Supporting Information

Willis et al. 10.1073/pnas.1222317110

SI Results

Liquid chromatography (LC)-MS analyses were performed three times with independent batches of *Escherichia coli* K1 capsular polysaccharide (CPS). The number of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residues was consistent, but one batch contained both monoacyl- and diacyl-phosphatidylglycerol (PG) (Fig. S4). As phospholipids in *E. coli* are made with two acyl chains, the possibility that the lyso derivatives might result from the action of phospholipase A (PLA) was considered. *E. coli* has two known PLAs, a detergent-sensitive cytoplasmic PLA, which acts preferentially on PG, and a detergent-resistant outer-membrane PLA (OMPLA) (1). Although the gene for the cytoplasmic form has not been identified, OMPLA has been well-characterized. The OMPLA gene (Δ*pldA*) was deleted in *E. coli* K1. Initially, only CPS-deficient mutants were obtained, but their transformation with a plasmid carrying *pldA* did not restore surface capsule assembly (Fig. S10), suggesting a second-site mutation. The nature of the second-site mutation(s) was not pursued. Instead, the *pldA* mutant was constructed in a Δ*kpsS* background, where CPS is synthesized but not exported to the cell surface (2). Introduction of a plasmid carrying *kpsS* into the Δ*kpsS* Δ*pldA* strain restored surface expression of CPS (Fig. S10), indicating that PldA is not essential for CPS assembly. The LC trace of the lipid terminus in this mutant contained no material at the elution time of the diacyl species seen in the K1 parent, and MS of the material from 22 to 25 min revealed only monoacylated PG, indicating that OMPLA is not responsible for forming the lyso-phosphatidylglycerol (lyso-PG) moiety (Fig. S10). The cytoplasmic PLA remains a candidate, but we are unable to test this hypothesis without the corresponding gene being identified.

SI Materials and Methods

Construction of Δ*kpsC*, Δ*kpsS*, and Δ*pldA* Mutants. These mutants were made in the *E. coli* EV36 (K1) background using the λ-red recombinase procedure (3, 4). Cells were transformed with the helper plasmid pSIM6. PCR products were made containing homologous ends and kanamycin resistance based on the pKD4 plasmid. Primers used were as follows: for Δ*kpsC*, 5′-CCCCAGCATGCCTGGAAAAAGTGCGCGCCCTGATGGCACAGGAACTGGCTGAAAACGCATAAGTGTAGGCTGGAGCTGCTTC-3′ and 5′-GTGCATTACCTTGCATAAATAATTTGCGTAATAGTCAACTATGAAATTGTCATATGAATATCCTCCTTAG-3′; for Δ*kpsS*, 5′-GGTCAGATTTGGCTAAACAATTTCATAGTTGACTATTACGCAAATTATTTATAAGTGTAGGCTGGAGCTGCTTC-3′ and 5′-TTATGTTACTTTTCTTTGAAGAGGATGGAAATGATTTTTTGGCTACTTAAAATTCAAAAGATATTGACTTGAAATCATATGAATATCCTCCTTAG-3′; and for Δ*pldA*, 5′-ATGCGGACTCTGCAGGGCTGGTTGTTGCCGGTGTTTATGTTGCCTATGGCAGTGTAGGCTGGAGCTGCTTC-3′ and 5′-TCAAAACAGGTCGTTTAGCATAACTCCCACACCAACGCGGGTCTGGTTGCATATGAATATCCTCCTTAG-3′. Cells containing pSIM6 were transformed with PCR products, and mutants were selected for on kanamycin. For Δ*kpsC* and Δ*kpsS*, the kanamycin resistance cassette was subsequently removed using pKD46. For Δ*pldA*, no complicating polarity issues were anticipated, so the cassette was left in place.

Purification of CPS. *E. coli* cells from 1 L of culture were resuspended in 300 mL 4 mM NH_4OAc and lysed using an EmulsiFlex-C5 (Avestin). Hexadecyltrimethylammonium bromide (Cetavlon) was added to a final concentration of 1% to precipitate the CPS, and the mixture was incubated at room temperature for 20 min. Insoluble material was collected by centrifugation at 11,000 × *g* for 20 min at 15 °C and then resuspended in 50 mL dH_2O. An equal volume of 2 M $CaCl_2$ was added to dissociate the CPS–Cetavlon complexes and the mixture was stirred for 1 h. Ethanol was added to a final concentration of 25% and the mixture was stirred for 1 h to precipitate nucleic acids before the debris was removed by centrifugation at 25,000 × *g* for 20 min. The supernatant was brought up to 80% ethanol, and the CPS was collected by centrifugation at 2,000 × *g* for 10 min. The CPS pellet was washed three times with ethanol and twice with acetone, centrifuging at 13,000 × *g* for 10 min between washes. After the final resuspension in acetone, the solvent was removed using a rotary evaporator. The dried material was resuspended in 20 mM Tris·HCl (pH 8) containing 2 mM $MgCl_2$, 10 μg/mL DNase I, and 10 μg/mL RNase A and incubated at 37 °C for 2 h. Proteinase K was then added to 20 μg/mL and the mixture was incubated for another 2 h. Following enzyme digestion, an equal volume of Tris-saturated phenol was added and the mixture was centrifuged at 300 × *g* for 5 min. The upper aqueous phase was removed and dialyzed for 48 h against 4 mM NH_4OAc using a Spectra/Por 3500 molecular weight cutoff membrane to remove residual phenol, and then lyophilized. To remove any remaining contaminants, the CPS was subjected to gel-filtration chromatography on a 120-mL Sephacryl S-200 column (GE Healthcare) eluted in 50 mM ammonium formate. Absorbance was monitored at 230 nm and the void volume was collected and lyophilized.

