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Regular Article

Construction of *Staphylococcus aureus* Mutant Deficient in the Undecaprenol Kinase Gene, *dgkA*, and Elucidation of the Role of Undecaprenol Kinase in the Metabolism of Undecaprenyl Phosphate

Naoki Kawakami[1], Kasumi Shimada[1], Takaaki Fujita[1], Izumo Kanesaka[1,2], Intetsu Kobayashi[2], and Shingo Fujisaki[1]*

[1]Department of Biomolecular Science, Faculty of Science, Toho University, Funabashi, Chiba 274-8510, Japan
[2]Department of Infection Control and Prevention Nursing, Faculty of Nursing, Toho University, Ohta-ku, Tokyo 143-0015, Japan

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A *dgkA* homolog was cloned from the *Staphylococcus aureus* genome and expressed in *Escherichia coli*. The purified DgkA homolog encoded by the cloned gene revealed undecaprenol (UOH) kinase activity. A deletion mutant of *dgkA* homolog was constructed to examine the undecaprenyl phosphate (UP) metabolism in *S. aureus*. Cellular levels of UOH and UP were determined using cells grown in the medium containing ^{14}C-labeled and ^{3}H-labeled mevalonolactone by measuring the radioactivities in UOH and UP that were extracted from cells and separated by thin-layer chromatography. The relative UOH level in the deletion mutant was significantly higher than that in the parental strain both in the exponential growth phase and in the stationary phase. Conversion of UOH accumulated in the stationary phase to UP in the recultivated cells occurred not only in the parental strain but also in the deletion mutant.

Keywords: undecaprenyl phosphate, undecaprenyl diphosphate phosphatase, undecaprenol kinase, *Staphylococcus aureus*

Undecaprenyl phosphate (UP) is an essential compound in the biosynthesis of bacterial cell surface polysaccharides such as cell wall peptidoglycan [1] (Fig. 1). UP is called a sugar carrier lipid because it is bound to a hydrophilic sugar moiety to form a lipid intermediate that is translocated from the inner side to the outer side of the cytoplasmic membrane in the polysaccharide biosynthesis pathway [2]. Then, the sugar unit is incorporated into the growing polysaccharide chain by the action of the specific polymerase, and undecaprenyl diphosphate (UPP) is released at the outer side of the cytoplasmic membrane [3]. UP is produced in both *de novo* synthesis and recycling pathways by dephosphorylation of UPP [2,4–6]. In the *de novo* synthesis pathway, UPP is synthesized by sequential condensations of isopentenyl diphosphate to farnesyl diphosphate by the action of UPP synthase [7–9] at the inner side of the cytoplasmic membrane. In the recycling pathway, UPP is released from the lipid intermediate at the outer side of the membrane as described above. Dephosphorylation of UPP is catalyzed by one of three enzymes, BacA and two lipid phosphatases belonging to the type 2 phophatidic acid phosphatase family, in *E. coli* at the periplasmic space [10–12]. Although MurJ is the flippase that translocate sugar lipid intermediate for peptidoglycan biosynthesis form the inner side of the cytoplasmic membrane to the outer side [13], the flippase that enhances transbilayer movement of UP and/or UPP is not yet known.

Gram-positive bacteria contain a remarkable amount of undecaprenol (UOH) [14–16], which is phosphorylated to UP, although UOH has not been found in Gram-negative bacteria [17,18]. The DgkA protein in *Bacillus subtilis* [19] and *Streptococcus mutans* [20], which is homologous to diacylglycerol kinase in *Escherichia coli* [21], catalyzes the phosphorylation of UOH. It is possible that phosphorylation of UOH is a third pathway to produce UP at least in Gram-positive bacteria [2,4–6]. To investigate the role of a DgkA homolog in UP metabolism in *Staphylococcus aureus*, we cloned the *dgkA* homolog of *S. aureus*, and constructed the deletion mutant. The expression of the cloned gene in *E. coli* and the characterization of enzyme activity of the purified DgkA homolog were carried out. Cellular levels of UOH and UP in the mutant and parental strain were estimated by tracer experiment using cells grown in the medium containing radiolabeled mevalonolactone, the precursor for isoprenoid biosynthesis [22].

*Corresponding author. Fax: 81 47 472 1188
Email: sfujisak@biomol.sci.toho-u.ac.jp (S. Fujisaki).

