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Requirement of phosphatidylglycerol for flagellation of *Escherichia coli*

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We report that phosphatidylglycerol is required for flagellation of *Escherichia coli*. Cells carrying the *pgsA3* mutation did not form swarm rings in semisolid agar. P1 transduction experiments revealed that the potential for phosphatidylglycerol synthesis and for the formation of swarm rings was co-transducible. The *pgsA3* mutant transformed with the wild type *pgsA*$^+$ gene cloned into the R-plasmid vector had the potential for both phosphatidylglycerol synthesis and cell motility. Electronmicroscopic and SDS-PAGE analyses showed that the *pgsA3* mutation causes the lack of flagellation.

Phosphatidylglycerol; Bacterial motility; Flagellum; Flagellin; *Escherichia coli*

1. INTRODUCTION

Phosphatidylglycerol is an acidic phospholipid ubiquitously present in biological membranes; the content in total phospholipids of *Escherichia coli* membrane reaching approximately 20% [1,2]. In a genetic background lacking Braun's lipoprotein (*lpp*$^-$), cells harboring the *pgsA3* allele that encodes a defective phosphatidylglycerophosphate synthase grow normally, despite a drastic reduction in the contents of phosphatidylglycerol (about 4% of the wild type strain) [3,4]. Miller and Koshland reported that factors altering the physical state of membrane lipids did not affect bacterial motility [5]. Here we report evidence that the *pgsA3* mutation resulted in the loss of flagellation.

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

The following *Escherichia coli* strains were used: JE5513Tc (*Hfr man-1 pps lpp-2 uvrC279::Tn10 pgsA*$^+$), YA5513Tc (JE5513Tc *pgsA3*) [3], CB64 (*trp75 cysB93 tfr8*) and CB64CLR (*cls::kan trp*$^+$) [6] were provided by Dr. I. Shibuya (Saitama University).

2.2. *Plasmids*

The *pgsA* gene was subjected to polymerase chain reaction (PCR) on pPG2 (pSC101 plasmid containing the *pgsA* gene) [7] as a template, *Eco*RI and *Hin*dIII linkers were added, and inserted into the *Eco*RI–*Hin*dIII fragment of mini-R plasmid vector (*Cm*^{r}) [8]. The constructed plasmid was named as pAT105.

2.3. *Motility assay in a swarm plate*

Formation of swarm rings was observed in semi-solid agar containing 1% tryptone, 0.5% NaCl, 50 μg/ml thymine, and 0.3% agar [9]. Each bacterial strain was cultured until the stationary phase in LB medium supplemented with 50 μg/ml thymine. Aliquots (2 ml) of full growth suspension were placed on the swarm plate, and incubated at 28°C for 15 h.

2.4. *P1 transduction*

P1 phages were grown in YA5513Tc (*Hfr man-1 pps lpp-2 uvrC279::Tn10 pgsA3*), and transduced into JE5513 (*Hfr man-1 pps lpp-2 pgsA*$^+$). Transductants were selected on agar plates containing 12.5 μg/ml tetracycline and the potential for phosphatidylglycerol synthesis and for cell motility was examined.

2.5. *Electron microscopic observation of flagella*

Bacterial cells were incubated in L medium (1% tryptone, 0.5% NaCl) supplemented with 50 μg/ml thymine at 28°C. Exponentially growing cells were fixed with 5% formaldehyde for 10 h at 4°C, stained with 1% phosphotungstic acid, and observed under an electron microscope (JEM, 1200EX) at a magnification of 12,000 (60,000 for insets).

2.6. *SDS-PAGE of flagella*

Bacterial cells were incubated in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 50 μg/ml thymine at 28°C until the OD_{600} value reached 1.0, then the cells were collected by centrifugation at 5,000 rpm for 5 min, suspended in 100 μl of 0.9% NaCl. Flagella was released from the cells by vigorous vortex mixing for 10 min [10]. Cells were removed by centrifugation twice at 5000 rpm for 3 min, and half a volume of SDS sample buffer (150 mM Tris-HCl, pH 6.8, 6% SDS, 2% 2-mercaptoethanol, 30% glycerol, and 0.04% Bromphenol blue) was added to the supernatant. The samples (45 μl) were heated at 100°C for 2 min, and loaded onto an SDS-polyacrylamide slab gel containing 8% polyacrylamide [11].

