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Membrane Phospholipid Composition of *Escherichia coli* Affects Secretion of Periplasmic Alkaline Phosphatase into the Medium

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Abstract—Secretion of alkaline phosphatase (PhoA) encoded by a gene constituent of plasmids has been studied in *Escherichia coli* strains with controlled synthesis of anionic phospholipids (phosphatidylglycerol and cardiolipin, strain HDL11) and zwitterionic phospholipid (phosphatidylethanolamine, strain AD93). Changing the phospholipid composition of the membrane of these strains leads to an increase in secretion of PhoA, which is usually localized in the periplasm, into the culture medium. This correlates with a higher secretion of exopolysaccharides and lower content of lipopolysaccharide in the outer membrane. The results show the possibility of coupling protein secretion into the medium with biogenesis of cell envelope components in which phospholipids are involved.

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

The envelope of *E. coli* cells consists of several compartments: cytoplasmic membrane, periplasm, peptidoglycan layer or cell wall, and outer membrane [1]. The cell envelope serves as a barrier between cell contents and the environment and also as the means of communication between them, providing the flows of nutrients, energy, and information. Most secreted soluble proteins of this gram-negative bacterium, with the exception of some protein toxins, are localized in the periplasm [2]. However, our previous works [3, 4] have shown that, during overproduction of periplasmic alkaline phosphatase (PhoA) encoded by the gene constituent of plasmids, cells of different *E. coli* strains secrete the above enzyme not only into the periplasm but also into the culture medium in the absence of cell lysis. The level and mechanism of secretion depends on the nature of a strain and on the character of changes in the envelope chemical composition and structure [5-8]. In some cases, the above changes were related to the content in the outer membrane of lipoprotein (LP) and/or lipopolysaccharide (LPS) [5], which are responsible for permeability of the outer membrane. This suggested that overproduction and secretion of periplasmic proteins result in a competition between them and cell envelope components either for the sites of their translocation across membranes or for the common metabolites involved in these processes, which leads to a change in the outer membrane chemical composition and increases its permeability for periplasmic proteins. The most important components of cell envelope are phospholipids. As a constituent of the cytoplasmic membrane, they form not only a barrier of permeability for many substances but also, being a microenvironment of many proteins and possessing metabolic and structural dynamics, are involved in many transmembrane processes [9, 10], including protein translocation across the membranes [11-13]. In particular, anionic phospholipids (APL) provide optimal conditions for the function of protein components of secretory machinery [14], participate in secretion initiation by interacting with signal peptide [15, 16], and the zwitterionic phospholipid may be involved in protein translocation by formation of non-bilayer structures [17, 18]. Besides, phospholipids serve as metabolic precursors of some envelope components: periplasmic oligosaccharides (MDO), lipoprotein, and lipopolysac-

Abbreviations: PhoA) alkaline phosphatase; APL) anionic phospholipids; IPTG) isopropyl-thio-β-D-galactopyranoside; CL) cardiolipin; PG) phosphatidylglycerol; PA) phosphatidic acid; PE) phosphatidylethanolamine; EDTA) ethylenediaminetetraacetate; EPS) exopolysaccharide; KDO) 2-keto-3-deoxy-D-mannooctonate; MDO) periplasmic oligosaccharides.

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charides of outer membranes [9, 10]. This not only provides the vector value of protein translocation across the cytoplasmic membrane but also enables the coupling of this process with biogenesis of cell envelope components on the level of phospholipid metabolism.

The goal of the current work was to reveal the effect of changes in membrane phospholipid composition due to genetic manipulations on protein secretion and biogenesis of some envelope components, in particular, lipopolysaccharides. The model was *E. coli* alkaline phosphatase encoded by the gene constituent of plasmids, which is secreted not only into the periplasm but also into the culture medium. Besides, it is known [19] that PhoA becomes enzymatically active only after the enzyme translocation into the periplasm, where conditions for the formation of disulfide bonds and dimerization of subunits are available. Therefore, the level of protein translocation across the cytoplasmic membrane may be assessed by PhoA activity in cell culture and the level of protein secretion into the medium—by activity in culture liquid. Secretion of the enzyme was studied in *E. coli* mutants with altered content of individual phospholipids.

