

Acinetobacter baumannii is an emerging Gram-negative pathogen that has quickly become pervasive throughout clinics and hospitals because it adapts to harsh environmental conditions and quickly develops resistance to antimicrobials. Multidrug resistant *A. baumannii* isolates, especially those resistant to carbapenems, have been isolated (5–7). Currently, colistin (polymyxin E) is prescribed as the last-resort antimicrobial to treat multidrug resistant *A. baumannii* infections. Colistin is a cationic lipopeptide that binds the negatively charged lipid A phosphate groups to perturb the membrane and kill the bacterial cell. However, *A. baumannii* has also evolved colistin resistance mechanisms (8–11).

Significance

Antimicrobial drug resistance is a major threat to public health. Gram-negative bacteria are exceptionally resistant to antibiotics because of their outer-membrane barrier. Glycolipids called lipopolysaccharide (LPS) or lipooligosaccharide (LOS) fortify the outer membrane from many antimicrobials and biocides and were thought to be essential for Gram-negative bacterial survival. The last-resort treatment for multidrug-resistant Gram-negative infections is colistin, which targets the lipid A domain of LPS/LOS to disrupt the membrane, but the emerging pathogen *Acinetobacter baumannii* can develop colistin resistance by inactivating lipid A biosynthesis. This analysis advances our understanding of lipid A/LOS essentiality in *A. baumannii* and identifies antimicrobial targets.

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A. baumannii amplifies colistin resistance through modification of the LOS lipid A anchor domain using three mechanisms. (*i*) Similar to lipid A modification strategies described in other Gram-negative pathogens (4), *A. baumannii* enzymatically adds phosphoethanolamine and/or galactosamine to lipid A to enhance antibiotic resistance (8–10). Incorporation of amine-containing chemical moieties neutralizes electrostatic interactions and reduces colistin binding to lipid A in the outer membrane. (*ii*) Whereas many bacterial pathogens respond to cellular assaults through acyl chain addition to the lipid A domain, *A. baumannii* encodes a mechanism that constitutively hyper-acylates the lipid A domain, presumably to alter host immune recognition and to strengthen the outer-membrane barrier (11). (*iii*) *A. baumannii* has also demonstrated a unique capacity to inactivate the lipid A biosynthetic pathway to develop resistance to colistin (12). In this work, we show that lipid A inactivation results not only in colistin resistance, but also in cross-resistance to other clinically important antibiotics.

A. baumannii is one of only three Gram-negative bacteria known to date that can survive after inactivation of lipid A. Whereas previous analyses indicated that lipid A was essential for survival of most Gram-negative bacteria (13), in vitro characterization of LOS-deficient isolates including *A. baumannii*, *Neisseria meningitidis*, and *Moraxella catarrhalis* demonstrate that some Gram-negative bacteria can persist without lipid A (12, 14, 15). Furthermore, LOS-deficient *A. baumannii* were isolated following colistin treatment from a patient in South Korea (16). We do not currently understand the mechanism that permits some Gram-negative bacterial species to survive without LOS whereas others cannot. Previous analysis suggested that LOS-deficient bacterial cells compensate for the absence of LOS through alterations to the cell envelope, but the molecular rearrangements have not been elucidated (17–19). Understanding how LOS-deficient pathogens remodel the cell envelope barrier without LOS will advance our understanding of lipid A essentiality and could identify targets to direct future therapeutic treatments.

To date, examination of LOS-deficient *A. baumannii* has been performed only in a single strain, designated ATCC 19606. Transcriptomic analysis (RNA-seq) of one isolate, which encoded a mutation that inactivated lipid A biosynthesis, suggested that LOS-deficient *A. baumannii* increased transcription of genes encoding cell-envelope biogenesis and transport proteins. Specifically, the ***m***aintenance of outer membrane ***l***ipid ***a***symmetry (Mla) retrograde phospholipid transporter, ***l***ocalization ***o***f ***l***ipoproteins (Lol) lipoprotein transport, and poly-β-1,6-*N*-acetylglucosamine (PNAG) transcripts were increased relative to the wild-type parent strain (17). However, the contribution of each pathway to fitness after inactivation of lipid A biosynthesis and production of LOS was not determined.

To understand the requirements for bacterial survival without lipid A/LOS, we examined 15 laboratory-adapted and clinical *A. baumannii* isolates. We confirmed that, although some clinical *A. baumannii* isolates including ATCC 19606 inactivated lipid A biosynthesis to develop colistin resistance, other isolates could not. We discovered that the penicillin-binding protein (PBP) 1A (encoded by *ponA* or *mrcA*), impeded isolation of LOS-deficient *A. baumannii*. Transcriptomic analysis of the PBP1A mutant and four LOS-deficient *A. baumannii* strains exemplified a significant conserved increase in transcripts encoding Lol lipoprotein transport proteins. Specific lipoproteins were overexpressed and enriched in the outer membrane to potentially compensate for the absence of lipid A/LOS. Here we show a link between the peptidoglycan assembly protein, PBP1A, and the survivability of LOS-deficient *A. baumannii* on the last-resort drug, colistin.

Results

Colistin Exposure Selects for LOS-Deficient *A. baumannii*. In a previous report, in vitro selection of *A. baumannii* strain ATCC 19606 on colistin (10 μg/mL) yielded resistant mutants. Each isolate contained a single mutation in one of three genes including *lpxA*, *lpxC*, or *lpxD*, which encode the first three steps in the conserved lipid A biosynthetic pathway. Characterization of a single *lpxA* mutant confirmed that ATCC 19606 *A. baumannii* survived without LOS and its precursor, lipid A (12). To determine if survival without LOS is a characteristic of only strain ATCC 19606 (12) or all *A. baumannii* strains, 15 clinical isolates including four multidrug resistant strains were plated on colistin (10 μg/mL), and isolated colonies were analyzed to determine the frequency of LOS inactivation. As shown in Table 1, 9 of the 15 strains (60%) propagated LOS-deficient *A. baumannii* after colistin exposure (as determined by colistin resistance/vancomycin sensitivity assays described here) with a mutation frequency comparable to previously published analysis (12). Importantly, six strains did not yield LOS-deficient colistin-resistant colonies, demonstrating that some *A. baumannii* clinical isolates could not survive without LOS (Table 1).

To ensure that colistin-resistant *A. baumannii* did not produce lipid A, three isolates were further characterized including the previously described ATCC 19606 and two multidrug-resistant *A. baumannii* strains, designated 5075 and AYE (12, 20, 21). Parent and colistin-resistant *A. baumannii* progeny were ^{32}P-radiolabeled, and isolated lipid A was chromatographically separated based on hydrophobicity. Quantitative TLC demonstrated that parent *A. baumannii* strains produced equivalent lipid A, but the colistin-resistant progeny were defective in lipid A assembly (Fig. 1*A*). The genomes of parent and LOS-deficient isolates were sequenced and mutations were mapped to *lpxC* (*SI Appendix*, Table S1). The antibiotic-susceptible ATCC 19606 LOS-deficient strain that encoded a single-nucleotide polymorphism in *lpxC* was complemented with a plasmid expressing LpxC (pLpxC) to restore lipid A biosynthesis (Fig. 1*A*). To ensure that LOS-deficient *A. baumannii* did not produce an alternative lipid A glycoform, ***m***atrix-***a***ssisted ***l***aser ***d***esorption ***i***onization (MALDI)–time of flight mass spectrometry (MS) analysis was performed on purified lipid A. Spectra collected from wild-type and complemented *A. baumannii* strains indicated major molecular ions at *m/z* 1728.0 and 1911.0 as previously described (8, 9, 11). Importantly, LOS-deficient *A. baumannii* lacked molecular ions indicative of lipid A (*SI Appendix*, Fig. S1*A*).

