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Mutant deletions in *Escherichia coli* affect the cellular levels of undecaprenyl phosphate and undecaprenyl diphosphate

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***Escherichia coli* multiple-deletion mutants were constructed to examine the lipid phosphatase genes. Ratio of cellular levels of undecaprenyl phosphate (UP) and undecaprenyl diphosphate (UPP) was determined using cells labeled with ^{14}C and measuring the radioactivities in these lipids. The labeled cells were obtained through feeding [^{14}C]isopentenyl diphosphate to an *E. coli* strain. The relative level of UP in the wild type strain W3110; in the YS1234 strain defective in *ynbD, yeiU, ybjG,* and *pgpB*; in the YS1235 strain defective in *ynbD, yeiU, ybjG,* and *bacA*; and in the YS1245 strain defective in *ynbD, yeiU, pgpB*, and *bacA* were 78.0% ± 3.5%, 74.7% ± 1.2%, 68.4% ± 6.8%, and 64.0% ± 7.5%, respectively. The quadruple mutant strain that possessed the wild-type *bacA* showed almost identical UP level as the wild type strain W3110, indicating that sufficient level of UPP phosphatse activity was conferred by a *bacA* product without the products of *ynbD*, *yeiU*, *ybjG*, and *pgpB*. However, the mutants defective in *bacA* showed a slight but significant ($p < 0.01$) decrease in the UP level.**

Keywords: Undecaprenyl phosphate; Undecaprenyl diphosphate; isopentenyl diphosphate; lipid phosphatase; deletion mutant; lyophilized cell

Undecaprenyl phosphate (UP) is a sugar carrier in the biosyntheses of peptidoglycan and other cell surface polymers (1). UP is essential in forming undecaprenyl diphosphoryl sugar (UPP-sugar), which transports hydrophilic monomeric units of polysaccharides across the cell membrane to the external polymerization sites (2). Undecaprenyl diphosphate (UPP), the precursor of UP, is synthesized by the sequential condensation of isopentenyl diphosphsate (IPP) to farnesyl diphosphate (3, 4). Lipid phosphatases are integral membrane proteins that dephosphorylate UPP to produce UP (5-8). BacA-type (5, 9) and the PAP-2 type (6, 7) lipid phosphatases are different classes of integral membrane proteins in *Escherichia coli* that dephosphorylate UPP. In the *E. coli* chromosome there are four genes that encode proteins with a PAP-2 type phosphatase motif (7) (Fig. 1). The chromosome also shows the presence of a *bacA* copy. Enzymatic activities of BacA, PgpB, YbjG, and YeiU dephosphorylate UPP to produce UP (5, 6, 8). The YeiU is also a phosphotransferase, which produces lipid A 1-diphosphate (10). As of now, there are no published reports that elucidate the function of YnbD.

El Ghachi *et al*. reported that an *E. coli* strain deficient in *bacA*, *pgpB*, and *ybjG* was lethal, and that strains deficient in both *bacA* and *pgpB* or in both *bacA* and *ybjG* could grow as efficiently as a wild type strain (6). However, disruption of *bacA* decreased UPP phosphatase activity by 75% *in vitro*

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Protein	Sequence				
YbjG	GHLFPHD**RP**	(18)	**PS**D**H**	(32)	**SR**VYLGV**H**WPL**D**
YeiU	**K**ENAPGR**R**R	(56)	**P**GD**H**	(32)	P**R**VMIGA**H**WFT**D**
PgpB	**K**DKVQEP**RP**	(54)	**PSGH**	(36)	**SR**LLLGM**H**WPR**D**
YnbD	PLKFSFI**RP**	(23)	**PS**L**H**	(32)	**S**TLTTWQ**H**HFI**D**
Phosphatase motif	**K**XXXXXX**RP**	(12-54)	**PSGH**	(31-54)	**SR**XXXXX**H**XXX**D**

Fig. 1. Consensus motif of the phosphatase domain and comparison with predicted lipid phosphatases. Bold letter indicates the conserved amino acids. The numbers in parentheses indicate the number of residues separating each motif.