For *Neisseria meningitidis*, the CPS was purified by resuspending cells from 20 plates in 300 mL 4 mM NH_4OAc containing 1% Cetavlon and incubating overnight with agitation, instead of lysing with the EmulsiFlex-C5.

Phylogenetic Analysis. Sequences were obtained from the National Center for Biotechnology Information. Multiple sequence alignments and trees were generated using bioinformatics tools available through the European Bioinformatics Institute (EBI) (www.ebi.ac.uk).

1. Raetz CRH (1978) Enzymology, genetics, and regulation of membrane phospholipid synthesis in *Escherichia coli*. *Microbiol Rev* 42(3):614–659.
2. Cieslewicz M, Vimr E (1996) Thermoregulation of kpsF, the first region 1 gene in the kps locus for polysialic acid biosynthesis in *Escherichia coli* K1. *J Bacteriol* 178(11): 3212–3220.
3. Datta S, Costantino N, Court DL (2006) A set of recombineering plasmids for Gram-negative bacteria. *Gene* 379:109–115.
4. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.

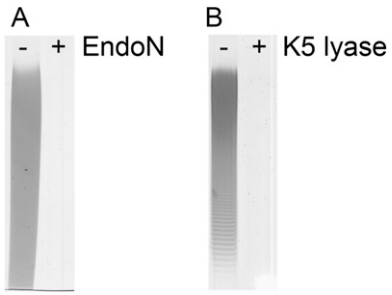

Fig. S1. Alcian blue/silver-stained Tris-borate-EDTA buffer (TBE) gel of the K1 endoneuraminidase (EndoN) (*A*) and K5 lyase (*B*) reactions. The – and + signs refer to without and with enzyme, respectively.

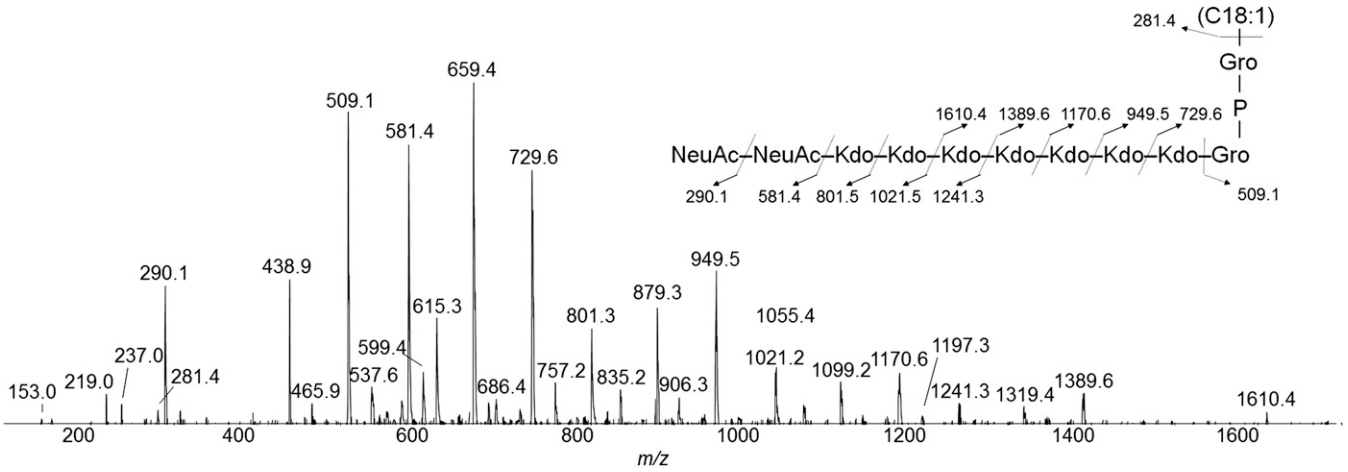

Fig. S2. LC-MS/MS of the *m/z* 877.5 ion from *E. coli* K1 polysialic acid (PSA) CPS (spectrum in Fig. 1*A*). Glycerol and phosphate are designated Gro and P, respectively.

