Supplemental Information

Molecular characterization and verification of azido 3,8-dideoxy-D-manno-oct-2-ulosonic acid incorporation into lipopolysaccharide

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Supplemental Methods

Chemicals and Materials

All chemicals were purchased from Sigma-Aldrich. Reactions were monitored by thin-layer chromatography (TLC; Silica Gel 60, F254, Merck EMD Millipore). The pH of the reaction mixture was monitored using a pH meter (Accumet basic AB15 pH meter with Accumet electrode 13-620-183A). TLC spots were made visible by dipping the TLC plates into a 10% ethanolic H_2SO_4 solution and charring with a heat gun for 3 min. Lyophilization was performed on a VirTis Sentry 2.0 Freezemobile system. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian 400-MR NMR instrument (400 MHz).

Synthesis of Kdo-N_3

5-azido-5-deoxy-D-arabinofuranose (Ara-N_3) was synthesized using the method described by Smellie et al.(1) Ammonium 8-azido-3,8-dideoxy-D-*manno*-octulosonate (Kdo-N_3) was synthesized with the procedure reported by Mikula et al. for the multi-gram scale synthesis of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) from D-arabinose.(2) It is noteworthy that the pH must be carefully monitored by a pH meter during the reaction. Furthermore, the preparation of the bicarbonate ion resin for the ion exchange chromatography must be performed precisely as described below before every usage.

10 M aqueous NaOH was added to a solution of $NaHCO_3$ (0.036 g, 0.43 mmol) in water (15 mL) at 0 °C until pH 10 was reached. To the reaction mixture, a suspension of oxaloacetic acid (1.13 g, 8.56 mmol) in water (5 mL) was simultaneously added with 10 M aqueous NaOH dropwise to keep the pH around 10 (pH 9-11) over a period of 30 min. A solution of Ara-N_3 (3.30 g, 18.82 mmol) in water (5 mL) was then added to the reaction mixture. The pH was adjusted to 11 by further addition of 10 M aqueous NaOH. The reaction mixture was stirred for 2 h at rt. During that time, the pH was checked occasionally and adjusted to pH 11 with 10 M NaOH if necessary. After 2 h, $NiCl_2 \cdot 6\ H_2O$ (16 mg, 0.068 mmol) in water (0.5 mL) was added to the solution. The reaction mixture was heated to 50 °C, and Amberlite IR120 (Sigma-Aldrich, H^+, strongly acidic) was added in portions until the pH reached 5.7 (gas evolution was visible). Stirring was continued for 1 h at 50 °C. Additional resin was added occasionally to keep the pH at 5.7. The reaction was deemed completed when the pH was stable at 5.7 for about 10 min.

The reaction mixture was filtered, and the yellow filtrate was applied onto an anion exchange column. Dowex 1X4 (Sigma-Aldrich, chloride form, 100-200 mesh) resin was converted freshly from chloride to bicarbonate form: the column was packed with resin (25 mL volume), washed with 13 column volumes of 0.63 M sodium bicarbonate solution (50 g/L) and then washed with 5 column volumes of deionized water. The pH of the eluent was checked by pH paper and was neutral after washing. After

applying the reaction mixture to the column, the column was washed with water (200 mL) and let sit overnight. On the following day, the column was eluted with aqueous $NaHCO_3$ (300 mL, gradient 0 M to 0.2 M). 10 mL fractions were collected and analyzed by TLC (MeOH-chloroform-water 10:10:3, R_f 0.42). Fractions containing Kdo-N_3 were combined and lyophilized for 24-48 h. Yield: 2.42 g (46%), white powder.

NMR analysis of Kdo-N_3 was complicated due to the several isomers (major isomers: α-pyranose ~55%, α-furanose ~20%, β-furanose ~20%, β-pyranose ~5%), but ^{1}H NMR and ^{13}C NMR data obtained were consistent with the NMR data published by Dumont et al.(3) and Winzar et al.(4) We further characterized Kdo-N_3 by LC-MS. The separation was performed on an Acquity UPLC BEH C_{18} column (gradient 5% B to 95% B, A: water with 0.1% formic acid, B: acetonitrile with 0.1% formic acid). ESI-MS data were recorded using a Synapt G2 HDMS (TOF mass spectrometer, Waters) in the negative ion mode. The resolution of the MS system was approx. 12000. $[M-H]^-$ peak: 100% observed 262.0500 *m*/*z* (calculated 262.0675 *m*/*z*), $[2M-H]^-$ peak: 10% obs. 525.1272 *m*/*z* (calc. 525.1277 *m*/*z*) and $[2M-2H+Na]^-$ peak: 5% obs. 547.1047 *m*/*z* (calc. 547.1096 *m*/*z*).

Fluorescence microscopy

Overnight cultures from *E. coli* K-12 (BW25113) and *E. coli* mCherry (TUT0018) were each inoculated to an OD_{600} of 0.05 into two different tubes and incubated at 37 °C at 225 rpm for 16 h. For each strain, one tube contained 1 mL fresh M9 medium lacking Kdo-N_3 and the other tube contained 1 mL fresh M9 medium with 5 mM Kdo-N_3 (Kdo-N_3 stock concentration 90 mM in water, sterile filtered). After 16 h incubation, two aliquots of 250 µL for each sample were transferred into 1.7-mL microcentrifuge tubes, and the cells were pelleted at 11,600 ×*g* for 2 min and washed three times with fresh M9 medium (200 µL). For the copper-free click reaction, the cells were resuspended in 20 µL fresh M9 medium and Click-IT AlexaFluor488-DIBO Alkyne (ThermoFisher, Alexa488-DIBO, 50 mM stock concentration in DMSO, Figure S1A) was added to a final concentration of 0.25 mM to one aliquot per strain (+/- Kdo-N_3). To the other aliquot (+/- Kdo-N_3), an equal amount of DMSO was added. All samples were shaken at 225 rpm at 37 °C for 1 h in the dark. After the click reaction, the cells were pelleted at 11,600 ×*g* for 2 min and washed three times with fresh M9 medium (200 µL) to remove excess click reagent. After the final wash, the samples were diluted in 250 µL of fresh M9 medium, and 3 µL of samples were deposited onto an agar pad (freshly prepared with 100 µL of 1.2% aqueous agarose). The cells were imaged using a Nikon Eclipse Ti inverted microscope with a Nikon halogen illuminator (D-LH/LC), a Sola light engine from Lumencor, and a Clara Interline CCD camera from Andor. A Nikon CFI Plan Apo Lambda DM ×100 Oil objective lens (1.45 NA) was used for phase contrast and fluorescent imaging. GFP images were taken by using the FITC-5050A-NTE-ZERO filter set (Semrock). mCherry images were taken by using the

