Cent. Eur. J. Biol. • 9(2) • 2014 • 131-138
DOI: 10.2478/s11535-013-0250-5

VERSITA

Central European Journal of **Biology**

Characterization of lipopolysaccharide transport protein complex

Research Article

Quanju Xiang[1], Haiyan Wang[1], Zhongshan Wang[1], Yizheng Zhang[1], Changjiang Dong[2]*

[1]*College of Life Sciences, Sichuan University, Sichuan Key Laboratory of Molecular Biology and Biotechnology, 610064 Chengdu, P. R. China.*

[2]*Norwich Research Park, NR4 7TJ Norwich, United Kingdom*

Received 15 April 2013; Accepted 10 August 2013

Abstract: Lipopolysaccharide (LPS) is an essential component of the outer membranes (OM) of most Gram-negative bacteria, which plays a crucial role in protection of the bacteria from toxic compounds and harsh conditions. The LPS is biosynthesized at the cytoplasmic side of inner membrane (IM), and then transported across the aqueous periplasmic compartment and assembled correctly at the outer membrane. This process is accomplished by seven LPS transport proteins (LptA-G), but the transport mechanism remains poorly understood. Here, we present findings by pull down assays in which the periplasmic component LptA interacts with both the IM complex LptBFGC and the OM complex LptDE *in vitro*, but not with complex LptBFG. Using purified Lpt proteins, we have successfully reconstituted the seven transport proteins as a complex *in vitro*. In addition, the LptC may play an essential role in regulating the conformation of LptBFG to secure the lipopolysaccharide from the inner membrane. Our results contribute to the understanding of lipopolysaccharide transport mechanism and will provide a platform to study the detailed mechanism of the LPS transport *in vitro*.

Keywords: *Protein Interactions • Protein complex • Pull down • Protease treatment • Crosslinker*

1. Introduction

The outer membrane of Gram-negative bacteria is a unique asymmetric lipid bilayer, with lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet [1]. The presence of LPS on the cell surface enhances the barrier function of the outer membranes (OM), making many antibiotics used to treat Gram-positive infections ineffective to control Gram-negative pathogens [2]. Although in certain Gram-negative bacteria, the LPS is absent or not necessary [3], it is essential in *Escherichia coli* and most of other Gram-negative bacteria including many pathogens.

LPS is synthesized in the cytoplasmic face of the inner membrane (IM). After synthesis, it is transported to the periplasmic leaflet by MsbA, which is an ATP-binding cassette transporter [4]. Genetic and cellular studies have shown that seven lipopolysaccharide transport (Lpt) proteins, *i.e.* LptA-G, are essential for the following LPS transport. LptB, -F, and -G form an ATP-binding cassette transporter LptBFG. The ATP hydrolysis within LptBFG is thought to provide energy for LPS transport and facilitate the release of mature LPS from the IM and enable its transfer to the periplasmic carrier molecule LptA [5-7]. The LptC anchors to the IM through an N-terminal transmembrane helix to form a complex with LptBFG and its periplasm domain was found to be the essential function region [8]. However, its function is not well understood within the complex LptBFGC [5,9-11]. LptA was proposed to act as a periplasmic chaperone for LPS transport across the periplasm and reported to interact with IM protein LptC [12,13] and OM protein LptD [13]. LptD and LptE form a stable complex at the OM which is proposed to serve as a translocon that facilitates the passage of LPS across the OM bilayer [14]. LptE stabilizes LptD by interacting with its C-terminal domain, and binds LPS, possibly serving as a substrate recognition site at the OM [14].

* *E-mail: c.dong@uea.ac.uk*

All the Lpt proteins could be cofractionated and copurified *in vivo*, suggesting these seven proteins can form a continuous complex spanning IM and OM [15]. Moreover, depletion of any Lpt protein resulted in the accumulation of LPS at the periplasmic face of IM [11], suggesting Lpt proteins function in a highly cooperative manner to guarantee the correct transport, targeting and assembly of LPS into outer membrane. However, the detailed mechanisms of how these Lpt proteins cooperate and coordinate the transport of large, amphipathic LPS molecules across the two membranes and the periplasmic space are not well understood.

Here we report the physical interactions between LptA and the membrane complexes *in vitro*, in which the periplasmic LptA physically interacted with both the IM complex LptBFGC and OM complex LptDE. Using purified Lpt proteins, we have reconstituted the seven proteins as a complex *in vitro*. These results provide a platform to study the detailed mechanism of LPS transport *in vitro*. In addition, we demonstrate that the inner-membrane component LptBFG undergoes a conformational change upon binding LptC.

