Characterization of the *Staphylococcus aureus* mprF gene, involved in lysinylation of phosphatidylglycerol

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Lysylphosphatidylglycerol (LPG) is a basic phospholipid in which L-lysine from lysyl-tRNA is transferred to phosphatidylglycerol (PG). This study examined whether the *Staphylococcus aureus* mprF gene encodes LPG synthetase. A crude membrane fraction prepared from wild-type *S. aureus* cells had LPG synthetase activity that depended on PG and lysyl-tRNA, whereas the membrane fraction from an mprF deletion mutant did not. When *S. aureus* MprF protein was trans-expressed in wild-type *Escherichia coli* cells, LPG synthesis was induced, whereas it was not observed in *E. coli* pgsA3 mutant cells in which the amount of PG is significantly reduced. In addition, LPG synthetase activity and a 93 kDa protein whose molecular size corresponded to that of MprF protein were co-induced in the crude membrane fraction prepared from *E. coli* cells expressing MprF protein. The $K_m$ values of the LPG synthetase activity for PG and for lysyl-tRNA were 56 μM and 6.9 μM, respectively, consistent with those of *S. aureus* membranes. These results suggest that the MprF protein is LPG synthetase.

INTRODUCTION

*Staphylococcus aureus* cells have three major phospholipids, phosphatidylglycerol (PG), cardiolipin (CL) and lysylphosphatidylglycerol (LPG) (White & Frerman, 1967). LPG is a basic lipid in which a lysyl group from lysyl-tRNA is transferred to PG (Lennarz et al., 1966, 1967; Nesbitt & Lennarz, 1968). This unusual lipid is identified not only in staphylococci, but also in other clinically important bacteria such as *Enterococcus faecalis* (Houtsomuller & van Deenen, 1965) and *Pseudomonas aeruginosa* (Kenward et al., 1979). Recently, it was reported that an *S. aureus* mprF gene mutant, in which LPG is absent, is sensitive to cationic antimicrobial peptides of the innate immune system (Peschel et al., 2001; Kristian et al., 2003). A decrease in the basic charge on the cell membrane was suggested to lead to a reduced repulsion against cationic peptides and sensitizes the mprF mutant cells to these peptides (Peschel, 2002). By protecting against cationic antimicrobial peptide-like bacteriocins, LPG also plays a role in the struggle for existence among soil micro-organisms (Staubitz & Peschel, 2002). Moreover, the mprF mutation also sensitized the *S. aureus* cells to vancomycin, β-lactams and bacitracin, suggesting that LPG has a role in multi-drug resistance in a series of methicillin-resistant *S. aureus* strains (Ruzin et al., 2003).

LPG is involved in the regulatory mechanisms of the initiation of DNA replication in *S. aureus* (Ichihashi et al., 2003). The initiation activity of DnaA protein, the initiator of bacterial DNA replication, is regulated by its adenine-nucleotide binding (Sekimizu et al., 1987; Katayama et al., 1998; Kurokawa et al., 1999; Nishida et al., 2002). The affinity of DnaA protein for adenine nucleotides is affected by acidic phospholipids (Sekimizu & Kornberg, 1988; Castuma et al., 1993; Xia & Dowhan, 1995; Mizushima et al., 1996; Kitchen et al., 1999). LPG inhibits the interaction between DnaA protein and acidic phospholipids, PG and CL, and the *S. aureus* mprF mutant exhibits an increased amount of replication origins (Ichihashi et al., 2003). These findings suggest that LPG is also involved in cell cycle regulation.

LPG synthetase activity in *S. aureus* exists in the cell membrane fraction and catalyses the transfer of L-lysine from lysyl-tRNA to PG (Lennarz et al., 1967; Nesbitt & Lennarz, 1968), but the gene encoding the enzyme has not yet been identified. Because the mprF deletion mutant did not synthesize LPG, the mprF gene was suggested to encode the LPG synthetase (Peschel et al., 2001). In the present study, we trans-expressed the *S. aureus* MprF protein in *Escherichia coli* cells and obtained biochemical evidence that the mprF gene product encodes LPG synthetase.

