Supplementary Materials for
Structure and mechanism of a proton-dependent lipid transporter involved in lipoteichoic acids biosynthesis

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Tandem MS/MS and parallel reaction monitoring (PRM) LC-MS analysis. Pure NBD-DAG (Cayman) and extracted NBD-DAG-Glc_2 (10 pmol/ul in 30% acetonitrile/69.9% water/0.1% formic acid) were prepared and analyzed by high-resolution mass spectrometry on a Q-Exactive mass spectrometer equipped with a Heated Electrospray Ionization (HESI-II) Probe (both Thermo Fisher Scientific) with direct infusion at a flow rate of 5 ul/min. For both molecules, full and tandem mass spectra were acquired at a resolution of 140,000 FWHM (at 200 *m/z*). Different normalized collision energies were tested and 30% was found to provide the most intense fragment ion signals. For both compounds the three most intense fragment ions were manually selected for quantitative parallel reaction-monitoring (PRM) MS analysis[1]. The setup of the μRPLC-MS system was as described previously[2]. Chromatographic separation of species was carried out using an EASY nano-LC 1000 system (Thermo Fisher Scientific), equipped with a heated RP-HPLC column (75 μm x 30 cm) packed in-house with 1.9 μm C18 resin (Reprosil-AQ Pur, Dr. Maisch). Compounds were analyzed per LC-MS/MS run using a linear gradient ranging from 95% solvent A (0.1% formic acid) and 5% solvent B (80% acetonitrile, 19.9% water, 0.1% formic acid) to 95% solvent B over 60 minutes at a flow rate of 200 nl/min. Mass spectrometry analysis was performed on a Q-Exactive mass spectrometer equipped with a nanoelectrospray ion source (both Thermo Fisher Scientific). Each PRM cycle consisted of one MS1 scan followed by high-collision-dissociation (HCD) of NBD-DAG and NBD-DAG-Glc2 ions. The mass list frequently contained ion masses of sodium adducts and water loss. The total cycle time was approximately 3 s. For MS1, $3x10^6$ ions were accumulated in the Orbitrap cell over a maximum time of 100 ms and scanned at a resolution of 60,000 FWHM (at 200 *m/z*) from 250 top 1600 *m/z*. MS2 scans were acquired at a target setting of $3x10^6$ ions, accumulation time of 100 ms and a resolution of 35,000 FWHM (at 200 *m/z*). The normalized collision energy was set to 30%, the mass isolation window was set to 0.4 m/z and one microscan was acquired for each spectrum. The acquired raw files were analyzed using QualBrowser (v. 4.3.73.11, Thermo Fisher Scientific). Here, the extracted ion chromatograms (XICs) were obtained for NBD-DAG and NBD-DAG-Glc2 using the most intense fragments selected before and employed for quantification.

***S. aureus* complementation assays.** The pLOW vector was used for the construction of *ltaA* complementary strains [3]. The *ltaA* fragment was amplified from genomic DNA with oligos OVL2243 and OVL2244 containing SalI and NotI digestion sites (**Supplementary Table 7**). The amplified *ltaA* fragment and vector pLOW-dCas9 [4] were digested with SalI and NotI, and ligated to produce pLOW-*ltaA*. The ligation product was then transformed into *E. coli* IM08B. To introduce the point mutations of *ltaA*, two partially reverse complementary oligos containing the point mutations were designed to couple with OVL2243 or OVL2244 for amplification of former part and the latter part of *ltaA* coding region, respectively (**Supplementary Table 7**). The former and latter part of *ltaA* were then fused by overlap PCR with oligos OVL2243 and OVL2244 to produce the whole *ltaA* fragment with point mutations, and then the fragments were cloned into pLOW with the same method described above. The pLOW vector carrying *ltaA* or its point mutation derivatives were then introduced into NCTC8325 Δ*ltaA* strain by electroporation transformation with erythromycin selection (5μg/ml) on LBA plates as described above [5].

***S. aureus* fluorescent microscopy.** LB medium pre-warmed at 37°C in the presence of 5% CO_2 overnight (causing acidification to about pH 6.5) or LB medium with low pH (adjusted by dissolving LB Broth powder in PBS pH 6.5) were used to grow cells for fluorescence microscopy experiments. A thawed frozen stock of cells was diluted into pre-warmed LB medium by 1:20 in 6-well plate (Nest Biotechnology, flat bottom, tissue culture treated). The bacterial culture was then incubated at 37°C in a 5% CO_2 incubator for 6 hours. 1ml of the bacterial culture was transferred into a 1.5-ml Eppendorf tube, and then 1μl of 1mg/ml nile red stock (DMSO as solvent) was added for 5-minutes staining. The bacterial cells were then centrifuged at 8000g for 4 minutes, and the supernatant was removed. Bacterial cells were washed with 1 ml of PBS (pH 7.4), followed with resuspension in fresh PBS. The resuspended bacterial cells were then spotted onto a PBS-agarose pad. Fluorescence microscopy was performed using a Leica DMi8 microscope with a 100x phase contrast objective (NA 1.40) with a SOLA Light Engine (Lumencor) light source. A chroma cube nr (Quad=Chroma 89000, mCT_LP=49017) was used. For nile red staining, light was filtered through external excitation filter 545/25 nm (Chroma ET545/25x), and the external filter ET605/70 nm was used for emission. An exposure time of 800 ms with 100% of light from SOLA Light Engine was used for capturing images. The images were obtained with LasX software (Leica) and processed with ImageJ (https://imagej.nih.gov).