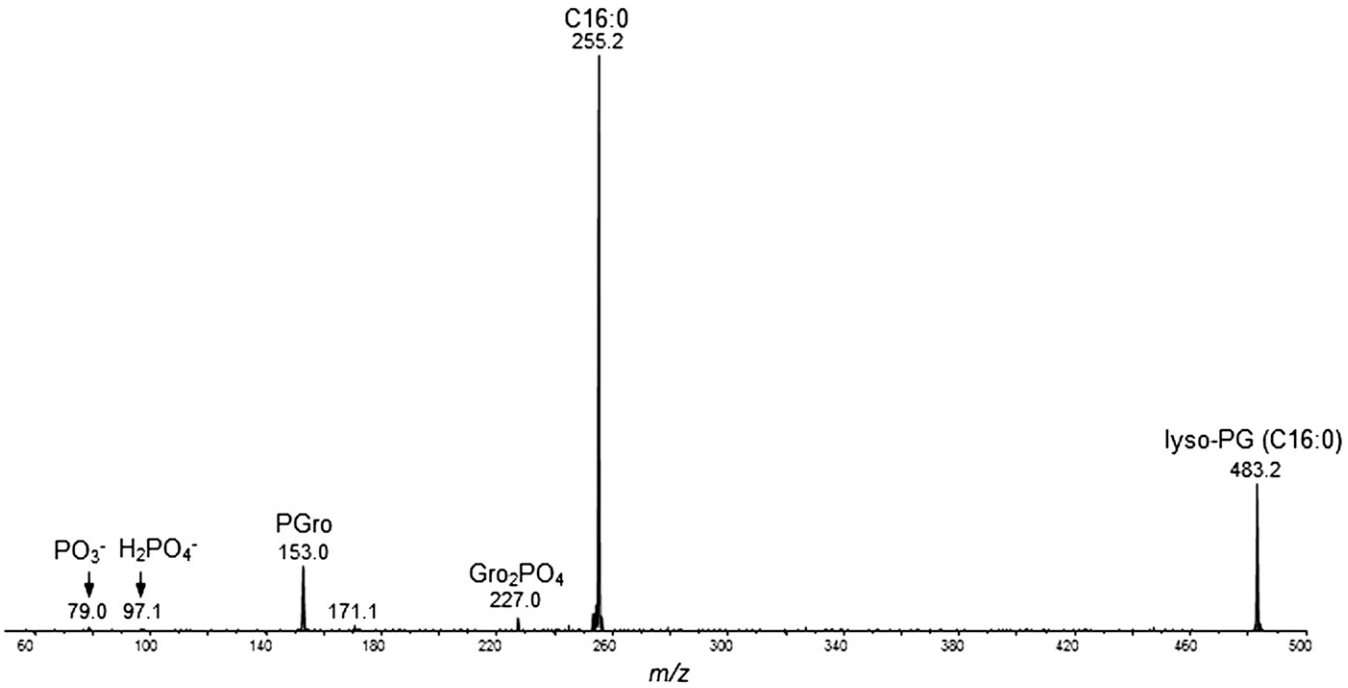

Fig. S3. LC-MS/MS/MS of the *m/z* 483 ion from *E. coli* K1 PSA CPS (spectrum in Fig. 1*B*). Lyso-phosphatidylglycerol is designated lyso-PG, with the acyl chain in parentheses.