METHODS

**Bacterial strains and plasmids.** *S. aureus* strains RN4220 and CK1001, an insertional deletion mutant of the mprF gene, were

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**Abbreviations:** CL, cardiolipin; LPG, lysylphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.
described previously (Novick et al., 1993; Ichihashi et al., 2003). E. coli strain MJ109 was obtained from Takara Shuzo. E. coli strains W3110, JE5513Tc and YAY511Tc (JE5513Tc pgaA3) were from our laboratory stock (Miyazaki et al., 1985; Tomura et al., 1993). An arabino-inducible expression plasmid in E. coli, pBAD24 (Guzman et al., 1995), was kindly provided by Dr S. Yasuda (Cloning Vector Collection of the National Institute of Genetics, Japan). The MpFR protein-expression vector, pBADmpFR, was constructed as follows. The reading frame of the mpFR gene was amplified by PCR using an S. aureus RNA4220 genomic DNA as a template with PfuTurbo DNA polymerase (Stratagene) and primers 5′-GAATCATGATCATCGGAAAGTAAAAACA-3′ and 5′-ACGGGTCGCCACTCAGGCCTCACGGCATAA-3′. The resultant PCR product was digested with BspHI and Sall, and cloned into pBAD24 at the Ncol and Sall sites.

Bacterial culture conditions. S. aureus and E. coli cells were grown at 37°C in Luria–Bertani (LB) medium [1% tryptone (Difco), 0.5% yeast extract (Difco) and 1% NaCl containing, where appropriate, 50 μg thymine ml⁻¹, 50 μg ampicillin ml⁻¹, 5 μg tetracycline ml⁻¹ or 12.5 μg chloramphenicol ml⁻¹. When bacterial cells were metabolically labelled with [¹⁴C]lysine (300 mCi mm⁻¹, Amersham Pharmacia Biotech) or [³²P]orthophosphate (10 mCi ml⁻¹, Amersham Pharmacia Biotech), LB medium containing 0.5 μCi (18.5 kBq) [¹⁴C]lysine ml⁻¹ or 10 μCi (0.37 MBq) [³²P]orthophosphate ml⁻¹ was used. For preparation of the membrane fraction from S. aureus RNA4220 or CK1001 cells, cells were grown at 37°C in 1 L LB medium to an OD₆₀₀ value of 0.9 and then harvested. For preparation of the membrane fraction from E. coli MJ109 cells harbouring either pBADmpFR or pBAD24, cells were grown at 37°C in 1 L LB medium to an OD₆₀₀ value of 0.5, then up to 1% L-(-)arabinose (Tokyo Kasei) was added; the cells were incubated further for 2 h, and then harvested.

Analysis of lipids of bacteria. For analysis of the E. coli membrane lipid composition, total lipid was extracted from 1 ml of each bacterial cell culture using a modified Bligh–Dyer method as described previously (Sekimizu & Kornberg, 1988). Extracted lipids were separated by TLC developed with chloroform/methanol/acetic acid [65:25:10 (by vol.)] using a silica gel plate (Silicagel 60, type PK6F, Whatman). To detect lipids, TLC plates were sprayed with 100 μg CuSO₄ ml⁻¹ containing 8% phosphoric acid and heated. To detect [¹⁴C]lysine- or [³²P]orthophosphate-labelled lipids, TLC plates were fluorographed using an image analyser (Fuji Film BAS2000).