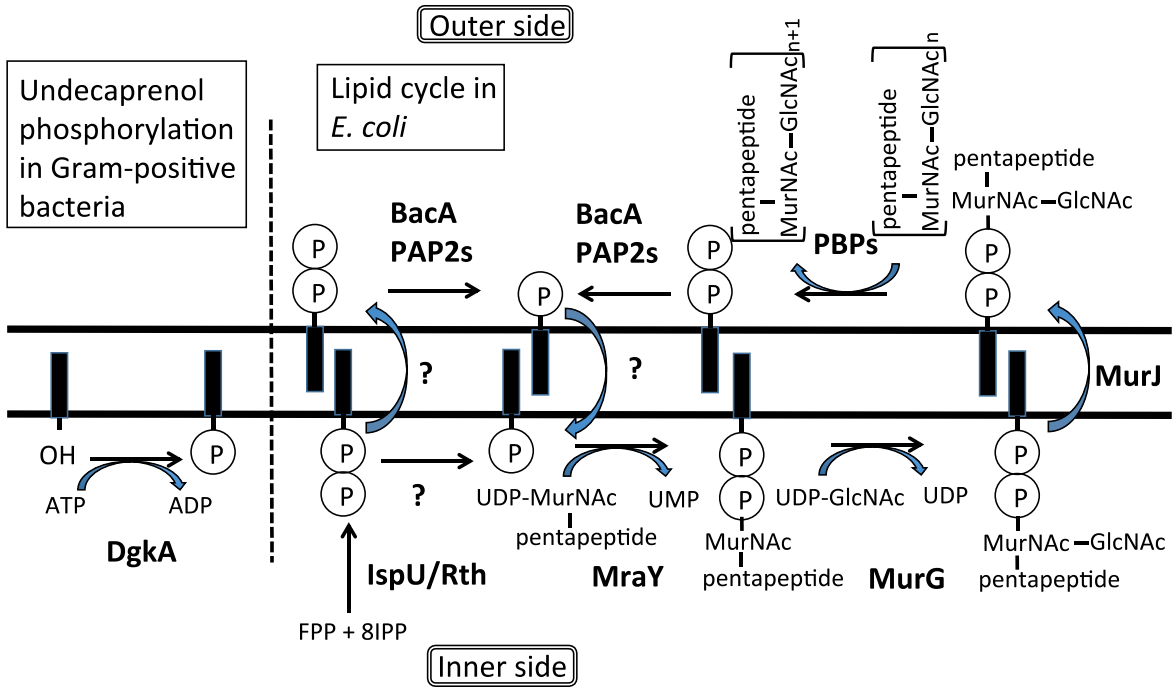


Fig. 1. Undecaprenyl phosphate (UP) metabolism in cell wall biosynthesis. The short black bar represents the undecaprenyl group, and the circled P represents the phosphate group. Undecaprenyl diphosphate (UPP) is synthesized by the sequential condensation of isopentenyl diphosphate (IPP) to farnesyl diphosphate (FPP) by the action of UPP synthase (IspU/Rth) [7–9]. Dephosphorylation of UPP is catalyzed by BacA and lipid phosphatases belonging to the type 2 phophatidic acid phosphatase (PAP2) family [10–12]. UPP disaccharide pentapeptide (lipid II) was synthesized by the sequential actions of MraY [31] and MurG [32] at the inner side of cytoplasmic membrane. The hydrophilic disaccharide pentapeptide moiety of lipid II is translocated from the inner side of the membrane to the outer side by MurJ [13]. Then the disaccharide pentapetide is incorporated into the growing chain of peptidoglycans by the action of penicillin-binding proteins (PBPs) [33], and UPP is released at the outer side of the cytoplasmic membrane [1–3]. The released UPP is dephosphorylated by BacA [10] or PAP2s [11,12]. The flippase that enhances transbilayer movement of UP and/or UPP is not yet known. A remarkable amount of undecaprenol (UOH) exists in Gram-positive bacteria [14–16]. DgkA protein, which is homologous to diacylglycerol kinase of *Escherichia coli*, catalyzes the phosphorylation of UOH [19,20].

Materials and methods

Bacterial strains and plasmids. The *E. coli* and *S.aureus* strains used are listed in Table 1. *E. coli* DH5α was used for plasmid construction and expression of the cloned *dgkA* homolog. *S. aureus* ATCC29213 was used for preparation of genomic DNA. Genomic DNA of ATCC29213 was extracted using RBC Genomic DNA

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics or derivation	Source or reference
Escherichia coli		
DH5a	Host strain for construction of recombinant plasmids and recombinant gene expression	Toyobo Co.
Staphylococcus aureus		
ATCC29213	Wild type strain	Laboratory stock
RN4220	Highly transformable strain	[23]
RN4220D*dgkA*	Constructed by allelic exchange	This work
pColdI	*E. coli* cloning vector for gene expression	Takara bio Co.
pCold-*dgkA*	Plasmid for *dgkA* expression	This work
pKOR1	Vector for allelic exchange	[24]
pKOR-D*dgkA*	Plasmid harboring disrupted *dgkA*	This work