The lipid A anchor of LPS/LOS is a PAMP that is bound with high affinity by the mammalian host ***t***oll-***l***ike ***r***eceptor-***4*** ***m***yeloid ***d***ifferentiation protein-***2*** (TLR-4/MD-2) complex (2). Lipid A recognition by TLR-4/MD-2 activates MyD88- and TRIF-dependent pathways to initiate an inflammatory response and clear the bacterial infection. Wild-type, LOS-deficient, and complemented ATCC 19606 *A. baumannii* were incubated with a human embryonic kidney reporter cell line (HEK-blue) that expressed the human TLR-4/MD-2 receptor complex. Whereas the wild-type and complemented strains stimulated TLR-4–dependent activation comparable to *Escherichia coli*, the LOS-deficient *A. baumannii* failed to stimulate the TLR-4/MD-2 receptor complex similar to the Gram-positive *Staphylococcus aureus*, which does not produce lipid A (Fig. 1*B*). Analogous TLR-4/MD-2 activation was detected when colistin-resistant 5075 and AYE multidrug-resistant *A. baumannii* strains were analyzed (*SI Appendix*, Fig. S2). Together, TLC, MALDI-MS, and TLR-4/MD-2 activation assays demonstrate not only that *A. baumannii* strain ATCC 19606 can survive without lipid A, but also that other *A. baumannii* strains are capable of surviving, including multidrug-resistant isolates.

To understand the drug resistance profiles of wild-type and LOS-deficient *A. baumannii* isolates, we determined the ***m***inimum ***i***nhibitory ***c***oncentration (MIC) of colistin and vancomycin, which are last-resort antimicrobials prescribed to treat either Gram-negative or Gram-positive infections, respectively. Following inactivation of lipid A biosynthesis, colistin resistance increased more than 500-fold, whereas vancomycin resistance decreased more than 500-fold relative to the wild-type parent *A. baumannii* (Fig. 1*C*). Our results demonstrated that removal of LOS through lipid A inactivation fostered colistin resistance, but also sensitized all

Table 1. Diverse *A. baumannii* strains develop resistance to colistin (polymyxin E) through inactivation of lipooligosaccharide biosynthesis

A. baumannii strain	Isolation date	Isolation source	Drug resistance	Mutation frequency (LOS-deficient)
5075	2008–2009	Bone infection	Multidrug	1.49×10^{-7}
ATCC 19606	1948	Urine	Susceptible	1.53×10^{-7}
ATCC 17978	1951	Meninges	Susceptible	NA
AYE	2003	Blood	Multidrug	5.09×10^{-8}
SDF	2003	Louse	Susceptible	9.22×10^{-8}
ACICU	2005	Cerebrospinal fluid	Multidrug	NA
Recent clinical #1	1/1/10	Blood	Susceptible	4.23×10^{-8}
Recent clinical #2	3/15/10	Vagina	Susceptible	NA
Recent clinical #3	4/30/12	Lungs	Susceptible	3.15×10^{-8}
Recent clinical #4	7/18/12	Pustule (hand)	Susceptible	3.13×10^{-8}
Recent clinical #5	8/19/12	Blood	Susceptible	NA
Recent clinical #6	1/24/13	Urine	Susceptible	NA
Recent clinical #7	2/26/13	Blood	Multidrug	NA
Recent clinical #8	3/28/13	Skin	Susceptible	1.56×10^{-7}
Recent clinical #9	4/11/13	Skin	Susceptible	7.522×10^{-8}

NA: indicates that no lipid A-deficient isolates were recovered.

strains including the multidrug-resistant strains to vancomycin, which is exclusively active against Gram-positive bacteria. The findings suggest that outer-membrane LOS is the major obstacle to vancomycin-dependent killing in *A. baumannii*, as previously shown in *E. coli* (22, 23). Furthermore, inactivation of lipid A biosynthesis in drug-susceptible *A. baumannii* ATCC 19606 resulted in multidrug resistance with increased resistance to ciprofloxacin and tigecycline, which are antibiotics prescribed to treat susceptible *A. baumannii* infections (*SI Appendix*, Fig. S3). These assays demonstrate that without acquiring antimicrobial resistance genes, drug-susceptible *A. baumannii* can develop resistance to clinically important antimicrobials. Notably, LOS-deficient *A. baumannii* demonstrated susceptibility to tobramycin, an aminoglycoside used to treat *Pseudomonas aeruginosa* infections (*SI Appendix*, Fig. S3).

Previous reports described alterations to the cellular glycerophospholipid composition after inactivation of lipid A biosynthesis (18, 19). To determine if phospholipids varied between wild-type and LOS-deficient *A. baumannii*, cells were ^{32}P-radiolabeled and phospholipids were isolated. Quantitative TLC illustrated that wild-type and LOS-deficient *A. baumannii* produced equivalent glycerophospholipids including the major lipids phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) (*SI Appendix*, Fig. S1*B*). Importantly, no discernible differences between primary phospholipid ratios of wild-type and LOS-deficient *A. baumannii* were observed. Although relative amounts did not vary, slight alterations to the acyl chains may not be obvious. Therefore, phospholipids were analyzed using normal-phase LC/MS/MS to discern subtle structural changes. One previous report described LOS-deficient *A. baumannii* producing phospholipids with shorter acyl chains (19), but our MS/MS analysis of two LOS-deficient *A. baumannii* strains did not indicate any notable differences in the ratio of phospholipid structures including PG (*SI Appendix*, Fig. S4*A*), CL (*SI Appendix*, Fig. S4*B*), PE (*SI Appendix*, Fig. S5*A*), or Lyso-PE and phosphatidic acid (*SI Appendix*, Fig. S5*B*) relative to the wild-type parent strains.

Inactivation of PBP1A Promotes Complete Loss of LOS After Exposure to Colistin. Importantly, 6 of 15 strains in our screen did not yield LOS-deficient colonies after colistin exposure, suggesting a mechanism that inhibits LOS-deficient *A. baumannii* survival (Table 1). We constructed a random transposon (Tn) library of ~250,000 mutants in *A. baumannii* strain ATCC 17978, a strain that did not produce LOS-deficient colonies after colistin exposure (Table 1). Selection of the ATCC 17978 Tn mutant library on colistin gave rise to colistin-resistant, vancomycin-sensitive isolates. Sequencing determined Tn insertion sites in the A1S_3196 and A1S_3197 (*ponA* or *mrcA*) genes, which encode the well-conserved penicillin-binding protein, PBP1A.

To confirm that the Tn-encoded isolates did not synthesize LOS, mutants were ^{32}P-radiolabeled and lipid A was extracted. Quantitative TLC confirmed that two *ponA::Tn* isolates did not synthesize the LOS precursor, lipid A (*SI Appendix*, Fig. S6*A*). E-strip analysis established that the MIC of colistin increased 64-fold, whereas the MIC to vancomycin decreased more than 1,000-fold (*SI Appendix*, Fig. S6*B*). In addition, LOS-deficient *ponA::Tn* mutants did not stimulate the host TLR-4/MD2 receptor complex (*SI Appendix*, Fig. S6*C*). Collectively, these data indicated that PBP1A inhibited LOS-deficient ATCC 17978 *A. baumannii* survival after colistin exposure.