(5). To estimate the contribution of an each gene product to the *in vivo* UPP phosphatase activity, we constructed multiple gene-deletion mutants for putative UPP phosphatases and measured the ratio of UP and UPP in the cells. High performance liquid chromatography (HPLC) can readily measure UP (4, 11), however, direct measurement of UPP by HPLC is difficult because UPP elutes in a crowded region (11). Therefore, we specifically labeled UP and UPP with ^{14}C through feeding of [^{14}C]IPP to an *E. coli* strain. The ratio of cellular levels of UP and UPP was then determined by measuring the radioactivities in these lipids. Because IPP is impermeable to growing cells, for labeling experiment, we prepared lyophilized cells that had been confirmed to retain normal metabolic activities (12**).**

Materials and Methods

Strains and plasmids – Strains used in this study are described in Table 1. A *recJ* strain KM354 (13) and deletion mutants (14) JWK1270, JWK2162, JWK3029, and JWK5112 were provided by Dr. Kenan C. Murphy from University of Massachusetts and Dr. Hirotada Mori from Nara Institute of Science and Technology, respectively. Plasmid pCP20 (15) and plasmid pKRP10 (16) were provided by Dr. Hirotada Mori and by Dr. Seiichi Yasuda from the National Insitute of Genetics, respectively. A cloning vector pCR-2.1TOPO was purchased from Invitrogen Co.

Chemicals – [^{14}C]IPP (specific activity, 2.2 TBq mol^{-1}) and bacitracin (>40,000 u g^{-1}) were acquired from Amersham Bioscience and Wako Pure Chemical Industries, Ltd., respectively.

Plasmid construction – The *ynbD* gene was PCR-amplified from an *E. coli* W3110 chromosome using oligonucleotides ynbDfw and ynbDrv (Table 2). The 1.7-kb PCR fragment was cloned into the pCR-2.1TOPO vector plasmid to construct a pCR-ynbD. pCR-ynbD was digested with *Sal*I and ligated with a chloramphenicol-resistant 0.9-kb *Sal*I fragment from pKRP10 to construct pCR-ynbD::Cm^R.

Strain construction – A strain carrying *ynbD* disrupted with the chloramphenicol-resistant cassette was constructed by transforming the *recJ* strain KM354 with the 2.6-kb PCR fragment that was amplified from pCR-ynbD::Cm^R, using oligonucleotides ynbDfw and ynbDrv. The disrupted *ynbD* was introduced into W3110 by P1 transduction. Multiple-deletion mutants were generated by transduction of kanamycin resistant markers with phage P1, followed by excision of the cassettes from the chromosome (15). P1 phages were prepared from strains of Keio collection (14), JW2162 (*ΔyeiU*::Km), JWK5112 (*ΔybjG*::Km), JWK1270 (*ΔpgpB*::Km), and JWK3029 (*ΔbacA*::Km). The disrupted gene was introduced into kanamycin-sensitive parental strains derived from W3110 by P1 transduction. Kanamycin-resistant clones were isolated and the disruption of the *yeiU*, *ybjG*, *pgpB*, and *bacA* genes in the chromosome was verified by PCR using oligonucleotides yeiUfw and yeiUrv, ybjGfw and ybjGrv, pgpBfw and pgpBrv, and bacAfw and bacArv, respectively (Table 2). Excision of the kanamycin-resistance genes from the chromosome of the mutant was obtained by the transformation with pCP20. Antibiotic-sensitive clones were isolated, and the excision of the cassette was confirmed by PCR analysis as described above.

Table 1. Bacterial strains.