TRITC-B-NTE-NEZO filter set (Semrock). Images were captured by using Nikon Elements software, and exported for figure preparation in ImageJ.(5)

Flow cytometry analysis of fluorescently-labeled E. coli

Kdo-N_3 dose-dependence for labeling *E. coli* K-12

An overnight culture of *E. coli* K-12 was inoculated to an OD_{600} of 0.002 into 6 × 1 mL LB medium supplemented with different concentrations of Kdo-N_3 (0 mM, 1 μM, 10 μM, 100 μM, 1 mM, 10 mM) and grown for 16 h at 37 °C at 225 rpm. Then, 250 μL per sample (2 × 250 μL for LB with 0 mM Kdo-N_3) were transferred into 1.7-mL microcentrifuge tubes, and the cells were pelleted at 11,600 ×*g* for 2 min and washed three times with fresh M9 medium (200 μL). For the copper-free click reaction, the cells were resuspended in 20 μL of M9 medium, and sulfo-dibenzocyclooctyne-biotin (Sigma-Aldrich, Sulfo-DBCO-biotin, stock 0.3 M in DMSO, Figure S1B) was added to a final concentration of 0.25 mM. All samples were shaken at 225 rpm at 37 °C for 2 h. After the click reaction, the cells were pelleted at 11,600 ×*g* for 2 min and washed three times with M9 medium (200 μL) to remove any excess click reagent. After the final wash, the samples were diluted in 50 μL M9 medium, and 0.5 μL mouse anti-biotin AlexaFluor488 antibody conjugate (AlexaFluor488 IgG fraction monoclonal mouse anti-biotin, Jackson ImmunoResearch, catalog 200-542-211, lot 101654, stock solution 2 mg/mL in M9 medium) was added to all samples, except for one control sample grown without Kdo-N_3. The samples were left in the dark for 30 min at rt and then pelleted at 11,600 ×*g* for 2 min, washed one time with M9 medium (200 μL) and resuspended in 250 μL M9 medium. 10× dilutions in M9 medium of all samples were aliquoted into a 96-well plate and analyzed on a FACS Canto-II (BD Biosciences), using the FITC channel to read AlexaFluor488 with excitation wavelength 488 nm and emission wavelength 530 nm.

Kdo-N_3 incorporation time-course for labeling *E. coli* K-12

An overnight culture from *E. coli* K-12 (BW25113) was inoculated to an OD_{600} of 0.05 into 2 × 2 mL fresh LB and grown at 37 °C at 225 rpm. One tube lacked Kdo-N_3, and the other tube contained 1 mM Kdo-N_3 (Kdo-N_3 stock concentration 90 mM in water, sterile filtered). After 30 min, 1 h, 2 h, 4 h, 6 h, and 19 h, aliquots of 200 μL per sample (for 0 h to 24 h, with Kdo-N_3) or 2 aliquots of 200 μL per sample (for 24 h, without Kdo-N_3) were transferred into 1.7-mL microcentrifuge tubes, and the cells were pelleted at 11,600 ×*g* for 2 min and washed three times with fresh M9 medium (200 μL). For the copper-free click reaction, cells were resuspended in 20 μL fresh M9 medium and Alexa488-DIBO (50 mM stock concentration in DMSO) was added to a final concentration of 0.25 mM. To one control sample (grown without Kdo-N_3), an equal amount of DMSO was added. All samples were shaken at 225 rpm at 37 °C for 1 h in the dark. After the click reaction, cells were harvested by pelleting at 11,600 ×*g* for 2 min and washed 3 times with fresh M9 medium (200 μL) to remove excess click reagent. After the final wash, the

samples were diluted in 250 µL of fresh M9 medium. 10× dilutions in M9 medium of all samples were deposited into a 96-well plate and analyzed on the FACS Canto-II as described above.

Alexa488-DIBO reaction time-course for labeling *E. coli* K-12

An overnight culture from *E. coli* K-12 (BW25113) was inoculated to an $OD_{600\ of}$ 0.002 into 2 × 2 mL fresh LB and grown at 37 °C at 225 rpm for 16 h. One tube lacked Kdo-N_3 and the other tube contained 1 mM Kdo-N_3 (Kdo-N_3 stock concentration 90 mM in water, sterile filtered). Then 2 × 200 µL per sample (without Kdo-N_3) and 5 × 200 µL (with Kdo-N_3) were transferred into 1.7-mL microcentrifuge tubes, and the cells were pelleted at 11,600 ×*g* for 2 min and washed three times with fresh M9 medium (200 µL). For the copper-free click reaction, the cells were resuspended in 20 µL fresh M9 medium, and Alexa488-DIBO (50 mM stock concentration in DMSO) was added to a final concentration of 0.25 mM. To one control sample (grown without Kdo-N_3) an equal amount of DMSO was added. All samples were shaken at 225 rpm at 37 °C in the dark. After 15 min, 30 min, 1 h, 2 h and 3 h the cells were harvested by pelleting at 11,600 ×*g* for 2 min and washed three times with fresh M9 medium (200 µL) to remove excess of click reagent. The samples grown without Kdo-N_3 were harvested after 3 h, pelleted and washed three times with fresh M9 medium. After the final wash, the samples were diluted in 250 µL fresh M9 medium. 10× dilutions in M9 medium of all samples were aliquoted into a 96-well plate and analyzed on the FACS Canto-II as described above.

SDS-PAGE of E. coli K-12, ΔwaaC E. coli and ClearColi whole cell samples

Novex 16% Tricine SDS gels showed the best separation for faster migrating LPS species that were abundant in rough and deep-rough *E. coli*. In other SDS gels, the faster-migrating LPS species were not readily separated. It is important to note that the AlexaFluor488 label could also be seen under the UV light used to image total LPS. This provided an in-gel confirmation of the co-migration of AlexaFluor488 Alkyne-labeled LPS and unlabeled LPS.

