Depletion of phosphatidylethanolamine affects secretion of *Escherichia coli* alkaline phosphatase and its transcriptional expression

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Abstract In this report we demonstrate that depletion of the major phospholipid phosphatidylethanolamine, a single non-bilayer forming phospholipid of *Escherichia coli*, significantly reduces the secretion efficiency of alkaline phosphatase in vivo. Secretion, however, is correlated with the content in membranes of cardiolipin, which in combination with selected divalent cations has a strong tendency to adopt a non-bilayer state indicating the possible involvement of lipid polymorphism in efficient protein secretion. Depletion of this zwitterionic phospholipid also inhibits expression of the protein controlled by the endogenous P_{PHO} promoter but not the P_{BAD} promoter, which is suggested to be due to the effect of unbalanced phospholipid composition on the orthophosphate signal transduction system (Pho regulon) through an effect on its membrane bound sensor. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Alkaline phosphatase; Protein expression; Protein translocation; Phosphatidylethanolamine; Pho regulon; *Escherichia coli*

1. Introduction

Phospholipids of *Escherichia coli* have been shown to play important roles in various membrane bound functions including protein translocation across the cytoplasmic membrane [1–4]. The requirement of anionic phospholipids for efficient protein membrane translocation in vivo and in vitro has been clearly demonstrated [1,5–7]. However, the studies addressing the involvement of the major phospholipid of *E. coli*, phosphatidylethanolamine (PE), in protein membrane translocation are limited to experiments in vitro [8], while the effect of this phospholipid on protein secretion in vivo has been partially demonstrated for only one protein, trimethylamine *N*-oxide reductase of the TAT secretory pathway [9]. The non-bilayer forming potential of PE [10,11] has been assumed to be very important for the process of protein translocation [12,13].

The requirement of PE for efficient membrane translocation in vivo of *E. coli* alkaline phosphatase (PhoA), which belongs to the Sec-dependent secretory pathway, has been analyzed in this study. Secretion of this protein is coupled to its biogenesis [14]. The latter includes: biosynthesis in the cytoplasm of precursor protein (prePhoA) containing an additional N-terminal signal peptide; translocation of prePhoA across the cytoplasmic membrane, its cleavage by membrane bound signal peptidase on the outer surface of the cytoplasmic membrane; release of a mature protein into the periplasm and assembly of an active enzyme macromolecule. The main strategy was to analyze the expression and secretion of PhoA in a strain of *E. coli* either containing wild type levels of PE (AD93/pDD72) or completely lacking PE (AD93). AD93 carries a null allele of *pssA* which is required for the synthesis of the precursor to PE. Plasmid pDD72 carries a functional copy of the *pssA* gene [15]. The results of the current work show that not only the secretion of PhoA but also its expression is suppressed when PE is lacking.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

In this study *E. coli* strain AD93 (*pss93::kan recA srl::Tn10 nadB*$^+$) with or without plasmid pDD72 carrying the *pssA* gene was used [15]. Plasmids pHI-7 and pSAP-1 carrying the *phoA* gene under control of its own P_{PHO} promoter [16] or the P_{BAD} promoter of the *araBAD* (arabinose) operon, respectively, were used for the overexpression of PhoA. Plasmid pSAP-1 was constructed in the current work by placing the *phoA* open reading frame behind P_{BAD} of plasmid pBAD18 [17]. The *phoA* gene was amplified by PCR by a mixture of Taq and Vent DNA polymerases using upstream (5′-AATGCTCGAGTACATGGAGAAAATAAAGTG-3′) and downstream (5′-ATCTGGATCCAAGTCTGGTTGCTAACAGC-3′) primers (introduced sites for *Xho*I and *Bam*HI, respectively, are underlined). The products were cloned into the *Xho*I–*Bam*HI sites of vector pBC (Stratagene). Then a *Kpn*I–*Xba*I DNA fragment was subcloned into vector pBAD18.

