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Phosphatidylcholine is Required for the Efficient Formation of Photosynthetic Membrane and B800-850 Light-Harvesting Complex in *Rhodobacter sphaeroides*

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Abstract No phosphatidylcholine (PC) was detected in the membrane of *Rhodobacter sphaeroides pmtA* mutant (PmtA1) lacking phosphatidylethanolamine (PE) *N*-methyltransferase, whereas PE in the mutant was increased up to the mole % comparable to the combined level of PE and PC of wild type. Neither the fatty acid composition nor the fluidity of membrane was altered by *pmtA* mutation. Consistently, aerobic and photoheterotrophic growth of PmtA1 were not different from wild type. However, PmtA1 showed an extended lag phase (15 h) after the growth transition from aerobic to photoheterotrophic conditions, indicating the PC requirement for the efficient formation of intracytoplasmic membrane (ICM). Interestingly, the B800-850 complex of PmtA1 was decreased more than twofold in comparison with wild type, whereas the level of the B875 complex comprising the fixed photosynthetic unit was not changed. Since *puc* expression is not affected by *pmtA* mutation, PC appears to be required for the proper formation of the B800-850 complex in the ICM of *R. sphaeroides*.

Key words: Phosphatidylcholine, photosynthetic complexes, *Rhodobacter sphaeroides*

Rhodobacter sphaeroides is a purple nonsulfur photosynthetic bacterium that has been used to study bacterial photosynthesis and the development of intracytoplasmic membrane (ICM) that houses all the photosynthetic complexes. Transcriptional regulations leading to the formation of ICM upon lowering the partial pressure of oxygen (pO_2) have been studied extensively (for a review, see [20]). Although the expression and function of the proteins comprising the photosynthetic complexes, which include B800-850 light-harvesting (LH) complex, B875 LH complex, and reaction center (RC) complex, have been extensively studied, the role of specific phospholipids in the formation of spectral complexes and ICM have received only limited attention. The major phospholipids found in *R. sphaeroides* membrane include phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), and sulfolipids (SL) [2]. Although PC is the major membrane-forming phospholipid in most eukaryotes, it is not present in many Gram-negative bacteria such as *Escherichia coli*. PC is found in only a small but increasing number of bacteria where PE serves as the major membrane phospholipid [19, 26].

When the steady-state level of phospholipids was altered in *E. coli*, various physiological phenomena including solute transport and protein translocation across the membrane, electron transfer in the membrane, and the initiation of DNA replication were significantly affected, implying the association of many important cellular processes with specific functions of phospholipids [22]. However, no such apparent phenotype was found by the mutations in either SL or PC synthesis of *R. sphaeroides* [2, 4]. Most phospholipid synthesis of *R. sphaeroides*, which rapidly ceased during the lag period after a non-gratuitous shift from aerobic to photoheterotrophic conditions, did not resume until the initiation of photoheterotrophic growth thereafter [5]. However, concomitant with the ICM formation through the lag period is PC synthesis [5], suggesting the important role of PC in ICM formation. In an effort to elucidate the role of PC in photosynthetic membrane development, we interrupted the *pmtA* coding for PE *N*-methyltransferase, and examined the mutation effect on cell growth and the formation of photosynthetic complexes. The results in this work clearly demonstrate that PC appears to be required for the efficient formation of ICM and the proper assembly of the B800-850 complex in *R. sphaeroides*.

R. sphaeroides 2.4.1, which was used as a wild-type strain, was grown at 28°C in Sistrom's succinate-based (Sis) minimal medium [25], as described previously [3, 7]. Cells were grown aerobically by shaking (250 rpm) on a

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gyratory shaker or by sparging (100 ml/min per 100-ml culture) with a defined gas mixture of 20% O_2, 79% N_2, and 1% CO_2. Cells were grown photoheterotrophically in completely filled vessels or by sparging a liquid culture with a gas mixture of 95% N_2 and 5% CO_2. Cell growth was monitored with a Klett-Summerson colorimeter (Manostat) equipped with a KS-66 filter. *E. coli* was grown at 37°C in Luria-Bertani (LB) medium. Antibiotics for *R. sphaeroides* and *E. coli* cultures were added as indicated previously [10]. For growth transition from aerobic to photoheterotrophic conditions, cells were first grown aerobically in Sis medium up to logarithmic growth phase (70–80 Klett units [KU]). An aliquot (0.5 ml) was then harvested and inoculated into a fresh Sis medium (KU up to 5.0), followed by photoheterotrophic growth.