Overnight cultures of *E. coli* K-12 (BW25113), Δ*waaC E. coli* (JW3596-1) and ClearColi K-12 were each inoculated to an OD_{600} of 0.05 into 3 × 1 mL LB and grown at 225 rpm to an OD_{600} ~1.2-1.5 (3-4 h for *E. coli* K-12 and 6-8 h for ClearColi). For *E. coli* K-12 and Δ*waaC E. coli* one tube lacked Kdo-N_3, the second tube contained 1 mM Kdo-N_3, and the third tube contained 5 mM Kdo-N_3. For ClearColi K-12 one tube lacked Kdo and Kdo-N_3, the second tube contained 5 mM Kdo, and the third tube contained 5 mM Kdo-N_3. To harvest the cells, 2 × 250 µL per sample were transferred into 1.7-mL microcentrifuge tubes, and the cells were pelleted at 11,600 ×*g* for 2 min and washed three times with fresh M9 medium (200 µL). For the copper-free click reaction, one aliquot of cells was resuspended in 20 µL of fresh M9 medium containing Alexa488-DIBO (ThermoFisher, 50 mM stock concentration in DMSO) at a final concentration of 0.25 mM, and the other aliquot was resuspended in 20 µL of fresh M9

medium containing the same amount of DMSO as a control. All samples were shaken at 225 rpm at 37 °C for 1 h. Beginning from the click reaction, all samples and SDS-PAGE gels were covered with aluminum foil to protect the fluorescent samples from light. After the click reaction, the cells were pelleted at 11,600 ×*g* for 2 min and washed three times with fresh M9 medium (200 μL) to remove any excess click reagent. After the final wash, the cells were resuspended in Tricine SDS sample buffer (BioRad, containing 4% β-mercaptoethanol [Sigma Aldrich]) and normalized to OD_{600} such that 50 μL of the resuspended samples contained the equivalent of 1 mL of cells at an OD_{600} of 0.5. The samples were heated at 100 °C for 10 min at 500 rpm. The SDS-PAGE gel (Novex 16% Tricine protein gel, ThermoFisher) was prepared with Tricine SDS buffer (Novex) and run for 1.5 h at 80 V. In addition to the samples, two controls were included: the Alexa488-DIBO in one lane and in a second lane LPS from *E. coli* Serotype 055:B5 conjugated to AlexaFluor488 (ThermoFisher) resuspended in Tricine sample buffer. Before fixing, the LPS gel was imaged on a Typhoon 9400 imager (GE Healthcare) using the FITC channel to read AlexaFluor488 with excitation wavelength 488 nm and emission wavelength 528 nm to visualize the AlexaFluor488 labeled LPS. Fixing and staining of the LPS were performed with the Pro-Q Emerald 300 LPS staining kit (ThermoFisher) according to the manufacturer's instructions.(25) For Δ*waaC E. coli* and ClearColi samples, the green fluorescence imaging was performed after fixing the gel with methanolic acetic acid and washing with 3% aqueous acetic acid overnight (as described in the manufacturer's manual) to remove excess Alexa488-DIBO. The Pro-Q Emerald 300 stain was visualized under UV light in a ChemiDoc XRS+ imager (BioRad, Qdots 525, UV translumination excitation, 525 nm monitored) with Image Lab 3.0 software. For protein staining, the gel was incubated for 1 h with CoomassieBlue (ThermoFisher) while gently agitating. After washing with water, the gel was imaged on the ChemiDoc imager. The Alexa488, ProQ Emerald 300 LPS-stained and CoomassieBlue-stained images of the gels are displayed in Figure S3.

Production of KdsB

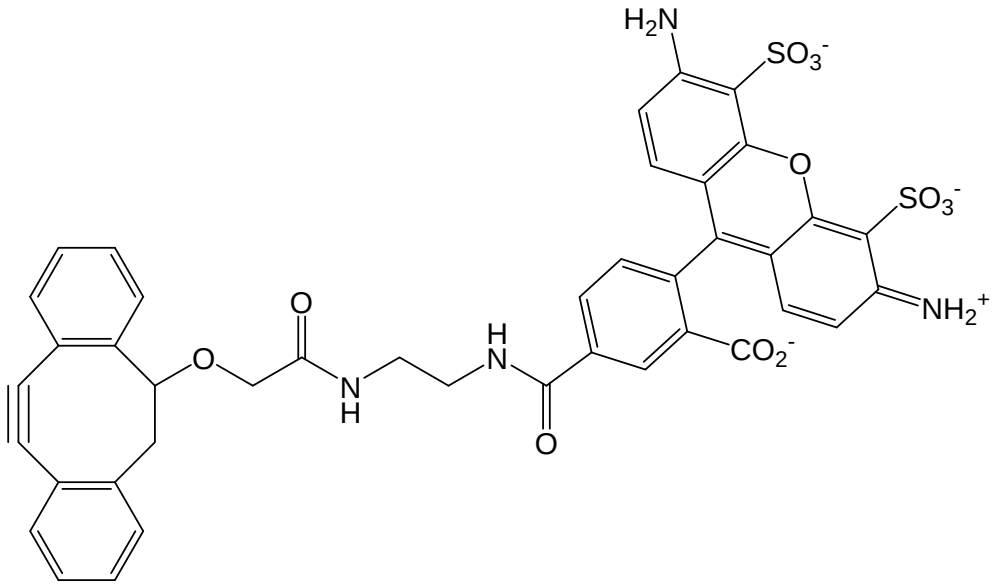
KdsB was cloned from *E. coli* and sub-cloned into a modified pETite vector (Lucigen) using Gibson assembly (NEB Gibson Assembly 2x Master Mix). This vector also added an N-terminal 8xHis tag followed by an HRV 3C protease cleavage site. C41(DE3) *E. coli* cells were then transformed with the resultant plasmid using standard techniques. A starter culture was inoculated with a single colony and grown in LB supplemented with 50 μg/mL kanamycin at 37 °C overnight. The following day, a 1 L culture of cells was then grown in Terrific Broth supplemented with 50 μg/mL kanamycin. The cells grew at 37 °C in 2.8 L Fernbach flasks while shaking at 225 rpm until OD_{600} reached 0.6-0.8, upon which the temperature was decreased to 20 °C and 1 mM IPTG was added to the cultures. Growth continued for 20 h. Cells were harvested by centrifugation at 6,000 ×*g* for 20 min, followed by resuspension in Lysis buffer (25 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.5, 200 mM NaCl, 10 mM imidazole, 1 mM