Bacterial strains were grown at 37°C and strains containing the temperature sensitive plasmid pDD72 at 30°C in mineral medium [18] supplemented with tryptophan (100 μg/ml). Because of the absolute requirement for divalent cations of AD93 strains, the latter were grown in the presence of $MgCl_2$ (50 mM) or $CaCl_2$ (50 mM) [19]. The cations were also added to control strains containing plasmid pDD72. As required, kanamycin (50 μg/ml), chloramphenicol (20 μg/ml), tetracycline (12 μg/ml), ampicillin (50 μg/ml), arabinose (0.5%) were added to the growth media.

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Abbreviations: PhoA and prePhoA, *Escherichia coli* alkaline phosphatase and its precursor, respectively; PE, phosphatidylethanolamine; CL, cardiolipin; Pi, orthophosphate; TCA, trichloroacetic acid; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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2.2. Alkaline phosphatase induction

PhoA belongs to the Pho regulon and is controlled by exogenous orthophosphate (Pi), like other proteins of the regulon, and induced under Pi starvation [20]. For PhoA induction, *E. coli* cells were grown in the mineral medium with 1 mM Pi to mid-log or stationary growth phase, harvested, washed in the medium without Pi (and containing cations for AD93) and then incubated in the same medium. For induction and secretion of PhoA during culture growth, cells were grown in the mineral medium with low Pi (0.01 mM) for 15 h. To induce PhoA encoded by the gene under P_{BAD} promoter control, 0.5% of arabinose was added to the culture that was grown in the mineral medium with excess Pi to the mid-log phase. It should be mentioned that PhoA expression from the chromosome is completely repressed under these conditions of growth (in the presence of Pi). Culture samples were taken at time intervals for analysis. Biosynthetic processes were stopped by 0.02% thiomersal.

2.3. Alkaline phosphatase secretion

Two approaches were used to examine PhoA secretion. One measures specific PhoA activity of the cell culture, since PhoA becomes active only after translocation into the periplasm [14]. The other analyzes the dynamics of conversion of pulse labeled prePhoA into the mature protein (maturation) in pulse chase experiments in vivo, because the above conversion also occurs after protein translocation across the membrane.

2.4. Alkaline phosphatase maturation

Pulse chase experiments were used to analyze prePhoA maturation according to the method of Michaelis et al. [21]. *E. coli* cells, incubated for 20 min with 0.5% arabinose to induce PhoA synthesis, were labeled with L-[^{35}S]methionine for 30 s and chased for various periods of time by addition of unlabeled methionine. PhoA and prePhoA were immunoprecipitated using affinity purified rabbit antibodies against PhoA and separated by 10% SDS–PAGE followed by autoradiography [22]. Quantification of the relative quantities of precursor and mature PhoA was made using a LKB UltroScan laser densitometer and calculated with the adjustments of additional methionine residues in the prePhoA.

2.5. Immunoblotting of alkaline phosphatase

After SDS–PAGE the gel was electroblotted on BA-85 nitrocellulose (Schleicher and Schuell), and PhoA was immunostained using rabbit antibodies against PhoA and protein A conjugated horseradish peroxidase (Bio-Rad) [23].

2.6. Phospholipid analysis

Lipids were extracted according to Ames et al. [24] and separated on boric acid impregnated thin layer silica gel plates (Kieselgel 60, Merck) in one dimension using chloroform:methanol:water:ammonium hydroxide (60:37.5:3:1 (v/v)) as described in [25]. Individual phospholipid spots were detected in iodine vapor, cut out, extracted from silica gel by chloroform:methanol:water (5:5:1 (v/v), and then quantified spectrophotometrically at 660 nm after staining with malachite green by analysis of lipid phosphorus content as described [26].

2.7. Synthesis of total protein and RNA

Synthesis of total protein and RNA was assayed by the incorporation of [^{35}S]methionine and [^{14}C]uracil, respectively, into trichloroacetic acid (TCA) insoluble material that was isolated on Microfibre GF/F filters (Whatman). Radioactivity was counted in a toluene scintillator in a gamma counter (SL-30, Intertechnique).

