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# Journal Pre-proof

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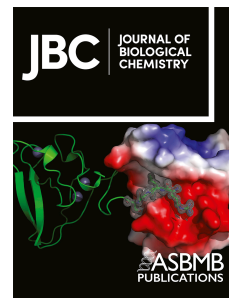
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**The lipopolysaccharide transporter complex LptB<sub>2</sub>FG also displays adenylate kinase activity *in vitro* dependent on binding partners LptC/LptA**

Tiago Baeta<sup>1</sup>, Karine Giandoreggio-Barranco<sup>1</sup>, Isabel Ayala<sup>1</sup>, Elisabete C. C. M. Moura<sup>2</sup>, Paola Sperandeo<sup>2</sup>, Alessandra Polissi<sup>2</sup>, Jean-Pierre Simorre<sup>1</sup>, Cedric Laguri<sup>1\*</sup>

<sup>1</sup>Univ. Grenoble Alpes, CNRS, CEA, IBS, F-38000 Grenoble, France

<sup>2</sup>Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, Italy

\*Corresponding author: Cedric Laguri

E-mail: [cedric.laguri@ibs.fr](mailto:cedric.laguri@ibs.fr)

**Running title:** Regulation of Adenylate Kinase of LptB<sub>2</sub>FG through LptCA recruitment

**Keywords:** cell wall, lipopolysaccharide (LPS), ABC transporter, adenylate kinase, nuclear magnetic resonance (NMR)

**ABSTRACT**

Lipopolysaccharide (LPS) is an essential glycolipid that covers the surface of gram-negative bacteria. The transport of LPS involves a dedicated seven-protein transporter system called the Lpt machinery that physically spans the entire cell envelope. The LptB<sub>2</sub>FG complex is an ABC transporter that hydrolyses ATP to extract LPS from the inner membrane (IM) for transport to the outer membrane. Here we extracted LptB<sub>2</sub>FG directly from the IM with its original lipid environment using Styrene-Maleic acid polymers (SMA). We found that SMA-LptB<sub>2</sub>FG in nanodiscs display not only ATPase activity but a previously uncharacterized Adenylate Kinase (AK) activity, as it catalyzed phosphotransfer between two ADP molecules to generate ATP and AMP. The ATPase and AK activities of LptB<sub>2</sub>FG were both stimulated by the interaction on the periplasmic side with the periplasmic LPS transport proteins LptC and LptA and inhibited by the presence of the LptC transmembrane helix. We determined that the isolated ATPase module (LptB) had weak AK activity in absence of transmembrane proteins LptF and LptG; one mutation in LptB that weakens its affinity for ADP led to AK activity similar to that of fully assembled complex. Thus, we conclude that LptB<sub>2</sub>FG is capable of producing ATP from ADP, depending on the assembly of the Lpt bridge, and that this AK activity might be important to ensure efficient LPS transport in the fully assembled Lpt system.

**Introduction**

Gram-negative bacteria possess a double membrane system delimiting an aqueous space, the periplasm. While the cytoplasmic inner membrane is a canonical phospholipid bilayer, the outer membrane (OM) is asymmetric with its outer layer mostly composed of the Lipopolysaccharide (LPS) glycolipid (1). These molecules are surface exposed, contribute to the high impermeability of the OM, and play a role in immune response, pathogenesis, and drug resistance (2). Maintenance of the OM asymmetry is essential for bacterial viability and a continuous flow of LPS molecules needs to cross the periplasm and reach the external layer to keep up with cell growth. A system of 7 essential proteins, the Lipopolysaccharide Transport System (Lpt), is dedicated to trafficking LPS across the cell envelope. These proteins (LptA-G)

are found in all cellular compartments (IM, periplasm and OM) and in the periplasm they associate through jellyroll domains (present in LptF,C,A, and D) to form a continuous periplasmic bridge connecting IM and OM (3, 4). LptB<sub>2</sub>FG inner membrane complex is an ATP-binding Cassette (ABC) transporter in which LptB<sub>2</sub> represents the Nucleotide Binding Domain (NBD) component which binds and hydrolyses Adenosine Triphosphate (ATP) to generate mechanical force to transport LPS. Upon ATP binding, LptB dimerizes in a closed structure, and ATP hydrolysis relaxes the dimer into an open conformation. Conformational changes are transmitted to LptFG through coupling helices and allow LPS extraction (5). LptB<sub>2</sub>FG associates with LptC via 2 distinct interactions: i) LptF/LptG interact with LptC transmembrane domain, which contributes to form the cavity that accommodates LPS into the complex and regulates LptB<sub>2</sub>FG ATPase activity (6), ii) LptF and LptC jellyroll domains associate in the periplasm. LptA jellyroll then bridges LptB<sub>2</sub>FGC to LptD/E OM complex that ultimately assembles the LPS to the OM outer leaflet (3).