To validate that PBP1A inactivation enabled ATCC 17978 survival without lipid A, targeted mutagenesis removed the *ponA*-coding sequence from the *A. baumannii* chromosome. Deletion of *ponA* alone in the wild-type background did not alter lipid A assembly. However, colistin selection yielded *ponA* mutants that lacked LOS (*SI Appendix*, Fig. S9*C*) due to genetic mutation in the lipid A biosynthesis pathway (Fig. 2*B*). Genomic sequencing mapped the mutation of ATCC 17978 Δ*ponA* LOS-deficient strain to *lpxA* (*SI Appendix*, Table S1). LpxA expression in the LOS-deficient mutant restored lipid A production to wild-type levels (Fig. 2*B*), and *ponA* deletion alone did not alter TLR-4/MD-2 recognition (Fig. 2*C*). Together, these assays suggest that the *ponA* mutation alone does not impact LOS in any way before colistin treatment, but primes the cell to grow without LOS upon challenge with colistin.

ATCC 19606 PBP1A is encoded by a single *ponA* gene and is orthologous to PBP1A in *E. coli*, which is a bifunctional enzyme with conserved glycosyltransferase and transpeptidase domains (*SI Appendix*, Fig. S7) (24, 25). However, the *A. baumannii* ATCC 17978 annotation predicted a guanine nucleotide insertion to result in a premature stop codon of *ponA*, resulting in two separate genes, annotated as A1S_3196 and A1S_3197 (21). The guanine nucleotide insertion was not identified by highly reliable Sanger sequencing analysis, which indicates that, like ATCC 19606, ATCC 17978 *ponA* is encoded by a single gene and produces a full-length PBP1A protein (*SI Appendix*, Fig. S8).

Deletion of PBP1A in *E. coli* had no significant effect on cell growth, but effected cell width and the timing of divisome assembly (26). Also, previous analysis performed on PBP1A distinguished two catalytic domains essential for glycosyltransferase and transpeptidase activities (25, 27). The glycosyltransferase activity catalyzes polymerization of peptidoglycan from lipid II, whereas the transpeptidase activity catalyzes peptide cross-links to form a rigid

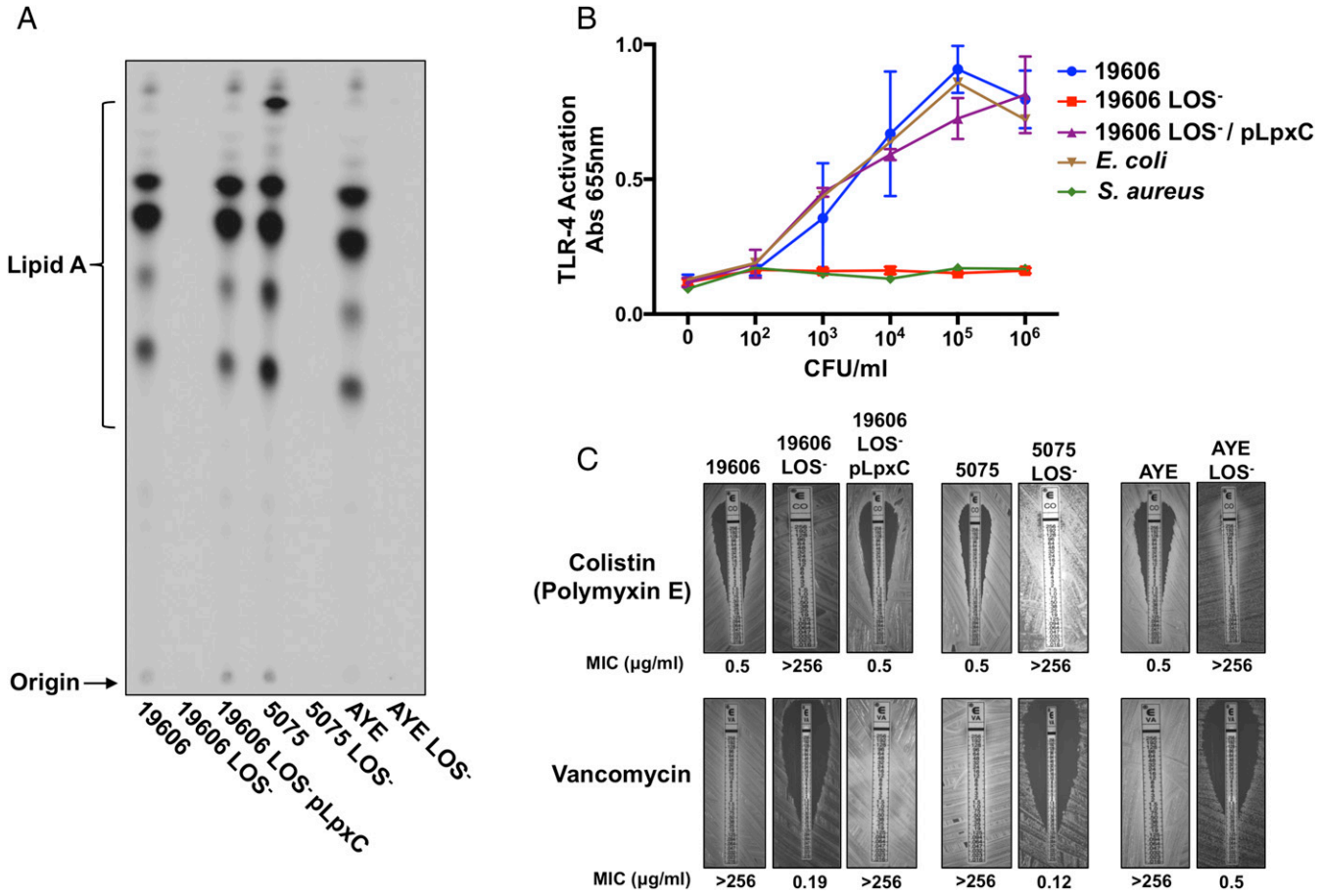

Fig. 1. *A. baumannii* inactivates lipooligosaccharide biosynthesis to alter immune recognition and susceptibility to clinically relevant antibiotics. (*A*) ^{32}P-radiolabeled lipid A was isolated from ATCC 19606, 5075, and AYE parent *A. baumannii* strains and their LOS-deficient progeny and separated based on hydrophobicity using TLC. (*B*) Stimulation of human TLR-4/MD2 complex following incubation of bacterial cells (cfu/mL) with HEK blue cells expressing the TLR-4 receptor complex is depicted. (*C*) MICs for parent and LOS-deficient *A. baumannii* strains.

cell wall (Fig. 2*A*) (28). We were curious to see if either the glycosyltransferase and/or transpeptidase catalytic domains of PBP1A influenced isolation of LOS-deficient *A. baumannii* on colistin. Catalytic residues for either the glycosyltransferase (E92Q) or transpeptidase (S459A) activities were inactivated via point mutation (Fig. 2*A*). It is worthwhile to note that the *E. coli* glycosyltransferase mutant lacks both activities as the transpeptidase activity is dependent on ongoing glycosyltransferase reactions in the same enzyme molecule (25). Similar to the wild-type strain, expression of PBP1A or PBP1A$_{S459A}$ from a multicopy plasmid in Δ*ponA* did not yield LOS-deficient ATCC 17978 *A. baumannii*. In contrast, LOS-deficient ATCC 17978 *A. baumannii* were isolated on colistin when PBP1A$_{E92Q}$ was expressed in Δ*ponA*, analogous to the Δ*ponA* mutant. Thus, the glycosyltransferase activity of PBP1A is sufficient to inhibit LOS-deficient survival (Fig. 2*D*, *Left*). Using PBP1A specific antisera, we confirmed that PBP1A and PBP1A$_{E92Q}$ were expressed (Fig. 3*B*). Unlike Δ*ponA*, deletion of the closely related bifunctional PBP1B (encoded by *ponB* or *mrcB*) did not produce LOS-deficient survival following colistin exposure, illustrating that LOS-deficient survival is specific to PBP1A activity (Fig. 2*D*, *Left*).