Strain	Genotype[a]	Source or reference
W3110		Laboratory stock
KM354	*recJ* pTP223*gam bet exo*	(13)
YS0	*ynbD*::Cm^R	Derived from KM354 by transformation with PCR fragment containing *ynbD*::Cm
JWK2162	*ΔyeiU*::Km^R	(14)
JWK5112	*ΔybjG*::Km^R	(14)
JWK1270	*ΔpgpB*::Km^R	(14)
JWK3029	*ΔbacA*::Km^R	(14)
YS1	*ynbD*::Cm^R	P1 (YS0) × W3110
YS2	*ΔyeiU*::Km^R	P1 (JWK2162) × W3110
YS3	*ΔybjG*::Km^R	P1 (JWK5112) × W3110
YS4	*ΔpgpB*::Km^R	P1 (JWK1270) × W3110
YS5	*ΔbacA::*Km^R	P1 (JWK3029) × W3110
YS12	*ynbD*::Cm^R, *ΔyeiU*::Km^R	P1 (JWK2162) × YS1
YS13	*ynbD*::Cm^R, *ΔybjG*::Km^R	P1 (JWK5112) × YS1
YS14	*ynbD*::Cm^R, *ΔpgpB*::Km^R	P1 (JWK1270) × YS1
YS15	*ynbD*::Cm^R, *ΔbacA*::Km^R	P1 (JWK3029) × YS1
YS20	*ΔyeiU*	pCP20 × YS2
YS23	*ΔyeiU*, *ΔybjG*::Km^R	P1 (JWK5112) × YS20
YS24	*ΔyeiU*, *ΔpgpB*::Km^R	P1 (JWK1270) × YS20
YS25	*ΔyeiU*, *ΔbacA::*Km^R	P1 (JWK3029) × YS20
YS30	*ΔybjG*	pCP20 × YS3
YS35	*ΔybjG*, *ΔbacA::*Km	P1 (JWK3029) × YS30
YS120	*ynbD*::Cm^R, *ΔyeiU*	pCP20 × YS12
YS123	*ynbD*::Cm^R, *ΔyeiU*, *ΔybjG*::Km^R	P1 (JWK2162) × YS120
YS124	*ynbD*::Cm^R, *ΔyeiU*, *ΔpgpB*::Km^R	P1 (JWK1270) × YS120
YS125	*ynbD*::Cm^R, *ΔyeiU*, *ΔbacA::*Km^R	P1 (JWK3029) × YS120
YS130	*ynbD*::Cm^R, *ΔybjG*	pCP20 × YS13
YS135	*ynbD*::Cm^R, *ΔybjG*, *ΔbacA::*Km^R	P1 (JWK3029) × YS130
YS140	*ynbD*::Cm^R, *ΔpgpB*	pCP20 × YS14
YS142	*ynbD*::Cm^R, *ΔpgpB*, *ΔyeiU::*Km^R	P1 (JWK2162) × YS140
YS143	*ynbD*::Cm^R, *ΔpgpB*, *ΔybjG::*Km^R	P1 (JWK5112) × YS140
YS145	*ynbD*::Cm^R, *ΔpgpB*, *ΔbacA::*Km^R	P1 (JWK3029) × YS140
YS230	*ΔyeiU*, *ΔybjG*	pCP20 × YS23
YS234	*ΔyeiU*, *ΔybjG*, *ΔpgpB*::Km^R	P1 (JWK1270) × YS230
YS235	*ΔyeiU*, *ΔybjG*, *ΔbacA*::Km^R	P1 (JWK3029) × YS230
YS240	*ΔyeiU*, *ΔpgpB*	pCP20 × YS24
YS245	*ΔyeiU*, *ΔpgpB*, *ΔbacA*::Km^R	P1 (JWK3029) × YS240
YS1230	*ynbD*::Cm^R, *ΔyeiU*, *ΔybjG*	pCP20 × YS123
YS1235	*ynbD*::Cm^R, *ΔyeiU*, *ΔybjG*, *ΔbacA*::Km^R	P1 (JWK3029) × YS1230
YS1240	*ynbD*::Cm^R, *ΔyeiU*, *ΔpgpB*	pCP20 × YS124
YS1243	*ynbD*::Cm^R, *ΔyeiU*, *ΔpgpB*, *ΔybjG*::Km^R	P1 (JWK2162) × YS1240
YS1245	*ynbD*::Cm^R, *ΔyeiU*, *ΔpgpB*, *ΔbacA*::Km^R	P1 (JWK3029) × YS1240

[a] Cm^R and Km^R represent inserted resistance genes to chloramphenicol and kanamycin, respectively.