Table 2. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'-3')[a]
dgkA_Fw1	CCACCCATATGAAAAGGTTTAAATATGCACTTG
dgkA_Rv1	TGGGGATCCGTGCTTTTCTAACTTCTTG
attB1_DdgkA_Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTATTGTATTAACAAGACCTGC
attB1_DdgkA_Rv	CATCCGCGGATTTTCAGCCCATCAAGTGC
attB2_DdgkA_Fw	CATCCGCGGATAGCGTTATTTTAGGGAGG
attB2_DdgkA_Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTAGGTCCTTCGGGTAAATACG
dgkA_Fw2	ATGAAAAGGTTTAAATATGCACTTG
dgkA_Rv2	GTGCTTTTCTAACTTCTTG

[a] Sequences of restriction sites are underlined. Sequences of specific recombination sites are double underlined.

Extraction Kit (RBC Bioscience Co.). pColdI (Takara Bio Co.) was used as a vector for expression of *dgkA* homolog in *E. coli*. The primers used are listed in Table 2. The primers dgkA_Fw1 and dgkA_Rv1 were used for amplification of a coding region of *dgkA* homologue from a genomic DNA of ATCC29123 by PCR (Fig. 2A). The amplified DNA fragment and pColdI were digested with *Nde*I and *Bam*HI and ligated. The resulting plasmid pCold-*dgkA* was used for expression of the fusion gene to produce hexahistidine-tagged DgkA homolog. *S. aureus*

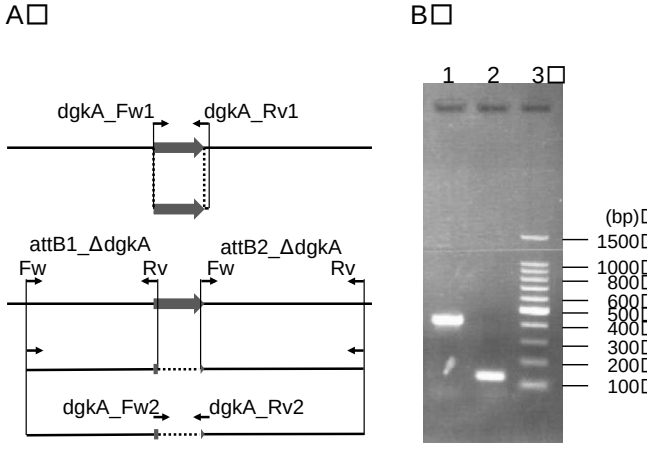


Fig. 2. Construction of the *dgkA* deletion mutant. (A) A thick line including a large arrow denotes a *dgkA* region in the chromosome of *S. aureus*, and a large arrow denotes the open reading frame of *dgkA*. Small arrows denote the primer binding sites for PCR. A sequence of the cloned fragment was deposited to GenBank/EMBL/DDBJ, and its accession number is LC365784. (B) PCR analysis of the chromosomes of the *dgkA* disruptant and the parental strain was carried out. DNA fragments of the *dgkA* region were amplified from chromosomal preparations of RN4220Δ*dgkA* and RN4220 on PCR. The resulting fragments were separated by electrophoresis in a 0.85% agarose gel. Lanes: 1, RN4220; 2, RN4220Δ*dgkA*, 3, molecular weight markers (100 bp ladder).

RN4220 [23] was used as a parental strain for construction of *dgkA* disruptant RN4220Δ*dgkA*. pKOR1 is a vector for allelic exchange [24]. Primers attB1_ΔdgkA_Fw and attB1_ΔdgkA_Rv were used for amplification of an upstream and an amino terminal region, and primers attB2_ΔdgkA_Fw and attB2_ΔdgkA_Rv were used for amplification of a downstream and a carboxyl terminal region of *dgkA* homolog in the *S. aureus* genome. The PCR amplified fragments were digested with *Sac*II and ligated to construct a truncated *dgkA* that lacked 82% of coding region. The ligated fragment was amplified again by PCR using primers attB1_ΔdgkA_Fw and attB2_ΔdgkA_Rv, and the amplified fragment was reacted with pKOR1 by BP clonase (Thermo Fisher Co.) [25] to construct pKOR-Δ*dgkA*.

