FEBS *Letters*

Biochemical characterization of an ABC transporter LptBFGC complex required for the outer membrane sorting of lipopolysaccharides

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ABSTRACT

Seven Lpt proteins (A through G) are thought to be involved in lipopolysaccharide transport from the inner to outer membrane of *Escherichia coli*. LptB belongs to the ATP-binding cassette transporter superfamily. Although the *lptB* gene lacks neighboring genes encoding membrane subunits, bioinformatic analyses recently indicated that two distantly located consecutive genes, *lptF* and *lptG*, could encode membrane subunits. To examine this possibility, LptB was expressed with LptF and LptG. We report here that both LptF and LptG formed a complex with LptB. Furthermore, an inner membrane protein, LptC, which had been implicated in lipopolysaccharide transport, was also included in this complex.

Structured summary:
MINT-7137021: *lptb* (uniprotkb:P0A9V1) *physically interacts* (MI:0914) with *lptc* (uniprotkb:P0ADV9), *lptg* (uniprotkb:P0ADC6) and *lptf* (uniprotkb:P0AF98) by *pull down* (MI:0096)
MINT-7137160: *lptb* (uniprotkb:P0A9V1) *physically interacts* (MI:0914) with *lptf* (uniprotkb:P0AF98) and *lptg* (uniprotkb:P0ADC6) by *pull down* (MI:0096)

1. Introduction

The outer membrane (OM) of gram-negative bacteria contains lipopolysaccharides (LPS) in the outer leaflet and phospholipids in the inner leaflet [1]. β-Barrel proteins span OM while most lipoproteins are anchored to OM through acyl chains attached to the N-terminal cysteine [2]. These components are transported from the inner membrane (IM) to OM through specialized targeting pathways [3].

The ATP-binding cassette (ABC) transporter MsbA flips LPS from the inner to outer leaflet of IM [4]. Analyses of *Escherichia coli* mutants revealed that IM proteins, LptB and LptC, and a periplasmic protein, LptA, are required for the LPS transport to the inner leaflet of OM [5,6], where an LptD-LptE (formerly Imp-RlpB) complex flips LPS to the outer surface [7,8]. Tran et al. [9] speculated that LPS is targeted to OM through a pathway analogous to the lipoprotein-targeting system comprising the ABC transporter LolCDE, the periplasmic carrier protein LolA, and the OM receptor protein LolB [2]. In contrast, LPS transport has been proposed to occur via sites connecting IM and OM [10].

The *lptB* gene located at 72.0 min on the *E. coli* chromosome encodes an ABC protein. Unlike other bacterial ABC transporters, *lptB* lacks neighboring genes that encode membrane subunits so as to form an ABC transporter complex. Bioinformatic exploration allowed identification of genes for the membrane subunits that might constitute the ABC transporter with LptB [11]. The two essential genes, *yjgP* and *yjgQ*, form an operon at 96.7 min on the *E. coli* chromosome. Depletion of either gene caused a defective envelope, the defect being similar to that caused by LptB depletion [11]. Thus, *yjgP* and *yjgQ* were suggested to encode membrane subunits, and were renamed *lptF* and *lptG*, respectively. However, it remained unclear whether their depletion directly impairs LPS transport and whether LptB forms a complex with them. Here we present the first biochemical evidence for the formation of an ABC transporter complex from LptB, LptF and LptG. Moreover, it was found that IM protein LptC is also included in the complex.

2. Materials and methods

2.1. Materials

ʟ-Rhamnose and ʟ-arabinose were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma, respectively. Isopropyl-thio-β-ᴅ-

galactopyranoside (IPTG) was from Wako Pure Chemical Industries (Osaka, Japan). n-Dodecyl-β-D-maltopyranoside (DDM) was obtained from Dojindo Laboratories (Kumamoto, Japan). Anti-Penta-His HRP conjugate was purchased from QIAGEN.