MATERIALS AND METHODS

Bacterial strains and plasmids. Two mutant *E. coli* strains with controlled phospholipid composition were used in the work: AD93 (*pss9::kan recA srl::Tn10 nadB*$^+$) and HDL11. The strain *E. coli* AD93 completely lacks the zwitterionic phospholipid phosphatidylethanolamine (PE) due to a mutation in the *pss* gene responsible for the synthesis of PE precursors [20]. The same strain carrying plasmid pDD72 with a cloned *pss* gene does not differ from the wild type strain by PE content [20]. In strain *E. coli* HDL11, the synthesis of anionic phospholipids—cardiolipin (CL) and phosphatidylglycerol (PG)—is determined by the presence in the medium of isopropyl-thio-β-D-galactopyranoside (IPTG), which is an inducer of the lac operon controlling the expression of genes responsible for PG synthesis in this strain [21]. Plasmid pSAP1 [22] carries the wild type *phoA* gene cloned in plasmid vector pBAD18 under the control of *ara* promoter P_{BAD} [23]. Plasmid pHI-7 carries the wild type *phoA* gene under the control of its own P_{PHO} promoter [24].

The strains *E. coli* AD93 and *E. coli* HDL11 were grown at 37°C on a mineral medium with glucose as a carbon source [25], and the strain *E. coli* AD93 containing thermosensitive plasmid pDD72 was grown at 30°C. The medium for the strain AD93 additionally contained tryptophan (100 μg/ml) and $MgCl_2$ (50 mM) [19]. The growth medium was supplemented with kanamycin (50 μg/ml) and tetracycline (14 μg/ml) for growing this strain, IPTG (100 mM) to induce the synthesis of anionic phospholipids (APL), chloramphenicol (25 μg/ml) to maintain plasmids pDD72 and pSAP1, and ampicillin (100 μg/ml) to maintain plasmid pHI-7.

Induction of alkaline phosphatase synthesis. Alkaline phosphatase is a member of the Pho regulon and induced under phosphorus starvation [25]. For induction of the enzyme synthesis controlled by their own promoter, cells were grown on a medium with orthophosphate (P_i) to the mid log growth phase, twice washed in medium containing no P_i (cells of strain AD93—in the presence of 50 mM $MgCl_2$), and placed on a P_i-lacking medium. The synthesis of PhoA controlled by the *ara* promoter P_{BAD} was induced by addition of 0.5% arabinose to a culture grown on the same medium to the mid log growth phase. Biosynthetic processes were stopped by adding 0.02% merthiolate.

Alkaline phosphatase secretion. Alkaline phosphatase secretion was assessed by its activity in the culture because the enzyme becomes active only after translocation of the polypeptide chain across the cytoplasmic membrane into the periplasm [26]. Alkaline phosphatase secretion into the medium was assessed by centrifugation of the culture at 5000*g* for 10-20 min and the activity of extracellular enzyme was determined in the supernatant.

Isoforms of alkaline phosphatase. Alkaline phosphatase isoforms were revealed by their activity in 7.5% polyacrylamide gel (after electrophoresis of periplasmic proteins under denaturing conditions) by gel treatment with α-naphthylphosphate and Fast Red RR [26].

Lipid analysis. Lipids were extracted and analyzed by thin layer chromatography as described previously [17].

Preparation of subcellular fractions. Spheroplasts were prepared from harvested and washed cells by cell wall lysis using lysozyme and EDTA in the presence of sucrose following Miura and Mitzushima [27]. Spheroplasts were centrifuged at 12,000*g* for 60 min, and the supernatant was used as the periplasmic fraction. Spheroplasts were lysed by osmotic shock and membranes were centrifuged at 12,000*g* for 60 min after the removal of non-lysed cells and spheroplasts. The supernatant was used as the cytoplasmic fraction. Cytoplasmic and outer membrane proteins were separated by selective solubilization of the membranes using 2% Triton X-100 following Schnaitmann [28].

Analytical methods. Protein electrophoresis was carried out in 10% polyacrylamide gel with 0.4% SDS [29] and in non-denaturing conditions according to Davis [30]. Alkaline phosphatase activity was determined by the rate of *p*-nitrophenylphosphate hydrolysis in 50 mM Tris-HCl buffer, pH 8.5, containing 5 mM $MgCl_2$, taking the amount of enzyme that hydrolyzes 1 μmol of *p*-nitrophenylphosphate per 1 min at 37°C as a unit of activity (U). Protein was assayed according to Lowry [31]. The content of exopolysaccharides (EPS) was determined by the anthrone method [32] and that of 2-keto-3-deoxy-D-mannooctonate as a component of membrane lipopolysaccharides—using thiobarbituric acid [33].