The superfamily of ABC transporter proteins comprises more than 500 members, and supports traffic of metabolites and molecules through ATP hydrolysis (7). Several prokaryotic ABC transporters (MsbA, TmrAB and LmrA) couple another reaction in their ATPase domain, Adenylate Kinase (AK) (8). AK catalyzes a phosphotransfer reaction between 2 Adenosine Diphosphate (ADP) molecules producing ATP and AMP (Adenosine mono-phosphate) with no energy consumption (9). MsbA in particular translocates LPS across the inner membrane prior to its transport by LptB<sub>2</sub>FG (10, 11). The additional active site for the AK has been suggested to be located proximal to the ATP binding site, since the reaction requires both ADP molecules close by in space (12).

LptB<sub>2</sub>FG was extracted directly from the *Escherichia coli* inner membrane with Styrene-Maleic Acids (SMA) polymers without the use of detergents. <sup>1</sup>H NMR on nucleotides showed the SMA-LptB<sub>2</sub>FG complex has ATPase activity but also Adenylate Kinase (AK) activity, and both activities are regulated by the assembly of LptB<sub>2</sub>FG with LptC and LptA partners. Point-mutations were introduced in LptB<sub>2</sub>FG complex and in isolated LptB to address their effect on

ATPase and AK activity by NMR and locate the AK site.

## Results

### LptB<sub>2</sub>FG extracted from the inner membrane by SMA polymers displays ATPase and Adenylate Kinase activity

LptB<sub>2</sub>FG complexes from several organisms have been expressed and purified in either detergent micelles (13–17), reconstituted nanodiscs (13) or liposomes (18). These protocols all involve solubilization and purification of LptB<sub>2</sub>FG complex in detergent micelles. The use of detergent-free protocols in preparation of membrane protein, to maintain their original lipid environments, becomes increasingly important to ensure functionality and stability. In that aspect the use of SMA polymers and its derivatives, that allow direct extraction of membrane proteins from purified membranes, provides a versatile tool to study membrane proteins as close as possible to their natural environment (19, 20). LptB<sub>2</sub>FG complex extraction directly from the inner membrane of *Escherichia coli* was tested using two SMA polymers with 2:1 and 2.3:1 styrene/maleic acids ratio. SMA-LptB<sub>2</sub>FG was successfully purified in nanodiscs of about 10 nm diameter only with SMA 2:1 polymer and with the expected 2:1:1 LptB/LptF/LptG stoichiometry (Fig. S1). LptB<sub>2</sub>FG ATP hydrolysis was checked in presence of LptC and LptA, its periplasmic partners, using NMR to probe the reaction (Fig. 1A). The monomeric version of LptA (LptA<sub>m</sub>) and the soluble version of LptC ( $\Delta$ TM-LptC), which are able to sustain activity and cell viability (4, 21, 22), were added to SMA-LptB<sub>2</sub>FG and ATP hydrolysis was followed by real-time <sup>1</sup>H-NMR (Fig. 1B). NMR is a spectroscopic technique that provides resonance frequency of active nuclei, in our case protons, depending on their chemical environments (23). It can discriminate between ATP and ADP as several <sup>1</sup>H shift frequencies upon phosphate loss, here hydrogen H4' on the ribose (Fig. 1B). SMA-LptB<sub>2</sub>FG shows ATP hydrolysis, with fast disappearance of peaks characteristic of ATP and concomitant appearance of ADP specific peaks in the NMR spectrum. The rate of ATP hydrolysis is 6.5 moles of ATP consumed/min/mole of LptB. After the initial rapid accumulation of ADP due to ATPase activity, ADP level decreases at the same rate as the appearance of a new set of NMR peaks (8.60 ppm in the H8 region, and 4.39 ppm in H4'

region). This suggests conversion of ADP into another specie over time and its <sup>1</sup>H NMR chemical shifts suggest it could correspond to AMP (24). <sup>31</sup>P NMR spectrum was collected at the end of the reaction and showed the characteristic peak of AMP at 3 ppm (Fig. S2). Conversion of ADP into AMP is compatible with an Adenylate Kinase reaction  $2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$ , already been observed for several ABC transporters (8, 12). ATP was replaced by ADP as a substrate and reaction was followed in identical conditions in real time (Fig. 1B). ADP decrease is seen immediately, together with appearance of AMP and ATP. Generation of ATP is consistent with AK activity and excludes hydrolysis of ADP into AMP. ATP levels observed are lower than those of AMP, as AK and ATPase activities occur simultaneously, and newly generated ATP is partly hydrolyzed back to ADP. The initial rates of AK activity are much slower than ATPase with 0.2 and 0.7 moles of AMP produced/min/mole of LptB with ATP or ADP as starting substrates (Fig. 1B).