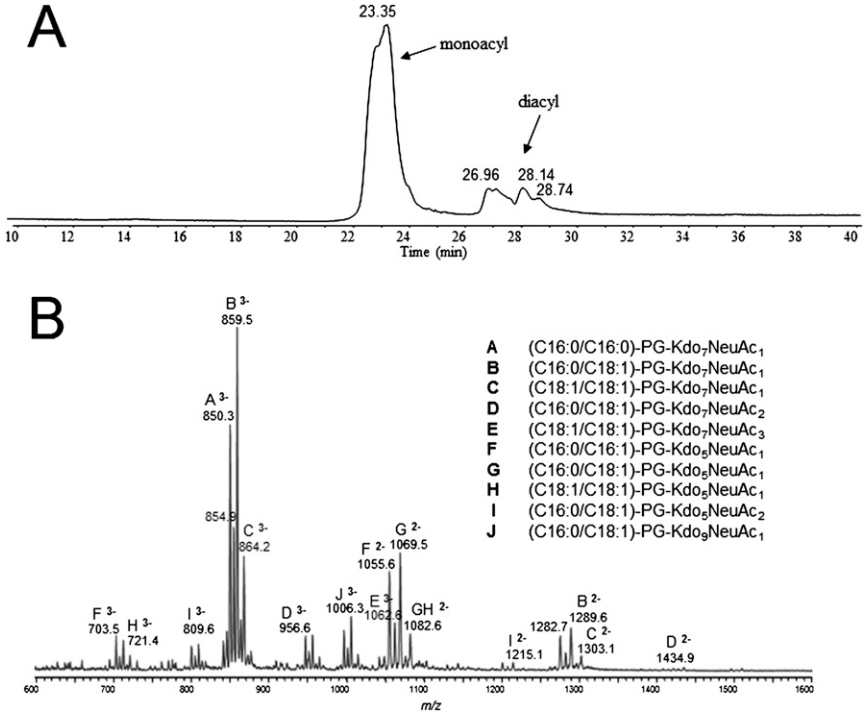

Fig. S4. (*A*) LC trace of the *E. coli* K1 PSA CPS from the only batch of CPS to contain both monoacyl and diacyl species. (*B*) LC-MS of 26–30 min (diacylated species). LC-MS of 22–25 min (monoacylated) is the same as in Fig. 1*A*. The composition of the acyl chain is in parentheses and the number of Kdo and α-(2→8)-linked sialic acid (NeuAc) residues in each ion is indicated. The charge of the ion is shown next to the letter identifying it.

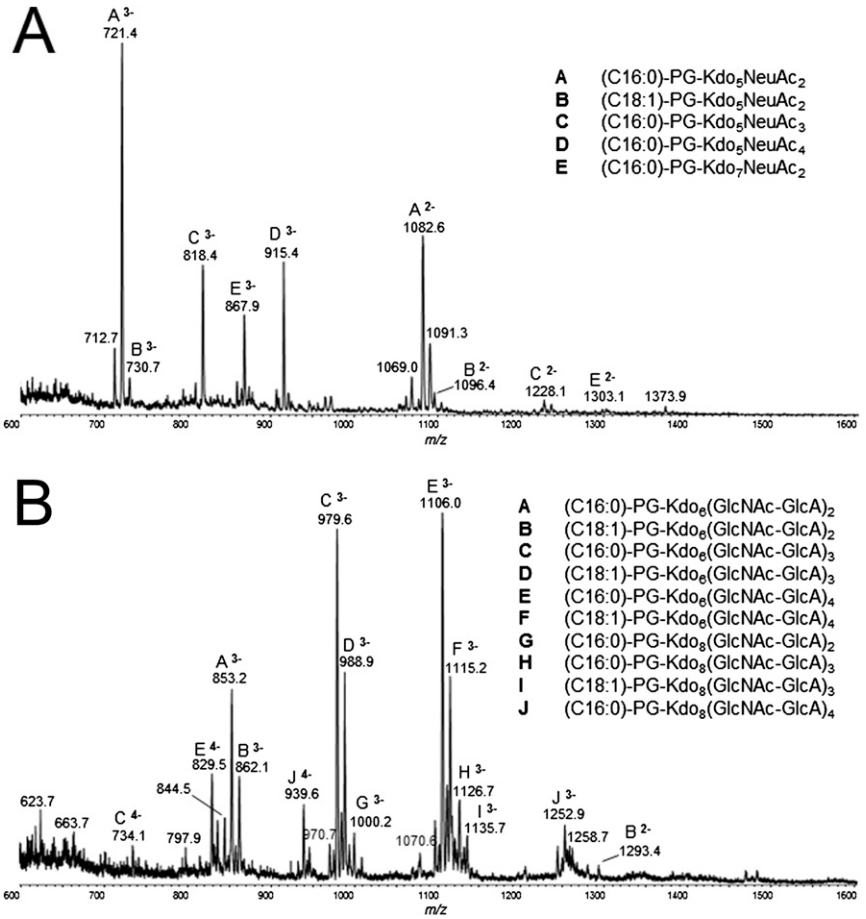

Fig. S5. LC-MS of the *N. meningitidis* 992B glycolipid (*A*) and *E. coli* K5 glycolipid (*B*). The composition of the acyl chain is in parentheses and the number of Kdo and NeuAc residues in each ion is indicated. The charge of the ion is shown next to the letter identifying it.