To verify that mutation of *ponA* alone had no effect on antibiotic resistance or lipid A assembly, we assessed resistance to colistin and vancomycin in the wild-type, Δ*ponA*, and Δ*ponA* LOS-deficient ATCC 17978 strains. Consistent with analysis in the *ponA::Tn* mutant, colistin resistance and vancomycin sensitivity in Δ*ponA* were altered only after colistin selection (*SI Appendix*, Fig. S9*A*). Analysis confirmed that Δ*ponA* and strains expressing all complementation plasmids produced lipid A (*SI Appendix*, Fig. S9*B*) whereas a colistin-resistant, vancomycin-sensitive Δ*ponA* ATCC 17978 *A. baumannii* isolate did not (Fig. 2*B*). Finally, LOS staining demonstrated that Δ*ponA* ATCC 17978 synthesized LOS, but the Δ*ponA* LOS-deficient strain did not (*SI Appendix*, Fig. S9*C*).

Inactivation of PBP1A in *A. baumannii* Strain ATCC 17978 Alters Expression of Lipoprotein Transport Genes. Expression of PBP1A prevented isolation of LOS-deficient ATCC 17978 *A. baumannii* (Fig. 2*D*, *Left*). However, we were curious to see if expression of native and mutant PBP1A proteins in ATCC 19606 *A. baumannii*, a strain known to survive without LOS, would block isolation of LOS-deficient colonies after colistin exposure. Selection of ATCC 19606 *A. baumannii* expressing PPB1A and PBP1A$_{S459A}$ from a multicopy plasmid yielded zero LOS-deficient isolates. Furthermore, PBP1A$_{E92Q}$ expression resulted in LOS-deficient isolates equivalent to wild-type levels (Fig. 2*D*, *Right*).

The *ponA* genes and respective promoters in ATCC 17978 and ATCC 19606 are nearly identical, indicating that each wild-type strain should produce similar levels of PBP1A. To understand why ATCC 19606, but not ATCC 17978, produces LOS-deficient isolates on colistin, we analyzed transcription of *ponA* in the two parent strains. Interestingly, an 80-fold lower abundance of *ponA* transcripts were detected in ATCC 19606 relative to ATCC 17978 *A. baumannii* (Fig. 3*A*). Furthermore, ATCC 17978 *A. baumannii* produced PBP1A, but ATCC 19606 *A. baumannii* did not (Fig. 3*B*).

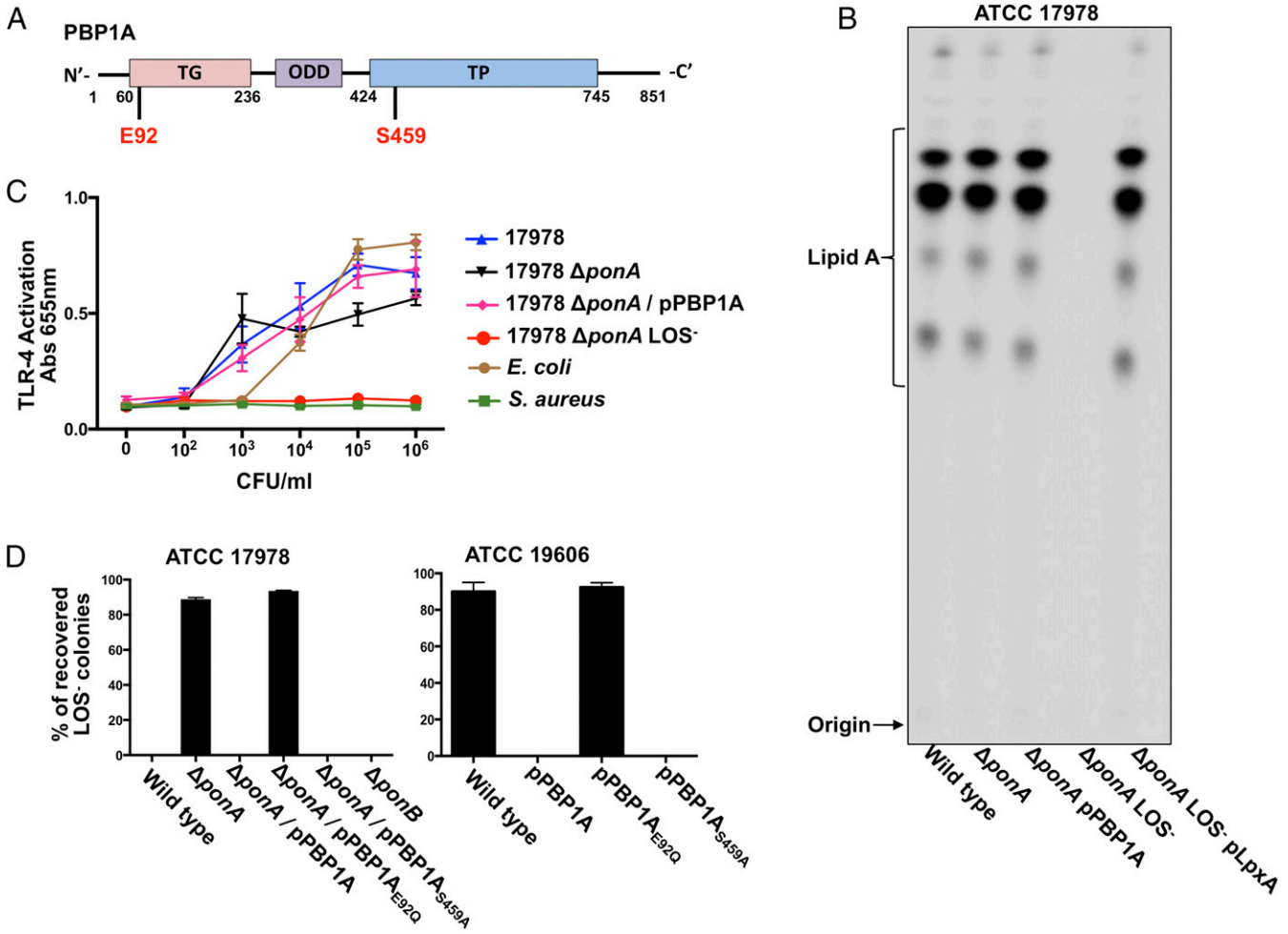

Fig. 2. Inactivation of *ponA* promotes complete loss of LOS in ATCC 17978 *A. baumannii*. (*A*) Illustration depicting PBP1A and its domains with respective catalytic residues (red). (*B*) ^{32}P-radiolabeled lipid A isolated from ATCC 17978 wild-type and *ponA* mutant *A. baumannii* was separated based on hydrophobicity using TLC. (*C*) Stimulation of human TLR-4/MD-2 following incubation of bacterial cells (cfu/mL) with HEK blue cells expressing the TLR-4 receptor complex is depicted. (*D*) Percentage recovery of LOS-deficient *A. baumannii* after selection of plating either 10^9 cfu ATCC 17978 (*Left*) or ATCC19606 (*Right*) on colistin.

To understand how peptidoglycan was altered in LOS-deficient *A. baumannii*, we isolated muropeptides from wild-type ATCC 17978, Δ*ponA*, the Δ*ponA* LOS-deficient strain, and Δ*ponA* expressing either PBP1A or PBP1A$_{E92Q}$ from a multicopy plasmid. The extracted muropeptide pool was separated using high-performance liquid chromatography to quantify the differences in the chemical composition (28). The peptidoglycan structure of wild-type *A. baumannii* contained significantly more peptide cross-links (~60%) than either *E. coli* (~40–50%) (28) or *Helicobacter pylori* (~40%) (29) with an average chain length of 30 disaccharides (*SI Appendix*, Table S2). Neither the average peptidoglycan cross-links nor the chain lengths were significantly altered in the Δ*ponA* or the LOS-deficient mutant compared with wild-type ATCC 17978 *A. baumannii* (*SI Appendix*, Table S2).

