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Influence of K^+-dependent membrane lipid composition on the expression of the *kdpFABC* operon in *Escherichia coli*

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ABSTRACT

The membrane-bound sensor kinase KdpD and the cytoplasmic response regulator KdpE regulate the expression of the *kdpFABC* operon coding for the high affinity potassium uptake system KdpFABC in *Escherichia coli*. The signal transduction cascade of this two component system is activated under K^+-limiting conditions in the medium, but is less sensitive to high osmolality. In order to test whether K^+ limitation affects membrane phospholipid composition and whether this change affects *kdpFABC* expression, we analysed the phospholipid composition of *E. coli* under these conditions. Our measurements revealed that there is an increase in the cardiolipin (CL) content during the exponential growth phase at the expense of the zwitterionic phospholipid phosphatidylethanolamine. The higher anionic phospholipid content occurs along with an increase of transcriptional activity of the *cls* gene coding for CL synthase. Furthermore, *in vivo* studies with *E. coli* derivatives carrying mutations in genes coding for enzymes involved in phospholipid biosynthesis revealed that the increase in the anionic lipid composition enhances the expression rate of the *kdpFABC* operon. Finally, we show that kinase activity of KdpD is stimulated in its native membrane environment by fusion with liposomes of anionic, but reduced with liposomes of zwitterionic phospholipids.
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1. Introduction

Since K^+ plays a major role in the cell, *Escherichia coli* has established several transport systems to adjust the intracellular K^+ concentration (150–300 mM) in response to changes in the environment. The KdpFABC complex (K^+-dependent ATPase), encoded by the *kdpFABC* operon [1], is an inducible, high affinity K^+ transporter synthesized by *E. coli* as an emergency system to scavenge K^+ when the other transporters cannot keep up with the cell's requirement for K^+ [2]. Expression of the *kdpFABC* operon is regulated by the products of the adjacent *kdpDE* genes [3]. The membrane bound sensor kinase KdpD and the cytosolic response regulator KdpE comprise a typical prokaryotic two component signal transduction system [4]. KdpD (99 kDa) consists of a cytoplasmic N-terminal domain and a cytoplasmic C-terminal domain interconnected by 4 transmembrane segments [5]. Upon stimulus perception KdpD undergoes autophosphorylation and subsequently, the phosphoryl group is transferred to the response regulator KdpE [6]. KdpE binds in its phosphorylated and dimerized form with high affinity at the *kdpFABC* promoter, thereby triggering *kdpFABC* transcription [7]. The unusually large and unique input domain of the sensor kinase KdpD (about 660 amino acids), which is responsible for stimulus perception, is composed of an extended cytoplasmic N-terminal domain, the four transmembrane helices followed by a cluster of arginine residues and about 140 amino acids of the cytoplasmic C-terminal domain. The other 230 amino acids of the cytoplasmic C-terminal part constitute a typical transmitter domain of histidine sensor kinases carrying all the conserved sequence motifs [8]. *In vitro*, KdpD exhibits not only kinase activity, but also phosphatase activity for phospho-KdpE [9]. Brandon et al. [10] have suggested that the initiation of signal transduction by KdpD is mediated by the inhibition of the phospho-KdpE specific phosphatase activity and a switch between kinase and phosphatase activity is proposed to be of electrostatic nature [11].

The *kdpFABC* operon is very effectively induced under K^+-limiting conditions in the medium, but is less sensitive to varying degrees by high osmolality [12]. It was proposed that KdpD probably integrates various parameters: changes in turgor, in phospholipid composition of the membrane, in extra- and/or intracellular K^+ concentration, in medium osmolality, in ionic strength of the cytoplasm, and in internal ATP concentration [for more details, see [13]]. However, the results of Hamann et al. [12] provide clear evidence that neither changes in turgor nor in the concentration of several cytoplasmic solutes, like K^+, ATP, putrescine, spermidine, trehalose, glutamate, and proline can be the stimulus for KdpD. Previously, it has been reported that the positive arginine cluster following the fourth transmembrane helix may interact with the anionic phospholipids by electrostatic interactions [11]. Zimmann et al. [14] showed that single amino acid replacements

Abbreviations: CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol

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in this arginine cluster of KdpD led to a constitutive phenotype with respect to *kdpFABC* expression under K^+ limitation probably caused by lower phosphatase activity. Therefore, it is conceivable that the KdpD derivatives adopt a different conformation that affects the protein–lipid interaction.

The anionic phospholipid content of the cytoplasmic membrane of *E. coli* is known to respond to high osmolality in such a way that the amount of the negatively charged phospholipid cardiolipin (CL) increases at the expense of the zwitterionic phosphatidylethanolamine (PE) [15]. Since K^+ limitation is another stimulus to which bacteria, including *E. coli*, get exposed to, the focus of this work was on the phospholipid composition of *E. coli* membranes under these conditions. For that purpose, the synthesis of phospholipids and the expression of the corresponding enzymes following a potassium downshift, the phospholipid compositions of various *E. coli* derivatives in comparison to their *kdpFABC* induction rate under K^+ limitation as well as activity studies of KdpD in different membrane environments were analysed to clarify their role in *kdpFABC* expression.