2.2. Bacterial strains and plasmids

E. coli K-12 KRX was purchased from Promega. *E. coli* B strain C43(DE3) [12] was provided by John Walker. The construction of plasmids is explained in Supplementary Fig. 1.

2.3. Purification of LptBFG and LptBFGC complexes

E. coli KRX carries a gene for T7 RNA polymerase under the control of the L-rhamnose-inducible $rhaP_{BAD}$ promoter. To suppress basal expression of T7 RNA polymerase, the KRX strain was transformed with pLysS [13]. KRX/pLysS cells were then transformed with pLptBFG encoding LptB/F/G and grown at 37 °C. When the culture OD reached 1.0, the expression of LptB/F/G was induced for 3 h by the addition of 0.02% L-rhamnose. To co-express LptC, KRX/pLysS/pLptBFG cells were transformed with pLptC and grown as described above except that 0.02% L-arabinose was added with the L-rhamnose. Cells were harvested by centrifugation at $5000 \times g$ for 10 min, and then resuspended in 50 mM Tris–HCl, pH 7.5, containing 10 μg mL^{-1} DNase I and 500 μg mL^{-1} protease inhibitor cocktail (Sigma). Harvested cells were frozen-thawed and disrupted by a single passage through a French Press cell at 10 000 psi. After removal of unbroken cells by centrifugation at $10\,000 \times g$ for 10 min, membranes were recovered by centrifugation at $100\,000 \times g$ for 1 h and then resuspended in 20 mM Tris–HCl, pH 7.5, containing 10% glycerol. Membranes were solubilized with 20 mM Tris–HCl, pH 7.5, containing 5 mM MgCl$_2$, 1% DDM, 10% glycerol and 2 mM ATP on ice for 30 min, followed by centrifugation at $100\,000 \times g$ for 30 min. The supernatant was applied to TALON metal affinity resin (Clontech), followed by elution with 20 mM Tris–HCl, pH 7.5, containing 300 mM NaCl, 0.05% DDM, 10% glycerol and 50 mM imidazole. The eluate was concentrated by filtration (Amicon Ultra, Millipore), and then subjected to size exclusion chromatography (Superdex 200, 10/300 GL, GE Healthcare). The column was pre-equilibrated with 20 mM Tris–HCl, pH 7.5, containing 300 mM NaCl, 0.05% DDM and 10% glycerol, and developed with the same buffer at a rate of 0.5 mL min^{-1} and fractions of 0.5 mL were collected.

2.4. Other techniques

SDS–PAGE was carried out as described [14]. Proteins were blotted onto polyvinylidene fluoride membranes, treated with antibodies and then developed with enhanced chemiluminescence substrate (ECL-Plus, GE Healthcare), followed by detection with a lumino-image analyzer (LAS-1000plus; Fujifilm).

3. Results

3.1. Co-expression of LptF and LptG stabilizes LptB

The chromosomal *lptF* and *lptG* genes are aligned and oriented in the same direction with a single overlapping base, indicating that they form an operon. To express ABC protein LptB and membrane proteins LptG and LptF, their genes were cloned into pCDFDuet-1 (Novagen). The plasmid thus constructed, pLptBFG, carries a gene for hexahistidine (His)-tagged LptB at the first multiple cloning site and the genes for LptG and LptF at the second site (Supplementary Fig. 1). C43(DE3)/pLysS cells were transformed with pLptB or pLptBFG, and then the expression of Lpt proteins was induced by

