Supplemental data for 'Alternative lipid synthesis in response to phosphate limitation promotes antibiotic tolerance in Gram-negative ESKAPE pathogens'

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Supplementary Figures

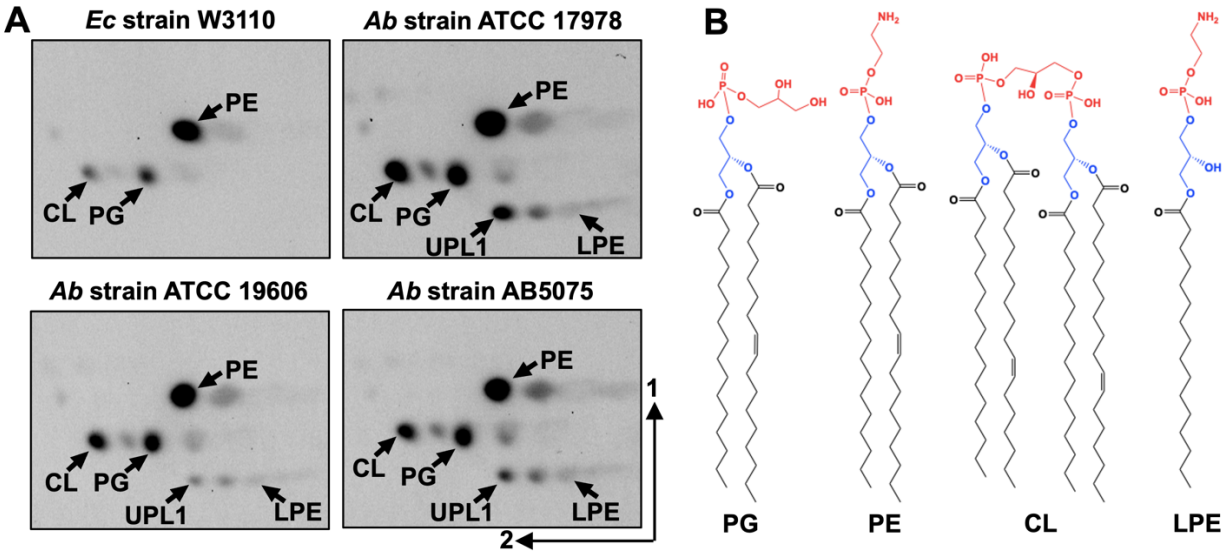

Figure S1: *Escherichia coli* (*Ec*) and *Acinetobacter baumannii (Ab)* lipid composition after growth in complex media. A. Strains were grown in complex media (LB broth) in the presence of ^{32}P-orthopohosphoric acid until mid-logarithmic growth phase. Cells were collected and total lipids were extracted using the Bligh and Dyer method and separated using 2-dimensional thin-layer chromatography. Labelled lipids include anionic cardiolipin (CL) and phosphatidylglycerol (PG), aminolipids phosphatidylethanolamine (PE) and lyso-PE (LPE), and unknown phospholipid 1 (UPL1). **B.** PG, PE, CL and LPE chemical structures. Head groups are red, the glycerol backbone is blue, and fatty acids are black.

