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# Regulation of *Bacillus subtilis* DesK thermosensor by lipids

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Temperature sensing is essential for the survival of living cells. The membrane-bound thermosensor DesK from *Bacillus subtilis* is a key representative of histidine kinases receptors able to remodel membrane lipid composition when the temperature drops below ~30 °C. Although the receptor is well studied, a central issue remains: how does the compositional and functional diversity of the surrounding membrane modulate receptor function? Reconstituting full-length DesK into proteoliposomes of well-defined and controlled lipid composition represents a minimal synthetic approach to systematically address this question. Thus DesK has been reconstituted in a variety of phospholipid bilayers and its temperature-regulated autokinase activity determined as function of fatty acyl chain length, lipid head-group structure and phase preference. We show that the head group structure of lipids (both *in vitro* and *in vivo*) has little effect on DesK thermosensing, whereas properties determined by the acyl chain of lipids, such as membrane thickness and phase separation into coexisting lipid domains, exert a profound regulatory effect on kinase domain activation at low temperatures. These experiments suggest that the non-polar domain of glycerolipids is essential to regulate the allosteric structural transitions of DesK, by activating the autophosphorylation of the intracellular kinase domain in response to a decrease in temperature.

Key words: DesK, histidine kinase, lipid regulation, membrane protein.

## INTRODUCTION

Cell membranes are composed of a lipid bilayer, containing proteins that either span the bilayer or interact with the lipids on either side of the two leaflets. Membrane proteins come in a huge structural variety, but they have one property in common: they contain one or more hydrophobic regions with which they span the membrane, most often as a single $\alpha$-helix or as a bundle of $\alpha$-helices. Many properties of membrane proteins are determined by interaction between these helices and the surrounding lipids, whereby the helices can act as sensors of the lipid environment [1]. Although analytical advances demonstrated the complexity of lipid molecular forms that make up the lipidome, the function of this diversity remains enigmatic. Moreover, the issue of how membrane proteins interact with lipids in the bilayer is generally a neglected area of research [2].

In the present study, we address the topic of how phospholipids affect the well-studied thermosensor DesK from *Bacillus subtilis* [3]. DesK is an integral membrane-associated histidine kinase which is at the top of a cascade of a regulatory pathway controlling the synthesis of unsaturated fatty acids in *B. subtilis* (Figure 1). *In vivo* and *in vitro* experiments have demonstrated that DesK acts as a kinase at cold temperatures, autophosphorylating a conserved histidine within the kinase domain [4–6]. The phosphoryl group is then transferred to the receiver aspartic acid in the DNA-binding response regulator DesR. Phosphorylated DesR activates the transcription of the *des* gene that encodes acyl lipid desaturase $\Delta^5$-Des [7,8]. Unsaturated fatty acids, the end products of $\Delta^5$-Des activity, promote a more fluid membrane that appears to switch DesK from a kinase to a phosphatase state [9]. Consequently, the concentration of DesR phosphate declines and transcription of the *des* gene is terminated. Two key discoveries that helped the elucidation of the pathway are: (i) a crystallographic study of the DesKC (DesK catalytic core), which has revealed how the domains of this protein can interact to assemble the three active sites that determine its regulatory state, providing an excellent baseline for understanding the mechanism by which DesK functions as a molecular switch, transducing bilayer deformations into protein motions [5]; and (ii) the establishment that DesK retains its functionality even when reconstituted in pure vesicles and hence that no other protein components are involved in either sensing or signalling [5,6]. Thus structural and biochemical approaches showed that DesK is cold-activated through specific interhelical rearrangements in its central four-helix bundle domain, known as DHp (dimerization and histidine phosphotransfer) [5]. In a fluid membrane, the TM (transmembrane) domain would stabilize a connecting coiled-coil and the catalytic core into a rigid conformation with the ATP-binding domains attached to the DHp domain. This conformation inhibits autokinase activity and the DHp surface is competent to interact with DesR phosphate, resulting in a phosphatase signalling state. On cold signal reception, the ensuing

Abbreviations used: AU, arbitrary units; DesKC, DesK catalytic core; DHp, dimerization and histidine phosphotransfer; di($C_{14:1}$)PC, 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine [14:1 ($\Delta^9$-*Cis*) PC]; di($C_{16:1}$)PC, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine [16:1 ($\Delta^9$-*Cis*) PC]; di($C_{20:1}$)PC, 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine [20:1 ($\Delta^9$-*Cis*) PC]; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine [14:0-PC]; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine [18:1 ($\Delta^9$-*Cis*) PC]; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine [18:1 ($\Delta^9$-*Cis*) PE]; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt) [18:1 ($\Delta^9$-*Cis*) PG]; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine [16:0 ($\Delta^9$-*Cis*) PC]; GL, glycolipid; LB, Luria–Bertani; $L_d$, liquid-disordered; MS, minimal sensor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; $S_o$, solid-ordered gel; TM, transmembrane.