### ATPase and AK activities are regulated by the assembly of the Lpt system.

LptB<sub>2</sub>FG ATPase activity is repressed by the TM domain of LptC (13, 18), we thus examined ATPase and AK activities of SMA-LptB<sub>2</sub>FG with/without  $\Delta$ TM-LptC/LptA<sub>m</sub> and in complex with full length LptC with/without LptA<sub>m</sub> (Fig. 2A and 2B). For the following experiments nucleotides were quantified by NMR at the end of incubation (Fig. S3). SMA-LptB<sub>2</sub>FG displays less ATPase and AK activity in absence of  $\Delta$ TM-LptC and LptA<sub>m</sub>. Proper assembly of the periplasmic part of LptC and its complex with LptA (22) is thus important in stimulating both ATPase and AK activity. SMA-LptB<sub>2</sub>FGC, containing full length LptC has no ATPase activity, but the repression is relieved in presence of LptA<sub>m</sub> (Fig. 2B). Similarly, SMA-LptB<sub>2</sub>FGC has little AK activity (Fig. 2B) which is increased by addition of LptA<sub>m</sub>. AK activity and ATPase activities are thus both stimulated by the assembly of LptB<sub>2</sub>FGCA complex without the transmembrane LptC segment. In intact LptB<sub>2</sub>FGC ATPase and AK are activated by assembly with LptA.

### Variants in periplasmic LptF domain and in cytoplasmic LptB differently affect AK activity.

Number of mutations in LptB<sub>2</sub>FG were studied for their effect on the complex activity, either

biochemically or for their *in vivo* functionality (25, 26). Two SMA-LptB<sub>2</sub>FG complexes carrying already characterized mutations were purified, one on LptF periplasmic side, and one in the ATPase domain (LptB). LptF<sup>R212G</sup> variant can overcome the absence of the essential LptC protein and allow cell viability (27). R212 is located in LptF periplasmic domain at the interface with LptC and is in the path of the LPS flow (28). SMA-LptB<sub>2</sub>F<sup>R212G</sup>G has impaired ATPase activity when compared to wild-type (wt) complexes (Fig. 2C), and  $\Delta$ TM-LptC and LptA<sub>m</sub> stimulate ATPase and AK activity (Fig. S3). With ADP as substrate SMA-LptB<sub>2</sub>F<sup>R212G</sup>G with and without  $\Delta$ TM-LptC and LptA<sub>m</sub>, show similar production of AMP as wt complexes (Fig. 2C). R212G mutation, while impairing the ATPase, does not affect the AK, suggesting different regulation mechanisms of the two activities.

LptB H195 is involved in  $\gamma$  phosphate binding of ATP (Fig. 3A) and LptB<sup>H195A</sup> variant has decreased ATPase activity in isolated LptB and is deleterious for cell growth (25). SMA-LptB<sub>2</sub><sup>H195A</sup>FG has almost knocked out ATPase activity, even in presence of  $\Delta$ TM-LptC and LptA<sub>m</sub>. AK activity is still activated by  $\Delta$ TM-LptC/A<sub>m</sub> but significantly decreased compared to wild type complex (Fig. 2D, Fig. S3). Alterations in the ATPase binding site of LptB affect ATPase activity and AK activity, but the latter to a lesser degree.

### Mutations in the A- and B- loops of LptB stimulate AK

The location of the AK site is shared with the ATPase site (29–31). One ADP molecule occupies the ATP binding site while the other must be presented «in line» with the first one for the phosphotransfer to occur. Current models for AK suggest the second ADP substrate molecule to be located around the Q-loop (12) (Fig. 3A). The location of the second ADP molecule was probed in LptB using mutagenesis on LptB isolated protein. In addition to H195A mutation in the H motif (or switch region), other mutations were inserted in canonical ABC motifs, specifically at the A-loop (Y13W), B-loop (E163Q and 163A), and Q-loop (A87Q) (Fig. 3A).

Purified soluble LptB wild-type and variants proteins were incubated with either ATP or ADP, and nucleotides were quantified by <sup>1</sup>H-NMR at endpoint of experiment (Fig. S4). LptB, similarly to SMA-LptB<sub>2</sub>FG complex (without  $\Delta$ TM-LptC