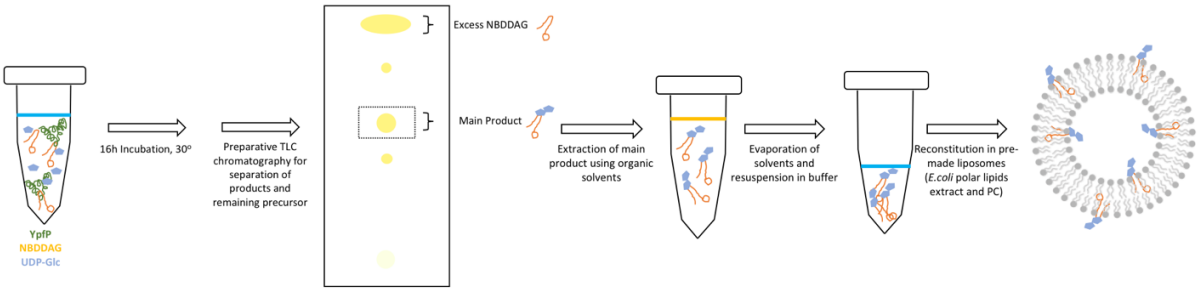
***S. aureus* transmission electronic microscopy.** The same bacterial cells for fluorescent microscopy were used for transmission electronic microscopy. Samples were fixed in 2.5% glutaraldehyde solution (EMS) in Phosphate Buffer (PB 0.1M pH7.4) (Sigma) for 1 hour at room temperature (RT). Then the samples were directly post-fixed by a fresh mixture of 1% osmium tetroxide (EMS) with 1.5% potassium ferrocyanide (Sigma) in PB buffer for 1 hour at RT. The samples were then washed three times with distilled water and spin down in 2% low melting agarose in water (Sigma), followed by solidifying on ice. The solidified samples were cut in 1mm3 cube and dehydrated in acetone solution (Sigma) at graded concentrations (30%-40 min; 50%-40 min; 70%-40 min; 100%-3x1h). This was followed by infiltration in Epon (Sigma) at graded concentrations (Epon 1/3 acetone-2 h; Epon 3/1 acetone-2 h, Epon 1/1-4 h; Epon 1/1-12 h) and finally polymerized for 48 hours at 60°C in oven. Ultrathin sections of 50 nm were cut on a Leica Ultracut (Leica Mikrosysteme) and picked up on a copper slot grid 2x1mm (EMS) coated with a polystyrene film (Sigma). Sections were poststained with 4% uranyl acetate (Sigma) for 10 minutes, and then rinsed several times with water followed by Reynolds lead citrate (Sigma) for

10 minutes, and finally rinsed several times with water. Micrographs were collected with a transmission electron microscope Philips CM100 (Thermo Fisher Scientific, Waltham) at an acceleration voltage of 80 kV with a TVIPS TemCam-F416 digital camera (TVIPS). The final images were processed with ImageJ (https://imagej.nih.gov).

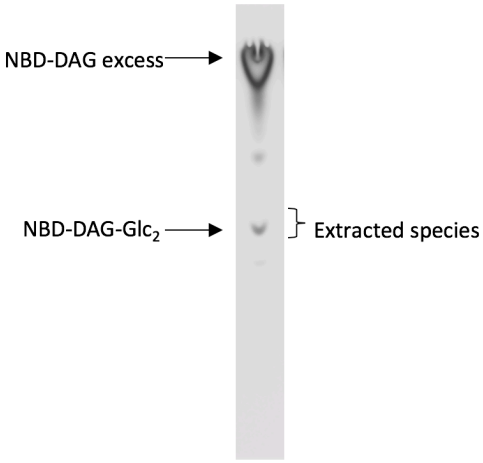
Targeted PRM-LC-MS analysis of LtaA and variants in *S. aureus* membranes. In a first step, parallel reaction-monitoring (PRM) assays[1] for all possible peptides of LtaA being 6 to 25 amino acid long comprising double and triple charged precursor ions were generated. In total, 5 peptides were found to match the length criteria leading to a total of 10 PRM assays. These were applied to identify LtaA in membrane fractions of wild-type *S. aureus*. The setup of the µRPLC-MS system was as described previously[2]. Chromatographic separation of peptides was carried out using an EASY nano-LC 1000 system (Thermo Fisher Scientific), equipped with a heated RP-HPLC column (75 µm x 30 cm) packed in-house with 1.9 µm C18 resin (Reprosil-AQ Pur, Dr. Maisch). Peptides were analyzed per LC-MS/MS run using a linear gradient ranging from 95% solvent A (0.15% formic acid, 2% acetonitrile) and 5% solvent B (98% acetonitrile, 2% water, 0.15% formic acid) to 45% solvent B over 60 minutes at a flow rate of 200 nl/min. Mass spectrometry analysis was performed on a Q-Exactive mass spectrometer equipped with a nanoelectrospray ion source (both Thermo Fisher Scientific). Each MS1 scan was followed by high-collision-dissociation (HCD) of the 10 LtaA precursor ions in PRM mode using a global isolation mass list. Total cycle time was approximately 3 s. For MS1, $3x10^6$ ions were accumulated in the Orbitrap cell over a maximum time of 50 ms and scanned at a resolution of 60,000 FWHM (at 200 m/z). MS2 scans were acquired at a target setting of $3x10^6$ ions, an accumulation time of 250 ms and a resolution of 120,000 FWHM (at 200 m/z). The normalized collision energy was set to 27%, the mass isolation window was set to 0.4 m/z and one microscan was acquired for each spectrum. The acquired raw-files were converted to the mascot generic file (mgf) format using the msconvert tool (part of ProteoWizard, version 3.0.4624 (2013-6-3)). Using the MASCOT algorithm (Matrix Science, Version 2.4.1), the mgf files were searched against a decoy database containing normal and reverse sequences of the predicted UniProt entries of *S. aureus* (strain STAA8, Uniprot, download date: 2019/06/25) and commonly observed contaminants (in total 6,562 sequences) generated using the SequenceReverser tool from the MaxQuant software (Version 1.0.13.13). The precursor ion tolerance was set to 10 ppm and fragment ion tolerance was set to 0.02 Da. The search criteria were set as follows: full tryptic specificity was required (cleavage after lysine or arginine residues unless followed by proline), 3 missed cleavages were allowed, carbamidomethylation (C), was set as fixed modification and oxidation (M) as a variable modification. Next, the database search results were imported to the Scaffold Q+ software (version 4.9.0, Proteome Software Inc., Portland, OR) and the protein false identification rate was set to 1% based on the number of decoy hits. Protein probabilities were assigned by the Protein Prophet program[6]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Using these strict identification criteria, three peptides ions of LtaA, LTNYNTRPVK (2+ and 3+ ion) and MQDSSLNNYANHK (2+) could be confidently identified and were used for label-free PRM quantification. Therefore, a spectral library was build using the data acquired above and imported to Skyline (version 19.1 (https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline/begin.view)). A

mass isolation lists containing the three identified peptide ion masses was exported form Skyline and imported into the Q-Exactive operating software for PRM analysis using the following settings: The resolution of the orbitrap was set to 140k FWHM (at 200 m/z) and the fill time was set to 500 ms to reach a target value of 3e6 ions. Ion isolation window was set to 0.4 Th and the scan range was set to 100-1500 Th. A MS1 scan using the same conditions as for DDA was included in each MS cycle. Each condition was analyzed in biological triplicates. Also, two DDA LC-MS/MS analysis were carried out to assign peptide sequences to the precursor ions quantified by label-free quantification. All raw-files were imported into Skyline for PRM based LtaA peptide quantification. To control for variation in sample amounts, the total ion chromatogram (only comprising peptide ions with two or more charges) of each sample was determined by label-free quantification using Progenesis QI (version 2.0, Waters) and used for normalization. The integrated peak areas of the 3 peptide ions quantified by PRM were summed and employed for LtaA quantification.