RESULTS

Changing membrane phospholipid composition has no significant effect on the growth of *E. coli* cells. Cell growth has been studied in the mutant *E. coli* strains HDL11 and AD93 having different contents of individual phospholipids and transformed by plasmid pSAP1 with the alkaline phosphatase gene *phoA* controlled by exogenous *ara* promoter P_{BAD}. Cells of the strain *E. coli* HDL11 contain 2-4% APL in the absence of IPTG and about 20% APL in the presence of this inducer, which corresponds to the APL level in the wild type strain [21]. The strain AD93 lacks the zwitterionic phospholipid PE, the predominant membrane phospholipid of *E. coli* [20], and the cells contain only APL. The same strain bearing plasmid pDD72 with the *pss* gene synthesized PE, the amount of which corresponded to that in the wild type strain (about 80%). These regularities in the phospholipid content of mutant strains have been supported in the current work (table) [34]. Phospholipids were extracted from cells grown to the mid log growth phase.

In spite of significant changes in the phospholipid content of the above strains, the cell growth rate did not change considerably compared with that of cells having the wild type level of anionic or zwitterionic phospholipids (Fig. 1). This is evidently because the strain HDL11 in the absence of IPTG on the background of the lower PG and CL content has higher content of phosphatidic acid (PA), which is a PG precursor and an anionic phospholipid and may partially compensate for their deficiency. The strain AD93 needs for its growth high concentrations of Mg^{2+} (50 mM), and the anionic phospholipid CL may functionally replace the lacking PE under these conditions, since CL in the presence of bivalent cations, like PE, is able to form non-bilayer structures [35, 36].

Changing membrane phospholipid composition affects the level of PhoA secretion into the culture medium. The dynamics of PhoA secretion into the periplasm and culture medium has been studied in mutant strains with different contents of individual phospholipids. The analysis of phosphatase activity of the culture of the strain HDL11 and in culture liquid showed that the rate of PhoA secretion across the cytoplasmic membrane slightly decreases in the absence of IPTG (Fig. 2a). This strain, however, efficiently secretes the enzyme into the culture medium, and about 70% of active enzyme is found in the extracellular environment in the presence of IPTG in 5 h after the induction of enzyme synthesis. In the absence of IPTG, the enzyme amount in the medium increases to 90% (Fig. 2b). The level of PhoA secretion across the cytoplasmic membranes of PE-depleted cells of *E. coli* AD93 is much lower (Fig. 3a). In spite of the decrease in the total level of PhoA secretion across the cytoplasmic membrane in strain AD93, enzyme secretion into the medium by cells of this strain increases much more compared with cells having the wild type level of PE (Fig. 3b). Whereas the content of extracellular enzyme in cells with the wild type level of PE does not exceed 10% in 6 h after PhoA induction, in PE-lacking cells it reaches 35% of the total amount of the enzyme. The higher level of secretion into the medium by HDL11 compared with AD93 is evidently associated with the absence of lipoprotein in HDL11, which is responsible for barrier properties of the outer membrane. As a result, secretion into the medium in this strain proceeds in the course of PhoA synthesis and secretion across the cytoplasmic membrane, without preliminary accumulation of the enzyme in the periplasm. We have already observed such secretion by the lky-type mutants in strain *E. coli* KS203, also lacking lipoprotein [37].

Proteins of different cell envelope compartments, e.g., periplasm and outer membrane, are also secreted proteins. With the purpose to assess the effect of phospholipid composition on secretion of such proteins, the protein composition of subcellular fractions of *E. coli* AD93 corresponding to these compartments has been studied. No significant effect of phospholipid composition on the protein spectrum of these fractions was revealed (data not presented).

PhoA secretion into the culture medium includes a periplasmic stage. It is known [38] that PhoA undergoes post-translocational proteolytic modification in the

Phospholipid composition of different *E. coli* strains depending on conditions of their cultivation

Strain	Content of phospholipids, mole %			
	PE	PG	CL	PA
AD93 (+50 mM $MgCl_2$)	0	61.6 ± 3.6	32.1 ± 3.0	6.3 ± 0.7
AD93/pDD72	78.6 ± 4.5	14.8 ± 1.7	4.0 ± 0.5	2.7 ± 0.3
HDL11 (+IPTG)	83.6 ± 2.1	10.7 ± 3.1	3.9 ± 1.3	1.8 ± 0.5
HDL11 (–IPTG)	93.6 ± 0.9	0.4 ± 0.2	1.3 ± 0.7	4.7 ± 0.7