2. Experimental Procedures

2.1 Cloning of the seven Lpt genes

The primers used in this study are listed in Table 1. The genomic DNA of *E. coli* K-12 MG1655 was used to amplify the Lpt genes. The gene for LptA was cloned to pET28b (+) vector with a C-terminal Strep-tag to generate expression plasmid pLptA. The genes for LptC and LptD with the predicted signal/TM sequences were cloned into pET28b (+) vector without any tags, producing plasmids pLptC and pLptD. The genes for the ABC transporter LptBFG were cloned into pACYCDuet-1vector (Novagen), carrying a C-terminally hexahistidine tagged LptB at the first multiple cloning site and the genes for LptG and LptF at the second site, resulting in the expression plasmid pLptBFG. The sequence of LptE was cloned to pACYCDuet vector with a C-terminal His-tag at the first multiple cloning site to obtain plasmid pLptE.

The recombinant plasmids were transformed into *E. coli* DH5α competent cells for amplification. The pLptA was transformed into BL21 (DE3) for expression of protein LptA-Strep. The C43 (DE3) cells transformed with pLptBFG, pLptC/pLptBFG or pLptD/pLptE were used to express complexes LptBFG-His_6, LptBFGC-His_6 or LptDE-His_6.

2.2 Protein overexpression and purification

The glycerol stocks were used to inoculate 10 ml sterile LB media containing 34 μg mL^{-1} kanamycin (Kan) and/or chloramphenicol (Chl) and grown overnight at 37°C. For the expression of LptA-Strep, 1 L of LB (34 μg mL^{-1} Kan) was inoculated with the starter culture, grown to OD_{600}~1 at 37°C, induced with 0.2 mmol L^{-1} IPTG overnight at 20°C. For the expression of LptBFG-His_6 and LptBFGC-His_6, 1 L of LB (34 μg mL^{-1} Chl for LptBFG, 34 μg mL^{-1} Chl

Primers	Sequence (5'~3')
LptA-F	CATGCC ATG GCAATGAAATTCAAAACAAACAAACTC
LptA-R	TATACTCGAGTTACTTTTCGAACTGCGGGTGGCTCCAATTACCCTTCTTCTGTGCCG
LptB-F	CATGCCATGGCAACATTAACTGCAAAG
LptB-R	ATCGGAATTCTTAATGATGATGATGATGATGCTCGAGTCTGAAGTCTTCCCCAAGG
LptC-F	CATGCCATGGGTAAAGCCAGACGTTGGGTTATC
LptC-R	TCGCAAGCTTAAGGCTGAGTTTGTTTGTTTTG
LptD-F	AGCTCCATGGTGAAAAAACGTATCCCCACTCTC
LptD-R	TTATGCGGCCGCTCACAAAGTGTTTTGATACGGCAGA
LptE-F	TATACATATGCGATATCTGGCAACATTG
LptE-R	TATACTCGAGTTAGTGGTGATGATGGTGATGGTTACCCAGCGTGGT GGAGAC
LptF-F	GGAATTCCATATGATAATCATAAGATATCTGGTGCGGGAGAC
LptG-N	TATACTCGAGTTACGATTTTCTCATTAACAGCCACAGG

Table 1. Oligonucleotide primers used in this study.

The restriction enzyme sites were underlined. The sequences for His6 - and sterp-tactin tags were boxed.

and Kan for LptBFGC) was inoculated with the starter culture, grown to OD_{600}~1 at 37°C, induced with 0.1 mmol mL^{-1} IPTG for another 3 h at 37°C. For the expression of LptDE-His_6, 1 L of LB (34 μg mL^{-1} Chl and Kan) was inoculated with the starter culture, grown to OD_{600}~0.6 at 26°C, induced with 0.1 mmol mL^{-1} IPTG for another 20 h at 26°C. The cells were harvested by centrifugation at 5,000 x *g* for 15 min and the pellets were dissolved in the lysis buffer (50 mmol mL^{-1} Tris-HCl pH 7.8, 5% (v/v) glycerol and 400 mmol mL^{-1} NaCl) containing 1 mmol mL^{-1} phenylmethylsulfonyl fluoride (PMSF, Sigma), 50 μg mL^{-1} DNase I (Sigma) and 1 tablet of protease inhibitor (Roche). The resuspended cells were disrupted by passage through a French Press (Thermo Electron) at 30,000 psi. The cell lysate of LptA-Strep was centrifuged at 15,000 × *g* for 30 min to remove unbroken cells, and the cell lysates of membrane proteins were centrifuged at 5,000 × *g* for 15 min to remove unbroken cells.

2.3 Protein purifications

The supernatant of LptA-Strep was loaded onto 0.5 mL Strep-tactin superflow plus beads (Qiagen) pre-equilibrated with the lysis buffer and incubated at 4°C for 30 min with rocking. The beads were washed twice with the lysis buffer. The LptA-Strep protein was eluted with buffer consisting of 50 mmol mL^{-1} Tris-HCl, pH 7.8, 400 mmol mL^{-1} NaCl, 5% (v/v) glycerol and 2.5 mmol mL^{-1} desthiobiotin (Sigma). The eluted protein was further purified by a pre-equilibrated Superdex 200 size exclusion column (SEC). The running buffer contained 20 mmol mL^{-1} Tris-HCl, pH 7.8, 100 mmol mL^{-1} NaCl and 5% (v/v) glycerol.