To understand how PBP1A hindered survival of some *A. baumannii* strains without LOS, we isolated total RNA transcripts from wild-type, Δ*ponA*, and Δ*ponA* LOS-deficient ATCC 17978 *A. baumannii* strains. RNA sequencing detected altered transcription of genes encoding important cell envelope pathways relative to the wild-type parent (Datasets S1 and S2). Pathway analysis determined by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations (*P* value ≤0.05) specified increased transcription of three pathways in Δ*ponA* and six pathways in Δ*ponA* LOS-deficient *A. baumannii*. Only increased transcription of genes encoding the Lol transport pathway was conserved, which included overexpression of *lolA* (Fig. 4*A*), where transcripts relative to the wild-type parent increased >4-fold in Δ*ponA* and >56-fold in the Δ*ponA* LOS-deficient strain (Datasets S1 and S2). In addition, analysis indicated reduced transcription of four pathways using a false discovery rate adjusted *P* value ≤0.05 in the Δ*ponA* strain and two pathways in the Δ*ponA* LOS-deficient strain relative to the parent ATCC 17978. Although not the focus of this article, transcripts in the phenylalanine biosynthesis pathway were reduced in both strains (Fig. 4*A*).

The Lol transport system is an essential pathway in Gram-negative bacteria (reviewed in ref. 27). LolA is a periplasmic chaperone that transports lipoproteins from LolCDE at the inner membrane to LolB at the outer membrane, where the lipoproteins are anchored. Consistent with global transcriptome analysis, qPCR analysis confirmed that *lolA* transcription increased ~8-fold in Δ*ponA* and ~110-fold in the Δ*ponA* LOS-deficient strain ATCC 17978 relative to wild type. Furthermore, *lolA* transcription in wild-type ATCC 19606 was ~10-fold higher relative to ATCC 17978 and >100-fold higher in LOS-deficient ATCC 19606 (Fig. 4*B*).

Global RNA-seq Analysis in Multiple *A. baumannii* Strains Highlights Pathways Important for Loss of LOS. Previously published analysis on a single LOS-deficient strain, a 19606 *A. baumannii lpxA* mutant,

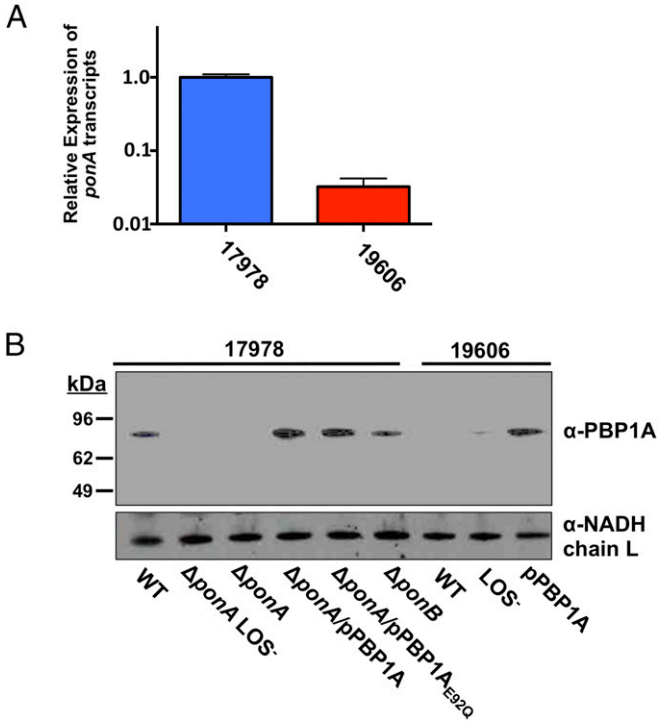

Fig. 3. Differential expression of *ponA* and PBP1A in ATCC 17978 and ATCC 19606 *A. baumannii*. (*A*) Relative concentration of *ponA* mRNA in ATCC 17978 and ATCC 19606 wild-type *A. baumannii*. (*B*) Immunoblot analysis of PBP1A and NADH chain L proteins in whole-cell lysates from wild-type and mutant ATCC 17978 and ATCC 19606 *A. baumannii* strains. Each protein was detected using specific polyclonal antiserum.

showed increased transcription of genes encoding Lol-lipoprotein transporters, Mla-retrograde phospholipid transporters, and carbohydrate transporters, but reduced transcription of genes encoding surface structures (17). Congruent with our multistrain analysis, we sought to understand if the transcriptional alterations in LOS-deficient *A. baumannii* were conserved. RNA was isolated from four LOS-deficient *A. baumannii* strains and their wild-type parent strains including ATCC 17978 Δ*ponA*, ATCC 19606, 5075, and AYE. The RNA transcripts were sequenced to ascertain the altered transcriptional profile of each isolate relative to the wild-type parent strain (Datasets S2–S5). Pathway analysis (P value ≤ 0.05) indicated that increased transcription of five pathways was conserved among the LOS-deficient strains (Fig. 5*A*), whereas no down-regulated pathways were conserved (Fig. 5*B*). Conserved up-regulated pathways that shared significant increased transcription among all LOS-deficient strains included Lol lipoprotein transport genes, putative lipoproteins, retrograde phospholipid transport genes, genes involved in drug efflux and CAMP resistance, and genes encoding the BaeSR two-component system. A heat map illustrates the fold change in expression for each gene transcript in the conserved pathways relative to the respective wild-type *A. baumannii* strain (Fig. 5*C*). Transcription of PNAG biosynthetic genes was not conserved in our analysis, but the genes were included in the heat map because a previous report suggested that these pathways could contribute to *A. baumannii* strain ATCC 19606 survival without LOS (17).

Lipoproteins Are Overexpressed and Surface-Displayed in LOS-Deficient *A. baumannii*. Together, the transcriptomic analysis performed on four LOS-deficient *A. baumannii* strains (Fig. 5*C*) and the previous analysis performed on ATCC 19606 (17) indicated that transcripts encoding lipoprotein transporters and putative lipoproteins are increased relative to the wild-type strain. Specifically, transcription of HMPREF0010_01944 (1944), HMPREF0010_01945 (1945), and HMPREF0010_02739 (2739), as annotated in ATCC 19606 *A. baumannii*, exhibited the largest conserved increased fold-change in the RNA-seq analysis among the four LOS-deficient isolates (Fig. 5*C*).

To evaluate lipoprotein localization, wild-type and LOS-deficient *A. baumannii* were treated with sulfo-NHS-LC-LC-biotin, which reacts with primary amines to conjugate a biotin molecule onto surface-exposed proteins. Previously, a derivative of this molecule (NHS-LC-LC-biotin) identified surface proteins of *E. coli* (30) and *Vibrio cholerae* (31). This compound does not permeate the outer membrane because of its size and the charge added by the sulfo-NHS groups. To confirm that the sulfo-NHS-LC-LC-biotin molecule does not enter *A. baumannii*, whole cells from wild-type *E. coli*, *A. baumannii*, and LOS-deficient *A. baumannii* were labeled. Western blotting with streptavidin-HRP showed biotinylation of membrane proteins, but no soluble proteins, illustrating that the molecule does not enter the cell (Fig. 6*A*). In contrast, when the cells were lysed before the addition of sulfo-NHS-LC-LC-biotin, both membrane and soluble proteins were biotinylated (Fig. 6*B*).