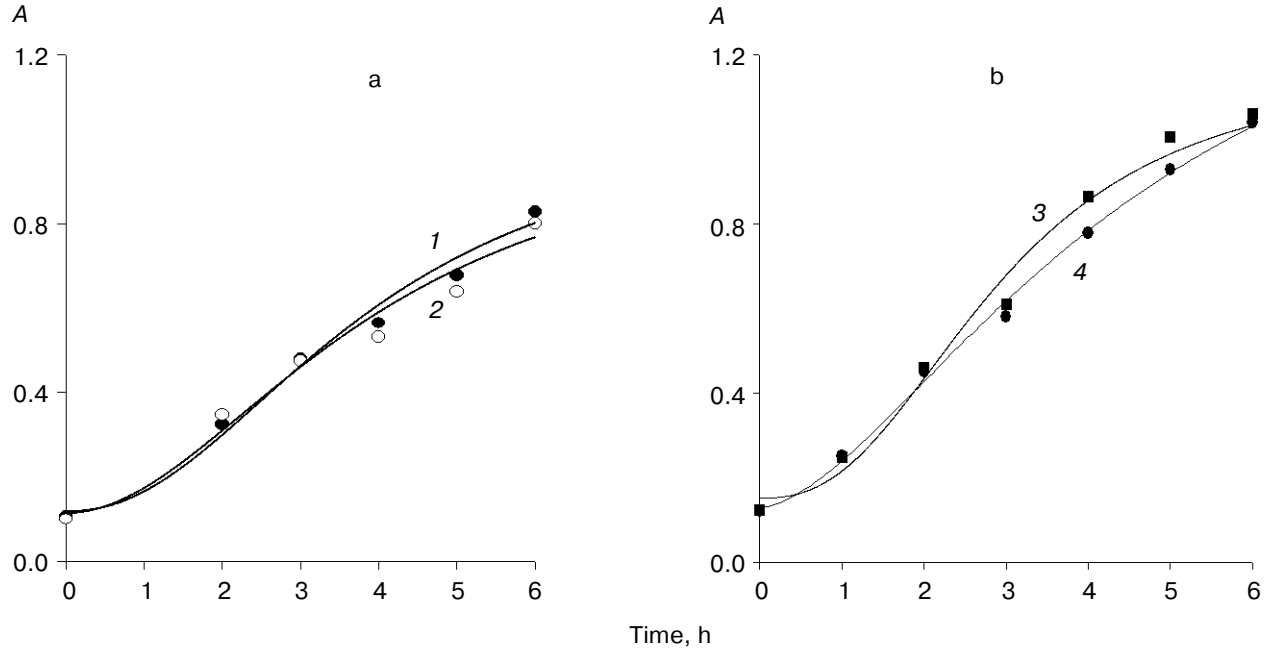

Fig. 1. Growth of cells of mutant *E. coli* strains with different content of anionic and zwitterionic phospholipids: a) strain HDL11 in the absence (*1*) and presence (*2*) of IPTG; b) strain AD93 without (*3*) and with (*4*) plasmid pDD72. The standard deviation is no more than 10%.