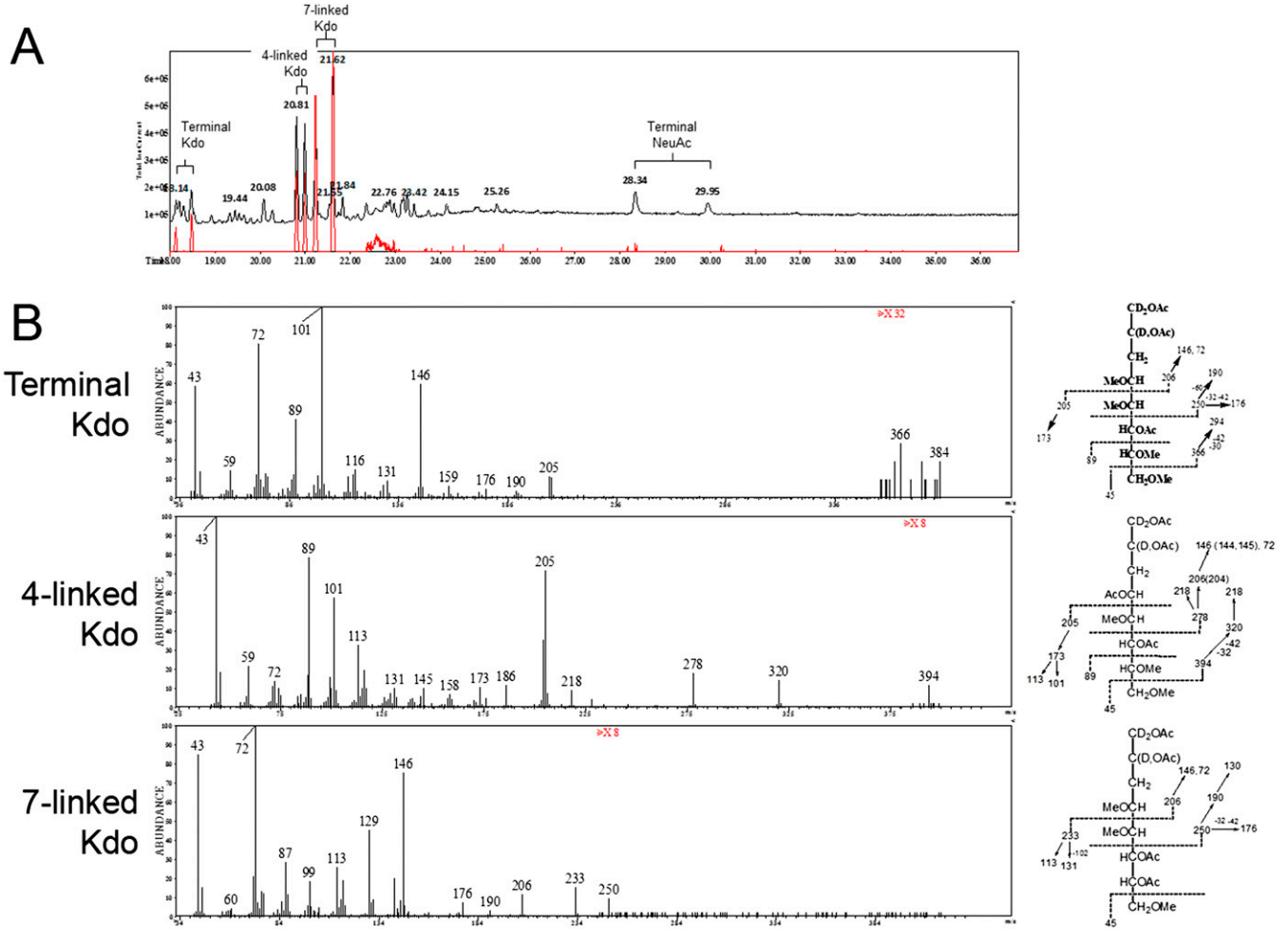

Fig. S6. Methylation analysis of the *E. coli* K1 poly-Kdo linker. (*A*) GC-MS spectrum of permethylated alditol acetate derivatives. The black line shows total ions, and the red line shows selected ion extractions for Kdo derivatives (*m/z* 206). (*B*) Electron-impact MS of terminal, 4-linked, and 7-linked Kdo species showing the fragment ions.

