Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*

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The outer membrane of most Gram-negative bacteria is made up of LPS, and in nearly all bacteria that contain LPS it is essential for the life of the organism. The lipid portion of this molecule, lipid A, also known as endotoxin, is a potent activator of the innate immune response. More than 50 genes are required to synthesize LPS and assemble it at the cell surface. Enormous progress has been made in elucidating the structure and biosynthesis of LPS, but until recently the cellular components required for its transport from its site of synthesis in the inner membrane to its final cellular location at the cell surface remained elusive. Here we describe the identification of a protein complex that functions to assemble LPS at the surface of the cell. This complex contains two proteins: Imp, already identified as an essential outer-membrane protein implicated in LPS assembly; and another protein, RlpB, heretofore identified only as a rare lipoprotein. We show that RlpB is also essential for cell viability and that the Imp/RlpB complex is responsible for LPS reaching the outer surface of the outer membrane.

essential lipoprotein | Gram-negative bacteria | outer-membrane biogenesis

The cell envelope of Gram-negative bacteria such as *Escherichia coli* is composed of the inner (cytoplasmic) membrane (IM), the outer membrane (OM), and the periplasmic space in between, where the bacterial peptidoglycan (cell wall) is located. The OM is an asymmetric lipid bilayer with phospholipids forming the inner leaflet and LPS forming the outer leaflet (1). β -Barrel OM proteins (OMPs) are inserted into the OM whereas lipoproteins are anchored to the inner leaflet of the OM through posttranslationally attached lipid moieties. The OM serves as a permeability barrier that protects the cells against toxic compounds such as antibiotics and detergents in their environment (2).

The components of the OM (proteins and lipids) are synthesized inside the cell or at the inner leaflet of the IM. They need to be transported to and assembled at the OM in the correct orientation to maintain this barrier function during cell growth and division. Proteins involved in transporting these components across the IM have been identified and characterized (3–5). Much less is known, however, about how the OM components are transported across the aqueous periplasmic space and inserted into the OM. The recently identified Lol system targets lipoproteins to the OM through a periplasmic carrier protein, LolA, and an OM receptor, LolB (6, 7). Periplasmic factors involved in OMP folding have also been identified (8, 9). However, the mechanism(s) of how these factors facilitate transport of the OMPs across the periplasmic space is not known. Recently, a multiprotein complex involved in OMP assembly was identified by using a combination of genetic and biochemical approaches (10–12). This complex contains an OMP, YaeT, and three previously uncharacterized lipoproteins, YfgL, YfiO, and NlpB.

An essential gene *imp* was recently shown to be involved in OM biogenesis. In *E. coli* when Imp was depleted a novel

membrane fraction with higher density was observed (13). Remarkably, LPS is not essential in *Neisseria meningitidis*. Moreover, in this organism Imp is not essential either (14). In this *imp* knockout strain LPS was not modified by enzymes expressed in the OM or added into the extracellular medium and thus was not surface-exposed. These results suggested that Imp is involved in assembling LPS in the outer leaflet of the OM (14). Here we identified a new protein, RlpB, which physically interacts with Imp. In *E. coli* the *rlpB* gene is essential, and RlpB depletion results in similar defects in OM biogenesis as Imp depletion. We show that Imp and RlpB are components of an OM complex required for LPS assembly.

Results

Imp Forms a Complex with RlpB. We have previously used a tagged lipoprotein, YfgL-His, to isolate a multiprotein complex that was shown to be involved in OMP assembly (11). Imp, the protein involved in LPS assembly, has also been suggested to exist in higher-molecular-weight complexes (13). To determine whether there were other proteins associated with Imp, we constructed a tagged version of Imp (Imp-His) and, in a copurification experiment, looked for proteins that might interact with Imp physically. Imp-His was enriched on a Ni-NTA column from solubilized OM extracts prepared from wild-type cells containing the gene for the tagged construct on a plasmid. When compared with the same experiment done with wild-type cells lacking the plasmid, Imp-His copurified with an additional protein. As shown in Fig. 1A, there is an additional protein with a molecular mass of ≈ 20 kDa that is not present in the control sample. We isolated the 20-kDa protein and determined the amino acid sequence of peptides generated by trypsin digestion using tandem MS. This protein was RlpB.

