JOURNAL OF BACTERIOLOGY, May 1995, p. 2926–2928 Vol. 177, No. 10
0021-9193/95/$04.00+0

Phosphatidylinositol Cannot Substitute for Phosphatidylglycerol in Supporting Cell Growth of *Escherichia coli*

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Received 24 October 1994/Accepted 12 February 1995

Escherichia coli **cells are dependent on the anionic phospholipid phosphatidylglycerol for cell growth. Introduction into cells of the ability to make phosphatidylinositol did not suppress the need for naturally occurring phosphatidylglycerol. Therefore, the requirement for phosphatidylglycerol must be more than maintenance of the proper membrane surface charge.**

Phospholipids are essential components of cell membranes defining the cell barrier, providing a matrix for membrane-associated processes, supplying precursors to other biosynthetic pathways, and acting as regulatory and effector molecules. *Escherichia coli* phospholipids are made up primarily of zwitterionic phosphatidylethanolamine (70%), anionic phosphatidylglycerol (PG; 20%), and anionic cardiolipin (CL; 5%) (18). Strains (HD30 and HL30) carrying a null allele of the *pgsA* gene (encoding phosphatidylglycerophosphate synthase, the committed step to synthesis of the major anionic phospholipids) are dependent on a plasmid-borne functional copy of the gene for growth. Curing such null mutants of the plasmid (pHD102, temperature sensitive for replication) at the restrictive temperature results in arrest of cell growth when the PG and CL contents drop to limiting levels of about 4 and 1%, respectively (5). Cell viability is unaffected at the above limiting levels of these two anionic phospholipids, as evidenced by the reversible arrest of cell growth dependent on the expression of the *pgsA* gene in strains in which the gene is under the regulation of *lacOP* (6). The limiting anionic phospholipid appears to be PG, since null alleles of the *cls* gene (encodes CL synthase) contain less than 0.1% CL (with compensating elevated levels of PG) and remain fully viable (17).

PG is a precursor for the final maturation of the periplasmically localized membrane-derived oligosaccharides (10) and the major outer membrane lipoprotein (20); however, none of these molecules appears to be absolutely essential to *E. coli*. Anionic phospholipids are also required for the SecA protein-dependent translocation of proteins across the inner membrane (2, 7, 12, 13) and appear to be required for the DnaA protein-dependent initiation of DNA replication (9, 22, 23). The latter two roles for PG are essential, but in vitro, almost any anionic phospholipid, including phosphatidylinositol (PI), which is not found in *E. coli*, can substitute for PG while zwitterionic phospholipids, such as phosphatidylcholine (not found in *E. coli*) and phosphatidylethanolamine, cannot.

The question posed by this report is whether the in vivo requirement for PG in supporting cell growth is specific to the PG molecule or is only a general requirement for anionic membrane surface charge normally provided by PG and/or CL. To answer this question, the yeast *PIS* gene, which encodes PI synthase and has been shown to effect the synthesis of PI by *E. coli* cells grown in the presence of *myo*-inositol (15, 16), was introduced into *pgsA* mutant strains to suppress the need for a functional *pgsA* gene.

E. coli cells were grown either in Luria broth (LB) liquid medium or on LB agar plates at either 30 or 42°C as the permissive (retention of plasmid pHD102) or restrictive (loss of plasmid pHD102) condition, respectively. Strains HD30 (*pgsA30*::*kan*), HD631 (*pgsA3*, point mutation), and HL30 (*pgsA30*::*kan lpp-2*) all require plasmid pHD102 for growth (5). Strain HL30 also carries the *lpp-2* allele, which is a suppressor of the point mutation (*pgsA3*) (1) but not the null allele (3). Plasmid pPIS18 was constructed by in-frame insertion of an *Eco*RI-*Hin*dIII fragment of the yeast *PIS* gene, which encodes PI synthase (4, 14, 15), into the *lacZ* gene in vector pUC18 (Amp^r). The N-terminal three amino acids of the new *PIS* gene product were replaced by the N-terminal five amino acids of β-galactosidase; such a construct has previously been reported to express functional PI synthase in *E. coli* (16). Plasmid pPIS18 was maintained in cells by the presence of 100 μg of ampicillin per ml in the growth medium.