2.8. Analytical methods

Protein separation was performed in 10% SDS–PAGE [27]. The PhoA activity was assayed by the rate of *p*-nitrophenylphosphate hydrolysis in 50 mM Tris–HCl, 5 mM $MgCl_2$ buffer pH 8.5, as described earlier [18], taking the amount of enzyme hydrolyzing 1 μmol of substrate per minute at 37°C as a unit (U) of enzymatic activity. The PhoA activity was measured in whole culture (cells+culture liquid) because PhoA encoded by gene cloned in plasmid is partially secreted into the medium [28]. Protein was assayed by the method of Lowry et al. as described [29].

3. Results

3.1. PE is required for E. coli alkaline phosphatase translocation across the cytoplasmic membrane

To estimate the requirement of PE for PhoA secretion, PhoA activity was determined in cells of strain AD93 with PE (carrying plasmid pDD72) or without PE (lacking plasmid pDD72 and grown with Mg^{2+} or Ca^{2+}). The phospholipid composition of the strains is shown in Table 1. Mg^{2+} grown PE deficient cells showed about 10-fold lower PhoA activity than cells of the control strains containing PE (Fig. 1a). When cells were grown in the presence of Ca^{2+}, they were even less active (15-fold and more) than PE containing cells. This was characteristic of PhoA encoded by the chromosomal gene and the plasmid pHI-7 encoded gene, both under the control of their own promoter.

The dynamics of conversion of pulse labeled precursor into a mature protein was studied in pulse chase experiments to obtain more strict evidence of the effect of PE on secretion. The cells of the AD93/pSAP-1 strain grown in the presence of Mg^{2+} and the same strain containing plasmid pDD72 were used for this analysis. Fig. 2 shows that PE depletion significantly decreases the rate of prePhoA conversion into the mature protein. In AD93 cells, non-translocated precursor is found even after 5 min of chase. The control strain AD93/pDD72 shows the presence of exclusively mature PhoA after 30 s of chase. The data clearly suggest that the zwitterionic phospholipid PE is required for efficient PhoA secretion.

However, the effect of PE depletion on PhoA secretion turned out to depend on cell growth and was weaker in growing cells (Fig. 1b). Indeed, the activity of PhoA (the product of both the chromosomal gene and the gene cloned in plasmid pHI-7) in PE deficient cells growing in medium with low Pi was only three-fold lower in the presence of Mg^{2+} and six-fold lower in the presence of Ca^{2+} than in PE containing cells (cf.

Table 1
Phospholipid composition of the *E. coli* AD93 strains

Strain (*E. coli*)	Cation (50 mM)	Phospholipid content (mol.%)			
		CL	PG	PA	PE
AD93	Mg^{2+}	41.7 ± 3.7	55.3 ± 3.9	3.0 ± 0.2	–
	Ca^{2+}	15.8 ± 1.9	81.3 ± 4.2	2.9 ± 0.3	–
AD93/pHI-7	Mg^{2+}	47.2 ± 3.5	47.6 ± 2.8	5.1 ± 0.7	–
	Ca^{2+}	14.6 ± 1.9	80.9 ± 4.7	4.5 ± 0.6	–
AD93/pSAP-1	Mg^{2+}	32.1 ± 3.0	61.6 ± 3.6	6.3 ± 0.7	–
	Ca^{2+}	16.5 ± 2.0	79.5 ± 4.7	4.0 ± 0.6	–
AD93/pDD72	–	2.8 ± 0.3	19.1 ± 1.8	1.6 ± 0.2	76.5 ± 4.8
AD93/pHI-7/pDD72	–	2.1 ± 0.2	18.8 ± 2.0	0.4 ± 0.2	78.7 ± 5.1
AD93/pSAP-1/pDD72	–	4.0 ± 0.5	14.8 ± 1.7	2.7 ± 0.3	78.6 ± 4.5

CL, cardiolipin; PG, phosphatidylglycerol; PA, phosphatidic acid. The data given are the mean values ± S.D. of five independent experiments.