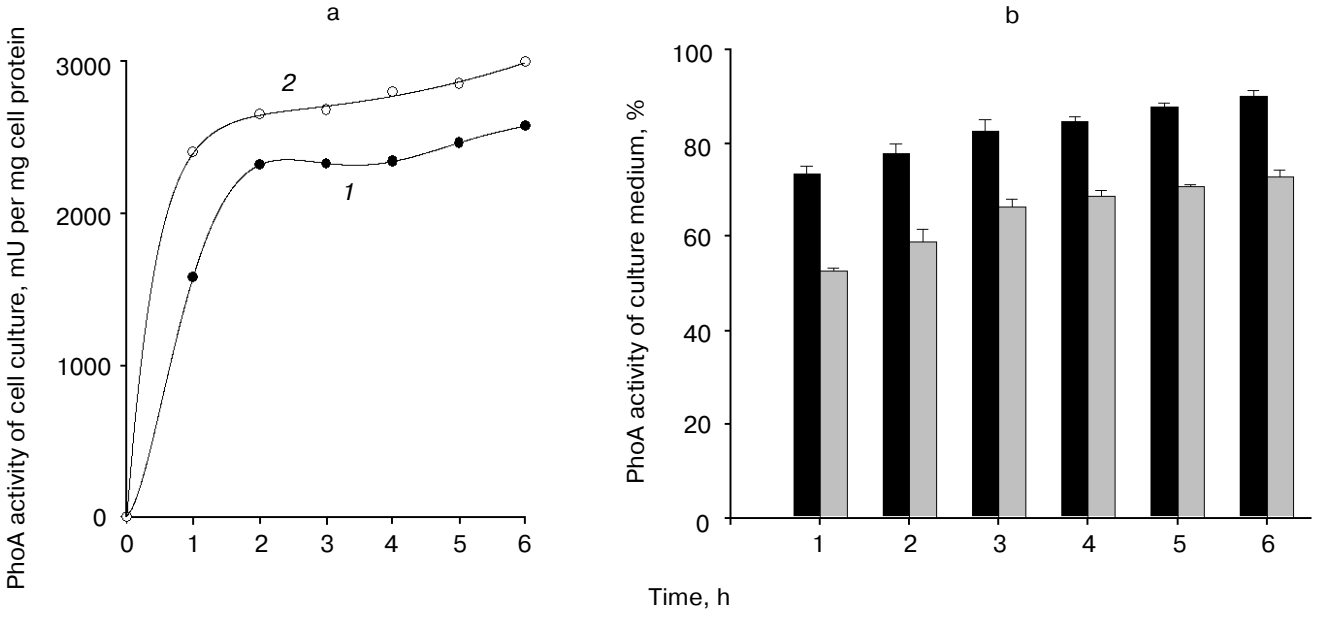

Fig. 2. PhoA activity of cell culture (mU per mg cell protein) (a) and culture medium (% of total) (b) of *E. coli* HDL11/pSAP1 in the absence (curve *1* in panel (a) and dark columns in (b)) and presence (curve *2* in panel (a) and light columns in (b)) of IPTG.

periplasm after translocation across the cytoplasmic membrane. This modification includes the cleavage of N-terminal Arg by modifying proteinase (the *jap* gene product) and dimerization of subunits. In the course of modification, three enzyme isoforms are formed: a dimer of identical subunits with uncleaved Arg (isoform I), a heterodimer lacking Arg in only one subunit (isoform II), and a dimer with both subunits lacking N-terminal Arg

