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 $\Delta uppP\Delta ybjG\Delta yeiU\Delta waaL$ 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.

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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-

phate (Und-PP), which in turn results from the addition of isoprene units onto farnesyl pyrophosphate, a reaction catalysed by the UppS synthase (Apfel *et al.*, 1999; Shimizu *et al.*, 1998). Since UppS is a soluble protein these biosynthetic steps must occur in the cytosol or in close proximity to the cytosolic face of the plasma membrane. The isoprene molecules isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the five-carbon universal basic building blocks of all isoprenoids (White, 1996). Two distinct biosynthetic pathways exist for the *de novo* synthesis of IPP and DMAPP: the mevalonate pathway in eukaryotes and archaea and the 2-C-methyl-erythritol 4-phosphate (MEP) pathway in eubacteria, green algae and plant chloroplasts (Kuzuyama, 2002; Rohmer *et al.*, 1993; Rohmer, 1999). The MEP pathway (Fig. 1) starts with the synthesis of 1-deoxy-xylulose 5-phosphate from

Abbreviations: DMAPP, dimethylallyl diphosphate; Fm, fosmidomycin; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-erythritol 4-phosphate; Und, undecaprenyl; Und-P, undecaprenyl phosphate; Und-PP, undecaprenyl pyrophosphate; XP, 5-bromo-3-chloro-indolyl phosphate.

pyruvate and glyceraldehyde 3-phosphate, a reaction catalysed by the 1-deoxy-xylulose 5-phosphate synthase encoded by the *dxs* 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 *dxr* 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 and DMAPP, the precursors of Und-PP (Fig. 1).

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.

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 & Aeby, 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.*, 2001). Fernandez *et al.* (2001) have shown that Cwh8, a membrane protein in the endoplasmic reticulum of *Saccharomyces cerevisiae*, has a dolichol-PP pyrophosphate phosphatase activity and possesses a lumenally oriented active site. Homologues of this protein are also found in mammalian cells (Rush *et al.*, 2002). The topology of Cwh8 agrees with its proposed function in the formation of dolichol-P from dolichol-PP intermediates arising from the oligosaccharyl transfer reaction during N-linked protein glycosylation, which occurs on the luminal side of the endoplasmic reticulum membrane. Cwh8 has a segment of approximately 122 amino acids that corresponds to an acid phosphatase domain (Fig. 2). This domain, shared among lipid phosphatases and non-specific acid phosphatases, comprises three motifs (Neuwald, 1997; Stucky & Carman, 1997). The conserved His from motif 3 is proposed to attack the phosphate group of the substrate, leading to the

	N	F	P	G	TT	N	E
		YGS	AS				Q
Consensus	KxxxxxxRP (12–54)	PSGH (31–54)	SRxxxxxHxxxD				
Cwh8	KxxxxxxRP (19)	PSAH (48)	SRxxxxxHxxx-D				
YgjG	K-xxxxxxRP (18)	PSDH (32)	SRxxxxxHxxxD				
YeiU	KxxxxxxTF (22)	PGDH (32)	PRxxxxxHxxxD				
PgpB	KxxxxxxRP (54)	PGDH (36)	SRxxxxxHxxxD				
		*	*	*	*		
	Motif 1	Motif 2	Motif 3				

Fig. 2. Consensus motif of the acid phosphatase domain (Neuwald, 1997; Stukey & Carman, 1997) and comparison with predicted acid pyrophosphate phosphatases. Residues in *italics* correspond to amino acids that can be found with less frequency at similar positions in some of the members of this acid phosphatases family. The numbers in parentheses indicate the number of residues separating each motif. 'x' indicates any non-relevant amino acids. Asterisks indicate the His (H) and Arg (R) residues from motif 3 that are proposed to attack the phosphate group of the substrate and stabilize the covalent phosphoenzyme intermediate, respectively, and the conserved H from motif 2 (Ishikawa *et al.*, 2000).

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 *E. coli* K-12 proteins YbjG, YeiU and PgpB have a similar motif to that found in Cwh8 (Fig. 1) (El Ghachi *et al.*, 2005). However, their function in the recycling of Und-PP remains to be determined. In this work, we show that the acid phosphatase motifs in YbjG and YeiU face the periplasmic space, and provide experimental evidence suggesting that these proteins are 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 (corresponding to a bacterial density of approximately 10⁴ c.f.u. ml⁻¹), and the growth was monitored every half hour in a 100-well microtitre plate using a Bioscreen C automated microbiology growth

curve analysis system (MTX Lab Systems). This system is a computerized bacterial incubator that measures growth continuously by optical density (Löwdin *et al.*, 1993). The results were analysed statistically by two-way ANOVA and the Bonferroni post-test using the Prism 4 software package (GraphPad Software).