3. RESULTS AND DISCUSSION

3.1. *Defect in motility in a pgsA3 mutant*

Bacterial motility was assayed in swarm plates with semi-solid agar according to the method described by Armstrong et al. [9]. We found that motility was drastically decreased in the *pgsA3* mutant, YA5513Tc [3], compared to the isogenic wild type strain, JE5513Tc (Fig. 1). Under the light microscope, the motility of the *pgsA3* mutant was much less than that of the wild type.

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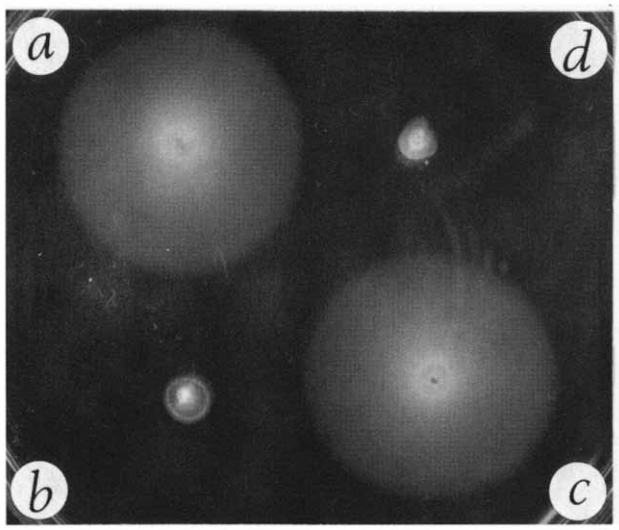

Fig. 1. Comparison of motility between the wild type and the *pgsA3* mutant in a swarm plate. (a) JE5513Tc (*Hfr man-1 pps lpp-2 uvrC279::Tn10 pgsA*$^+$), (b) YA5513Tc (JE5513Tc *pgsA3*); (c) YA5513Tc transformed with pAT105 (*pgsA*$^+$ *Cm*^{r}); (d) YA5513Tc transformed with mini-R plasmid vector (*Cm*^{r})

These observations imply that phosphatidylglycerol is required for bacterial motility. Cardiolipin, another major acidic phospholipid in *Escherichia coli*, is synthesized from phosphatidylglycerol with cardiolipin synthase. Cells deficient in the cardiolipin synthase gene (*cls*) grow normally, despite the very low content of cardiolipin [6]. We found that the mutant cells CB64CLR (*cls::kan trp*$^+$) formed a swarm ring (data not shown), as seen in the isogenic parent CB64 (*trp75 cysB93 tfr8*), thereby suggesting that cardiolipin is not essential for bacterial motility.

3.2. *Co-transduction of phosphoatidylglycerol synthesis and motility*

To determine whether the *pgsA3* mutation would explain the decrease in motility of YA5513Tc, we first tested the co-transducibility of phosphatidylglycerol synthesis and motility (Table I). A P1 lysate prepared on YA5513Tc, a *uvrC::Tn10 pgsA3* mutant, was used to transduce a tetracycline-sensitive *pgsA*$^+$ strain, JE5513 [12]. The 112 tetracycline-resistent transductants had lost both the potential for phosphatidylglycerol synthesis and for motility (107 transductants), or retained the potential for phosphatidylglycerol synthesis and for motility (5 transductants). A transductant with the ability for phosphatidylglycerol synthesis without motility, or with motility but without ability for phosphatidylglycerol synthesis was not obtained. This observation strongly suggests that the decrease in motility in YA5513Tc was due to the *pgsA3* mutation.

3.3. *Recovery of motility by plasmid complementation*

To confirm the requirement of the *pgsA* gene for motility, we examined whether or not the wild type *pgsA* gene cloned on a mini-R plasmid would recover motility of the *pgsA3* mutant. The *pgsA* gene (from guanine residue 26 bases upstream from the potential promoter for the *pgsA* gene to adenine residue at 11 bases downstream from the last codon of the *pgsA* gene [13]) was amplified and added to *Hin*dIII and *Eco*RI linkers by polymerase chain reaction (PCR) from pPG2 [7], a pSC101 plasmid harboring the wild type *pgsA* gene, and cloned between the *Hin*dIII and *Eco*RI sites of a low copy mini-R vector pKP1673 [8]. The *pgsA3* mutant YA5513Tc harboring the resultant plasmid pAT105 recovered both the potential for phosphatidylglycerol synthesis and for motility, while YA5513Tc harboring the miniR vector, pKP1673, did not (Fig. 1). Thus we concluded that the functioning of the *pgsA* gene is required for motility.

