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Transmembrane transport of peptidoglycan precursors across model and bacterial membranes

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Summary

Translocation of the peptidoglycan precursor Lipid II across the cytoplasmic membrane is a key step in bacterial cell wall synthesis, but hardly understood. Using NBD-labelled Lipid II, we showed by fluorescence and TLC assays that Lipid II transport does not occur spontaneously and is not induced by the presence of single spanning helical transmembrane peptides that facilitate transbilayer movement of membrane phospholipids. MurG catalysed synthesis of Lipid II from Lipid I in lipid vesicles also did not result in membrane translocation of Lipid II. These findings demonstrate that a specialized protein machinery is needed for transmembrane movement of Lipid II. In line with this, we could demonstrate Lipid II translocation in isolated *Escherichia coli* inner membrane vesicles and this transport could be uncoupled from the synthesis of Lipid II at low temperatures. The transport process appeared to be independent from an energy source (ATP or proton motive force). Additionally, our studies indicate that translocation of Lipid II is coupled to transglycosylation activity on the periplasmic side of the inner membrane.

Introduction

Almost all bacteria are surrounded by a cell wall. Being essential for cell survival, the cell wall provides maintenance of shape and prevents the cell from lysis because of a high osmotic pressure inside the cell. The main compound of the bacterial cell wall is the peptidoglycan layer, which is a macromolecular structure consisting of a 3-dimensional network of repeating subunits composed of the amino sugars *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). A pentapeptide consisting most often of L-alanyl-γ-D-glutamyl-diaminopimelyl (or L-lysyl)-D-alanyl-D-alanine is attached to the carboxyl group of MurNAc subunits. These peptides are necessary for interlinking adjacent glycan chains, resulting in a rigid structure (van Heijenoort, 2001).

The first membrane-located step in the biosynthesis of peptidoglycan starts at the cytoplasmic side of the plasma membrane with the transfer of UDP-activated MurNAc-pentapeptide from the cytoplasm to a lipid carrier, undecaprenyl phosphate, which serves as an anchor for the peptidoglycan subunits in the membrane. This transfer is catalysed by the transmembrane protein MraY and results in Lipid I. The addition of GlcNAc to Lipid I results in Lipid II and is catalysed by the MurG enzyme (Mengin-Lecreulx *et al*., 1991). MurG is peripherally associated with the inner surface of the cytoplasmic membrane (Bupp and van Heijenoort, 1993; van den Brink-van der Laan *et al*., 2003). In order to get the peptidoglycan subunits incorporated into the growing cell wall, Lipid II has to be efficiently transferred through the cytoplasmic membrane. After translocation, the subunits are polymerized and inserted into the growing peptidoglycan chain by transglycosylation and transpeptidation. These steps are catalysed by penicillin binding proteins (PBPs) (Rogers *et al*., 1980; Terrak *et al*., 1999). After transglycosylation, the undecaprenyl carrier moves back to the cytoplasmic side of the membrane, via a yet unknown mechanism, where it becomes available again for synthesis of Lipid II.

Despite decades of research on cell wall synthesis, the mechanism by which membrane transport of Lipid II occurs remains unknown. This transport is a key step in the synthesis of the bacterial cell wall. Peptidoglycan synthesis occurs at a rate of 1000 units s^{-1} in *Escherichia coli* (van Heijenoort *et al*., 1992) and the rate of Lipid II transport has to match this rate. An *E. coli* cell possesses approximately 1000 copies of Lipid II so the rate of Lipid II transport across the cytoplasmic membrane must be very fast (van Heijenoort *et al*., 1992). This is supported by the observation that in *Micrococcus lysodeikticus* a frequency

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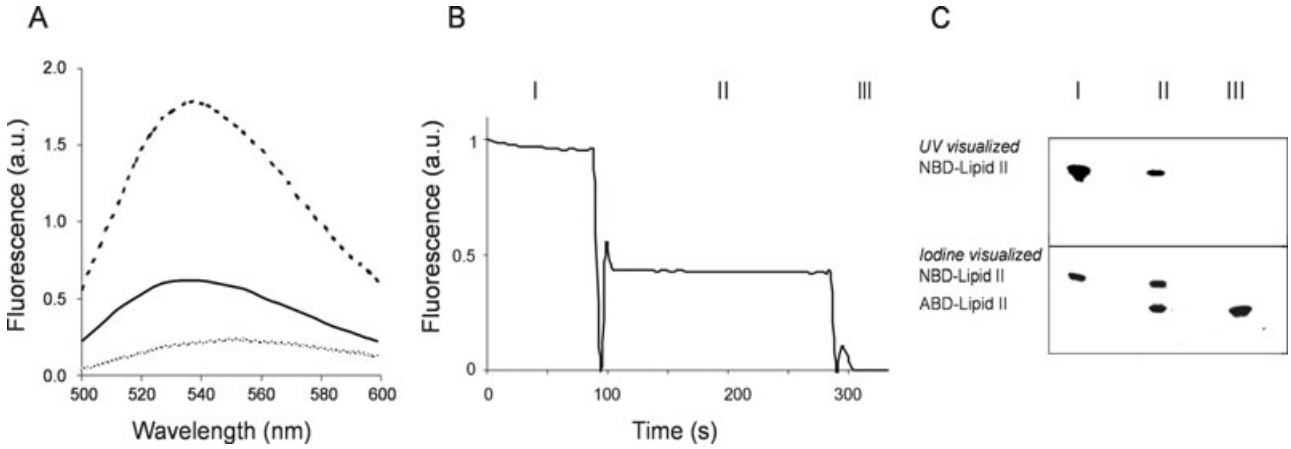