Strain HD30/pHD102 with and without plasmid pPIS18 was grown in 3 ml of LB medium containing 15 μCi of *myo*-[2-^{3}H]inositol (20 μCi/nmol; Amersham) at 30°C to the stationary phase. Lipids were extracted in the presence of unlabeled authentic PI (Sigma Chemical Co.) as the carrier and separated by two-dimensional thin-layer chromatography (5). The areas corresponding to authentic PI (detected by I_2 vapor) were collected and counted with a liquid scintillation counter. No radiolabel (97 dpm, corresponding to the background) was found in the PI area derived from cells lacking plasmid pPIS18, while this area from cells carrying plasmid pPIS18 contained 34,000 dpm. Therefore, *E. coli* cells carrying the yeast *PIS* gene and grown in the presence of *myo*-inositol were able to synthesize authentic PI.

The phospholipid composition of cells was determined after growth in LB medium containing 5 μCi of $^{32}PO_4$ (Amersham) per ml for at least five generations to attain uniform labeling of the phospholipid pool (5); distribution of radiolabel in the extracted phospholipids after thin-layer chromatography was determined with a Betagen to quantify the radiolabel. With added *myo*-inositol (1.1 mM) in the growth medium, cells of strain HD30 (grown at 30°C) carrying the *PIS* gene contained about 20% of their phospholipid as PI (Fig. 1 and Table 1). Growth without *myo*-inositol supplementation still resulted in 3% of the phospholipid as PI, presumably derived from the low levels of *myo*-inositol in LB medium. However, HD30 cells lacking the *PIS* gene did not synthesize detectable levels of PI under either growth condition. HD30 cells carrying the *PIS*

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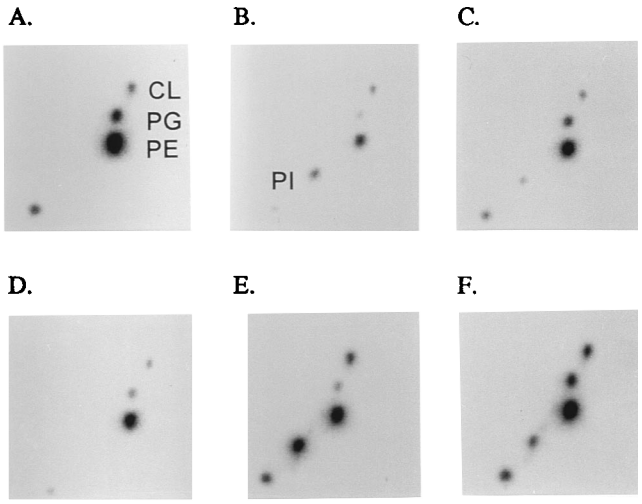

FIG. 1. Distribution of phospholipids in strains carrying plasmid pPIS18. Shown are images from scans (Betascope in the log setting) of thin-layer chromatograms of ^{32}P-labeled phospholipids extracted from strain HD30/pHD102 grown under the following conditions in LB medium either with or without plasmid pPIS18: A, minus plasmid pPIS18, 30°C, plus inositol; B, plasmid pPIS18, 30°C, plus inositol; C, plasmid pPIS18, 30°C, minus inositol; D, minus plasmid pPIS18, 42°C, plus inositol; E, plasmid pPIS18, 42°C, plus inositol; F, plasmid pPIS18, 42°C, minus inositol. PE, phosphatidylethanolamine.

gene and grown at 42°C for 4 h contained levels of PI similar to those of the cells grown at 30°C.

Introduction of plasmid pPIS18 into strains HL30 and HD631 also resulted in synthesis of PI (Table 1). Growth of all three mutant strains at 42°C resulted in the expected reduction of PG levels, while PI levels reached about 20% or more of total phospholipid. Even growth at 30°C resulted in a significant reduction in PG levels from that expected under permissive conditions (5) which had been noted before in cells synthesizing PI (16). This reduction may be due to increased competition for the CDP-diacylglycerol pool which is the common substrate for the *pssA*, *pgsA*, and *PIS* gene products. In strains carrying the *PIS* gene, CL levels increased, especially with growth at 42°C, most likely because of the turnover of residual PG to CL which occurs as cells reach the stationary phase (8).

