Requirement for phospholipids of the translocation of the trimethylamine *N*-oxide reductase through the Tat pathway in *Escherichia coli*

Nathalia I. Mikhaleva[a,b], Claire-Lise Santini[a], Gérard Giordano[a], Marina A. Nesmeyanova[b], Long-Fei Wu[a,*]

[a] *Laboratoire de Chimie Bactérienne, UPR9043 CNRS, Institut de Biologie Structurale et Microbiologie, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France*
[b] *Laboratory of Protein Secretion in Bacteria, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region 142292, Russia*

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Abstract Trimethylamine *N*-oxide reductase (TorA) is an anaerobically synthesized molybdoenzyme. It is translocated across the cytoplasmic membrane in a folded conformation via the Tat pathway of *Escherichia coli*. The requirement for phospholipids for the export of this enzyme was analyzed in the *pgsA* and *pss* mutants lacking anionic phospholipids and phosphatidylethanolamine, respectively. Anaerobic growth did not influence phospholipid composition of the *pgsA* and *pss* mutants. Interestingly, both *pgsA* and *pss* mutations severely retarded the translocation of TorA into the periplasm. Therefore, translocation of proteins through the Tat pathway is dependent on the anionic phospholipids and on lipid polymorphism.

Key words: Trimethylamine *N*-oxide reductase; Twin arginine signal sequence; Folded conformation; Protein translocation; Phospholipid; Anaerobic growth

1. Introduction

Proteins synthesized in the cytoplasm but destined for the periplasmic space or outer membrane have to cross the plasma membrane. This process can be divided into several steps: efficient targeting to the membrane, translocation across this membrane, and processing into the mature form by cleavage of an N-terminal signal sequence. Beside their function in establishing the permeability barrier for cells, phospholipids play a very important role in various steps of protein translocation. The inner membrane of *Escherichia coli* consists of a major zwitterionic phospholipid, phosphatidylethanolamine (PE), and two anionic phospholipids, phosphatidylglycerol (PG) and diphosphatidylglycerol or cardiolipin (CL) [1]. Analysis of the interrelation between the secretion of PhoA and phospholipid exchange led to the suggestion of a dynamic involvement of phospholipids in protein translocation [2]. Mutants with defects in the biosynthesis of particular classes of phospholipids provide a powerful tool to study this involvement. The *pss* mutation abolishes the synthesis of PE, which is a non-bilayer preferring lipid, and affects membrane protein assembly [3]. The membrane of this mutant strain consists of large amounts of PG and CL and these cells require millimolar concentrations of specific divalent cations for growth. These divalent cations shift the phase preference of CL towards a non-bilayer phase and allow the strain to maintain a balance between bilayer and non-bilayer forces in the membrane in the absence of PE. In this mutant, the Sec-dependent protein translocation system appears to function normally in the absence of PE [4]. Moreover, by adjustment of the level of CL with the divalent cations it was shown that non-bilayer lipids are essential for efficient protein transport across the cytoplasmic membrane of *E. coli* [3]. The *pgsA* mutation strongly reduces the level of the major anionic phospholipid PG and results in a significant retardation in the Sec-dependent translocation of outer membrane proteins such as OmpA and PhoE [5], and in the SecA-independent translocation of M13 procoat [6]. In addition, the anionic phospholipids are determinants of membrane protein topology [7].

Recently, a novel Sec-independent, ΔpH-driven protein export pathway was identified in *E. coli* K-12 [8–12]. Proteins exported by this pathway are synthesized with peculiar cleavable targeting signals with an essential twin arginine motif. This pathway has been thus designated the twin arginine translocation (Tat) pathway. The particularity of the Tat pathway is that it is capable of translocating protein in folded conformation, even as an oligomeric complex across the cytoplasmic membrane [8,13–15]. The mechanism used for exporting folded proteins across the phospholipid bilayer raises a fundamental question of biology. In this study, we analyzed the involvement of phospholipids in the translocation of trimethylamine *N*-oxide reductase (TorA), which is a very well characterized substrate of the Tat pathway of *E. coli*.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