and LptA<sub>m</sub>) displays ATPase activity and little AK activity (Fig. S5). All mutations introduced in LptB decrease ATPase activity and E163A is catalytically fully inactive, with no ATPase or AK activity. Two LptB variants show increased AK activity, E163Q and more significantly Y13W, the latter accumulating AMP at levels similar to SMA-LptB<sub>2</sub>FG/ $\Delta$ TM-LptC/LptA<sub>m</sub> (Fig. 3B). To assess how binding of nucleotides relates to the activity of LptB, Saturation Transfer Difference (STD) experiments were recorded on wild-type (wt) and LptB variants. STD is an NMR technique that allows observation of the free ligand in transient binding to a protein (32). The protein is selectively saturated, and part of the saturation is transferred to the ligand during binding and a decrease of intensity is observed on the free ligand. STD on ADP by wild-type LptB shows saturation on protons H2, H8 and H1' of the ADP, showing significant interaction with LptB. LptB<sup>E163Q</sup> variant shows no difference of STD with respect to wt. STD measurement of Y13W variant with ADP is not possible, since AK activity is too fast relative to the experimental time (Fig. S6). ADP- $\beta$ S, which is not a substrate of LptB, showed STD similar as ADP with wild-type and LptB<sup>E163Q</sup> variant (Fig. S6). LptB<sup>Y13W</sup>, on the other hand, showed a highly reduced STD (Fig. 3C Fig. S6,S7). A reduction in STD can be ascribed to a reduced affinity of LptB<sup>Y13W</sup> for ADP- $\beta$ S, with a reduced residence time of the ligand, or on the contrary to a reduction in  $k_{off}$  leading to less saturation of the free ligand. A Y-W mutation in this position has already been found to reduce ATPase activity and ATP binding in other ABC transporters (33, 34). We thus conclude that LptB<sup>Y13W</sup> variant increase in AK activity can be assigned to a reduction in the affinity for ADP, likely in the canonical ATPase binding site considering the position of Y13 (Fig. 3A). Apart from the E163A mutant, largely impaired in ATPase and AK activities, we could find no mutation that selectively affects Adenylate Kinase activity, suggesting that a defined binding site for the second ADP molecule does not exist in LptB.

### Discussion

Understanding bacterial mechanisms that coordinate envelope assembly is critical to harness ways to challenge persistent and clinically relevant infections. Here, LptB<sub>2</sub>FG and LptB<sub>2</sub>FGC complexes were extracted directly from *E. coli* inner membrane in their natural lipid context, without the need for detergent extraction.

LptB<sub>2</sub>FG complex ATPase activity is stimulated by interaction on the periplasmic side of LptB<sub>2</sub>FG with LptC and LptA and fully repressed when full-length LptC is coextracted with LptB<sub>2</sub>FG. SMA-LptB<sub>2</sub>FG LptB subunit is also an Adenylate Kinase. This activity is marginal until LptB<sub>2</sub>FG interacts with the jellyroll domains of LptC and LptA, thus forming an almost complete Lpt bridge (missing LptD/E OM complex). Establishment of the bridge must be transmitted by conformational changes through the lipid bilayer to the cytoplasmic LptB and activate both ATPase and AK activity. LptB<sub>2</sub>FGC complex that contains full-length LptC with its N-terminal transmembrane domain has no ATPase activity and little AK activity until it interacts with LptA which increases both activities. Level of activity of LptB<sub>2</sub>FGC is much lower due to the presence of the transmembrane LptC domain. This domain interacts with LPS into the LptB<sub>2</sub>FG complex and is more dynamic in LptB<sub>2</sub>FG bound to ATP analogs (13). This could suggest a role of TM-LptC in the synchronization of LPS entry and ATP hydrolysis.

The Lpt system possesses several mechanisms that regulate its activity to ensure efficient LPS transport when the Lpt system is assembled. A mutant discovered in LptF periplasmic jellyroll domain which can complement  $\Delta lptC$  cells is particularly interesting. SMA-LptB<sub>2</sub>F<sup>R212G</sup> ATPase activity is highly reduced compared to wild-type complex while AK activity is identical. This suggests that the regulation of the ATPase and AK activities by the assembly of the Lpt system are somewhat different. Nevertheless, the regulation mechanisms are currently not characterized at the molecular level.

Adenylate kinase reaction involves 2 ADP molecules, one located in the ATPase canonical site, and one located nearby. Our mutagenesis study could not pinpoint the location of a binding site for the second ADP. LptB<sup>E163Q</sup> showed increased AK activity, and H195A in the full complex showed reduced AK activity. While those residues could be important for the AK activity, they are all involved in the ATPase reaction, either by binding ATP  $\gamma$ -phosphate, the magnesium ion or coordinating water molecules (25). Our data suggest that, on the contrary to pure Adenylate Kinase (35), there is no well-defined binding site for the AK activity in LptB, apart from the canonical ATP binding site. The high AK activity of LptB<sup>Y13W</sup> variant cannot be easily explained. The consequence of this

mutation is to strongly reduce STD, likely due to decreased affinity/residence time of the ligand in the canonical ATPase site. This could in turn accelerate the turnover of the reaction, but we cannot exclude that this variant in isolated LptB somehow mimics the conformation of LptB<sub>2</sub>FG in presence of the jellyroll domains of LptC and LptA.