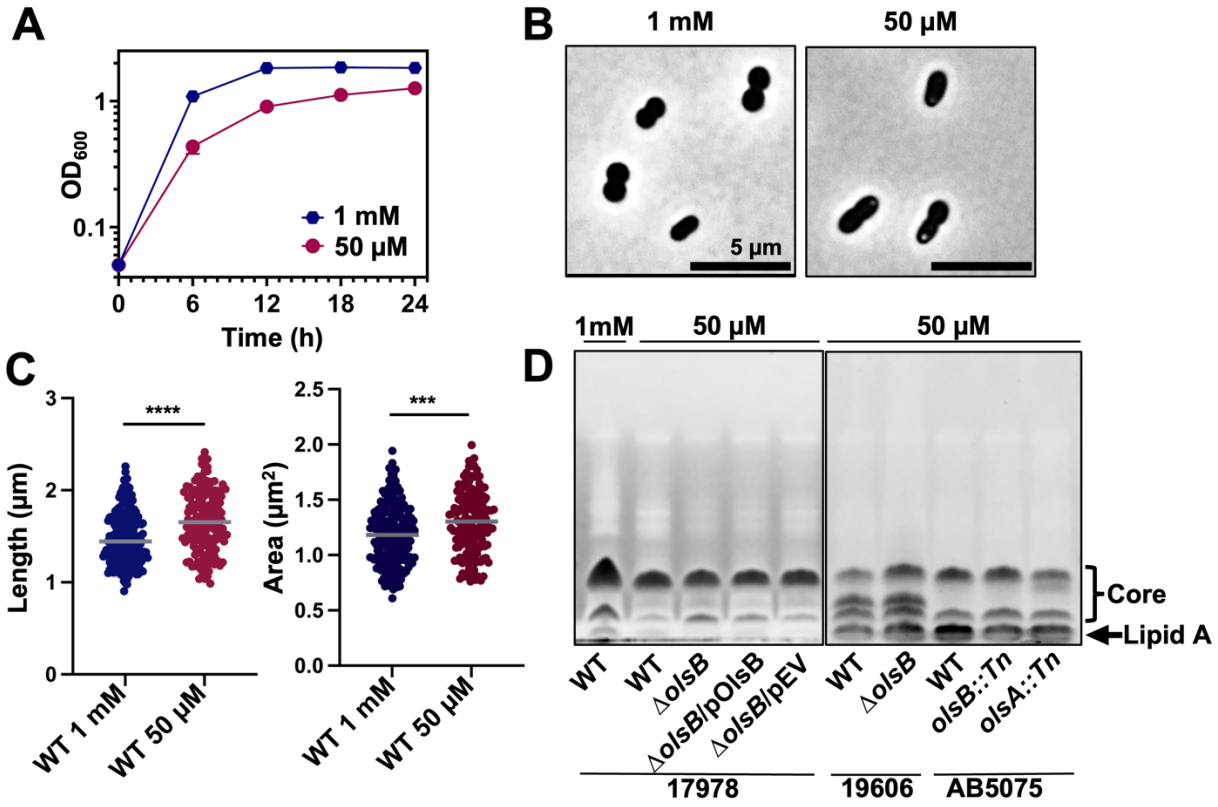

Figure S2: Effect of phosphate availability on growth, cell morphology, and LOS production. **A.** *A. baumannii* ATCC 17978 was cultured in minimal medium containing either excess (1 mM) or limiting (50 µM) phosphate for 24 hours. **B.** Phase-contrast images of *A. baumannii* grown under these conditions, captured during the exponential phase. Scalebar is 10 µm. **C.** Cell length and area were quantified for each population ($n \geq 150$) using ImageJ software. Each point represents an individual cell. The experiment was repeated twice, and one representative dataset was reported. Significance testing conducted using Student *t* test with two-tailed distribution assuming equal variance. ***$P < 0.001$, ****$P < 0.0001$. **D.** Proteinase K-treated whole-cell lysate from wild-type *A. baumannii* strains ATCC 17978, ATCC 19606, and AB5075, as well as from aminolipid-deficient mutants grown under excess (1mM) or limiting (50 µM) phosphate conditions. The LOS samples were separated using SDS-PAGE and stained using Pro-Q Emerald 300.