Bacterial strains HDL11 (*pgs::kan*, φ(*lacOP-pgsA*$^+$)1 *lacZ′ lacY*::Tn*9* *lpp2 zdg*::Tn*10*) [16] and AD93/pDD72 (*pss93::kan recA srl*::Tn*10 nadB*$^+$/*pss*$^+$ *cam*) [17] were routinely grown at 37°C and 30°C in LB and 2YT media, respectively [18]. Microaerobic and anaerobic growths were achieved normally in stoppered bottles or tubes filled to the top and on plates in GasPak anaerobic jars (BBL Microbiology Systems), respectively. $MgCl_2$ (50 mM), $CaCl_2$ (50 mM), kanamycin (50 μg/ml), chloramphenicol (20 μg/ml), tetracycline (10 μg/ml), isopropylthiogalactoside (IPTG) (50 mM), maltose (0.2%) and trimethylamine *N*-oxide (TMAO) (1 mg/ml) were added to the growth media as required. For curing plasmid pDD72, culture was inoculated in 1 ml 2YT medium from an overnight preculture at 50-fold dilution and incubated at 42°C for 4 h. About 10^6 cells were then spread on a 2YT plate. The absence of the plasmid from the mutant AD93 was confirmed by its growth only in the presence of magnesium.

*Corresponding author. Fax: (33)-491-718 914.
E-mail: wu@ibsm.cnrs-mrs.fr

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2.2. Preparation of subcellular fractions, immunoblotting and enzyme assays

Periplasmic and cytoplasmic fractions were prepared by lysozyme/EDTA/cold osmoshock and ultracentrifugation as described previously [8,13]. Cellular fractionation was monitored by following the distribution of the cytoplasmic protein NarJ by immunoblot. Immunoblot was performed using the ECL method according to the manufacturer's instructions (Amersham Corp.). Quantification of the chemiluminescence-generated bands was performed using the ImageQuant program (Molecular Dynamics). TorA activity was measured by following the TMAO-linked oxidation of benzyl viologen spectrophotometrically at 600 nm or by activity staining as described [8].

2.3. Phospholipid analysis

Lipids were extracted from cells by the method of Ames [19] and separated on boric acid-impregnated thin-layer silica gel plates (Kieselgel 60, Merck) in one dimension using chloroform:methanol:water:ammonium hydroxide (60:3.75:3.38:0.62) as described in [20]. Individual phospholipid spots were cut out, and extracted with chloroform:methanol:water (5:5:1) and then quantified spectrophotometrically at 660 nm after staining with malachite green as described [21].

2.4. Pulse chase and immunoprecipitation

Cells were grown anaerobically in 5–10 ml M9 minimal medium supplemented with 19 amino acids (50 μg/ml for each one, without methionine), 0.2% maltose, 1 mg/ml TMAO, 1 μM sodium molybdate and thiamine (16.5 μg/ml) with or without IPTG. One ml cultures of a cell density of 2.5×10^8 cells/ml were pulse-labeled for 1 min by the addition of 50 μCi [^{35}S]methionine and cysteine per ml and then chased by the addition of non-radioactive methionine to a final concentration of 3.4 mM for the times as indicated in Section 3. Immunoprecipitation and separation of sample by SDS-PAGE were performed according to [8]. Dried gels were digitized by Storm PhosphorImager and signals were analyzed by ImageQuant program (Molecular Dynamics).

3. Results

3.1. Influence of anaerobic growth condition on the phospholipid composition of E. coli cells

To date, the phospholipid composition of *E. coli* has been analyzed only for aerobically grown cells. The phospholipids of *E. coli* K-12 consist of ~75% PE, ~18% PG and ~5% CL [19]. The *pgsA* gene encodes the PG synthase which catalyzes the committed step in the biosynthesis of PG and CL. The strain HDL11 bears a ϕ(*lacOP-pgsA*$^+$) gene fusion in a *pgsA::kan* null allele background, its anionic phospholipid content can be controlled by the inducer of the *lac* operon IPTG. In the absence of IPTG, the level of PE of HDL11 increases slightly to 91% while the levels of PG and CL drop to 2 and 1% of total phospholipids [16]. The addition of 50 mM IPTG restores the levels of PE, PG and CL to 79%, 16% and 3% respectively. A strain carrying a null allele of *pssA* completely lacks PE (< 0.1%) and is viable dependent on divalent cation (Ca^{2+}, Mg^{2+} and Sr^{2+}) supplementation [3]. In the *pssA* mutant, PG and CL account for about 90% of the total phospholipid in about equal molar amounts when grown in Mg^{2+} or Sr^{2+} and in about a 2:1 molar ratio when grown in Ca^{2+} [3].