Table 2. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'-3')
ynbDfw	AACTCACTCGCAGGTCTTGC
ynbDrv	GCACAGTCTGACGCAAAAGC
yeiUfw	GTGAAGTTTTCGGTGATGATCTG
yeiUrv	CCCTGATGATGTTAATTACTG
ybjGfw	CTTGAGGGAAATAAGACGATG
ybjGrv	AAATGGCGTTGCTGGCGTGA
pgpBfw	ACAAGCGGACTTCATTGACG
pgpBrv	TATGGTCAACTTACCGCAAT
bacAfw	CTGCTCCCTTGCCACCGATA
bacArv	AGCGTCGCATCAGGCGTTGA

Preparation of lyophilized cells and ^{14}C labeling of cellular lipids – A previously described protocol (12) was slightly modified to prepare the lyophilized cells. *E. coli* cells which were grown under shaking conditions in 5 mL of L-broth (17) for 16 h at 37˚C were harvested, washed once with 0.1 M potassium phosphate buffer (pH 7.4), and then suspended in 200 µL of 3% sodium glutamate. The suspensions in the tubes were frozen in liquid nitrogen and lyophilized under a pressure of less than 20 Pa for two hours. The lyophilized cells were rehydrated with 0.1 mL of 45 mM potassium phosphate buffer (pH 7.5) containing 9,200 Bq of [^{14}C]IPP and incubated for 20 min at 30˚C. The cells were washed with 0.1 M potassium phosphate buffer and suspended in 0.85% NaCl for the extraction of the labeled lipids.

Extraction and analysis of labeled lipids – Lipids were extracted with chloroform–methanol (1:1, v/v) according to the method of Bligh and Dyer (18), and analyzed by thin-layer chromatography on silica gel 60 plate (Merck Co.) using diisobutyl ketone–acetic acid–water (8:5:1, v/v/v) as a mobile phase (12). Radioactivity on the plate was detected by BAS-1000 (Fuji film Co.). For radioactivity quantification, the area containing each product was scraped off of the plate into a counting vial containing scintillator.

Results

Construction of multiple deletion mutants – Strains carrying multiple gene deletions were constructed by successive rounds of transduction of strains derived from W3110 by phage P1 obtained from deletion mutants of Keio collection containing integral kanamycin resistance markers, followed by excision of the markers from the chromosome. Although multiple-deletion mutants shown in Table 1 were obtained without any difficulty, transduction of the *ybjG* and *pgpB* double-deficient mutant by phage P1 containing *bacA*::Km^R was unsuccessful. This result confirmed the lethality of the inactivation of *bacA*, *ybjG*, and *pgpB* genes (6). Every mutant strain that was obtained grew as efficiently as the parental strain W3110. The growth of the triple and quadruple mutants defective in *bacA*, YS235, YS245, YS1235, and YS1245 was slightly inhibited in the bacitracin medium (120 u mL^{-1}), whereas the growth of the wild type strain in the same medium was not affected.

Determination of UP and UPP level by measurement of radioactivity – In the labeling experiment described in Materials and Methods, about 10% of radioactivity of added [^{14}C]IPP was found in the chloroform-methanol extract. The ^{14}C-labeled lipids were separated on a silica gel plate and the radioactivities of the UP (Rf=0.5) and UPP (Rf=0.33) spots were measured. The ratio of radioactivity in UP to the sum of those in UP and UPP was calculated and considered to be a relative level of UP. Because the relative levels of UP in all single and double disruption mutants (Table 1) were not significantly different from that in the parental strain, we constructed triple and quadruple mutants and measured the UP levels in them. The relative level of UP in the wild type strain W3110; in the YS1234 strain defective in *ynbD, yeiU, ybjG,* and *pgpB*; in the YS1235 strain defective in *ynbD, yeiU, ybjG,* and *bacA*; and in the YS1245 strain defective in *ynbD, yeiU, pgpB*, and *bacA* were 78.0% ± 3.5%, 74.7% ± 1.2%, 68.4% ± 6.8%, and 64.0% ± 7.5%, respectively (Table 3). The quadruple mutant YS1234 that possessed the wild-type *bacA* showed almost identical UP level as W3110, indicating that a sufficient level of UPP phosphatase activity was conferred by a *bacA* product without the products of *ynbD*, *yeiU*, *ybjG*, and *pgpB*. However the quadruple mutants YS1235 and YS1245 that possessed wild-type *pgpB* and *ybjG*, respectively, showed slight but significant ($p < 0.01$) decrease in the UP level, indicating that either a product of *pgpB* or *ybjG* alone could confer the UPP phosphatase activity slightly lower than that in the wild type strain, which would prove enough for its growth in an antibiotic-free medium.

