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Phospholipid Composition and Phenotypic Correction of an *envC* Division Mutant of *Escherichia coli*

G. MICHEL, D. DI SAVINO, AND J. STARKA*

Laboratoire de Physiologie Microbienne, Centre Universitaire de Luminy, 13009 Marseille, France

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The cytoplasmic and outer membranes of a nonconditional chain-forming mutant, *Escherichia coli* PM61 *envC*, were separated by sucrose density gradient centrifugation. The phosphatidylglycerol/cardiolipin ratio in both membrane fractions was about one-third as high as in the parental strain P678. The increased level of cardiolipin in PM61 membranes is the result of an alteration of the polyglycerophosphatide cycle. It was found that the turnover rate of phosphatidylglycerol is more rapid in PM61 than in the parental strain but that its cardiolipin turnover is not significantly different. The *envC* mutation can be corrected phenotypically by increasing the osmolarity of the medium. In the presence of 0.6 M sucrose, the population of PM61 is composed of short rods, and the phosphatidylglycerol/cardiolipin ratio is shifted to that of the parent. The phosphatidylglycerol turns over more slowly, whereas the cardiolipin turns over more rapidly in both strains. Thus, the increase of external osmolarity acts on phospholipid metabolism as well as on an unknown step involved in the mechanism of cell division of the *envC* mutant.

It is generally admitted that the completion of a round of deoxyribonucleic acid (DNA) replication is a necessary condition for septum formation, although apparently other events are also involved. As pointed out by Pardee et al. (24), the link between DNA completion and septation should be perturbed by modification of metabolic activities other than those related directly to DNA synthesis. Several classes of *Escherichia coli* mutants that form minicells (1), or fail to form septa at a nonpermissive temperature (11), or produce chains during normal growth (19, 28) have been described. In all three classes septum formation is perturbed, but cell growth and DNA synthesis continue normally.

In our initial studies with *E. coli* PM61 (*envC*), a nonconditional chain-former of the third class of mutants, we found that the cells were more sensitive to detergents, antibiotics, and other antibacterial agents, and that their envelope contained relatively more cardiolipin (CL) and less phosphatidylglycerol (PG) than the parental strain P678 (*envC*$^+$) (27, 28). Moreover, the mutation mapping at 71 to 72 min (26) was phenotypically corrected when the cells were grown in a high-osmolarity medium containing 0.4 M sucrose (30). This mutant could be particularly useful for the analysis of the process of septation.

In the present study, we examine the phospholipid composition of the cell envelope of *E. coli* PM61 after separating the outer and cytoplasmic membrane fractions. It will be shown that the relative CL content in both membrane fractions of the mutant is about three times as high as in the parental strain. In phenotypically corrected PM61 cells, the CL content decreases. The turnover of PG, which under normal conditions is more rapid in PM61 than in the parent, slows down when osmolarity is increased.

MATERIALS AND METHODS

Bacteria, growth, and media. The characteristics and sources of *E. coli* P678 (*envC*$^+$) and PM61 (*envC*$^-$) have been described previously (26). Bacteria were grown at 37°C in a gyratory shaker on complete medium containing 5 g of tryptose (Difco Laboratories), 5 g of yeast extract (Difco), and 8 g of NaCl per liter. Absorbance was measured at 450 nm with a Spectronic 20 spectrophotometer. The cell number and cell size distributions were monitored with a Coulter counter (model Z 2, Coultronics, Margency, France) equipped with a 50-μm aperture probe.

Preparation of membrane fractions. The total membrane was prepared and fractionated into inner and outer membrane by the method of Osborn et al. (23). Cells were grown in 2,000 ml of complete medium supplemented with about 2 μCi of [^{32}P]phosphate per ml (obtained from the Radiochemical Centre, Amersham, United Kingdom) to an absorbance of 0.80. Harvested cells were suspended in 0.75 M sucrose (grade I, Sigma Chemical Co.) in 0.01 M tris(hydroxymethyl)aminomethane-