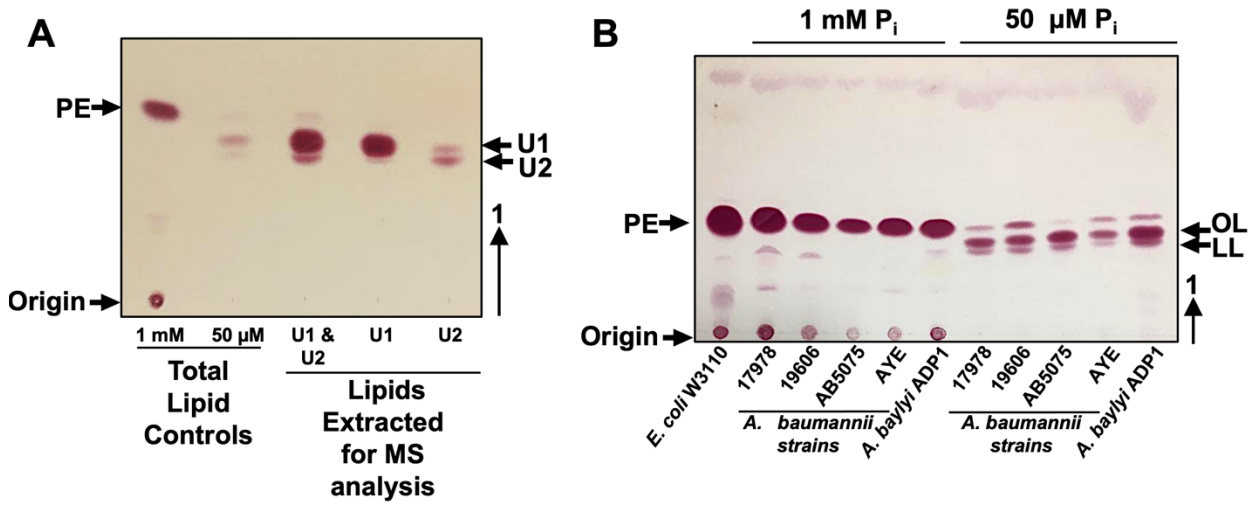

Figure S3: Thin-layer chromatography of aminolipids in *Acinetobacter* strains. A. To produce samples for MS analysis, total lipids were separated using thin-layer chromatography and scraped from the plate, extracted using the Bligh and Dyer method, and run alongside lipid controls. Extractions resulted in isolation of U1 & U2, U1, or U2. Lipids were stained with ninhydrin to visualize aminolipids. Specific lipids are labelled: PE, phosphatidylethanolamine; U1, unknown lipid 1; U2, unknown lipid 2. **B.** Total lipids were grown in minimal medium supplemented with excess (1 mM) or limiting (50 µM) phosphate conditions from indicated *Acinetobacter* strains. Total lipids were spotted on thin-layer chromatography and separated based on hydrophobicity. Plates were stained with ninhydrin to visualize aminolipids. Specific lipids are labelled: PE, phosphatidylethanolamine; OL, ornithine lipid; LL, lysine lipid.