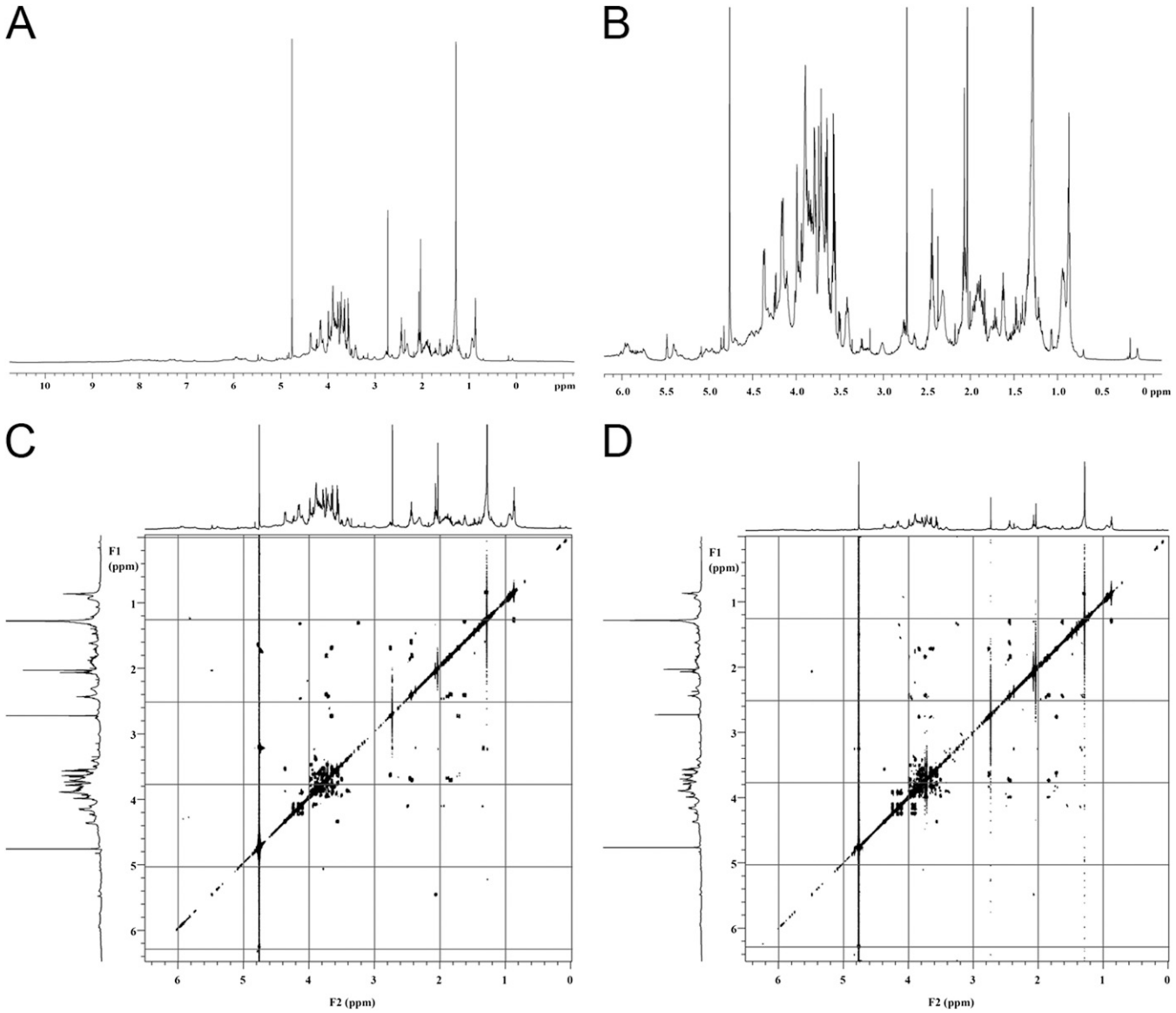

Fig. S7. NMR spectra of the *E. coli* K1 CPS terminus in D_2O at 700 MHz. ^1H NMR spectrum (*A*), expanded ^1H NMR spectrum (*B*), ^1H-^1H gradient correlation spectroscopy (gCOSY) spectrum (*C*), and ^1H-^1H gradient total correlation spectroscopy (gTOCSY) spectrum (*D*) of the CPS terminus.