Preparation of membrane fraction. Membrane fractions from S. aureus or E. coli were prepared as described previously (Lennarz et al., 1966) with slight modifications. S. aureus cells (wet weight 11.2 g), harvested as described above, were suspended in 2–2.4 ml 20 mM Tris/HCl (pH 6–8), and incubated with 200 μg lysostaphin (Wako) ml⁻¹ at 4°C for 30 min. The resulting lysate was sonicated for 30 s at output 2.0 using a Branson Sonifier 450. Sonicated lysate was centrifuged at 15,000 g for 15 min at 4°C, followed by centrifugation at 100,000 g for 60 min at 4°C. The resultant pellet was suspended in 250 μl buffer containing 20 mM Tris/HCl and 1 mM 2-mercaptoethanol, and suspended at 100,000 g for 60 min at 4°C; the pellet was suspended in 250 μl buffer containing 20 mM Tris/HCl (pH 6-8) and 1 mM 2-mercaptoethanol, and used as the membrane fraction. To prepare E. coli cell membrane fractions, harvested cells (wet weight 2.8 g) were suspended in 560 μl 20 mM Tris/HCl (pH 6–8), and lysed with 200 μg lysozyme ml⁻¹ at 4°C for 30 min, followed by the addition of spermidine hydrochloride to 20 mM. Sonication and centrifugation of samples from E. coli were performed as described above. Protein concentration was determined by the Bradford method (Bradford, 1976) using BSA as a standard.

Preparation of [¹⁴C]lysyl-tRNA. [¹⁴C]Lysyl-tRNA was prepared as described by von Ehrenstein (1967) with slight modification. To prepare the tRNA synthetase enzyme fraction, E. coli W3110 cells (wet weight 5.4 g) were suspended in 10–4 ml buffer A [1 mM Tris/HCl (pH 7.2) and 10 mM MgCl₂] and incubated with 200 μg lysosome ml⁻¹, followed by the addition of spermidine hydrochloride up to 20 mM. The supernatant was centrifuged at 105,000 g for 1 h. The resultant supernatant was passed through a DE52 (Whatman) column equilibrated with buffer A to remove endogenous tRNAs, and flowthrough fractions were pooled as the tRNA synthetase enzyme fraction. The reaction mixture (0–5 ml) for [¹⁴C]lysyl-tRNA synthesis contained 100 mM Tris/HCl (pH 7.2), 10 mM MgCl₂, 10 mM KCl, 1 mM ATP, 0.4 μM [¹⁴C]lysine (100 μCi mm⁻¹, Amersham Pharmacia Biotech), 200 μg E. coli tRNA ml⁻¹ (Sigma), and 50 μg tRNA synthetase enzyme fraction. Synthesized [¹⁴C]lysyl-tRNA was purified by salt-ethanol precipitation with two washes. To make sure that free [¹⁴C]lysine was removed in the [¹⁴C]lysyl-tRNA fraction, we simultaneously performed experiments omitting the tRNA synthetase fraction and washed the fractions until the background radioactivities disappeared.

In vitro LPG synthetase activity assay. This assay was performed as described by Lennarz et al. (1967) with minor modifications. The reaction mixture (50 μl) contained 17-4 mM Tris/maleate (pH 7.0), 94 mM KCl, 7 mM MgCl₂, 0.24 mM l-lysine, 1.5 mM phosphatidylglycerol (Sigma), 30 μM [¹⁴C]lysyl-tRNA prepared above, and 0–40 μg protein from the membrane fraction. The reaction was performed at 30°C for 10 min and terminated by the addition of 550 μl chloroform/methanol [2:1 (v/v)]. The mixture was vigorously vortexed, incubated at 55°C for 3 min to denature proteins, vortexed again, incubated at 55°C for 7 min, and filtered through a paper filter (no. 2, Toyo Roshi). The filter was washed with 100 μl chloroform/methanol [2:1 (v/v)] and 250 μl 0.9% NaCl to get reproducible recovery of LPG in the organic phase. The filtrate was mixed, vigorously vortexed for 1 min, placed on ice for 5 min, and centrifuged at 3000 r.p.m. for 5 min at 4°C. The chloroform layer was washed once with 0.9% NaCl to extract [¹⁴C]lysyl-tRNA that distributed in the chloroform layer independently of enzyme reaction. The amount of radioactivity incorporated into the chloroform layer was measured with a liquid scintillation counter (LS3801, Beckman).