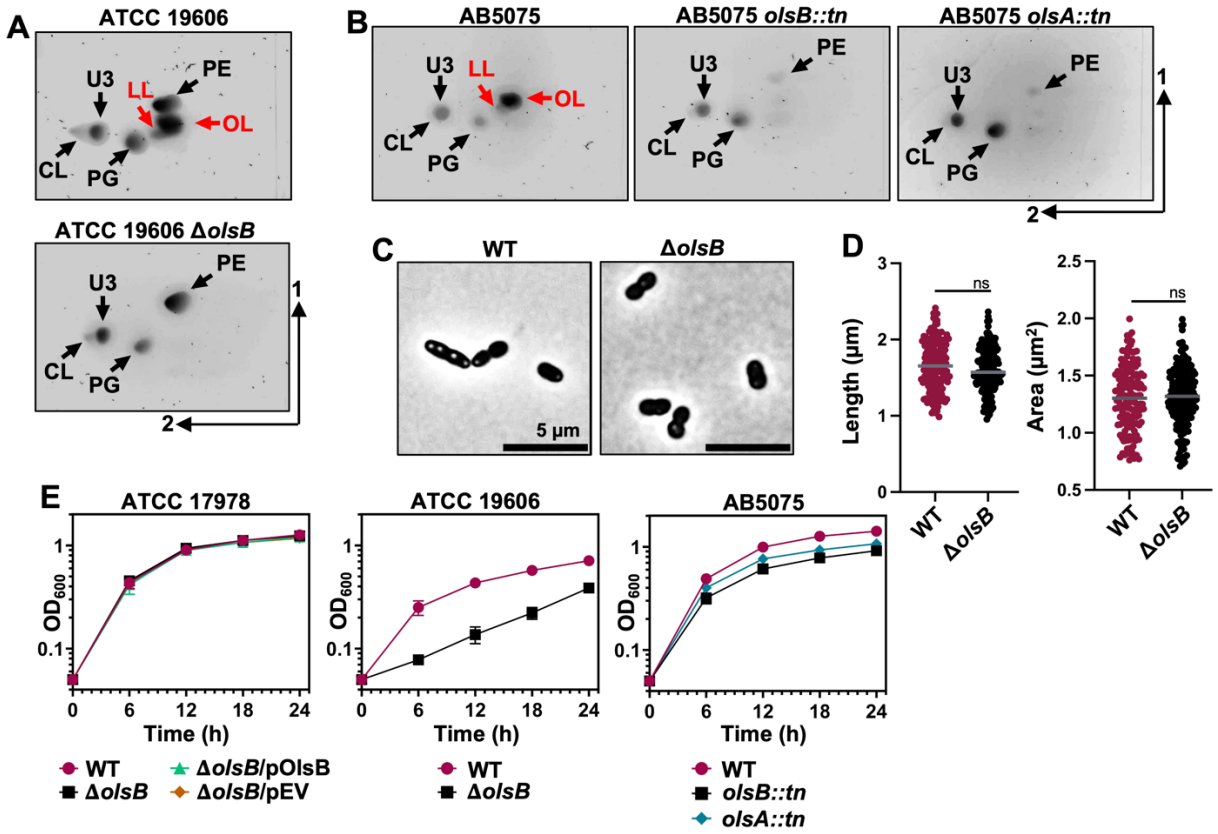

Figure S4: *olsB* and *olsA* are required for ornithine and lysine lipid biosynthesis in *A. baumannii*. **A.** 2D thin-layer chromatography lipid analysis in ATCC 19606 wild type and the Δ*olsB* mutant strain after growth in limiting (50 µM) phosphate concentrations. Lipids were stained with sulfuric acid. **B.** Analysis in AB5075 wild type and transposon (Tn*101*) mutant strains after growth in limiting (50 µM) phosphate concentrations. Specific lipids are labelled: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; OL, ornithine lipid; LL, lysine lipid. OL and LL aminolipids are labelled in red. **C.** Phase-contrast images of *A. baumannii* strain ATCC 17978 grown under phosphate limiting conditions, captured during exponential phase growth. Scalebar is 10 µm. **D.** Cell length and area of strain ATCC 17978 were quantified for each population ($n \geq 150$) using ImageJ software. Each point represents an individual cell. Significance testing conducted using Student *t* test with two-tailed distribution assuming equal variance. ns = not significant. **E.** Optical density (OD_{600}) measurements of *A. baumannii* strains grown in 50 µM phosphate over 24 h.

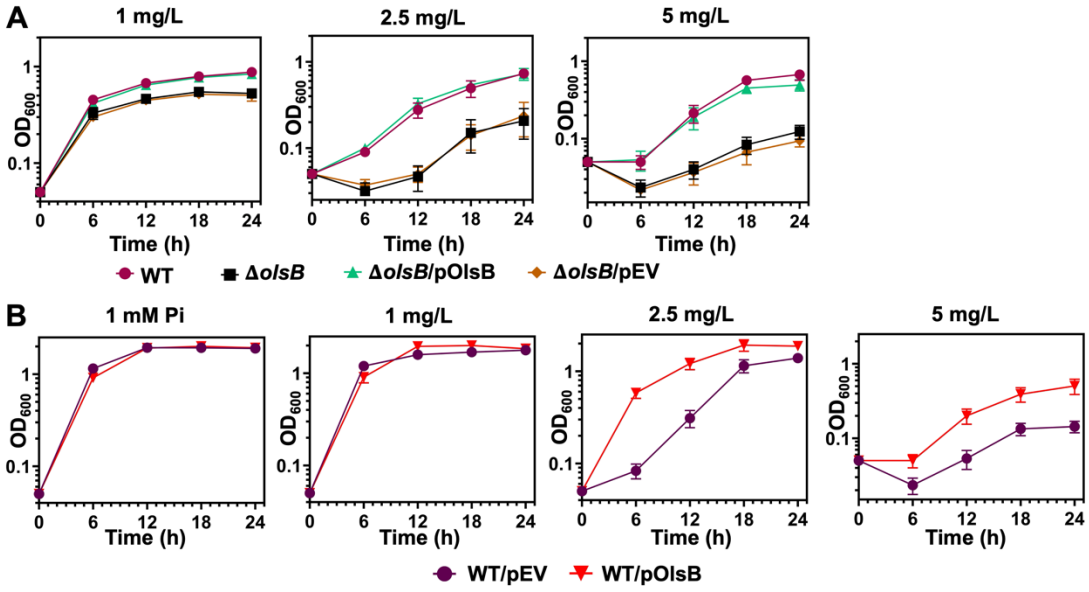

Figure S5: Aminolipid formation promotes *A. baumannii* tolerance to colistin. Growth (OD_{600}) of *A. baumannii* ATCC 17978 strains was measured at 37 °C in minimal medium with limiting phosphate (**A**) or excess phosphate (**B**), and in the presence of colistin at concentrations of 1, 2.5, or 5 mg/L.

Supplementary Tables

Please See the Excel Spreadsheet for Tables S1-3

Table S4: Strains and plasmids used in this study.