To provide further evidence that the interaction between Imp and RlpB is physiologically relevant, we cloned the gene for RlpB and then modified it to add a C-terminal tag (RlpB-His). The plasmid carrying the tagged gene was transformed into wild-type cells, and an immunoprecipitation experiment was performed by using a monoclonal anti-His tag antibody. In addition to RlpB-His, the anti-His tag antibody also immunoprecipitates a protein of higher molecular mass (≈ 110 kDa in an SDS/PAGE run under nonreducing conditions) (Fig. 1B). We confirmed that this protein was Imp by immunoblot using a polyclonal anti-Imp antibody. The results demonstrate that RlpB physically interacts with Imp.

Conflict of interest statement: No conflicts declared.

Abbreviations: IM, inner membrane; OM, outer membrane; OMP, OM protein.

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Fig. 1. Imp copurifies with RlpB. (A) Ni-NTA column-enriched proteins from OM extracts of wild-type cells (lane 2) and wild-type cells containing the *pImp-His* plasmid (lane 3). The band appearing in lane 3 that is not present in lane 2 was determined to be RlpB by tandem MS. (B) Immunoprecipitated samples from whole-cell extracts prepared from wild-type cells (lane 2) and wild-type cells containing the *pRlpB-His* plasmid (lane 3) by using a monoclonal anti-His antibody. The protein labeled as Imp was confirmed by immunoblot. Samples were subjected to SDS/PAGE and stained with silver (A) or Coomassie blue (B). The relevant proteins that are described are labeled with arrows. Molecular mass markers (lane 1) are indicated in kDa.

RlpB Is Essential. Takase *et al.* (15) characterized RlpB as a low-abundance lipoprotein in *E. coli*. Sequence characteristics (7) suggest that RlpB localizes to the OM, a prediction supported by the copurification of Imp with RlpB (Fig. 1). Because of its strong interaction with an essential OMP, we sought to determine whether RlpB is essential. Gerdes *et al.* (16) and Baba *et al.* (17) predicted that RlpB is essential, whereas another genetic screen for essential genes did not identify RlpB (18).

To test whether RlpB is essential, we placed an inducible copy of *rlpB* at the *latt* site using the λ InCh method (19). This method allowed us to disrupt the endogenous *rlpB* locus by deletion-substitution using recombinering (20). The genetic structure of the resulting strain, AM689, is depicted in Fig. 2A. This strain's behavior is similar to that of wild-type strains when grown in the presence of arabinose. After subculturing in the absence of arabinose, cell growth stopped after approximately five generations (Fig. 2B), and then cell death was observed as measured

Fig. 2. Test to determine whether RlpB is essential. (A) Strain AM689 expresses *rlpB* from an arabinose-inducible promoter at the *latt* site, allowing knockout of the endogenous *rlpB* gene. (B) Growth curve of AM689 grown in the presence (diamonds) or absence (squares) of arabinose as measured by optical density.

Fig. 3. Fractionation of wild-type, Imp, RlpB, and YaeT depletion strains. Strains indicated were fractionated by sucrose density gradients as described and submitted to immunoblots using antibodies recognizing LPS, Imp, and LamB as indicated. (A) Wild-type cells grown in LB. (B) MB215 cells depleted of Imp. (C) AM689 cells depleted of RlpB. (D) JCM166 cells depleted of YaeT. Fractions were collected and loaded such that denser fractions are on the left with fractions of lighter density on the right. The 55-kDa protein is an IM protein that reacts with the Imp antibody (13). The 32-kDa protein that reacts with the Imp antibody is OmpA. The 55-kDa protein remains visible in the LamB blots because they were performed sequentially.

by colony-forming units (data not shown). We therefore conclude that RlpB is essential.

Imp/RlpB Depletion Leads to Increased OM Density. Previously, Braun and Silhavy (13) observed a novel dense membrane fraction when Imp was depleted, and they concluded that Imp is involved in OM biogenesis. Because RlpB interacts with Imp, we predicted that RlpB is also involved in OM biogenesis. To test this prediction we fractionated the membranes isolated from strains depleted of Imp or RlpB as well as control strains using sucrose density gradient centrifugation.

