An *Escherichia coli* undecaprenyl-pyrophosphate phosphatase implicated in undecaprenyl phosphate recycling

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Undecaprenyl phosphate (Und-P) is a universal lipid carrier of glycan biosynthetic intermediates for carbohydrate polymers that are exported to the bacterial cell envelope. Und-P arises from the dephosphorylation of undecaprenyl pyrophosphate (Und-PP) molecules produced by *de novo* synthesis and also from the recycling of released Und-PP after the transfer of the glycan component to other acceptor molecules. The latter reactions take place at the periplasmic side of the plasma membrane, while cytoplasmic enzymes catalyse the *de novo* synthesis. Four Und-PP pyrophosphatases were recently identified in *Escherichia coli*. One of these, UppP (formerly BacA), accounts for 75 % of the total cellular Und-PP pyrophosphatase activity and has been suggested to participate in the Und-P *de novo* synthesis pathway. Unlike UppP, the other three pyrophosphatases (YbjG, YeIu and PgpB) have a typical acid phosphatase motif also found in eukaryotic dolichyl-pyrophosphate-recycling pyrophosphatases. This study shows that double and triple deletion mutants in the genes *uppP* and *ybjG*, and *uppP*, *ybjG* and *yeiU*, respectively, are supersensitive to the Und-P *de novo* biosynthesis inhibitor fosmidomycin. In contrast, single or combined deletions including *pgpB* have no effect on fosmidomycin supersensitivity. Experimental evidence is also presented that the acid phosphatase motifs of YbjG and YeIu face the periplasmic space. Furthermore, the quadruple deletion mutant Δ*uppP-ΔybjG-ΔyeiU-ΔwaaL* has a growth defect and abnormal cell morphology, suggesting that accumulation of unprocessed Und-PP-linked O antigen polysaccharides is toxic for these cells. Together, the results support the notion that YbjG, and to a lesser extent YeIu, exert their enzymic activity on the periplasmic side of the plasma membrane and are implicated in the recycling of periplasmic Und-PP molecules.

INTRODUCTION

The assembly of glycan intermediates onto isoprenoid lipid carriers is a common theme in the biogenesis of glycoproteins and cell-surface polysaccharides of prokaryotes and eukaryotes (Bugg & Brandish, 1994; Burda & Aebi, 1999; Helenius & Aebi, 2002; Helenius *et al.*, 2002; Valvano, 2003). A 55-carbon isoprenoid, undecaprenyl phosphate (Und-P), is the lipid carrier of biosynthetic intermediates for cell wall peptidoglycan, O antigen, enterobacterial common antigen, teichoic acids and other bacterial carbohydrate polymers. Und-P arises from the dephosphorylation of its precursor undecaprenyl pyrophos-
pyruvate and glyceraldehyde 3-phosphate, a reaction catalysed by the 1-deoxy-xylulose 5-phosphate synthase encoded by the ddx gene (Lois et al., 1998; Sprenger et al., 1997). In the second step, MEP is synthesized by the 1-deoxy-xylulose 5-phosphate reductoisomerase encoded by the ddx gene (Takahashi et al., 1998). This reaction is specifically inhibited by the antibiotic fosmidomycin (Kuzuyama et al., 1998a; Steinbacher et al., 2003). Subsequent enzymic steps result in the formation of IPP (Fig. 1).

Und-PP is not only synthesized de novo by UppS at the cytosolic side of the plasma membrane (or the inner membrane in Gram-negative bacteria), but also regenerated at the other side of the membrane because of its release from the Und-PP-linked glycans, which after translocation across the plasma membrane are transferred onto other appropriate acceptors to complete the formation of mature cell-envelope polymers. The dephosphorylation of Und-PP is an obligatory step before this molecule can be used (or reused) as a lipid sugar acceptor for polymer biosynthesis (Anderson et al., 1966; Goldman & Strominger, 1972). The availability of Und-P is a limiting factor in the biosynthesis of O polysaccharides, since this lipid carrier is made in very small amounts, and it is also required for the biosynthesis of multiple carbohydrate polymers. Therefore, the recycling pathway for Und-P synthesis from preformed Und-PP released at the periplasmic side of the plasma membrane may also contribute to the total Und-P pool available for the initiation of lipid-linked glycan biosynthesis (Fig. 1).

The de novo synthesis and regeneration of Und-PP at opposite sides of the membrane suggests the requirement for pyrophosphatases whose active sites are in cytosolic and periplasmic environments, respectively. Recently, El Ghachi et al. (2004, 2005) have identified four genes in Escherichia coli K-12 encoding membrane proteins with Und-PP pyrophosphatase activities. Among these, UppP (formerly BacA) accounts for 75% of the cellular Und-PP pyrophosphatase activity (El Ghachi et al., 2004), while the proteins YbjG, YeiU and PgpB account for the remaining activity (El Ghachi et al., 2005). However, it is not clear which of these proteins function in Und-PP recycling.