hydrochloride (pH 7.5), treated with lysozyme (700 μg/ml), and diluted by adding carefully 2 volumes of 0.0015 M ethylenediaminetetraacetic acid in 0.01 M tris(hydroxymethyl)aminomethane (pH 7.5). The resulting spheroplasts were disintegrated by sonic extraction with a Branson Sonifier B-12. After the intact cells were removed by centrifugation at 1,500 × *g* for 15 min, the total membrane was sedimented at 220,000 × *g* for 2 h and treated further as described by Osborn et al. (23). Sucrose density gradient centrifugation was carried out at 96,000 × *g* for 15 h with an SW25 rotor (Beckman Instruments, Inc.). Fractions (1 ml each) were collected with a coarse needle and peristaltic pump (Autodensiflow II, G. D. Searle and Co.).

Extraction of phospholipids. Total phospholipids were extracted from membrane fractions or from rapidly chilled cells by the method described previously (31). Dialyzed extracts were chromatographed on Silica Gel G (Merck and Co., Inc.) plates in one dimension (17). The spots were transferred quantitatively to scintillation vials and counted (31).

Quantitative determinations and enzyme assays. Protein content was determined by the method of Lowry et al. (15) with bovine serum albumin (fraction V, Sigma Chemical Co.) as the standard. Determination of 2-keto-3-deoxyoctonic acid (KDO) was carried out by the thiobarbituric acid method (22). Reduced nicotinamide adenine dinucleotide (NADH) oxidase activity was assayed as described by Osborn et al. (23).

Chase experiments. Exponentially growing bacteria, labeled for several generations with [^{32}P]phosphate (2 μCi/ml) in complete medium, were centrifuged and washed with prewarmed medium without ^{32}P. Resuspended cells were incubated at 37°C, samples were withdrawn at time intervals and precipitated with perchloric acid (final concentration, 0.3 M), and the phospholipids were extracted.

RESULTS

Relative amounts of phospholipids in cytoplasmic and outer membrane fractions. A separation of outer and cytoplasmic membranes of *E. coli* P678 and PM61 was performed by sucrose density gradient centrifugation as described by Osborn et al. (23). The protein profiles of both strains showed four bands not significantly different from those observed in other strains of *E. coli* (14) or *Salmonella typhimurium* (2, 23). The fractions were assayed for NADH oxidase activity and KDO content (Table 1). The lighter band was identified as the cytoplasmic membrane fraction (ρ = 1.151), the intermediary band as a mixture of cytoplasmic and outer membranes, and the two heavy bands as the outer membrane fractions (ρ = 1.202 and 1.229). It should be noted that the total membrane fraction of the mutant PM61 contained only approximately 30% as much KDO as the parent.

Total membrane preparations and cytoplasmic and outer membrane fractions obtained from [^{32}P]phosphate-labeled cells of both strains were used for the analysis of phospholipids. The relative content of chromatographically separated phospholipids is shown in Table 2. It appears that the cytoplasmic membrane of both strains contains relatively less phosphati-

TABLE 1. *NADH oxidase activity and KDO content of fractionated membranes*

Strain	Membrane fraction			Total membranes
	Cytoplasmic	Intermediary	Outer	
NADH oxidase[a]				
P678	5.63	1.01	0.68	0.67
PM61	2.77	0.62	0.20	0.66
KDO[b]				
P678	0.65	1.58	2.86	1.17
PM61	0.10	0.21	1.20	0.38

[a] Specific activities expressed as micromoles per minute per milligram of protein.

[b] Expressed as absorbance units (at 550 nm) per milligram of protein obtained by the thiobarbituric acid assay (22).

TABLE 2. *Relative amounts of phospholipids in cells and membranes of E. coli P678 and PM61*

Membrane prepn	cpm (%)[a]				
	PS[b]	PE	PG	CL	PG/CL
Strain P678					
Total	4.7	84.1	8.2	3.3	2.48
Outer	5.4	86.0	7.8	2.4	3.25
Cytoplasmic	6.2	74.8	14.4	4.9	2.94
Strain PM61					
Total	3.7	79.2	9.8	7.3	1.34
Outer	3.3	81.5	8.5	6.7	1.27
Cytoplasmic	4.6	65.3	12.8	17.4	0.73

[a] Percentage of total counts per minute in [^{32}P]phosphate-labeled phospholipids separated by thin-layer chromatography.