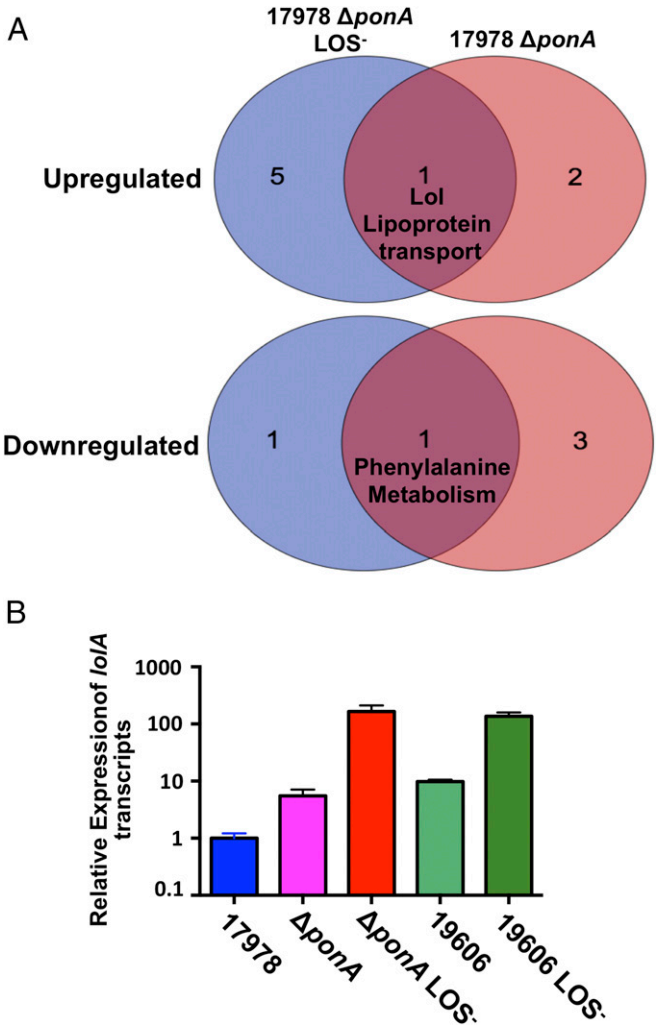

Fig. 4. Inactivation of *ponA* in ATCC 17978 *A. baumannii* increases expression of lipoprotein transport genes. (*A*) Venn diagram showing altered pathway expression in ATCC 17978 Δ*ponA* LOS-deficient and ATCC 17978 Δ*ponA A. baumannii* strains (P value ≤ 0.05). Gene expression was calculated relative to the wild-type parent ATCC 17978 *A. baumannii*. (*B*) Relative concentration of *lolA* mRNA in wild-type and mutant ATCC 17978 and ATCC 19606 *A. baumannii* strains.

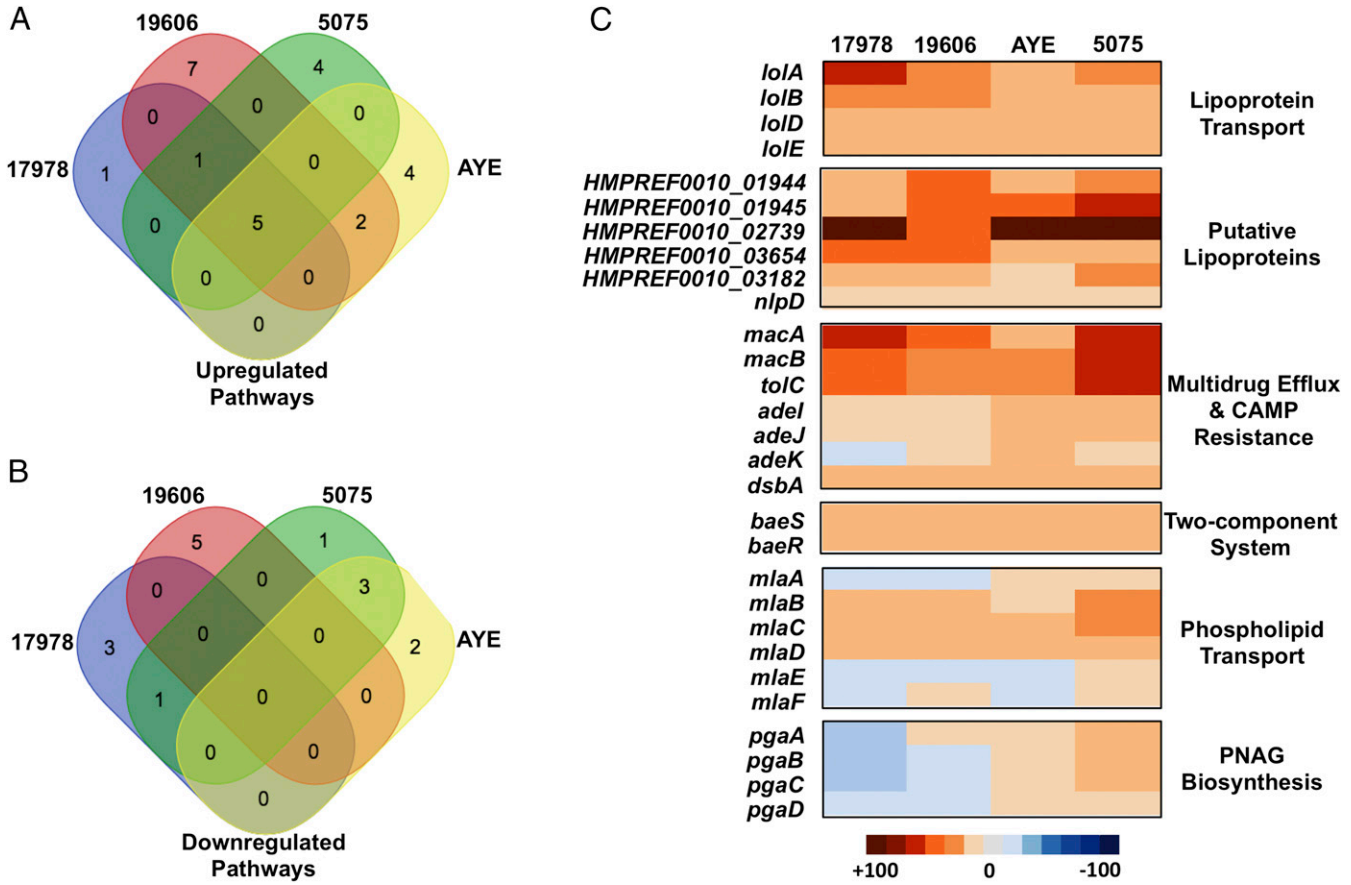

Fig. 5. Global transcriptional analysis from multiple *A. baumannii* strains highlights conserved pathways important for loss of LOS. Venn diagrams showing up-regulated (*A*) and down-regulated (*B*) pathways in four LOS-deficient *A. baumannii* strains (P value ≤ 0.05). Gene expression was calculated relative to the parent wild-type *A. baumannii* strains. The five conserved significantly up-regulated pathways in LOS-deficient *A. baumannii* included lipoprotein transport, putative lipoproteins, retrograde phospholipid transport, multidrug efflux pumps/CAMP resistance proteins, and the BaeSR two-component system. (*C*) Heat map illustrating the altered expression of each gene in the five conserved pathways and for PNAG biosynthesis. Our statistical analysis did not highlight the PNAG biosynthesis as conserved responses to loss of LOS, but genes were included in the heat map because this pathway was previously thought to be important for *A. baumannii* survival without LOS.