Strain or Plasmid	Description	Reference or Source
Strains		
A. baumannii ATCC 17978	Wild type	(1)
A. baumannii ATCC 19606	Wild type	(2)
A. baumannii AB5075	Wild type	(3)
A. baumannii AYE	Wild type	(4)
A. baylyi ADP1	Wild type	(5)
P. aeruginosa PAO1	Wild type	(6)
K. pneumoniae KPNIH1	Wild type	(7)
E. cloacae ATCC 13047	Wild type	(8)
A. baumannii ATCC 17978	*ΔolsB (A1S_0889)::deletion*	This study
A. baumannii ATCC 17978	*ΔphoR (A1S_3376)::deletion*	This study
A. baumannii ATCC 19606	*ΔolsB (HMPREF0010_01383)::deletion*	This study
A. baumannii AB5075	*ΔolsB (ABUW_3039)::tn101*	(9)
A. baumannii AB5075	*ΔolsA (ABUW_0502)::tn101*	(9)
A. baumannii AB5075	*ΔphoR (ABUW_0105)::tn26*	(9)
E. coli W3110	Wild type, F- *λ-, rph-1 IN (rrnD, rrnE)1*	(10)
E. coli DH5α	recA1, ϕ80 *lacZ*ΔM15, host for cloning	(11)
Plasmids		
pAT03	pMMB67EH with FLP recombinase, Tet^R	(12)
pAT04	pMMB67EH with REC_{Ab} system, Tet^R	(12)
pKD4	Kan^R	(13)

pMMB67EHKn	pMMB67EH with the Kan^R gene from pKD4 inserted into the PvuI site, Kan^R	(14)
pROB01	pMMB67EHKn carrying *olsB* (*A1S_0889*)	This study
pROB02	pMMB67EHKn carrying *phoR* (*A1S_3376*)	This study

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145
146 **Table S5: Primers used in this study.**

Oligo Name	**Sequence (5' to 3')**
Deletion Primers	
17978 *olsB*Ab Kan FRT 5'	AGTGGGTGTCACTAGGAGCGTTCATTATGCTGGAAAAA TTTAATCAATATCGCCAAACCTGGACTTTACCTTTAAAT CGCCATAAGGCTAACAATCAAACACAATTCCGCTTTGA ATGGGTTGATAGCGATTGTGTAGGCTGGAGCTGCTTCG
17978 *olsB*Ab Kan FRT 3'	TTGTTCATTACAAACGAAGTGGCAATTTTATTCACTTCT AAAAATACAAAGTAATCGAGACAGTTAAATTCAGCATC AAAGAAAGCATCTTTAGATAATTTAGACTGCATACTCA AATACATTTGATATCCTCCTTAGTTCCTATTCCG
17978 *olsB*Ab confirm 5'	GTGGTGTTGTGAGCGCACATATTG
17978 *olsB*Ab confirm 3'	GCCTTGAGTCGCCTTACGAATATG
17978 *phoR*Ab Kan FRT 5'	CGTTTGCTAAACAAGATTTACGACTTTTATTATTTTTCCT GATTATTGCAGGTTTAGTCGGTTTAGGAATTGGGTATTT CTGGAGCTGTATTTTTATTGCCTTTGTGGTGTTTTTTACA CTTCAGAGCGATTGTGTAGGCTGGAGCTGCTTCG
17978 *phoR*Ab Kan FRT 3'	ATGTTATAGAGTCTTTCTTTTGGAAAAACTGCGGTAAAG GTTGATCCTTCATTTTCTTTAGATTGCACATCTAAGTAG GCGCCGTGTTGCATGAGTACATGTTTTACAATCGCCAAG CCTAAACCATATCCTCCTTAGTTCCTATTCCG
17978 *phoR*Ab 5' confirm	GGACCAACAGAATACCGTCTGCTTG
17978 *phoR*Ab 3' confirm	GATGGTGGAGATCATCGTGATGCAC
Complementation Primers	
17978 *olsB*Ab KpnI 5'	CGCGGTACCATGCTGGAAAAATTTAATCAATATCGC
17978 *olsB*Ab SalI 3'	CGCGTCGACTTATCGCTGAGCCATTTTGTTC
17978 *phoR*Ab SacI 5'	CGCGAGCTCATGTATGAACCCTACCCCGTCC
17978 *phoR*Ab BamHI 3'	CGCGGATCCTTAAGTCATGTTATAGAGTCTTTCTTTTGG
Transposon mutant primers	
AB5075 *olsB::tn101* 5'	CGACACAAGAAGTTGACCGTTTAATCG

confirm	
AB5075 *olsB::tn101* 3' confirm	CAATTGGTGATTCAACTGCAGACTGG
AB5075 *olsA*::*tn101* 5' confirm	GCTCAAGAATATCTGCACCAGCTGAC
AB5075 *olsA::tn101* 3' confirm	GGATGAGCTGGCAACTAAAGCG
AB5075 *phoR::tn26* 5' confirm	GTCTGGATGCTGGTGCAGATGAC
AB5075 *phoR::tn26* 3' confirm	GGTGCCGACTGTACCGTTAATG

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