The recycling of pyrophosphoryl polyisoprenols is not only confined to bacteria. In eukaryotes and also in archaea, dolichol phosphate serves as a lipid anchor for the assembly of glycan moieties that are further added to proteins (Bugg & Brandish, 1994; Burda & Aebi, 1999). After the transfer of the glycan to the glycosylation site of the protein, released dolichol-PP is recycled to dolichol-P by dephosphorylation (Abeijon & Hirschberg, 1992; Fernandez et al., 2004, 2005). However, it is not clear which of these proteins function in Und-PP recycling.

Und-PP synthesis and recycling in E. coli. The precursors and products of this pathway indicated are: G3P, glyceraldehyde 3-phosphate; DXP, deoxy-xylulose 5-phosphate; MEP, 2-C-methyl-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; Und-PP, undecaprenyl pyrophosphate; Und-P, undecaprenyl phosphate; ECA, enterobacterial common antigen; LTA, lipoteichoic acid. Open rectangles denote some of the key enzymes for the synthesis of Und-PP: DXS, deoxy-xylulose-5-phosphate synthase; DXR, deoxy-xylulose-5-phosphate reductoisomerase; UppS, Und-PP synthase; UppP, Und-PP pyrophosphate phosphatase (formerly BacA; El Ghachi et al., 2004). The reaction inhibited by fosmidomycin (FM, black rectangle) is also indicated. Dotted arrows after MEP and Und-P indicate that several other reactions, not specified in this scheme, take place before the synthesis of IPP and the synthesis of the surface glycans, respectively. The dashed arrow indicates that Und-PP released after the synthesis of the surface glycans is recycled by an as yet unknown pathway.

**Fig. 1.** Scheme of Und-P synthesis and recycling in E. coli. The precursors and products of this pathway indicated are: G3P, glyceraldehyde 3-phosphate; DXP, deoxy-xylulose 5-phosphate; MEP, 2-C-methyl-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; Und-PP, undecaprenyl pyrophosphate; Und-P, undecaprenyl phosphate; ECA, enterobacterial common antigen; LTA, lipoteichoic acid. Open rectangles denote some of the key enzymes for the synthesis of Und-PP: DXS, deoxy-xylulose-5-phosphate synthase; DXR, deoxy-xylulose-5-phosphate reductoisomerase; UppS, Und-PP synthase; UppP, Und-PP pyrophosphate phosphatase (formerly BacA; El Ghachi et al., 2004). The reaction inhibited by fosmidomycin (FM, black rectangle) is also indicated. Dotted arrows after MEP and Und-P indicate that several other reactions, not specified in this scheme, take place before the synthesis of IPP and the synthesis of the surface glycans, respectively. The dashed arrow indicates that Und-PP released after the synthesis of the surface glycans is recycled by an as yet unknown pathway.
formation of a covalent phosphoenzyme intermediate that is stabilized by the neighbouring Arg in motif 3, while Lys and Arg in motif 1 together with Ser, Gly and His in motif 2 have an important role in holding the phosphate group of the substrate close to the His from motif 3 (Ishikawa et al., 2000; Neuwald, 1997).

A similar enzyme has not been directly characterized in bacteria, but homologues of Cwh8 are widely conserved in prokaryotes, suggesting they may have an analogous function in the recycling of Und-PP. The growth of these proteins is implicated in the recycling of periplasmic Und-PP molecules.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids used in this work are described in Table 1. Plasmids were introduced into recipient cells by calcium chloride transformation (Cohen et al., 1972) or electroporation (Dower et al., 1988). Bacteria were grown at 30, 37 or 42 °C in Luria–Bertani (LB) medium supplemented with 100 μg ampicillin ml⁻¹, 40 μg kanamycin ml⁻¹, 80 μg spectinomycin ml⁻¹ and 0.2% or 0.02% (w/v) arabinose, as appropriate. For growth curves, overnight cultures of mutants and parental E. coli strains were diluted to an OD600 of 0.03 and 0.02% (w/v) arabinose were added to induce recombinant protein expression. In all cases survival data were analysed at the 6 h time point from multiple repeats.