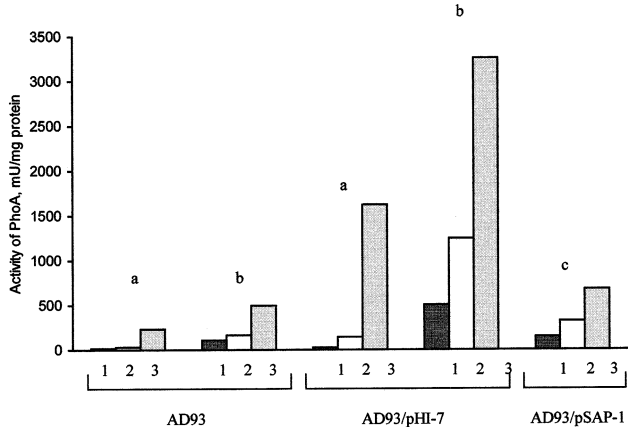

Fig. 1. Effect of PE depletion on secretion of PhoA in the *E. coli* AD93 strains. PhoA synthesis was induced by complete Pi starvation (no culture growth) (a), during culture growth in the medium with low Pi (b), and by arabinose during culture growth in the medium under Pi excess (c). AD93 strains were grown in the presence of Ca^{2+} (1) or Mg^{2+} (2). AD93 strains containing an additional plasmid pDD72 (3) were used as a control. Addition of Mg^{2+} to the control strains (AD93 containing plasmid pDD72) had no effect on PhoA activity; Ca^{2+} decreased it two-fold, but its effect was much weaker than the effect of PE depletion. The data given are the mean values of three independent experiments, S.D. values never exceeding 10%.

10- and 15-fold decrease, respectively, under no culture growth). Besides, when the *phoA* gene was under the control of the P_{BAD} promoter and induced during active culture growth in the complete medium with Pi, PhoA activity was only 2–4-fold lower in Mg^{2+} and Ca^{2+} grown cells of AD93/pSAP-1, respectively, in contrast to the strain containing PE (Fig. 1c). Thus, cell growth is an important factor necessary for protein secretion in the cells lacking PE. The interrelationship between culture growth and the effect of PE depletion on PhoA secretion was also supported in experiments with cells containing PE or lacking PE, where growth was inhibited by the antibiotic cephalexin [30]. This antibiotic had no effect on PhoA secretion in the strain containing PE but suppressed secretion almost two-fold in the mutant strain lacking PE and grown with Mg^{2+} (data not presented).

Earlier it was shown that the membrane of strain AD93 lacking PE contains only anionic phospholipids: phosphatidylglycerol and cardiolipin (CL), and their relative contents strictly depend on the nature of cations in the rich growth medium [15,19]. In addition, we obtained such dependence under conditions of cell growth in a mineral medium, as well as the dependence on the age of a culture [31]. This allowed us first to study the protein secretion efficiency in vivo depending on the content of CL, which in the presence of selected cations was shown to be able to form non-bilayer structures in vitro [11,19]. Indeed, the current work has revealed a distinct correlation between CL content regulated by the above factors and the activity of PhoA in strains AD93 (data are not presented) and AD93/pHI-7 (Fig. 3). This correlation was shown for the PhoA encoded by a gene under its own promoter control and synthesized in conditions of complete Pi starvation. AD93/pHI-7 cells grown in the presence of Ca^{2+} to the mid-log or stationary phase and containing only 13 and 15% of CL, respectively, have nine- and six-fold lower PhoA activities than Mg^{2+} grown cells of the same age containing 37 and 47% of CL, respectively.