The exact role of the AK activity in LptB<sub>2</sub>FG ABC transporter remains unknown. In one ABC transporter, cystic fibrosis receptor (CFTR), the AK activity was shown to be important for transport(37). Our characterisation of AK activity was performed at high substrate concentration (5mM), about 10 times higher than the concentration of ADP in bacteria. AK reaction was thus tested in physiologic concentrations with a mix of ATP, ADP and AMP (Fig. S8). In these conditions, AK activity is similar as with 5mM ADP as substrate (Fig. 2). Similarly, LptB<sub>2</sub>FG has AK activity starting with only 500 $\mu$ M of ADP, the concentration found in cells (Fig. S8). While this reaction is possible in concentrations of nucleotides found *in vivo*, its contribution to LPS transport has not been tested yet. Nevertheless the capacity of the AK reaction to generate ATP, which can be used to transport LPS, and at a negligible energy cost must be considered in future studies, and specifically in conditions of bacterial stress.

## Experimental Procedures

### Used Plasmids

Plasmids used for soluble LptB, SMA-LptB<sub>2</sub>FG,  $\Delta$ TM-LptC and LptA<sub>m</sub> purifications were previously described: pET22-42-LptB-His8 (25), pCDF-Duet1-LptB<sub>2</sub>FG (36), expressing LptB-His6/LptF/LptG, (36), pQESH LptC $_{\Delta[1-23]}$  ( $\Delta$ TM-LptC) (37) and pET21b-LptA $_{\Delta 160}$  (LptA<sub>m</sub>) (38). LptB point mutations were performed by Genewiz using pET22-42-LptB-His8 as template. *lptF*<sup>R212G</sup> mutation in pCDF-Duet1-LptB<sub>2</sub>FG was introduced by site-directed mutagenesis using Q5 Site-Directed Mutagenesis Kit (New England Biolabs) and AP613 (5'-ACCAGGGAACGGGCTTCGAAGGC-3') - AP614 (5'-TGAGAGTGACGACCTGGGAG-3') oligonucleotides. Plasmids pBAD/HisA-LptC-His (encoding LptC with a C-terminal His8 tag) and pCDF-Duet1-utLptB<sub>2</sub>FG (encoding untagged LptB, LptF and LptG) were used for the purification of SMA-LptB<sub>2</sub>FGC. pBAD/HisA-LptC-His is a derivative of pBAD/HisA-LptC (Narita 2009) obtained by replacing the *lptC* gene

(cloned into *NcoI*-*HindIII* sites) with the PCR-amplified *lptC*-*His8* gene using pET23/42-LptC-*His* (Villa et al., 2013) as template and AP656 (5'-catgcatggGTAAAGCCAGACGTTGGGTT-3') - AP657 (5'-cccaagcttTTAGTGGTGGTGGTGGTG-3') oligonucleotides. pCDF-Duet1-utLptB<sub>2</sub>FG is a derivative of pCDF-Duet1-LptB<sub>2</sub>FG obtained by replacing of *lptB*-*His6* gene (cloned into *NcoI*-*EcoRI* sites) with the PCR-amplified *lptB* gene using *E. coli* MG1655 genomic DNA as template and AP599 (5'-catgcatggCAACATTAAGTCAAAG-3') and AP655 (5'-gagaggaattcTCAGAGTCTGAAGTCTTCCC-3') oligonucleotides.

### Strains used and Protein Expression

LptB expression was done in *E. coli* BL21 (DE3) strain (Novagen), and LptB<sub>2</sub>FG in *E. coli* C43 (DE3) strain (Novagen). Co-expression of LptB<sub>2</sub>FG and LptC was done in *E. coli* KRX cell as described (36). LptA<sub>m</sub> and ΔTM-LptC were expressed and purified in *E. coli* BL21 (DE3) as described (22). For LptB and LptB<sub>2</sub>FG, bacterial cells were grown in Luria Broth (LB), supplemented with the correct antibiotic (ampicillin 100 μg ml<sup>-1</sup> and spectinomycin 50 μg ml<sup>-1</sup>) at 37°C, until optical density of 600 nm (OD<sub>600</sub>) around 0.7. For both sets of proteins, induction was done with isopropyl β-D-thiogalactopyranoside (IPTG): for LptB<sub>2</sub> proteins with 0.1 mM at 20°C for 16 h, while for LptB<sub>2</sub>FG proteins with 0.5 mM at 37°C for 3 h. Cells were harvested by centrifugation at 6000 xg for 20 min at 4°C, and frozen at -20°C until purification. Co-expression of LptB<sub>2</sub>FG with LptC and cell harvesting was done as before, except induction which was done with 0.02% (w/v) L-Rhamnose and 0.02% (w/v) L-Arabinose.

