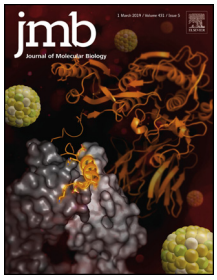

Reverse Engineering of a Thermosensing Regulator Switch

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Abstract

To address the mechanism of thermosensing and its implications for molecular engineering, we previously deconstructed the functional components of the bacterial thermosensor DesK, a histidine kinase with a five-span transmembrane domain that detects temperature changes. The system was first simplified by building a sensor that consists of a single chimerical transmembrane segment that retained full sensing capacity. Genetic and biophysical analysis of this minimal sensor enabled the identification of three modular components named determinants of thermodetection (DOTs). Here we combine and tune the DOTs to determine their contribution to activity. A transmembrane zipper represents the master DOT that drives a reversible and activating dimerization through the formation of hydrogen bonds. Our findings provide the mechanism and insights to construct a synthetic transmembrane helix based on a poly-valine scaffold that harbors the DOTs and regulates the activity. The construct constitutes a modular switch that may be exploited in biotechnology and genetic circuitry.

Introduction

The DesK–DesR two-component system maintains membrane fluidity in *Bacillus subtilis* and may be regarded as a representative cell sensor. In response to a decrease in environmental temperature, a dimeric DesK endowed with its histidine kinase domain phosphorylates the response regulator DesR, which in turn controls the enzymatic formation of double bonds in fatty acids, promoting recovery of membrane fluidity (Fig. 1A [1]). A truncated DesK variant lacking the transmembrane region shows high activity at any temperature, suggesting that the sensor is the transmembrane region. To identify the trigger of the signaling process, we first determined how the transmembrane segments (TMSs) sense temperature changes and transduce this variation into a conformational rearrangement that will steer the catalytic state [2–5]. The five-pass TM domain from DesK was re-engineered into a chimerical single TMS named Minimal Sensor (MS-DesK) that retains wild-type sensing and transmission capacity *in vivo* and *in vitro* (Fig. 1A [2]). The MS-DesK sensor provides the ideal setting to apply genetic analysis coupled with biophysical insights to identify the operative structural components of the sensing mechanism. In previous independent studies, we found three such motifs hereby named determinants of thermodetection (DOTs), which are located at the N- and C-terminus of the MS-DesK. Each DOT contributes to elicit a conformational transition in response to temperature changes that alter the membrane properties. At high temperatures (T 37 °C), the membrane gets thinner and more hydrated [6], which has been proposed to suppress the kinase state of the cytoplasmic domain, while at low temperatures (T 25 °C), the membrane becomes thicker and dehydrated [6], promoting the kinase state [2,4,5]. We first genetically dissected the system to delineate the mechanistic underpinnings of the signal transmission from the extracellular into the cytosolic space [2–5].

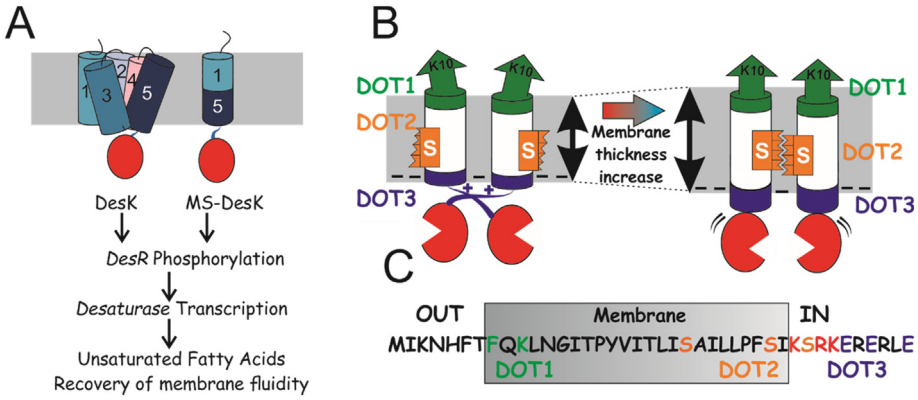


Fig. 1. Dissection of sensing-signal transduction mechanism in DesK. (A) DesK (with 5 TMS) or MS-DesK (with TMS chimera) phosphorylates the response regulator DesR upon a temperature decrease (for simplicity, the other DesK monomer is not shown). (B) DOTs in action. At 37 °C (left panel), the ammonium group of K10 (DOT1, green) snorkels into the aqueous phase, locking the TMS into the lipid phase, while the hydroxyl groups of the serine zipper (DOT2, orange) get partially hydrated and the linker (DOT3, blue line), fully embedded into the aqueous cytosol, adopts a random coil conformation. At lower temperature (25 °C, right panel), the membrane becomes thicker and dehydrated, DOT2 engages in dimerization-promoting intermolecular hydrogen bonding and DOT3 penetrates the lipid phase by adopting a helical conformation. (C) Sequence of MS-DesK. Amino acids highlighted with green, orange or red/blue correspond to DOT1, DOT2 and DOT3, respectively.