2. Materials and methods

2.1. Materials

^{32}P-phosphate and [γ-^{32}P] ATP were purchased from Hartmann Analytic (Braunschweig, Germany), IPTG from PeqLab (Erlangen, Germany), synthetic phospholipids from Avanti Polar Lipids (Alabaster, USA), the molybdenum blue reagent from Sigma-Aldrich (Taufkirchen, Germany), Ni-NTA and RNeasy Mini Kit from Qiagen (Hilden, Germany), DNAse I (RNase free) from New England Biolabs (Frankfurt am Main, Germany), RevertAid First Strand cDNA Synthesis Kit from Fermentas (St. Leon-Rot, Germany), Bio-Beads AM-2 and IQ Sybr Green Supermix from BioRad (München, Germany). Primers were obtained from Operon (Köln, Germany). All other reagents were reagent grade and obtained from commercial sources.

2.2. Bacterial strains and growth conditions

Table 1 summarizes the strains and plasmids used in this work. All *E. coli* strains except for strain AD93 were grown at 37 °C in complex KML medium (1% KCl, 1% tryptone, 0.5% yeast extract) or in minimal phosphate-buffered media, identified by the K^+ content in mM, which have been described previously [16]. *E. coli* strain AD93 was grown at 37 °C in GM56 medium [17] modified with different K^+ concentrations which were designated by the K^+ content in mM. In minimal media glucose (0.4%) served as carbon and energy source. Other compounds, e.g. IPTG (0.7 mM or 1 mM) or $MgCl_2$ (50 mM) were added to the media as needed. Compounds like thiamine (0.0002%), casamino acids (0.2%), bactopeptone (0.145%), proline (20 μg/ml), nicotinic acid (2 μg/ml) and tryptophane (1 mM) were added to the minimal phosphate-buffered media as needed.

Table 1
E. coli strains and plasmids.

E. coli strains	Relevant characteristics	Reference or source
K12	*kdp*$^+$ *trk*$^+$	47
TKR2000	Δ*kdpFABCDE thi rha lacZ nagA trkA405 trkD1 atp706*	48
WC3899	*F*$^-$ *nadB7 supE rfbD1 cls::Tc*	W. Dowhan
AD93	*pss93::kan recA srl::Tn10 nadB*$^+$	49
HDL1001	*pgsA30::kan Φ(lacOP-pgsA*$^+$*)1 lacZ' lacY::Tn9 recA srl::Tn10*	28
UE54	*lpp-2 Δara714 rcsF::mini-Tn10 cam ΔpgsA::FRT-kan-FRT*	29
SOH92	*bla kan T1*$_4$ *lacY*$^+$ *lacA*$^+$ *imm21 P90C Φ(cls-lacZ*$^+$*)*	34
Plasmids		
pDD72	*pssA*$^+$ / Cm^R	49
pPV5-6His	*kdpD-6His* / Ap^R	9

2.3. Medium shift and determination of phospholipid head group composition

Cells were grown overnight in the different media supplemented with 25 μCi/ml ^{32}P-orthophosphate and then transferred into the same medium. When the cells had reached early exponential growth phase, they were harvested by centrifugation. To monitor changes in the lipid composition directly after the cells were exposed to K^+ limitation, the cells were grown to OD 0.5, filtered, washed, and transferred into fresh and preheated medium with appropriate K^+ concentrations supplemented with 25 μCi/ml ^{32}P-orthophosphate according to Hamann et al. [12]. After the transfer, samples were taken at different time points by centrifugation of the cells. To determine phospholipid composition, polar lipids were extracted with chloroform/methanol and thin layer chromatography was performed as described by Tsatskis et al. [15]. Radiolabeled lipids were visualized and quantified by image analysis using the Phosphoimager Storm 820 (Molecular Dynamics), and expressed as mole % adjusting PO_4. As a control, non-radioactive lipid extract was separated by thin layer chromatography and the plate was sprayed with molybdenum blue reagent to detect phospholipids by comparison with synthetic lipids.

2.4. Determination of cls expression in vivo by β-galactosidase activity

In vivo expression of the cardiolipin synthase (*cls*) gene was determined after growth of *E. coli* SOH92 (*cls-lacZ* transcriptional fusion) in minimal phosphate-buffered media containing the indicated amounts of K^+. Cells were grown to different growth phases and harvested by centrifugation. β-galactosidase activity was determined as described [18] and is given in Miller units. β-galactosidase activity corresponds indirectly to the CL synthase content of the cell, because the *lacZ* gene is expressed instead of *cls*.