### Purification of LptB<sub>2</sub> proteins

Cells were mixed with buffer A containing 20 mM Tris-HCl, 150 mM NaCl, 20% (v/v) glycerol, pH 8.0, 0.5 mM Tris(2-CarboxyEthyl)Phosphine [TCEP]) and cOmplete™ EDTA-free (Sigma) and lysed by sonication. Soluble fraction was separated by centrifugation at 10.000 xg for 20 min at 4°C and loaded after addition of 10mM imidazole into Ni-NTA Agarose (QIAGEN). Resin was washed with buffer A with 20 mM Imidazole and eluted with buffer A with 300 mM Imidazole. LptB was then purified on a HiLoad® 16/600 Superdex® 200 pg column (GE Healthcare) in Tris-Buffered Saline (TBS) (50

mM Tris-HCl, 150 mM NaCl, pH 8.0) supplemented with 0.5 mM TCEP. LptB was concentrated with a 10 kDa cut-off Amicon® Ultra Centrifugal Filter (Merck) and sample concentration determined by running a 15% SDS-PAGE with known concentration samples of Bovine Serum Albumin (BSA). The yields of purified proteins for the various LptB proteins were 30, 24, 15, 5, 3 and 1 mg/ liter of culture for wild-type, H195A, E163A, E163Q, A87Q, Y13W respectively.

### Purification of LptB<sub>2</sub>FG and LptB<sub>2</sub>FGC

Cells in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 1 mM MgCl<sub>2</sub>, pH 8.0 and cOmplete™ EDTA-free [Sigma]), were lysed on a microfluidizer at 15000 psi. Cell debris are removed by centrifugation at 10000 xg for 20 min at 4°C, and membranes collected by centrifugation at 100.000 xg for 1 h at 4°C. Membranes are resuspended in 50 mM Tris-HCl, 250 mM NaCl, pH 8.0 with 0.5% SMA 25010 (Xiran SL25010 Polyscope) for 17 h at Room Temperature (RT). Soluble SMALP particles were obtained by ultracentrifugation at 100.000 xg for 30 min at 4°C and loaded into a HisTrap™ 1 ml column equilibrated in 20 mM Tris-HCl, 150 mM NaCl, 30 mM Imidazole, pH 8.0. Elution was done in a gradient with the previous buffer supplemented with 170 mM Imidazole. Fractions containing the proteins were dialyzed against TBS buffer (20 mM Tris, 150 mM NaCl, pH 8.0) at RT and concentrated with a 100 kDa cut-off Amicon® Ultra Centrifugal Filter (Merck). Sample concentration was determined by running a 15% SDS-PAGE with known concentrations of BSA.

### Electron Microscopy

SMA-LptB<sub>2</sub>FG at 59μg/ml was prepared by Negative Stain Mica-carbon Flotation Technique (MFT). Samples were absorbed to the clean side of a carbon film on mica, stained with 2% Na<sub>4</sub>O<sub>40</sub>SiW<sub>12</sub> in distilled water, then transferred to a 400-mesh copper grid. Images were taken under low dose conditions (<10 e<sup>-</sup>/Å<sup>2</sup>) with defocus values between 1.2 and 2.5 μm on a Tecnai 12 LaB6 electron microscope at 120 kV accelerating voltage using CCD Camera Gatan Orius 1000.

### NMR spectroscopy

Experiments were recorded on Bruker 600, 700, 850 and 950 MHz spectrometers equipped with triple <sup>1</sup>H, <sup>13</sup>C, <sup>15</sup>N resonance cryoprobes, at 25°C for <sup>1</sup>H and <sup>31</sup>P, and at 37°C for real-time kinetic



analysis, in TBS Buffer with 10% D<sub>2</sub>O. All 1D <sup>1</sup>H experiments were based on Bruker zgpg30 experiment with water suppression using sculpting with gradients and 15 seconds interscan delay. Data was processed using TopSpin 3.5 and Ccpnmr Analysis 2.4.2. ATPase and AK activity were checked by supplying ATP or ADP as substrate. For LptB<sub>2</sub>FG/C 5 μM of complex was incubated with 5 mM of nucleotide and 1 mM MgCl<sub>2</sub> in TBS buffer. When necessary, LptC<sub>Δ[1-23]</sub> and LptA<sub>Δ160</sub> were added at 10 μM. These activity assays, were incubated at 37°C for 7 h, flash frozen and transferred to 3 mm NMR tubes to be analyzed. For LptB, 2 μM of protein was incubated with 5 mM of nucleotide /2.5 mM MgCl<sub>2</sub> in TBS buffer at 25°C for 17 h, flash frozen and transferred to 3 mm NMR tubes. STD experiments were recorded on a 700 MHz cryoprobe at 25°C, with Bruker stddiffesg3 pulse sequence alternating on and off resonance at 0.5ppm and -40ppm, with a 60ms spinlock to suppress protein signals. 500 μM of nucleotide (ADP or ADPβS) is mixed with 12.5 μM LptB (wt or mutant) with 50 μM MgCl<sub>2</sub> in TBS buffer with 1.7% Glycerol and 10% D<sub>2</sub>O.