The DOTs are identified as follows:

DOT1—sunken buoy

The dyad F8/K10 located at the N-terminus of the TMS functions as a "sunken buoy" due to the hydrophilicity of the K10 ammonium group coupled with the lipophilicity of F8, and anchors the TMS to the extracellular water–lipid interphase (Fig. 1B–C, green arrow and letters [2]). Thus, K10 is located in the hydrophobic membrane core, while its side chain snorkels toward the water phase so the ammonium gets hydrated. At high temperatures, membrane thinning favors lysine hydration, while at low temperature, membrane thickening forces the hydrated polar ammonium of K10 to pull the TMS toward the water phase. This piston-like motion induces downstream conformational changes that promote the kinase state [2,5].

DOT2—serine zipper

The zipper controls TMS dimerization and involves three serine residues (S23, S30 and S33) located on the same face of the TMS C-terminus helix [4] (Fig. 1B–C, orange boxes and letters). Through formation of intermolecular hydrogen bonds, this motif becomes a dimerization promoter, sensitive to the membrane hydration level, which in turns depends on temperature. At higher temperatures, the membrane becomes thinner and more hydrated at the water–lipid interphase [5,6]. We propose that membrane hydration at higher temperature would allow the serine hydroxyls, in particular S30 and S33, to form hydrogen bonds with water. On contrary, at lower temperature, the membrane becomes thicker and less hydrated [5,6]; therefore, serine hydroxyl groups would form hydrogen bonds with the serines in the partner monomer to compensate for hydroxyl dehydration. It has been proposed that this intermolecular hydrogen bonding at low temperature leads to reorientation of the TMS helices, a motion transduced into the cytoplasmic domain to turn on the kinase state by enabling cross-phosphorylation [4].

DOT3—dual linker

The charged region (K_{32}–K_{43}) connects the transmembrane sensor domain with the cytoplasmic catalytic domain and may adopt two conformational states in response to changes in membrane thickness (Fig. 1B–C, blue lines and blue/red letters). At higher temperature, the membrane gets thinner and the linker is fully hydrated, adopting a random coil conformation. Positive charges of the linker orient to interact electrostatically with the negatively charged phospholipid head groups at the water membrane interface. At low temperature, membrane thickening triggers linker folding into a helix to increase the length of the TMS and to avoid the hydrophobic mismatch. Specifically, the linker detaches from the membrane surface forming a continuous helix, with charges neutralized in the register (i, i + 4). This conformation promotes the kinase state. The helix/coil conformational duality triggered by membrane thickness changes enables the linker to function like a transmission switch [3].

The key issue is now to understand how the three DOTs interplay to determine the MS DesK signaling state. This prompts us to ask whether each DOT contributes equally or there is a master DOT that exerts the strongest influence, to the extent of overwriting the effect of the other DOTs in accord

with a functional hierarchy. Identifying the key components of thermosensing allowed us to reverse engineer the sensor by modularly inserting DOTs into a poly-valine transmembrane scaffold, which surprisingly controls gene expression in response to cold. Our findings contribute to fundamental research as we unravel the necessary and sufficient building blocks of the mechanism of cold thermosensing. This contributes to bioengineering design as our enhanced DOTs may be incorporated modularly into genetic circuits, allowing for reprogramming of biological signaling processes for biotechnological applications that require control of gene expression with minimal invasiveness.

Results

DOTs interplay

According to the model presented in Fig. 1B, kinase activity may be promoted in three ways: (i) by hampering hydration of K10 in DOT1, (ii) by stabilizing the serine zipper (DOT2) through formation of hydrogen bonds across the dimer interface and (iii) by disrupting the electrostatic interaction between the linker and membrane and stabilizing the linker helix (DOT3). The key issue is to find which effect (if any) exerts the strongest influence in defining the signaling state.