3.2. Depletion of PE inhibits the expression of PhoA

PhoA immunoblotting showed (Fig. 4) that PE depletion significantly decreases the total amount of the enzyme protein which correlates with a decrease in enzyme activity (Fig. 1). The incorporation of radioactively labeled protein precursor [^{35}S]methionine into cells showed no significant difference in the rate of total protein synthesis depending on the PE content (data not presented). However, the relative content of immunoprecipitated radioactive PhoA expressed under its own promoter control in the total pool of radioactive protein was 2–5-fold lower in cells lacking PE than in cells of the control strain. This could be evidence of an inhibitory effect of PE depletion on the synthesis of PhoA and probably of other proteins of the Pho regulon at the level of RNA transcription. Indeed, the incorporation of RNA precursor [^{14}C]uracil into a TCA insoluble fraction after induction of the *phoA* gene controlled by its own P_{PHO} promoter in cells lacking PE was suppressed about six-fold as compared with that of PE containing cells (Fig. 5A). No significant inhibition of the total RNA synthesis was found when the *phoA* gene was expressed under the P_{BAD} promoter control at Pi excess (Fig. 5B). If PhoA was induced in this strain under Pi starvation, the effect of depletion of PE was higher, about two-fold. This indicates the inhibition of expression of other proteins of the Pho regulon induced under these conditions (Fig. 5A). All the above data suggest that unbalanced membrane phospholipid composition affects the expression of the Pho regulon, to which alkaline phosphatase belongs.

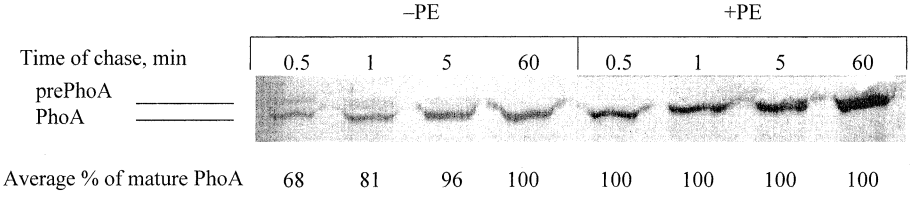

Fig. 2. Effect of PE depletion on PhoA maturation in *E. coli* AD93/pSAP-1. After PhoA induction by 0.5% arabinose for 20 min, cells were pulse labeled with L-[^{35}S]methionine (50 μCi/ml) for 30 s, and radioactivity was chased for the indicated period of time by addition of unlabeled methionine to a final concentration of 0.05%. Pulse labeled PhoA and its precursor were immunoprecipitated and resolved by 10% SDS–PAGE followed by autoradiography and densitometry. The total amount of PhoA and prePhoA was set at 100% for each point of the chase time. The relative quantity of mature PhoA was calculated at the indicated times with adjustments of additional methionine residues in the prePhoA. Radioautograph presents the data of the typical experiment of three independent experiments, the data of the relative quantity (in %) of mature PhoA are the mean values of these experiments; S.D. values do not exceed 10%.

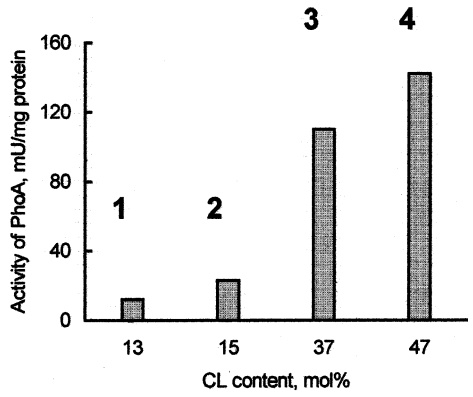

Fig. 3. The efficiency of PhoA secretion in strain AD93/pHI-7 depending on CL content in the membranes. Cells were grown with Ca^{2+} (1) or Mg^{2+} (3) to the mid-log phase and with Ca^{2+} (2) or Mg^{2+} (4) to the stationary phase. The data given are the mean values of three independent experiments, S.D. values never exceeding 10%.