**LPS analysis.** LPS was prepared as described earlier (Marolda et al., 1993) and samples were separated by 14% Tricine SDS-PAGE. The gels were stained with silver nitrate (Marolda et al., 1990) and samples were separated by 14% Tricine SDS-PAGE. The membrane was treated with either anti-O16 (The Gastroenteric Disease Center, Wiley Laboratory, University Park, PA, USA) or anti-LPS core monoclonal antibodies (HyCult Biotechnologies). Fluorescent secondary antibodies (IRDye800CW affinity-purified anti-rabbit IgG antibodies) were used to detect the positive bands by monitoring the fluorescence with an Odyssey infrared imaging system (LI-COR Biosciences).

**Preparation of crude membrane extracts.** Bacterial cultures were grown overnight in 5 ml LB with the appropriate antibiotic.

**Construction of chromosomal gene deletions.** Chromosomal gene deletions were obtained by the method of Datsenko & Wanner (2000). PCR fragments for mutagenesis were obtained using primers containing 40–45 nucleotides corresponding to regions adjacent to the gene targeted for deletion, plus 20 more nucleotides that annealed to the template DNA from plasmid pKD4, which carries a kanamycin-resistance gene flanked by FLP recognition target sites. PCR products were transformed into E. coli strains carrying pKD46 that were grown in LB plus 0.5% (w/v) arabinose. Kanamycin-resistant colonies were screened for the insertion of the kanamycin-resistance cassette into the targeted gene by PCR with primers annealing to regions outside of the mutated gene. This resulted in the complete replacement of the parental gene by the kanamycin phosphotransferase gene (aph). If required, the antibiotic gene cassette was excised using pCP20, which encodes the FLP recombinase. Plasmids pKD46 and pCP20 are thermosensitive for replication and they were cured at 42 °C.

**Cloning strategies.** PCR products were cloned into pBADHis (Table 1) using specific restriction sites included in the cloning primers. The primers used in this work are listed in Table 2. We used the Stratagene Quick Change kit and appropriate mutagenesis primers to replace His145 by Ala in YbjG. The correct replacement was confirmed by DNA sequencing.

**Fosmidomycin (Fm) survival assay.** The survival of parental and mutant strains in the presence of subinhibitory concentrations of Fm was determined by a microdilution assay using the Bioscreen C automated microbiology growth curve analysis system. This system facilitates easy and reproducible screening of multiple bacterial strains for the growth effects of antibiotics at subinhibitory concentrations (Löwdin et al., 1993). Overnight cultures grown at 30 °C on LB plates were used to inoculate Mueller–Hinton (MH) broth followed by incubation at 30 °C until an OD600 of approximately 1. As indicated above for the growth curves, aliquots of these cultures were adjusted to an OD600 of 0.03 in 100-well microtitre plate containing 300 μl MH broth with various Fm concentrations ranging from 0 to 480 ng ml⁻¹. The Bioscreen C was set at low constant shaking at 37 °C and OD600 measurements were automatically recorded every 30 min during 18 h. The percentage survival for each strain was calculated at each time point using the optical density at 0 ng ml⁻¹ Fm as 100% growth. For complemenation experiments with strain LDT30, cells were grown as indicated before in MH broth also containing 100 μg ampicillin ml⁻¹ and 0.02% (w/v) arabinose to induce recombinant protein expression.
diluted in 15 ml fresh medium to an OD$_{600}$ of 0.05 and grown to an OD$_{600}$ between 0.5 and 0.7. At this point recombinant protein expression was induced by adding arabinose to a final concentration of 0.2 % or 0.02 % (w/v) and cultures were incubated for another 3 h. Bacteria were harvested by centrifugation at 7741 g for 10 min at 4 °C and pellets stored at −20 °C. Bacterial pellets were resuspended in 4 ml 20 mM phosphate buffer pH 7.2 containing an EDTA-free cocktail of protease inhibitors (Roche), and lysed by sonic disruption with a Branson Digital Sonifier model 450 on ice using 10–15 s pulses at an amplitude of 40 %. Cell lysates were cleared of cell debris by centrifugation in a tabletop centrifuge at full speed (13 000 r.p.m.) for 15 min at room temperature. The membranes were collected from the clear lysate by another centrifugation at 39 191 g for 30 min at 4 °C using a Beckman high-speed refrigerated centrifuge, and total protein

### Table 1. Strains and plasmids used in this study

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*Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin.
**Table 2. Primers used for mutagenesis and cloning**

Bold and italicized nucleotides indicate the codon encoding alanine for the His replacement in ybjG<sub>H145A</sub>-His<sub>6</sub>. Underlined nucleotides indicate restriction endonuclease sites (shown in parentheses) incorporated into the primer sequence. The designations in the right column indicate the genes targeted for deletion or for cloning.

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Concentration was determined by the Bradford assay (Bio-Rad), using BSA as a protein standard.