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 complementation 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. 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.*, 1990) and samples were separated by 14% Tricine SDS-PAGE. The gels were stained with silver nitrate (Marolda *et al.*, 1990). For some experiments, bands were transferred to nitrocellulose membranes for immunoblot analysis. The membranes were 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 grown overnight in 5 ml LB with the appropriate antibiotic were

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties*	Source or reference
Strains		
CC118	$\Delta(\text{ara leu}) \Delta\text{lac phoA galE galK thi rpsL rpsB argE recA}$	Laboratory stock
DH5 α	F [−] $\phi 80\text{lacZ}\Delta\text{M15 endA recA hsdR}(\text{r}_\text{K}^- \text{m}_\text{K}^-) \text{supE thi gyrA}$	Laboratory stock
W3110	$\text{relA } \Delta(\text{lacZYA-argF})\text{U169}$	Laboratory stock
LDT5	$\text{rph-IIN}(\text{rrnD-rrnE})1$	This study
LDT6	W3110, ΔuppP	This study
LDT18	W3110, $\Delta\text{uppP } \Delta\text{pgpB}::\text{Km}$	This study
LDT21	W3110, $\Delta\text{uppP } \Delta\text{yeiU}$	This study
LDT30	LDT21, $\Delta\text{uppP } \Delta\text{yeiU } \Delta\text{ybjG}::\text{Km}$	This study
LDT37	LDT6, $\Delta\text{yeiU } \Delta\text{ybjG}::\text{Km}$	This study
LDT38	LDT5, $\Delta\text{uppP } \Delta\text{ybjG}::\text{Km}$	This study
LDT39	W3110, $\Delta\text{ybjG}::\text{Km}$	This study
LDT40	W3110, $\Delta\text{pgpB}::\text{Km}$	This study
LDT53	W3110, $\Delta\text{uppP } \Delta\text{yeiU } \Delta\text{waaL } \Delta\text{ybjG}::\text{Km}$	This study
Plasmids		
pANP1	yeiU_{GFP} cloned into pBAD24; Ap ^R	This study
pANP3	$\text{yeiU}_{\text{I93-PhoA}}$ cloned into pBAD24; Ap ^R	This study
pANP4	$\text{yeiU}_{\text{T123-PhoA}}$ cloned into pBAD24; Ap ^R	This study
pANP5	$\text{yeiU}_{\text{D144-PhoA}}$ cloned into pBAD24; Ap ^R	This study
pANP7	$\text{yeiU}_{\text{H190-PhoA}}$ cloned into pBAD24; Ap ^R	This study
pANP9	$\text{yeiU}_{\text{PhoA}}$ cloned into pBAD24; Ap ^R	This study
pANP10	$\text{ybjG}_{\text{PhoA}}$ cloned into pBAD24; Ap ^R	This study
pBAD24	Expression vector carrying $\text{araC-P}_{\text{araB}}$; inducible with arabinose; $\text{ori}_{\text{ColE1}}$; Ap ^R	Guzman <i>et al.</i> (1995)
pBADHis	pBAD24 encoding a His ₅ tag for N-terminal fusions; Ap ^R	This study
pBADGFP	gfpmut3 cloned into pBAD24; Ap ^R	This study
pCP20	Flp^+ , λd857^+ , λ p _R Rep ^{ts} ; Ap ^R Cm ^R	Datsenko & Wanner (2000)
pDDV1	ybjG_{GFP} cloned into pBAD24; Ap ^R	This study
pDDV14	$\text{ybjG}_{\text{Q55-PhoA}}$ cloned into pBAD24; Ap ^R	This study
pDDV15	$\text{ybjG}_{\text{V85-PhoA}}$ cloned into pBAD24; Ap ^R	This study
pDDV16	$\text{ybjG}_{\text{H105-PhoA}}$ cloned into pBAD24; Ap ^R	This study
pDDV17	$\text{ybjG}_{\text{R121-PhoA}}$ cloned into pBAD24; Ap ^R	This study
pDDV19-1	$\text{ybjG}_{\text{H145-PhoA}}$ cloned into pBAD24; Ap ^R	This study
pKD4	Template plasmid for mutagenesis; Ap ^R Km ^R	Datsenko & Wanner (2000)
pKD46	γ , β and exo from λ phage, $\text{araC-P}_{\text{araB}}$; Ap ^R	Datsenko & Wanner (2000)
pLDT64	yeiU_{GFP} cloned into pBAD24; Ap ^R	This study
pLDT67	$\text{ybjG}_{\text{H145A-His6}}$ cloned into pBADHis with addition of one His by PCR mutagenesis; Ap ^R	This study
pLDT68	$\text{ybjG}_{\text{His6}}$ cloned into pBADHis with addition of one His by PCR mutagenesis; Ap ^R	This study
pLDT76	$\text{ybjG}_{\text{R139-PhoA-V140}}$ cloned into pBAD24; Ap ^R	This study
pPR1474	wbbL_{O16} cloned into pBR322; Ap ^R	Liu & Reeves (1994)

*Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin.

diluted in 15 ml fresh medium to an OD₆₀₀ of 0.05 and grown to an OD₆₀₀ 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 2. Primers used for mutagenesis and cloning

Bold and italicized nucleotides indicate the codon encoding alanine for the His replacement in *ybjG*_{H145A-His6}. 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.

Primer	Sequence (5'–3')	Gene
Mutagenesis		
2021	cgcgttttcttggcgctc gcat ggccgctggatgctcgg	<i>ybjG</i> _{H145A}
2022	ccgagcatatccagcgg ccat gacgccaagataaacgcg	<i>ybjG</i> _{H145A}
Cloning		
1574	cgattactcgagttgccgttgctgtaa (<i>Xho</i> I)	<i>ybjG</i> _{Q55-PhoA}
1575	cgattactcgagttcgacaaagggtcggtc (<i>Xho</i> I)	<i>ybjG</i> _{V85-PhoA}
1576	cggttactcgaggtgatcgcttgggaatga (<i>Xho</i> I)	<i>ybjG</i> _{H105-PhoA}
1577	caattactcgaggcgatgccagcataa (<i>Xho</i> I)	<i>ybjG</i> _{R121-PhoA}
1578	caattactcgaggtggacgccaagata (<i>Xho</i> I)	<i>ybjG</i> _{H145-PhoA}
1579	caattactcgagaatcacgatacgtcgt (<i>Xho</i> I)	<i>ybiU</i> _{193-PhoA}
1580	caattactcgagagtcattgttgggct (<i>Xho</i> I)	<i>ybiU</i> _{T123-PhoA}
1581	aaattactcgagatctcgtgagcgtc (<i>Xho</i> I)	<i>ybiU</i> _{D144-PhoA}
1583	ctttactcgaggtgtgcgccaatcat (<i>Xho</i> I)	<i>ybiU</i> _{H190-PhoA}
1704	cttaatctcgagcctgttctggaacaa (<i>Xho</i> I)	<i>phoA</i>
1705	tttattgaagctttttatttcagccccagagc (<i>Hind</i> III)	<i>phoA</i>
766	cattaagcttgcatgcctgcagg (<i>Hind</i> III)	<i>gfpmut3</i>
1570	caataactcgaggcatgagtaaaggagaa (<i>Xho</i> I)	<i>gfpmut3</i>
1571	gcaactcttactgtttctccataccggt	pBAD
1667	caataactcgaggatgagtaaaggagaa (<i>Xho</i> I)	<i>gfpmut3</i>
765	caattactcgaggcatgagtaaaggagaa (<i>Pst</i> I)	<i>gfpmut3</i>
DNA sequencing		
1827	ccgagtcctccatcccat	<i>phoA</i>
1664	gctctggtctgtgattaccgtca	<i>gfp</i>

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 2 h 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 His₆ epitope was detected with rabbit polyclonal (Sigma) anti-His₆ 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⁻¹ 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 3 h to an OD₆₀₀ of 0.5–0.6 and then induced with 0.02% arabinose for 3 h, after which the cells were harvested and assayed as described by Manoil (1991) with the addition of 1 mM iodoacetamide in all the buffers to avoid folding of cytoplasmic PhoA (Derman & Beckwith, 1995).

Microscopy. Overnight cells expressing GFP fusions were diluted in LB to OD₆₀₀ 0.1, grown to OD₆₀₀ 0.5 and then induced with 0.2% arabinose for 3 h. At this point the cells were kept on ice for 1–2 h 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 (Sonnhammer *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 YbjG_{GFP} 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 YbjG_{PhoA} or YeiU_{PhoA} 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

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

Protein	Alkaline phosphatase (units)*
YbjG _{Q55-PhoA}	ND
YbjG _{V85-PhoA}	1487 ± 306 (284)
YbjG _{H105-PhoA}	624 ± 22 (475)
YbjG _{R121-PhoA}	ND
YbjG _{H145-PhoA}	ND
YbjG _{R139-PhoA-V140}	1613 ± 24 (245)
YbjG _{PhoA}	ND
YeiU _{I93}	1 ± 3.5 (0.7)
YeiU _{T123}	3730 ± 1314 (905)
YeiU _{D144}	3585 ± 838 (1495)
YeiU _{H190}	695 ± 214 (198)
YeiU _{PhoA}	ND

*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.