IPTG. Subcellular fractionation revealed that the level of LptB in the membrane fraction significantly increased on the co-expression of LptF/LptG (Fig. 1A). We then examined the level of LptB after the
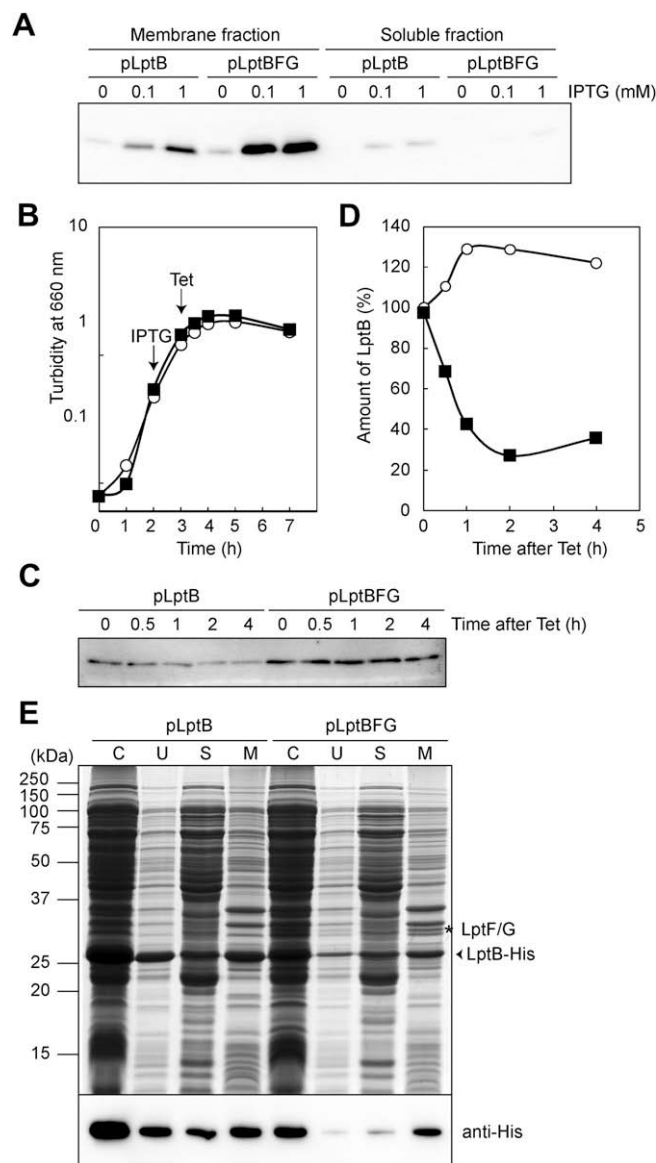

Fig. 1. Co-expression of LptF and LptG stabilizes LptB. (A) C43(DE3)/pLysS cells harboring pLptB or pLptBFG were grown at 37 °C to the early logarithmic phase. Expression of LptB or LptBFG was induced by the addition of the specified concentrations of isopropyl-thio-β-D-galactopyranoside (IPTG) at 37 °C for 2 h. Cells were harvested and disrupted by sonication. After removal of unbroken cells, cell lysates were fractionated into membranes and supernatants by centrifugation at $100\,000 \times g$ for 1 h. Equivalent amounts of the respective fractions were analyzed by SDS–PAGE and immunoblotting with anti-His tag antibodies to detect LptB-His. (B) C43(DE3)/pLysS cells harboring pLptB (squares) or pLptBFG (circles) were grown as described in A. IPTG (1 mM) and tetracycline (Tet, 50 μg mL^{-1}) were added as indicated. The growth of cells was monitored by measuring the turbidity at 660 nm. (C) Aliquots (10 μL) of the culture shown in B were withdrawn at the indicated times after the addition of tetracycline and then subjected to SDS–PAGE followed by immunoblotting with anti-His tag antibodies. (D) The densities of the bands shown in C were determined and plotted as a function of time after the addition of tetracycline. (E) Expression of Lpt proteins was induced by the addition of 0.02% L-rhamnose at 37 °C for 3 h in KRX/pLysS cells harboring pLptB or pLptBFG. Cells were harvested and disrupted as described under Materials and Methods. Equivalent amounts of proteins from whole cells (C), unbroken cells (U), soluble fractions (S) and membranes (M) were analyzed by SDS–PAGE and Coomassie Brilliant Blue R-250 (CBB) staining (upper panel) or immunoblotting (lower panel) with anti-His tag antibodies. The asterisk indicates LptF and/or LptG.
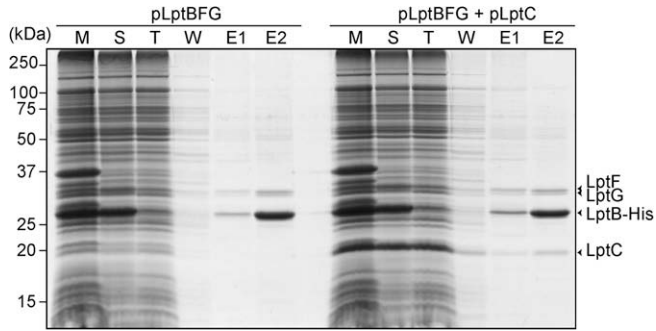
Fig. 2. LptF, LptG and LptC are co-purified with LptB. Membrane fractions were prepared from KRX/pLysS cells harboring pLptBFG or pLptBFG plus pLptC, and then solubilized with *n*-dodecyl-β-D-maltopyranoside (DDM), followed by metal affinity chromatography as described under Section 2. Proteins in membranes (M), DDM-soluble fractions (S), pass-through fractions (T), and fractions eluted with buffer containing 0, 10 and 50 mM imidazole (W, E1 and E2, respectively) were analyzed by SDS–PAGE with staining with CBB. N-Terminal sequencing allowed identification of the bands, as indicated at the right of the gel.