Fig. 1. A. Emission spectra of 0.2 mol% NBD-Lipid II at an excitation wavelength of 478 nm incorporated in LUVs composed of 75% DOPE and 25% DOPG (solid black line), 0.2 mol% NBD-PG incorporated in LUVs in buffer A (dotted line) and NBD-labelled UDP-MurNAc pentapeptide directly in buffer A (grey line). Equal amounts of the different NBD-labelled compounds were used.
B. The online tracing of NBD-Lipid II quenching upon dithionite addition. LUVs with 0.2 mol% NBD-Lipid II incorporated in 75 mol% DOPE, 25 mol% DOPG LUVs were used. *I* represents LUVs with NBD-Lipid II symmetrically incorporated not treated with dithionite, *II* represents NBD-Lipid II from dithionite treated LUVs and in *III* LUVs were dissipated by 0.2% Triton X-100 in the presence of dithionite.
C. TLC analysis of the online tracing of NBD-Lipid II quenching as shown in B. In *lane I* LUVs consisting of 75 mol% DOPE, 25 mol% DOPG and 0.2 mol% NBD-Lipid II were subjected to phospholipids extraction without addition of dithionite. *Lane II* represents NBD-Lipid II from dithionite treated LUVs and in *lane III* LUVs were treated with 0.2% Triton X-100 in the presence of dithionite. Dithionite quenching was stopped by addition of a 2.5-fold excess of $K_3Fe(CN)_6$ prior to phospholipids extraction. For all TLCs depicted in this study, the point of origin is at the bottom.

of transbilayer passage of the undecaprenyl carrier per molecule was calculated to be approximately 1–3 s^{-1} (McCloskey and Troy, 1980). The transport of newly synthesized membrane phospholipids across the cytoplasmic membrane of *E. coli* is a process that also occurs rapidly, with rates of transport with half-times in the order of minutes (Donohue-Rolfe and Schaechter, 1980). It was proposed that the membrane spanning parts of proteins are responsible for fast phospholipids transport (McLean and Phillips, 1981; Jones and Thompson, 1989). This hypothesis was supported by the observation that incorporation of transmembrane peptides into the bilayer induced translocation of some phospholipids (Kol *et al*., 2001). This raises the possibility that the transmembrane parts of proteins are also responsible for rapid translocation of Lipid II. We tested this possibility by analysing the influence of transmembrane peptides on translocation of Lipid II across a bilayer composed of synthetic phospholipids.

Alternatively, translocation of Lipid II might be coupled to synthesis. This is an attractive possibility because of its efficiency and because the Lipid II synthesizing enzyme MurG possesses a hydrophobic patch which enables the protein to penetrate the membrane (Mengin-Lecreulx *et al*., 1991; Ha *et al*., 2000). Moreover, MurG is the last enzyme known to be in contact with Lipid II on the cytoplasmic side of the inner membrane of *E. coli*. We therefore investigated transmembrane transport of newly synthesized Lipid II using a MurG-membrane system.

The transbilayer movement of Lipid II was analysed via a well described fluorescence assay (McIntyre and Sleight, 1991; Huijbregts *et al*., 1996; Kol *et al*., 2001) for determination of phospholipid membrane asymmetry using the fluorescent label 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) attached to the lysine residue of the MurNAc pentapeptide moiety of Lipid II. Its accessibility for dithionite, that leads to reduction from NBD to the non-fluorescent 7-amino-2,1,3-benzoxadiol-4-yl (ABD), was determined by both fluorimetry and TLC and used as a tool to determine Lipid II topology.

Results

Characterization of NBD-labelled compounds

First, the fluorescence characteristics of the NBD-labelled compounds were investigated. NBD-Lipid II that was incorporated in vesicles composed of DOPE/DOPG (75:25 mol%) at 0.2 mol% gave a fluorescence spectrum with a maximum located at 540 nm (Fig. 1A, solid line). When the NBD-labelled phospholipid phosphatidyl glycerol (NBD-PG) was incorporated in an identical way the fluorescence spectrum also gave a maximum at the same wavelength but the intensity was increased almost threefold (Fig. 1A, dotted line). In contrast, NBD-labelled UDP-MurNAc pentapeptide in buffer A gave a fluorescence spectrum with a maximum at 548 nm with an intensity of about threefold lower than the NBD-Lipid II emission

(Fig. 1A, grey line). This demonstrates that the NBD labels of Lipid II and PG are located in a more hydrophobic environment when incorporated in the membrane. Moreover, the threefold increased intensity of the NBD-PG emission indicates that its NBD label is more deeply incorporated into the membrane as compared with the NBD label of NBD-Lipid II.

When NBD-labelled Lipid II is treated with dithionite, the nitroxide of the NBD moiety is reduced to an amine resulting in the loss of fluorescence (McIntyre and Sleight, 1991). The fluorescence experiment shown in Fig. 1B demonstrates that when dithionite was added to a suspension of large unilamellar vesicles (LUVs) with NBD-Lipid II homogeneously incorporated (Fig 1B, *I*), the fluorescence signal decreased to 47 ± 1.5% of the initial level in approximately 10 s (Fig 1B, *II*). This is in marked contrast to the behaviour of NBD-PG, of which the fluorescence took about 300 s to quench to the same level in a similar experiment (data not shown). This supports the more accessible location of the NBD label of NBD-Lipid II versus that of NBD-PG as suggested above. The fact that the fluorescence remained at a stable level for several minutes after addition of dithionite demonstrates that NBD-Lipid II was distributed symmetrically over the two leaflets of the bilayer and that no rapid redistribution occurred. When Triton X-100 was added to a final concentration of 0.2% to disintegrate the LUVs, the fluorescence was quenched almost directly to zero levels (Fig 1B, *III*). This demonstrates that the amount of dithionite added to the vesicles was enough to quench all fluorescence.