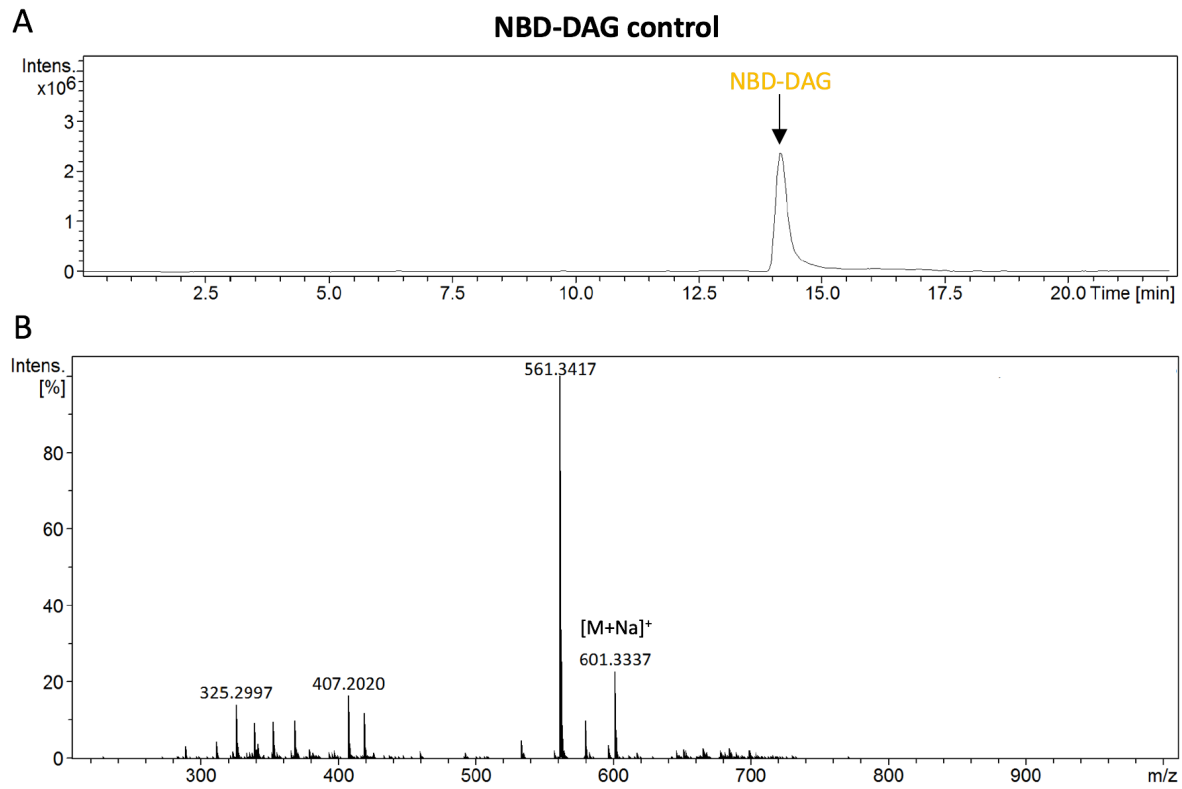
A



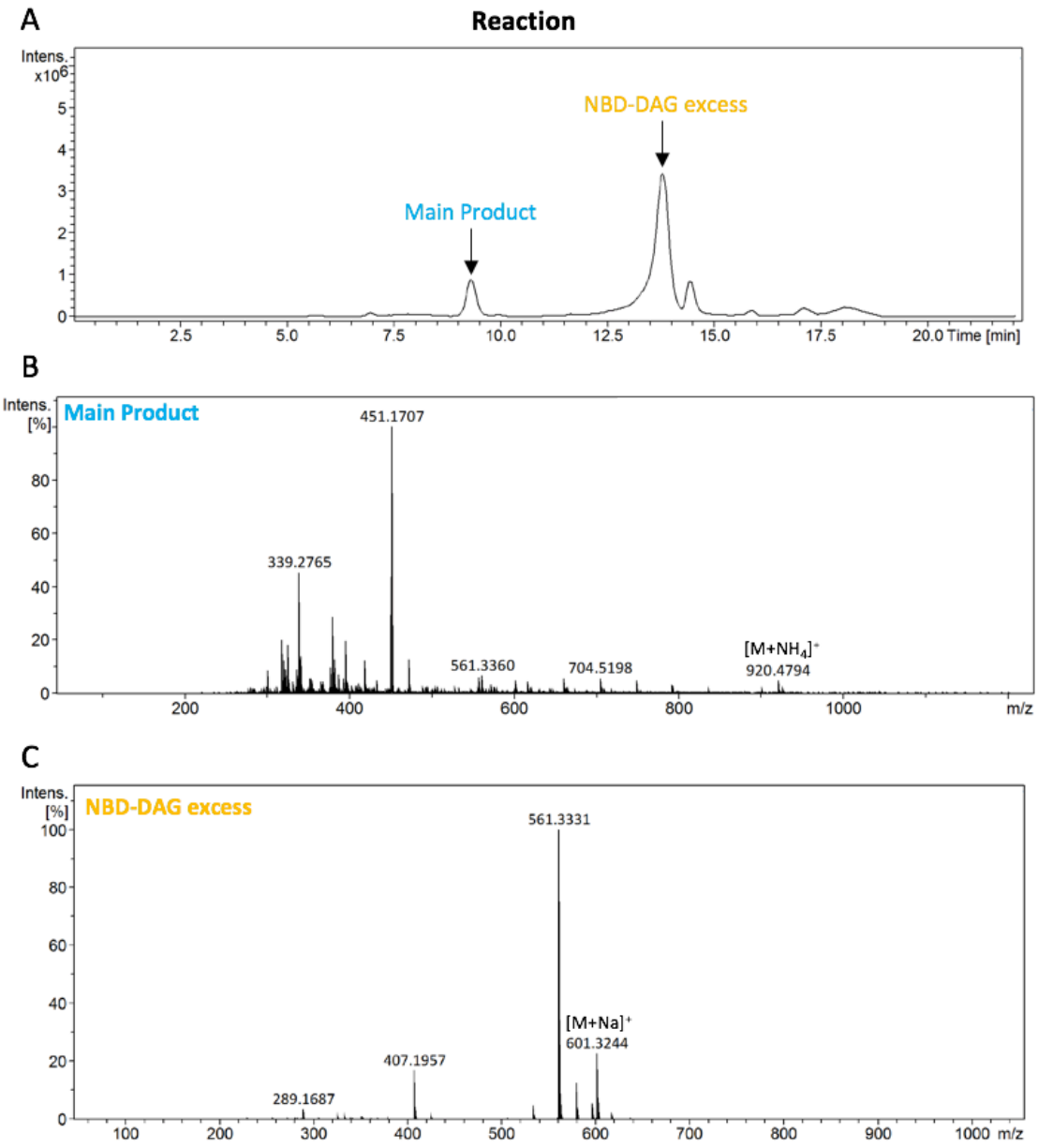
B



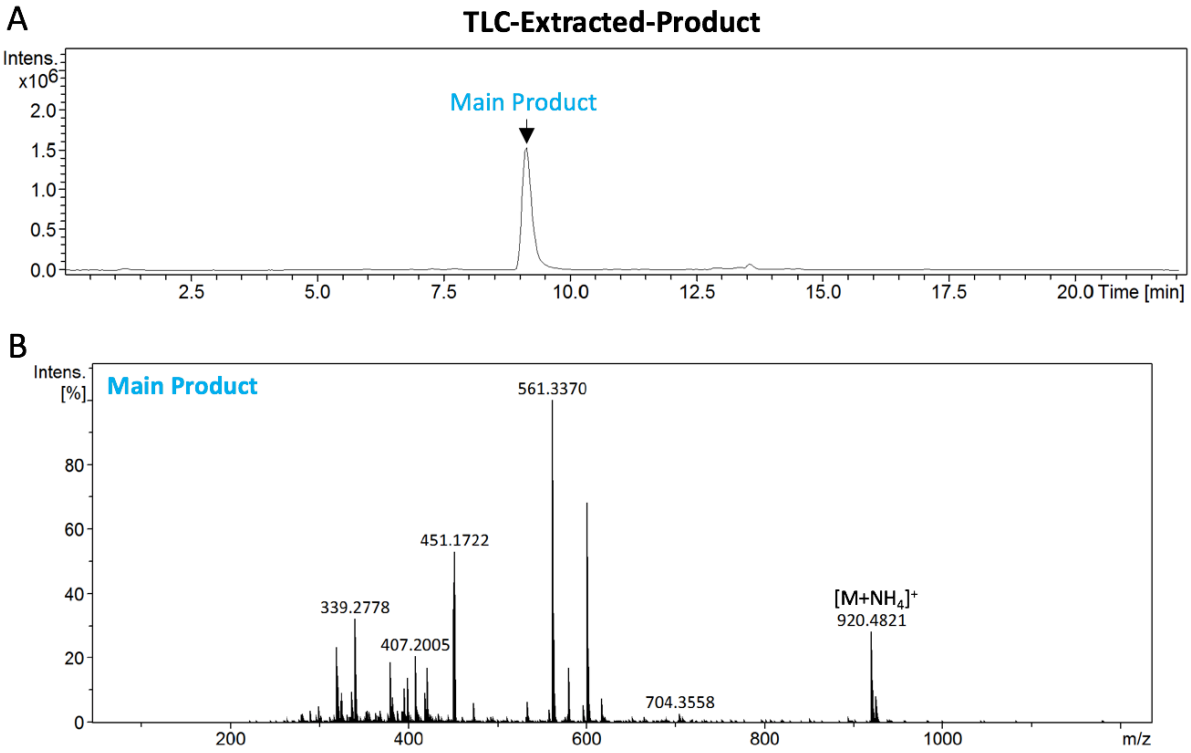
Supplementary Figure 1. Preparation of NBD-Anchor-LLD. **A**. Products and remaining precursor lipids from the YpfP catalysed reaction are separated by preparative TLC. NBD-Anchor-LLD (NBD-DAG-Glc_2) is extracted and resuspended in an aqueous buffer before reconstitution in pre-made liposomes for functional assays. **B**. Fluorescence