[b] Phosphatidylserine.

dylethanolamine (PE) and more PG and CL than the outer membranes. Moreover, the higher CL content found in total PM61 cells is observed in both membrane fractions of that strain.

The consistently lower PG/CL ratio found in total membranes and in both membrane fractions of PM61 suggested that the turnover of these phospholipids might be modified in the mutant. In fact, the chase experiments (Fig. 1 and 2) indicate that the turnover rate of CL is not significantly different in both strains, but that PG turns over faster in PM61 than in the parent. Moreover, there is a transient increase of radioactivity in parental PG, undoubtedly due to the intracellular pool of ^{32}P that was not detected in the mutant.

Effect of sucrose on phospholipid content. The *envC* mutation can be corrected phenotypically by increasing the osmolarity of the medium by sucrose or other sugars (30). We sought, therefore, to determine the relative amounts of phospholipids in osmotically remedied cells.

A culture of strain PM61 growing exponentially in complete medium containing [^{32}P]phosphate (1 μCi/ml) was centrifuged at 37°C and suspended in prewarmed medium of the same composition that had been supplemented with sucrose (final concentration, 0.6 M). After 3 h of incubation, two samples were withdrawn. One was used for determination of phospholipids; the other was washed with and suspended in prewarmed complete medium and incubated for another 3 h, and then its phospholipids were assayed. The incubation temperature was always 37°C.

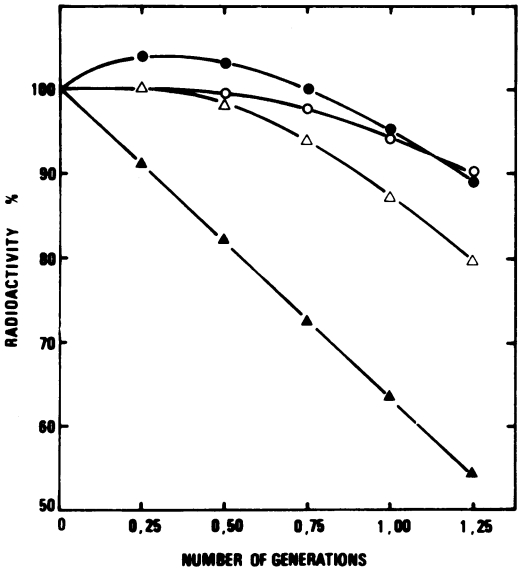

FIG. 1. *Turnover of PG in media of low and high osmolarity. ^{32}P-labeled bacteria were suspended in ^{32}P-free medium, and phospholipids were extracted and analyzed as described in the text. The change of labeled phospholipid in each sample was expressed as a percentage of initial radioactivity. One generation unit of each culture represents one doubling of absorbance at 450 nm. Symbols: ●, strain P678 without sucrose; ▲, strain PM61 without sucrose; ○, strain P678 with sucrose; △, strain PM61 with sucrose.*

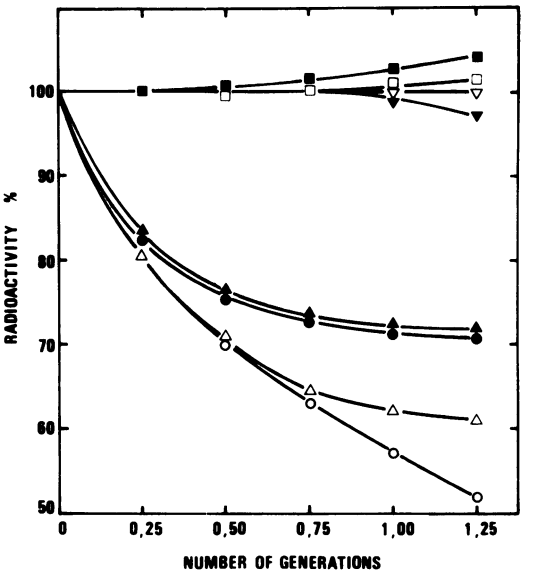

FIG. 2. *Effect of external osmolarity on turnover of PE and CL. Experimental data are those given in Fig. 1. PE turnover: ■, strain P678 without sucrose; ▼, strain PM61 without sucrose; □, strain P678 with sucrose; ▽, strain PM61 with sucrose. CL turnover: ●, strain P678 without sucrose; ▲, strain PM61 without sucrose; ○, strain P678 with sucrose; △, strain PM61 with sucrose.*