2.5. Determination of the fatty acid composition of the phospholipids

Cells of *E. coli* K12 were grown in phosphate-buffered minimal media with the appropriate K^+ concentrations and harvested at different optical densities by centrifugation. Saponification, methylation and extraction of the fatty acid methyl esters (FAMEs) were carried out according to Sasser [19]. Identification of the FAMEs was performed by GC-MS as described previously [20].

2.6. Determination of kdpFABC expression by Q-RT-PCR

RNA was extracted from cells in the exponential growth phase cultivated in minimal media according to the manufacturer's protocol of the RNeasy Mini Kit. After the DNAse treatment, the cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit. The sequences of the primers used for the Q-RT-PCR (quantitative reverse transcriptase PCR) as well as the run protocol in an iCycler thermal cycler (BioRad) were described previously [12]. Primer pair kdpAfor2/kdpArev2 was used for *kdpA* detection (*kdpFABC* expression) and as an internal standard primer pair gapAfor1/gapArev1 for the *E. coli* house-keeping gene *gapA*. To avoid sampling errors, the levels of expression of *kdpA* gene, as determined from their cycle threshold C_T values, were normalized to the level of the housekeeping gene. For statistical analysis the data were averaged from different measurements. The induction of the *kdpFABC* expression is given as the ratio of the values of the normalized expression rate of the induced state (0.1 mM K^+) divided by the values of the normalized expression of the non-induced state (115 mM K^+). This indicates the extent of

kdpFABC expression after a shift of cells from non-inducing to inducing conditions. It should be mentioned that although variations of the data set are caused by the extremely low non-induced-state values, the overall magnitude of the induction ratio stayed quite constant.

2.7. Over-expression of KdpD-6His and preparation of inverted membrane vesicles

Cells of *E. coli* TKR2000 transformed with plasmid pPV5-6His were cultivated in KML complex medium supplemented with ampicillin (100 μg/ml) for plasmid selection. Cells were harvested at an optical density at 600 nm of ~1.0 and were disrupted by using the Basic Z machine (IUL Instruments, Königswinter). Membranes were prepared according to Siebers and Altendorf [21] with the following modification: Tris–HCl was used in the same concentration instead of HEPES-Tris. Membrane vesicles in TG-buffer [50 mM Tris/HCl, pH 7.5, 10% (v/v) glycerol] were frozen in liquid nitrogen and stored at −80 °C until use.

2.8. Fusion of inverted membrane vesicles with liposomes

Liposomes of synthetic phospholipids (10 mg/ml) were made according to Knol et al. [22] by dissolving lipids in K^+ buffer (50 mM KP_i, pH 7.0) to obtain a final concentration of 10 mg/ml, followed by sonication, three freeze/thaw cycles and extrusion through polycarbonate filters with 400 nm pore size. Liposomes of 100% CL or 50% PE and 50% PC were made to fuse anionic or zwitterionic phospholipids with membrane vesicles. Inverted *E. coli* membrane vesicles were mixed with these liposomes in different ratios to vary the anionic phospholipid content of the vesicles according to Özcan et al. [23].

2.9. Autophosphorylation assay for KdpD-6His

To test autophosphorylation activity, membrane-liposome fusions containing wild type KdpD were incubated at room temperature in standard phosphorylation buffer (50 mM Tris/HCl, pH 7.5, 10% glycerol, 0.5 M NaCl, 0.02 mM $MgCl_2$ and 2 mM DTT). Phosphorylation was initiated by addition of 20 μM [γ-^{32}P]ATP (2.38 Ci/mmol). At various time points, aliquots were removed and mixed with an equal volume of 2× concentrated SDS sample buffer. All samples were immediately subjected to 11% SDS-polyacrylamide gel electrophoresis (PAGE) [24]. Shortly before stopping SDS-PAGE, a [γ-^{32}P]ATP standard was loaded on the gel. The gels were dried and exposed to a Storage Phosphor Screen. Phosphorylated proteins were quantified by image analysis using the Phosphoimager Storm 820 (Molecular Dynamics).

2.10. Analytical procedures

Protein was assayed by a modified Lowry method [25], using bovine serum albumin as a standard. Immunodetection of KdpD was performed with polyclonal antibodies against KdpD as described [6].

3. Results

3.1. Influence of K^+ limitation on the lipid composition of E. coli K12

Cells of the *E. coli* strain K12 were grown in minimal media with different potassium concentrations to exponential growth phase and the lipid compositions were determined. The data revealed that the CL content increased at the expense of PE under K^+ limitation while the PG content stayed constant at 15% (Fig. 1). Comparison of the lipid composition of cells grown at 0.02 mM K^+ versus 115 mM K^+ revealed that a 1.44 fold CL increase was detected in minimal medium