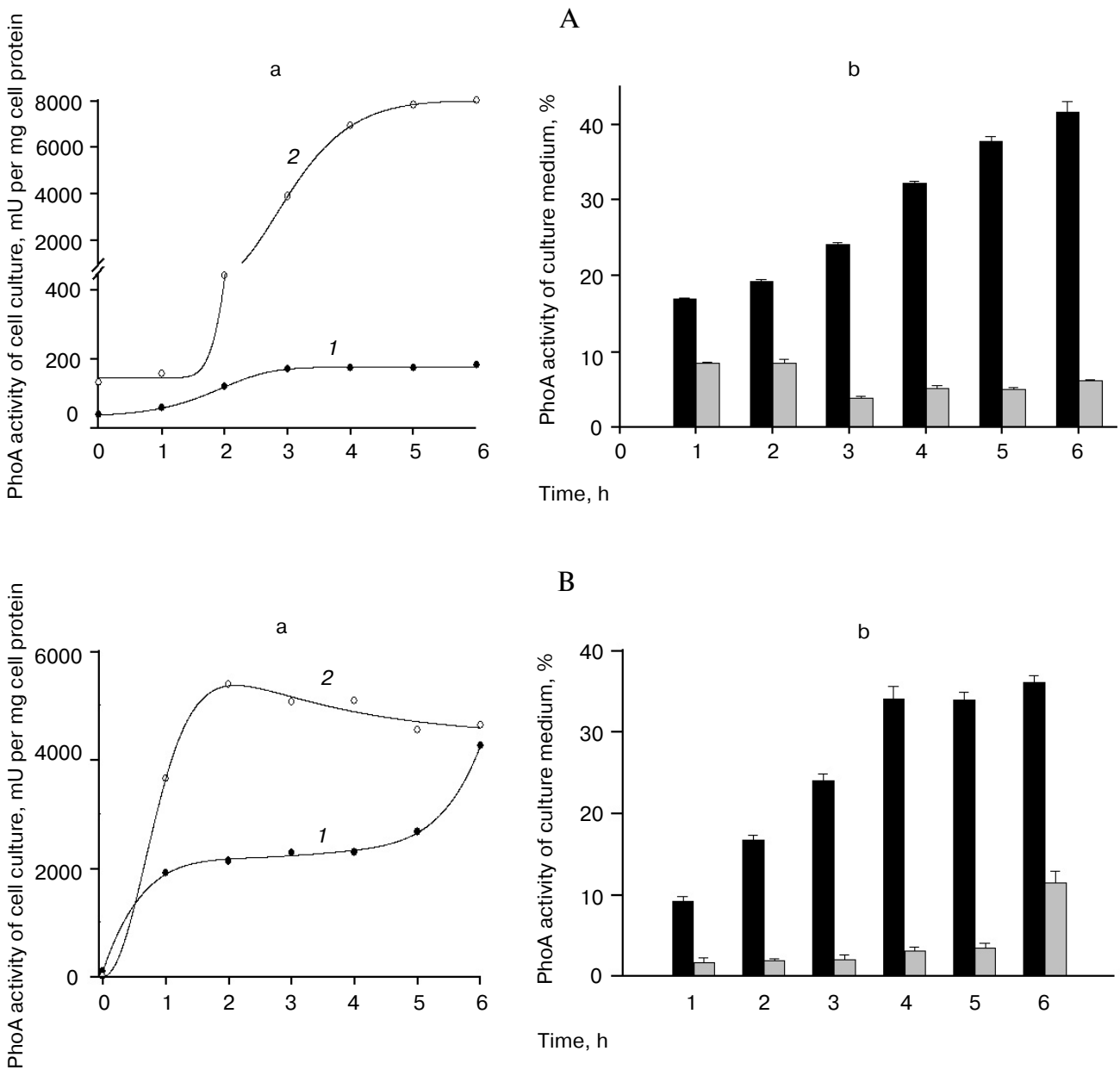

Fig. 3. PhoA activity of cell culture (mU per mg cell protein) (a) and of culture medium (% of total) (b) of *E. coli* AD93 without (curve *1* in panel (a) and dark columns in (b)) and with (curve *2* in panel (a) and light columns in (b)) plasmid pDD72. The enzyme was encoded by the *phoA* gene under control of its own promoter (plasmid pHI-7) (A) and under control of exogenous P_{BAD} promoter (plasmid pSAP1) (B).

(isoform III) [38, 39]. The ratio of these isoforms depends on cultivation conditions and the level of enzyme expression [40, 41]. The comparative analysis of the spectrum of PhoA isoforms and their relative contents in the periplasm and in the medium might answer the question whether protein secretion has a periplasmic stage, where the protein undergoes modification, or whether it proceeds by-passing the periplasm, without post-translocational modification with the formation of protein isoforms. In the current work we have studied the spectrum of periplasmic and extracellular PhoA in strain AD93. The analysis showed that the spectrum of PhoA isoforms depends on the content of PE controlled by plasmid pDD72. Cells of AD93/pDD72 contain three enzyme isoforms, while PE-deficient AD93 cells do not reveal the completely modified form III. This is in accordance with our previous data that the absence of PE depresses not only secretion [17, 18] but also post-translocational modification of PhoA [42], as we suppose, due to depression of secretion of the modifying proteinase, localized in the

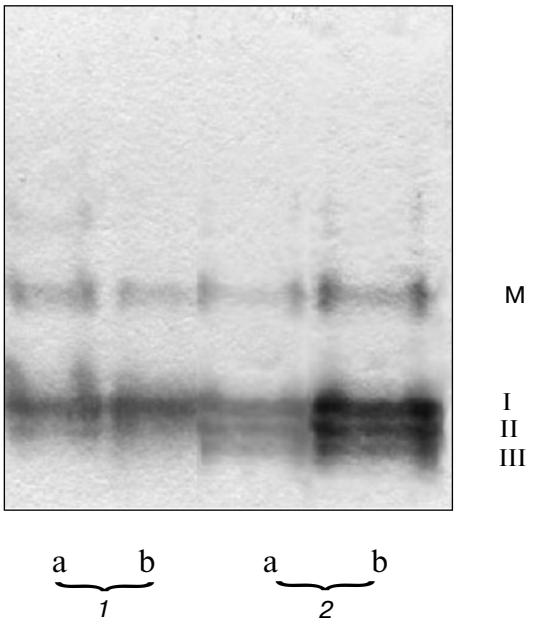

Fig. 4. Isoform spectrum of alkaline phosphatase encoded by the gene constituent of plasmids and localized in the periplasm (a) and culture liquid (b) of PE-deficient cells of *E. coli* AD93 (*1*) and cells of *E. coli* AD93/pDD72 with normal phospholipid composition (*2*). I, II, and III are isoforms; M are multimers of the enzyme.

periplasm like PhoA [43]. The comparison of isoforms in the periplasm and culture medium of cells with both normal and changed phospholipid compositions showed their absolute identity (Fig. 4). This means that the enzyme completes its modification in the periplasm and is secreted into the medium with the same isoenzyme spectrum, evidencing the presence of a periplasmic stage in the secretory pathway to the culture medium. Thus, the change in membrane phospholipid composition has no effect on the mechanism of protein secretion into the medium, influencing only its level.