The purification of LptDE-His_6 was performed as previously described with minor changes [14]. The supernatant was ultracentrifuged at 100,000 × *g* for 1 h. The pellet was extracted with TBS (20 mmol mL^{-1} Tris-HCl, pH 8.0, 150 mmol mL^{-1} NaCl)/1% N-lauroylsarcosine (sodium salt) (Sigma) at 20°C for 3 h and re-ultracentrifuged as above. The obtained pellet was extracted with TBS-B (20 mmol mL^{-1} Tris-HCl, pH 8.0, 300 mmol mL^{-1} NaCl, 5% glycerol and 10 mmol mL^{-1} imidazole) / 2% SB-314 (Sigma) overnight at 4°C and ultracentrifuged as above to harvest the supernatant.

The supernatant of LptDE-His_6 was loaded to 4 mL Ni-NTA resin (Sigma) pre-equilibrated with TBS-B/0.016% DDM (Anatrace) and incubated at 4°C with rocking for 1 h, and the beads were washed with wash buffer (20 mmol mL^{-1} Tris-HCl, pH 8.0, 300 mmol mL^{-1} NaCl, 5% (v/v) glycerol, 30 mmol mL^{-1} imidazole and 0.016% DDM). The LptDE-His_6 protein was eluted with buffer consisting of 20 mmol mL^{-1} Tris-HCl, pH 8.0, 300 mmol mL^{-1} NaCl, 5% (v/v) glycerol, 300 mmol mL^{-1} imidazole and 0.016% DDM). The LptDE complex was further purified by a pre-equilibrated Superdex 200 size exclusion column using 20 mmol mL^{-1} Tris-HCl, pH 7.8, 100 mmol mL^{-1} NaCl, 5% (v/v) glycerol and 0.016% DDM as the running buffer.

The supernatants of LptBFG(C)-His_6 were ultracentrifuged at 100,000 × *g* for 1 h and the pellets were extracted with buffer containing 20 mmol mL^{-1} Tris-HCl, pH 7.5, 5 mmol mL^{-1} $MgCl_2$, 1% DDM, 10% (v/v) glycerol and 2 mmol mL^{-1} ATP at 4°C overnight with rocking. The rest purification steps are the same as for the LptDE-His_6. All the proteins were analyzed by SDS-PAGE and confirmed by mass spectrometry (MS).

2.4 Protease sensitivity assays

Trypsin was ordered from Sigma-Aldrich and the stock solution was made up as previously described [16]. Purified LptBFG and LptBFGC complexes were incubated with proteases at room temperature at a mass ratio of 1:50. Samples were analyzed by BN-PAGE or SDS-PAGE after 4–5 h.

2.5 Protein and protein interactions by pull-down assays

In order to investigate LptA-LptDE interaction, we use 0.5 mg LptDE-His6 to bind 0.2 mL Ni-NTA beads. Then the beads were washed with 10 times column volume of the lysis buffer and loaded with 0.5 mg LptA-Strep. We incubated the mixture for 1 h at 4°C with constant agitation. Subsequently, we washed the beads again with 10 column volumes of the lysis buffer. Finally we got the bound proteins with a buffer containing 50 mmol mL^{-1} Tris-HCl, pH 7.8, 100 mmol mL^{-1} NaCl, 5% (v/v) glycerol, 300 mmol mL^{-1} imidazole and 0.016% DDM. The reverse test was carried out with 0.5 mg of LptA-Strep bound to 0.2 mL Strep-tactin superflow plus beads, washed and 0.5 mg of LptDE-His_6 added for 1h at 4°C, washed again and the proteins were eluted with a buffer containing Tris-HCl, pH 7.8, 100 mmol mL^{-1} NaCl, 5% (v/v) glycerol, 2.5 mmol mL^{-1} desthiobiotin and 0.016% DDM.

The interactions of LptA-LptBFGC and LptA-LptBFG were investigated by the same steps as for LptA-LptDE. The results were analyzed by SDS-PAGE and the corresponding protein bands were verified by mass spectrum.

2.6 Formation of Seven Lpt Protein complex by cross-linking

Purified proteins, namely LptA, LptBFGC and LptDE were mixed together at the same molar ratio. The buffer was changed to PBS containing 10% glycerol and 0.0016% DDM using a buffer change column (Pierce). The crosslinker reagent Dithiobis (succinimidyl

propionate, DSP) was added to the mixture to a final concentration of 1mM and incubated at 25°C for 30 to 40 min. The reaction was stopped by directly adding non-reducing protein loading buffer and samples were run on 4-12% Bis-Tris SDS-PAGE gel (Invitrogen). Meanwhile, samples with reducing protein loading buffer were run on the same gel as a control.