tris(2-carboxyethyl)phosphine (TCEP)). Cells were lysed by 3 passes through a cell homogenizer (Microfluidics M110-P) at 18,000 psi. Cell lysate was clarified by centrifugation at 45,000 ×*g* for 45 minutes. Clarified lysate was flowed through theresin (5 mL bed volume, gravity-fed). The resin was pre-equilibrated with 2 column volumes of Elution buffer (25 mM Tris pH 7.5, 200 mM NaCl, 500 mM imidazole, 1 mM TCEP), followed by 2 column volumes of Lysis buffer. After binding of the lysate, the resin was washed with 5 column volumes of Lysis buffer, followed by 5 column volumes of Wash buffer (25 mM Tris pH 7.5, 200 mM NaCl, 40 mM imidazole, 1 mM TCEP), followed by elution with 5 CV of Elution buffer. This protein expressed so highly that the resin turned pure white, with a clear elution front upon addition of Elution buffer. SDS-PAGE analysis of the purification indicated that KdsB protein was the primary constituent of the eluate, at >99% purity after this one purification step (data not shown). The eluate was treated with HRV 3C protease with supplementation of an additional 1 mM TCEP. This reaction mixture was placed into a 3,500 Da Molecular Weight Cutoff dialysis cassette (Thermo Scientific) and dialyzed against Lysis Buffer overnight at 4 °C. Cleavage was confirmed by electrospray ionization liquid chromatography mass spectrometry (ESI-LC/MS) the following morning. The protein was then subjected to reverse-IMAC purification by flowing through 5 mL of Ni-NTA resin pre-equilibrated with Lysis buffer. Upon flow-through, an additional 5 mL of Lysis buffer was flowed over the resin to recover the remainder of the protein. The flow-through was then concentrated with a 10,000 MWCO concentrator (EMD Millipore) to approximately 1.5 mL. The protein was lastly subjected to Size Exclusion Chromatography (SEC) utilizing a Superdex 200 16/60 column (GE Healthcare Life Sciences), pre-equilibrated with SEC Buffer (20 mM Bis-Tris pH 7.0, 150 mM NaCl, 1 mM TCEP). Protein was loaded and run at 1 mL/min. KdsB eluted as a large, trailing peak, most likely due to overloading of the column. The first half of the peak was pooled and the concentration was found to be 7.75 mg/mL, so there was no further concentration. The protein was free of any detectable contaminants by SDS-PAGE. The protein was aliquoted into 1 mL aliquots and flash frozen in liquid nitrogen.

Table S1. Bacterial Strains used in this study .

Strain	**Genotype / description**	**Reference or source**
BW25113	F^- λ^- Δ*(araD-araB)567* Δ*lacZ4787(::rrnB-3) rph-1* Δ*(rhaD-rhaB)568 hsdR514* *E. coli* K-12, rough LPS	Coli Genetic Stock Center, CGSC 7636
JW3596-1	BW25113 Δ*rfaC733::Kan*^{R} (Δ*waaC)* Deep-rough LPS	Coli Genetic Stock Center, CGSC 11805
ClearColi® K-12	F^- λ^- Δ*endA* Δ*recA msbA52 frr181* Δ*gutQ* Δ*kdsD* Δ*lpxL* Δ*lpxM* Δ*pagP* Δ*lpxP* Δ*eptA* *Defective in* Kdo biosynthesis, truncated LPS (lipid IV_A)	Lucigen 60850-1
MG1655	F^- λ^- *ilvG rfb-50 rph-1* *E. coli* K-12 parent strain of TUT0018	ATCC® 47076™
TUT0018	*MG1655* Δ*lacI::frt attλ* (P*lac*::mCherry, Cam^R) mCherry-expressing strain	This study

A



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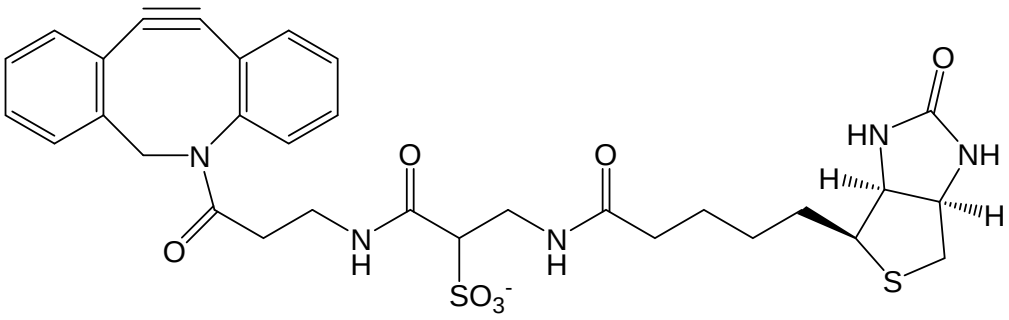

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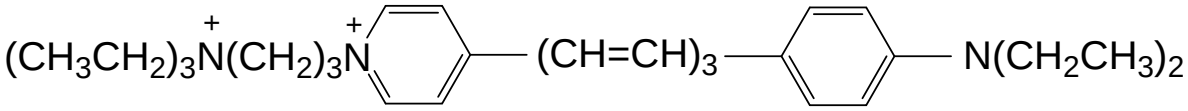

Figure S1: Structures of copper-free click reagents and membrane stain used in this study. A) Click-IT AlexaFluor488-DIBO Alkyne (ThermoFisher, Alexa488-DIBO), B) Sulfo-dibenzocyclooctyne-biotin (Sigma-Aldrich, Sulfo-DBCO-biotin), C) FM 4-64 Dye (ThermoFisher, *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide).