Figure 1C shows the TLC analysis of the experiments shown in Fig. 1B. Both UV light (Fig. 1C, upper panel) and iodine (Fig. 1C, lower panel) were used to visualize the spots. TLC analysis of the untreated vesicles showed that a spot could be visualized with UV light, representing fluorescent NBD-Lipid II (Fig. 1C, upper panel, *I*). Treatment with dithionite resulted in a spot at similar Rf-value that was approximately half as intense when visually inspected (Fig. 1C, upper panel, *II*). In presence of Triton X-100 no spot could be visualized with UV light (Fig. 1C, upper panel, *III*). Staining with iodine revealed the non-fluorescent pools on the TLC. One spot was visible when no dithionite was added (Fig. 1C, lower panel, *I*), but after addition of dithionite two spots were visible (Fig. 1C, lower panel, *II*). The other spot corresponded to NBD-Lipid II that was reduced by dithionite (ABD-Lipid II), and thus lost its fluorescence properties. This observation corresponds to the decrease in intensity of the fluorescence signal of approximately 47 ± 1.5% (Fig. 1B). ABD-Lipid II typically had a lower Rf-value compared with its unquenched variant (Fig. 1C, lower panel, *I*). In Fig. 1C, lower panel, *III* only a spot at the position of ABD-Lipid II could be seen, which corresponds to the decrease of fluorescence to zero levels in Fig. 1B. Thus, by combining the results of online fluorescence measurements with TLC analysis information, the location of all the Lipid II molecules can be retrieved.

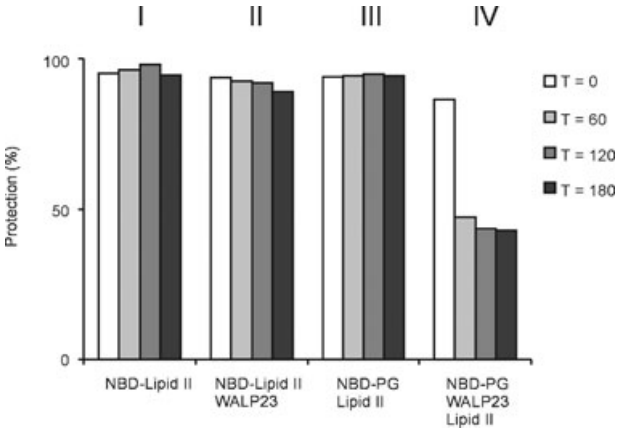

Fig. 2. NBD-Lipid II does not flip-flop spontaneously or in presence of transmembrane helix WALP23. *I*, NBD-Lipid II; *II*, NBD-Lipid II; WALP23; *III*, NBD-PG, Lipid II; *IV*, NBD-PG, WALP23, Lipid II. Flip-flop was measured at 0, 60, 120 and 180 min of incubation at 30°C. 0.2 mol% of NBD- labelled compounds and 0.4 mol% of WALP23 were incorporated symmetrically in 75 mol% DOPE, 25 mol% DOPG LUVs.

Lipid II flop does not occur spontaneously nor in the presence of transmembrane helices

To examine the occurrence of spontaneous transport of Lipid II across the membrane, asymmetrically labelled vesicles consisting of DOPE/DOPG/NBD-Lipid II were prepared. For this purpose, vesicles were treated with dithionite to quench all fluorescent NBD-groups located in the outer leaflet of the membrane. Subsequently, vesicles were treated with excess $K_3Fe(CN)_6$ to remove dithionite and incubated at 30°C. At several time points, the vesicles were subjected to another dithionite treatment to examine whether fluorescent NBD-Lipid II had moved from the inner to the outer leaflet of the membrane. After 0, 1, 2 and 3 h of incubation almost all NBD-Lipid II in the LUVs was protected against dithionite (Fig 2, *I*) demonstrating that all NBD-Lipid II remained on the inside of the LUVs and thus is unable to spontaneously move across the bilayer at an appreciable rate. In membranes composed of an *E. coli* phospholipid extract, flip-flop of the major phospholipids PE and PG was shown to be dependent on the presence of transmembrane helices (Kol *et al*., 2001). Therefore, we also considered the possible transmembrane helix induced flop of Lipid II. For this, the transmembrane peptide WALP 23 was used, as it was shown to facilitate transmembrane movement of some phospholipids and especially of the negatively charged PG (Bugg and Brandish, 1994; Kol *et al*., 2001). However, the pres-

ence of WALP23 is not enough to facilitate transport of NBD-Lipid II across the lipid bilayer. The protection remained almost complete after 3 h of incubation (Fig 2, *II*). Even after 20 h of incubation the protection was only decreased to approximately 82% (not shown), which implicates a half-time for translocation of about 60 h in this system. In contrast, while NBD-PG did not flop spontaneously in pure lipid bilayers (Fig 2, *III*), its transmembrane transport was significantly enhanced in the presence of WALP23 (Fig 2, *IV*) confirming the observation of Kol *et al*. (Kol *et al*., 2001). Additionally, our results show that the presence of Lipid II did not affect peptide induced transbilayer movement of NBD-PG. For all measurements addition of Triton X-100 to the vesicles suspension typically led to a total loss of fluorescence, demonstrating that the amount of dithionite added was enough to reduce all NBD groups present in the system. Our findings demonstrate that Lipid II does not flip-flop spontaneously or in the presence of the membrane spanning helices.

Synthesis of Lipid II from Lipid I by MurG is not coupled to translocation of Lipid II across the membrane

Next we tested whether synthesis of Lipid II from Lipid I by MurG is coupled to transport. For this purpose LUVs were prepared with NBD-Lipid I incorporated symmetrically. These LUVs were incubated with UDP-GlcNAc and MurG. Thus, if MurG transfers NBD-Lipid II across the bilayer during its synthesis, an increased protection towards dithionite should be the result.