3 Results and Discussion

3.1 LptC may cause the conformational change of LptBFG

LptBFGC is a unique ABC transporter in the sense that unlike most ABC transporters it employs an additional subunit LptC to facilitate the LPS transport and the subunit ratios of LptBFG and LptBFGC were reported to be $LptB_2F_1G_1$ and $LptB_2F_1G_1$ C_1 respectively [5]. The molecular masses of protein-detergent complex of LptBFG and LptBFGC determined by SEC were approximately 130 kDa and 150 kDa in the solution we used (Figure 1). In this case, they seemed to exist as monomers. However, BN-PAGE analysis of the SEC fractions revealed two separate bands (Figure 2A), implying they migrated as both monomer and dimer in BN-PAGE gel, although the monomer was the majority. Since there was only one peak appeared in the curve (Figure 1), this monomer/dimer complex could not be separated by SEC. LptC can dimerize both *in vivo* and *in vitro* [17]. It is possible that LptC interacts with

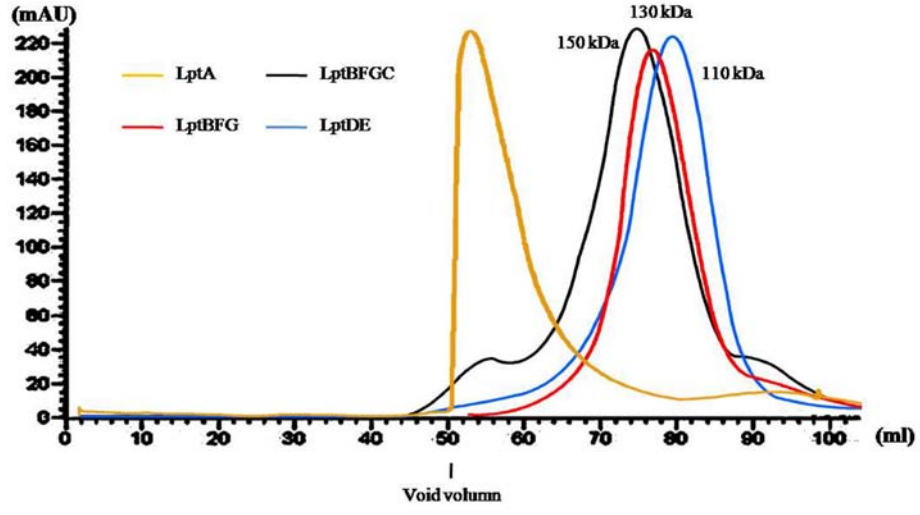

Figure 1. Size-exclusion chromatograph of LptA (yellow curve), LptBFGC (black curve), LptBFG (red curve) and LptDE (blue curve). The SEC of LptBFG, LptBFGC and LptDE indicated approximate molecular weights of 130 kDa, 150 kDa and 110 kDa respectively, corresponding to monomers. The SEC of LptA showed that LptA eluted at the void volume, indicating that the LptA exists as oligomers in the solution.

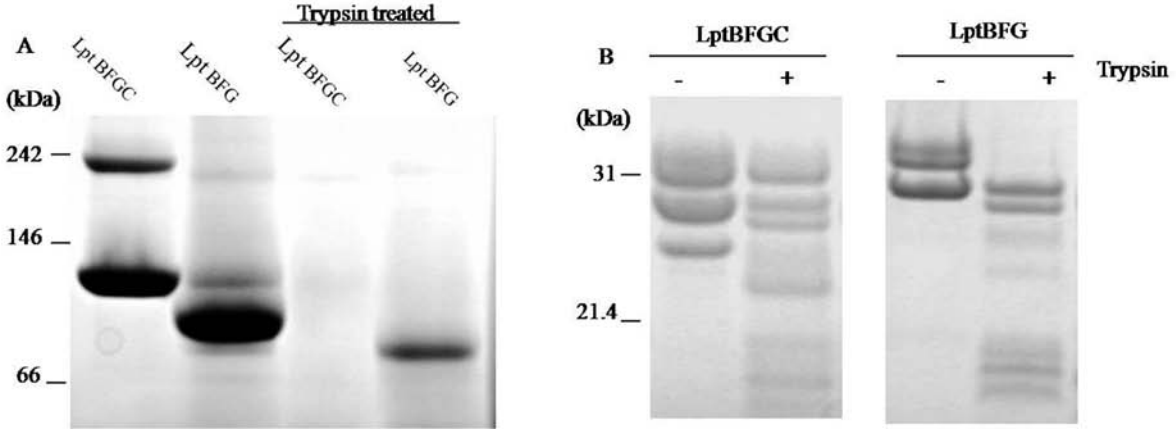

Figure 2. BN-PAGE (A) and SDS-PAGE (B) analyses of purified LptBFGC and LptBFG complexes. (A) BN-PAGE analysis of purified LptBFGC and LptBFG complexes with or without trypsin. (B) SDS-PAGE analysis of purified LptBFGC and LptBFG complexes with or without trypsin. The LptBFG and LptBFGC were treated with trypsin at the molar ration of 1:50 at room temperature for 3 h and analyzed by BN-PAGE or SDS-PAGE. The protein bands were visualized by staining with Coomassie-blue and positions of relevant molecular weight markers are indicated in kDa.

itself and results in dimerization of LptBFGC in solution, which may explain why more dimer of LptBFGC than that of LptBFG was observed on the BN-PAGE gel. Previous studies reported that LptC also existed as a monomer in solution [18], and this discrepancy might be caused by the use of different experimental approaches or conditions.