scan of TLC plate showing separation of NBD labelled lipids. The extracted main product is indicated (NBD-Anchor-LLD; NBD-DAG-Glc2).



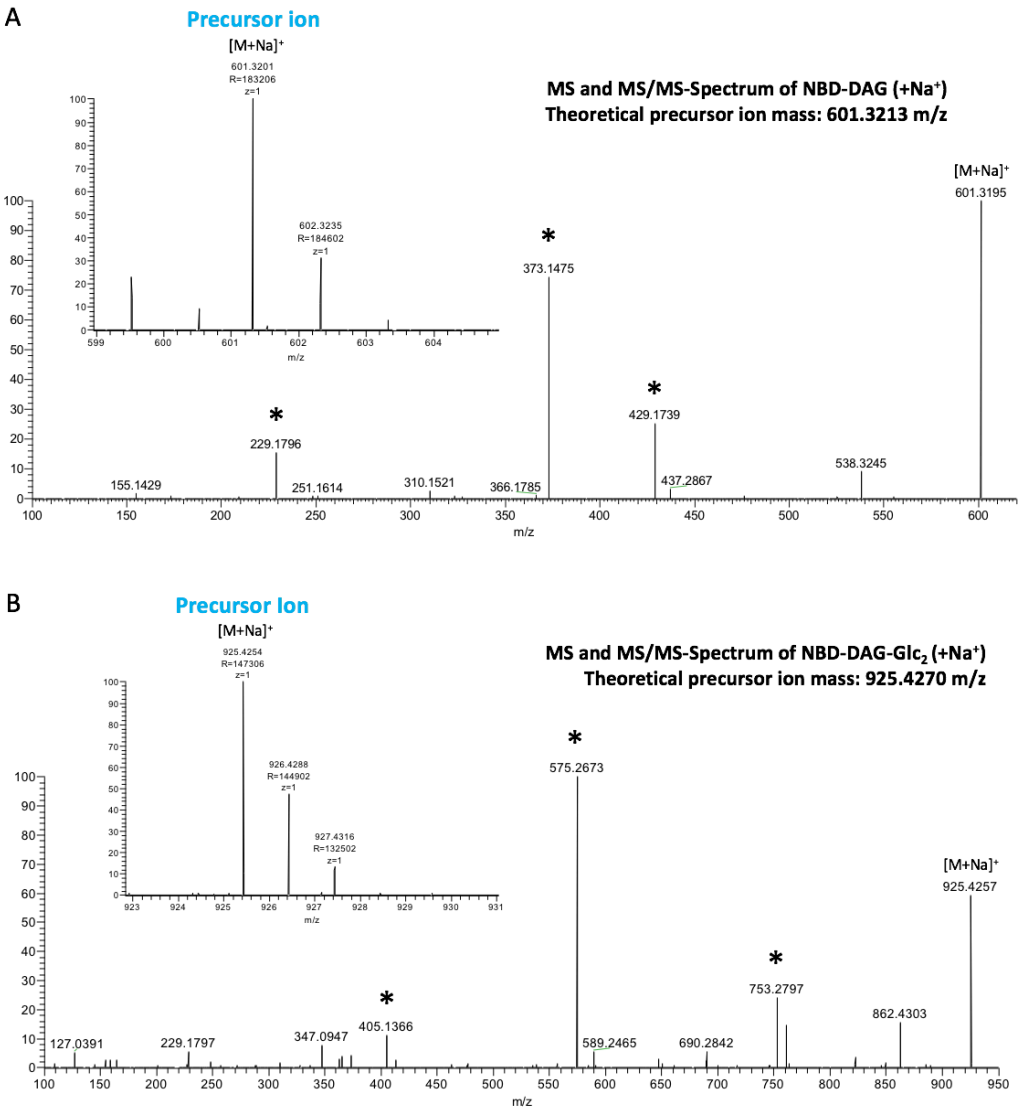
Supplementary Figure 2. HPLC-MS analysis of control NBD-DAG sample. **A**. Total ion chromatogram (TIC). **B**. ESI mass spectra of the species in the indicated peak (A). M indicates molecular ion. For peaks assignment see Supplementary Figure 7 and Supplementary Table 1.