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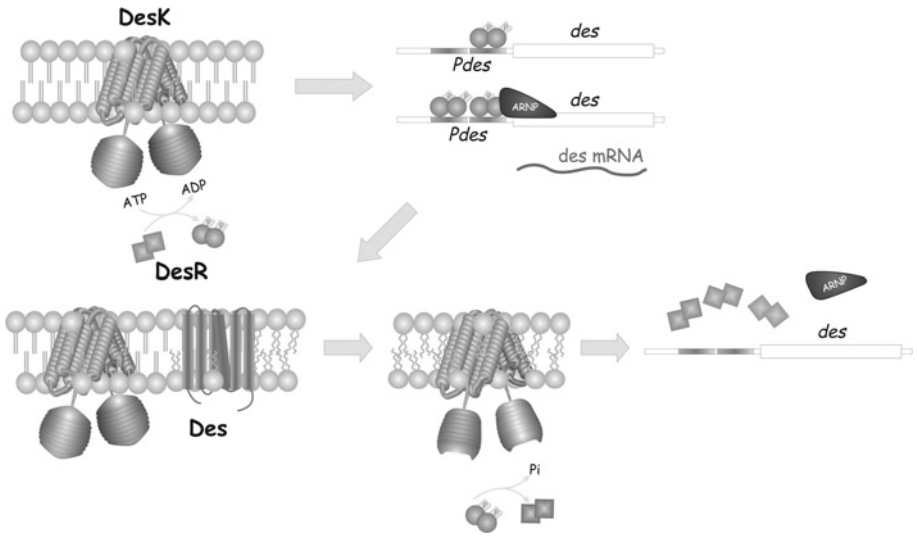



**Figure 1 DesK is the master switch for the *B. subtilis* Des pathway**

An increase in the proportion of ordered membrane lipids promotes a kinase-dominant state of DesK, which autophosphorylates and then transfers the phosphate group to DesR. Two DesR-phosphate dimers interact with the *des* promoter (P*des*) and the RNA polymerase, resulting in *des* transcriptional activation. As a consequence, Δ5-Des is synthesized and desaturates the acyl chains of membrane phospholipids, increasing the fluidity of the membrane. This change favours the phosphatase-dominant state of DesK, leading to DesR-phosphate dephosphorylation and turning off the transcription of the *des* gene.

structural reorganization would release the ATP-binding domains for histidine phosphorylation (kinase state). Phosphorylation of DesKC induces an as yet different, asymmetric conformation capable of interacting with DesR (phosphotranferase state) [5]. These structural changes of the catalytic domain are promoted by the sensor domain of DesK. One or more of the five TM segments in DesK [10] could undergo a conformational change, in the form of helix rotations and asymmetric helical bends, induced by a modification in the physical state of the membrane lipid bilayer. This information is transmitted to the cytoplasmic domain by the membrane-connecting two-helical coiled-coil, ultimately controlling the alternation between output autokinase and phosphatase activities.

Remarkably, the multimembrane-spanning domain from DesK can be simplified into a chimaerical single-membrane-spanning MS (minimal sensor)-DesK that fully retains, *in vivo* and *in vitro*, the sensing properties of the parental system [10]. Mutational and biochemical analysis of this membrane-bound chimaera supported the hypothesis that the N-terminal domain of MS-DesK is able to detect membrane thickness to adjust the signalling state of the catalytic cytoplasmic domain of DesK [10]. Although this minimalist approach shed light on the role of the N-terminal TM domain of DesK on signal transduction, the sensor domain of DesK is composed of five TM-spanning segments [10]. Thus the mechanism by which full-length DesK discriminates the surrounding lipid environment to adjust its signalling state could be much more complex.

In the present study, reconstitution of full-length DesK into proteoliposomes [6] enabled us to follow DesK autophosphorylation in single-component lipid bilayers that, at a given assay temperature, either form a $S_o$ (solid-ordered) phase or $L_d$ (liquid-disordered) phase or can phase separate into coexisting $L_d$ or $S_o$ domains [11]. Our data show that, in the bilayer system, the autophosphorylation of full-length DesK is sensitive to membrane thickness and is activated in phase-separated $L_d/S_o$ proteoliposomes. We also show that both *in vivo* and *in vitro* the regulation of DesK autophosphorylation by temperature is largely independent of the polar head of membrane phospholipids.

**Table 1 Strains and plasmids used in this study**

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *B. subtilis* strains | | |
| JH642 | *trpC2 pheA1* | IBR laboratory stock |
| AKP3 | JH642 *amyE*::P*des-lacZ* | [15] |
| HB5361 | CU1065 *pssA*::*Spc* | [14] |
| HB5346 | CU1065 *ugtP*::MLS | [14] |
| MM04 | JH642 *pssA*::*Spc* | The present study |
| MM05 | JH642 *ugtP*::MLS | The present study |
| MM06 | JH642 *pssA*::*Spc ugtP*::MLS | The present study |
| MM07 | JH642 *pssA*::*Spc amyE*::P*des-lacZ* | The present study |
| MM08 | JH642 *ugtP*::MLS *amyE*::P*des-lacZ* | The present study |
| MM09 | JH642 *pssA*::*Spc ugtP*::MLS *amyE*::P*des-lacZ* | The present study |
| *E. coli* strain | | |
| DH5α | *supE44 thi-1* Δ*lacU169*(ϕ*80lacZ*_M15) *endA1 cA1 hsdR17 gyrA96 relA1 trp6 cysT329*::*lac inm*$^{\lambda pI(209)}$ | IBR laboratory stock |
| Plasmids | | |
| pAD231 | *desK* cloned into pIVEX2.3d | [4] |
| pAR11 | *Pdes* cloned into pJM116 | [15] |