**Protein expression analysis.** Membrane proteins were separated by electrophoresis in 14% SDS-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes by standard procedures. Membranes were blocked overnight with 5% skim milk dissolved in TBS pH 7.6 (50 mM Tris and 150 mM sodium chloride), incubated for 2h with monoclonal anti-alkaline phosphatase antibodies (Sigma-Aldrich) at a dilution of 1:12 000. Specific bands were detected by fluorescence using Alexa Fluor 680 anti-mouse IgG antibodies (Molecular Probes) in the Odyssey infrared imaging system. The GFP fusions were detected with rabbit anti-GFP polyclonal antibodies (Chemicon International; 1:1000). The protein with a C-terminal His<sub>6</sub> epitope was detected with rabbit polyclonal antibody (Sigma) anti-His<sub>6</sub> antibodies. Alexa Fluor IRDye800 CW affinity-purified anti-rabbit IgG antibodies (Rockland) were used as the secondary antibody for the detection of specific bands by fluorescence.

**Alkaline phosphatase assay.** The expression of hybrid proteins with alkaline phosphatase (PhoA) activity was qualitatively assessed by examining the blue-colony phenotypes on LB plates containing 40 μg ml<sup>−1</sup> of the chromogenic substrate 5-bromo-3-chloro-indolyl phosphate (XP, Sigma). To quantify the alkaline phosphatase activity, 1:100 culture dilutions were grown for 3h to an OD<sub>600</sub> of 0.5–0.6 and then induced with 0.02% arabinose for 3h, after which the cells were harvested and assayed as described by Manoil (1991) with the addition of 1 mM isosaccharamide in all the buffers to avoid folding of cytoplasmic PhoA (Derman & Beckwith, 1995).

**Microscopy.** Overnight cells expressing GFP fusions were diluted in LB to OD<sub>600</sub> 0.1, grown to OD<sub>600</sub> 0.5 and then induced with 0.2% arabinose for 3h. At this point the cells were kept on ice for 1–2h to facilitate GFP folding, and then visualized using an AxioScope 2 (Carl Zeiss) microscope with an X100/1.3 numerical aperture Plan-Neofluor objective and a 50 W mercury arc lamp with a GFP band pass emission filter set (Chroma Technology) with a 470±20 nm excitation range and a 525±25 nm emission range. Images were digitally processed using the Northern Eclipse version 6.0 imaging analysis software (Empix Imaging).

**RESULTS AND DISCUSSION**

The acid phosphatase domain of YbjG and YeiU proteins faces the periplasmic side of the plasma membrane

Since the transfer reactions of Und-PP-linked polymers to their acceptor molecules (lipid A-core oligosaccharide or peptidoglycan) occur at the periplasmic side of the plasma membrane, we hypothesized that a recycling Und-PP phosphatase must have an active site oriented towards the periplasm. Topological models for PgpB, YbjG and YeiU produced using the TMHMM program (Sonhammer et al., 1998) predicted six, four and five transmembrane domains, respectively, with the C-terminal end of PgpB...
facing the cytosol and the C-terminal ends of YbjG and YeIU located towards the periplasmic space (data not shown). The computer-generated model of PgpB placed the acid phosphatase motif facing the periplasmic space. However, the models generated for YbjG and YeIU placed approximately half of the acid phosphatase motif shown in Fig. 2 within a cytosolic loop and the other half within a periplasmic loop (data not shown). Such an arrangement would render the enzyme inactive, suggesting the model was not accurate. We constructed C-terminally fused derivatives of YbjG and YeIU proteins with GFP to experimentally confirm the orientation of their C-termini. E. coli W3110 cells expressing the recombinant proteins YbjGGFP or YeIU GFP exhibited fluorescence with varying degrees of intensity, which was distributed around the perimeter of each individual cell (Fig. 3a, b). In contrast, the fluorescence of cells transformed with the control plasmid expressing soluble GFP was homogeneously distributed within the cytoplasm (Fig. 3c). The distribution of fluorescence around the cell perimeter agrees with a membrane location of the chimeric proteins. Since GFP cannot fold into a chromophore within the periplasm (Drew et al., 2002; Feilmeier et al., 2000) we concluded that the C-terminus in both proteins faces the cytosolic compartment. This conclusion was supported by a fusion experiment with PhoA, which folds into an enzymically functional form only in the periplasmic space. Bacteria expressing YbjGPhoA or YeIUPhoA had a white phenotype on agar plates containing the colour indicator XP, and cell lysates did not show any detectable alkaline phosphatase activity. Together, absence of PhoA activity and GFP fluorescence demonstrated unequivocally the cytoplasmic location of the C-terminal segment of YbjG and YeIU. This experimental observation, contrary to the computer prediction of the topologies for YbjG and YeIU, prompted us to examine more closely the topology of the internal loops in these proteins. PhoA fusions were constructed to amino acids Gln55, Val85, His105, Arg121 and His145 in YbjG, and amino acids Ile93, Thr123, Asp144 and His190 in YeIU (Table 3). We confirmed that the fusion proteins were properly expressed by Western blotting with an anti-PhoA monoclonal antibody (data not shown). Recombinant plasmids expressing the PhoA fusion proteins were transformed into the PhoA-deficient strain E. coli CC118 (Table 1) and transformants examined for a blue-colony phenotype, which results from the degradation of the indicator dye XP by those bacteria with periplasmic exposed fusions. The PhoA enzymic activity was quantified as described in Methods and normalized to the amount of PhoA protein detected densitometrically from Western blots. PhoA fusions to amino acids Val85 and His105 in YbjG (YbjGVal85-PhoA and YbjGH105-PhoA), and Thr123 and Asp144 in YeIU (YeIUThr123-PhoA and YeIUD144-PhoA) resulted in proteins with detectable normalized PhoA activities in YeIU (Table 3). We confirmed that the fusion proteins were properly expressed by Western blotting with an anti-PhoA monoclonal antibody (data not shown). Recombinant plasmids expressing the PhoA fusion proteins were transformed into the PhoA-deficient strain E. coli CC118 (Table 1) and transformants examined for a blue-colony phenotype, which results from the degradation of the indicator dye XP by those bacteria with periplasmic exposed fusions. The PhoA enzymic activity was quantified as described in Methods and normalized to the amount of PhoA protein detected densitometrically from Western blots. PhoA fusions to amino acids Val85 and His105 in YbjG (YbjGVal85-PhoA and YbjGH105-PhoA), and Thr123 and Asp144 in YeIU (YeIUTHr123-PhoA and YeIUAsp144-PhoA) resulted in proteins with detectable normalized PhoA activities