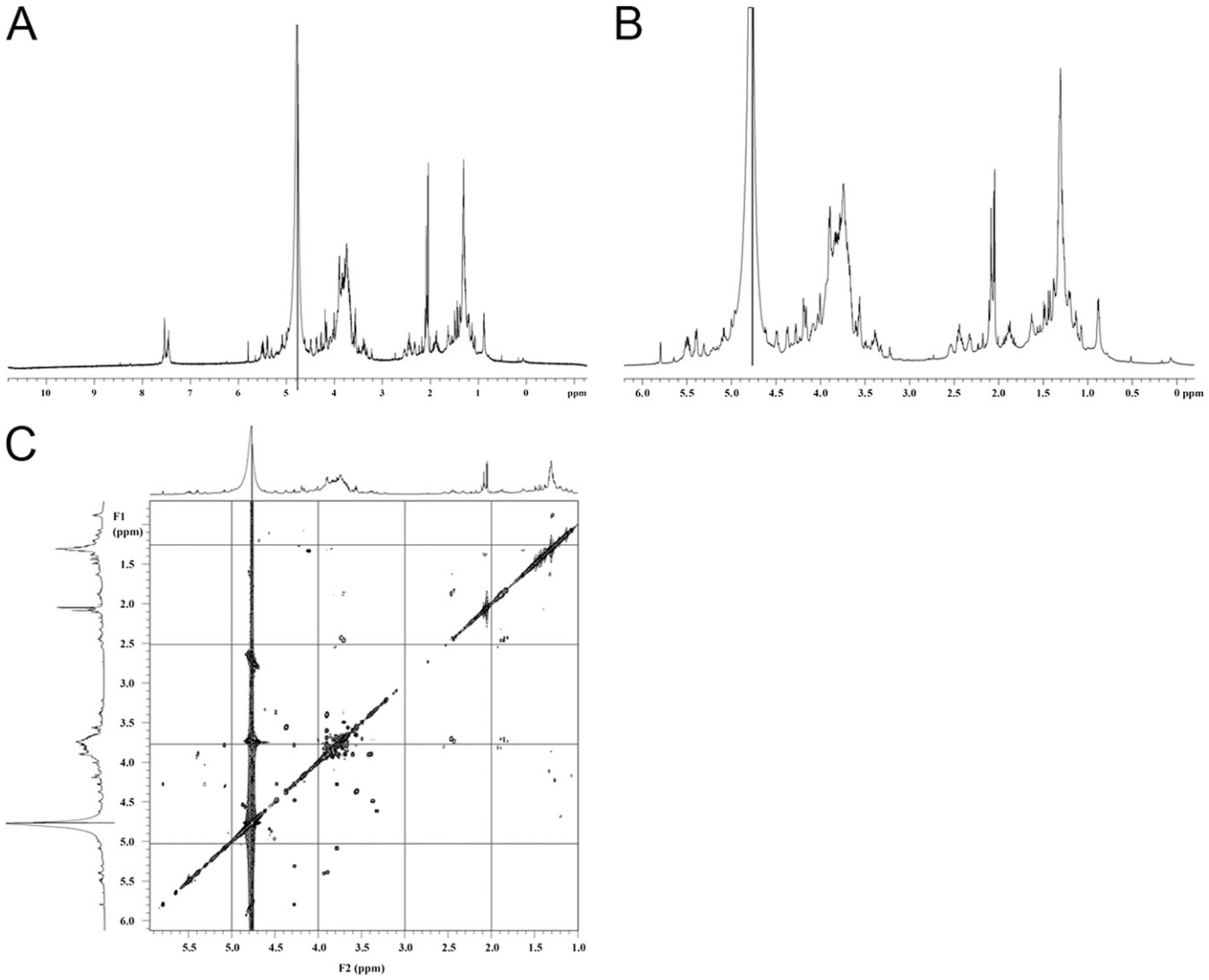

Fig. S8. NMR spectra of the *E. coli* K5 CPS terminus in D_2O at 700 MHz. ^1H NMR spectrum (*A*), expanded ^1H NMR spectrum (*B*), and ^1H-^1H gCOSY spectrum (*C*) of the CPS terminus.

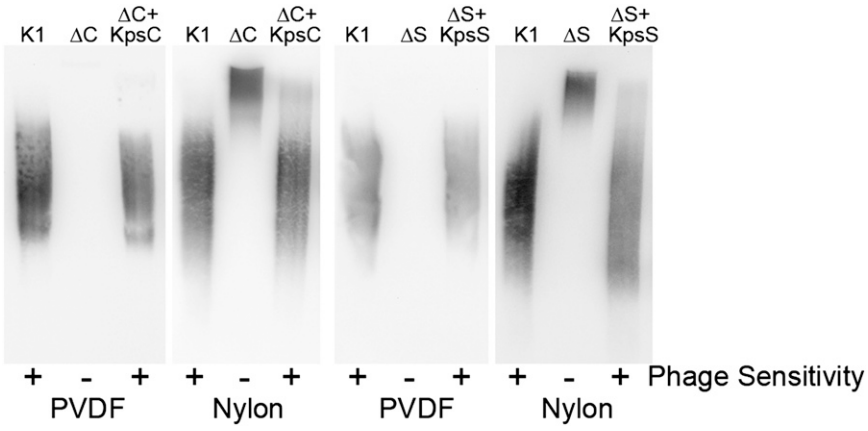

Fig. S9. Complementation of the *E. coli* K1 Δ*kpsC* (ΔC) and Δ*kpsS* (ΔS) mutants. Cloned genes were expressed from an arabinose-inducible pBAD24 vector. Immunoblots are of whole-cell lysates on either PVDF or positively charged nylon membranes. Phage sensitivity indicates whether the CPS is on the surface of the cells.