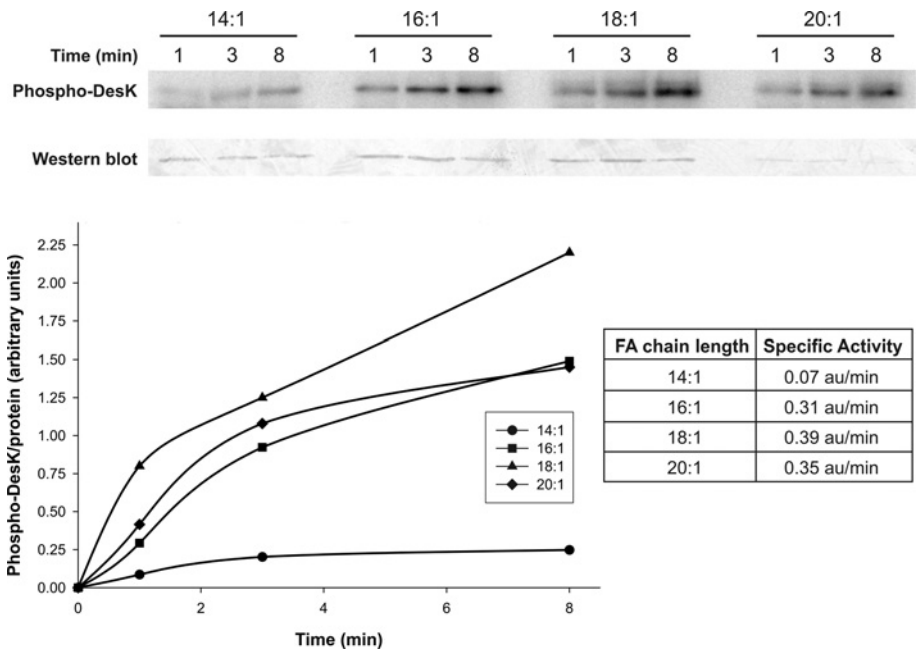
## MATERIALS AND METHODS

### Bacterial strains and growth conditions

Bacterial strains used in the present study are listed in Table 1. *Escherichia coli* and *B. subtilis* strains were routinely grown in LB (Luria–Bertani) broth media [12]. Spizizen salts [13], supplemented with 0.4 % glucose, trace elements, 0.5 mM $Mg_2SO_4$ and 0.01 % each of tryptophan and phenylalanine were used as the minimal medium for *B. subtilis*. Antibiotics were added to medium at the following concentrations: 100 μg/ml ampicillin, 5 μg/ml chloramphenicol, macrolides (1 μg/ml erythromycin and 25 μg/ml lincomycin) and 100 μg/ml spectinomycin.

### Strain constructions

The parental bacterial strain was JH642 (*trpC2 pheA1*) (Table 1). In order to obtain *pssA* and *ugtP* mutants (strains MM04




| FA chain length | Specific Activity |
|---|---|
| 14:1 | 0.07 au/min |
| 16:1 | 0.31 au/min |
| 18:1 | 0.39 au/min |
| 20:1 | 0.35 au/min |

**Figure 2 Effect of PC fatty acid chain length on DesK autophosphorylation**

DesK was reconstituted with PCs of a given chain length and autophosphorylation of the proteoliposomes was measured at 25 °C at the indicated times. Quantification of the level of phosphoprotein (Phospho-DesK autoradiography) as well as of protein (Western blot using anti-His antibodies) was performed by densitometry and plotted as AU against time. The table shows the values of DesK autokinase-specific activities obtained and are representative of at least two independent experiments. FA, fatty acid.

and MM05 respectively), the parental strain JH642 was transformed with chromosomal DNA of HB5361 or HB5346 strains respectively [14] (Table 1), and selected on LB plates supplemented with the proper antibiotics. To obtain strain MM06, strain MM05 was transformed with chromosomal DNA of the HB5361 strain. Then, the three resultant strains (MM04, MM05 and MM06) were transformed with pAR11 plasmid (containing a transcriptional fusion between P*des* and *lacZ* [15]) linearized previously with ScaI. This allowed integration of P*des-lacZ* by a double cross-over event at the *amyE* locus, yielding strains MM07, MM08 and MM09.