For spectrophotometric assay of spectral complexes, the membrane fraction of *R. sphaeroides* grown exponentially under photoheterotrophic conditions was prepared as described previously [12, 28]. The amount of B800-850 LH complex was determined from the spectral data by using $A_{849\text{-}900}$ with an extinction coefficient (ε) of 96 mM^{-1} cm^{-1}, normalized for three molecules of bacteriochlorophyll *a* per complex, whereas the amount of B875 complex was measured from $A_{878\text{-}820}$ with ε of 73 mM^{-1} cm^{-1}, normalized for two molecules of bacteriochlorophyll *a* per complex [18].

The nucleotide sequence of *R. sphaeroides* 2.4.1 was obtained from the genome site at http://mmg.uth.tmc.edu/sphaeroides/, and an 853-bp DNA fragment containing *pmtA* was PCR amplified using PMT-U (5'-CCA TCG TGA CCT ACC TGC-3') and PMT-D (5'-CCT GCT GAA GAT CGT GGC-3') primers. The BalI site (Fig. 1) within the PCR-amplified DNA was inserted with a 2.0-kb transcription and translation stop Ω (Sm^r/Sp^r) DNA [21], and the resulting DNA fragment was cloned into the suicide plasmid pLO1 [8, 14] to generate pLO-pmtA. The recombinant plasmid was mobilized from *E. coli* S17-1 into *R. sphaeroides* by conjugation as previously described [6]. The Km^r exconjugant, which was generated from a single crossover, was isolated and subjected to segregation to double crossover (PmtA1: Km^s and Sm^r/Sp^r; Fig. 1) on the Sis agar plate supplemented with Sm/Sp and sucrose (20%). The genomic structures of the mutants were confirmed by Southern hybridization analysis [24].

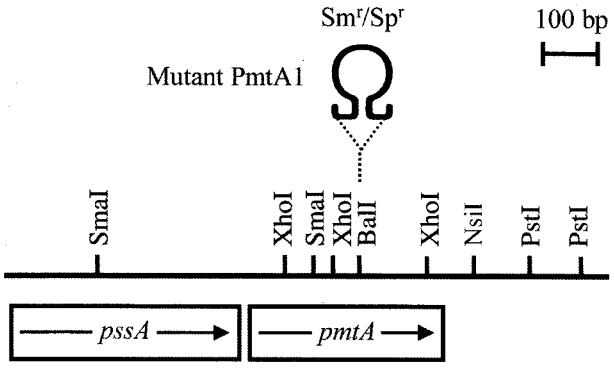

Fig. 1. *pmtA* mutant, PmtA1, of *R. sphaeroides*.
pmtA coding for PE *N*-methyltransferase was interrupted with a 2.0-kb transcription and translation stop Ω (Sm^r/Sp^r) DNA. *pssA* coding for phosphatidylserine synthase is also shown.

pCF200Km [14], a plasmid harboring a transcriptional fusion construct of *puc::lacZ*, was mobilized into *R. sphaeroides*, and the β-galactosidase activities (Miller units) of the cells grown exponentially were determined as described previously [11, 27].

Lipid analysis of membrane was performed as described previously [4]. Cells were extracted with chloroform-methanol (1:1, vol/vol), followed by addition of 1 M KCl-0.2 M H_3PO_4 for phase partitioning of the lipids into the chloroform phase. Lipids were concentrated by evaporation under a stream of N_2, and then spotted onto activated silica thin-layer chromatography (TLC) plates (Si250, Baker). The plates were developed in two dimensions, first with chloroform-methanol-water (65:25:4, by volume) and then with chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by volume). Separated lipid spots were visualized with iodine vapor, and then scraped into screw-cap tubes. Pentadecanoic acid was added to the samples as an internal standard. Fatty acid methyl esters (FAMEs) were prepared after 1-h incubation with 1 M methanolic HCl at 79°C [25]. Resulting FAMEs were analyzed with gas chromatography (GC-17A, Shimadzu), which was equipped with a capillary column (30 m×0.25 mm) coated with polyethylene glycol (VB-WAX, VICI), and a flame ionization detector.

Membrane fluidity was measured using a spectrofluorometer (QM-4, Photon Technology International) equipped with a temperature-controlled cuvette holder and two polarizers set in either a vertical or a horizontal position, as described previously [1, 29]. Cells from logarithmic-phase culture were harvested and then resuspended in 50 mM Tris-HCl buffer (pH 7.0) to an A_{600} of 0.4. Fluorescence anisotropy was measured at 28°C followed by incubation for 10 min with 4×10^{-6} M 1,6-diphenyl-1,3,5-hexatriene (DPH), which was used as a lipophilic probe to determine membrane dynamics. Excitation and emission wavelengths were set at 358 and 428 nm, respectively. The samples were excited with vertically polarized light, while vertical and horizontal emission intensities were recorded. Results are expressed as fluorescence anisotropy (A, unitless), which is defined as the ratio of polarized components to the total intensity by the equation $A=I_{II}-I_{\perp}/I_{II}+2I_{\perp}$, where I_{II} and $I_{\perp}$ are the fluorescence intensities parallel and perpendicular to the direction of the excitation light beam, respectively.