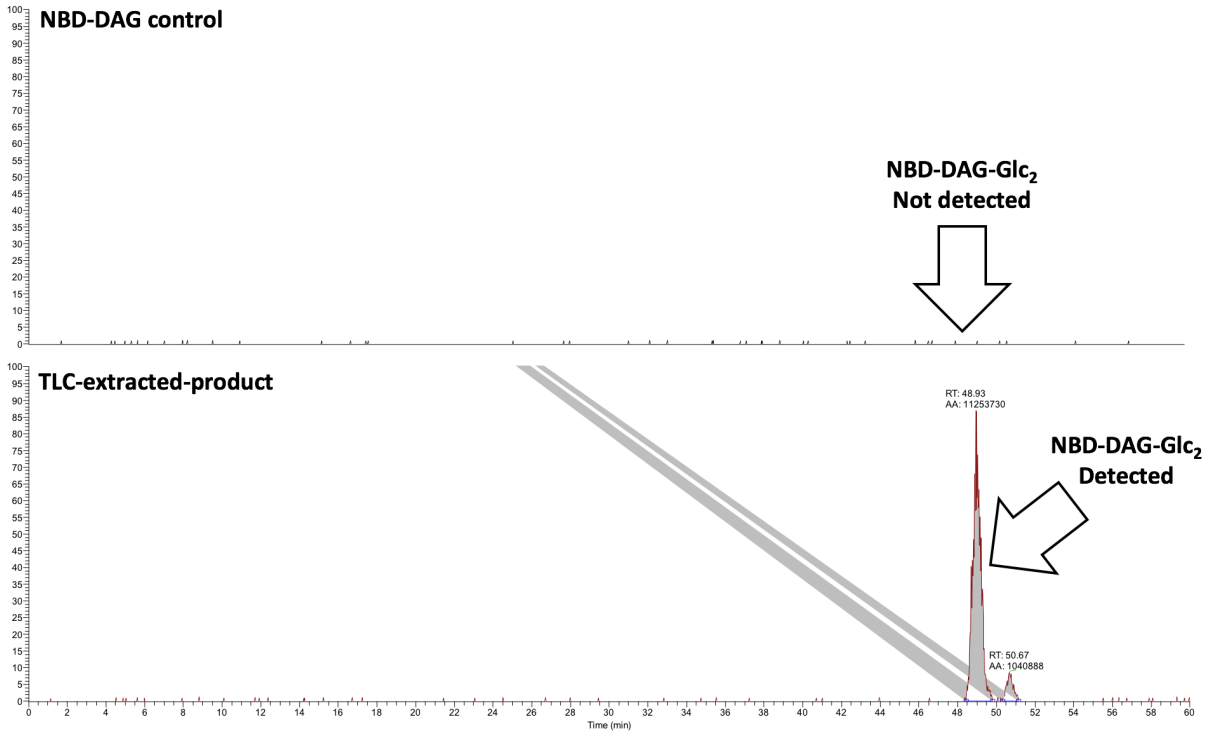
Supplementary Figure 3. HPLC-MS analysis of reaction after completion. **A**. Total ion chromatogram (TIC). **B** and **C**. ESI mass spectra of the species in the indicated peaks (A). Main product: NBD-Anchor-LLD (NBD-DAG-Glc_2). M indicates molecular ion. For peaks assignment see Supplementary Figure 7 and Supplementary Tables 2 and 3.