In Fig. 3A, lane 1 shows that in the absence of MurG and UDP-GlcNAc no synthesis occurs and only NBD-Lipid I can be seen on the TLC. When MurG and UDP-GlcNAc were added to these LUVs, immediately after addition of MurG ($t = 0$ min, with the dead time of the experiment being ~20 s) already more than 25% of the NBD-Lipid I was converted to NBD-Lipid II (Fig. 3A, *lane 2*). After 1, 2 and 3 h of incubation the amount of NBD-Lipid I converted into NBD-Lipid II remained around 50% (Fig. 3A, *lanes 3–5*). This observation indicates that only the outer NBD-Lipid I pool is accessible for NBD-Lipid II synthesis and confirms the symmetric distribution of NBD-Lipid I over the two leaflets of the bilayer. To check if the stable levels of conversion between 1 and 3 h of incubation were not due to inactivation of MurG in time, Triton X-100 was added to an overnight-incubated sample to make the protected NBD-Lipid I pool also accessible for MurG. After another hour of incubation the enzymatic activity after overnight incubation was sufficient to convert the reminiscent NBD-Lipid I pool completely into NBD-Lipid II (Fig. 3, *lane 6*). For both the vesicles incubated with MurG and UDP-GlcNAc as well as the vesicles incubated without these compounds, the fluorescence level after dithionite treatment was approximately 50% of the initial fluorescence level for all incubation times. This implicates that the NBD-labelled compounds were distributed equally over both sides of the membrane, suggesting that MurG did not translocate Lipid II across the bilayer.

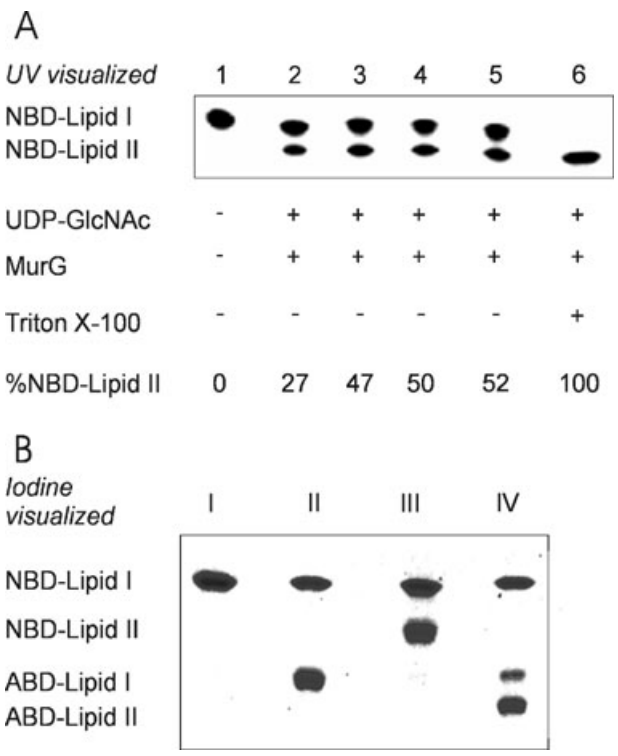

Fig. 3. A. Quantification of newly synthesized NBD-Lipid II. The amount of Lipid II in a sample is quantified as a percentage of the total NBD-compounds in the sample. Percentages are an average of 12 values derived from four different incubations. The UV-visualized spots of a typical corresponding TLC are shown. *Lanes 1* and *2* represent the sample at $t = 0$ min, without and with addition of UDP-GlcNAc and MurG respectively. *Lanes 3–5* represent samples after, respectively, 1, 2 and 3 h of incubation. After 20 h Triton X-100 was added to the sample followed by lipid extraction 1 h later (*lane 6*).
B. TLC analysis of MurG catalysed NBD-Lipid II synthesis after 3 h of incubation at 30°C. Spots were visualized by iodine vapour. *Lanes I* and *II* show NBD-Lipid I extracted from LUVs incubated in the absence of MurG and UDP-GlcNAc. *Lanes III* and *IV* show NBD-Lipid I and II extracted from LUVs incubated in the presence of MurG and UDP-GlcNAc. The samples used for *lanes II* and *IV* were subjected to dithionite quenching, followed after 5 min by addition of $K_3Fe(CN)_6$ in a 2.5-fold excess before extraction to quench the outer NBD pool.

To exactly localize the position of NBD-labelled compounds we used the TLC method as described in Fig. 1C. Figure 3B shows the analysis on TLC of vesicles with NBD-Lipid I incubated for 3 h with or without MurG and UDP-GlcNAc visualized with iodine. The vesicles incubated without UDP-GlcNAc and without MurG show only an NBD-Lipid I spot as expected (Fig. 3B, *lane I*). When the outer NBD pool of these vesicles was treated with dithionite before phospholipid extraction, two spots became visible: NBD-Lipid I and ABD-Lipid I. (Fig. 3B,

lane II). Addition of UDP-GlcNAc and MurG resulted in synthesis of NBD-Lipid II. Figure 3B, *lane III* shows one spot at a typical NBD-Lipid I position and one spot at a typical NBD-Lipid II position. Addition of dithionite after MurG catalysed synthesis of NBD-Lipid II resulted in a faint spot of ABD-Lipid I, where a more intense spot appeared for the ABD-Lipid II (Fig. 3B, *lane IV*). This demonstrates that almost all NBD-Lipid I on the outside of the membranes was converted into NBD-Lipid II. More importantly, even after 3 h, no newly synthesized NBD-Lipid II appeared in the inner pool of the LUVs that was protected from dithionite (Fig. 3B, *lane IV*), indicating that no NBD-Lipid II was transported across the membrane.

As MurG appeared to synthesize Lipid II more efficiently when associated with membrane vesicles containing cardiolipin (van den Brink-van der Laan *et al.*, 2003), the effect of cardiolipin was tested in a similar experiment. For this purpose the experiment was carried out on vesicles containing 80% DOPE/15% DOPG/5% cardiolipin/0.2% NBD-Lipid I, mimicking the *E. coli* inner membrane phospholipids composition (Dowhan, 1997). Similar to the experiment above, also no translocation of NBD-Lipid II could be measured under these conditions (not shown).