Since TorA is an anaerobic enzyme, we first analyzed the influence of anaerobic growth condition on the phospholipid composition of the *pgsA* and *pss* mutants. When HDL11 (*pgsA*) was grown anaerobically without IPTG, its phospholipid composition was 80.9% PE, 5.3% PG and 2.3% CL (Table 1). The addition of 50 mM IPTG increased the levels of PG and CL to 13.7% and 5.7%, respectively. These results are close to those obtained for aerobically grown cells. As expected, the phospholipid composition of the *pss* mutant carrying a wild type *pss* allele on plasmid pDD72 was the same as its parental wild type strain. The curing of plasmid pDD72 completely abolished the synthesis of PE in the *pss* mutant, and PG and CL represented 57.8% and 39.2% of total phospholipids of the cells grown with 50 mM Mg^{2+} and 77.4% and 19.5% of total phospholipids of those grown with 50 mM Ca^{2+}, respectively. As a consequence, the change in phospholipid composition of anaerobically grown mutants showed the same trends as in aerobically grown cells.

3.2. Anionic phospholipids are required for efficient transport of TorA

To assess the influence of the anionic phospholipids on the biosynthesis and translocation of TorA, HDL11 was grown with or without IPTG to the early exponential phase. Phospholipid contents of the cells were analyzed, only the incubation in the presence of IPTG allowed a normal biosynthesis of the anionic phospholipids. After washing the cells, we then induced the biosynthesis of TorA by the addition of TMAO in the growth medium with or without IPTG. The results are presented in Fig. 1A. TorA was normally translocated into the periplasm of cells that were previously incubated with IPTG, regardless of whether TorA was synthesized in the presence or in the absence of IPTG (Fig. 1A, lanes 1 and 5 compared to lanes 2 and 6). However, the amount of TorA was reduced in the periplasm of the cells that were pre-incubated in the absence of IPTG, especially when TorA was subsequently synthesized without concurrent synthesis of the anionic phospholipids (Fig. 1A, lane 4 versus lane 3). In addition, the decrease of periplasmic TorA was in parallel with an accumulation of TorA in the cytoplasm (Fig. 1A, lanes 3, 4, 7 and 8) and the total TorA activity remained the same under all growth conditions. Therefore, the depletion of anionic phospholipids apparently has no influence on the biosynthesis of TorA. However, it severely impairs the translocation of TorA. The necessary concomitant biosynthesis of anionic phospholipids and TorA for an efficient TorA translocation suggests that the anionic phospholipids play a significant role in the initiation step of the export.

Table 1
Phospholipid content of *pgsA* and *pss* mutants grown under anaerobic condition

Strain	Growth condition	Phospholipid content (in mol %)[a]			
		PE	PG	CL	PA
HDL11 (*pgsA*)	−IPTG	80.9	5.3	2.3	11.5
	+IPTG	77.2	13.7	5.7	3.4
AD93 (*pss*)	+pDD72	73.2	16.8	6.0	4.0
	+$MgCl_2$	< 0.1	57.8	39.2	3.0
	+$CaCl_2$	< 0.1	77.4	19.5	3.1

[a]PE: phosphatidylethanolamine; PG: phosphatidylglycerol; CL: cardiolipin; PA: phosphatidic acid.

To compare the effect of depletion of the anionic phospholipids on the Tat pathway with that on the Sec pathway, we studied the translocation of NikA which is a periplasmic nickel-binding protein involved in specific nickel uptake [22]. It is synthesized under anaerobic condition and contains the typical Sec-dependent signal sequence. Using the same fraction as used in the study of the TorA translocation, we found equal amounts of NikA in the periplasm of the mutant whether its anionic phospholipids were depleted or not (Fig. 1B, lanes 1–4). Therefore, unlike the translocation of proteins via the Tat pathway, the retardation of the translocation of NikA through the Sec pathway was not observed under these conditions.