Fractions collected from the gradient were subjected to immunoblot analysis using antibodies recognizing IM and OM markers. Wild-type strains display a bimodal fractionation pattern corresponding to IM and OM. As shown in Fig. 3A, wild-type OM equilibrates around fraction 9 in the gradient, and IM equilibrates around fraction 20. LPS and the OMPs OmpA, LamB, and Imp all equilibrate around fraction 9, whereas a 55-kDa IM protein (13) recognized by the Imp antibody equilibrates around fraction 20. Thus, our protocol achieves good membrane separation.

The OM assembly defect of Imp-depleted cells manifests itself as an increase in the density of the OM. Fig. 3B depicts a fractionation performed on Imp-depleted cells. In this case the OM equilibrates around fraction 3, whereas the IM remains

Fig. 5. LPS is modified by PagP when Imp/RlpB is depleted. Negative ion MS of lipid A species were obtained from different strains. (A) Wild-type strain MC4100 was treated with EDTA during growth following the protocol of Jia *et al.* (26). (B) The Imp depletion strain MB215 was grown in the absence of IPTG. (C) The RlpB depletion strain AM689 was grown in the absence of arabinose. Lipid A extraction and MS analysis followed the protocol of Zhou *et al.* (27).

occurs because ectopically expressed RlpB is not at physiological levels. Taken together these results suggest that when Imp or RlpB is depleted the OM becomes defective, making it possible for phospholipids to reach the outer leaflet of the OM and activate PagP for LPS modification.

De Novo Synthesized LPS Does Not Reach the Cell Surface in Imp/RlpB-Depleted Cells. The observation that LPS is modified by PagP in Imp- and RlpB-depleted cells suggests that we might be able use this modification as an OM outer leaflet marker to determine the localization of *de novo* synthesized LPS in these cells (29).

We prepared four radiolabeled cultures of each depletion strain, of which two were depleted and two were grown in the presence of inducer. One of the cultures was pulse-labeled with [^{14}C]acetate after depletion to label *de novo* synthesized LPS, and the other three cultures were labeled from the beginning; thus, they report steady-state distribution of LPS. One of the nondepleted cultures was treated briefly with EDTA before the cells were harvested to serve again as a positive control. Lipid A was isolated from all four cultures and analyzed by thin layer chromatography followed by autoradiography by using the method of Zhou *et al.* (27).

As shown in Fig. 6A, when Imp was not depleted lipid A was present mainly in the hexa-acyl form (lane 1), which is consistent with the MS results. When the culture was treated with EDTA a small amount of hepta-acyl lipid A was detected (lane 2). This finding is also consistent with our MS results. However, when Imp was depleted a significant amount of lipid A became hepta-acylated, as shown in lane 3. In contrast, after Imp depletion only the hexa-acyl form of lipid A was detected from *de novo* synthesized LPS. This finding indicates that the newly synthesized LPS did not reach the outer leaflet of the OM and thus could not be modified by PagP. Similar results were seen for RlpB depletion strains grown under the same set of conditions, except that here again we detect the hepta-acyl form of lipid A in the nondepleted strain (Fig. 6B). These data clearly show that

Fig. 6. Cell-surface accessibility of *de novo* synthesized LPS in Imp/RlpB-depleted cells. The Imp depletion strain MB215 (A) or the RlpB depletion strain AM689 (B) was grown with (lanes 1 and 2) or without (lanes 3 and 4) inducer. [^{14}C]Acetate was added at the beginning of subculturing to label steady-state LPS (lanes 1–3) or added after Imp/RlpB was depleted (lane 4) to label *de novo* synthesized LPS. Of the nondepleted cultures, one was treated with EDTA following the protocol of Jia *et al.* (26) (lane 2) to serve as a positive control for the position of the hepta-acyl lipid A. Lipid A was isolated and analyzed by thin layer chromatography, followed by autoradiography as in Zhou *et al.* (27). Equal amounts of radioactive material were spotted for each sample.

in the absence of Imp or RlpB *de novo* synthesized LPS does not reach the outer leaflet of the OM.