Lipid II translocation across E. coli *membrane vesicles*

Next, we tried to measure the translocation of Lipid II across biological membranes using the same approach. Unfortunately, the dithionite protection assay could not be used because of the permeability of the membrane for dithionite (data not shown). Instead, we made use of the intrinsic property of antibodies against dinitrophenyl groups to quench fluorescence of NBD amines (Lancet and Pecht, 1977), and it is not to be expected that these antibodies are membrane permeable. As shown in Fig. 4A, the anti-DNP antibody is indeed capable of quenching the fluorescence of NBD-Lipid II in a concentration-dependent manner and thus it was used to determine the topology of Lipid II in bacterial membranes. Inner membrane vesicles of *E. coli* strain W3899 with a right side-out (RSO) orientation were isolated to measure the membrane-translocation of NBD-Lipid II. Using a freeze-thaw procedure, the substrates NBD-UDP-MurNAc-pentapeptide and UDP-GlcNAc were brought into the lumen of the vesicles. We found that Lipid II synthesis, but not translocation, already occurs while the samples are standing on ice. Subsequently, the temperature of the NBD-Lipid II containing membrane vesicles was raised to 14°C and fluorescence spectra were recorded in the presence of anti-DNP antibody. A gradual decrease in the NBD-fluorescence was observed (Fig. 4B), indicating translocation of the NBD-Lipid II from the inside of the vesicles to the outside, followed by quenching of the NBD label by the antibody. To ascertain that the fluorescence quenching is not caused by leakage or desintegration of the vesicles, a control reaction was carried out. Tunicamycin, a specific inhibitor of MraY function, was added to the reaction during the freeze-thaw procedure. This prevented the synthesis of Lipid I/II. We argued that the UDP-MurNAc-pentapeptide would not be a substrate for translocation and should remain in the lumen of the membrane vesicles. Upon recording of the spectra it was observed that the fluorescence emission maximum was red-shifted almost 20 nm (compare Fig. 4B and C). This indicates that indeed no NBD-Lipid II is synthesized and only NBD-UDP-MurNAc-pentapeptide is present (see also Fig. 1) as also is indicated by TLC analysis (not shown). When fluorescence spectra of this reaction were recorded, only a small decrease in NBD-fluorescence was observed after addition of the anti-DNP antibody (Fig. 4C, antibody added at $t = 2$ min). After this small initial decrease, the signal remained stable, indicating that the initial quenching was probably caused by some NBD-UDP-MurNAc-pentapeptide sticking to the outside of the membranes. Only after addition of 0.2% Triton X-100, which dissolves the vesicles, the liberated NBD-UDP-MurNAc-pentapeptide was quenched by the antibody. This shows that NBD-Lipid II is specifically translocated across the membrane and the bacterial membrane vesicles are not permeable for either UDP-MurNAc-pentapeptide or the anti-DNP antibody.

Subsequently, we incubated bacterial vesicles in the absence of anti-DNP antibody. Under these conditions it appeared that, upon incubation of the vesicles at elevated temperatures after synthesis of NBD-Lipid II on ice, we could detect a decrease of the fluorescence in time (Fig. 4D). The rate of this decrease was dependent on the temperature, as increasing the temperature from 14 to 24°C resulted in a faster decrease of the fluorescence. In a parallel experiment we analysed the bacterial membrane vesicles for the presence of NBD-Lipid II by TLC analysis after extraction of the lipids with butanol-pyridine pH 4.2 (Fig. 4E). The results show that in time the NBD-Lipid II pool decreases concomitant with this decrease in fluorescence, indicating that NBD-Lipid II is processed presumably by transglycosylase activities. Addition of α-DNP antibody resulted at both temperatures in an even faster decrease in fluorescence (Fig. 4D). These observations indicate that the translocation of NBD-Lipid II may be directly linked to its polymerization by the transglycosylases, as discussed further below.

Discussion

In this study we demonstrated by fluorimetry and TLC that Lipid II does not translocate spontaneously across a DOPE/DOPG membrane. The long undecaprenyl chain