Table 3. Relative level of UP in parental strain and deletion mutants.

Strain	Remaining gene for phosphatase	Relative level of UP (%)[a]
W3110	*bacA*$^+$, *pgpB*$^+$, *ybjG*$^+$, *yeiU*$^+$, *ynbD*$^+$	78.0 ± 3.5
YS1243	*bacA*$^+$	74.7 ± 1.2
YS143	*bacA*$^+$, *yeiU*$^+$	80.0 ± 1.0
YS234	*bacA*$^+$, *ynbD*$^+$	82.7 ± 1.0
YS1235	*pgpB*$^+$	68.4 ± 6.8 **
YS135	*pgpB*$^+$, *yeiU*$^+$	67.8 ± 7.9*
YS235	*pgpB*$^+$, *ynbD*$^+$	74.6 ± 7.8
YS1245	*ybjG*$^+$	64.0 ± 7.5 **
YS145	*ybjG*$^+$, *yeiU*$^+$	66.8 ± 6.0 **
YS245	*ybjG*$^+$, *ynbD*$^+$	67.5 ± 14.5

[a] Values shown represent averages and standard deviations of results obtained at least three assays.
t-test * P < 0.05; **p <0.01

Discussion

We prepared lyophilized cells and labeled UP and its derivatives in this study. We had previously confirmed that the labeled cells retained normal metabolic activity (12). We had been able to grow the labeled cells in L-broth again, and to trace the metabolism of the labeled UP derivatives (12, 19). In the growing cells, radioactivity in UPP-sugar had been as high as that in UP and UPP, whereas before growing in L-broth, radioactivity in UPP-sugar had been much lower than that in UP and UPP (12). Because the labeled cells were not grown again in this study, radioactivity should primarily be in UP and UPP.

The UP level in multiple deletion mutants showed that BacA plays an essential role in dephosphorylating UPP. UP levels were maintained in the triple or quadruple mutants that lacked both the *ybjG* and *pgpB* genes. Although UP levels maintained by PgpB or YjbG alone were sufficient for growth in an antibiotic-free medium, these levels were slightly lower than that in the wild type strain. Thus, it is possible that this decrease in UP level causes hypersensitivity of the deletion mutants in *bacA* to bacitracin, the antibiotic that inhibits UPP dephosphorylation.

This is the first time that multiple-deletion mutants, including the *ynbD* deletion, have been constructed. The contribution of *ynbD* to UPP dephosphorylation was small, but the negligible difference in the UP level between YS1235 and YS235 may be due to the phosphatase activity of YnbD. Although we tried to detect the enzymatic activity of YnbD, we never obtained this protein because its expression was very small and probably deleterious to the cells (not shown).

Barreteau et al. (11) reported that 80% of the total undecaprenol derivatives in growing cells of *E. coli* were UPP. UPP was calculated by analyzing the difference of UP contents determined by HPLC before and after alkaline hydrolysis because direct measurement of UPP by HPLC was difficult. Barreteau et al.'s estimated UPP value included that of UPP-sugar, which led to the over-estimation of UPP. They assumed that the UPP-sugar content was low; whereas, our previous results suggested that the UPP-sugar content in growing cells was as high as that of UPP and UP (12). Thus, direct measurement of UPP or UPP-sugar by liquid chromatography-mass spectrometry analysis would solve the difference of the results of two groups.