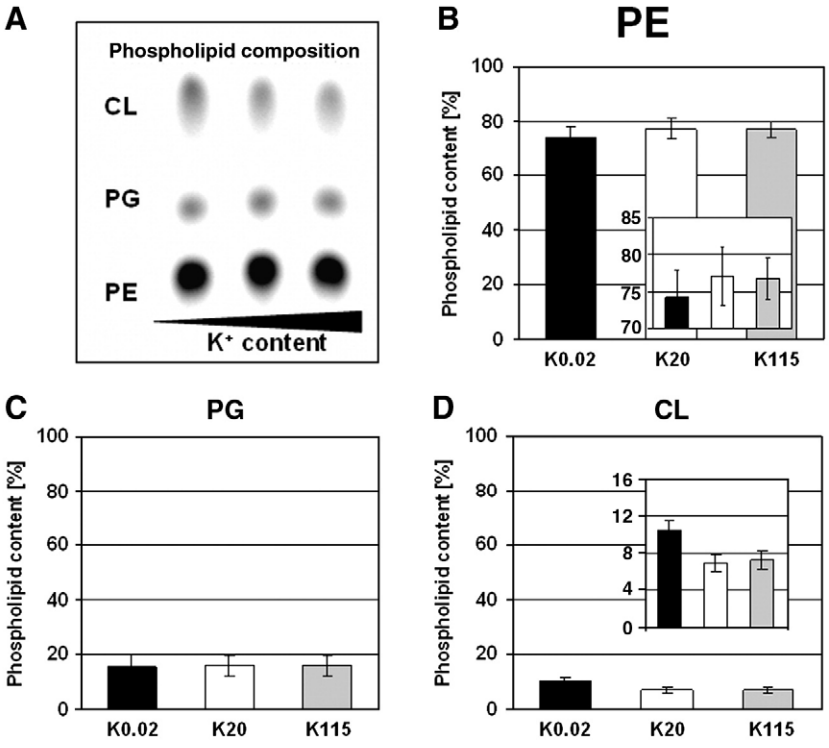


Fig. 1. The phospholipid head group composition of a polar lipid extract of *E. coli* K12. The composition was determined at exponential growth phase as described in Materials and methods. Bacteria were grown in phosphate-buffered minimal media of increasing potassium concentrations. Panel A: image of a representative chromatogram showing the relative positions and intensities of spots formed by the major phospholipid species in a polar lipid extract. The PE content (Panel B), the PG content (Panel C) and CL content (Panel D) is shown in percent of the total lipid composition in phosphate-buffered minimal medium with 0.02 mM K^+ (black bars), 20 mM K^+ (white bars) and 115 mM K^+ (grey bars). Error bars represent the range of four independent determinations. Please note the different scale of the smaller figure in Panel B and D.

containing 0.02 mM potassium (Fig. 1D). Furthermore, this CL increase was observed immediately (1 min) after a shift of *E. coli* K12 cells to medium with nominally no potassium (Fig. 2A). This change in lipid composition was not observed when cells were transferred to fresh minimal medium with the same potassium content (Fig. 2B). Independent of a medium shift, the CL content increased at the expense of PG when the cells had reached late stationary phase (Fig. 2C) as already reported [26,27]. Furthermore, it is important to mention that in Fig. 1 the proportion of CL among total lipids is indicated whereas in Fig. 2 the proportion of CL among newly synthesized lipids after the medium shift is given.