RESULTS AND DISCUSSION

Decrease in the LPG synthetase activity of membrane fractions prepared from the S. aureus mpFR deletion mutant

Because LPG was not detectable in the membrane lipid fraction of an S. aureus mpFR deletion mutant, the MpFR gene is considered indispensable for LPG synthesis (Peschel et al., 2001). The function of the mpFR gene product, however, is not clear. Possible functions of the MpFR protein other than LPG synthetase would be as an inhibitory factor for LPG degradation or an essential factor for the expression of LPG synthetase. In this study, we hypothesized that MpFR protein might function as the LPG synthetase. If this is correct, LPG synthetase activity in the membrane fraction of the mpFR deletion mutant should be lost. We constructed an mpFR insertional deletion mutant by homologous recombination (Ichihashi et al., 2003), and compared the LPG synthetase activity in the membrane fractions of the mutant and wild-type cells. The mpFR deletion mutant did not have a detectable amount of LPG (Ichihashi et al., 2003), consistent with a previous report (Peschel et al., 2001). A membrane fraction prepared from
the parent *S. aureus* strain RN4220 had LPG synthetase activity (Fig. 1). This activity was dependent on PG and lysyl-tRNA (data not shown), consistent with previous reports (Lennarz *et al.*, 1967; Nesbitt & Lennarz, 1968). On the other hand, [3H]lysine incorporation into the organic phase by a membrane fraction from the *mprF* deletion mutant was not significantly different from the background incorporation (Fig. 1). The results indicate that the *mprF* deletion mutant lacks LPG synthetase.

**Induction of LPG synthesis in *E. coli* cells via expression of *S. aureus* MprF protein**

If the *S. aureus* MprF protein encodes LPG synthetase, trans-expression of MprF protein in *E. coli* cells, which do not have either a homologue to the *mprF* gene or LPG, should induce LPG synthesis in their membranes. To examine this possibility, an open reading frame of the *S. aureus mprF* gene was inserted into plasmid pBAD24 under the control of the arabinose promoter (Guzman *et al.*, 1995), and *E. coli* JM109 was transformed by the resultant plasmid, pBADmprF. The cells were cultured in the presence or absence of arabinose, and total lipid was extracted and analysed by TLC. A lipid whose migration was consistent with LPG extracted from *S. aureus* cells and whose production was stimulated by the addition of arabinose to the medium was identified in total lipid from JM109/pBADmprF cells (Fig. 2a). The lipid stained positive for both the Dittmer–Lester reagent and ninhydrin reagent (data not shown), indicating that it contained phosphates and amino groups. This lipid was not present in the total lipid from JM109 harbouring the vector plasmid (Fig. 2a).

To confirm that this lipid was LPG, the *E. coli* strains were cultured in the presence of [14C]lysine, and total lipid was extracted and separated by TLC. A lipid labelled with 14C, whose *Rf* value corresponded to that of LPG extracted from *S. aureus* cells, was detected in the *E. coli* cells harbouring pBADmprF (Fig. 2b). In addition, when membrane lipids

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**Fig. 1.** Loss of *in vitro* LPG synthetase activity in a membrane fraction from an *S. aureus mprF* gene deletion mutant. Membrane fractions prepared from *S. aureus* RN4220 (wild-type, □) or CK1001 (*ΔmprF*, ○) were assayed for LPG synthetase activity *in vitro*. The indicated amount of membrane fraction (0, 10, 20, 40 μg protein) was incubated at 30°C for 10 min in the presence of 24 μM [3H]lysyl-tRNA and 1-55 mM PG. Reactions were terminated by the addition of chloroform, and lipids were extracted. The amount of [3H]lysine in the chloroform (organic) phase was determined. Results are mean ± SD of three independent experiments. Analyses from different membrane preparations did not differ qualitatively from these.