4. Discussion

At present, the role of individual phospholipids in specific membrane functions is not completely understood. Mutants with defects in the biosynthesis of particular classes of phospholipids provide a powerful tool to study different membrane associated processes and specifically the involvement of phospholipids in protein translocation across membranes [1–4]. The *pss93* mutant of *E. coli* lacks PE due to interruption of the *pssA* gene [15] that encodes phosphatidylserine synthase. It was shown with the use of this mutant that PE acts as a molecular chaperone in the folding of the polytopic membrane protein lactose permease [32,33]. In *E. coli*, PE is the major constituent of the cytoplasmic membrane as well as the major lipid that strongly prefers adopting a non-bilayer structure [10,11]. CL in the presence of selected divalent cations also has a strong tendency to adopt a non-bilayer state [11,19], furthermore, Ca^{2+} is more effective than Mg^{2+}. Besides, the non-bilayer structure is formed preferably in membrane regions with the higher curvature of lipid bilayer, located near the cell poles in the places of septum formation at cell division where CL is assumed to be preferably localized [30]. It has been proposed that the non-bilayer structure of membranes, in particular the balanced presence of bilayer and non-bilayer forming lipids, may be necessary to facilitate protein translocation across the membrane [12,13]. This idea was first confirmed in experiments in vitro with membrane vesicles from strains lacking PE [8]. In the current study, the first evidence of a requirement of PE for protein secretion was obtained in vivo by two independent methods (including pulse chase experiments). In addition, the secretion assayed by enzymatic activity of PhoA depends on culture growth and the content of CL in the membranes. The data of the current work allow us to suggest a possible involvement of lipid polymorphism in efficient secretion. In bacterial cells, non-bilayer phospholipid PE was suggested to facilitate membrane insertion of preproteins due to the larger headgroup spacing of anionic phospholipids in PE containing membrane [8] and its effect on the lateral membrane pressure and/or optimal matching of the protein–lipid interface [34].

Surprisingly, the depletion of PE has been shown here to profoundly affect not only PhoA secretion as a membrane function but also the central and apparently non-membrane event of regulation of protein expression by transcriptional level. The causal relationship between phospholipid mutations and the lower rate of transcription of the *flhD* master operon [35] resulting in impairment of flagellin formation [35–37], or activation of *micF* transcription that decreases the OmpF level, despite the nature of the phospholipid depleted [38], and functioning of the Cpx system [39] was shown earlier. However, the possible mechanism of this relationship has not been