Discussion

Here we describe a protein complex that is present in the OM and functions in LPS assembly. The complex contains a large β -barrel protein, Imp, which had previously been shown to function in OM biogenesis (13, 14), and a small lipoprotein, RlpB, which heretofore was known only as a rare lipoprotein (15). In *E. coli*, and probably many other Gram-negative bacteria, both of these proteins are essential. Cells depleted of Imp or RlpB have similar phenotypes; the density of the OM is altered, total cellular LPS is increased, abnormal membrane structures accumulate in the periplasm, and phospholipids appear in the outer leaflet of the OM. Moreover, we demonstrated by pulse-labeling analysis that in cells lacking Imp or RlpB newly synthesized LPS does not reach its final cellular location. Because RlpB does not function simply to stabilize or target Imp, we conclude that both of these proteins function together in what is likely to be the last step in LPS assembly, localization to the outer leaflet of the OM.

Although we have identified two essential components of the LPS assembly complex at the OM, we do not know whether there are other protein components in this complex, what the stoichiometry of the proteins is in the complex, or what each protein does in molecular terms. It is known that LPS is synthesized on the inner leaflet of the IM and is then flipped to the outer leaflet by MsbA (5, 30, 31). LPS must then be transported across the periplasm and delivered to an OM assembly site. LPS could cross the periplasm at membrane adhesion sites known as Bayer's bridges (5, 29, 32, 33). Alternatively, LPS may be escorted through the periplasm by dedicated chaperone components in a manner analogous to OM lipoproteins (7). Regardless of how LPS transits the periplasm, Imp/RlpB could function in receiv-

ing LPS at the inner leaflet of the OM, in flipping LPS across the OM, or in both of these processes. Logically, we could expect that, if the OM receiving step is blocked LPS would accumulate at the outer surface of the IM, and if the OM flipping step is blocked LPS should accumulate at the inner leaflet of the OM. We have shown that in the Imp and RlpB depletion strains the OM fractions are heavier than normal and cellular LPS levels increase. We do see extra membrane structures that appear to be located in the periplasm in strains depleted of Imp or RlpB. However, we cannot determine whether the extra membrane originated from the IM or from the OM, and thus we cannot distinguish between these potential functions. Indeed, we cannot yet rule out the possibility that one of the proteins is involved in receiving and the other is involved in flipping.

We have previously shown that the YaeT/YfiO/YfgL/NlpB complex is required for the assembly of OMPs (11). Cells depleted of YaeT have defects in OMP assembly (Fig. 3D). YaeT depletion dramatically reduces the amount of protein in the OM, and this decrease in the protein:lipid ratio reduces OM density. In our Imp- or RlpB-depleted cells OmpA and LamB are readily detectable. Moreover, OmpA and LamB are properly assembled and folded in the depletion strains (ref. 13 and data not shown). Thus, it appears that the processes of LPS and OMP assembly are separable. Similar results have been obtained by Doerrler and Raetz (21) in *E. coli* and Bos *et al.* (14) in *Neisseria*. So OMPs are targeted through the YaeT complex, and LPS is targeted through the Imp/RlpB complex independently. Because cells maintain a nearly constant protein:lipid ratio in the OM regardless of growth condition (34), and because there are genetic interactions between *imp* and genes specifying the YaeT complex (10), a challenge for the future is to understand how these two separate systems coordinate their functions.

Materials and Methods

Bacterial Strains. All strains are derived from MC4100 [*F*[−]*araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 flb5301 deoC1 ptsF25 thi*]. The Imp depletion strain (MB215) is described by Braun and Silhavy (13). The YaeT depletion strain (JCM166) is described by Wu *et al.* (11). The RlpB depletion strain (AM689) is described here. In both JCM166 and AM689 the *araD139* mutation has been reverted to *ara*⁺. All media were prepared and cells were grown as described previously (11). Antibiotics used were kanamycin (25 μg/ml) and carbenicillin (50 μg/ml).