**Fig. 2.** Induction of LPG synthesis by expression of the *S. aureus* MprF protein in *E. coli* cells. *E. coli* JM109 cells transformed either with pBAD24 (vector) or with pBAD24 harbouring the *S. aureus mprF* gene (*mprF*) under the control of the arabinose promoter were cultured in LB medium without (a) or with [14C]lysine (b) to an OD600 value of 0-1. Arabinose (1%) was then added and the cultures were further incubated for 2 h. Total lipids were extracted and then separated by TLC. Lipids on TLC plates were visualized by spraying with CuSO4 and heating (a). 14C-labelled lipids were detected using an image analyser (Fuji Film Bas2000) (b). Total lipid extracted from *S. aureus* RN4220 cells (Sau) grown in LB medium without (a) or with [14C]lysine (b) was loaded on the far right lanes as a standard for LPG.
were metabolically labelled with $^{32}$P orthophosphate and analysed by TLC, the lipid in the *E. coli* cells expressing MprF protein, whose *R*$_f$ value corresponded to that of LPG extracted from *S. aureus* cells, was also radiolabelled (Fig. 3). These results indicate that MprF protein expression in *E. coli* cells induces synthesis of a phospholipid to which lysine was transferred, that is, LPG.

In *vitro* LPG synthetase activity requires PG as a substrate (Lennarz *et al.*, 1967; Nesbitt & Lennarz, 1968). We examined whether LPG synthesis via MprF protein expression in cells requires PG as a substrate. When MprF protein was expressed in the *E. coli* pgsA3 mutant cells, which had a significantly reduced amount of PG in their cell membrane (Miyazaki *et al.*, 1985), LPG synthesis of MprF protein was not induced (Fig. 3). The results are consistent with the notion that MprF protein catalyses LPG synthesis from PG.

When LPG was synthesized via MprF protein expression in *E. coli* cells, the relative amount of CL increased, and that of phosphatidylethanolamine (PE) decreased (Fig. 2a). One interpretation of the result is that a homeostatic control of the net charge might exist on the cell membrane. The present *E. coli* strain, in which negatively charged LPG can be induced, will be a useful tool to elucidate novel regulatory mechanisms for phospholipid biosynthesis in bacterial membranes.

Identification of MprF protein and LPG synthetase activity in the membrane fraction of *E. coli* cells expressing MprF protein

MprF protein is proposed to be a transmembrane protein containing 13 putative transmembrane domains (Peschel *et al.*, 2001). Membrane fractions were prepared from *E. coli* cells transformed with pBADmprF or pBAD24 vector, and proteins were separated by SDS-polyacrylamide (7.5%) gel electrophoresis. An approximately 93 kDa protein, whose molecular size was consistent with the predicted mass of the MprF protein, was induced by the addition of arabinose with dependence on plasmid pBADmprF (Fig. 4a). Repeated washing of the membrane fractions increased the content of the 93 kDa protein (data not shown). The results support the prediction that MprF protein is a 93 kDa membrane protein.

We then analysed the *in vitro* LPG synthetase activity in the membrane fractions prepared above. The membrane fraction from the *E. coli* cells harbouring pBADmprF with arabinose induction had LPG synthetase activity measured by the transition of $^3$Hlysine into the organic phase (Fig. 4b). To confirm the *in vitro* synthesis of LPG, the lipids in the organic phase containing $^3$Hlysine were separated by TLC. Staining of the plate with rhodamine 6G, which detects phospholipids (Dittmer & Lester, 1964), revealed a
spot whose $R_f$ value corresponded to that of LPG from *S. aureus* cells (data not shown). Incorporation of reasonable amounts of $^3$H radioactivity into LPG was confirmed by scraping the lipids off the plate and measuring their radioactivity by liquid scintillation counting (data not shown). This LPG synthetase activity in the *E. coli* membrane fraction depended on both PG and lysyl-tRNA (Fig. 5), consistent with observations in the membrane fraction of *S. aureus* (Lennarz et al., 1967; Nesbitt & Lennarz, 1968). The $K_m$ values for PG and lysyl-tRNA were 56 $\mu$M and 6-9 $\mu$M, respectively, corresponding to those from *S. aureus* (Lennarz et al., 1967; Nesbitt & Lennarz, 1968).