Media and growth conditions. *E. coli* strains were grown in L-broth [26]. Tryptic soy broth (TSB) for cultivation of *S. aureus* was a product of Becton Dickinson Co. L-agar and TSB agar (TSA) were prepared by adding 1.5% of agar to L-broth and TSB, respectively. For the selection of *E. coli* transformant, antibiotics were added to 50 μg/mL for ampicillin and to 20 μg/mL for chloramphenicol (Cm). For the selection of *S. aureus* transformant, Cm was added to 10 μg/mL.

Chemicals. [γ-^{32}P]ATP (specific activity 111 TBq/mmol) was a product of Perkin Elmer Co. RS-[5-^{3}H(N)]mevalonolactone (specific activity 1.26 TBq/mmol) and RS-[2-^{14}C]mevalonolactone (specific activity 2.00 GBq/mmol) were products of Perkin Elmer Co. C_{95}-dolichol and C_{95}-polyprenol were provided by Collection of Polyprenols of the Institute of Biochemistry and Biophysics, the Polish Academy of Science. UOH, solanesol (all-*E*-nonaprenol), all-*E*-farnesol, and dipalmitoyl glycerol were

purchased from Sigma Chemical Co. Geraniol was purchased from Nacalai Tesque Co. Nerolidol and nerol were purchased from Wako Pure Chemicals Co. The thin-layer chromatography (TLC) plates (silica gel 60 F_{254} and LKC-18) were products of Merck Co. and Whatman Co., respectively.

Expression and purification of hexahistidine- tagged DgkA homolog. *E. coli* DH5α cells harboring pCold-*dgkA* were grown in L-broth medium deprived of glucose and containing ampicillin at 37°C. At the point in which A_{600} reached 0.5, the culture was chilled and kept at 15°C for 30 min. After addition of isopropyl β-D-thiogalactopyranoside (final concentration 0.2 mM), cells were grown for 24 h at 15°C. Cells from 100 mL of medium were collected, suspended in 2.5 mL of ice-cold 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole (buffer A), and disrupted by sonication. The mixture was centrifuged at 21,000×g for 15 min and removed supernatant. The pellet was suspended in 2 mL of buffer A containing 0.3% of Triton X-100, and the suspension was centrifuged at 21,000×g for 15 min. One milliliter of the supernatant containing solubilized DgkA homolog was collected and mixed with 0.25 mL of Ni-nitrilotriacetic acid agarose (Qiagen Co.). Purification of the hexahistidine-tagged DgkA homolog was carried out per the manufacturer's protocol with buffer containing 0.1% of Triton X-100. The eluted fraction from the Ni-nitrilotriacetic acid agarose column was dialyzed against 0.1 M Tris-HCl (pH 8.5) containing 0.1% of Triton X-100.

***UOH kinase activity*.** The activities of purified DgkA homolog were measured as described previously [27] with slight modifications. The reaction mixture for the phosphotransfer contained, in a final volume of 100 μL, 10 μmol of $MgCl_2$, 0.3% (w/v) Triton X-100, 10% (w/v) dimethylsulfoxide, 5 μmol of Tris-HCl buffer (pH 8.5), the indicated amount of a lipid, the indicated amount of [γ-^{32}P]ATP as described in a legent to Figure 4, and 50 ng of the purified enzyme. After incubation for 10 min at 25°C, 50 μL of 0.1 M EDTA trisodium salt (pH 8.0) was added to stop the enzyme reaction. The products of the reaction were extracted with chloroform-methanol or 1-butanol, and the radioactivity in the extract was measured after the extract was washed with chloroform-methanol-water (3:48:47 v/v/v) or water saturated with 1-butanol, respectively.

***Construction of the* dgkA *deletion mutant of* S. aureus.** *S. aureus* RN4220 was transformed with pKOR-Δ*dgkA* by electroporation [28], and transformants were selected on TSA containing 10 μg/mL of Cm at 30°C. A colony on the selection plate was picked up and streaked on the TSA containing Cm, and the plate was incubated over night at 43°C (integration process). The resulting colony was inoculated in TSB and grown over night at 30°C (resolution process). The culture was diluted 10^5 times with TSB and inoculated on TSA containing 1 μg/mL of anhydrotetracycline. The plate was incubated over night at 37°C (counter selection). The resulting colonies were inoculated in TSB and grown overnight. The genomic DNA was prepared from grown cells to examine allelic replacement at the *dgkA* site. The primers, dgkA_Fw2 and dgkA_Rv2 were used for PCR to amplify the fragment including *dgkA* coding region.