Construction of an RlpB Depletion Strain. To create an RlpB depletion strain, primers ACM143 (5′-GTAAAGTGATTTA-CGTACCAGGTAACTCCTCAATCTGGTTCGTTGGCT-AAGTGTAGGCTGGAGCTGCTTC-3′) and ACM145 (5′-CTAATCGGGTAGATATCACGGCCGGGGATCAACACGGTCGCATTAACCGTCATATGAATATCCTCCTTAG-3′) were used to amplify the kanamycin resistance cassette from pKD4 (35) by PCR. The product of this reaction has the *kan* cassette flanked by 50-bp regions with homology to the *rlpB* locus. This oligonucleotide was used in a recombineering reaction to replace the chromosomal *rlpB* gene following the protocol of Copeland *et al.* (20). Insertion of the *kan* cassette was verified by PCR and sequencing. Concomitantly, pBADRlpB was integrated into the *λ-att* site of AM604 (MC4100 *ara*⁺) by using the *λ*InCh procedure of Boyd *et al.* (19), which allowed introduction of the *rlpB::kan* allele by P1 transduction in the presence of arabinose. The resulting strain, AM689, has the first 306 bases of the endogenous *rlpB* locus replaced by the *kan* cassette but is viable because it expresses RlpB from the exogenous gene integrated at the *λ-att* site.

Plasmids. The multiple cloning sites of pET24a(+) vector (Novagen) between XbaI and Bpu1102I restriction sites was moved into pET23a(+) vector (Novagen) to create pET2342 vector by

using the above two restriction enzymes. The *imp* gene was amplified by PCR from genomic DNA of the MC4100 strain by using primers Imp-N (5′-ACACCATATGAAAAACG-TATCCCCACT CT C-3′) and Imp-C (5′-ACACCCATG-GCAAAGTGTGTTTGATACGGCAGAAT G-3′). The PCR product was inserted into pET2342 vector digested by NdeI and NcoI enzymes (New England Biolabs) to create *pImp-His*. The *rlpB* gene was amplified by PCR from genomic DNA of MC4100 strain using primers RlpB-N (5′-ATGACATATGCGATATCT-GGCAACAT TGTTGTTATCTCTG-3′) and RlpB-C (5′-ACGTCTCGAGGTTACCCAGCGTGGTGG AGAC-3′). The gene was inserted into pET23a(+) vector digested with NdeI and XhoI enzymes (New England Biolabs) to create *pRlpB-His*. Plasmid pBADRlpB was constructed as follows. Primers ACM146 (5′-AAAGAATTTCGCGCGGGAGGAAGC-3′) and ACM147 (5′-TTATCTAGACGCGGAGTTGTTCC-3′) were used to amplify the *rlpB* gene from MC4100. The PCR product was digested with EcoRI and XbaI and ligated into pBAD18 that had been cut with the same enzymes. The construct was transformed into AM604. The resulting plasmid has the *rlpB* gene cloned under control of an arabinose-inducible promoter. These constructs were verified by DNA sequencing.

Affinity Purification. Culture (1.5 liters) was grown in the LB broth to mid-logarithmic phase (OD₆₀₀ ≈ 0.6). Cells were pelleted by centrifugation at 5,000 × *g* for 10 min and then resuspended in 25 ml of TBS (20 mM Tris-HCl, pH 7.4/150 mM NaCl) with 100 μg/ml lysozyme and 50 μg/ml DNase I. The cells were lysed by two passages through a French press (Thermo Electron) at 16,000 psi (1 psi = 6.89 kPa). The cell lysate was centrifuged at 5,000 × *g* for 10 min to remove unbroken cells, and the supernatant was centrifuged at 100,000 × *g* for 1 h in an ultracentrifuge (Model XL-90, Beckman). The pellet was extracted with 15 ml of TBS/2% Triton X-100 at room temperature for 20 min and recentrifuged as above. The obtained pellet was then extracted with 5 ml of BugBuster Reagent (Novagen) and ultracentrifuged again. The final supernatant was supplemented with 20 mM imidazole and loaded into a column packed with 0.5 ml of Ni-NTA resin (Qiagen), which had been preequilibrated with TBS, 20 mM imidazole, and 0.1% Triton X-100. The column was washed with 10 ml of equilibration buffer and eluted with 5 ml of TBS/200 mM imidazole. The eluate was concentrated in an ultrafiltration device (Amicon Ultra, Millipore) by centrifugation at 5,000 × *g* for 30 min. A 10-μl sample was used for SDS/PAGE analysis. Silver stain was conducted according to the protocol of the Bio-Rad silver stain plus kit.