Table 3. Topological mapping of YbjG and YeIU as determined by analysis of PhoA fusions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Alkaline phosphatase (units)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>YbjGQ55-PhoA</td>
<td>ND</td>
</tr>
<tr>
<td>YbjGV85-PhoA</td>
<td>1487 ± 306 (284)</td>
</tr>
<tr>
<td>YbjGH105-PhoA</td>
<td>624 ± 22 (475)</td>
</tr>
<tr>
<td>YbjGR121-PhoA</td>
<td>ND</td>
</tr>
<tr>
<td>YbjGH145-PhoA</td>
<td>ND</td>
</tr>
<tr>
<td>YbjGr139-PhoA-V140</td>
<td>1613 ± 24 (245)</td>
</tr>
<tr>
<td>YbjGPhoA</td>
<td>ND</td>
</tr>
<tr>
<td>YeIUThr93</td>
<td>1 ± 3.5 (0.7)</td>
</tr>
<tr>
<td>YeIUT123</td>
<td>3730 ± 314 (905)</td>
</tr>
<tr>
<td>YeIUAsp144</td>
<td>3585 ± 838 (1495)</td>
</tr>
<tr>
<td>YeIUThr123</td>
<td>695 ± 214 (198)</td>
</tr>
<tr>
<td>YeIUH190</td>
<td>695 ± 214 (198)</td>
</tr>
</tbody>
</table>

*Units of activity were determined as described in Methods. The results indicate the mean ± SD from three experiments. The relative units of activity, normalized for protein expression as determined from the intensities of fusion protein bands detected by Western blot with anti-PhoA antibodies, are indicated in parentheses. ND, Not detectable.
ranging from 284 to 1495 units (Table 3), indicating that these residues are exposed to the periplasmic space. However, a PhoA fusion to the predicted catalytic His145 residue in YbjG did not show detectable enzymic activity, while a fusion to the His190 in YeiU (corresponding to the catalytic His residue in this protein) gave 198 units. The expression of both protein fusions as detected by Western blotting was low, suggesting the possibility of folding problems leading to protein degradation. We therefore constructed a PhoA ‘sandwich’ fusion in YbjG such that the PhoA protein was inserted between Arg139 and Val140 (YbjG<sub>R139-PhoA-V140</sub> Table 3). This recombinant protein gave 245 units of activity, demonstrating a periplasmic location for these two residues, and suggesting that the entire loop containing the catalytic His145 is exposed to the periplasm. From these results, together with the analysis of the hydrophobicity of the intervening regions and the distribution of positively charged amino acids that are usually located in cytosolic loops (Heijne, 1986), we constructed a revised topological model for YbjG and YeiU (Fig. 4). The location of the essential residues of the conserved acid phosphatase domain in both proteins indicates that the catalytic site is oriented towards the periplasmic side of the plasma membrane, supporting the notion that the enzymic activity of YbjG and YeiU resides in the periplasmic compartment.