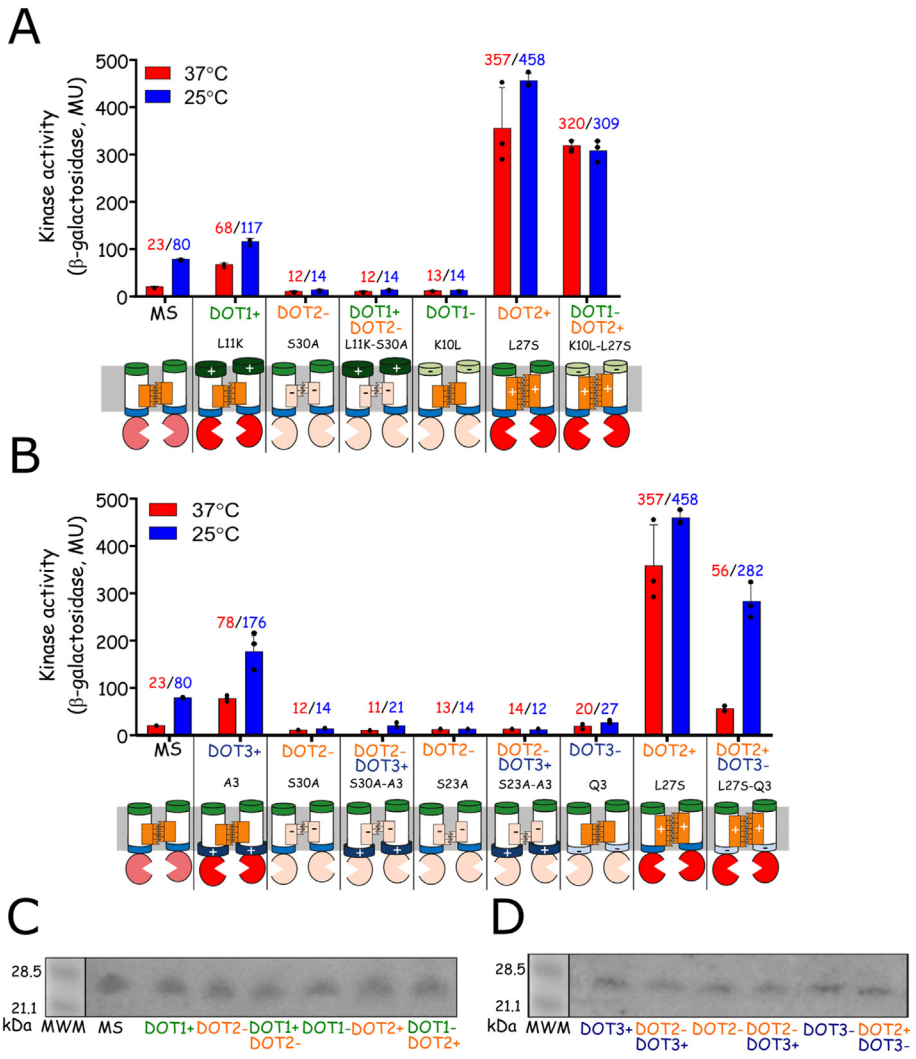


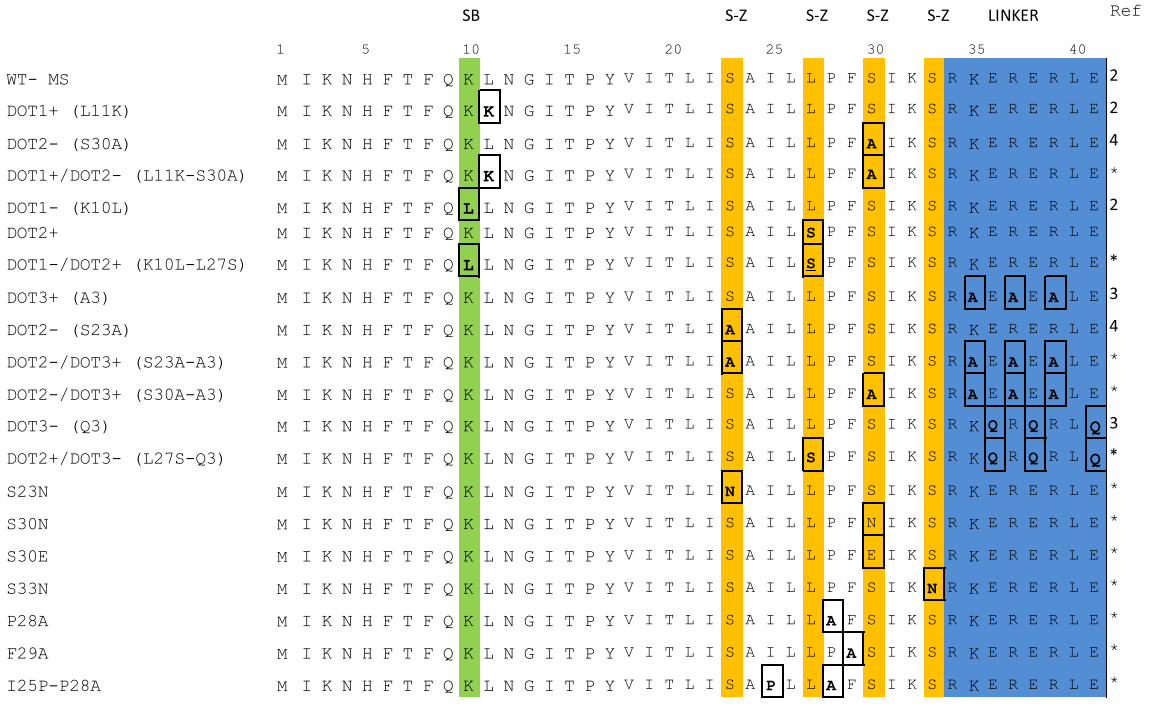
Fig. 2. The serine zipper is the master DOT. Mutations in different DOTs that have opposite effects on DesK activity were combined in one protein, and the effect at 25 °C was analyzed using the β-galactosidase reporter system (Miller units). (A) DOT1 *versus* DOT2. (B) DOT2 *versus* DOT3. Lower panels show representations of the different mutants. The green part of the cylinder represents DOT1; the orange squares on the sides, the serine zipper (DOT2); and the blue part, the linker (DOT3). Plus (+) and minus (–) symbols, which are accompanied by an increase or decrease in the color intensity of DOTs, mean that a single mutation in this region increases or decreases the kinase activity respectively. Western blots of the membrane fraction show that the levels of expression and integration of each DesK variants shown in panels A and B are similar (C and D, respectively).

First, we confronted DOT1 with DOT2. To that purpose, we first created DOT1^{+} mutant L11K, endowed with higher kinase activity because two consecutive lysine residues, K10 and K11, pull more strongly the TMS toward the water phase (Fig. 2A, Table 1). The DOT1^{+} variant was further modified to harbor DOT2^{-} mutant S30A, which suppresses kinase activity because the zipper is destabilized due to loss of one intermolecular hydrogen bond. Double mutant DOT1+/DOT2– (L11K/S30A) is in fact a kinase-inactive protein, suggesting that a stable zipper is essential to promote the kinase state (Fig. 2A). DOT2 dominant effect is apparent as serine-to-alanine substitution hampers the quaternary rearrangement required to recruit the kinase state. Similarly, DOT1^{-} mutant K10L, in which the pulling force of the sunken buoy is removed, was combined with DOT2^{+} L27S variant, promoting higher zipper stability through expansion of the dimerization interface. As it turns out, the double mutant DOT1–/DOT2+ (K10L/L27S) is kinase biased regardless of temperature, implying again that DOT1 is subordinated by DOT2 (Fig. 2A).