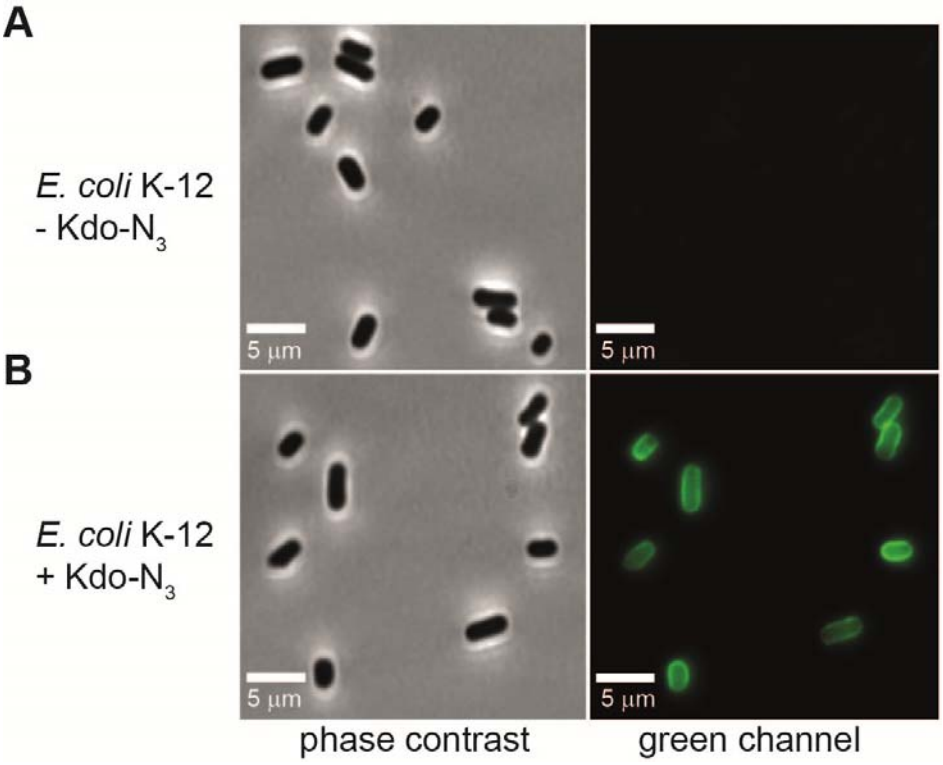

Figure S2: Microscopy images of *E. coli* K-12 grown in LB without Kdo-N_3 (A) and with 1 mM Kdo-N_3 (B). Bright field (left) and fluorescence images (right). Only *E. coli* K-12 grown in the presence of Kdo-N_3 and reacted with Alexa488-DIBO (B) are visible by fluorescence microscopy. The bar represents 5 μm. Images are representative of three independent experiments.

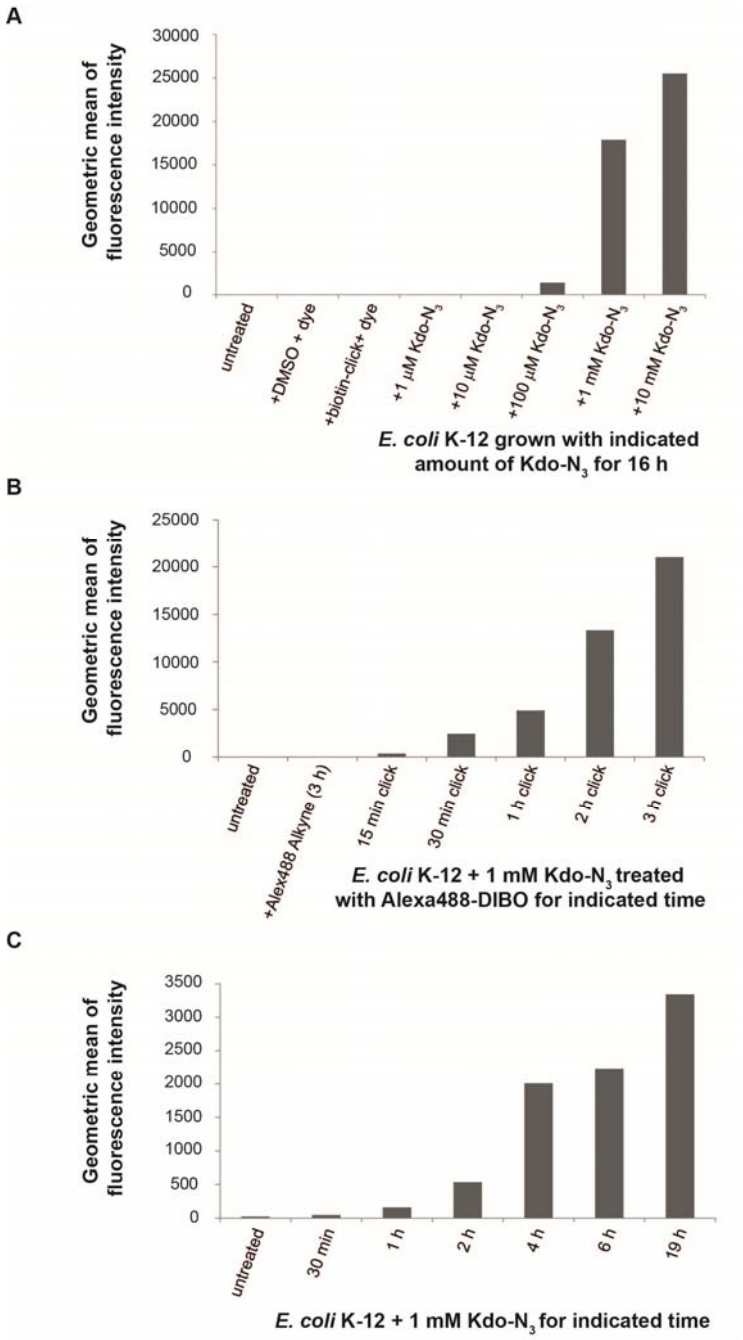

Figure S3: Flow cytometry analysis of *E. coli* K-12 samples labeled with Kdo-N_3. A) Comparison of geometric mean of fluorescence intensities of *E. coli* samples grown with different concentrations of Kdo-N_3 (0 mM to 10 mM Kdo-N_3) followed by copper-free click-reaction with sulfo-DBCO-biotin and binding to mouse anti-biotin AlexaFluor488 antibody conjugate. B) Comparison of geometric mean of fluorescence intensities of *E. coli* samples grown in the presence of 1 mM Kdo-N_3 for 16 h followed by treatment with Alexa488-DIBO for different periods of time. C) Comparison of geometric mean of fluorescence intensities of *E. coli* samples grown with 1 mM Kdo-N_3 for different periods of time (30 min to 19 h) followed by treatment with Alexa488-DIBO for 1 h. Approx. 200000 bacteria were sampled for each condition. Samples were ungated. These dose- and time-range finding experiments were performed once.