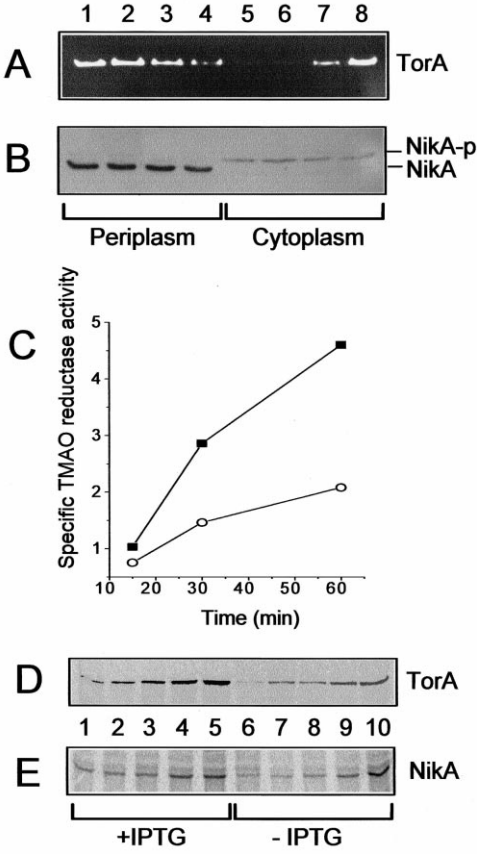

Fig. 1. Effect of PG and CL content on the in vivo synthesis and translocation of *E. coli* periplasmic enzyme TorA. A and B: HDL11 was grown anaerobically at 37°C in LB medium with (lanes 1, 2, 5 and 6) or without (lanes 3, 4, 7 and 8) IPTG until $OD_{600} = 0.3$. Cells were washed and resuspended in LB medium plus 1 mg/ml TMAO with (lanes 1, 3, 5 and 7) or without (lanes 2, 4, 6 and 8) IPTG for an additional 1.5 h incubation. Cells were harvested and the periplasm (8 μg of protein each) and cytoplasm (50 μg of protein each) were separated on a 10% native gel for detection of TorA activity by activity staining (A) or on a 10% SDS gel for analyzing NikA by immunoblotting (B). TorA, precursor (NikA-p) and mature form of NikA are indicated on the right. C: The biosynthesis of TorA was induced by the addition of TMAO in the culture without IPTG at the mid-exponential growth phase with (closed square) or without (open circle) concomitant addition of IPTG. Cells were harvested at different times as indicated and the specific TorA activity (μmol of TMAO reduced/min/mg protein) detected in the periplasm is presented. Pulse-labeled cells grown with (+IPTG) and without (−IPTG) IPTG were harvested and fractionated. The periplasmic fractions were immunoprecipitated using antisera against TorA (D) or NikA (E) and quantified with the ImageQuant program as described in Section 2. The chase times are 0 (lanes 1 and 6), 1 (lanes 2 and 7), 5 (lanes 3 and 8), 10 (lanes 4 and 9) and 20 min (lanes 5 and 10) in D; and 0 (lanes 1 and 6), 0.5 (lanes 2 and 7), 1 (lanes 3 and 8), 5 (lanes 4 and 9) and 10 min (lanes 5 and 10) in E.

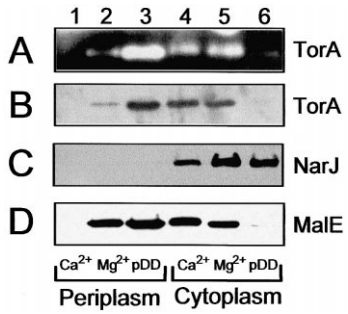

Fig. 2. Translocation of TorA in strain AD93 lacking PE. The *pss* mutant carrying plasmid pDD72 (lanes 3 and 6) or without *pss*$^+$ allele was grown in 2YT medium with 50 mM $CaCl_2$ (lanes 1 and 4) or 50 mM $MgCl_2$ (lanes 2, 3, 5 and 6) at 30°C or 37°C under anaerobic condition for 12 h. The periplasm (5 μg of protein each) and cytoplasm (25 μg of protein each) were separated on a 10% native gel (A and B) or 10% SDS gel (C and D). The native gel was first used to analyze TorA activity by activity staining (A), and the same gel was then used to quantify TorA protein by immunoblotting using antibody against TorA (B). In addition, the specific TorA activity was quantified by enzyme assay with data obtained from at least three sets of independent experiments and indicated in the text. The cellular distribution of MalE and NarJ was analyzed by immunoblotting using antibody against NarJ (C) and MalE (D), respectively.