addition of tetracycline in cells harboring pLptB or pLptBFG. Growth was completely arrested soon after the addition of tetracycline (Fig. 1B). When expressed alone, the level of LptB rapidly decreased whereas it remained at high level in the presence of LptF and LptG (Fig. 1C and D), indicating that LptF and/or LptG stabilize LptB, presumably through complex formation.

Since antibodies against LptF or LptG were not available, detection of these proteins expressed in C43(DE3)/pLysS cells harboring pLptBFG was difficult on SDS–PAGE with staining with Coomassie Brilliant Blue R-250 (CBB) (data not shown). To improve their expression, the Lpt proteins were expressed in the KRX strain, which carries a gene for T7 RNA polymerase under the tight control of the *rha*P$_{BAD}$ promoter and is known to improve the expression of some proteins. When cells harbored pLptBFG, a band corresponding to an apparent molecular mass of 32 kDa was detected on SDS–PAGE of the membrane fraction, whereas no such band was detected when the cells harbored pLptB, suggesting that the band

represents LptF and/or LptG (Fig. 1E). The total level of LptB was higher in KRX/pLysS cells harboring pLptB than ones harboring pLptBFG. However, most LptB molecules expressed alone were insoluble and recovered with unbroken cells, indicating the formation of inclusion bodies (Fig. 1E). In contrast, the majority of LptB molecules expressed with LptF and LptG were recovered in the membrane fraction.

3.2. Isolation of the LptBFG and LptBFGC complexes

Membranes prepared from KRX/pLysS cells harboring pLptBFG were solubilized and then subjected to immobilized metal affinity chromatography (IMAC). Preliminary experiments suggested that the LptBFG complex was stably solubilized with DDM as the Lol-CDE complex was [15]. DDM could efficiently solubilize LptBFG although some LptB molecules remained insoluble, presumably as an inclusion body. Eluate fractions E1 and E2 gave two bands at positions corresponding to 32 kDa in addition to the LptB-His band detected at about the 26 kDa position (Fig. 2). The N-terminal sequences were found to be MIIIR and MQPFG for the slower and faster migrating bands, respectively, which were identical to those of LptF and LptG. Thus, both LptF and LptG were biochemically found to form a stable ABC transporter complex with LptB, which has been reported to form a complex with uncharacterized partners [16]. Both LptF (40.4 kDa) and LptG (39.6 kDa) molecules migrated faster than expected from their molecular masses, presumably because of their hydrophobic properties.