The phenotypic correction of strain PM61 was monitored by phase-contrast microscopy and by measuring the particle volume in a Coulter counter (Fig. 3A). After 3 h of growth in a high-osmolarity medium, virtually all chains disappeared, and the volume distribution approached that found in cultures of strain P678 growing in sucrose-free medium. Three hours after elimination of sucrose, the chains reappeared and the volume distribution shifted to that observed under normal conditions of growth (Fig. 3B).

The phenotypic correction of PM61 was accompanied by a considerable decrease in relative amount of CL and by an increase in PG (Table 3). When the phenotypically corrected

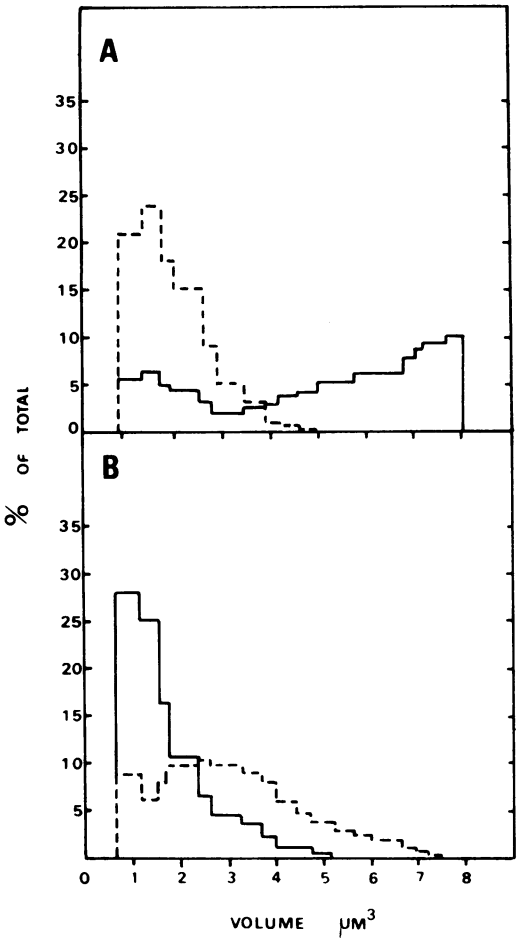

FIG. 3. *Size distributions of E. coli P678 and PM61. Appropriately diluted suspensions of cells were examined in a Coulter counter. (A) Exponentially growing cells of strain P678 (- - -) and strain PM61 (——). (B) Phenotypically corrected strain PM61 after 3 h of incubation in complete medium with 0.6 M sucrose (——), and 3 h after elimination of sucrose (- - -).*

cells were transferred to the medium without sucrose, the amounts of PG and CL returned to their initial values. It should be noted that the phospholipid composition of strain P678 was also modified in the presence of sucrose and that the PG/CL ratios of both strains were comparable under these conditions.

Effect of sucrose on phospholipid turnover. Turnover of the phosphate in the phospholipids was compared during incubation with and without sucrose. Bacteria allowed to grow in the presence of ^{32}P and then washed free of radioactive substrate with prewarmed medium were suspended in a medium not containing ^{32}P but supplemented with 0.6 M sucrose. The control cells were suspended in a medium without sucrose. The decrease of radioactivity of chromatographically separated phospholipids extracted from strains PM61 and P678 in a chase experiment is shown in Fig. 1 and 2. Since the growth rates of these strains are different (doubling time of strain PM61, 53 min, and of strain P678, 36 min [28]), doubling time was used as the unit on the abscissa. As stated above, chase experiments without sucrose revealed that turnover of PG is faster in the mutant. The addition of sucrose to the medium decreased the turnover rate of PG in the mutant (Fig. 1) whereas the turnover of CL increased in both strains (Fig. 2). Slight variations of PE turnover were not analyzed further.