Fig. 4. Topological model for YbjG and YeiU, as determined from the PhoA and GFP fusion experiments. The amino acids fused to PhoA are circled. Open circles indicate fusions with no or very low levels of alkaline phosphatase activity. Shaded circles indicate fusions with high alkaline phosphatase activity. The conserved amino acids of the three motifs corresponding to the acid phosphatase catalytic site are indicated by open squares. An asterisk indicates the catalytic His residue.
Fosmidomycin hypersensitivity suggests that YbjG and YeiU are involved in Und-PP metabolism

Fosmidomycin (Fm) is an antibiotic that specifically inhibits the conversion of deoxy-xylulose 5-phosphate into methlerythritol 4-phosphate (Kuzuyama et al., 1998a; Zeidler et al., 1998), the second step in the Und-PP de novo synthesis pathway (Kuzuyama et al., 1998b; Rodriguez-Conception & Boronat, 2002; Takahashi et al., 1998). We reasoned that in comparison to the parental strain, E. coli uppP mutants with additional defects in the Und-PP recycling pathway would exhibit higher susceptibility to Fm since de novo synthesis of Und-P and recycling of Und-PP would both be compromised. Therefore, we constructed in W3110 a series of mutants containing single, double and triple deletions of pyrophosphatase genes implicated in the metabolism of Und-P (Table 1) and grew them in increasing concentrations of Fm as described in Methods. The antibiotic concentrations used in these experiments were subinhibitory for the parental strain W3110, which has a MIC50 of 900 ng Fm ml⁻¹. Table 4 shows the percentage survival of these strains at 6 h in the presence of Fm concentrations of 160, 320 and 480 ng ml⁻¹. This time point was chosen as it allowed for detection of maximal differences among the mutant strains. The results show that single deletion mutants in uppP and ybjG genes, and double deletion mutants in either uppP and yeiU or uppP and ybjG, exhibited less than 50% survival, especially at 480 ng Fm ml⁻¹ (Table 4). Furthermore, a triple ΔuppP-ΔyeiU-ΔybjG deletion mutant reproducibly exhibited 35% survival, indicating that lack of these genes creates an additive effect on the mutants’ viability in the presence of Fm. In contrast, the survival of mutants with single deletions in either yeiU or pgpB was much higher than 50% and even closer to that of the parental strain (Table 4). The observed differences in percentage survival among parental and mutant strains were not due to growth rate differences or general changes in bacterial cell permeability, as the parental E. coli W3110 and all mutant derivatives grew similarly in the absence of antibiotic and did not differ with respect to their sensitivity to other antibiotics (data not shown). From these data we concluded that ybjG, and yeiU to a lesser extent, are required for Und-P synthesis when the de novo pathway is partially blocked by Fm. In contrast, the function of pgpB appears not to be required under similar experimental conditions.

To test whether the ybjG function is required for survival under subinhibitory Fm concentrations, the ΔuppP-ΔyeiU-ΔybjG triple mutant (LDT30) was transformed with pLDT68, which expresses the YbjGHis₆ protein under the control of the arabinose-inducible promoter. Fig. 5(a) shows that the percentage survival at 6 h of LDT30- (pLDT68) exposed to various Fm concentrations was similar to that of the parental strain W3110. In contrast, LDT30 transformed with pLDT67 expressing YbjG(ΔHis₆) which has an amino acid substitution of the phosphatase catalytic site (Ala replacing His), exhibited poor survival under the same conditions, suggesting that functional complementation required an enzymically active protein. The lower survival found with LDT30(pLDT67) relative to LDT30 with no plasmid is probably due to a general growth defect caused by protein expression under arabinose induction. Indeed, LDT30 cells carrying pLDT67 or pLDT68 displayed a growth rate delay in the presence of arabinose in the medium, compared to the growth rate of the same strains in arabinose-free medium (Fig. 5b). The growth rate delay was maximal at 6 h, which is the same as the time point chosen for the Fm survival experiments.