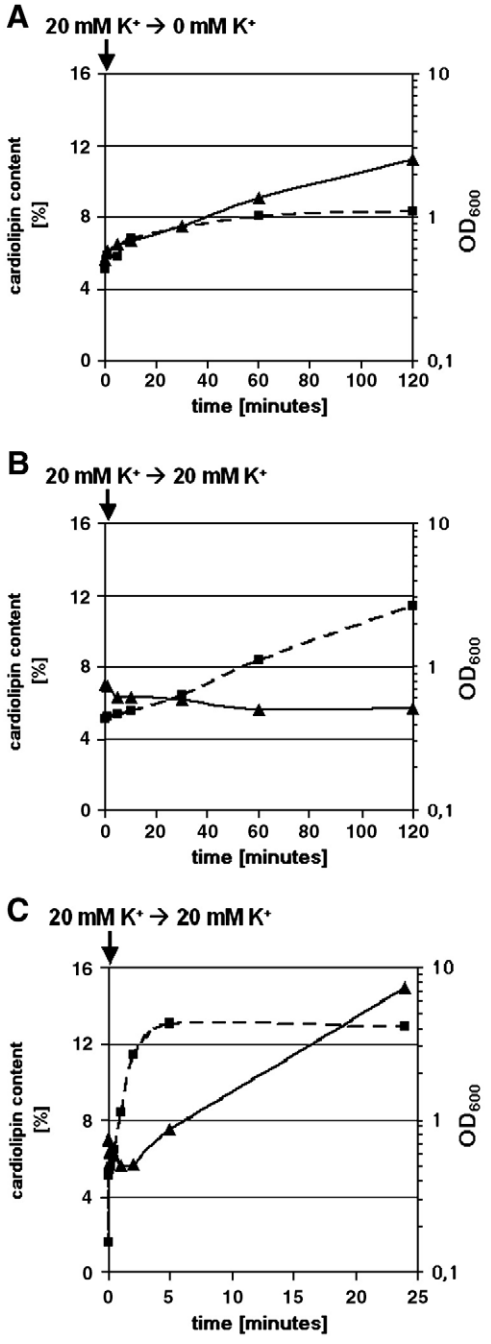


Fig. 2. CL content of *E. coli* K12 upon a medium shift. Cells were grown in phosphate-buffered minimal media with 20 mM K^+ to OD 0.5 and transferred at time point 0 in medium with 0 mM K^+ (Panel A) or in medium with 20 mM K^+ (Panels B and C). The determination of the newly synthesized CL molecules (triangles) was carried out as described in Materials and methods. The growth curve of each culture is shown in squares. Please note the different time scale between panel B and C to compare the exponential with the stationary phase.

3.2. Influence of K^+ limitation on the induction of *cls* expression

The reason for the observed higher level of CL could be due to an increase in the *cls* expression or a higher activity of the CL synthase under K^+ limitation. Therefore, we tested *cls* expression in cells of the strain SOH92 (*cls-lacZ* fusion) under K^+ limitation (Fig. 3). At any time in medium with 0.1 mM K^+ the *cls* expression was higher than in medium with 115 mM K^+. During the exponential growth phase in both media the β-galactosidase activity was higher followed by a decrease in the stationary phase. The induction ratio (K0.1/K115) for the *cls* expression determined by the β-galactosidase activities shown in Fig. 3 was 2- to 3-fold and stayed quite constant during growth.

3.3. Influence of K^+ limitation on the fatty acid composition of the phospholipids

Cells of *E. coli* K12 were analysed with respect to their phospholipids fatty acid content by gas chromatography after growth in media with different K^+ concentrations. These data as well as results for cells grown to different optical densities (Table 3) are similar to those previously described for complete media [26]. The detected changes in the amount of 16:1 *cis* 9 and 17:0 *cyclo* 9–10 between K0.1 and K115 may be due to the fact that cells under K^+ limitation reached stationary phase earlier. It is known that under stress conditions cells increase their content of 17:0 *cyclo* 9–10 at the expense of 16:1 *cis* 9 [26]. However, this change has no influence on the properties of the lipid bilayer of the cytoplasmic membrane because both fatty acids have the same chemical characteristics. In any case under K^+ limitation only the head group and not the fatty acid composition of the membrane phospholipids changed.

3.4. Influence of K^+ limitation on the lipid composition of cells carrying mutations in genes coding for enzymes involved in phospholipid biosynthesis

Extensive data exist about mutant strains with alterations in lipid biosynthesis [28–30]. However, the lipid composition of these strains under K^+ limitation has so far not been studied and is now reported in Table 2. Under K^+ limitation strain AD93, carrying a deletion in the *pssA* gene showed the expected lipid composition lacking PE, which results in exclusively anionic phospholipids primarily composed of PG and CL. In the case of *E. coli* strain WC3899 under K^+ limitation, traces of CL were found although the *cls* gene is deleted. For other *cls*$^-$ strains similar CL contents were observed [30]. The strain WC3899 contains a

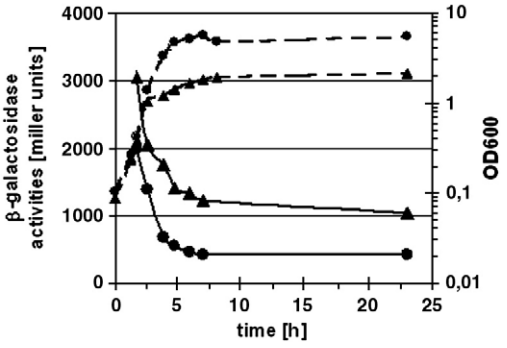


Fig. 3. β-galactosidase activities of *E. coli* SOH92 under K^+ limitation. The activities are an indirect detection for the level of the *cls* expression and measured as described in Materials and methods. The *cls* induction (lines) and growth (dashed lines) of cells in K0.1 (triangles) or K115 (circles) is shown.

Table 2
Response of *kdpFABC* expression under K^+ limitation in different *E. coli* strains in correlation to their phospholipid composition.

Strain	Genotype	Phospholipid content [%] in K0.1				*kdpFABC* induction ratio (*fold*)	
		CL	PG	Anionic CL + PG	PE	Minimal medium	GM56 medium
UE54	*pgsA::Kn*^{R}	0.0	0.0	0.0	100.0	640 (*100*)	–
HDL1001	*pgsA::Kn*^{R} *lacOP-pgsA* - IPTG	1.1	2.7	3.8	96.2	365 (*100*)	–
HDL1001	*pgsA::Kn*^{R} *lacOP-pgsA* +IPTG	4.2	13.9	18.1	81.9	2005 (*1000*)	–
WC3899	*cls::Tc*^{R}	0.9	25.0	25.9	74.1	2028 (*1000*)	–
K12	WT	9.8	15.1	24.9	75.1	5870 (*1000*)	909 (*100*)
AD93	*pss93::Kn*^{R}	49.6	50.4	100	0.0	–	2325 (*1000*)