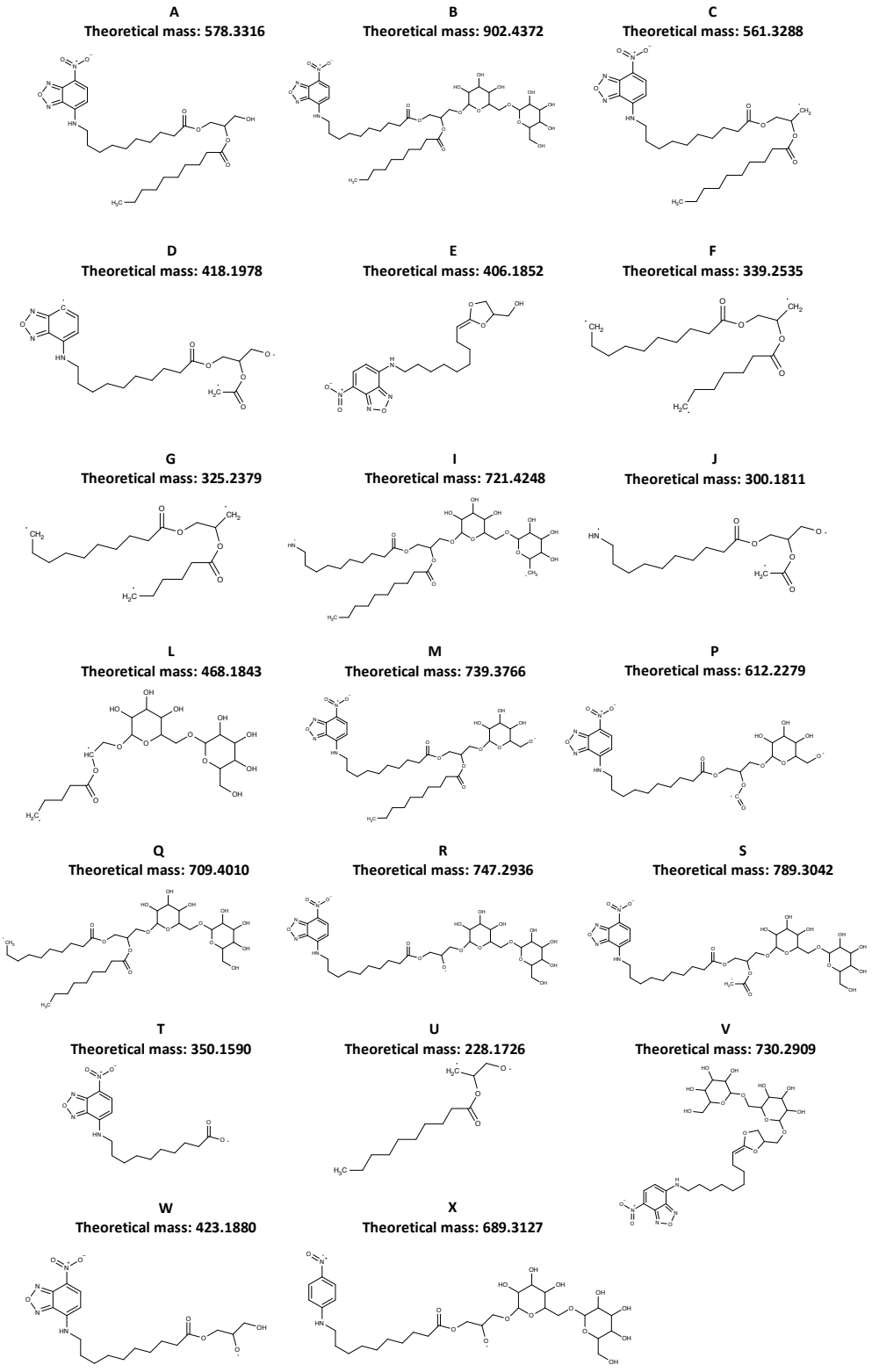
Supplementary Figure 4. HPLC-MS analysis of TLC-extracted product. **A**. Total ion chromatogram (TIC). **B**. ESI mass spectra of the species in the indicated peak (A). Main product: NBD-Anchor-LLD (NBD-DAG-Glc_2). M indicates molecular ion. For peaks assignment see Supplementary Figure 7 and Supplementary Table 4.



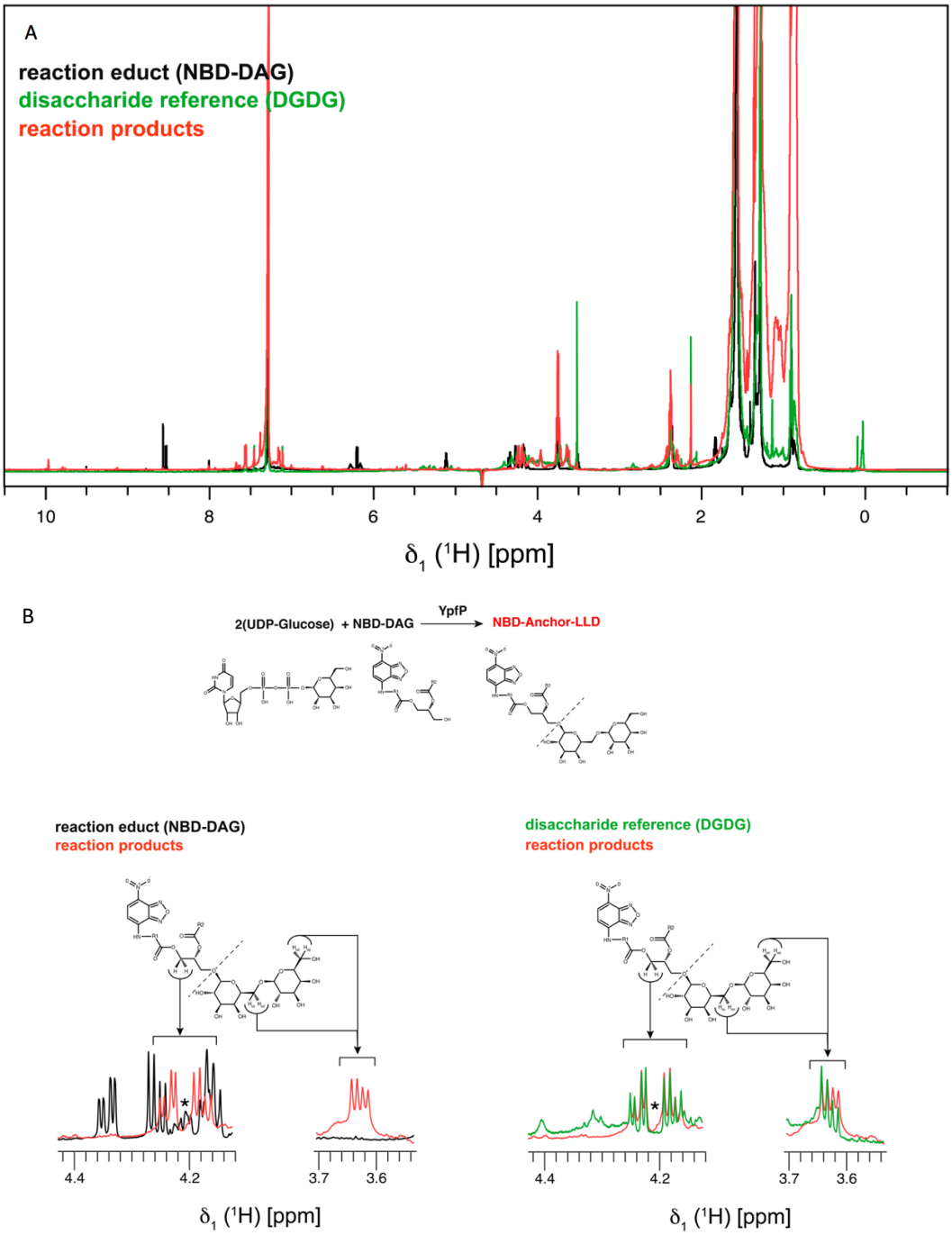
Supplementary Figure 5. High resolution MS analysis of NBD-DAG (Control) and TLC-extracted NBD-Anchor-LLD (NBD-DAG-Glc$_2$). **A** and **B**. Tandem mass spectra were acquired by direct infusion MS/MS analysis. From these, the most intense fragment ions were selected for subsequent PRM analysis as indicated (*) (Supplementary Figure 6). M indicates molecular ion. For peaks assignment see Supplementary Figure 7 and Supplementary Table 5 and 6.