An important additional control for the complementation experiments was to demonstrate that the YbjG(ΔHis₆) and YbjG(ΔHis₆-His₆) proteins are indeed expressed and localized in the bacterial membrane. Since the proteins were expressed as His₆ C-terminal fusions, we investigated their expression by Western blot analysis of membrane preparations, which were reacted with anti-His₆ epitope-specific antibodies. Fig. 5(c) shows that the parental and mutant proteins are expressed and migrate as a 24 kDa polypeptide, in agreement with the predicted molecular mass of YbjG. Polypeptides of higher molecular mass were detected; these are likely the result of protein aggregates due to the mild denaturing conditions used for electrophoretic separation of membrane proteins (see Methods), which are required to prevent complete protein aggregation that would preclude detection by Western blotting. From these results we concluded that the lack of complementation by the plasmid expressing the YbjG(ΔHis₆) polypeptide is not due to lack of protein expression or membrane localization, suggesting that YbjG(ΔHis₆) does indeed have a defect in enzymic activity. Collectively, the results of the experiments described in this section suggest that a functional YbjG protein is required to restore.

Table 4. Percentage survival of the parental strain and deletion mutants in the presence of various concentrations of fosmidomycin

Survival was determined as described in Methods. Data were taken from the bacterial survival at 6 h.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Deleted gene(s)</th>
<th>Survival at Fm concn (ng ml⁻¹) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>160</td>
</tr>
<tr>
<td>W3110 Wild-type</td>
<td></td>
<td>108 ± 1.4</td>
</tr>
<tr>
<td>LDT40 uppP</td>
<td></td>
<td>95 ± 1.6</td>
</tr>
<tr>
<td>LDT6 yeiU</td>
<td></td>
<td>94 ± 2.9</td>
</tr>
<tr>
<td>LDT37 yeiU, ybjG</td>
<td></td>
<td>92 ± 2.0</td>
</tr>
<tr>
<td>LDT18 uppP, pgpB</td>
<td></td>
<td>82 ± 2.2</td>
</tr>
<tr>
<td>LDT21 uppP, yeiU</td>
<td></td>
<td>81 ± 3.1</td>
</tr>
<tr>
<td>LDT39 ybjG</td>
<td></td>
<td>90 ± 1.8</td>
</tr>
<tr>
<td>LDT38 uppP, ybjG</td>
<td></td>
<td>86 ± 0.7</td>
</tr>
<tr>
<td>LDT5 uppP</td>
<td></td>
<td>77 ± 1.1</td>
</tr>
<tr>
<td>LDT30 uppP, yeiU, ybjG</td>
<td></td>
<td>77 ± 0.8</td>
</tr>
</tbody>
</table>
survival of strain LDT30 exposed to subinhibitory Fm concentrations to similar levels to those of the parental strain, supporting the notion that YbjG might be required for Und-PP metabolism.

O16 LPS expression in a ΔwaaL-ΔuppP-ΔyeiU-ΔybjG mutant causes a growth defect

E. coli K-12 W3110 does not normally produce O antigen due to an insertion element inactivating the rhamnosyltransferase gene wbbL (Stevenson et al., 1994; Yao & Valvano, 1994). Complementation of the wbbL defect in trans with plasmid pPR1474 (wbbL+) reconstitutes the synthesis of O16 LPS in E. coli W3110 (Feldman et al., 1999; Liu & Reeves, 1994), as detected by silver staining and Western blots with antibodies specific for O16 and the E. coli lipid A-core oligosaccharide (Fig. 6). In contrast, a deletion of the O antigen ligase waaL gene in strain CLM24 (W3110 ΔwaaL) results in the synthesis of O16 antigen polymers that are poorly detectable by silver staining but clearly detected with anti O16 antibodies (Fig. 6, top and central panels). However, these polymers do not react with the lipid A-core oligosaccharide-specific antibody (Fig. 6, bottom panel). This indicates that the O16-specific material produced by the ligase-deficient control strain CLM24 (ΔwaaL) containing the high-copy number plasmid pPRI474 (wbbL+) corresponds to polymerized O units not linked to lipid A-core oligosaccharide, which most likely remain linked to Und-PP. Therefore, we reasoned that the introduction of the waaL gene deletion into the triple deletion ΔuppP-ΔyeiU-ΔybjG strain would increase the synthesis of Und-PP-linked O units that cannot be processed any further, resulting in reduction of the available pool of Und-PP for recycling into Und-P. This prediction is supported by the results in Fig. 6 that show increased amounts of O16 polymeric bands only detectable by silver staining and anti-O16 antibodies.