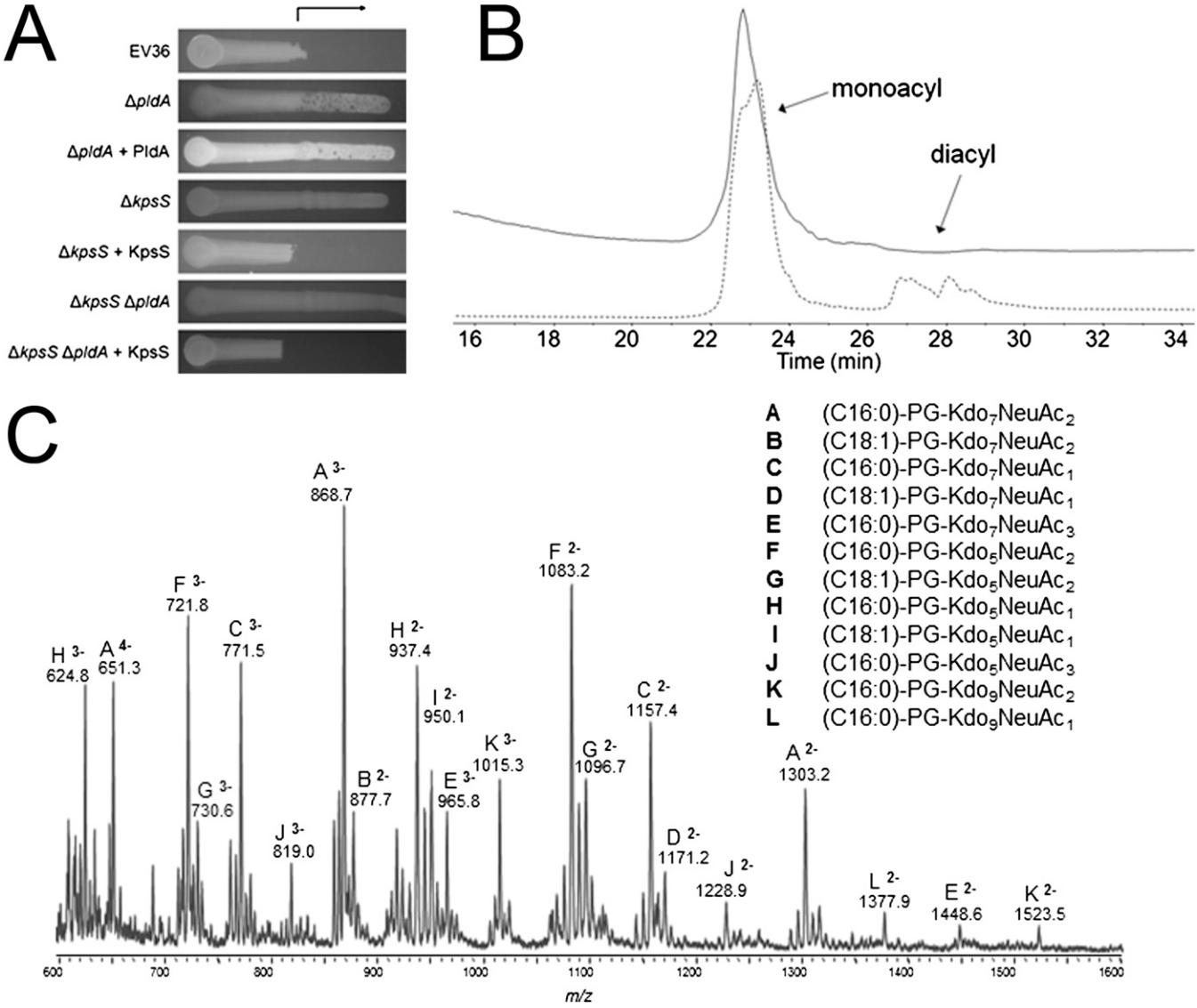

Fig. S10. OMPLA is not responsible for generating the lyso-PG moiety. (*A*) Diagram showing sensitivities of *E. coli* K1 and the Δ*kpsS* and Δ*pldA* mutants to bacteriophage K1F. Overnight cultures were spotted on plates that had PSA-specific K1F phage streaked over half the plate. The plates were tipped to allow the culture to flow into the phage and then were incubated until the streaks appeared. Cells that are positive for extracellular PSA do not grow on the side of the plate containing phage. The bent arrow denotes the region of the petri plate containing phage. (*B*) Overlay of the LC traces showing that CPS from the Δ*pldA* mutant is monoacylated. The K1 LC trace from the only batch of CPS that contained diacyl-PG in addition to monoacyl-PG is shown as a dotted line and the LC trace for the complemented Δ*kpsS* Δ*pldA* mutant is shown as a solid line. (*C*) LC-MS of the CPS terminus from the *kpsS*-complemented Δ*pldA* Δ*kpsS* mutant. MS was performed on the material that eluted at 22–25 min. The composition of the acyl chain is identified in parentheses and the number of Kdo and NeuAc residues in each ion is indicated. The charge of the ion is shown next to the letter identifying it.