### $\beta$-Galactosidase assays

*B. subtilis* strains harbouring P*des-lacZ* fusions were grown overnight in minimal medium. The following day, cells were collected by centrifugation (3000 ***g*** at 4 °C for 15 min) and diluted in fresh medium of the same composition as described above. When cultures reached an attenuance of 525 nm of 0.4–0.5, cells were divided into two cultures and incubated at 25 °C and 37 °C. Samples were taken at the times indicated in Figure 5(B) and assayed for $\beta$-galactosidase activity as described previously [16]. Specific activity was expressed in MU (Miller units).

### Liposome preparation

To obtain large multilamellar vesicles, 20 mg of each lipid or lipid mixture (Avanti Polar Lipids) were hydrated in 1 ml of hydration buffer [20 mM Tris/HCl (pH 8.0), 250 mM sucrose and 100 mM $K_2SO_4$], and the suspension was incubated at 37 °C {or at 45 °C when DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) {also known as 16:0 [$\Delta^9$-*Cis*] PC (phosphatidylcholine)} was used} in a vortex-mixer for 3 h. The suspension was then extruded as described previously [6].

The lipids used include: di($C_{14:1}$)PC (1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine; also known as 14:1 [$\Delta^9$-*Cis*] PC), di($C_{16:1}$)PC (1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine; also known as 16:1 [$\Delta^9$-*Cis*] PC), DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine; also known as 18:1 [$\Delta^9$-*Cis*] PC), di($C_{20:1}$)PC (1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine; also known as 20:1 [$\Delta^9$-*Cis*] PC), DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; also known as 14:0 PC), DPPC, DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; also known as 18:1 ($\Delta^9$-*Cis*) PE) and DOPG (1,2-dioleoyl-*sn*-glycero-3-phospho-1′-*rac*-glycerol sodium salt; also known as 18:1 [$\Delta^9$-*Cis*] PG).

### Proteoliposome collection and biochemical characterization

Plasmid pAD231 [6] was incubated in Roche RTS 100 HY Kit reactions or RTS 500 ProteoMaster *E. coli* HY Kit (Roche) in the presence of 4 mg/ml of the corresponding liposomes according to the manufacturer's instructions. To separate DesK-containing proteoliposomes from the reaction mixture, sucrose gradient ultracentrifugation was performed as described previously [6]. Finally, proteoliposomes containing DesK were used for protein concentration determinations [17] and biochemical characterization.

All autokinase phosphorylation assays were carried out in P buffer [50 mM Tris/HCl (pH 8), 200 mM NaCl, 1 mM dithiothreitol, 20 % (v/v) glycerol, 50 mM KCl, 1 mM $MgCl_2$ and 25 $\mu$M ATP] containing 0.25 $\mu$Ci/$\mu$l [$\gamma$-$^{32}$P]ATP (NEN Life Science Products) [5,6]. DesK proteoliposomes were incubated in P buffer at the indicated temperatures and at different time-points aliquots were taken in five loads of sample buffer and separated by SDS/PAGE (12 % gels), followed by electroblotting [18] to a nitrocellulose membrane (GE Healthcare). Radioactivity of phosphorylated DesK was visualized using a (Typhoon 9200) PhosphorImager screen in a STORM840 (GE Healthcare) and the

same membrane was probed using anti-His antibodies (QIAGEN). Both signals were quantified using ImageQuant software (version 5.2). The values obtained were expressed as AU (arbitrary units). All results shown are representative of at least two independent experiments.

### Lipid analysis

To determine the lipid composition of *B. subtillis*, cells were grown at 37 °C in LB medium. The cultures were harvested in exponential phase and total lipids were extracted by the method of Bligh and Dyer [19]. The lipids were concentrated by evaporation, separated on Silica Gel 60 TLC plates (Merck) in methyl acetate, isopropyl alcohol, chloroform, methanol and 0.25 % KCl (at a ratio of 25:25:25:10:9 by vol.), and visualized by copper phosphoric stain. Lipids were identified by the level of mobility relative to known standard lipids, and by a positive colour reaction with orcinol or ninhydrin.

## RESULTS

To address the role of lipids in the activity of the *B. subtilis* DesK histidine kinase, we bypassed cell membrane complexity by reconstituting DesK into proteoliposomes with defined lipid composition and employed this simplified system to study the role of the lipid environment on autokinase activity. We produced full-length DesK in an *E. coli*-based *in vitro* transcription–translation system and inserted the protein co-translationally into liposomes [6]. We first tested phospholipids containing fatty acyl chains of different lengths, focusing our work on previously well-characterized lipids [20,21] that display different phase states at the DesK autokinase assay temperatures. We then analysed the effect of phospholipid head groups.

### Effects of phospholipid chain length on DesK autokinase activity

DesK was reconstituted into bilayers of PCs containing monounsaturated fatty acyl chains of lengths between 14 and 20 carbons: di($C1_{4:1}$)PC, di($C_{16:1}$)PC, DOPC and di($C_{20:1}$)PC. Phase transition temperatures for all these lipids are $<0$ °C [20] so that all the lipids tested are in the $L_d$ crystalline phase at the assay temperature (25 °C). Reconstitution of DesK into bilayers of di($C_{16:1}$)PC, DOPC and di($C_{20:1}$)PC gave almost the same autokinase activity (Figure 2). Nevertheless, when DesK was reconstituted into bilayers of di($C_{14:1}$)PC, its activity was approximately 5-fold lower than DesK reconstituted into PCs containing longer acyl chains. The present results indicate that, as reported for MS-DesK [10], the thickness of the bilayer is an important parameter regulating the activity of full-length DesK.