BN-PAGE analysis of trypsin-digested LptBFGC showed that the bands completely disappeared from the gel (Figure 2A), implying that the linkages among the four subunits might be digested by trypsin and the three-dimensional organization of LptBFGC was destroyed. Consequently, the four subunits migrated separately (Figure 2B). However, a part of trypsin-digested LptBFG remained intact, migrating as one single band in the BN-PAGE gel (Figure 2A). These results suggested that LptBFG might undergo a conformational change upon binding LptC, presumably through complex formation. The different responses to trypsin treatments might be caused by the exposure of trypsin–sensitive regions which become the protease substrates when LptBFG binds LptC.

Although the crystal structure of LptC has been described in detail [18], the molecular mechanisms of LptC in LPS transport can be elucidated only when LptC is reconstituted into proteoliposomes as LptBFGC. The functions of ABC transporters have been clarified by detailed studies of crystal structures [19,20]. It would be interesting and important to know how the accessory protein LptC affects the three-dimensional organization of LptBFG complex and thereby contributes to LPS transport. Understanding the structure of the complex LptBFGC will undoubtedly help us to elucidate the specific function of LptC.

3.2 LptA interacts with both IM and OM Lpt complex

LptA, the only periplasmic Lpt component without a membrane tether, has been proposed to physically link the IM and OM fractions [15]. Moreover, an intermolecular interaction that LptA can be co-purified with overexpressed LptC has already been established [17], and the two proteins can be stably associated when purified separately [12]. Recent studies showed that LptA interacts with the C-terminal part of LptC at the IM and the N-terminal part of LptD at the OM *in vivo* [13]. To investigate whether these interactions still remain when LptC and LptD bind to their partners to form complex LptBFGC and LptDE, the pull-down assays between LptA and LptDE (Figure 3A), LptA and LptBFGC (Figure 3B) were performed under the conditions as described above. The assays were carried out respectively using LptA-Strep, or LptBFGC-His_6 and LptDE-His_6 as baits. The LptA remained bound to LptBFGC and LptDE, indicating that LptA interacted with both LptBFGC and LptDE. As a control, the interaction between LptA and LptBFG was also investigated. LptA and LptBFG were found only in the Strep-tactin (Figure 3C, Lane 15) or Ni-NTA (Figure 3C, Lane 11) elutions, suggesting that LptA did not bind to LptBFG.

Previous studies showed that LptA remained bound to LptC even after a wash with 1 m mL^{-1} NaCl