Cells were grown in minimal medium or in GM56 medium with 0.1 mM or 115 mM K^+ to exponential growth phase as described in Materials and methods. The content of each phospholipid is determined as described in Materials and methods. Strains are listed in the table in order of their increasing anionic phospholipid content (CL and PG). *kdpFABC* induction ratio was measured by Q-RT-PCR as described in Materials and methods. The induction ratio was calculated by division of the amount of normalized mRNA under inducing conditions (0.1 mM K^+) by the amount under non-inducing conditions (115 mM K^+). Each data of this table represents values from 3 to 6 independent measurements. The orders of magnitude are also indicated as "*fold*".

similar anionic phospholipid content compared to wild type K12 caused by a higher PG content. The *E. coli* strain HDL1001 with the *pgsA* gene under the control of an IPTG inducible promoter showed in the absence of IPTG a reduced anionic phospholipid level. However in the presence of IPTG the anionic phospholipid level of HDL1001 resembles that of wild type strain K12. In addition, under K^+ limitation strain UE54, null in *pgsA*, did not contain the major anionic phospholipids PG and CL, as expected, and contained almost exclusively PE. It is worth mentioning that these "lipid strains" contained the identical spectrum of PE, PG and CL content in the presence of K^+ (data not shown) as well as for K^+-limitation (Table 2). In contrast, with the "parental strains" of the "lipid strains", containing no mutation in genes coding for lipid synthesis enzymes, we recorded changes in the lipid composition in dependence of the K^+ content in the medium. Like wild type *E. coli* K12 (Fig. 1), these "parental strains" showed a CL increase under K^+ limitation at the expense of PE (data not shown). These results led to the suggestion that mutations in genes coding for lipid synthesis enzymes prevented changes in the lipid composition in dependence of the K^+ content.

3.5. Influence of K^+ limitation on kdpFABC expression in cells carrying mutations in genes coding for enzymes involved in phospholipid biosynthesis

Based on the data presented thus far it is conceivable that a correlation between the activity of the membrane-bound sensor kinase KdpD resulting in *kdpFABC* expression and the lipid composition exists. To clarify this point, cells of strains with different lipid compositions were analysed by Q-RT-PCR to compare the amount of synthesized *kdpFABC* mRNA. This method allows us to measure the *kdpFABC* expression *in vivo* more directly in comparison to KdpFABC detection by immunoblot analysis of whole cells. In medium with 0.1 mM K^+ *kdpFABC* expression is induced, whereas in medium containing 115 mM K^+ no *kdpFABC* expression occurs. As presented in Table 2 the induction ratios (K0.1/K115) for the *kdpFABC* expression of the different strains varied. The wild type strain K12 showed a high induction ratio which means that under K^+ limitation the expression of *kdpFABC* is several thousand fold induced in contrast to high potassium concentrations as already reported [12]. To test if the amount of anionic phospholipids has an influence, the *kdpFABC* expression of the *E. coli* strains HDL1001, WC3899, UE54 and AD93 were analysed. Strain HDL1001 has the *pgsA* gene under the control of an inducible promoter and showed in the absence of IPTG (3.8% PG/CL) a reduced induction ratio of *kdpFABC* expression (100 fold). In the presence of 1 mM IPTG, which leads to an almost restored PG/CL content, a higher induction ratio in the range of the wild type K12 was detected. The induction ratio of strain WC3899, which has a deletion in the *cls* gene but synthesized increased amounts of PG for compensation, was also comparable to K12 in order of magnitude (1000 fold). Furthermore, we analysed the *kdpFABC* expression under K^+ limitation of the strain UE54 which has a deletion in the *pgsA* gene resulting in a membrane lacking both anionic phospholipids, PG and CL. This strain showed a low induction ratio of *kdpFABC* expression like HDL1001 without IPTG indicating an influence of the total amount of anionic phospholipids. Strain AD93 is the opposite extreme of UE54 due to the lack of the zwitterionic phospholipid PE resulting in a cytoplasmic membrane consisting of 100% anionic phospholipids. The Q-RT-PCR data of this strain, in comparison to K12, showed a 2.5-fold higher induction ratio for *kdpFABC* expression measured in GM56 medium which also confirms the anionic phospholipid effect.

Table 3
Fatty acid composition of *E. coli* K12 in the phosphate-buffered minimal media containing high (K115) or low (K0.1) K^+ concentrations.