Radiolabeling of* S. aureus *strains. *S. aureus* RN4220 and RN4220Δ*dgkA* were inoculated in 1 mL of TSB, grown for 1 h at 37°C followed by addition of 37 kBq of RS-[2-^{14}C]mevalonolactone, and grown for 24 h at 37°C. Cells in the stationary phase were collected by centrifugation and suspended in 1 mL of fresh TSB. The suspensions were divided into two 0.5 mL of aliquots. Then the first aliquots were centrifuged, and the collected cells were used for the extraction of radiolabeled products. The remaining aliquots were added to 10 mL of TSB, grown for 1 h followed by the addition of 185 kBq of RS-[5-^{3}H(N)]mevalonolactone, and grown for 2 h at 37°C. Cells grown to the exponential growth phase were collected, and used for the extraction of radiolabeled products.

Extraction and analysis of radiolabeled polyprenols and polyprenyl phosphates.
The radiolabeled cells were suspended in 2 mL of methanol-0.3% NaCl (10:1 v/v), and 1 mL of 60% (w/w) KOH was added to the mixture and the mixture was heated in boiling water for 1 h. Polyprenols were extracted twice with 2 mL of hexane and then once with 1 mL of hexane. The hexane layer was rinsed with 1 mL of methanol-0.3% NaCl (10:1 v/v), and 100 μL of hexane layer was used for measuring radioactivity by liquid scintillation counter, Tri-Carb 2810 TR (Perkin Elmer Co.), using the single label ^{14}C DPM measurement mode or the double label DPM measurement mode. The residual solution was dried under N_2, dissolved with 20 μL of hexane, and analyzed by normal phase and reversed phase TLC. Normal phase TLC was carried out on a silica gel 60 plate with benzene-ethyl acetate (4:1, v/v). Reversed-phase TLC was carried out on an LKC-18 plate with acetone-water (19:1 v/v). Radioactivity on the plate was detected by FLA-7000 (Fuji Film Co.). For radioactivity quantification, the area containing each product was scraped off of the plate, and radioactive compounds were dissolved in methanol for measurement by liquid scintillation counter. Polyprenyl phosphates were extracted twice with 2 mL of diethyl ether and once with 3 mL of diethyl ether from the residual suspension in alkaline methanol-water layer after the extraction with hexane. The

diethyl ether extract was washed with 5% acetic acid and dried under N_2. The residue was dissolved with chloroform-methanol (2:1 v/v) and 100 µL of solution was used for measuring radioactivity. Residual polyprenyl phosphates were treated with acid phosphatase as described previously [29]. The resulting polyprenols were extracted with hexane and analyzed as described above.

Results

Properties of hexahistidine-tagged DgkA homolog of S. aureus

E. coli cells expressing DgkA homolog were disrupted by sonication in the buffer without a detergent at first. SDS-polyacrylamide gel electrophoresis (PAGE) analysis revealed a specific 15-kDa band in both supernatant and pellet fraction in centrifugation. Hexahistidine-tagged DgkA homolog could be solubilized and purified by Ni-nitrilotriacetic acid agarose chromatography using the buffer containing Triton X-100 (Fig. 3). The analysis of fractions eluted with the buffer containing 250 mM of imidazole in SDS-PAGE revealed a major 15-kDa band and a minor 20-kDa band. The second and third fractions

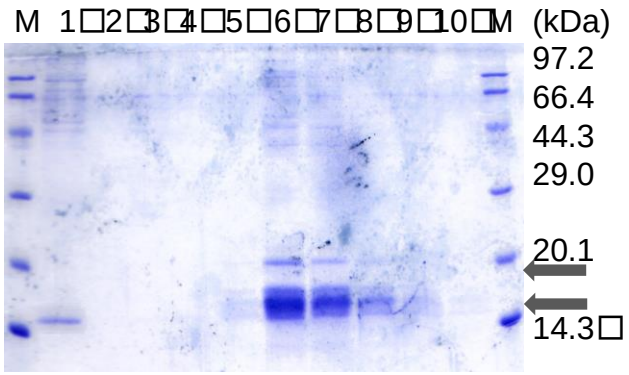


Fig. 3. SDS-polyacrileamide gel electrophoresis of DgkA homolog. Fractions in the purification of the hexahistidine-tagged DgkA homolog were analyzed on 12.5% acrylamide gel. Lane M, moleculare weight marker: mixture of phosphorylase b (97,200), bovine-serum albumin (66,400), ovalbumin (45,000), carbonic anhydrase (29,000), trypsin inhibitor (20,100), and lysozyme (14,300). The solubilized fraction was applied to a Ni-nitriloacetic acid agarose column as described in Materials and Methods. Lane 1, flow through; lanes 2–4, fractions of 2.5 mL each eluted with 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 0.1% (w/v) Triton X-100, and 20 mM imidazole; lanes 5–10, fractions of 0.25 mL each eluted with 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 0.1% (w/v) Triton X-100, and 250 mM imidazole.