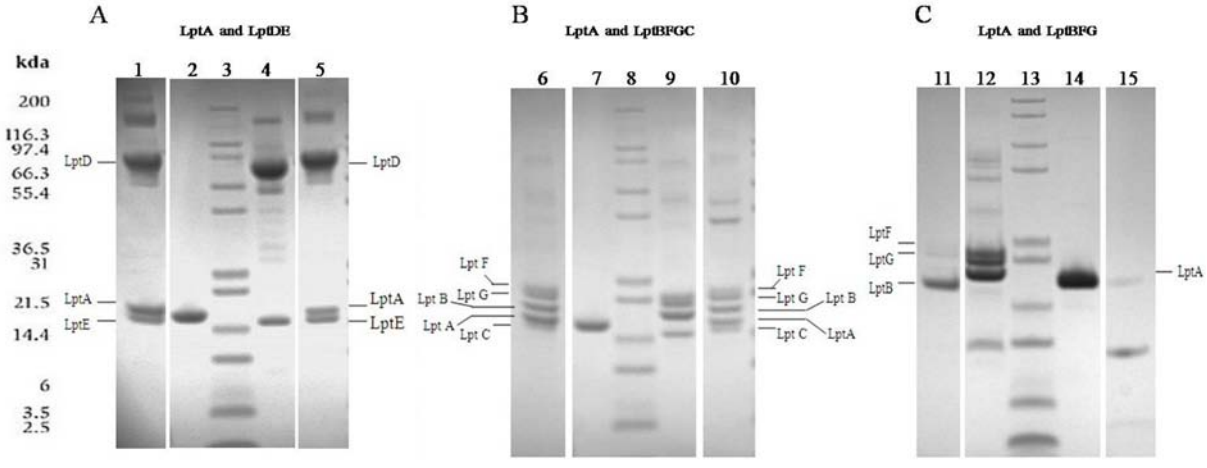

Figure 3. Complex formations between LptA and LptDE (A), LptA and LptBFGC (B), LptA and LptBFG (C) as shown by pull-down assays and visualized on SDS-PAGE. (A) Purified LptA-Strep (Lane 2) and LptDE-His_6 (Lane 4) were mixed and run on both Ni-NTA and Strep-tactin beads. In Strep-tactin (Lane 1) and Ni-NTA (Lane 5) elution, a complex was eluted. (B) Purified LptA-Strep (Lane 7) and LptBFGC-His_6 (Lane 9) were mixed and run on both Ni-NTA and Strep-tactin beads. In Strep-tactin (Lane 6) and Ni-NTA (Lane 10) elution, a complex was eluted. (C) Purified LptA-Strep (Lane 14) and LptBFG-His_6 (Lane 12) were mixed and run on both Ni-NTA and Strep-tactin beads. In Strep-tactin (Lane 15) and Ni-NTA (Lane 11) elution, only LptA or LptBFG was eluted. Lane 3, 8 and 13 represent the protein marker. The gels were stained with Coomassie Blue. Molecular masses (in kDa) and protein components are indicated to the left or right of the gels.

[12], indicating there are strong interactions between LptA and LptC. However, this was not the case in our study as no interaction was detected between LptA and LptBFGC when a higher concentration of NaCl was used (300 mmol mL^{-1}, data not shown). This result suggests that the three-dimensional organization of LptC may be changed when forming complex with LptBFG as shown above and the interaction between the LptA and LptBFGC becomes weaker.

We show here by pull down assays that *in vitro* LptA still binds to IM complex LptBFGC and OM complex LptDE. Although the IM component LptFG possesses periplasmic loops as well [9], no interaction was detected between LptA and LptBFG, further confirming that LptC may act as a dock site for LptA oligomers at the IM and thereby assist with LPS extraction from the inner membrane and its subsequent export to the surface. Since LptA, LptC, and N-LptD all belong to the OstA structural superfamily [6,21,22], and that LptA physically interacts with the N-terminal of LptD [13], the N-LptD protein might be the docking site for LptA at the OM. LptC was predicted to have a single transmembrane helix (Trp^{7}-Asp^{29}) and a large soluble domain [11,18], and its C-terminal part is involved in the interactions with LptA [13] and the N-terminal region is responsible for binding to LptBFG [8]. An earlier study suggested that Due to the structural similarity between LptC and the periplasmic loop of LptF, it is possible that LptF is the candidate part for interactions in this complex [17].

3.3 Lpt protein complexes formed *in vitro*

As discussed above, LptA physically interacted with both LptBFGC and LptDE and the seven Lpt proteins were reported to form a trans-envelope complex *in vivo* [15]. However, whether these Lpt proteins can form a complex *in vitro* is still unknown. In this study, we put the seven Lpt proteins together and used Strep-LptA as the bait, but no interactions were detected among them by pull down assay (Figure 4B). In addition, when the seven Lpt proteins were mixed together, no corresponding band or peak were observed by BN-PAGE or SEC, which suggest that the seven proteins neither form a complex nor formed an unstable complex *in vitro*.

In order to check whether Lpt proteins could form a complex *in vitro*, the crosslinker DSP was introduced into the reactions to stabilize interactions. The same molar mass of LptBFGC, LptDE and LptA were put together and cross-linked as described in the Materials and methods. Two separate bands were observed from SDS-PAGE gel with non-reducing protein loading buffer (Figure 4A, lane 3), and Lpt proteins with reducing protein loading buffer migrated individually (Figure 4A, lane 1). MS of the two protein bands (Figure 4A, lane 3) revealed that six of the seven Lpt proteins (except LptC) were detected in each band, indicating that they assembled into the predicted complex. According to the molecular masses of the complex, it migrated faster than expected. One possible explanation is that the hydrophobic