3.4. *Lack of flagellation in the pgsA3 mutant*

To determine whether a decrease in motility in the *pgsA3* mutant was due to loss of flagella formation or to loss of flagella function, we examined these strains under the electron microscope; the flagella in the *pgsA3* mutant were poorly developed (Fig. 2). A number of cell populations in both strains had fimbriae [14] on their cell surfaces and structures were indistinguishable between the *pgsA* mutants and the wild type (Fig. 2, insets).

Flagella of *Escherichia coli* are a polymerized form of flagellin subunits. We searched for the presence of flagella in the *pgsA3* mutant, using SDS-PAGE. Flagella can be easily removed from cells by vigorous vortex mixing [10]. A major band with a molecular mass of 63 kDa was observed in JE5513Tc (*pgsA*$^+$) (Fig. 3a, lane 1), while the protein was not present in YA5513Tc (*pgsA3*) (Fig. 3a, lane 2). We recovered the protein from the gel and determined the amino acid sequence of the N-terminal of this protein, using an automatic protein sequencer (Applied Biosystems 473A). The sequence of 10 residues completely matched that of flagellin [15].

Table I

Transduction potential for phosphatidylglycerol synthesis and for cell motility in P1 transduction

Production of phosphatidylglycerol[a]	Cell motility[b]	Number of transductants	(%)
+	+	5	(4.5)
+	−	0	(0)
−	+	0	(0)
−	−	107	(95.5)

[a] Total phospholipids were extracted by the method described by Ames [1] and analyzed by thin layer chromatography developed with chloroform/methanol/acetic acid (55:25.10). Phospholipids on the plates were stained with iodine and the presence of phosphatidylglycerol was determined.

[b] Cell motility was determined by a swarm plate assay [9].