We then analyzed the kinetics of TorA translocation by monitoring the amount of TorA found in the periplasm of HDL11. Biosynthesis of TorA was induced by adding TMAO in cultures of HDL11 at mid log phase. Samples were prepared at 15, 30 and 60 min after the induction, and assayed for total TorA activity and for periplasmic TorA activity. The same increasing levels of total TorA activity were detected in the mutant grown with IPTG compared to that without IPTG at all time points. These results thus confirm that depletion of the anionic phospholipids has no influence on TorA biosynthesis. However, the amount of TorA translocated into the periplasm of HDL11 depleted of anionic phospholipids was severely hampered compared with that in the same strain containing a normal quantity of anionic phospholipids (Fig. 1C). Taken together, these results clearly show that depletion of the anionic phospholipids severely affects the translocation of TorA.

To gain further kinetic information on the translocation of TorA and NikA in the *pgsA* mutant, we performed pulse chase and immunoprecipitation experiments. The depletion of anionic phospholipids considerably slowed down the translocation of both TorA and NikA (Fig. 1D,E, lanes 6–10 compared to lanes 1–5). However, a difference in the retardation was observed. The TorA found in the periplasm of the cells depleted of anionic phospholipids amounted to less than 50% of the level of the cells with anionic phospholipids after a 20 min chase (Fig. 1D, lane 10 compared to lane 5). In contrast, NikA detected in the periplasm of the cells depleted of anionic phospholipids increased to 60% of the level of cells with normal phospholipid content after a 5 min chase (Fig. 1E, lane 9 compared to lane 4). At the 10 min chase point, the same amount of NikA was found in the periplasm of both types of cells (lanes 10 and 5). These results show that the

slight retardation of NikA translocation can be observed by the sensitive pulse chase experiment, but not by the immunoblot. Most importantly, they suggest a higher dependence on anionic phospholipids for an efficient translocation of proteins through the Tat system than that via the Sec pathway.

3.3. Non-bilayer phospholipid is important for efficient translocation of TorA

The *pss* mutant lacks PE and its viability depends on the addition of specific divalent cations in the growth medium, which regulate the lipid polymorphism [4]. We assessed the functional consequences of the depletion of PE and of regulation of lipid polymorphism in the export of TorA. In AD93 complemented by the wild type *pss* allele, specific TorA activity was found at 3.56 ± 0.10 units in the periplasm and 0.02 ± 0.01 unit in the cytoplasm. In this case, more than 95% of the total TorA activity was normally translocated into the periplasm (Fig. 2A,B, lanes 3 and 6). After curing the mutant of the wild type gene, the specific TorA activity decreased to 0.23 ± 0.10 unit and 0.61 ± 0.24 unit in the periplasm of the mutant grown with 50 mM $MgCl_2$ and 50 mM $CaCl_2$, respectively. These values correspond to 6.5% and 17% of the level of TorA activity found in the periplasm of the mutant complemented by the pss^+ allele. Importantly, the specific TorA activity increased to 0.12 ± 0.01 and 0.12 ± 0.05 units in the cytoplasm of the *pss* mutant grown with 50 mM $MgCl_2$ and 50 mM $CaCl_2$, respectively. Further quantification by analyzing the immunoblot signals confirmed the change of cellular distribution of TorA as measured by enzyme assays (Fig. 2B). Therefore, the decrease of TorA in the periplasm with its parallel increase in the cytoplasm of the mutant grown with the cations clearly shows a defect of TorA translocation under these conditions. Moreover, the lower level of TorA found in the periplasm of the $CaCl_2$-grown cells than that of the $MgCl_2$-grown cells shows a dependence of TorA translocation on the CL/PG ratio. The regulation of lipid polymorphism of membrane is thus essential for the export of TorA.

A small amount of TorA was found in the periplasm of the *pss* mutant grown with $CaCl_2$ and $MgCl_2$ (Fig. 2A,B, lane 2). It is possible that the *pss* mutation may lead to a cytoplasmic leakage resulting in the detection of TorA. Therefore, we monitored the cellular fractionation by following the distribution of the cytoplasmic protein NarJ by immunoblot. The absence of NarJ in all periplasmic fractions (Fig. 2C, lanes 1–3) excludes the possibility of a cytoplasmic leakage. Consistently, it had been reported that the *pss* mutation does not affect membrane potential, thus does not seem to lead to a cytoplasmic leakage [23,24].