Increase in PhoA secretion into the medium by altered phospholipid composition correlates with changes in composition and structure of the cell envelope. The most important component of *E. coli* outer membranes, responsible for their barrier properties, is lipopolysaccharide [42]. The analysis of its content depending on membrane phospholipid composition and efficiency of PhoA secretion into the medium revealed a certain correlation between these parameters. The higher efficiency of PhoA secretion into the medium at a changed membrane phospholipid composition correlates with the lower content of lipopolysaccharide under these conditions (Fig. 5). Its content in cells depends on strain nature and growth conditions. The strain AD93 under conditions of phosphorus starvation, with PhoA induction controlled by its own P_{PHO} promoter, had the higher content of lipopolysaccharide than the strain grown in a P_i-containing medium, when PhoA induction proceeded under the control of exogenous P_{BAD} promoter. The highest content of lipopolysaccharide was found in strain HDL11. As stated above, this strain does not contain lipoprotein, and its higher lipopolysaccharide content may be explained by competitive interrelation between the content of these two outer membrane components, which we revealed previously [37]. So, at a change in phospholipid composition, independent of strain and growth conditions, the content of lipopolysaccharide in cells credibly decreases (Fig. 5a), which correlates with the increased level of PhoA secretion into the medium (Fig. 5b). Just the decreased lipopolysaccharide content may be the reason for higher outer membrane permeability for periplasmic proteins on a change in phospholipid composition. The increase of such permeability is supported by the fact that EPS secretion into the medium under the above conditions also

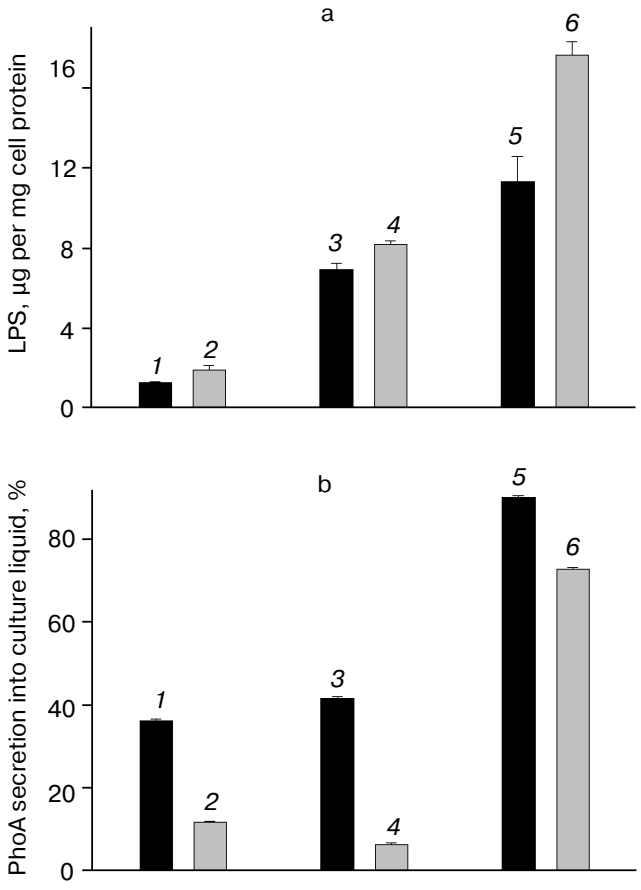

Fig. 5. Content of lipopolysaccharide (LPS, μg per mg cell protein) (a) and PhoA secretion into culture liquid (b) in cells of *E. coli* AD93/pSAP1 (*1*), AD93/pDD72/pSAP1 (*2*), AD93/pHI-7 (*3*), AD93/pDD72/pHI-7 (*4*), HDL11/pSAP1 (–IPTG) (*5*), and HDL11/pSAP1 (+IPTG) (*6*).

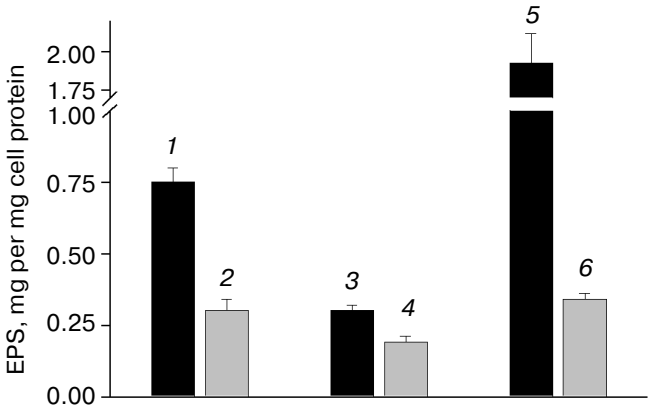

Fig. 6. Content of exopolysaccharide (EPS, mg per mg cell protein) in culture liquid of *E. coli* AD93 (*1*), AD93/pDD72 (*2*), AD93/pSAP1 (*3*), AD93/pDD72/pSAP1 (*4*), AD93/pHI-7 (*5*), and AD93/pDD72/pHI-7 (*6*).