### Regulation of DesK by lipid phase transition

To examine the role of the lipid phase behaviour on DesK activity, we reconstituted the sensor in bilayers containing lipids that display different phase states at the assay temperature. To this end, we reconstituted DesK into selected PCs that undergo phase transitions between the $L_d$ and $S_o$ phase at $-20$ °C (DOPC), 23 °C (DMPC) or 42 °C (DPPC) [21]. DesK autokinase activity was assayed at 25 °C. At this temperature, DPPC and DOPC are in the $S_o$ and $L_d$ phase respectively, whereas in liposomes of DMPC $S_o$ and $L_d$ phases coexist. The highest activity was observed in DMPC, whereas reconstitution of DesK into DOPC and DPPC leads to a gradual decrease in activity (Figure 3A).

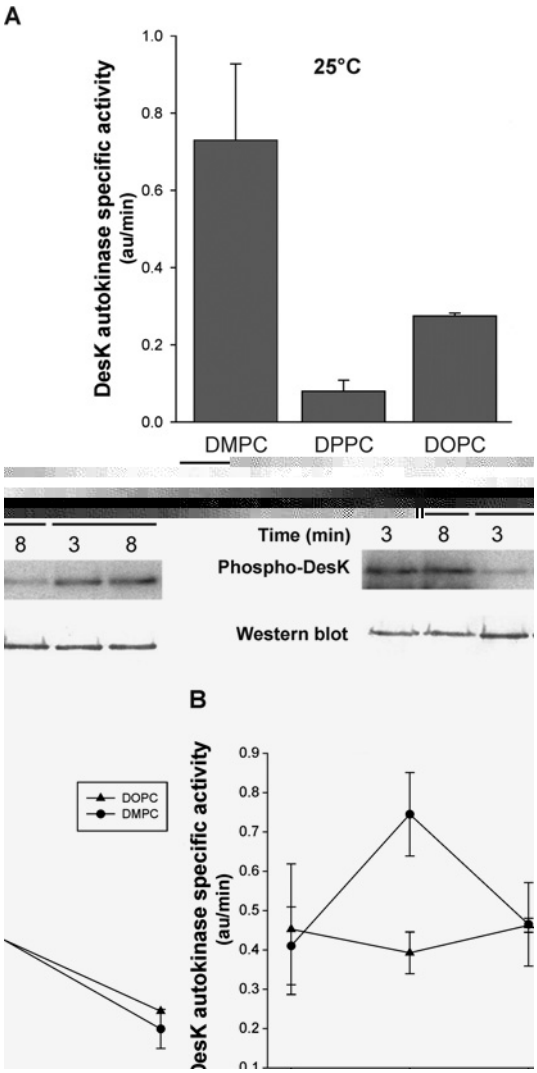
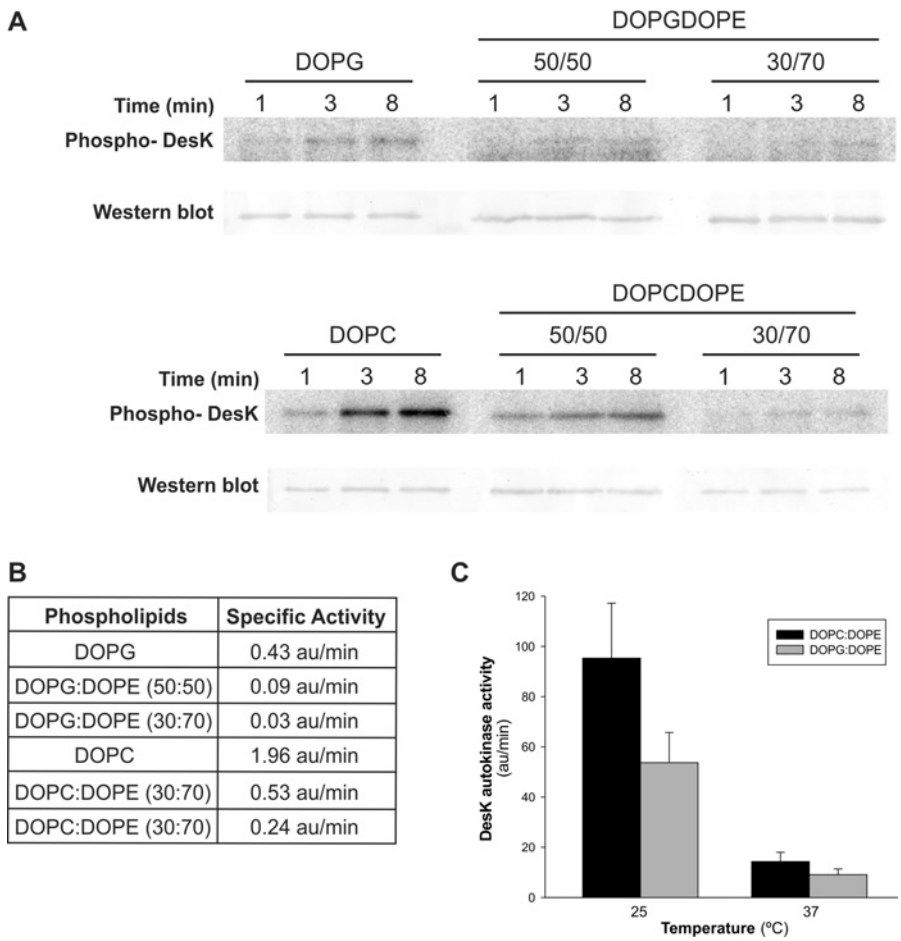