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References

1. Higashi, Y., Strominger, J.L., and Sweely, C.C. Structure of a lipid intermediate in cell wall peptidoglycan synthesis: a derivative of a C_{55} isoprenoid alcohol. *Proc. Natl. Acad. Sci. U.S.A.* 57, 1878-1884 (1967).
2. Bouhhs, A., Trunkfield, A.E., Bugg, T.D., and Mengin-Lecreulx, D. The biosynthesis of

peptidoglycan lipid-linked intermediate. *FEMS Microbiol. Rev.* 32, 208-233 (2008).
3. Apfel, C.M., Takacs, B., Fountoulakis, M., Stieger, M., and Keck, W. Use of genomics to identify bacterial undecaprenyl pyrophosphate synthetase: Cloning, expression, and characterization of the essential *uppS* gene. *J. Bacteriol*. 181, 483-492 (1999).
4. Kato, J., Fujisaki, S., Nakajima, K., Nishimura, Y., Sato, M., and Nakano, A. The *Escherichia coli* homologue of yeast Rer2, a key enzyme of dolichol synthesis, is essential for the carrier lipid formation in bacterial cell wall synthesis. *J. Bacteriol*. 181, 2733–2738 (1999).
5. El Ghachi, M., Bouhss, A., Blanot, D., and Mengin-Lecreulx, D. The *bacA* gene of *Escherichia coli* encodes an undecaprenyl pyrophosphate phosphatase activity. *J. Biol. Chem.* 279, 30106-30113 (2004).
6. El Ghachi, M, Derbise, A., Bouhss, A., and Mengin-Lecreulx, D. Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in *Escherichia coli. J. Biol. Chem.* 280, 18689-18695 (2005).
7. Stukey, J. and Carman, G.M. Identification of a novel phosphatase sequence motif. *Protein Sci.* 6, 469-472 (1997).
8. Touze, T., Blanot, D., and Mengin-Lecreulx, D. Substrate specificity and membrane topology of *Escherichia coli* PgpB, an undecaprenyl pyrophosphate phosphatase. *J. Biol. Chem.* 283, 16573-16583 (2008).
9. Cain, B.D., Norton, P.J., Eubanks, W., Nick, H.S., and Allen, C.M. Amplification of the *bacA* gene confers bacitracin resistance to *Escherichia coli*. *J. Bacteriol.* 175, 3784-3789 (1993).
10. Touze, T., Tran, A.X., Hankins, J.V., Mengin-Lecreulx, D., and Trent, M.S. Perilasmic phosphorylation of lipid A is linked to the synthesis of undecaprenyl phosphate. *Mol. Microbiol.* 67, 264-277 (2008).
11. Barreteau, H., Magnet, S., El Ghachi, M., Touze, T., Arthur, M., Mengin-Lecreulx, D., and Blanot, D. Quantitative high-performance liquid chromatography analysis of the pool levels of undecaprenyl phosphate and its derivatives in bacterial membranes. *J. Chromatogr., B Analyt. Technol. Biomed. Life Sci.* 877, 213-220 (2009).
12. Fujisaki, S., Nishino, T., and Katsuki, H. Biosynthesis of isoprenoids in intact cells of *Escherichia coli. J. Biochem.* 99, 1137-1146 (1986).
13. Murphy, K.C. Use of bacteriophage l recombination functions to promote gene replacement in *Escherichia coli*. *J. Bacteriol.* 180, 2063-2071 (1998).
14. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Wanner, B.L., and Mori, H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* Doi:10.1038/msb4100050 (2006).
15. Datsenko, K.A. and Wanner, B.L. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci., U.S.A*. 97, 6640-6645 (2000).
16. Reece, K.S. and Phillips, G.J. New plasmids carrying antibiotic-resistance cassettes. *Gene* 165, 141-142 (1995).
17. Lennox, E.S. Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* 1, 190-206 (1955).
18. Bligh, E.G. and Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911-917 (1959).
19. Fujisaki, S., Nishino, T., Izui, K., and Katsuki, H. Specific ^{14}C-labeling of isoprenoid of intact *E. coli* cells. *Biochem. Int.* 8, 779-785 (1984).

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