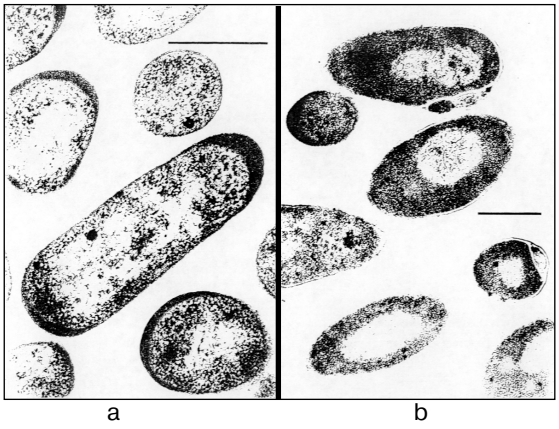

Fig. 7. Electron microscopy of thin sections of *E. coli* AD93/pDD72 cells with normal phospholipid composition (a) and *E. coli* AD93 cells with unbalanced phospholipid composition (b). Both strains secrete alkaline phosphatase encoded by the gene constituent of pHI-7. The scale length is 1 μm.

increases. The content of EPS in PE-lacking cells growing on P_i-containing medium increases 1.5-fold, and on P_i-lacking medium it increases 6-fold (Fig. 6).

The morphology of cells with significant alterations in membrane phospholipid composition changes as well. Cells with the wild type phospholipid composition have a typical envelope, where the outer membrane with peptidoglycan layer is closely attached to the cytoplasmic membrane all along. The periplasm of these cells (particularly on their poles) is filled with homogeneous substance (Fig. 7a), which was previously identified cytochemically and immunocytochemically as alkaline phosphatase under conditions of its induction [4, 6]. However, in cells of PE-depleted strain the periplasm has a considerable volume and is not filled with the above substance, while the outer membrane is far apart from the cytoplasmic membrane (Fig. 7b). Probably, natural contact between outer and inner membranes is disturbed in these cells due to absence of PE, and the periplasm is deficient of proteins because of their excretion into the medium. Attention is drawn to large inclusions localized in the periplasm of these cells, which probably are secondary metabolites of phospholipid exchange formed under its disturbance due to mutations.

DISCUSSION

The current work has revealed for the first time a correlation between the change in membrane phospholipid composition of *E. coli* cells and the level of secretion of periplasmic alkaline phosphatase, as well as EPS, into the culture medium. This effect does not depend on whether the changes are in the content of anionic or zwitterionic phospholipids. Both the decrease in APL content in strain HDL11 and the presence of only anionic phospholipids with complete absence of zwitterionic phospholipid in strain AD93 result in increased secretion of the enzyme from periplasm into the medium. Previously we have observed similar "pseudosecretion" of PhoA during oversynthesis of the enzyme encoded by the gene constituent of plasmids [3-7]. In that case, various mechanisms of secretion were revealed. They included outer membrane fragmentation [8], formation of outer membrane vesicles [4, 5], and formation of zones of close contact between the cytoplasmic and outer membrane [6]. These mechanisms of secretion correlated with specific changes in the envelope chemical composition: increase in the content of lysophospholipids in the membranes by an order of magnitude [8], decrease in the content of lipoprotein and/or lipopolysaccharide [4, 5], or were accompanied by only structural changes [6], respectively. It was assumed that there is a competition between protein secretion and biogenesis of envelope components for the common metabolites involved in both processes, e.g., phospholipids, which results in a changed chemical composition of the envelope and its higher permeability for proteins. The results of the current work support this assumption. A change in membrane phospholipid composition caused by genetic manipulations [20, 21] actually enhances the secretion of periplasmic protein, as well as EPS, into the medium, indicating the higher permeability of the outer membrane. The reason for such increase in permeability was the lower content under the above conditions of lipopolysaccharide, which is known to be responsible [42] for barrier properties of the outer membrane. Metabolic precursors of many envelope components, including those responsible for its permeability, are phospholipids [9, 10]. It is interesting that we have observed an increase in secretion into the

medium even on the background of significant decrease in protein expression and secretion across the cytoplasmic membrane. In this case, increased synthesis or secretion of proteins could no longer be the reason for changes in cell envelope, as shown in all previous works [3-7]. The main reason for a change increasing the outer membrane permeability was, first of all, a significant change in metabolism and composition of phospholipids as metabolic precursors of outer membrane components [44]. The results obtained favor the assumption of a relationship between protein secretion into the medium and cell envelope biogenesis on the level of phospholipid metabolism.

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