LptC is a bitopic IM protein encoded by a gene located immediately upstream of *lptA* and *lptB*. Depletion of LptC impairs LPS transport, as observed when LptA, LptB, LptD or LptE was depleted [6]. It therefore seemed possible that LptC functions with the LptBFG complex. To determine whether or not LptC is included in the ABC transporter complex, membranes prepared from KRX/pLysS cells harboring pLptBFG and pLptC were solubilized and then subjected to IMAC as described above. The eluate fraction gave an extra band migrating to a position corresponding to 19 kDa (Fig. 2). The N-terminal sequence of this material was MGKAR, which was
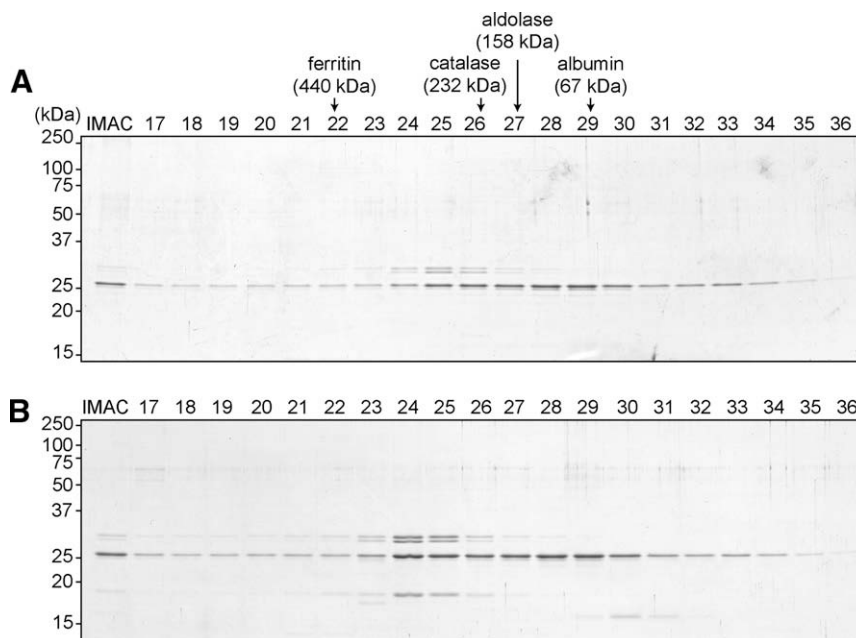

Fig. 3. Size exclusion chromatography of LptB-containing complexes. The LptBFG (A) and LptBFGC (B) complexes purified in Fig. 2 (fraction E2) were subjected to size exclusion chromatography as described under Section 2. Aliquots of the fractions (20 μl) were analyzed by SDS–PAGE and CBB-staining. The elution positions of molecular mass markers and fraction numbers are indicated above the gel. The input protein was analyzed in the left lane.

identical to that of LptC encoded by pLptC although about 20% of the LptC molecules lacked the N-terminal M. To create a restriction site, S at position 2 of LptC was mutated to G (Supplementary Fig. 1). These results indicate that the LptBFG complex is a unique ABC transporter possessing an additional subunit, LptC, and catalyzes the LPS transport to OM.

3.3. Subunit ratio of the LPS-specific ABC transporter

The LptBFG complex with or without LptC was analyzed by size exclusion chromatography. Since the complex was purified with His-tag affinity resin, the purified fractions contained excess LptB-His, which was mainly eluted as homo-oligomers in fractions corresponding to 60–120 kDa whether LptC was co-expressed or not. Both the LptF and LptG subunits were eluted with LptB in a fraction corresponding to 280 kDa in the absence of LptC (Fig. 3A, fraction 25). On the other hand, these two membrane subunits and LptC were eluted with LptB in fractions corresponding to slightly larger molecular masses (Fig. 3B, fractions 24 and 25). The elution profiles of LptC, LptF and LptG were essentially identical, indicating strongly that LptC is an intrinsic subunit of the complex.