Total membrane lipids of *R. sphaeroides* were isolated and analyzed by TLC. No significant difference in lipid composition between wild-type cells grown aerobically

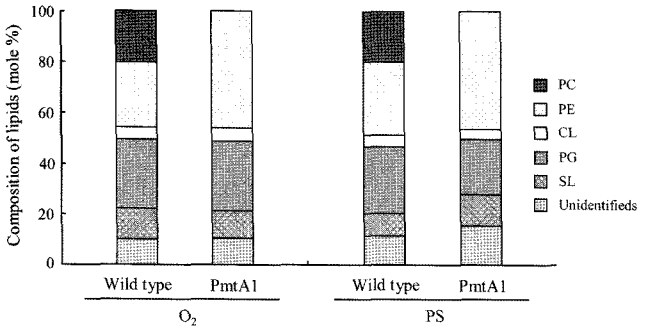

Fig. 2. Membrane lipid composition of wild type and PmtA1 grown aerobically (O_2) and photoheterotrophically (PS).
PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; PG, phosphatidylglycerol; and SL, sulfoquinovosyldiacylglycerol.

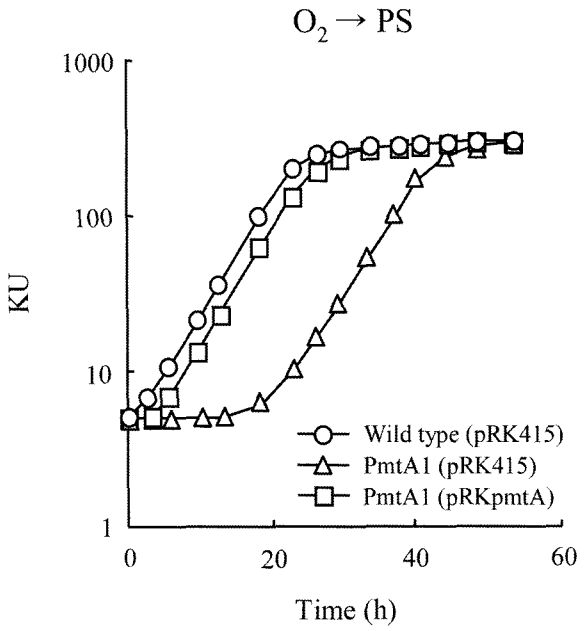

Fig. 3. Growth of wild type and PmtA1 after transition from aerobic to photoheterotrophic conditions.
Wild-type containing pRK415 [9, 15] (○), PmtA1 containing pRK415 (△), and PmtA1 containing pRKpmtA, a pRK415 harboring the *pmtA* gene as an insert (□).

and photoheterotrophically was observed (Fig. 2); PE was the most abundant, and PG, PC, SL, and cardiolipin (CL) were detected in a decreasing order (Fig. 2). No PC was present in the membrane of *R. sphaeroides* PmtA1. Instead, PE in the mutant was increased up to the mole % comparable to the combined level of PE and PC of wild type (Fig. 2). PC of *R. sphaeroides* is synthesized by the methylation pathway in which PE is sequentially methylated three times using a methyl donor *S*-adenosylmethionine (SAM) [2]. The methylation pathway, catalyzed by one or several phospholipid *N*-methyltransferases (pmt), is universally employed in both eukaryotes and prokaryotes. Although there is another pathway in which choline is condensed with CDP-diacylglyceride to form PC in a reaction catalyzed by PC synthase, it is not found in *R. sphaeroides* [17]. Thus, the lack of PE *N*-methyltransferase results in the accumulation of PE in the membrane (Fig. 2).

It was examined whether the lack of PC affects the composition of fatty acids. Total cellular fatty acids of wild type and PmtA1 were extracted with chloroform-methanol and subsequently methyl esterified with methanolic HCl. The resulting methyl esters of fatty acids were analyzed with gas chromatography. The fatty acid composition of PmtA1 was not different from that of wild type (Table 1), indicating that the cellular fatty acid composition was regulated irrespective of the relative level of PC and PE in the membrane of *R. sphaeroides*. The membrane fluidity of wild type and PmtA1 were measured at 28°C with a spectroflurometer after membrane labeling with DPH. The anisotropy value reflecting membrane fluidty was 0.505 for PmtA1 and 0.569 for wild type. Thus, the membrane fluidity was not significantly affected by the lack of PC.