#### Data Availability

All data is contained within this paper.

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**Conflict of Interest:** The authors declare that the project was conducted in absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

#### Supporting information.

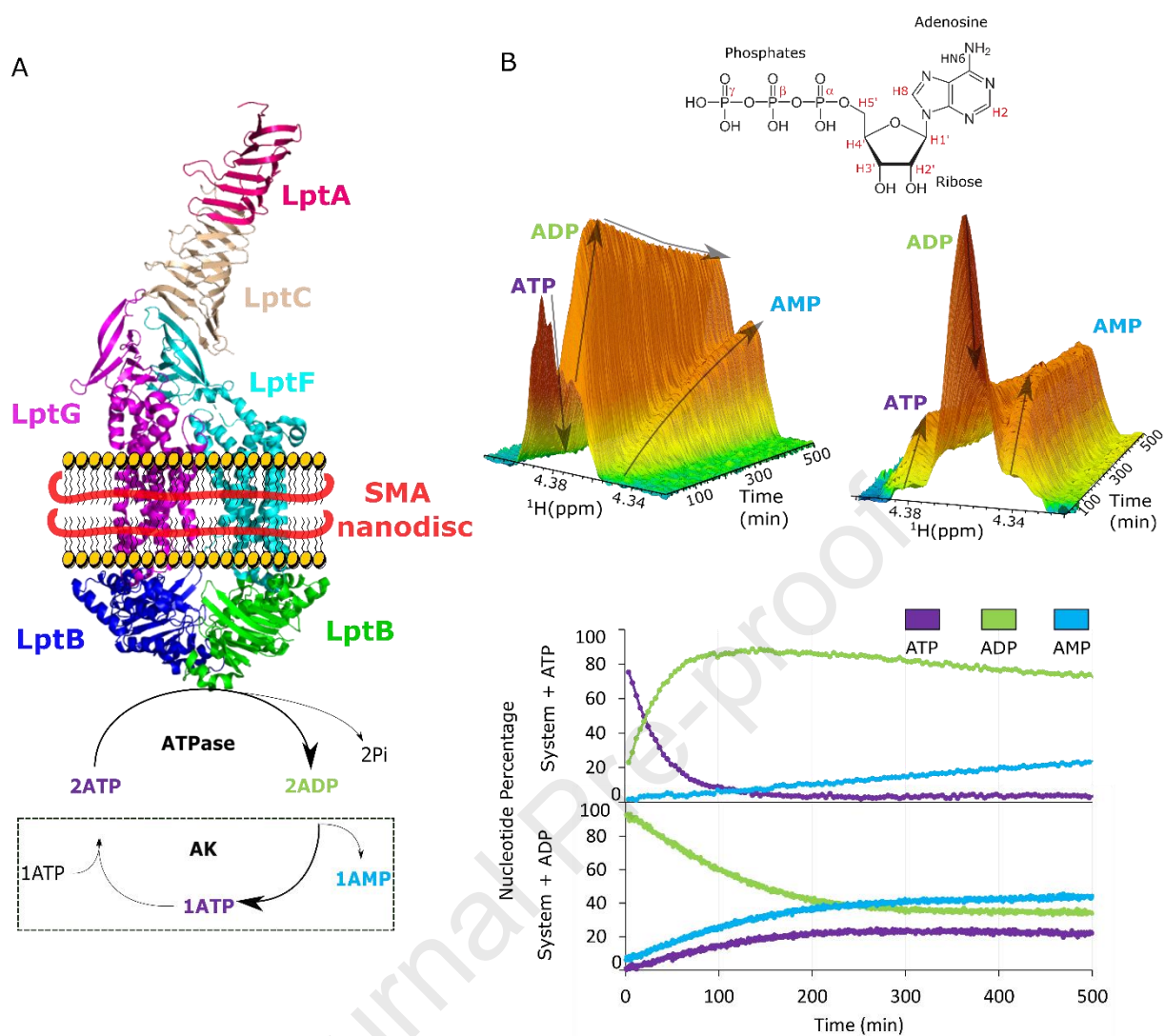
This article contains supplementary information.

