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EXPERIMENTAL ARTICLES

Changes in the Composition of Anionic Membrane Phospholipids Influence Protein Secretion and Cell Envelope Biogenesis in *Escherichia coli*

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Abstract—The secretion of alkaline phosphatase (PhoA) and peculiarities of biogenesis of the cell envelope were studied in *Escherichia coli* strains HD30/pHD102 and HDL11 with controlled synthesis of the anionic phospholipids, phosphatidylglycerol and cardiolipin. Inactivation of the *pgsA* gene responsible for the synthesis of anionic phospholipids or changes in the regulation of its expression by an environmental factor caused changes in the metabolism and composition of membrane phospholipids, which resulted in a decrease in the secretion of alkaline phosphatase through the cytoplasmic membrane and an increase in PhoA secretion from the periplasm into the culture medium. An increase was observed in exopolysaccharide secretion, as well as a decrease in the contents of the outer membrane lipopolysaccharides and lipopolyproteins, which determine its barrier properties. The results obtained show that anionic phospholipids play a significant role in protein secretion and are probably involved in the interrelation between protein secretion and biogenesis of cell envelope components.

Key words: Escherichia coli, alkaline phosphatase, secretion, phospholipids, outer membrane.

As we demonstrated earlier [1], enhanced synthesis of plasmid-encoded periplasmic alkaline phosphatase (PhoA) in *Escherichia coli* resulted in PhoA secretion into the culture broth without cell lysis. The level and mechanism of PhoA secretion depend on the specific strain and the pattern of modifications in the chemical composition and structure of the cell envelope, which usually take place in the case of enhanced protein production [2–4]. In a number of cases, modifications of the cell envelope are determined by changes in the composition of lysophospholipids [2] and lipoproteins (LP) and/or lipopolysaccharides (LPS) [3] responsible for the barrier properties of the outer membrane. The metabolic precursors of these components are phospholipids [5], which also participate in protein secretion and constitute an integral part of the cell secretory system [6, 7]. This allows the assumption that protein secretion and the biogenesis of the cell envelope are interconnected at the level of phospholipid metabolism [6]. Construction of mutant strains the phospholipid composition of which can be significantly changed by varying the culture conditions [8–10] makes it possible to test this assumption.

Previously, we showed that the loss of the predominant membrane phospholipid phosphatidyl ethanolamine (PE) in *E. coli* AD93 resulted in a significant inhibition of PhoA secretion through the cytoplasmic membrane and, at the same time, in an increase in PhoA secretion into the culture broth, which was correlated with changes in the composition of the outer membrane [11]. However, we did not estimate how specific the effect of the changes in phospholipid composition on these processes was; it remained unknown whether they were related only to the lack of PE or also to changes in the composition of other phospholipids.

The purpose of this work was to study the influence of the changes in the composition of membrane phospholipids caused by the inactivation of the *pgsA* gene responsible for the synthesis of anionic phospholipids (APLs) or by the regulation of its expression on protein secretion and on biogenesis of some components of the cell envelope. *Escherichia coli* PhoA encoded by the gene cloned in a plasmid to enhance protein synthesis was used as a model. Parent strains with only chromosomal *phoA* were used as a control. Secretion was studied in *E. coli* strains HD30/pHD120 and HDL11 with controlled APL synthesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. In this study, we used *Escherichia coli* strain HD30/pHD120

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Table 1. Phospholipid composition of *E. coli* cells as dependent on cultivation conditions

E. coli strain	Cultivation conditions	Phospholipids*, mol %				
		PE	PG	CL	PA	APL
HD30/pHD102	30°C	68.5 ± 1.5	24.2 ± 0.6	3.2 ± 0.4	4.2 ± 0.4	31.6 ± 1.4
	42°C	75.2 ± 0.9	14.8 ± 1.6	5.4 ± 0.4	4.8 ± 1.1	25 ± 3.1
HDL11	+ IPTG	84.0 ± 2.0	11.0 ± 3.0	3.9 ± 1.3	1.8 ± 0.5	16.7 ± 4.8
	– IPTG	94.0 ± 1.0	0.4 ± 0.2	1.3 ± 0.7	4.7 ± 0.7	6.4 ± 4.5

Note: PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; CL, cardiolipin; PA, phosphatidic acid; APL, anionic phospholipids.
* Phospholipids were extracted from cultures in the middle of the log phase.