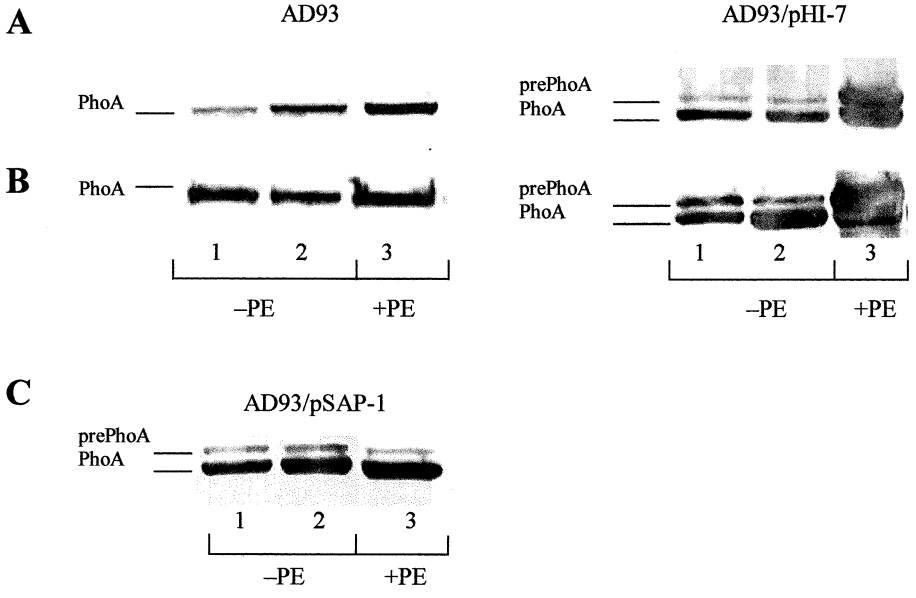

Fig. 4. Effect of PE depletion on PhoA expression in *E. coli* AD93 strains by PhoA immunoblotting analysis. PhoA synthesis was induced by complete Pi starvation (no culture growth) (A), during culture growth in the medium with low Pi (B), and by arabinose during culture growth in the medium under Pi excess (C). Cells were grown in the presence of Ca^{2+} (1) or Mg^{2+} (2). AD93 strains containing an additional plasmid pDD72 (3) were used as a control. Equivalent amounts of cell protein (10–20 μg) were analyzed by PAGE. Data of a typical experiment out of three to six independent experiments are presented.

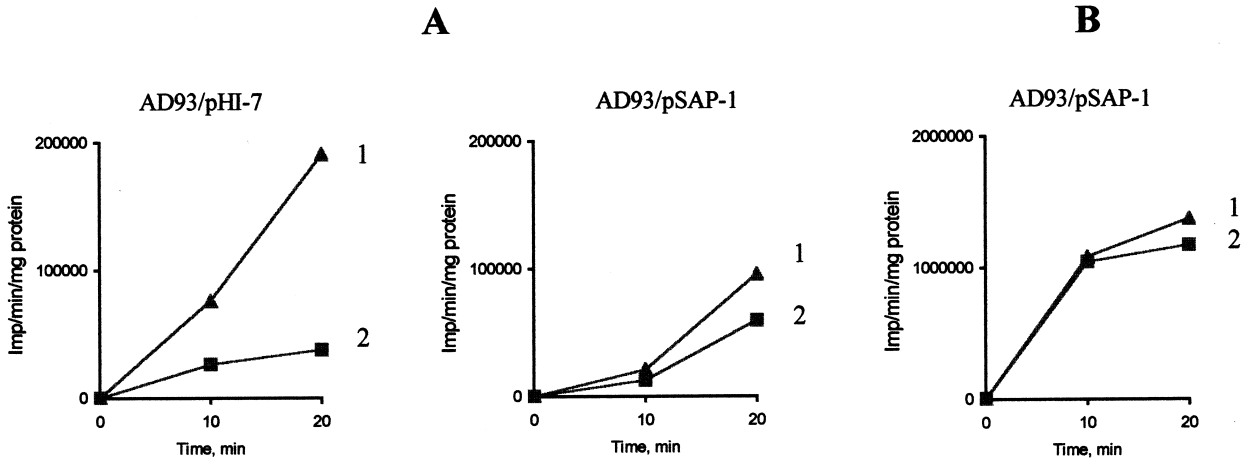

Fig. 5. Effect of PE depletion on total RNA synthesis in *E. coli* AD93 strains. Cells were labeled with [^{14}C]uracil (0.05 mCi/ml) simultaneously with PhoA induction. Samples were taken at the indicated periods of time and precipitated by equal volumes of cold 20% TCA followed by filtration and counting of radioactivity. AD93 strains were grown in the presence of Mg^{2+} (2). AD93 strains containing an additional plasmid pDD72 were used as a control (1). Cells were grown under Pi starvation (A) and Pi excess (B). The data given are the mean values of three independent experiments, S.D. values never exceeding 10%.

explained. In the present study we have shown the effect of unbalanced phospholipid composition on the expression of a protein encoded by a gene controlled not by the P_{BAD} promoter but by the P_{PHO} promoter, which belongs to the Pho regulon, a member of the two component regulatory system of signal transduction. In this connection, it is interesting and important to note that all the above described cases of the effect of unbalanced phospholipid composition also concern members of the two component regulatory systems. The latter are known to consist of membrane bound sensor proteins (receptors/transmitters) and cytoplasmic regulators (regulatory proteins) interacting with DNA operators [40]. Probably, dramatic changes in the phospholipid composition of phospholipid mutants, independently of the nature of these changes, may affect the conformation of membrane bound sensors or their assembly and thus affect the signal transduction needed for the function of regulatory genes.

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