Determination of the respective band densities revealed that the subunit ratio of LptBFG was LptB:LptF:LptG = 2:1:1. The subunit ratio of the LptBFGC complex was LptB:LptF:LptG: LptC = 2:1:1:1. The predicted molecular masses of the $LptB_2F_1G_1$ and $LptB_2F_1G_1C_1$ complexes were 135.5 kDa and 157.2 kDa, respectively. LptB was reported to form a ~140 kDa complex with uncharacterized partners [16]. In contrast, the molecular masses of LptBFG and LptBFGC complexes determined on the size exclusion chromatography were ~280 kDa and ~330 kDa, suggesting that they exist as dimers of $LptB_2F_1G_1$ and $LptB_2F_1G_1C_1$, respectively, under the current conditions, although binding of DDM micelles is not completely excluded. A half-type human ABC transporter, ABCG2, has been reported to exist as a tetramer in a detergent solution [17]. However, it seems more likely that both LptBFG and LptBFGC complexes exist as monomers in the cells.

The ATPase activities of the LptBFG and LptBFGC complexes (Supplementary Fig. 2A) exhibited similar Km (1.94 ± 0.27 and 1.54 ± 0.16 mM, respectively) and V_{max} (129.3 ± 7.7 and 119.5 ± 5.0 ATP mol min^{-1} mol^{-1}, respectively) values. These values were considerably different from those of LolCDE in proteoliposomes [15] and MsbA in a DDM solution [18], both of which have been reported to exhibit similar Km (~0.3 mM) and V_{max} (~60 ATP mol min^{-1} mol^{-1}) values. Both LptBFG and LptBFGC complexes were sensitive to orthovanadate (Supplementary Fig. 2B) as reported with many other ABC transporters [19]. The ATPase activities of many ABC transporters are stimulated by their substrates [20]. In contrast, those of LptBFG and LptBFGC complexes were unaffected by LPS, phospholipids and lipid A (Supplementary Fig. 2C).

4. Discussion

The four domains of bacterial ABC transporters, i.e. two nucleotide-binding domains and two membrane-spanning ones, are frequently located in separate polypeptides [19]. However, the genes for the subunits of ABC transporters usually form an operon. The *E. coli* chromosome has been predicted to encode 79 ABC proteins and 57 ABC transporters [21]. Some ABC proteins are not involved in transport reactions and therefore lack neighboring genes encoding membrane subunits. LptB was identified as an ABC protein required for LPS transport but apparently lacked membrane subunits. However, we biochemically proved that LptF and LptG are the membrane subunits of the LPS-specific ABC transporter

complex. Other orphan ABC proteins might have membrane subunits encoded by genes distantly located in a bacterial genome.

Although LptC is essential for the transport of LPS to OM, the kinetic parameters of the ATPase activity were hardly affected by LptC (Supplementary Fig. 2). The gene organization of *lptC–lptA–lptB* is highly conserved among gram-negative bacteria. It seems likely that LptC plays a distinct role in the LPS transport. To elucidate the specific function of LptC, the LPS transport activity must be reconstituted into proteoliposomes with LptBFGC. Molecular mechanisms underlying the functions of ABC transporters have been significantly clarified by the crystal structures of both ABC importers and exporters [22,23]. It is of great interest how the accessory protein LptC affects the function of the LptBFG complex and thereby contributes to LPS transport.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.05.051.