Fatty acid [%]	K0.1	K115
12:0	2.6	3.1
14:0	6.5	7.4
16:0	38.8	36.4
16:1 *cis* 9	29.8	34.8
18:0	0	0
18:1 *cis* 11	11.0	10.4
17:0 *cyclo* 9–10	6.1	3.7
19:0 *cyclo* 11–12	0	0
Others	5.2	4.2

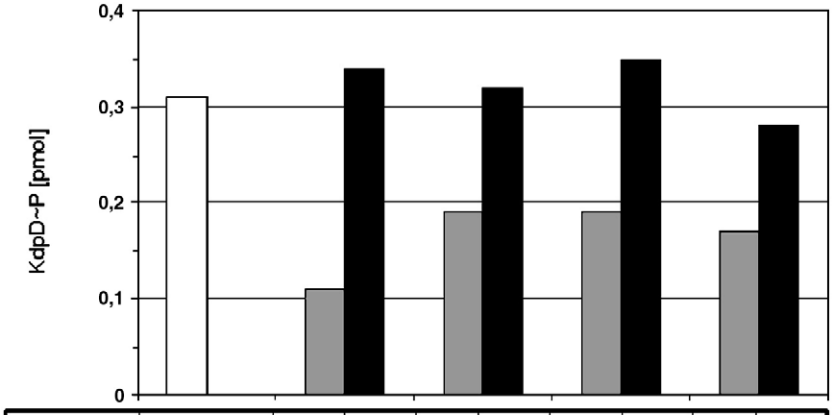
The fatty acid content is given in percent and is determined as described in Materials and methods.

3.6. Influence of anionic phospholipids on the autophosphorylation of KdpD

It has been shown that the activation of the sensor kinase KdpD can be regulated by anionic phospholipids [31]. To analyse this influence in more detail, we tested the kinase activity of wild type KdpD in inverted membrane vesicles fused with synthetic liposomes containing different amounts of the anionic phospholipid CL (Fig. 4). This method was chosen to retain KdpD in its native membrane environment. The addition of CL stimulates the autophosphorylation of KdpD while the addition of the same amount PE/PC liposomes results in a reduced kinase activity. Even at various time points after addition of radioactive ATP the same effects with respect to the kinase activity were observed (data not shown).

4. Discussion

It is well established that the lipids of bacterial cytoplasmic membranes vary with growth medium osmolality and growth phase. In *E. coli* CL increases 2- to 3-fold at the expense of PE in exponential growth phase in high osmolal medium independent of the osmolyte [15]. In stationary phase the CL content rises 2- to 3-fold at the expense of PG [26,27] with no net increase in the anionic lipid content. In this study



Ratio MV:PE/PC MV:CL	1:0 -	1:3 -	- 1:3	1:2 -	- 1:2	1:1 -	- 1:1	2:1 -	- 2:1
CL	5	1.3	76.3	1.7	67.7	2.5	52.5	3.3	36.6
Anionic	25	6.3	81.3	8.3	74.3	12.5	62.5	16.6	49.9

phospholipid content (%)

Fig. 4. Phosphorylation activity of KdpD-6His in inverted membrane vesicles (white bar) fused with synthetic liposomes containing CL (black bars) or PE/PC (grey bars). Membrane-liposome fusions containing wild type KdpD were incubated with [γ-^{32}P] ATP and after 5 min the phosphorylation was stopped as described in Materials and methods. Proteins were separated by SDS-PAGE, and the amount of phosphorylated KdpD (KdpD~P) was quantified with a phosphoimager. The activities were averaged from at least six different measurements.

we focused on changes of the phospholipid composition of the membrane under K^+ limiting conditions in order to study their influence on *kdpFABC* expression in *E. coli*. We showed for the first time that the head group composition of the phospholipids of *E. coli* K12 changes under K^+ limitation by increasing the content of the anionic phospholipid CL at the expense of PE not only during exponential growth under potassium limitation but also directly after a potassium downshift. Romantsov et al. [32] suggest that the CL increase under high osmolality observed by Tsatskis et al. [15] is due to an increase in PG synthesis and PG conversion to CL although the detectable PG level was unchanged. This hypothesis may also explain the CL increase under K^+ limitation presented in this study.

The effect of cytoplasmic K^+ on the activity of the CL synthase was analysed in earlier studies where it was shown that K^+-phosphate acts as an activator for the CL synthase [33]. In addition, we provided new experimental evidence that under K^+ limitation the amount of CL increases. There are at least two possible explanations for this observation during exponential growth. On the one hand there might be a higher activity of the CL synthase and on the other hand there might be a higher expression of the *cls* gene. At least during exponential growth our results with a *cls-lacZ* transcriptional fusion strain are consistent with the second explanation, indicating that the CL synthesis is indeed regulated at the genetic level as already suggested by Heber and Tropp [34]. This conclusion doesn't rule out an additional activation of the CL synthase at the enzymatic level as observed during the stationary growth phase [35]. These results show that the synthesis of CL is regulated at the genetic and enzymatic level, lending support to the notion that modulation occurs at both levels. These multiple modes of regulation may enable the cell to fine-tune its CL and/or anionic phospholipid content, thereby avoiding negative influences on essential processes and functions in the cell.