The growth of PmtA1 under aerobic and photoheterotrophic conditions was not different from the wild type (data not shown). However, PmtA1 showed an extended lag phase of approximately 15 h after the growth transition from aerobic to photoheterotrophic conditions, whereas no such growth lag was observed with the wild type (Fig. 3). The extended lag by PmtA1 was abolished by complementation with the *pmtA* gene *in trans* (Fig. 3). It was previously shown that the synthesis of all the phospholipids except PC stopped during the lag after the same shift from aerobic to photoheterotrophic conditions [5]. Only PC synthesis resumed midway through the lag period and coincided with the

Table 1. Fatty acid composition of membrane lipids of wild type and PmtA1 grown under aerobic or photoheterotrophic conditions.

Strains	Fatty acid composition (mole %)				
	Palmitic acid	Palmitoleic acid	Stearic acid	*cis*-Vaccenic acid	Cyclopropane fatty acid[a]
Wild type O_2[b]	6.1±0.6	1.3±0.0	9.2±0.7	79.7±1.4	3.7±1.3
PmtA1 O_2	8.2±0.6	1.4±0.1	9.0±0.1	78.2±0.9	3.2±0.1
Wild type PS[c]	5.7±1.2	1.5±0.6	10.1±2.3	79.5±3.2	3.2±0.1
PmtA1 PS	7.6±0.1	1.0±0.1	12.1±0.5	75.9±0.8	3.3±0.3

[a]*cis*-11,12-Methyleneoctadecanoic acid.
[b]Cells grown aerobically.
[c]Cells grown photoheterotrophically (10 W/m^2).

Table 2. Specific level of LH complexes of wild type and PmtA1 grown photoheterotrophically at 10 W/m^2.

Strains	LH complex level (nmoles per mg of protein)	
	B800-850 complex	B875 complex
Wild type (pRK415)	15.1±0.5	9.6±0.3
PmtA1 (pRK415)	6.7±0.3	9.5±0.5
PmtA1 (pRKpmtA[a])	14.5±0.4	9.8±0.3

[a]pRK415 harboring the *pmtA* gene as an insert.

ICM formation, suggesting the important role of PC in ICM formation. The extension of lag by *pmtA* mutation further corroborates the requirement of PC for the efficient invagination of cytoplasmic membrane, which leads to the formation of ICM. The results are consistent with known differences in membrane-forming properties between PC and PE. Vesicles are easily formed with PC, whereas a bilayer to non-bilayer physical transition is observed with PE, since PE has a smaller head group than PC [26]. Thus, PC appears to be required for the efficient formation of ICM.

It was examined whether the levels of photosynthetic complexes are affected by *pmtA* mutation, although no difference in growth rate was observed between the wild type and PmtA1 during exponential growth under photoheterotrophic conditions. The B800-850 complex of PmtA1 was decreased more than twofold in comparison with the wild type, whereas the level of B875 complex comprising the fixed photosynthetic unit (B875 complex and RC complex) was not changed (Table 2). The reduced level of the B800-850 complex of PmtA1 was restored to the wild-type level after complementation with the *pmtA* gene (Table 2). The transcriptional expression of *pucBA* coding for structural polypeptides of B800-850 β and α was examined after mobilization of pCF200Km [14], a plasmid harboring *puc::lacZ* transcriptional fusion construct, into the wild type and PmtA1. The β-galactosidase activities from *puc::lacZ* in the wild type was not different from that measured in PmtA1 (Table 3). Thus, PC is required for the posttranscriptional expression of the *puc* operon, possibly at the level of B800-850 complex formation of *R. sphaeroides*. Although the specific role of PC in the formation of the B800-850 complex remains to be determined, the phospholipids composition

Table 3. β-Galactosidase activity from *puc::lacZ* transcriptional fusion construct pCF200 Km in wild type and PmtA1.

Strains	β-Galactosidase activity (Miller units)	
	O_2[a]	PS[b]
Wild type	505±33	3,952±136
PmtA1	549±16	4,078±121

[a]Cells grown aerobically.
[b]Cells grown photoheterotrophically (10 W/m^2).

of the B800-850 complex from *Rhodopseudomonas acidophila* revealed two major phospholipids of PC (42%) and PE (43%) in addition to CL (15%) [23]. Accordingly, PC appears to be required for the proper assembly of the LH complex in purple nonsulfur photosynthetic bacteria.

Taken together, PC is required not only for the efficient membrane invagination forming ICM but also for the proper formation of the B800-850 complex therein.

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