References

[1] Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. 67, 593–656.
[2] Tokuda, H. (2009) Biogenesis of outer membranes in gram-negative bacteria. Biosci. Biotechnol. Biochem. 73, 465–473.
[3] Ruiz, N., Kahne, D. and Silhavy, T.J. (2006) Advances in understanding bacterial outer-membrane biogenesis. Nat. Rev. Microbiol. 4, 57–66.
[4] Doerrler, W.T., Gibbons, H.S. and Raetz, C.R. (2004) MsbA-dependent translocation of lipids across the inner membrane of *Escherichia coli*. J. Biol. Chem. 279, 45102–45109.
[5] Sperandeo, P., Cescutti, R., Villa, R., Di Benedetto, C., Candia, D., Dehò, G. and Polissi, A. (2007) Characterization of *lptA* and *lptB*, two essential genes implicated in lipopolysaccharide transport to the outer membrane of *Escherichia coli*. J. Bacteriol. 189, 244–253.
[6] Sperandeo, P., Lau, F.K., Carpentieri, A., De Castro, C., Molinaro, A., Dehò, G., Silhavy, T.J. and Polissi, A. (2008) Functional analysis of the protein machinery required for transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. J. Bacteriol. 190, 4460–4469.
[7] Bos, M.P., Tefsen, B., Geurtsen, J. and Tommassen, J. (2004) Identification of an outer membrane protein required for the transport of lipopolysaccharide to the bacterial cell surface. Proc. Natl. Acad. Sci. USA 101, 9417–9422.
[8] Wu, T., McCandlish, A.C., Gronenberg, L.S., Chng, S.S., Silhavy, T.J. and Kahne, D. (2006) Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 103, 11754–11759.
[9] Tran, A.X., Trent, M.S. and Whitfield, C. (2008) The LptA protein of *Escherichia coli* is a periplasmic lipid A-binding protein involved in the lipopolysaccharide export pathway. J. Biol. Chem. 283, 20342–20349.
[10] Tefsen, B., Geurtsen, J., Beckers, F., Tommassen, J. and de Cock, H. (2005) Lipopolysaccharide transport to the bacterial outer membrane in spheroplasts. J. Biol. Chem. 280, 4504–4509.
[11] Ruiz, N., Gronenberg, L.S., Kahne, D. and Silhavy, T.J. (2008) Identification of two inner-membrane proteins required for the transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 105, 5537–5542.
[12] Miroux, B. and Walker, J.E. (1996) Over-production of proteins in *Escherichia coli*: Mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J. Mol. Biol. 260, 289–298.
[13] Moffatt, B.A. and Studier, F.W. (1987) T7 lysozyme inhibits transcription by T7 RNA polymerase. Cell 49, 221–227.
[14] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
[15] Ito, Y., Kanamaru, K., Taniguchi, N., Miyamoto, S. and Tokuda, H. (2006) A novel ligand bound ABC transporter, LolCDE, provides insights into the molecular mechanisms underlying membrane detachment of bacterial lipoproteins. Mol. Microbiol. 62, 1064–1075.
[16] Stenberg, F., Chovanec, P., Maslen, S.L., Robinson, C.V., Ilag, L.L., von Heijne, G. and Daley, D.O. (2005) Protein complexes of the *Escherichia coli* cell envelope. J. Biol. Chem. 280, 34409–34419.

[17] Xu, J., Liu, Y., Yang, Y., Bates, S. and Zhang, J.T. (2004) Characterization of oligomeric human half-ABC transporter ATP-binding cassette G2. J. Biol. Chem. 279, 19781–19789.
[18] Dong, J., Yang, G. and McHaourab, H.S. (2005) Structural basis of energy transduction in the transport cycle of MsbA. Science 308, 1023–1028.
[19] Holland, I.B. and Blight, M.A. (1999) ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. J. Mol. Biol. 293, 381–399.
[20] Higgins, C.F. and Linton, K.J. (2004) The ATP switch model for ABC transporters. Nat. Struct. Mol. Biol. 11, 918–926.
[21] Linton, K.J. and Higgins, C.F. (1998) The *Escherichia coli* ATP-binding cassette (ABC) proteins. Mol. Microbiol. 28, 5–13.
[22] Davidson, A.L. and Chen, J. (2004) ATP-binding cassette transporters in bacteria. Annu. Rev. Biochem. 73, 241–268.
[23] Dawson, R.J., Hollenstein, K. and Locher, K.P. (2007) Uptake or extrusion: Crystal structures of full ABC transporters suggest a common mechanism. Mol. Microbiol. 65, 250–257.