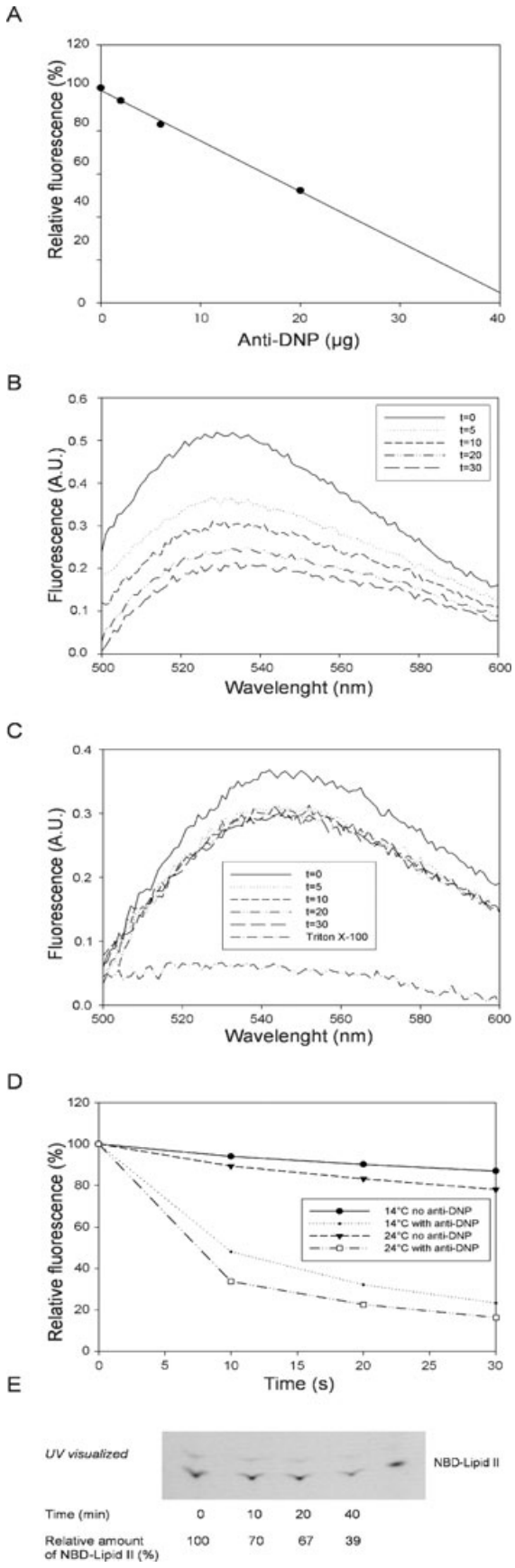

Fig. 4. NBD-quenching demonstrates Lipid II translocation in *E. coli* membrane vesicles.
A. Addition of anti-DNP antibody to NBD-labelled Lipid II in Triton X-100 containing buffer quenches the NBD-fluorescence in a linear fashion.
B. Translocation of NBD-Lipid II across the cytoplasmic membrane is shown by NBD-quenching using external anti-DNP antibody. NBD-labelled UDP-MurNAc-pentapeptide was brought into membrane vesicles and Lipid II was synthesized as described in *Experimental procedures*. Fluorescence spectra were recorded in the presence of 40 µg anti-DNP antibody at 14°C for 30 min as indicated in the figure.
C. UDP-MurNAc-pentapeptide is not translocated or leaking across the membrane. NBD-labelled UDP-MurNAc-pentapeptide was brought into membrane vesicles of which MraY was inhibited by tunicamycin, thus blocking the synthesis of Lipid I/II. Fluorescence spectra were recorded at 14°C for 35 min as indicated in the figure. After 2 min, 40 µg anti-DNP antibody was added to the reaction. After 32 min, 0.2% Triton X-100 was added to the reaction.
D. Temperature-dependent fluorescence decrease in the absence of NBD-quenching antibody indicates polymerization of translocated NBD-Lipid II. Reactions were incubated at 14°C or 24°C in the presence or absence of anti-DNP antibody as indicated. The relative fluorescence at 534 nm was measured at 0, 10, 20 and 30 min.
E. A decrease in the NBD-Lipid II pool indicates polymerization by PBPs after translocation. Reactions were incubated at room temperature and samples were taken at the times indicated and analysed by TLC and UV detection. The relative amounts of NBD-Lipid II were quantified and indicated above each lane. NBD-Lipid II: control NBD-Lipid II.

has been proposed to enable spontaneous diffusion of Lipid II across the bacterial membrane (Bugg and Brandish, 1994), but even after 3 h of incubation we did not observe Lipid II flop (Fig. 2). The presence of transmembrane helices in a bilayer system enables several phospholipids to flip-flop across the membrane (Kol *et al*., 2001; Kol *et al*., 2004). However, we showed that in contrast to phospholipids, Lipid II does not flop through the bilayer in the presence of the transmembrane peptide WALP23. This can be understood by the observations of Kol *et al.* who showed that transmembrane peptide induced phospholipids translocation was strongly dependent on the charge of the lipid head group (Kol *et al*., 2003). A high number of total charges in the head group resulted in a lower rate of flip-flop in these experiments. The negatively charged phosphatidyl glycerol and phosphatidic acid were translocated quickly, while the zwitterionic phosphatidyl choline (two opposing charges) and phosphatidyl serine (zwitterionic plus an extra negative charge) showed a much slower flop (Kol *et al*., 2003). More charges on a phospholipid head group would require a higher energy to bring the head group into the hydrophobic interior of the bilayer. Apparently, the presence of four charges in the NBD-labelled Lipid II head group blocks this transmembrane helix-mediated translocation. This charge effect would be even larger for unlabelled Lipid II that carries an additional positive charge. It can be estimated that the half-time of translo-

cation of Lipid II across the peptide containing bilayer was about 60 h (Fig. 2), which is several orders of magnitude slower than the half-time of translocation of Lipid II in biological membranes which has to be in the order of seconds (McCloskey and Troy, 1980; van Heijenoort *et al*., 1992). This difference in behaviour between phospholipids and Lipid II can be rationalized from the difference in function of the phospholipids and Lipid II. The phospholipids need to form a bilayer and therefore will require a fast and relatively a specific translocation mechanism. In contrast, a unique and highly dynamic molecule as Lipid II needs to undergo a very fast and unidirectional transport to allow the peptidoglycan layer to grow. This most likely will require a more specific translocation mechanism. Such a specific mechanism has been proposed for instance for the analogous transport of LPS precursors across the inner membrane of *E. coli* (Doerrler *et al*. 2001).

Using the NBD-quenching property of the anti-DNP antibody, we directly demonstrated NBD-labelled Lipid II translocation in biological membranes (Fig. 4B). This shows that an intact translocation machinery is still present in isolated *E. coli* inner membrane vesicles. Although we have used a labelled lysine-version of the peptidoglycan precursor, we expect that neither labelling, nor the use of a lysine version as opposed to a diaminopimelic acid version that is common to *E. coli* affect the translocation to a large extent. In several publications the peptidoglycan synthesizing machinery was observed to exhibit a high tolerance towards variants of the peptide moiety of Lipid II and even towards chemically modification of the pentapeptide. Sadamoto *et al*. (Sadamoto *et al*., 2002; Sadamoto *et al*., 2004), for example, showed that when using a fluorescein labelled UDP-MurNAc-pentapeptide derivative, *E. coli* was able to incorporate the fluorescent precursor into its cell wall. Likewise, incubation of *E. coli* cells with NBD-labelled substrates for the cell wall recycling machinery resulted in incorporation of the fluorescent label into the cell wall (N.K. Olrichs and E. Breukink, unpublished). Therefore, we conclude that our results obtained with the NBD-labelled Lipid II can be extrapolated to unlabelled, wild-type Lipid II.