of this buffer (lanes 6 and 7 in Fig. 3) were combined and used for characterization of lipid kinase activity. Approximately 42 µg of protein was obtained in these fractions. The most effective lipid substrate was *Z,E*-mixed UOH (Fig. 4A). The activities of phosphorylation of *Z,E*-mixed C_{95}-polyprenol and C_{95}-dolichol were 45% and 12% of that of UOH, respectively. The activities of phosphorylation of solanesol and all-*E*-farnesol were both 6% of that of undecaprenol. Phosphorylation of nerolidol, geraniol, nerol, and dipalmitoyl glycerol was not detected. To examine whether the various nucleotide triphosphates were substrates for the DgkA homolog, competition assay was carried out (Fig. 4B). Only cold ATP competed with the incorporation of radioactivity into lipid fraction from

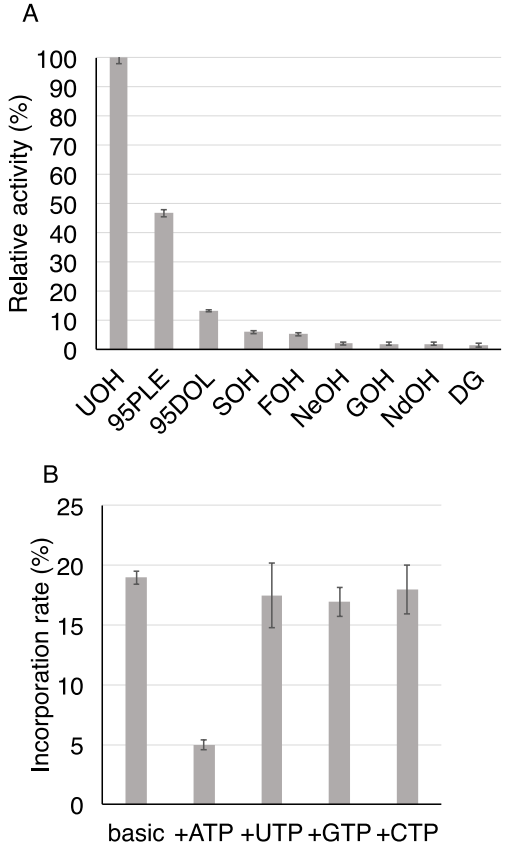


Fig. 4. Substrate specificity of the DgkA homolog. (A) Enzyme reactions with 150 µM ATP and various alcohols were carried out, and radioactivities in reaction products extracted with 1-butanol were measured. UOH, undecaprenol; 95PLE, C_{95}-polyprenol; 95DOL, C_{95}-dolicohol; SOH, solanesol (all-*E*-nonaprenol); FOH, all-*E*-farnesol; NeOH, nerol; GOH, geraniol; NdOH, nerolidol; DG, dipalmitoyl glycerol. C_{95}-polyprenol and C_{95}-dolicohol were added to 75 µM, and the other alcohols were added to 150 µM. (B) Enzyme reactions with 150 µM UOH, 30 µM [γ-^{32}P]ATP , and various nucleoside triphosphate were carried out, and radioactivities in a reaction product extracted with chloroform-methanol were measured. basic, without additional nucleoside triphosphate; +ATP, 300 µM of ATP was added; +UTP, 300 µM of UTP was added; +GTP, 300 µM of GTP was added; +CTP, 300 µM of CTP was added.

[γ-^{32}P]ATP, and the other nucleotides UTP, GTP, and CTP did not affect the incorporation of radioactivity. Thus the enzyme was shown to be specific to ATP. The K_m value for UOH that was obtained while holding the concentration of

ATP constant (150 μM) was 41 μM. The K_m value for ATP that was obtained while holding the concentration of UOH constant (150 μM) was 56 μM.

Construction of dgkA *disruptant*

After the integration and resolution process, counter-selection was carried out as described in Materials and Methods. Four colonies were selected to examine whether the disrupted *dgkA* homolog (Δ*dgkA*) was introduced into a chromosome. PCR analysis showed that three colonies gave a 0.1-kb band resulting from Δ*dgkA*, and that one colony gave a 0.4-kb band resulting from wild type *dgkA* (Fig. 2B). Thus, we were able to obtain gene disruptant RN4220Δ*dgkA*. The growth rate of the disruptant was same as that of the parental strain. The doubling times in TSB at 37°C of both strains were 30 min.