A number of biotinylated proteins detected in the LOS-deficient cell surface were absent in wild-type cells (Fig. 6 *A* and *B*), especially lower-molecular-weight proteins (<17 kDa). To determine if the lipoproteins 1944, 1945, and 2739 were enriched on the surface of LOS-deficient *A. baumannii*, we generated three separate strains where each lipoprotein was fused to a C-terminal His_6-tag. The theoretical mass of 1944-His_6 and 1945-His_6 is 11.52 and 11.83 kDa, respectively, whereas the theoretical mass of 2739-His_6 is 14.76 kDa. Intact whole cells were treated with sulfo-NHS-LC-LC-biotin and lysed. The proteins in each lysate were separated using SDS/PAGE. Streptavidin-HRP blotting indicated that protein(s) at the indicated theoretical masses of each lipoprotein were biotinylated in LOS-deficient cells, but not the wild-type *A. baumannii* (Fig. 6 *C–E*, lanes 1 and 2, *Top*). To determine if the biotinylated proteins represented the target lipoproteins, blots were probed with an anti-his antibody. Biotinylated and His_6-tagged proteins had equivalent masses, suggesting that the biotinylated proteins were the target lipoproteins (Fig. 6 *C–E*, lanes 1 and 2, *Top* and *Middle*). Furthermore, each respective lipoprotein mutant (Δ*2739* and Δ*1944–45*) did not conjugate biotin in the LOS-deficient mutant, confirming that the three lipoproteins are enriched in the outer membrane of LOS-deficient *A. baumannii* (Fig. 6 *C–E*, lanes 3 and 4). Surprisingly, the three lipoproteins (1944, 1945, and 2739) were not detected in the wild-type *A. baumannii* strain, suggesting that expression and/or outer membrane localization transpires only in the LOS-deficient mutant (Fig. 6 *C–E*, lane 1). Next, we questioned if lipoprotein overexpression would result in outer-membrane accumulation in wild-type *A. baumannii*. Each overexpressed His_6-tagged lipoprotein was produced (Fig. 6 *C–E*, lane 5, *Middle*), and localized to the outer membrane in the wild-type strain (Fig. 6 *C–E*, lane 5, *Top*). Therefore, lipoprotein overexpression in the LOS-deficient strain does not redirect lipoprotein transport. Instead, overexpressed lipoproteins likely accumulate at the surface-exposed face of the outer membrane to potentially compensate for the absence of LOS. Importantly, LOS-deficient *A. baumannii* was isolated in each lipoprotein mutant, suggesting that no individual lipoprotein was essential for survival without LOS.

Discussion

Gram-negative bacteria are defined by a second membrane bilayer in the cell envelope that encases the peptidoglycan and acts as a fortified barrier to protect the cell from environmental toxins. Unlike the cytoplasmic membrane, the outer membrane is asymmetric with the surface-exposed monolayer enriched in either LPS or LOS glycolipids, which prevent entry of many antibiotics. Whereas the LOS/LPS precursor, lipid A, was thought to be essential for survival of most Gram-negative bacteria (13), recent reports confirm that *A. baumannii* also persists after inactivation of lipid A biosynthesis (12, 14, 15). Importantly, colistin selection in vitro enables isolation of LOS-deficient *A. baumannii* that are colistin resistant due to inactivation of lipid A biosynthesis (12). As previously reported, a growth defect is associated with

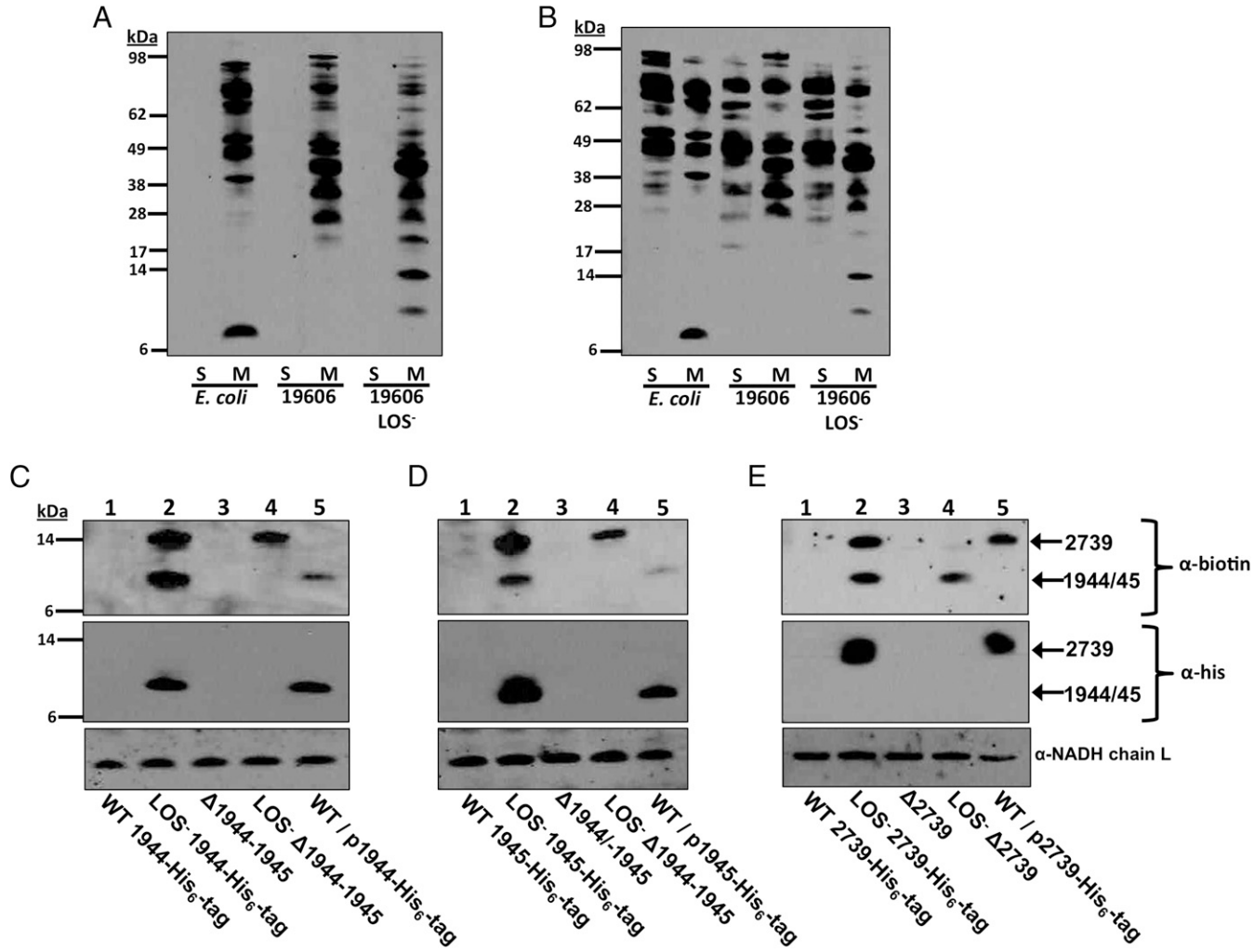

Fig. 6. Lipoproteins are overexpressed and surface-displayed in LOS-deficient *A. baumannii*. Intact whole cells (*A*) or cell lysates (*B*) were incubated with sulfo-NHS-LC-LC-biotin to biotinylate accessible proteins. After labeling, proteins from the soluble (S) and membrane (M) fractions were subjected to SDS/PAGE and Western blotted using streptavidin-HRP. Intact whole cells containing chromosomal His_6-tag fusions including (*C*) HMPREF0010_1944 (1944-His_6) (11.52 kDa), (*D*) HMPREF0010_1945 (1945-His_6) (11.83 kDa), and (*E*) HMPREF0010_2739 (2739-His_6) (14.76 kDa) were treated with sulfo-NHS-LC-LC-biotin, and proteins were separated using SDS/PAGE. Wild-type, LOS-deficient for each respective mutant, and overexpression strains were blotted using a streptavidin-HRP conjugate (*Top*), anti-his antibody (*Middle*), or a polyclonal NADH chain L antibody (*Bottom*).