(*pgsA30::kan*), which lacked the *pgsA* gene responsible for phosphatidyl glycerol (PG) synthesis but contained plasmid pHD102 that carried this gene and whose replication was temperature–dependent. Culturing at different temperatures allowed regulation of the content of APL in HD30/pHD120 cells. We also used *E. coli* strain HDL11 containing the *pgsA* gene under the control of the *lac* promoter, with APL production depending on the presence of the inducer of this promoter, isopropyl thio-β-D-galactopyranoside (IPTG) [10]. For enhanced PhoA synthesis, we used strains HD30/pHD102/pHI-7 and HDL11/pHI-7, which contained plasmid pHI-7 carrying the *phoA* gene under the control of its own P_{PHO}-promoter [12].

Culture conditions. Cultures were grown until the middle of the log phase on mineral medium containing glucose as the carbon source [13]. The *E. coli* strain HD30/pHD120 was grown at 30 and 42°C, and the *E. coli* strain HDL11, at 37°C in the presence or absence of IPTG (100 mM), an APL synthesis inducer. For the maintenance of plasmids pHD102 and pHI-7, chloramphenicol (25 μg/ml) and ampicillin (100 μg/ml) were used, respectively. For the induction of PhoA synthesis, cells were aseptically harvested by centrifugation at 5000 *g* for 10 min, washed with 0.14 M NaCl, and transferred to a medium without orthophosphate. At regular time intervals, samples for further tests were taken, and merthiolate was added to a final concentration of 0.005% for blocking biosynthesis.

PhoA secretion. PhoA secretion through the cytoplasmic membrane was judged from its activity in the culture, since the enzyme becomes active only after the translocation of the polypeptide chain through the cytoplasmic membrane into the periplasm [14]. For the estimation of PhoA secretion into the culture broth, cells were precipitated by centrifugation at 12000 *g* for 5 min; the supernatant was removed and used for determination of the activity of extracellular enzyme.

PhoA isoforms. The activities of PhoA isoforms were determined in 7.5% PAAG after electrophoresis of proteins under nondenaturing conditions (the gel was treated with α-naphthyl phosphate and stained with Resistant Red (RR)) [15].

Immune-enzymatic detection of PhoA and LPs was carried out after electroblotting of proteins onto nitrocellulose BA-85 (Schlicher und Schull), using rabbit antibodies against denatured *E. coli* PhoA and lipoprotein and protein A conjugated to horse-radish peroxidase (Bio-Rad, United States) by the standard method.

Phospholipid analysis. Lipids were extracted by the Ames method. The extraction and thin-layer chromatography were carried out as described earlier [16].

Analytical methods. The electrophoresis of proteins was carried out in 10% PAAG containing 0.4% SDS [17]; electrophoresis under nondenaturing conditions was carried out as described by Davis [18]. PhoA activity was estimated by the rate of the hydrolysis of *n*-nitrophenyl phosphate in 50-mM Tris–HCl buffer containing 5 mM $MgCl_2$, pH 8.5. The amount of the enzyme that hydrolyzed 1 μmol of substrate in 1 min at 37°C was taken to be one unit of activity (U). The protein content was determined by the Lowry method [19]. The exopolysaccharide concentration in the culture broth was measured using the anthranone method [20]. Membrane LPS was assayed by determining the concentration of its component 2-keto-3-deoxy-D-mannooctonate (KDO) using thiobarbituric acid from the formula 0.5 mM KDO = 1 mg LPS [21].

RESULTS AND DISCUSSION

1. Phospholipid Composition of Mutant E. coli Strains HD30/pHD102 and HDL11 Depends on Culture Conditions

To modify the phospholipid composition of membranes in the *E. coli* HD30/pHD102 strain, cells were grown at temperatures of 30 and 42°C, which permitted and precluded, respectively, the replication of plasmid pHD102 carrying the *pgsA* gene. At 30°C, HD30/pHD102 cells had a phospholipid composition typical of this bacterium (Table 1). At 42°C, the PG concentration in HD30/pHD102 cells was 1.6 times lower. Control experiments with wild-type *E. coli* strain K12 showed that the modifications of PG composition were determined by the inactivation of the *pgsA* gene at 42°C (the elimination of plasmid pHD102 carrying this gene) rather than by a mere change in cultivation temperature. The APL content of *E. coli* HDL11