Quantification of UOH and UP derivatives in S. aureus *strain by tracer experiment*

To investigate UP metabolism in RN4220 and RN4220Δ*dgk*, a tracer experiment was carried out using radiolabeled precursor for isoprenoids. ^{14}C-labeled compounds extracted from cells in the stationary phase were analyzed by TLC (Fig. 5). Analysis of hexane extract

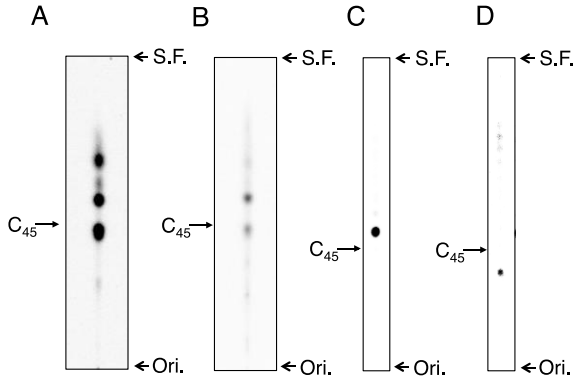
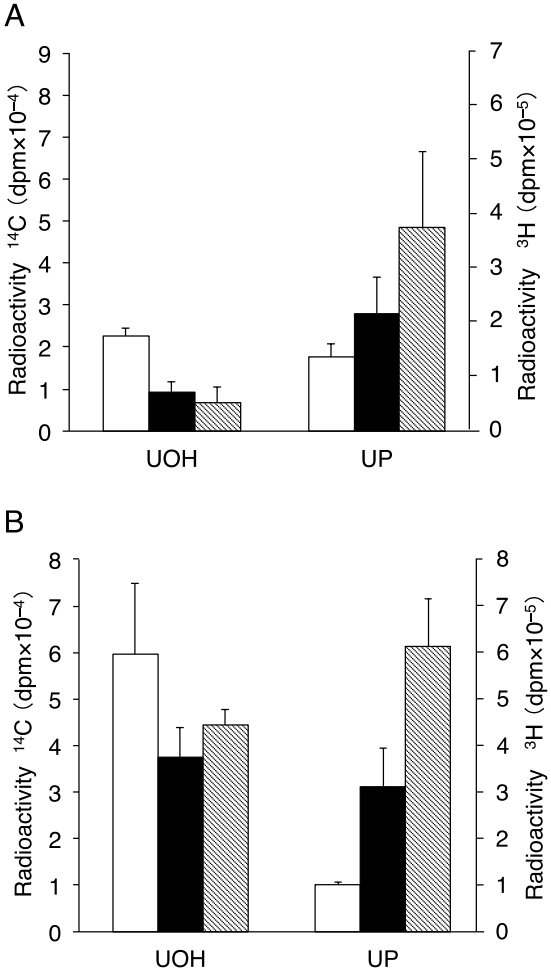


Fig. 5. Autoradiograms of radiolabeled products from *S. aureus* cells grown in the presence of ^{14}C-labeled mevalonolactone. Radiolabeling of cells of *S. aureus* and analysis of radioactive products were carried out as described in Materials and Methods. Ori., Origin; SF, solvent front; C_{45}, solanesol. (A) The hexane extract from the stationary cells of RN4220 was analyzed by normal phase TLC. (B) The diethylether extract from the stationary cells of RN4220 was treated with acid phosphatase, and resulting products were analyzed by normal phase TLC. (C) The compound in the spot of R_f= 0.44 in the normal phase TLC (A) was eluted from the silica gel and analyzed by reversed phase TLC. (D) The compound in the spot of R_f= 0.54 in the normal phase TLC (A) was eluted from the silica gel and analyzed by reversed phase TLC.

in normal phase TLC revealed three radioactive spots (Fig. 5A). A spot that had a slightly lower R_f value (0.44) than that of solanesol (R_f=0.45) was considered to be octaprenol, as it had a higer R_f value (0.45) than that of solanesol (R_f=0.38) in reversed phase TLC (Fig. 5C). A spot that had a modestly higher R_f value (0.54) than that of solanesol was considered to be UOH, as it showed lower R_f value (0.32) than that of solanesol in reversed phase TLC (Fig. 5D). A spot that showed highest R_f value (0.67) was considered to be menaquinone-8. Diethylether extract treated with acid phosphatase also contained octaprenol and UOH (Fig. 5B), showing that diethyether extract contained octaprenyl phosphate and UP.



Radioactivities in UOH and UP are shown in Figure 6.