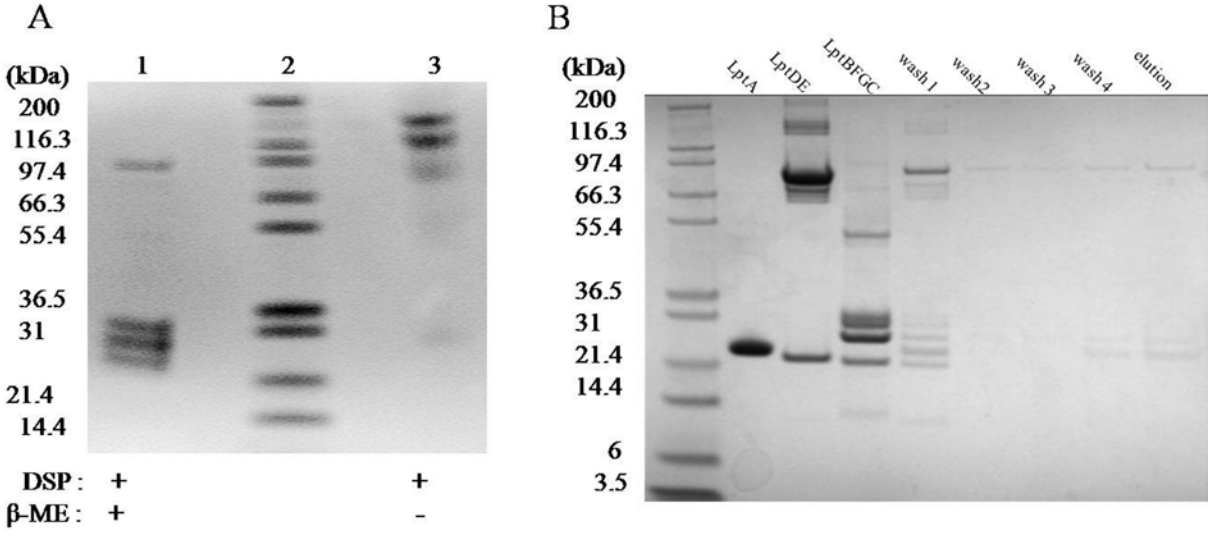

Figure 4. SDS-PAGE analyses of complex formation of the Lpt proteins. (A) Cross-linked Lpt proteins and visualized on SDS-PAGE. Seven Lpt proteins and 1mM DSP were mixed together and incubated at 25°C for 30 min. Lane 1: Lpt proteins with reducing protein loading buffer (β-ME); Lane 2: marker protein; Lane 3: Lpt proteins with non-reducing protein loading buffer. (B) Complex formation of the Lpt proteins as shown by pull-down assays and visualized on SDS-PAGE. Purified LptA-Strep, LptDE-His6 and LptBFGC-His6 were mixed and LptA-Strep was used as the bait. The mixture was washed with the lysis buffer four times (wash 1, 2, 3 and 4) and proteins were eluted (elution).

properties of the membrane proteins enable them to bind more SDS than soluble proteins.

The reason that LptC was not detected in the two bands might be due to the use of the crosslinker. Since crosslinker DSP reacts with lysines in the proteins, and the trypsin enzyme that we standardly use to digest the proteins into peptides digests the proteins at lysines and arginines. So if the lysines are blocked by the crosslinker, the only available digestion site for trypsin is arginine. This makes the peptides being produced much larger. Additionally, the extra mass of the crosslinker will make the peptide bigger and potentially outwith the mass spectrum analysable range. These reasons add up to poor coverage of proteins of interest, or no coverage in the case of LptC, since LptC is a relatively small protein with only 166 residues.

Previous findings showed that the LptA monomers are packed as a linear filament when crystallized in the presence of LPS [6], suggesting that oligomerization of LptA is the functional status. Since Strep-LptA seems to exist in an oligomeric state (Figure 1, yellow curve) in the current solution, the two bands may contain different amounts of LptA. As both the N- and the C-terminus of LptA are involved in the protein and protein interactions in the seven protein complex and LptA existed as oligomers, one of the LptA termini may be occluded by the affinity tag and thus prevent the proper 7 protein complex assembly. The findings that LptA proteins migrated as single complexes in SDS-PAGE gels when cross-linked suggest that in vitro the Lpt proteins can form a single complex.

The permeability barrier function of Gram-negative organisms mainly depends on the proper assembly of LPS, and therefore the Lpt proteins provide new opportunities to discover new antibiotics [23,24]. A better understanding of the mechanism of how Lpt proteins facilitate LPS transport and assembly would help us finding ways to interfere with the pathway. The fact that the Lpt complex spans two membranes poses a challenge for studying the mechanism of LPS transport. In this study, we reconstituted the Lpt protein complex *in vitro*, which provides a platform to study the LPS transport mechanism. Further studies are needed to reveal the underlying mechanism, as well as the specific roles played by the seven essential Lpt proteins in this process.

Acknowledgement

We thank Prof. Jim Naismith for providing the experimental instruments, Dr. Sally Shirran for processing the mass spectrum sequencing, and Dr. Ping Li for providing the reagent DSP.

This study was supported by grants from the National Science Foundation of China (No. 30911130136) and Welcome Trust Career Development Fellowship (WT083501MA) and The Royal society.