We then compared the translocation of TorA with the translocation of proteins through the Sec pathway in the *pss* mutant. For an unknown reason, NikA was hardly detectable in the strains used. Therefore, we analyzed the export of MalE, an extensively studied substrate of the Sec pathway. Similar to that was observed for TorA, translocation of MalE occurred normally in the complemented cells (Fig. 2D, lanes 3 and 6), but it was abolished in the cells grown with $CaCl_2$ (Fig. 2D, lanes 1 and 4). Unlike the poor translocation of TorA, about 50% MalE was translocated into the periplasm of the *pss* mutant grown with $MgCl_2$ (Fig. 2D, lanes 2 and 5). The same results were observed in the study of PhoA translocation through the Sec system of the *pss* mutant (N.I. Mikhaleva, V.V. Golovastov and M.A. Nesmeyanova, unpublished data). Therefore, polymorphism of the lipids plays a more important role in the translocation of proteins through the Tat pathway than that via the Sec pathway.

4. Discussion

Numerous proteins are exported through the general secretory (Sec) pathway, which recognizes N-terminal signal peptides in newly synthesized proteins and directs their translocation across the plasma membrane. It was shown that the *pgs* mutation retards the Sec-dependent translocation of precursors of the outer membrane proteins PhoE and OmpA across the inner membrane [5]. This is consistent with the observation that SecA, a key enzyme in the Sec pathway, requires PG for membrane binding and ATPase activity [25]. Phage M13 procoat protein is translocated across membranes in a signal sequence-dependent manner. This process is independent of SecA, but it also requires PG [6]. Therefore, PG plays a dual role in protein translocation in the bacterial secretion pathway. The interaction between the negative charge on the lipid headgroup and the basic amino acids at the signal sequence was proposed as the origin of the common anionic lipid dependence of efficient protein translocation [6,26]. Our results presented in this study provide new completely independent evidence which supports this hypothesis. TorA bears a twin arginine-containing targeting signal [27], and it is translocated via a novel Sec-independent Tat pathway [8–12]. The hallmark of this pathway is its ability to transport fully folded proteins or enzyme complex [8,13,14]. Translocation of TorA was deleteriously retarded when the level of anionic phospholipids was reduced. This finding generalizes the involvement of anionic phospholipids in protein translocation through all known pathways.

Accumulating evidence strengthens the idea that polymorphism of phospholipid may play a fundamental role in the dynamics of membrane biology, including protein translocation across the membranes of chloroplasts, of endoreticulum, of mitochondria and *E. coli* inner membrane via the Sec pathway [28]. The *pss* mutant of *E. coli* lacks PE and its growth is strictly dependent upon the presence of specific divalent cations such as Mg^{2+} and Ca^{2+}, which promote the formation of non-bilayer structures by CL. In this strain, the synthesis of CL under anaerobic condition is significantly upregulated by Mg^{2+} compared to Ca^{2+} (39.2% versus 19.5%). Consistently, larger amounts of TorA or MalE were found in the periplasm of cells grown with Mg^{2+} than that of cells grown with Ca^{2+}. Therefore, regulation of membrane lipid polymorphism is also essential for the translocation of proteins through the Tat pathway. Moreover, the recovery of the translocation of TorA was much less important than that of MalE in the *pss* mutant grown with $MgCl_2$, suggesting a higher dependence of the Tat than the Sec pathway on the non-bilayer phospholipids.

Since the Tat pathway is capable of exporting tightly folded proteins, even enzyme complexes [8,13,14], the gating mechanism and the operation of the channel for the proteins across the membrane should be fundamentally different from those of the Sec pathway. Phospholipids may play more important, active roles in the translocation of folded proteins through the Tat pathway than the translocation of proteins in extended

conformation via the Sec pathway. Our preliminary results show that the depletion of PE or anionic phospholipids has a more deleterious effect on the translocation of TorA than the effect on the export of substrates of the Sec pathway. The increasing dependence on the phospholipids might be related to the fact that the Tat pathway, by analogy to the thylakoid ΔpH-driven protein import pathway, may be energized by the proton-motive force [8,29]. Besides, anionic phospholipids participate in the Sec-dependent protein export process by an electrostatic interaction with the positively charged signal sequence and by an indirect effect on the activities of SecA or leader peptidase [3]. The slow process of translocating folded protein through the Tat pathway may increase the opportunity for a direct, active interaction between the phospholipids and the passenger proteins. Further investigation should be oriented toward the analysis of the involvement of phospholipids in (i) targeting of the twin arginine signal sequence to the membrane; (ii) insertion of Tat subunits into the membrane; (iii) folding of the Tat subunits in the membrane; and finally (iv) assembly and operation of the Tat translocase.

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