Immunoprecipitation. Immunoprecipitation experiments were performed according to Wu *et al.* (11).

Membrane Fractionation. Sucrose density gradient centrifugation was performed according to Braun and Silhavy (13).

Electron Microscopy. Samples for electron microscopy were prepared according to the method of Ogura *et al.* (25). Thin 70-nm sections were obtained by using a diamond knife on a Leica UC6 Ultramicrotome and observed at 80 kV on a Zeiss 912AB Transmission Electron Microscope equipped with an Omega Energy Filter. Micrographs were captured by using a digital camera from Advanced Microscopy Techniques and saved as TIFF files onto a Dell PC computer.

Isolation of Lipid A and MS Characterization. Five-milliliter cultures of wild-type strain MC4100 and the depletion strains AM689 or MB215 were inoculated with overnight cultures to an OD₆₀₀ of 0.01 in LB broth. The cells were grown at 30°C for 5 h and then harvested. One MC4100 culture was treated with 25 mM EDTA (pH 8.0) for 10 min before harvesting. Comparable amounts of

cells of the same strain, grown under different conditions, were pelleted. Lipid A was then extracted from these samples following the procedure described by Zhou *et al.* (27). The solid obtained was redissolved in 10 μ l of 4:1 chloroform/methanol, and 1 μ l of this solution was mixed with 1 μ l of MALDI matrix on a MALDI plate and allowed to dry. The MALDI matrix was prepared as a mixture of saturated 6-aza-2-thiothymine in 50% acetonitrile and 10% tribasic ammonium citrate (9:1, vol/vol) (27). Spectra were acquired in the negative reflector mode by using a time-of-flight MALDI Voyager DE Pro mass spectrometer (Applied Biosystems). Each spectrum was the average of 100 shots.

Labeling de Novo Synthesized LPS. Four cultures of the AM689 and MB215 strain were inoculated with overnight cultures to an OD₆₀₀ of 0.01 in 50 ml of fresh LB broth. Two of the cultures were grown under depletion conditions, and two were grown in the presence of the appropriate inducers. For the nondepletion controls and one of the depletion cultures, 50 μ l of 1 mCi/ml (1 Ci = 37 GBq) sodium [1-¹⁴C]acetate was added to give a final concentration of 1 μ Ci/ml. The four cultures were grown at 30°C until the depletion and nondepletion samples started to diverge ($\approx$ 3 h for MB215 and 3.5 h for AM689). The cultures were

harvested and resuspended in 50 ml of fresh LB broth with appropriate antibiotics and inducers. A total of 50 μ l of 1 mCi/ml sodium [1-¹⁴C]acetate was added to the depletion culture grown in the absence of radiolabel to give a final concentration of 1 μ Ci/ml. All four cultures were grown for another 30 min. For one of nondepleted cultures, 25 mM EDTA (pH 8.0) was then added and the culture was grown for another 10 min, after which all four cultures were harvested. The cell pellets were washed with 10 ml of PBS (pH 7.2) and resuspended in 1.6 ml of PBS, and 2 ml of chloroform and 4 ml of methanol were added. From here, lipid A was extracted according to the procedure of Zhou *et al.* (27). The remaining solids were dissolved in 100 μ l of a 4:1 chloroform/methanol mixture and spotted onto a TLC plate. (Four times as much solution was spotted for the pulse-labeled sample.) The TLC plate was developed in a chloroform/pyridine/96% formic acid/water (50:50:14.6:4.6) solvent system. The plates were dried under vacuum for 1 h and then visualized by using a PhosphorImager.

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