DISCUSSION

The turnover of polyglycerophosphatides in *E. coli* occurs in a cyclic manner involving the conversion of two PGs to one CL and the hydrolysis of CL to PG plus phosphatidic acid (3). Although the cycle is under energy control (6) and cellular levels of PG and CL respond sensitively to various factors, the physiological function of this interconversion is unknown. However, phospholipid metabolism appears to have a vital role in cell growth and division (8, 9, 21). Thus, the transition of *E. coli* cells from the exponential to the stationary phase, or treatment with inhibitors of cell division, induces a striking increase of CL and a decrease of PG (7, 31). Similarly, *E. coli* B treated with nalidixic acid, washed free of drug, and resuspended in the culture medium accumulates CL in the interval preceding the renewal of cell division (17). Furthermore, different types of division mutants including chain-forming *envA* (20) and *envC* (27) have been shown to have a higher content of CL than normally dividing *E. coli* cells (21).

Our results demonstrate that the modified phospholipid composition of *envC* cells is found in both the outer and the cytoplasmic membrane. This observation suggests that the metabolism of phospholipids and particularly the PG-CL cycle may be regulated by a mechanism common to both membranes. Furthermore, it might be argued that this postulated regulatory mechanism is osmotically sensitive, and that it could be linked in some way with the division process since the phenotypic correction of *envC*, by increasing the osmolarity of the medium, results in the division of cells of normal length as well as the shift of the PG-CL ratio to the value found in the parent. It is interesting to note that other substances like glucose, lactose,

TABLE 3. *Effect of 0.6 M sucrose on relative amounts of phospholipids in E. coli P678 and PM61 grown in complete medium (CM)*

Growth conditions	cpm (%)[a]				
	PS[b]	PE	PG	CL	PG/CL
Strain P678					
Without sucrose[c]	3.8	75.5	15.2	5.2	2.8
3 h in CM + sucrose	1.8	78.1	16.0	3.9	4.1
3 h in CM after elimination of sucrose	2.2	77.0	14.6	5.5	2.7
Strain PM61					
Without sucrose[c]	6.3	71.0	10.9	11.6	0.9
3 h in CM + sucrose	4.3	74.9	16.7	4.2	4.0
3 h in CM after elimination of sucrose	5.7	74.7	12.0	6.8	1.8

[a] Percentage of total counts per minute in [^{32}P]phosphate-labeled phospholipids separated by thin-layer chromatography.

[b] Phosphatidylserine.

[c] Extract from perchloric acid-precipitated exponentially growing bacteria.

mannitol, and polyethylene glycol (molecular weight, 4,000), but not sodium chloride, are also capable of phenotypically correcting *envC* (30).

The observed shifts of PG-CL ratio appear to be the result of a modified turnover rate of these phospholipids. Chase experiments showed that PG turned over faster in growing *envC* chains than in parental cells, but their rates of CL turnover were comparable. In the presence of 0.6 M sucrose, the turnover rate of PG in the mutant was slowed down to a value approaching that found in the parent whereas the turnover of CL of both strains was accelerated.

In strain P678, the turnover rates of PG and CL were also affected by shifts of osmolarity although to a lesser extent. These observations are consistent with the evidence presented by Munro and Bell (18) showing that high external osmolarity decreases the turnover rates of PE and PG in *E. coli* (CL was not determined). The changes of balance between CL and PG described in this paper could have as a consequence the modifications of the overall ion permeability and membrane selectivity. A number of conditional mutants have been described that can grow and divide under nonpermissive conditions if the media are of sufficiently high osmolarity (5, 12, 25, 29). In most cases, osmotically "remediable" mutants have altered envelopes and show hypersensitivity to various antibiotics, detergents, and other agents (4, 10). However, the response of their phospholipid metabolism to changes of medium osmolarity has not been studied.

In conclusion, it appears that increased external osmolarity acts on phospholipid metabolism as well as on an unknown step involved in the mechanism of cell division of the *envC* mutant. We do not know how phospholipid metabolism might be related to the postulated event in the process of septum formation. Because the mechanism of bacterial cell division is still obscure at the biochemical and molecular levels, it is not yet feasible to show by direct evidence that such a relation exists.

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