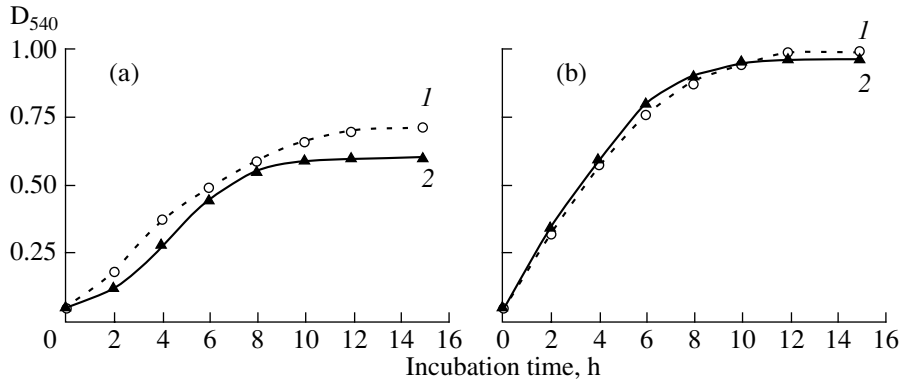

Fig. 1. Dynamics of cell growth in *E. coli* strains (a) HD30/pHD102/pHI-7 and (b) HDL11/pHI-7 with (*1*) a normal and (*2*) a decreased APL content.

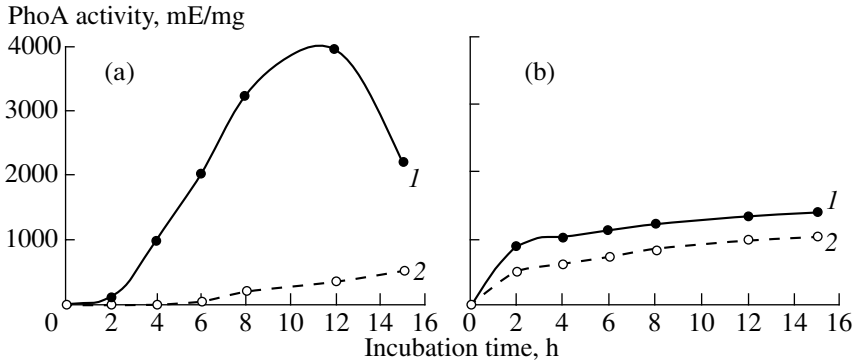

Fig. 2. Dynamics of PhoA secretion (*1*) through the cytoplasmic membrane and (*2*) into the culture broth in *E. coli* strains (a) HD30/pHD102/pHI-7 and (b) HDL11/pHI-7 under PhoA–inducing conditions. PhoA activity is indicated in mU/mg cell protein.

cells depended on IPTG availability in the culture medium. HDL11 cells growing in the absence of IPTG contained only 0.4 ± 0.2% of PG (total APL accounted for 6.4 ± 0.2%), whereas in the presence of this inducer, the PG content was 11.0 ± 3.0% (total APL accounted for 16.7 ± 1.6%) (Table 1). Thus, phospholipid imbalance expressed in a decreased APL content occurred in *E. coli* HD30/pHD102 and HDL11 cells because of the inactivation of the *pgsA* gene and the regulation of its expression, respectively.

Cell transformation with plasmids pHI-7 carrying the *phoA* gene and the expression of this gene had no significant effect on the phospholipid composition (data not shown). Nor did APL the content affect cell growth significantly (Fig. 1).

2. Decrease in the APL Content Affects PhoA Secretion through Both Cytoplasmic Membrane and Outer Membrane of E. coli

The PhoA secretion through cytoplasmic membrane in strain HD30/pHD102/pHI-7 was observed 2 h after the induction of PhoA synthesis, and the enzyme was secreted linearly for 12 h (Fig. 2). Secretion into the medium started much later, and the level of extracellular PhoA did not exceed 10% of the total active PhoA. In strain HDL11, PhoA secretion into the medium was observed immediately after the synthesis induction, in parallel with PhoA secretion through the cytoplasmic membrane. As much as 50% of the active protein was found in the medium. This indicates that the mechanisms of PhoA secretion in strain HDL11 are similar to those in leaky mutants; this agrees with the fact that the outer membrane of HDL11 cells does not contain LP [10], which determines its barrier properties.