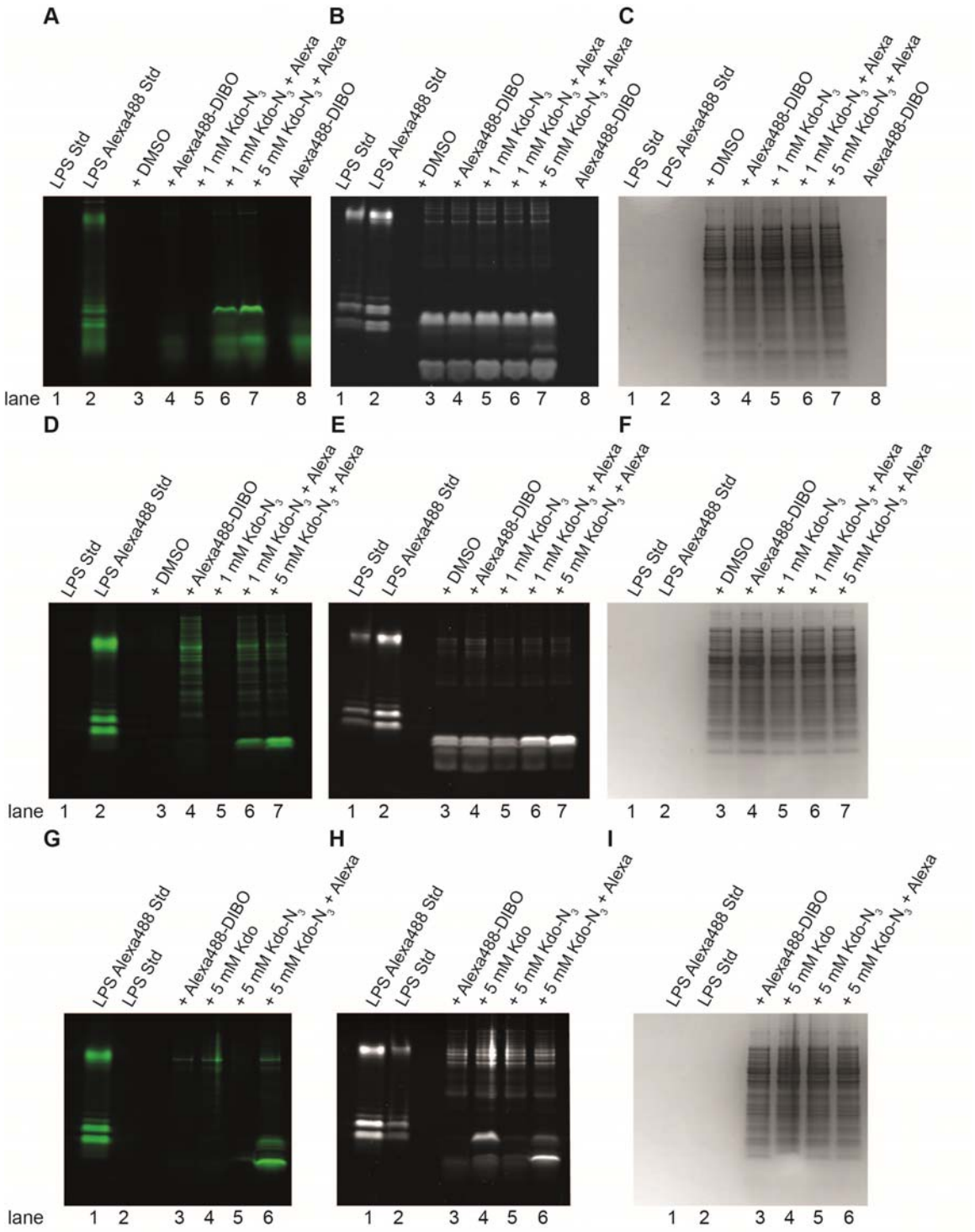

Figure S4: Images of the *E. coli* K-12 SDS-PAGE gel (A-C), Δ*waaC E. coli* SDS-PAGE gel (D-F) and ClearColi K-12 SDS-PAGE gel (G-I). The gels were visualized by fluorescent imaging for AlexaFluor488 (A, D, G), after staining LPS with the Pro-Q Emerald 300 LPS staining kit (B, E, H) and after staining proteins with Coomassie Blue (C, F, I). The data are representative of three independent biological replicates.