Next, we assessed whether mutations in the pyrophosphatases affect bacterial growth. The quadruple mutant ΔuppP-ΔyeiU-ΔybjG-ΔwaaL (strain LDT53), containing plasmid pPRI474 (wbbL+), grew significantly slower than the parental strain, the triple ΔuppP-ΔyeiU-ΔybjG mutant (strain LDT30) with or without pPRI474, and the LDT53 quadruple mutant without pPRI474 (Fig. 7 and data not shown). No growth defects were observed in the pyrophosphatase single, double and triple mutants in strains not expressing O16 antigen, or in the ΔwaaL mutant containing pPRI474 (data not shown). Phase-contrast microscopy also revealed changes in the morphology of bacterial cells, especially cells transformed with pPRI474. Unlike W3110(pPRI474), LDT30 and LDT53 cells containing pPRI474 were very long with a filamentous appearance, unlike W3110(pPRI474), LDT30 and LDT53 cells containing pPRI474 were very long with a filamentous appearance, and abundant empty sacculi plus cellular debris were noticed, particularly in LDT53 (data not shown). Together, these results suggest that in cells expressing O16 antigen but lacking the O16 antigen ligase WaaL and the UppP, YigG and YeIu pyrophosphatases, sequestration of Und-PP-linked O16 that cannot be ligated to the lipid A-core oligosaccharide delays growth rate and compromises growth.
cell viability. This phenotype would be consistent with an expected reduction in the Und-PP pool available to the recycling pathway, which cannot be compensated by \textit{de novo} synthesis due to the deletion of the \textit{uppP} gene.

**Concluding remarks**

The experiments in this work support the hypothesis that the proteins YbjG and to a lesser extent YeiU function in the recycling of periplasmic Und-PP, presumably released from peptidoglycan and O antigen biosynthesis pathways. This notion is based on the following observations: (i) both proteins have an acid phosphatase motif topologically oriented toward the periplasmic side of the plasma membrane, suggesting the enzyme is active on the periplasm; (ii) deletions of the genes encoding these proteins, combined with a \textit{uppP} gene deletion, result in a supersensitive phenotype to Fm, which inhibits the \textit{de novo} synthesis of Und-P; (iii) accumulation of Und-PP-O16-specific polymers that cannot be ligated to the rest of the LPS is linked to a partial growth defect, abnormal bacterial cell morphology and bacterial lysis. Und-P is synthesized at a very low level \textit{in vivo}, which makes it difficult to determine whether or not the Und-PP form is accumulated on the periplasmic side of the plasma membrane in the mutants. Also, the analysis of accumulated Und-PP at either side of the plasma membrane is not trivial. These difficulties are further compounded because peptidoglycan synthesis is essential for bacterial viability and requires Und-P-linked intermediary steps. Therefore, further experiments are required to conclusively demonstrate that lack of YbjG and YeiU is associated with an accumulation of Und-PP molecules oriented towards the periplasmic side of the plasma membrane.

PgpB is also a membrane pyrophosphate phosphatase of the same family as YbjG and YeiU (Fig. 2). However, this protein may have another function, since deletions in the \textit{pgpB} gene did not cause any observable phenotype under the same conditions in which deletions of \textit{ybjG} and \textit{yeiU}
did. In contrast to the other three phosphatases, UppP lacks any features of a typical acid phosphatase. However, this protein accounts for approximately 75% of the cellular Und-PP phosphatase activity, and it has been suggested to catalyse the conversion of de novo-synthesized Und-PP into Und-P (El Ghachi et al., 2004). This conclusion also agrees with the observation that the enzymes for the earlier steps of Und-PP synthesis are all present in the cytosolic compartment, including UppS, which catalyses the condensation of isopentenyl-P units into Und-PP. A predicted topological model for UppP reveals a large cytosolic loop containing the motif IGxxQ(E)xxA(S,G)xxxPGxSRS(A,G)G that is conserved in more than 500 bacterial homologues of this protein (M. A. Valvano, unpublished observations). No other proteins are detected from PSI-BLAST and of this protein (M. A. Valvano, unpublished observations). No other proteins are detected from PSI-BLAST and of this protein (M. A. Valvano, unpublished observations). No other proteins are detected from PSI-BLAST and of this protein (M. A. Valvano, unpublished observations). No other proteins are detected from PSI-BLAST and of this protein (M. A. Valvano, unpublished observations).

The topography of Und-P synthesis by de novo and recycling pathways is indicated using the synthesis of O16 LPS as an example of a surface polymer that requires Und-P as a lipid carrier. The question mark denotes that it is presently unknown how periplasmic Und-P becomes available at the cytosolic side of the membrane to reinitiate lipid-linked glycan synthesis.

**Fig. 8.** Working model for Und-P biogenesis in *E. coli*. The topography of Und-P synthesis by de novo and recycling pathways is indicated using the synthesis of O16 LPS as an example of a surface polymer that requires Und-P as a lipid carrier. The question mark denotes that it is presently unknown how periplasmic Und-P becomes available at the cytosolic side of the membrane to reinitiate lipid-linked glycan synthesis.

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