The decrease in the APL content of HD30/pHD102 cells at 42°C or in HDL11 cells growing in the absence of IPTG resulted in the inhibition of PhoA secretion through the cytoplasmic membrane (Fig. 3a). This effect was less pronounced but also statistically significant in HDL11 cells. PhoA secretion into the culture broth (Fig. 3b) inversely depended on the APL content. Cells with normal phospholipid composition secreted into the medium no more than 10–20% of the active enzyme, versus 40–50% secreted by cells with an imbalance of phospholipid composition. At the chromosomal level of expression, the PhoA secretion into the medium was very low, but it was also increased in

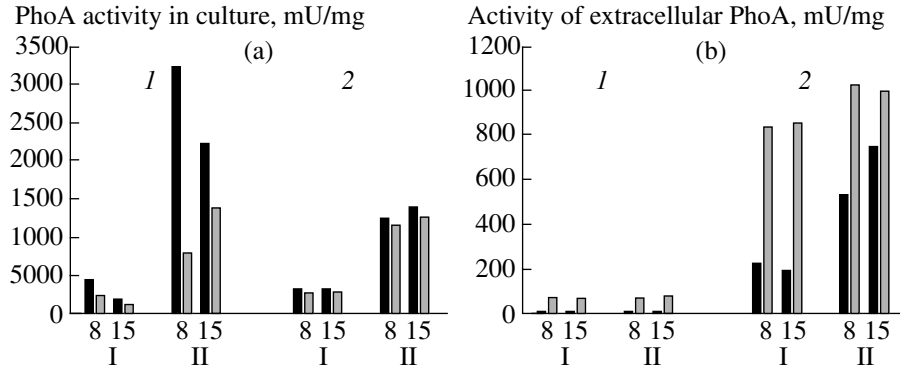

Fig. 3. PhoA secretion (a) through the cytoplasmic membrane and (b) into the culture broth in cases of the expression of (I) the chromosomal *phoA* gene and (II) the *phoA* gene cloned into a plasmid after 8 and 15 h of cultivation in *E. coli* strains (*1*) HD30/pHD102 and (*2*) HDL11 with a normal (dark columns) and a decreased (light columns) APL content.

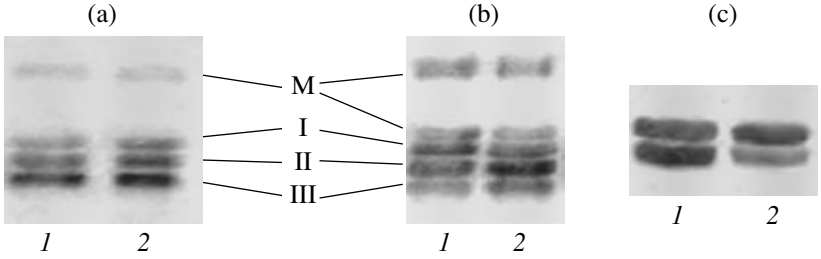

Fig. 4. The spectrum of isoforms in *E. coli* strains (a) HD30/pHD102/pHI-7 and (b) HDL11/pHI-7 with (*1*) a normal and (*2*) a decreased APL content and (c) PhoA immunoblotting in strain HD30/pHD102/pHI-7. I, II, and III, isoforms; M, multimers.

cells with the imbalance in phospholipid content. In strain HDL11 (lacking LP in the outer membrane), as much as 20% of PhoA was secreted into the medium even at the chromosomal level of expression, this amount reaching 40–60% in cells with an enhanced PhoA synthesis. In addition, an increase in secretion into the medium was observed in cells with imbalance in phospholipid composition, though it was less pronounced than in strain HD30/pHD102.

The APL-generated imbalance in phospholipid composition did not have a strong effect on the spectrum of PhoA isoforms (Figs. 4a, 4b). The relative content of PhoA isoforms differing in specific activities [22] did not contribute to the activity that was used to estimate PhoA secretion. PhoA immunoblotting (Fig. 4c) showed that the decrease in APL content did not influence PhoA synthesis significantly. However, it resulted in inhibition of PhoA secretion through the cytoplasmic membrane (since the pre-PhoA to PhoA ratio was higher), which confirmed the results of the analysis of PhoA secretion on the basis of PhoA activity.