Although sufficient PI was made under restrictive growth conditions by each of the *pgsA* mutant strains (Table 1) to substitute for PG on a molar basis, these strains carrying the *PIS* gene could not form colonies when plated at 42°C on LB plates containing *myo*-inositol. The presence of high levels of PI in these strains also did not alter the number of generations before cell arrest occurred in liquid culture after a shift to growth at 42°C (data not shown). Therefore, these strains were still dependent on a functional *pgsA* gene (plasmid pHD102) for viability, and high levels of PI could not substitute for the loss of PG biosynthetic capability.

TABLE 1. Phospholipid compositions of *PIS*-containing strains

Strain	Composition (%)[a]							
	30°C[b]				42°C[c]			
	PE	PG	CL	PI	PE	PG	CL	PI
HD30	82	13	5	0	90	5	2	0
HD30/pPIS18	60	6	11	23	58	2	12	28
HL30/pPIS18	68	13	5	14	67	5	7	21
HD631/pPIS18	77	12	3	8	63	5	10	22
Deviation	±1.9	±0.5	±1.0	±2.0	±0.4	±0.2	±1.2	±0.5

[a] Determined as described in the legend to Fig. 1 after growth in LB medium containing *myo*-inositol. PE, phosphatidylethanolamine.
[b] Containing plasmid pHD102.
[c] Cured of plasmid pHD102 by growth at 42°C for 4 h before the lipids were extracted. The HD30 cells without plasmid pPIS18 also contained 3% phosphatidic acid.

This study provides direct in vivo evidence that an anionic phospholipid environment in the membrane of *E. coli* is not in itself sufficient to replace the requirement for PG in sustaining cell growth. The total anionic phospholipid pool either remains the same or increases with the loss of PG biosynthetic capability by growth at 42°C in cells carrying the *PIS* gene (Table 1). It is unlikely that the high levels of PI were toxic to cells, since similar levels of PI do not affect the growth of the cells at 30°C. Since the majority of the PG is associated with the inner membrane of *E. coli* (19), most of the critical functions of PG probably reside within this membrane. Certainly, the suppressive effect (1) on "leaky" PG synthesis mutants of lack of the major lipoprotein of *E. coli* (utilizes PG from the inner membrane during maturation [20]), the bypass (22, 23) of the need for PG for initiation of DNA replication (specifically, the need for the DnaA protein), and the involvement of PG (12, 13) in forming the membrane-associated complex (specifically, with the SecA protein) necessary for translocation of proteins across the inner membrane are consistent with this point. A common theme in the latter two functions for PG is in providing an organizational site on the inner surface of the inner membrane for cytoplasmic proteins (DnaA and SecA proteins) which hydrolyze ATP.

E. coli cells can tolerate large changes in both the anionic and zwitterionic phospholipid compositions of their membranes with only minor effects on cell viability and growth (18). However, these changes do have specific and dramatic effects on cellular functions and, in the extreme, do result in arrest of cell growth. Possibly, as suggested by model systems (11, 21), PG and/or CL act as proton donors and acceptors, forming proton conductance networks along the membrane surface which would be compromised at low levels of these anionic phospholipids; it is not known whether PI has similar properties. Since suppressors of only leaky mutants and not null mutants in PG synthesis have been found (1, 3, 22), a continuing supply of newly synthesized PG may be more critical than a steady-state level of PG or other anionic phospholipids. The properties of such suppressors also indicate that there may be a hierarchy in the critical levels of PG required for a variety of functions; bypassing the need for one essential function may uncover a lower critical level for other functions.

The results reported here continue to support a specific role for PG in critical functions of the cell which now appear to be more than simply providing an anionic character to the cell membrane. Since these requirements remain unidentified, continued isolation and characterization of suppressors of PG biosynthesis mutants should lead to the uncovering of new roles for PG in cell function.

This work was supported by National Institutes of Health Public Health Service grant GM20487.

We are grateful to S. Yamashita for providing us with a clone of the *PIS* gene.

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