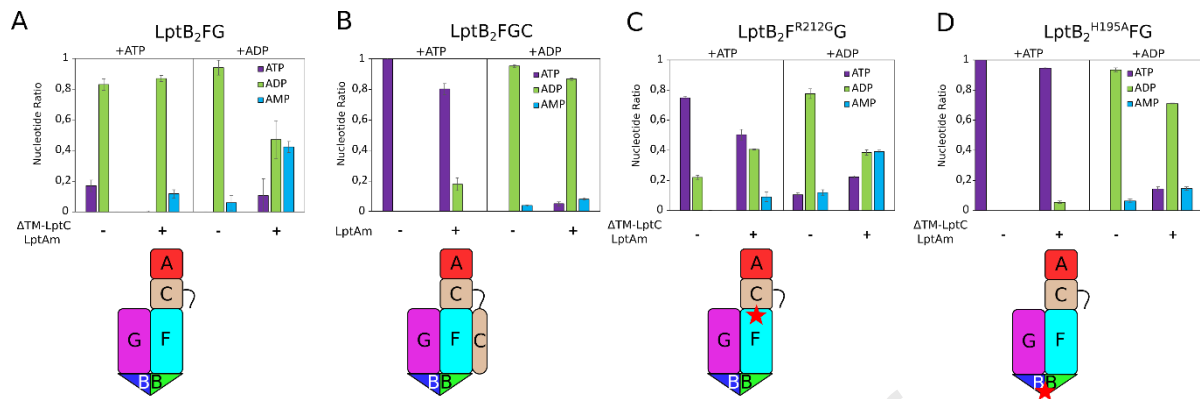
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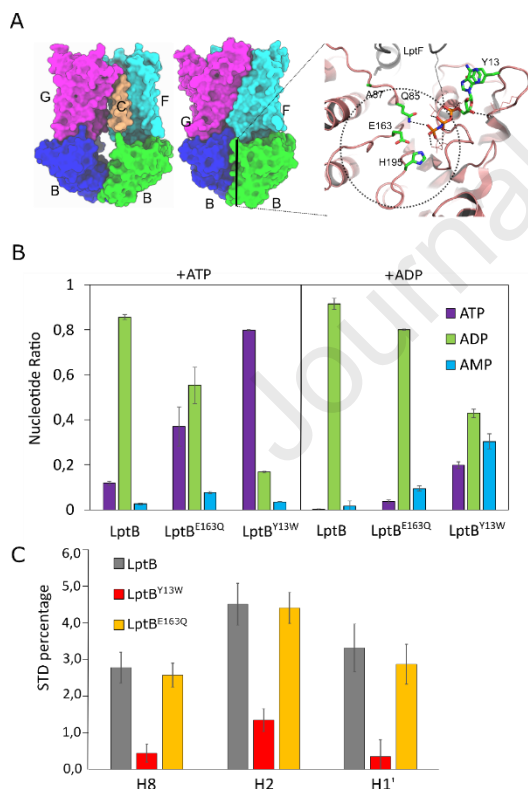
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**Figure 2 – (Top Panel) Assembly on the periplasmic or membrane level with LptC and LptA influence ATPase and AK activity of LptB<sub>2</sub>FG.** SMA-LptB<sub>2</sub>FG is incubated with ATP or ADP and nucleotides levels (ATP, ADP and AMP) quantified by <sup>1</sup>H NMR from two or three independent experiments and the standard deviation is shown. (A) SMA-LptB<sub>2</sub>FG without/with  $\Delta$ TM-LptC/LptA<sub>m</sub>, (B) SMA-LptB<sub>2</sub>FGC complex without/with LptA<sub>m</sub>. (C and D) SMA-LptB<sub>2</sub>F<sup>R212G</sup> and SMA-LptB<sub>2</sub>F<sup>H195A</sup> without/with  $\Delta$ TM-LptC/LptA<sub>m</sub>. A schematic representation of the complexes used is shown at the bottom panel, with stars marking the location of mutations.



**Figure 3 – (A) Structures of LptB<sub>2</sub>FGC in open (apo form PDB 6S8N) and closed conformation (with bound ATP analog PDB 6S8G).** The jellyroll domains were not resolved in these structures as well as the LptC transmembrane helix in the open conformation. Right panel shows ATPase site with putative location of the second ADP in the AK reaction (dashed line area). Residues mutated in this study are shown (B) ATPase/AK of LptB<sub>2</sub> and LptB<sub>2</sub><sup>E163Q</sup> and LptB<sub>2</sub><sup>Y13W</sup> mutants, with ATP or ADP as substrate. Nucleotide levels (ATP, ADP and AMP, in colour-code) were detected by <sup>1</sup>H-NMR experiment in 2 independent experiments and the standard deviation is shown; (C) Saturation Transfer Different (STD) experiments of LptB<sub>2</sub> and LptB<sub>2</sub><sup>E163Q</sup> and LptB<sub>2</sub><sup>Y13W</sup> on ADP- $\beta$ S. The three resonances shown are from H8, H2 and H1' on the adenosine of ADP- $\beta$ S. Error bars represent two times the standard deviation of the NMR experiments' noise.