3. Changes in APL Content Affect the Biogenesis of Some Components of the Cell Envelope

LPS and LP are the most important components of the *E. coli* outer membrane, which determine the membrane barrier properties [23]. According to our data, the level of LPS in cells with a normal phospholipid composition increased upon the increase in PhoA synthesis and secretion determined by the plasmid gene, especially in the HD30/pHD102/pHI-7 strain (Table 2). However, in cells with an imbalance in phospholipid composition (decrease in APL content), LPS content dropped significantly irrespective of the level of PhoA synthesis.

EPS secretion also correlated with PhoA secretion through the cytoplasmic membrane. The enhanced PhoA synthesis resulted in an increase in EPS level, especially in strain HDL11. The decrease in APL content did not have a significant effect on EPS secretion in HD30/pHD102, but resulted in its increase in HDL11. The EPS content of HDL11 was two orders of magnitude higher than in HD30/pHD102, presumably because of the absence of LP in its membrane. Earlier, we reported competitive interaction between LPS and LP content in the outer membrane [11].

Immunoblotting of HD30/pHD102 LP (Fig. 5) showed that LP content, unlike LPS content, decreased upon enhancement in the PhoA synthesis and secretion determined by the plasmid gene. The imbalance in phospholipid composition resulted in even greater changes in LP biogenesis. The immunoblotting revealed two LP forms: mature LP and, presumably, its precursor. In cells with the imbalance in phospholipid

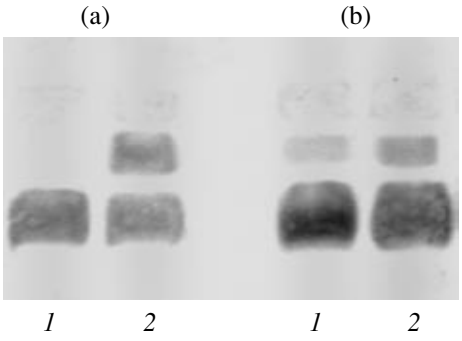

Fig. 5. Immunoblotting of LP in *E. coli* strains (a) HD30/pHD102/pHI-7 and (b) HDL11/pHI-7 with (*1*) a normal and (*2*) a decreased APL content.

composition, the level of mature LP decreased, whereas the level of its precursor increased.

Thus, the results obtained confirm the interaction between protein secretion and biogenesis of the components of the cell envelope. PhoA secretion, in the case of its enhanced synthesis, was positively correlated with LPS biogenesis, which indicates that PhoA secretion and the transport of LPS are coupled. At the same time, PhoA secretion through the cytoplasmic membrane was inversely correlated with LP biogenesis. This may have resulted from the competition between PhoA and LP (which are secreted via the same pathway) for the sites of translocation through the cytoplasmic membrane. The decrease in APL level due to the inactivation or decrease in the expression of the *pgsA* gene resulted in the inhibition of both PhoA secretion through the cytoplasmic membrane and biogenesis of LP and LPS. It is known that LP and LPS determine the barrier properties of the outer membrane, and a decrease in the levels of these components results in the leaky phenotype, i.e., in an increase in membrane permeability for periplasmic proteins and their efflux into the culture broth [24]. The results obtained confirm these data. Decrease in LP and LPS levels resulted in an increase in PhoA secretion into the culture broth. Since the decrease in LP and LPS levels was observed in cells with modified metabolism and composition of APLs, which are known to be LP and LPS precursors [5], it is plausible to assume that phospholipids contribute to the interaction between PhoA secretion and biogenesis of the cell envelope components.

Evidently, this interaction is ensured not only via changes in PE metabolism and concentration, as described previously [11], but also by APLs.

Table 2. The LPS and EPS contents of *E. coli* strains

Strain	Cultivation conditions	LPS, mg/mg protein	EPS, mg/mg protein
HD30/pHD102	30°C	0.35	0.30
HD30/pHD102/pHI-7		0.57	0.39
HD30/pHD102	42°C	0.29	0.28
HD30/pHD102/pHI-7		0.35	0.42
HDL11	+ IPTG	0.23	29.5
HDL11/pHI-7		0.28	129.7
HDL11	– IPTG	0.14	63.7
HDL11/pHI-7		0.26	160.2

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