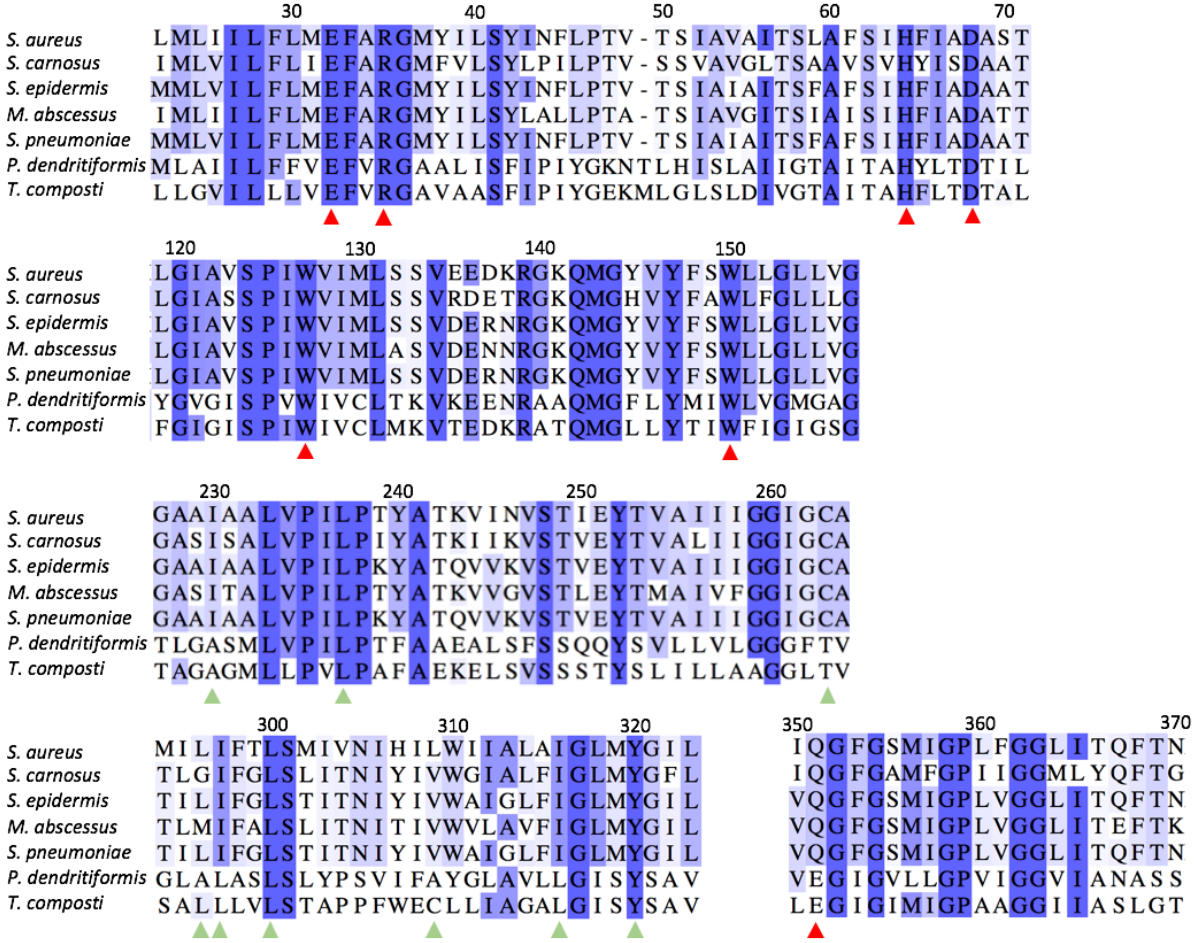
Supplementary Figure 6. Quantitative parallel-reaction monitoring (PRM)-LC-MS analysis of NBD-Anchor-LLD (NBD-DAG-Glc_2). The extraction ion chromatograms for the NBD-DAG control and the TLC-extracted-product samples are shown. No signal for NBD-DAG-Glc_2 was observed in the NBD-DAG control sample.



Supplementary Figure 7. Proposed fragmentation of NBD-DAG and NBD-DAG-Glc$_2$ (NBD-Anchor-LLD) in MS experiments.



Supplementary Figure 8. NMR analysis of NBD-Anchor-LLD synthesis product. **A** and **B**. 1D ^{1}H NMR spectra of the reaction products (red); NBD-DAG reference compound (black); reference digalactosyl-diacylglycerol (green) (DGDG) (Avanti). The resonance assignments of the protons at the positions sn1 of the glycerol moiety and the carbohydrate headgroup are indicated. Since the chemical shift of the protons at the sn1 of the glycerol moiety are highly sensitive to the local chemistry, their spectral overlay with the DGDG reports on the presence of the lipid-glycerol ester bond.



Supplementary Figure 9. Sequence conservation analysis. A multiple sequence alignment of 76 LtaA homologues found in related *Staphylococcus* species or other Gram-positive bacteria was generated. The figure shows representative results of the multiple sequence alignment showing *S. aureus* LtaA and 6 homologues. Red triangles show conserved residues in the N-terminal hydrophilic pocket. Green triangles show residues participating in formation of the C-terminal hydrophobic pocket.

Supplementary Table 1: Masses assignment for mass spectra shown in Suppl. Fig. 2

Fragment*	Adduct	z	Experimental mass	Theoretical mass
G	[G]	+1	325.2997	325.2379
F	[F]	+1	339.2795	339.2535
E	[E+H]	+1	407.2020	407.1932
D	[D + H]	+1	419.0972	419.2058
C	[C]	+1	561.3417	561.3289
A	[A + Na]	+1	601.3335	601.3213

*See structures of fragments in Supplementary Fig. 7

Supplementary Table 2: Masses assignment from mass spectra shown in Suppl. Fig. 3B†

Fragment*	Adduct	z	Experimental mass	Theoretical mass
P	**[P + H + Na]**	**+2**	**318.2459**	**318.1122**
F	[F]	+1	339.2765	339.2535
M	**[M + H + Na]**	**+2**	**381.2862**	**381.6865**
S	**[S + 2H]**	**+2**	**395.1078**	**395.6600**
D	[D + H]	+1	419.0944	419.2058
L	**[L -H2O + H]**	**+1**	**451.1707**	**451.1817**
C	[C]	+1	561.3360	561.3288
I	**[I -H2O + H]**	**+1**	**704.5198**	**704.4222**
R	**[R + H]**	**+1**	**748.5462**	**748.3016**
Q	**[Q + 2ACN + H]**	**+1**	**792.5721**	**792.4614**
B	**[B + NH4]**	**+1**	**920.4794**	**920.4710**