References

[1] Funahara Y., Nikaido H., Asymmetric localization of lipopolysaccharides on the outer membrane of Salmonella typhimurium, J. Bacteriol., 1980, 141, 1463-1465

[2] Nikaido H., Molecular basis of bacterial outer membrane permeability revisited, Microbiol. Mol. Biol. Rev., 2003, 67, 593-656

[3] Steeghs L., Den Hartog R., Den Boer A., Zomer B., Roholl P., van der Ley P., Meningitis bacterium is viable without endotoxin, Nature, 1998, 392, 449-449

[4] Zhou Z., White K.A., Polissi A., Georgopoulos C., Raetz C.R., Function of Escherichia coli MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis., J. Biol. Chem.,1998, 273, 12466-12475

[5] Narita S., Tokuda H., Biochemical characterization of an ABC transporter LptBFGC complex required for the outer membrane sorting of lipopolysaccharides, FEBS Lett., 2009, 583, 2160-2164

[6] Suits M.D.L., Sperandeo P., Dehò G., Polissi A., Jia Z., Novel structure of the conserved gram-negative lipopolysaccharide transport protein A and mutagenesis analysis, J. Mol. Biol., 2008, 380, 476-488

[7] Okuda S., Freinkman E., Kahne D., Cytoplasmic ATP hydrolysis powers transport of lipopolysaccharide across the periplasm in E. coli, Science, 2012, 338, 1214-1217

[8] Villa R., Martorana A.M., Okuda S., Gourlay L.J., Nardini M., Sperandeo P., et al., The Escherichia coli Lpt transenvelope protein complex for lipopolysaccharide export is assembled via conserved structurally homologous domains, J. Bacteriol., 2013, 195, 1100-1108

[9] Ruiz N., Gronenberg L.S., Kahne D., Silhavy T.J., Identification of two inner-membrane proteins required for the transport of lipopolysaccharide to the outer membrane of Escherichia coli, Proc. Natl. Acad. Sci., 2008, 105, 5537-5542

[10] Sperandeo P., Cescutti R., Villa R., Di Benedetto C., Candia D., Dehò G., et al., Characterization of lptA and lptB, two essential genes implicated in lipopolysaccharide transport to the outer membrane of Escherichia coli, J. Bacteriol., 2007, 189, 244-253

[11] Sperandeo P., Lau F.K., Carpentieri A., De Castro C., Molinaro A., Dehò G., et al., Functional analysis of the protein machinery required for transport of lipopolysaccharide to the outer membrane of Escherichia coli, J. Bacteriol., 2008, 190, 4460-4469

[12] Bowyer A., Baardsnes J., Ajamian E., Zhang L., Cygler M., Characterization of interactions between LPS transport proteins of the Lpt system, Biochem. Biophys. Res. Commun., 2011, 404, 1093-1098

[13] Freinkman E., Okuda, S. Ruiz N., Kahne D., Regulated assembly of the transenvelope protein complex required for lipopolysaccharide export, Biochemistry, 2012, 51, 4800-4806

[14] Chng S.S., Ruiz N., Chimalakonda G., Silhavy T.J., Kahne D., Characterization of the two-protein complex in Escherichia coli responsible for lipopolysaccharide assembly at the outer membrane, Proc. Natl. Acad. Sci., 2010, 10, 5363-5368

[15] Chng S.S., Gronenberg L.S., Kahne D., Proteins required for lipopolysaccharide assembly in Escherichia coli form a transenvelope complex, Biochemistry, 2010, 49, 4565-4567

[16] Wernimont A., Edwards A., In situ proteolysis to generate crystals for structure determination: an update, PLoS One, 2009, 4, e5094

[17] Sperandeo P., Villa R., Martorana A.M., Šamalikova M., Grandori R., DehòG., et al., New insights into the Lpt machinery for lipopolysaccharide transport to the cell surface: LptA-LptC interaction and LptA stability as sensors of a properly assembled transenvelope complex, J. Bacteriol., 2011, 193, 1042-1053

[18] Tran A. X., Dong C., Whitfield C., Structure and functional analysis of LptC, a conserved membrane protein involved in the lipopolysaccharide export pathway in Escherichia coli, J. Biol. Chem., 2010, 285, 33529-33539

[19] Davidson A.L., Chen J., ATP-binding cassette transporters in bacteria, Annu. Rev. Biochem., 2004, 73, 241-268

[20] Dawson R.J.P., Hollenstein K., Locher K.P., Uptake or extrusion: crystal structures of full ABC transporters suggest a common mechanism, Mol. Microbiol., 2007, 65, 250-257

[21] Bos M.P., Robert V., Tommassen J., Biogenesis of the gram-negative bacterial outer membrane, Annu. Rev. Microbiol., 2007, 61, 191-214

[22] Bateman A., Coin L., Durbin R., Finn R. D., Hollich V., Griffiths-Jones S., et al., The Pfam protein families database, Nuc. Acids Res., 2004, 32, D138-D141

[23] Gronenberg L.S., Kahne D., Development of an activity assay for discovery of inhibitors of lipopolysaccharide transport, J. Am. Chem. Soc., 2010, 132, 2518-2519

[24] Srinivas N., Jetter P., Ueberbacher B.J., Werneburg M., Zerbe K., Steinmann J., et al., Peptidomimetic antibiotics target outer-membrane biogenesis in Pseudomonas aeruginosa, Science, 2010, 327, 1010-1013