**Figure 3 Effect of phospholipid phase on DesK autophosphorylation**

(**A**) DesK was reconstituted in bilayers of DOPC ($T_m$: $-20$ °C), DPPC ($T_m$: 41 °C) and DMPC ($T_m$: 23 °C) and autophosphorylation of the proteoliposomes assayed at 25 °C. Quantification of the level of phosphoprotein (Phospho-DesK autoradiography) as well as of protein (Western blot using anti-His antibodies) was performed by densitometry and plotted as AU against time in order to calculate specific activities. Bars show DesK autokinase-specific activities obtained and error bars represent the S.D. for the values obtained. (**B**) Effect of temperature on DesK activity in DOPC or DMPC lipids. DesK was reconstituted in bilayers of DOPC or DMPC and autophosphorylation of the proteoliposomes assayed at different temperatures. Specific DesK activities were calculated as described in (**A**). The values are presented as means ± S.D. for least two independent experiments.

To further investigate the effect of lipid phase on DesK signalling state, we analysed the activities of DesK as a function of temperature in lipid bilayers composed of either DMPC or DOPC. As shown in Figure 3(B), the DesK activities in proteoliposomes of DMPC and DOPC were almost similar at 20 °C. However, at 25 °C, the activity of DesK in DMPC increased 2-fold, whereas in DOPC, DesK activity remained similar to when assayed at 20 °C. Finally, the activity of DesK in DMPC strongly decreased at 37 °C, reaching values similar to those showed by DesK in DOPC at the same temperature. The present results show that DesK activity is stimulated when the bilayer is in the transition region between the $L_d$ and the $S_o$ phase. On the other hand, the low activity of DesK in DPPC at 25 °C could reflect some conformational constraint in the rigid environment provided by the $S_o$ phase




| Phospholipids | Specific Activity |
|---|---|
| DOPG | 0.43 au/min |
| DOPG:DOPE (50:50) | 0.09 au/min |
| DOPG:DOPE (30:70) | 0.03 au/min |
| DOPC | 1.96 au/min |
| DOPC:DOPE (30:70) | 0.53 au/min |
| DOPC:DOPE (30:70) | 0.24 au/min |

**Figure 4 Effect of lipid head groups on DesK autophosphorylation**

(**A**) DesK was reconstituted into DOPC, DOPG or mixtures of either DOPC or DOPG containing 50 % (50:50) or 70 % (30:70) of the non-bilayer DOPE and autophosphorylation of the proteoliposomes was assayed at 25 °C at the indicated times. Quantification of the level of phosphoprotein as well of protein was performed by densitometry and plotted as AU against time to estimate specific activities. (**B**) The table shows the values of DesK autokinase-specific activities obtained, which are representative of at least two independent experiments. (**C**) Effect of temperature on DesK activity in equimolar mixtures of DOPC:DOPE or DOPG:DOPE lipids.

bilayer. Many membrane proteins exhibit low activity when the surrounding lipid is in the $S_o$ phase, for example, the $Ca^{2+}$-ATPase of the sarcoplasmic reticulum [22], the glucose transporter from red blood cells [23] and diacylglycerol kinase of *E. coli* [24].

### Effects of lipid head group on DesK regulation

The functional characterization of DesK was performed in PC vesicles, because in this phospholipid DesK is efficiently inserted and thermoregulated [6]. However, PC is absent in *B. subtilis* membranes, which instead contains PE (phosphatidylethanolamine) and PG (phosphatidylglycerol) as major phospholipids. Thus we decided to test *in vitro* the effects of PG and PE on DesK activity and its regulation by temperature. To this end, we reconstructed DesK in mixed phospholipid bilayers composed of either DOPC or DOPG or in liposomes composed of DOPC and DOPE or DOPG and DOPE. As shown in Figure 4, the autokinase activity at 25 °C of DesK reconstituted in DOPG was approximately 5-fold lower than in DOPC. Furthermore, the presence of DOPE has a marked inhibitory effect on DesK autophosphorylation (Figure 4A). In DOPC or DOPG, the addition of DOPE to a molar ratio of 0.5 lead to a decrease of approximately 3-fold in autokinase activity with higher concentrations of DOPE resulting in significant lower activities (Figures 4A and 4B).