We examined whether non-denaturing detergents could solubilize functional MprF protein from the membrane fraction. When the membrane fraction from *E. coli* cells expressing MprF protein was treated with 0-5% Triton X-100 and centrifuged, a significant amount of the LPG synthetase activity was recovered in the supernatant fraction (data not shown). This result demonstrated that MprF protein could be solubilized by non-denaturing detergent and therefore supported a membrane localization of MprF protein.

**Conclusion**

The present study examined the LPG synthetase activity of the *S. aureus mprF* gene product. The membrane fraction

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**Fig. 4.** Co-induction by arabinose of a 93 kDa protein and LPG synthetase activity in the membrane fraction of *E. coli* cells harbouring pBADmprF. Membrane fractions were prepared from *E. coli* JM109 harbouring either pBAD24 (vector, ■, □) or pBADmprF (mprF, ●, ○), which were grown to an OD$_{600}$ value of 0.5 and further incubated for 2 h in the presence (■, ●) or absence (□, ○) of 1% arabinose. (a) Analysis of the membrane fractions (40 $\mu$g protein) by SDS-polyacrylamide (7.5%) gel electrophoresis with Coomassie Brilliant Blue R-250 staining. The arrow indicates an approximately 93 kDa protein, which is consistent with the predicted molecular mass of *S. aureus* MprF protein. The migration positions and sizes (in kDa) of molecular size markers are shown on the left. (b) In vitro LPG synthetase activity of each membrane fraction. Assays were performed as described in the legend for Fig. 1. Results are representative of three independently performed experiments.

**Fig. 5.** Lysyl-tRNA- and phosphatidylglycerol-dependent synthesis of LPG in vitro by the membrane fraction prepared from *E. coli* JM109 cells expressing the *S. aureus* MprF protein. In vitro LPG synthetase activity of the membrane fraction (10 $\mu$g) prepared from these cells was assayed as described in the legend for Fig. 1 except for the $[^3H]$lysyl-tRNA concentration of 0, 5, 10, 20 or 40 $\mu$M in (a) or except for the phosphatidylglycerol concentration of 0, 0-02, 0-04, 0-09, 0-19, 0-39, 0-77 or 1.55 mM in (b). Results are representative of three independently performed experiments.
prepared from the *S. aureus mprF* deletion mutant cells had lost LPG synthetase activity (Fig. 1). *E. coli* cells that expressed the *S. aureus* MprF protein synthesized LPG (Figs 2 and 3). Co-induction of a 93 kDa protein whose molecular size corresponds to that of MprF protein and LPG synthetase activity was demonstrated in the membrane fraction prepared from the *E. coli* cells in which MprF protein was expressed (Fig. 4). The enzyme reaction mediated by this *E. coli* cell membrane fraction was dependent on both PG and lysyl-tRNA, consistent with that from *S. aureus* cells. *Kₚ* values obtained from the *E. coli* membrane fraction for PG and lysyl-tRNA were consistent with the values of the LPG synthetase activity from *S. aureus* cells. These results suggest that MprF protein functions as the LPG synthetase in the *S. aureus* cell membrane.

Although this study strongly suggests that MprF protein is LPG synthetase, we cannot deny the possibility that there is a polypeptide(s) other than the MprF protein that is essential for the enzyme activity. To exclude this possibility, purification and characterization of the LPG synthetase must be performed. LPG is suggested to be involved in resistance against cationic antimicrobial peptides (Peschel et al., 2001; Kristian et al., 2003), cationic antimicrobial peptide-like bacteriocins (Staubitz & Peschel, 2002), or antibiotics (Ruzin et al., 2003), and to have a role in cell cycle regulation (Ichihashi et al., 2003). The cellular content of LPG varies depending on the growth phase and culture conditions (Houtsmuller & van Deenen, 1965; Kenward et al., 1979; Ichihashi et al., 2003). To understand the roles of LPG in various aspects of cell physiology, it is critical to elucidate the mechanism that controls the membrane LPG content. LPG localization in cells is an important factor in the regulation of LPG function. The *E. coli* strain expressing MprF protein constructed in the present study will serve as a useful tool for further research on this basic phospholipid.

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