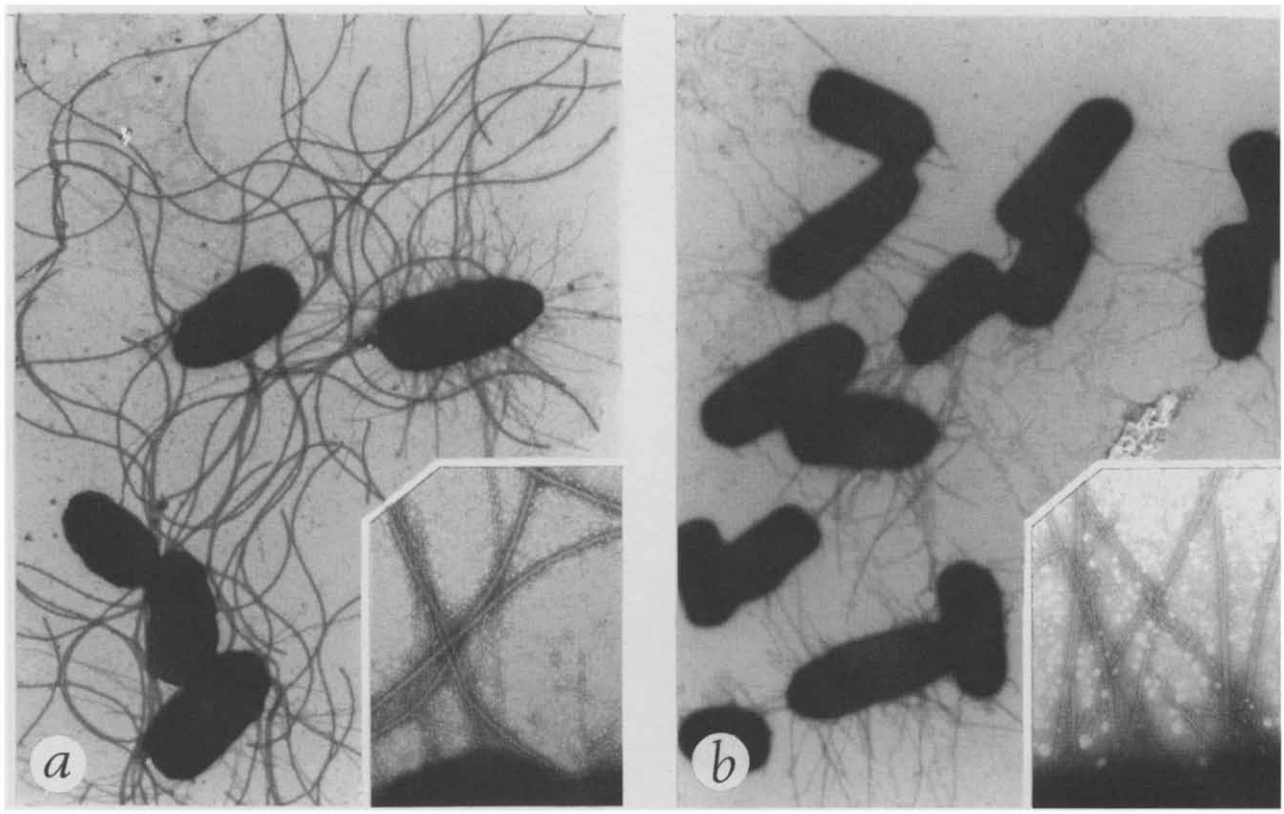

Fig. 2. Electron microscopic observations of flagella. Bacterial cells were stained with phosphotungstic acid, and observed under an electron microscope at a magnification of 12,000 (60,000 for insets). (a) JE5513Tc (*pgsA*$^+$); (b) YA5513Tc (JE5513Tc *pgsA3*).

Furthermore, the protein band appeared in the flagella fraction when YA5513Tc was transformed with pAT105, R plasmid containing the wild type *pgsA* gene (Fig. 3a, lane 3), whereas the protein did not appear when the vector was transformed (Fig. 3(a), lane 4). These observations strongly suggest that phosphatidylglycerol synthesis is necessary for flagella formation in *Escherichia coli*. Analysis of total cell proteins by SDS-PAGE showed that flagellin did not accumulate in the *pgsA3* mutant (Fig. 3b). Therefore, the synthesis of

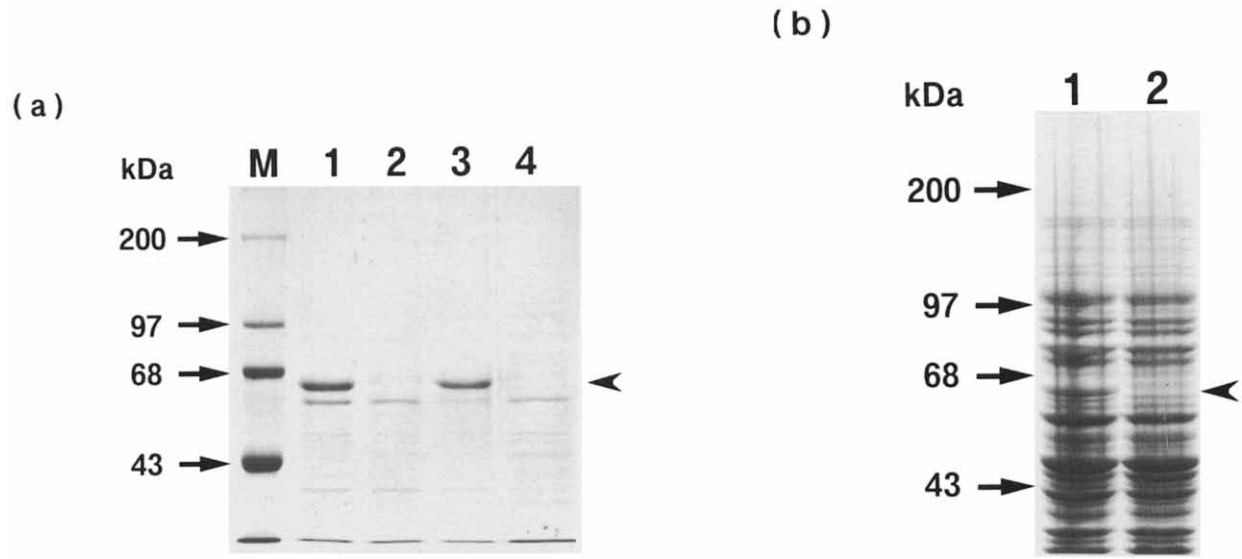

Fig. 3. SDS-PAGE of the flagella fraction (a) and total proteins (b). Lanes M, molecular mass standards, myosin (200kDa), phosphorylase b (97kDa), bovine serum albumin (68kDa), and ovalbumin (43kDa); 1, JE5513Tc (*pgsA*$^+$); 2, YA5513Tc (*pgsA3*); 3, YA5513Tc transformed with pAT105 (*pgsA*$^+$ *Cm*^{r}); 4, YA5513Tc transformed with mini-R plasmid vector (*Cm*^{r}). The positions of flagellin (63kDa) are shown by arrowheads.

flagellin is presumably blocked in the *pgsA3* mutant. The possibility that flagellin is rapidly degraded in the *pgsA3* mutant would need to be ruled out.

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