The effect of DOPE on DesK in DOPC or DOPG liposomes is exerted on autophosphorylation rather than in thermosensing since the DesK autokinase activity was up-regulated approximately 6-fold when the assay temperature was decreased from 37 °C to 25 °C (Figure 4C). Thus we conclude that DesK is properly folded in either PG- or PE-containing liposomes and that the TM domain of DesK is able to sense and transmit the temperature signal regardless of the glycerolipid head group used to reconstitute the sensor kinase.

### Temperature regulation of DesK in *B. subtilis* cells with altered membrane phospholipid composition

The results described above suggest that up-regulation of DesK autophosphorylation at low temperatures is largely independent of the polar head of membrane phospholipids. To rule out an artefact introduced by reconstitution conditions and to determine whether or not the regulatory properties of DesK are affected *in vivo* by the lipid head groups, we took advantage of the fact that the membrane of *B. subtilis* can be greatly simplified by removal of PE and GL (glycolipids) without affecting growth proficiency [14]. To this end, we disrupted the *pssA* and *ugtP* genes that are involved in the

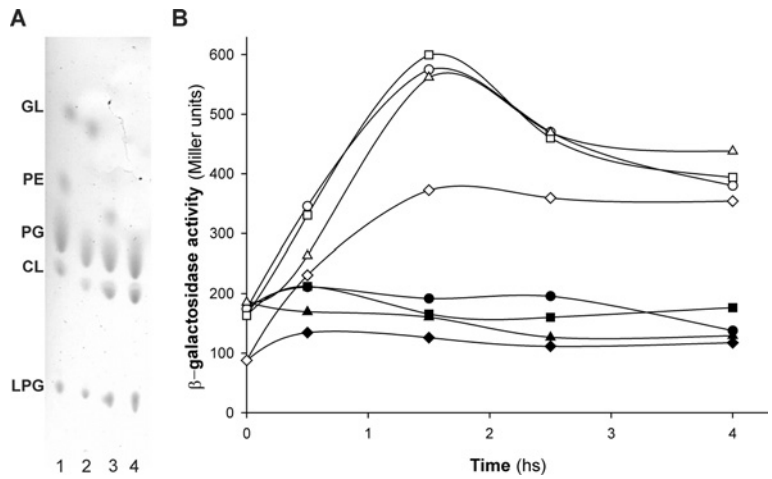



**Figure 5 Regulation of DesK in *B. subtilis* lipid-deficient mutants**

(**A**) TLC analysis of lipid-deficient mutants. Lipids from cells grown in LB media were extracted, separated by TLC (methyl acetate system) and developed by copper phosphoric stain as described in the Materials and methods section. Lane 1, strain JH642; lane 2, MM04 (PE-minus); lane 3, MM05 (GL-minus) and lane 4, MM06 (PEGL-minus). (**B**) Effect of complex lipid composition on P*des-lacZ* expression. *B. subtilis* lipids mutant cells harbouring a wild-type DesK and a P*des-lacZ* transcriptional fusion located in the *amyE* locus were grown at 37 °C to an attenuance of 0.3–0.4 (at 525 nm) and then divided into two samples. One sample was transferred to 25 °C (open symbols), and the second was kept at 37 °C (closed symbols). Aliquots were taken at the indicated times and $\beta$-galactosidase activity was determined. (●, ○), strain JH642; (■, □), strain MM07 (PE-minus); (▲, △), strain MM08 (GL-minus) and (◆, ◇), strain MM09 (PEGL-minus). The values are representative of three independent experiments. CL, cardiolipin; LPG, lysyl-phosphatidylglycerol.

biosynthesis of PE and GL respectively, by allelic replacement with antibiotic resistance cassettes. The absence of PE and GL in the mutant strains was verified by TLC analysis (Figure 5A). We also constructed a *pssA ugtP* double mutant (strain MM06) that was unable to produce both PG and GL (Figure 5A). Then, to monitor the signalling states of DesK in membranes with different lipid composition, we introduced a *lacZ* reporter gene fused to the desaturase promoter (P*des*) into the different strains, which is activated only when there is flux of phosphate from autophosphorylated DesK to the DesR transcription factor. When a *B. subtilis* strain containing a wild-type lipid composition carrying the P*des-lacZ* fusion was grown at 37 °C, the levels of $\beta$-galactosidase were very low (Figure 5B, ●). However, when cells containing this fusion growing at 37 °C were shifted to 25 °C, $\beta$-galactosidase synthesis began approximately 30 min after the temperature downshift reaching maximum induction levels 3-fold higher than the levels at 37 °C after approximately 1.5 h at 25 °C (Figure 5B, ○). When a similar experiment was performed with *pssA* (strain MM07, Figure 5B, ■ and □) or *ugtP* (strain MM08, ▲ and △) strains, the same pattern of $\beta$-galactosidase expression was obtained, indicating that the lack of PE or GL in *B. subtillis* membrane did not affect the capacity of DesK to sense the cold stimulus that control the transcription of the desaturase gene (Figure 5B). A similar pattern of P*des-lacZ* induction was also observed in the *pssA ugtP* double mutant (strain MM09, Figure 5B, ◆ and ◇), although the levels of $\beta$-galactosidase in this strain were lower than those observed in *pssA* or *ugtP* single mutants (Figure 5B). Altogether, we conclude that in a *B. subtilis* membrane with grossly altered phospholipid composition, the TM domain of DesK is still able to discriminate the surrounding lipid environment to promote membrane remodelling following a drop in growth temperature.