Thus, the translocation machinery is likely composed of one or more integral membrane proteins or membrane associated proteins. What would then be this specific machinery? Our experiments exclude the possibility that the Lipid II synthesis enzyme MurG is responsible for membrane translocation. Despite the fact that the enzyme is inserted into the membrane (van den Brink-van der Laan *et al*., 2003), the conversion of Lipid I to Lipid II does not result in translocation across model membrane vesicles. In addition to this, while MurG catalysed the synthesis of Lipid II in the isolated *E. coli* membrane vesicles on ice, no transmembrane movement of Lipid II could be observed yet. This demonstrates that synthesis and translocation of Lipid II are not obligatory coupled, and suggests that translocation is carried out by a specific protein. Moreover, because we have shown Lipid II transport in *E. coli* membrane systems lacking ATP or a proton motive force (pmf), we can reason that no ABC-like transporter or any other energy source is needed for the translocation to occur.

The translocated Lipid II should in principle become a substrate for the transglycosylase activities of the high molecular weight PBPs or the monofunctional transglycosylases that are present in the bacterial membrane. This process should lead to a reduction in fluorescence. Assays for measuring transglycosylase activity exploit this phenomenon using dansylated versions of Lipid II (Schwartz *et al*., 2002). As shown in Fig. 1A, also the fluorescence intensity of the NBD label is influenced by the environment, thereby allowing us to test if the NBD-labelled Lipid II that appears on the outside of the vesicles is a substrate of the transglycosylases. Any transglycosylase activity would polymerize Lipid II. This polymerized Lipid II is then likely to be degraded by the lysozyme activity remaining in our vesicle preparation. During these processes the NBD label is transferred from a membranous environment, the Lipid II head group, to an aqueous environment. The temperature- and time-dependent decrease of fluorescence in combination with the diminishing NBD-Lipid II pool in Fig. 4D and E show indeed that the NBD label is transferred to a more polar environment. These observations clearly indicate that there is a link between Lipid II transport and transglycosylation activity. Considering our results, we hypothesize that the translocation of Lipid II is coupled to ongoing transglycosylation by the PBPs, which is an important novel insight in the process of Lipid II transport.

Experimental procedures

Materials

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-phosphoglycerol (DOPG) and 1-oleoyl-2-[6-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphoglycerol C_6NBD-PG were purchased from Avanti Polar Lipids. UDP-N-acetylglucosamine (UDP-GlcNAc) was purchased from Sigma. WALP23 (AcGWWLALA LALALALAL-d_4-Ala-LALWWANH_2) was synthesized and purified as described (Ozdirekcan *et al*., 2005). Phospholipids and WALP23 stock solutions were prepared in chloroform/methanol 1:1 (v/v) and stored at –20°C under nitrogen. MurG was purified as described (van den Brink-van der Laan *et al*., 2003) and stored at –20°C in a concentration of 0.142 mg ml^{-1} in 20 mM Tris-HCl, pH 8 in the presence of 20% glycerol. Anti-dinitrophenyl (DNP) polyclonal rabbit antibodies were purchased from Molecular Probes. Tunicamycin was obtained from Sigma.

Isolation, purification and labelling of UDP-MurNac pentapeptide

The lysine form of UDP-MurNAc pentapeptide was purified from *Staphylococcus simulans* as described (Kohlrausch and Holtje, 1991). It was essentially labelled with NBD-chloride at the lysine of the pentapeptide moiety residue in an identical way as described for the labelling of UDP-MurNAc pentapeptide with pyrenesulfonylchloride (Breukink *et al*., 2003).

Synthesis and purification of cell wall precursors

Lipid II, NBD-labelled Lipid II and NBD-labelled Lipid I were synthesized and purified (with yields > 80%) as described before (Breukink *et al*., 2003), with slight adaptations for NBD-labelled Lipid I. In this case *Micrococcus flavus* membrane vesicles (16–32 μmol lipid Pi) were incubated with 3 μmol NBD-labelled MurNAc pentapeptide, 6 μmol undecaprenylphosphate in 60 ml of buffer containing 100 mM Tris-HCl, pH 8, 5 mM MgCl and 1% Triton X-100. The mix was incubated at room temperature for 1 h followed by extraction of the lipids with 1.5-volume butanol/6 M pyridine-acetate, pH 4.2. After brief centrifugation, the butanol phase containing NBD-Lipid I was recovered and washed with 60 ml of water. Purification of NBD-Lipid I was performed using a DEAE-cellulose column as described (Breukink *et al*., 2003) and monitored by high performance thin layer chromatography (HPTLC) on silica 60 plates using chloroform/methanol/water/ammonia, 88:48:10:1 (v/v/v/v) as eluent. NBD-labelled Lipid I/II spots could be visualized by both UV and staining with iodine vapour.

Preparation of LUVs containing Lipids I/II

Large unilamellar vesicles with NBD-labelled compounds incorporated, were prepared by mixing 75 mol% DOPE, 25 mol% DOPG and 0.2 mol% of either NBD-Lipid I, NBD-Lipid II or NBD-PG from stock solutions. The percentages of DOPG and DOPE were chosen, because they roughly mimic the membrane phospholipid composition of the *E. coli* inner membrane (Dowhan, 1997). When indicated, WALP23 was incorporated into the LUVs by adding it to the lipid solution from a stock solution to a concentration of 0.4 mol% prior to making the lipid film. The concentrations of phospholipids in the experiments, expressed as lipid-Pi, were determined by quantification of the phosphorus concentration as described by Rouser *et al*. (Rouser *et al*., 1970).

Lipids were dried under a N2 flow and subsequently under vacuum for 2 h. The lipid films were hydrated with a buffer composed of 10 mM Tris-HCl, pH 8, 100 mM NaCl (buffer A) supplemented with 20 mM $K_3Fe(CN)_6$ to a lipid-Pi concentration of approximately 10 mM. $K_3Fe(CN)_6$ was incorporated to protect the NBD-labelled Lipid II situated on the inside of the LUVs against possible dithionite leakage into the vesicles (Kol *et al*., 2001). The vesicle suspension was freeze-thawed 10 times. Subsequently, unilamellar vesicles were formed by manually extruding the suspension 10 times through 200 nm membrane filters (Anotop 10, Whatman, Maidstone, UK). Excess $K_3Fe(CN)_6$ was removed by gel filtration on a Sephadex G 50 medium spin column in buffer A.