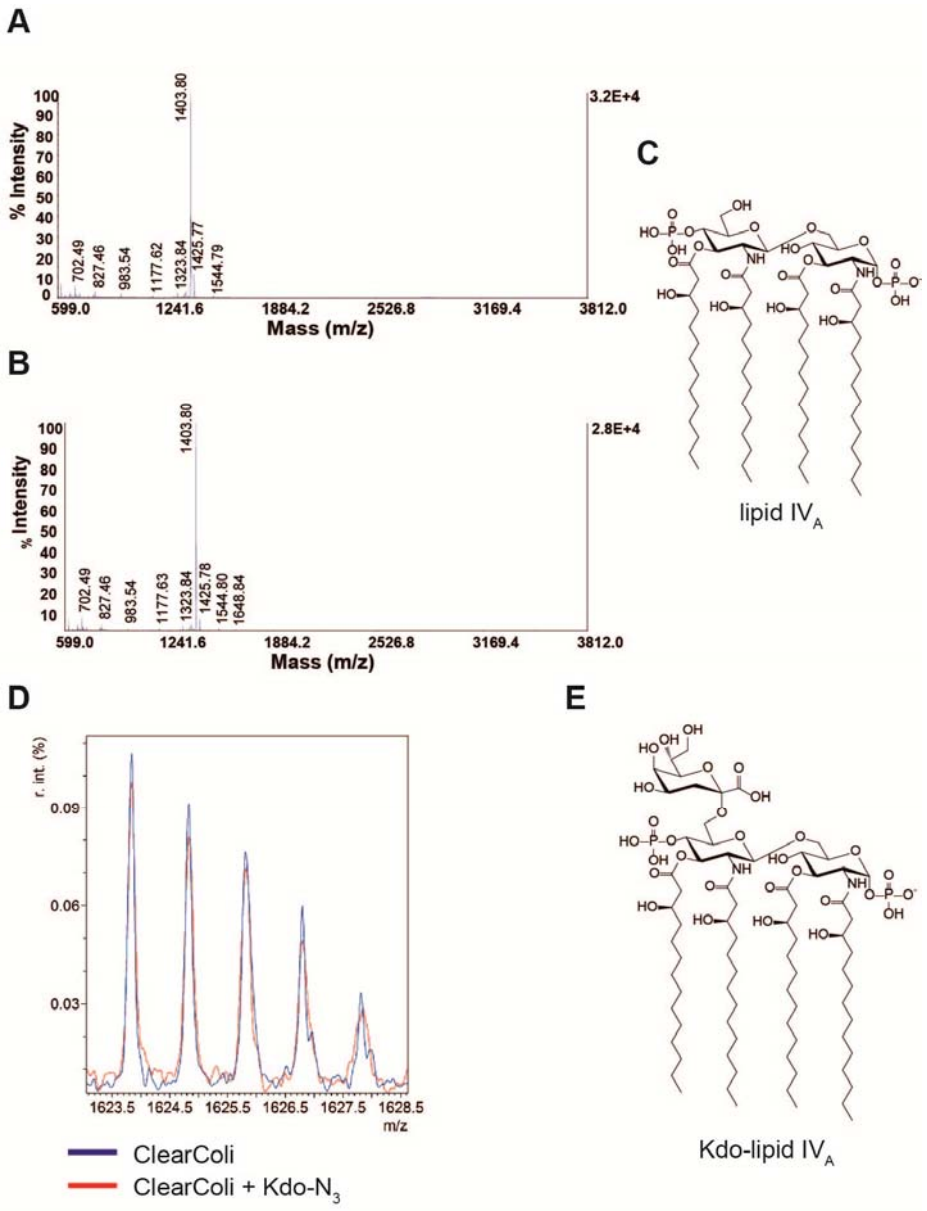

Figure S5: MALDI-TOF MS spectra (negative ion mode) of total lipid extracts from ClearColi K-12. Full spectra of ClearColi K-12 samples grown in the absence (A) or presence (B) of 1 mM Kdo-N_3. C) Structure of lipid IV_A, the most abundant species in both spectra (A and B), respectively, at *m/z* 1403.8. D) Zoomed-in MALDI-TOF MS spectrum. The spectra were normalized to the highest abundant ion (lipid IV_A) and overlaid. The mass window shown corresponds to the $[M-H]^-$ ion of Kdo-lipid IV_A. The red spectrum corresponds to cells grown with Kdo-N_3 and blue corresponds to cells grown without Kdo-N_3. E) Structure of Kdo-lipid IV_A, present in both samples (red and blue trace) at *m/z* 1623.8. The spectra shown are representative of two independent experiments.