## DISCUSSION

An unresolved issue concerning the Des pathway [3] is how the lipid environment could influence DesK regulation. Whereas the structure of full-length DesK has not yet been solved, structural studies of the catalytic core of DesK highlights the structural plasticity of the central DHp domain and suggest an important role of these changes in catalysis regulation, either by modifying the mobility of the ATP binding domains for autokinase activity or by modulating binding of DesR to sustain the phosphotransferase and phosphatase activity [5]. Albanesi et al. [5] suggested a model in which the TM sensor domain of DesK promotes these structural changes through conformational signals transmitted by the membrane-connecting two-helical coiled-coil. The question now becomes how the balance between output kinase and phosphatase activities is regulated. Previous studies have suggested that the lipid environment plays a key role in dictating the signalling state of the sensor [5,6,9,10,15], but because of *Bacillus* membrane composition diversity, unambiguous assignment of lipid-mediated DesK modulation has not found its way into the signal transduction canon. Our results suggest that, in addition to an increase in membrane thickness (Figure 2), DesK is specifically activated by phase-separated $L_d/S_o$ liposomes (Figure 3). How this finding corresponds to DesK behaviour when *B. subtilis* living cells are exposed to low temperatures is not clear. One possibility is that DesK could preferentially associate in a dynamic fashion with nanoscale membrane domains induced during *B. subtilis* cold adaptation. Evidence of lipid-dependent functional domains has been reported for *B. subtilis* [25] and several other bacteria [26]. Thus we could speculate that the compartmentalization of DesK in phase-separated areas might facilitate its activation. For example, it has been suggested that *B. subtilis* membranes contain lipid-dependent domains that are functionally similar to eukaryotic lipid rafts, in that they harbour and organize a variety of signalling proteins such as KinC (a sensor kinase involved in biofilm production) [25]. Interestingly, compromising the integrity of rafts by disrupting the synthesis of a polyisoprenoid, a lipid that is thought to assemble lipid rafts in *B. subtilis*, leads to mislocalization of KinC and inhibition of the signal transduction pathway triggered by this sensor kinase. In concert with the concept of the formation of microdomains in prokaryotes, it was recently shown that hopanoids, which are structurally similar to sterols, have the ability to order

saturated lipids and to form a $L_d$ phase in model membranes [26]. Although the physiological relevance of this property in bacteria remains unaddressed, the hopanoids could potentially confer the ability to subcompartamentalize bacterial membranes into functional domains. In summary, the regulation of DesK by coexisting lipid phases could be analogous to the orchestration of physiological process in microdomains promoted by bacterial 'sterol surrogates'.

Another important aspect of DesK regulation by membrane lipids is that whereas the hydrophilic domains of lipids do not affect DesK thermosensing, both *in vitro* and *in vivo* (Figures 4 and 5), the interactions between the sensor TM domain and the hydrocarbon domains of lipids seem to be essential for perceiving the cold signal. The observation in the present study is in agreement with a previous report showing that alterations in membrane lipid head group composition are generally well-tolerated by *B. subtilis* cells, retaining viability and even rapid growth when the membrane is comprised predominantly, if not exclusively, of PG [14]. However, a surprising result was that the naturally occurring PG and PE have a marked inhibitory effect on DesK activity when they are mixed with vesicles composed of PC, which is absent in *B. subtilis* membranes [27]. It could be possible that the high negative charge of PG or the intermolecular H-bonds formed by either PE or PG could affect the interactions between the lipid head groups and protein functional groups during catalysis. These differences between PC and PE on supporting the function of reconstituted membrane proteins have been previously observed in several secondary transport proteins [28,29]. Therefore the molecular basis for lipid requirements by DesK is more complex than previously thought, involving the physical and chemical properties of a multi-component membrane bilayer. It should be noted that the complexity of *B. subtilis* cellular membrane follows in part from the wide variety of fatty acyl chains (i.e. branched fatty acid) contained in the lipids and that the immediate microenvironment of membrane-bound DesK may be different from the bulk membrane properties. Clearly, further studies are necessary to understand the molecular basis of lipid requirement for DesK activity and temperature regulation.

## AUTHOR CONTRIBUTION

Mariana Martín and Diego de Mendoza designed the work. Mariana Martín performed all the experimental work. Mariana Martín and Diego de Mendoza analysed the data and wrote the final paper.

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