Fig. 6. Radioactivity of undecaprenol and undecaprenyl phosphate in the cells of *S. aureus* RN4220 and RN4220Δ*dgkA* grown in the presence of ^{14}C-labeled mevalonolactone and/or ^{3}H-labeled mevalonolactone. Radiolabeling of *S. aureus* RN4220 (A) and RN4220Δ*dgkA* (B) and analysis of radioactive products were carried out as described in Materials and Methods. Bar graphs show averages of three assays. Error bars show standard deviations. White bar, ^{14}C radioactivity in the stationary phase cells; black bar, ^{14}C radioactivity in the recultivated cells; hatched bar, ^{3}H radioactivity in the recultivated cells.

Radioactivities of ^{14}C in the compound extracted from cells in the stationary phase are considered to reflect the levels of these compounds in the cells in the stationary phase, and radioactivities of ^{3}H in the compound extracted from

regrown cells are considered to reflect the levels of these compounds in the cells in the exponential growth phase.

The relative UOH level to the sum of UOH level and UP level in the stationary phase calculated from ^{14}C radioactivities was 85.3%±2.2% in the *dgkA* deletion mutant and was significantly higher ($p<0.01$) than that in the parental strain (56.4% ±3.7%). The relative UOH level in the exponential growth phase calculated from ^{3}H radioactivities was 42.2%±5.3% in the *dgkA* deletion mutant and was also significantly higher ($p<0.01$) than that in the parental strain (13.8%±6.6%). Radioactivities of ^{14}C in the compounds extracted from regrown cells are considered to reflect the result of conversion of radioactive compounds that had accumulated in the stationary phase during recultivation. The relative UOH level significantly decreased ($p<0.01$) during recultivation of cells that had reached the stationary phase (from 56.4% ±3.7% to 26.1%±7.5%) in the parental strain, indicating that the conversion of UOH to UP occurred. The relative UOH level in the deletion mutant also decreased ($p<0.01$) during the regrowth of cells (from 85.3%±2.2% to 54.8%±9.7%), indicating that the conversion of UOH to UP occurred also in the deletion mutant.

Discussion

In the early study of cell wall biosynthesis, UOH kinase was purified from *S. aureus*. It is an extremely hydrophobic protein with a molecular mass of 17 kDa in SDS-PAGE that catalyzes the phosphorylation of UOH with ATP in the presence of magnesium ion [27]. The DgkA homolog purified in this study showed similar properties to that of previous purified UOH kinase. DgkA homolog located mainly in precipitate fraction was solubilized with Triton X-100 and gave a 15-kDa band in SDS-PAGE. K_m values of DgkA homolog (56 μM for ATP and 41 μM for UOH) were almost the same as that of previous purified UOH kinase (57 μM for ATP and 57 μM for UOH). Thus, *dgkA* of *S. aureus* was confirmed to be the gene for UOH kinase as that of *B. subtilis*[19] and *S. mutans*[20].

We constructed a *dgkA* deletion mutant using a plasmid for allelic exchange. A tracer experiment showed that the relative UOH levels in the mutant were higher both in the exponential growth phase and in the stationary phase than that in the parental strain. These results indicate that the DgkA homolog has a role in maintaining the cellular UP level by phosphorylation of UOH both in the exponential growth phase and in the stationary phase. Recently, Radeck et al. constructed a *dgkA* deletion mutant of *B. subtilis* [30]. The upregulation of the expression of UPP phosphatase gene in the deletion mutant was observed, though the mutant did not show remarkable growth or morphological phenotype. This result suggests that lack of UOH kinase causes modest shortage of UP also in the cell of *B. subtilis* as well.

Relative UOH levels in the stationary phase were higher than in the exponential growth phase, both in the deletion mutant and in the parental strain. By the recultivation of the ^{14}C-labeled stationary phase cells, metabolism of accumulated ^{14}C-labeled lipids was examined. Surprisingly conversion of UOH to UP was observed in the *dgkA* deletion mutant as well as in the wild type parental strain. Although DgkA homologue is UOH kinase that maintain cellular UP level, there should be another enzyme to phosphorylate UOH in *S. aureus*. The possible candidate of phosphate donor is not only ATP but also the other compounds such as phospholipids.

Because all known UPP phosphatases examined for membrane topology have substrate binding sites at the outer side of the membrane [10–12], there should be a flippase to translocate UPP from the inner side of the membrane to the outer side or a novel UPP phosphatase that act at the inner side of the membrane for the *de novo* UP synthesis (Fig. 1). However both of them have not been found yet. Since transbilayer movement of UOH is much faster than that of UP and UPP, UOH can move across the membrane without any flippase. If the transfer of phosphate from UPP to UOH occurred at the cytoplasmic side of the membrane, it would provide a means of *de novo* synthesis of UP from UPP. The enzyme that catalyzes phosphotransfer to UOH in the *dgkA* mutant should be investigated in future studies.

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