With respect to the induction of expression under K^+ limitation, the promoters of the *cls* gene and of the *kdpFABC* operon are up-regulated or activated. Therefore, an alignment of both promoter sequences was carried out (data not shown), but no homologies were observed. Additionally, both genes showed clearly different induction ratios with respect to low K^+ concentrations in the medium, suggesting different mechanisms for the regulation of the *cls* and *kdpFABC* expression under K^+ limitation.

Therefore, the question arose whether anionic phospholipids are a stimulus for KdpD activation and, in turn, for *kdpFABC* induction. For some sensor kinase/response regulator systems it was already observed that the lipid composition had an effect on the activation of these systems. The Cpx two-component signal transduction pathway for example was found to be activated in *E. coli* mutant strains lacking PE [36]. In the case of the KdpD/KdpE system we showed in this work that *in vitro* the kinase activity of the sensor kinase KdpD was stimulated in its native membrane environment by increasing amounts of CL, but a lower activity was observed by the same amount of PE/PC. A correlation between the induction ratio of the *kdpFABC* expression and the total anionic phospholipid content (Table 2) is also supported by the observation that strains with a higher anionic lipid content (18–25%, given in strains K12, WC3899 and HDL1001+IPTG) had higher induction ratios of the *kdpFABC* expression than strains with a lower anionic lipid content (0–4%, strains UE54, HDL1001 IPTG). Furthermore, the data from the Q-RT-PCR lead to the suggestion that *in vivo* the total anionic phospholipid content rather than the CL content has an influence on the *kdpFABC* expression. Strain WC3899 (Δ*cls*) for example had a comparable induction ratio of the *kdpFABC* expression as K12 (wild type) although the composition of the anionic lipids differs due to a lower CL content. The observation that the highest *kdpFABC* expression was measured in a strain consisting of a membrane with only anionic phospholipids (AD93) further supports the stimulating effect of anionic phospholipids on *kdpFABC* expression. However, the net charge of the membrane surface as influenced by the phospholipid composition is not solely responsible for *kdpFABC* expression because in *E. coli* strain UE54 *kdpFABC* expression still occurs although the membrane lacks the anionic phospholipids PG and CL. Although the residual activity might be the result of accumulation of the precursor molecules phosphatidic acid and CDP-diacylglycerol in this strain that would provide some negative charge character to the membrane surface [15] it is unlikely that anionic phospholipids serve as primary stimulus for KdpD activation and subsequent KdpFABC synthesis.

The results of this work demonstrate a correlation between the anionic phospholipid content (especially CL) and the signalling state of the membrane bound sensor kinase KdpD *in vivo* and *in vitro*. As already mentioned, the positive arginine cluster following the fourth transmembrane helix may interact with the anionic phospholipids. Such an interaction was also suggested for the RCK domain of the K^+

channel MjK2 of *Methanococcus jannaschii* [37]. In addition, Schmidt et al. [38] showed that the function of a voltage-dependent K^+ channel is dependent on the negatively charged phospholipids by a stabilizing interaction with the positively charged arginine residues of the voltage sensor. Mutation analysis revealed that KdpD derivatives probably adopt conformations that affect the protein–lipid interaction [14]. Such an effect was already observed for the light harvesting complex II, where a single-site mutation alters the protein–lipid interaction [39]. Instead of a direct interaction with phospholipids, it is possible that KdpD has a thus far undetected protein interaction partner, as indicated for KdpD of *Mycobacterium tuberculosis* [40]. It is important to note that the bacterial membrane exhibits a heterogeneous lipid distribution [41, and references therein]. In *E. coli*, the anionic phospholipid CL is enriched in the septal region and at the cell poles thereby forming CL-rich domains [42]. Huang et al. [43] suggested that this clustering is a curvature-mediated mechanism. With respect to the heterogeneous distribution of lipids, several membrane proteins are localized at the septum and/or at the poles in bacterial cells [44]. This localization indicates that these membrane proteins prefer the vicinity of anionic phospholipids. Furthermore, it was suggested that membrane proteins with a high affinity for CL stabilize the patches of CL [41, and references therein] or that CL promotes polar localization of these proteins as proposed for the osmosensory transporter ProP in *E. coli* [32]. With respect to this, it is possible that the membrane protein KdpD is also clustered in these CL-rich domains or even interacts with CL. Microdomains containing CL and KdpD might explain why a CL increase under K^+ limitation is not absolutely necessary as found in some strains. UE54, which lacks PG and CL, phosphatidic acid and a newly identified anionic lipid, N-acyl PE, were found enriched in the polar regions of the cell [45]. Another reason for the different KdpD activities in mutant strains with alterations in lipid biosynthesis may be that the phospholipids have different lateral packing pressure [46].

In summary, our studies revealed that the increase of anionic phospholipids in the cytoplasmic membrane of *E. coli* affects *kdpFABC* expression under *in vivo* growth conditions. In addition, the overall lipid distribution may be a parameter to which the signalling state of KdpD is sensitive.

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