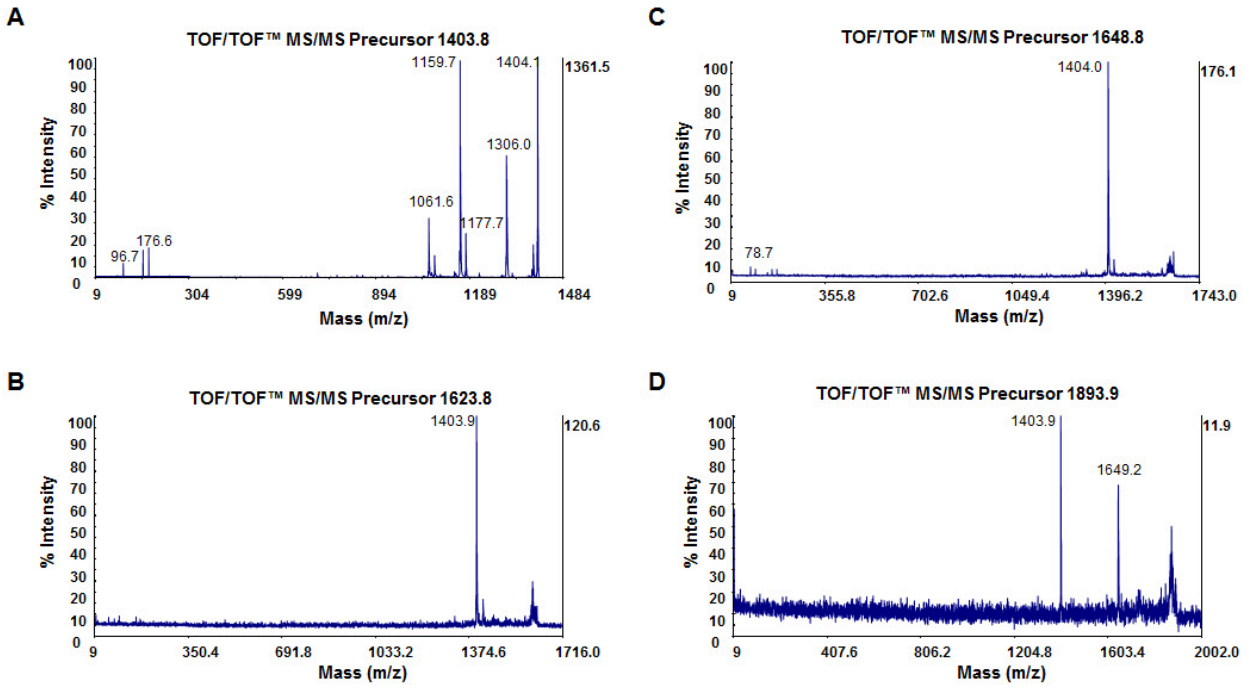

Figure S6: MALDI-TOF/TOF MS/MS spectra of lipid IV_A species observed in the total lipid extracts of ClearColi K-12 total lipid extracts. A) MS/MS fragmentation of the most abundant ion *m/z* 1403.8 (present in both ClearColi K-12 total lipid extracts) is consistent with the expected fragmentation of lipid IV_A, B) MS/MS fragmentation of the ion *m/z* 1623.8 present in both ClearColi K-12 total lipid extracts is consistent with the expected fragmentation of Kdo-lipid IV_A into lipid IV_A upon loss of one Kdo unit, C) MS/MS fragmentation of the ion *m/z* 1648.8 present only in the total lipid extract of ClearColi grown in the presence of 1 mM Kdo-N_3 is consistent with the expected fragmentation of Kdo-N_3-lipid IV_A into lipid IV_A upon loss of one Kdo-N_3 unit, D) MS/MS fragmentation of the ion *m/z* 1893.9 present only in the total lipid extract of ClearColi grown in the presence of 1 mM Kdo-N_3 is consistent with the expected fragmentation of (Kdo-N_3)$_2$-lipid IV_A into Kdo-N_3-lipid IV_A and lipid IV_A upon loss of one or two Kdo-N_3 units, respectively. The spectra shown are representative of two independent experiments.

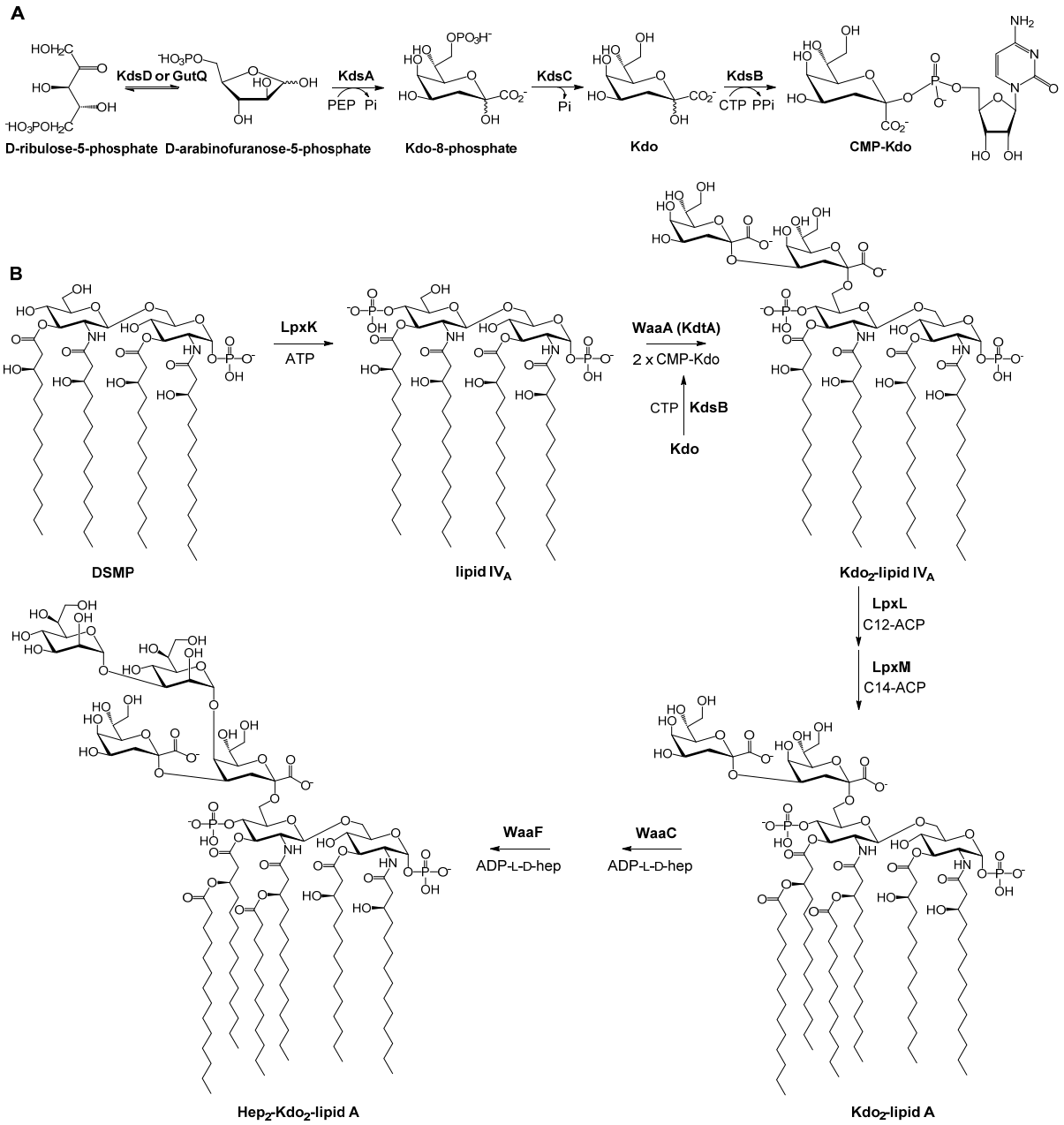

Figure S7: Pathway of CMP-Kdo biosynthesis (A) and Hep_2-Kdo_2-lipid IV_A biosynthesis (B) in *E. coli* K-12. KdsD and GutQ: D-arabinose 5-phosphate isomerases, KdsA: 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase, KdsC: D-arabinose 5-phosphate isomerase, KdsB: 3-deoxy-D-*manno*-octulosonate cytidylyltransferase, PEP: phosphoenolpyruvate, Pi: phosphate, PPi: pyrophosphate, CTP: cytidine triphosphate, CMP: cytidine monophosphate. LpxK: Tetraacyldisaccharide 4′-kinase, WaaA/KdtA: Kdo transferase, LpxL: lauroyl acyltransferase, LpxM: myristoyl-acyl carrier protein-dependent acyltransferase, WaaC: ADP-heptose:LPS heptosyltransferase I, WaaF: ADP-heptose:LPS heptosyltransferase II, ATP: Adenosine triphosphate, CMP: Cytidine monophosphate, ACP: Acyl carrier protein, ADP: Adenosine diphosphate, Hep: heptose.

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