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 (YbjG_{V85-PhoA} and YbjG_{H105-PhoA}), and Thr123 and Asp144 in YeiU (YeiU_{T123-PhoA} and YeiU_{D144-PhoA}) resulted in proteins with detectable normalized PhoA activities

Fig. 3. Fluorescence microscopy of *E. coli* cells expressing (a) YbjG_{GFP}, encoded by pDV1, (b) YeiU_{GFP}, encoded by pLDT64, and (c) soluble GFP, encoded by pBADGFP. Bacteria were immobilized in glass plates with agarose and imaged by fluorescence microscopy at 1000× magnification as described in Methods.

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_{R139-PhoA-V140}; 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 methylerythritol 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-Concepcion & 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 *ybiU* or *uppP* and *ybjG*, exhibited less than 50 % survival, especially at 480 ng Fm ml⁻¹ (Table 4). Furthermore, a triple $\Delta uppP\Delta ybiU\Delta ybjG$ 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 *ybiU* 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 *ybiU* 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 $\Delta uppP\Delta ybiU\Delta ybjG$ triple mutant (LDT30) was transformed with pLDT68, which expresses the YbjG_{His6} 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_{H145A}, 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_{His6} and YbjG_{H145A-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_{H145A} polypeptide is not due to lack of protein expression or membrane localization, suggesting that YbjG_{H145A} 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.

Strain	Deleted gene(s)	Survival at Fm concn (ng ml ⁻¹) of:		
		160	320	480
W3110	Wild-type	108 ± 1.4	87 ± 0.87	73 ± 1.0
LDT40	<i>pgpB</i>	95 ± 1.6	86 ± 1.01	83 ± 1.0
LDT6	<i>ybiU</i>	94 ± 2.9	78 ± 0.61	68 ± 0.5
LDT37	<i>ybiU</i> , <i>ybjG</i>	92 ± 2.0	73 ± 0.69	64 ± 1.1
LDT18	<i>uppP</i> , <i>pgpB</i>	82 ± 2.2	61 ± 0.86	54 ± 2.3
LDT21	<i>uppP</i> , <i>ybiU</i>	81 ± 3.1	60 ± 0.94	48 ± 0.4
LDT39	<i>ybjG</i>	90 ± 1.8	60 ± 0.55	48 ± 0.2
LDT38	<i>uppP</i> , <i>ybjG</i>	86 ± 0.7	57 ± 1.10	44 ± 0.8
LDT5	<i>uppP</i>	77 ± 1.1	53 ± 1.13	43 ± 0.5
LDT30	<i>uppP</i> , <i>ybiU</i> , <i>ybjG</i>	77 ± 0.8	50 ± 0.32	35 ± 1.3

Fig. 5. (a) Complementation of the survival defect of the triple deletion mutant $\Delta uppP\text{-}\Delta yeiU\text{-}\Delta ybjG$ (strain LDT30) exposed to various Fm concentrations. Strains W3110 and LDT30 containing pLDT68 (encoding YbjG), pLDT67 (encoding the catalytic site replacement mutant YbjG_{H145A}) or no plasmid were grown in the presence of 100, 250, 500 and 750 ng Fm ml⁻¹ and the percentage survival was calculated as described in Methods. Each data point represents the mean and standard deviation of five independent determinations (SD bars not shown were smaller than symbols). (b) Growth curve of strains LDT30(pLDT67) and LDT30(pLDT68) without arabinose and with 0.005 % arabinose (Ara). The data points represent the mean of five repeated determinations (SD bars are smaller than symbols and are not shown). (c) Expression of parental YbjG_{His6} and YbjG_{H145A-His6} proteins. Total membranes were prepared from strain LDT30 with no plasmid or LDT30 transformed with pLDT68 (YbjG_{His6}) or pLDT67 (YbjG_{H145A-His6}). Bacteria were grown in LB containing 0.02 % arabinose. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane and reacted with anti-His polyclonal antibodies as described in Methods. Thick and thin arrows denote the location of the 24 kDa YbjG monomer and higher molecular mass aggregates, respectively. Molecular mass standards (kDa) were: 99, BSA; 54, ovalbumin; 38, carbonic anhydrase; 29, soybean trypsin inhibitor; and 20, lysozyme.