*See structures of fragments in Supplementary Fig. 7

†Fragments in blue letters correspond to species with saccharide moieties

Supplementary Table 3: Masses assignment from mass spectra shown in Suppl. Fig. 3C†

Fragment*	Adduct	z	Experimental mass	Theoretical mass
E	[E+H]	+1	407.1957	407.1932
C	[C]	+1	561.3331	561.3289
A	[A + Na]	+1	601.3244	601.3213

*See structures of fragments in Supplementary Fig. 7

Supplementary Table 4: Masses assignment for mass spectra shown in Suppl. Fig. 4B†

Fragment*	Adduct	z	Experimental mass	Theoretical mass
P	**[P + H + Na]**	**+2**	**318.2465**	**318.1122**
F	[F]	+1	339.2778	339.2535
M	**[M + H + Na]**	**+2**	**381.2917**	**381.6865**
E	[E+H]	+1	407.2005	407.1932
L	**[L -H2O + H]**	**+1**	**451.1722**	**451.1817**
C	[C]	+1	561.3370	561.3288
J	[2J + H]	+1	601.3303	601.3701
I	**[I -H2O + H]**	**+1**	**704.3558**	**704.4222**
B	**[B + NH4]**	**+1**	**920.4821**	**920.4710**

*See structures of fragments in Supplementary Fig. 7

†Fragments in blue letters correspond to species with saccharide moieties

Supplementary Table 5: Masses assignment for MS/MS spectra shown in Suppl. Fig. 5A

Fragment*	Adduct	z	Experimental mass	Theoretical mass
U	[U + H]	+1	229.1796	229.1805
T	[T + Na]	+1	373.1475	373.1488
E	[E + Na]	+1	429.1739	429.1750
A	[A + Na]	+1	601.3195	601.3213

*See structures of fragments in Supplementary Fig. 7

Supplementary Table 6: Masses assignment for MS/MS spectra shown in Suppl. Fig. 5B†

Fragment*	Adduct	z	Experimental mass	Theoretical mass
U	[U + H]	+1	229.1797	229.1805
W	[W – H_2O + H]	+1	405.1366	406.1853
X	**[X + H]**	**+1**	**690.2842**	**690.3207**
V	**[V + Na]**	**+1**	**753.2797**	**753.2807**
B	**[B + Na]**	**+1**	**925.4257**	**925.4270**

*See structures of fragments in Supplementary Fig. 7

†Fragments in blue letters correspond to species with saccharide moieties

Supplementary Table 7: Oligos used in this study.

Oligo name	5'-3' sequence
OVL2243_LtaA-NotI-R	GAGAGCGGCCGCAATAGTATTGTTAATCGTAGTATGTTTGAATTAATAAGA
OVL2244_LtaA-SalI-F	GAGAGTCGACGGTCATTCATCACAACCACAAGAGA
OVL2253_LtaA-up-F1	CATTAATATTCCAGTTGCTACAGTGATGAC
OVL2254_LtaA-up-GA-R	TTGCTCATTATAACCCTCTTTTATTTAACGAAGAATCTTGCATATAAAGGAA
OVL2255_LtaA-dn-GA-F	TAGAAACTTCTCTCAATTAGTAAGGTTAAAACGTATTTTAAAATAAAGAAAATG
OVL2256_LtaA-dn-R1	TTTATGAATAATTTACATTAGTAGATTTAGTATGAAT
OVL2257_Spc-KO-F	AAGAGGGTTATAATGAGCAATTTGATTAACG
OVL2258_Spc-KO-R	CTAATTGAGAGAAGTTTCTATAGAATTTTTCATATAC
OVL2259_LtaA-up-NcoI-F2	GAGACCATGGGTGACAGGTATTCCTATTGATAACAAATTTGA
OVL2260_LtaA-dn-BamHI-R2	GAGAGGATCCAATGTAACGGTTTTCAAAGAAATTTGATATAAT
OVL2751_LtaA-D68A-F	CATTTTATTGCTGCTGCATCTACAAAC
OVL2752_LtaA-D68A-R	GTTTGTAGATGCAGCAGCAATAAAATG
OVL2753_LtaA-W127A-F	CAGTTAGTCCTATTGCGGTCATTATGTTAT
OVL2754_LtaA-W127A-R	ATAACATAATGACCGCAATAGGACTAACTG
OVL2755_LtaA-W150A-F	ATGTTTATTTTTCAGCGTTGTTAGGCCTAT
OVL2756_LtaA-W150A-R	ATAGGCCTAACAACGCTGAAAAATAAACAT
OVL2757_LtaA-Y320A-F	ATCGGTCTAATGGCTGGCATCTTATTA
OVL2758_LtaA-Y320A-R	TAATAAGATGCCAGCCATTAGACCGAT
OVL2759_LtaA-Q351A-F	GTTTTTAATAGTATCGCAGGATTCGGCTCAAT
OVL2760_LtaA-Q351A-R	ATTGAGCCGAATCCTGCGATACTATTAAAAAC
OVL3240_E32A-F	TTATTTTTAATGGCATTTGCGAGAGGC
OVL3241_E32A-R	GCCTCTCGCAAATGCCATTAAAAATAA
OVL3242_R35A-F	TGGAATTTGCGGCAGGCATGTATA
OVL3243_R35A-R	TATACATGCCTGCCGCAAATTCCA

References

1. Peterson, A.C., Russell, J.D., Bailey, D.J., Westphall, M.S. & Coon, J.J. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol Cell Proteomics* **11**, 1475-88 (2012).
2. Ahrne, E. et al. Evaluation and Improvement of Quantification Accuracy in Isobaric Mass Tag-Based Protein Quantification Experiments. *J Proteome Res* **15**, 2537-47 (2016).
3. Liew, A.T. et al. A simple plasmid-based system that allows rapid generation of tightly controlled gene expression in Staphylococcus aureus. *Microbiology* **157**, 666-76 (2011).
4. Stamsas, G.A. et al. CozEa and CozEb play overlapping and essential roles in controlling cell division in Staphylococcus aureus. *Mol Microbiol* (2018).
5. Lofblom, J., Kronqvist, N., Uhlen, M., Stahl, S. & Wernerus, H. Optimization of electroporation-mediated transformation: Staphylococcus carnosus as model organism. *J Appl Microbiol* **102**, 736-47 (2007).
6. Nesvizhskii, A.I., Keller, A., Kolker, E. & Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem* **75**, 4646-58 (2003).