LOS-deficient *A. baumannii* under standard growth conditions, and cells are smaller and round during exponential growth (32). We observed a similar growth phenotype in our study. In addition, LOS-deficient *A. baumannii* displayed a virulence defect in a murine model (32), but, importantly, LOS-deficient *A. baumannii* were isolated from a patient after colistin treatment (16). A more detailed understanding how *A. baumannii* modifies its outer membrane to persist in the host without LOS will elucidate a novel drug resistance mechanism.

We have confirmed not only that selection of drug-susceptible *A. baumannii* strain ATCC 19606 on colistin results in isolation of resistant LOS-deficient colonies, but also have demonstrated the resistance of the strain to other clinically relevant antibiotics including ciprofloxacin and tigecycline in the ATCC 19606 LOS-deficient isolates (*SI Appendix*, Fig. S3). Resistance acquisition to multiple antimicrobials following colistin treatment has not been described and is extremely worrisome because drug-susceptible cells acquired multidrug resistance after only one exposure to colistin without acquisition of new genes. Although characterization of these drug resistance mechanisms is outside the scope of this work, overexpression of resistance pumps and transporters reported in our transcriptomic analysis could indicate resistance mechanisms (Datasets S1–S5). Interestingly, this study also showed that removal of lipid A/LOS molecules from the outer membrane increased *A. baumannii* sensitivity to vancomycin and tobramycin (an aminoglycoside) (Fig. 1*B* and *SI Appendix*, Fig. S3). This important finding demonstrates that alterations to the outer-membrane permeability barrier sensitize otherwise drug-resistant bacteria to specific classes of antibiotics, which could be useful for antimicrobial synergism to treat bacterial infections.

To date, bacterial survival after inactivation of the lipid A biosynthetic pathway is limited to *N. meningitidis*, *M. catarrhalis*, and *A. baumannii*, which are all Gram-negative mucosal pathogens that synthesize the LOS glycoform (12, 14, 15). We discovered that, although some *A. baumannii* strains can survive without lipid A, other strains cannot, providing a model system to explore lipid A essentiality. Screening of an *A. baumannii* ATCC 17978 Tn mutant library directed us to examine PBP1A, a major cell-wall synthase. Confirming our mutagenesis screen, removal of the *ponA* (which encodes PBP1A) coding sequence permitted *A. baumannii* ATCC 17978 to develop colistin resistance through inactivation of the lipid biosynthesis pathway. Furthermore, expression of PBP1A

from a multicopy plasmid in *A. baumannii* ATCC 19606 impeded isolation of LOS-deficient colonies, further supporting a role for PBP1A. Unexpectedly, our results link two fundamental cell-envelope processes, lipid A biosynthesis and biogenesis of the peptidoglycan cell wall. Interestingly, pathways involved in cell-wall maintenance/biogenesis and the steps in lipid biosynthesis do not directly impact each other, but previous analysis showed that mutations resulted in convergence of the two distinct biogenesis pathways (23).

Curiously, *A. baumannii* strains such as ATCC 17978, which produce PBP1A, and ATCC 19606, which does not (Fig. 3 *A* and *B*), encode almost identical *ponA* genetic coding sequences and promoters. The varied *ponA* expression between the two wild-type strains suggests complex genetic regulation among *A. baumannii* isolates. Although we cannot conclude why PBP1A is detrimental to LOS-deficient *A. baumannii*, we hypothesize that small changes in the cell wall that we could not detect act as a signal to initiate increased transcription of the gene encoding the lipoprotein transporter, LolA. Our analysis demonstrated that increased transcription of lipoprotein transporters and lipoproteins in LOS-deficient *A. baumannii* resulted in outer-membrane remodeling that could promote *A. baumannii* survival without lipid A/LOS. Regulation of *lolA* in *E. coli* is controlled by the Rcs phosphorelay system, which detects cell-envelope stress (33). However, *A. baumannii* does not encode an orthologous system, and regulatory mechanisms controlling *lolA* gene expression in *A. baumannii* are not known. It is within reason to speculate that increased LolA-dependent lipoprotein transport could result in accumulation of lipoproteins in the outer membrane where they assemble onto the cell surface of *A. baumannii*. Increased localization of lipoproteins at the cell surface could compensate for the absence of LOS. Although it is unknown how lipoproteins in Gram-negative bacteria flip from the inner leaflet to the cell-surface leaflet of the outer membrane, a recent report demonstrated that a protein termed SLAM is required for surface localization of key lipoproteins in *N. meningitidis*, and a similar mechanism may exist in *A. baumannii* (34).

The pathogenic spirochetes *Treponema pallidum* and *Borrelia burgdorferi* do not encode genes to synthesize lipid A (35, 36). Instead, the surface-exposed monolayer of their outer membrane is enriched in lipoproteins and devoid of outer-membrane proteins (37). Although *T. pallidum* is an obligate intracellular human pathogen (38), *B. burgdorferi* adapts to dramatic environmental fluctuations (39), suggesting that Gram-negative bacteria lacking surface-exposed LPS/LOS, such as *A. baumannii*, can potentially survive in a variety of environments including the human host.

Furthermore, *A. baumannii* encodes an *O*-linked glycosylation system that adds glycans to many proteins (40). If the surface-exposed lipoproteins were glycosylated in *A. baumannii*, they could partially mimic the biophysical properties of LPS/LOS in LOS-deficient *A. baumannii* to maintain the outer-membrane barrier. Although previous analysis determined the *O*-glycosylated proteome of *A. baumannii* strain ATCC 17978 (40), the lipoproteins in this analysis are not expressed in wild-type *A. baumannii* and could have been overlooked. Although we have shown that three putative lipoproteins, including 1944, 1945, and 2739, are overexpressed in the outer membrane and are potentially surface-localized, no single lipoprotein was required for isolation of LOS-deficient *A. baumannii* (Fig. 6 *A*–*C*). Interestingly, higher-molecular-weight (>17 kDa) proteins were biotin-labeled in the LOS-deficient *A. baumannii* cells (Fig. 6 *A* and *B*), indicating that other outer membrane proteins could contribute to survival of LOS-deficient *A. baumannii*.

To better understand how the outer-membrane glycolipids LPS or LOS protect Gram-negative bacteria from various environmental niches, we focused on *A. baumannii* not only because it is one of only three bacterial pathogens that survives after lipid A inactivation, but also because it shuts off LOS biosynthesis to promote colistin resistance. This study illustrates the plasticity of the Gram-negative outer membrane, which tolerates dramatic alterations in cell-envelope composition to protect the cell in the presence of toxic antibiotics. Furthermore, our work demonstrates that inactivation of lipid A increases expression of genes encoding lipoproteins and their transporters and that the lipoproteins accumulate on the cell surface when LOS is not produced, likely to maintain the outer-membrane barrier. Altogether our work indicates that the outer membrane of Gram-negative bacteria is a dynamic barrier that is modified to protect the cell from detrimental environmental conditions.

Materials and Methods

Description of all strains, plasmids, and primers can be found in *SI Appendix, Materials and Methods*, for all experiments including strain construction and isolation of LOS-deficient *A. baumannii*, lipid extractions, MS analysis, TLR-4–signaling assays, RNA and genomic sequencing, surface biotin labeling, and MICs can be found in *SI Appendix*. The data discussed in this publication have been deposited in the National Center for Biotechnology's Gene Expression Omnibus (41) (accession no. GSE84282).

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