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 $\Delta waaL\text{-}\Delta uppP\text{-}\Delta yeiU\text{-}\Delta ybjG$ mutant causes a growth defect

E. coli K-12 W3110 does not normally produce O antigen due to an insertion element inactivating the rhamnosyl-transferase 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 $\Delta waaL$) 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 ($\Delta waaL$) containing the high-copy number plasmid pPR1474 (*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 $\Delta uppP\text{-}\Delta yeiU\text{-}\Delta ybjG$ 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 $\Delta uppP\text{-}\Delta yeiU\text{-}\Delta ybjG\text{-}\Delta waaL$ (strain LDT53), containing plasmid pPR1474 (*wbbL*⁺), grew significantly slower than the parental strain, the triple $\Delta uppP\text{-}\Delta yeiU\text{-}\Delta ybjG$ mutant (strain LDT30) with or without pPR1474, and the LDT53 quadruple mutant without pPR1474 (Fig. 7 and data not shown). No growth defects were observed in the pyrophosphate phosphatase single, double and triple mutants in strains not expressing O16 antigen, or in the $\Delta waaL$ mutant containing pPR1474 (data not shown). Phase-contrast microscopy also revealed changes in the morphology of bacterial cells, especially cells transformed with pPR1474. Unlike W3110(pPR1474), LDT30 and LDT53 cells containing pPR1474 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, YgjG and YeiU pyrophosphatases, sequestration of Und-PP-linked O16 that cannot be ligated to the lipid A-core oligosaccharide delays growth rate and compromises

Fig. 6. O16 antigen production by the quadruple deletion mutant $\Delta uppP\text{-}\Delta yeiU\text{-}\Delta waaL\text{-}\Delta ybjG$ (strain LDT53). LPS was prepared and separated by Tricine-SDS-PAGE as described in Methods. The strains used for this experiment, LDT53, CLM24 (W3110 $\Delta waaL$) and W3110, carry pPR1474, encoding the WbbL rhamnosyltransferase that allows the synthesis of O16 LPS in derivatives of strain W3110 (Liu & Reeves, 1994). The gel in the top panel was stained with silver nitrate, while the other panels are Western blots of a similar gel that were reacted with anti-O16 and anti-lipid A-core oligosaccharide specific antibodies as described in Methods. The location of the lipid A-core oligosaccharide band (core-lipid A), the lipid A-core oligosaccharide plus one O16 unit (core+1 OAg), and the region containing highly polymerized O units (OAg) are indicated.

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 *de novo* synthesis due to the deletion of the *uppP* gene.

Concluding remarks

The experiments in this work support the hypothesis that the proteins YbjG and to a lesser extent YeiU function in

Fig. 7. Growth rate analysis of wild-type and pyrophosphate phosphatase mutants. Overnight cultures were diluted to OD₆₀₀ 0.01 and the growth was monitored every 15 min in a Bioscreen C apparatus as indicated in Methods. The strains used in this experiment were LDT30 ($\Delta uppP\text{-}\Delta yeiU\text{-}\Delta ybjG$), LDT53 ($\Delta uppP\text{-}\Delta yeiU\text{-}\Delta waaL\text{-}\Delta ybjG$) and W3110, all containing the plasmid pPR1474. Data points represent the mean and standard error of four replicates (SE bars not shown where smaller than symbols). Data for each time point were statistically analysed by two-way ANOVA as indicated in Methods.

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 *uppP* gene deletion, result in a supersensitive phenotype to Fm, which inhibits the *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 *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 *pgpB* gene did not cause any observable phenotype under the same conditions in which deletions of *ybjG* and *yeiU*

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.

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 linear hidden Markov models analyses (Hughey & Krogh, 1996), suggesting that UppP belongs to a unique family of Und-PP pyrophosphatases. Therefore, the biochemical evidence demonstrating the phosphatase activity on Und-PP (El Ghachi *et al.*, 2004) together with the predicted membrane topology of UppP suggests that this protein carries out its function in the cytosolic compartment. Based on the available genetic and biochemical evidence we suggest a model as depicted in Fig. 8, which implicates UppP in the *de novo* synthesis pathway of Und-P while YbjG and YeiU are implicated in the recycling pathway of periplasmic Und-PP. How dephosphorylated periplasmic Und-P molecules return to the biosynthesis pathway on the cytosolic side of the plasma membrane also remains a mystery and awaits further investigations.

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