Flop of NBD-Lipid II

All measurements were performed in 1.25 ml of buffer A in a quartz cuvette at 20°C on an SLM Aminco SPF 500C fluorimeter. The excitation wavelength was set on 478 nm while emission was recorded at 534 nm or between 495 and 595 in case of recording the emission spectra.

Flop of Lipid NBD-Lipid II was measured by determining the percentage of fluorescence not accessible for dithionite reduction after different times of incubation in LUVs with an asymmetrical distribution of fluorescent Lipid II.

First, vesicles with fluorescent label only on the protected inside of the membrane were prepared by adding dithionite to a suspension of LUVs containing 0.2 mol% NBD-Lipid II (10 mM lipid-Pi) in a final concentration of 8 mM, always from a 1 M stock solution in 1 M Tris pH 11, after which the suspension was incubated for 10 min at room temperature. This treatment quantitatively converted all the NBD-labelled Lipid II located in the outer leaflet of the membranes to the non-fluorescent ABD form. Subsequently, the excess of dithionite was inactivated by adding $K_3Fe(CN)_6$ from a 1 M stock solution to a final concentration of 20 mM, after which the vesicles were stored on ice until use.

Appearance of fluorescent NBD-Lipid II in the outer monolayer (flop) during an incubation at 30°C was tested by taking aliquots at different time points corresponding to 125 nmol lipid-Pi. These aliquots were diluted 100-fold in a fluorescence cuvette with buffer A followed by the addition of dithionite (8 mM final concentration). The amount of fluorescent Lipid II that remained on the protected inside of the LUVs in these samples was determined by calculating the percentage of fluorescence remaining 100 s after addition of dithionite.

The effect of MurG-catalysed synthesis of Lipid II from Lipid I and UDP-GlcNAc on the transmembrane movement of Lipid II was measured as follows. Vesicles containing 0.2 mol% NBD-Lipid I were incubated with UDP-GlcNAc (in 20-fold molar excess over NBD-Lipid I) in Buffer A containing 1 mM $MgCl_2$, in the presence of 0.001% Triton X-100. MurG was added in a molar ratio of 1:150, MurG to Lipid I, in a total volume of 1 ml, from which at several time points samples were taken for both determination of the amount of protection of NBD-labelled lipids as described above and for TLC analysis. Each sample for TLC analysis was divided into two pools. The first pool was subjected to lipid extraction without further treatment where the other half was treated with dithionite to quench the outer NBD pool as described above, prior to lipid extraction. Lipids were extracted with butanol/6 M pyridine-acetate, pH 4.2. The butanol phase was washed once with water, and subsequently evaporated. The lipid film was dissolved in 10 μl of chloroform/methanol (1:1) and applied on the HPTLC silica 60 plates and developed in chloroform/methanol/water/ammonia, 88:48:10:1 (v/v/v/v).

The amount of newly synthesized NBD-Lipid II as percentage of the total NBD-Lipid I and II pool was quantified by scratching the fluorescent spots from TLC plates. The silica powder samples containing NBD-Lipid I or NBD-Lipid II were extracted from the silica with chloroform/methanol (1:1). After evaporation of chloroform/methanol, samples were hydrated with 80 μl of buffer A containing 1 vol% Triton X-100. Fluorescence spectra were taken from these samples and the fluorescence intensity at 535 nm (bandwidth 5 nm) was

determined and assumed to be representative of the amount of NBD-Lipid I/II in the spot.

Determination of the quenching efficiency of anti-DNP-antibodies on the fluorescence of NBD-labelled Lipid II

The influence of increasing amounts of anti-DNP antibodies on the fluorescence of 15 pmol NBD-Lipid II was assayed in 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM NaCl, 1 mM $MgSO_4$ and 0.2% Triton X-100. The fluorescence was recorded as described above.

NBD-Lipid II translocation in isolated E. coli *membrane vesicles*

Quenching of NBD-labelled Lipid II by anti-DNP antibody was used as a means of measuring Lipid II translocation across the cytoplasmic membrane. RSO vesicles were isolated from *E. coli* strain W3899 (DeChavigny *et al*., 1991) and prepared in 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM NaCl and 1 mM $MgSO_4$ as described (Huijbregts *et al*., 1997). NBD-UDP-MurNAc-pentapeptide (250 pmol) and UDP-GlcNAc (10 nmol) were brought into the membrane vesicles (125 nmol lipid Pi) by freezing and thawing on ice twice in a volume of 45 μl of 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM NaCl and 1 mM $MgSO_4$. This procedure resulted in the incorporation of 4% of the NBD-UDP-MurNAc-pentapeptide and UDP-GlcNAc in the vesicles, as determined using radiolabelled versions of the precursors. NBD-Lipid II synthesis occurred while the samples were standing on ice. After washing, the membrane pellet was resuspended into 1.25 ml of the same buffer and fluorescence spectra were recorded in the presence or absence of 40 μg anti-DNP antibody at 14°C and 24°C at different time intervals for a total of 30 min. A control reaction was carried out (now using 4 nmol NBD-UDP-MurNAc-pentapeptide) where 2 μg tunicamycin was added during the freeze-thaw procedure to prevent synthesis of NBD-Lipid I/II. During the recording of the fluorescence spectra, 40 μg anti-DNP antibody was added 2 min after the first measurement and 0.2% Triton X-100 was added after 32 min to disrupt the membranes. Direct evidence of PBP activity was obtained by quantification of the NBD-Lipid II pool in the RSO vesicles after incubation at room temperature for up to 40 min in the absence of anti-DNP antibody. For good visualization and quantification, triple volumes of all components were used. At the times indicated, samples (25%) were taken and lipids were extracted with 1.5-volume butanol/6 M pyridine-acetate, pH 4.2 as described above. Samples were analysed by TLC as described above and NBD-Lipid II spots were quantified using a Bio-Rad Gel Doc 2000 imager with the Quantity One software.

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