Similarly, DOT2 was confronted with DOT3. DOT3 mutant A3, in which three positively charged residues of the linker were replaced by alanine residues, is locked in the kinase state because the electrostatic interaction between linker and membrane surface is impaired. The DOT3^{+} variant was further modified to harbor DOT2^{-} mutant S23A (or S30A), which suppresses kinase activity because the zipper is destabilized due to loss of one intermolecular hydrogen bond. The DOT2^{-}/DOT3^{+} double mutants S23A–A3 and S30A–A3 are kinase-inactive proteins (Fig. 2B). Thus, while DOT3 is genetically modified to promote the kinase state, this engineered functionality is overwritten by the weakening of the dimerization interface obtained by the genetic re-engineering of DOT2 to decrease the number of H-bonds between the TMS molecules. DOT3 mutant Q3, in which three glutamates of the linker were replaced by glutamine residues, is an inactive protein because these mutations result in a net positive charge of the linker that reinforce its electrostatic interaction with the negatively charged membrane surface. DOT3^{-} was combined with DOT2^{+} mutation L27S, which stabilizes the serine zipper due to incorporation of an extra serine to the dimerization interface. Although the temperature regulation of activity in the double mutant DOT2+/DOT3– (L27S/Q3) is maintained, the levels are 3.5 and 2.4 times higher than MS-DesK at 25 °C and 37 °C, respectively (Fig. 2B). This suggests that the engineered stabilization of the dimer interface engaging DOT2 supersedes the engineered linker propensity to promote the kinase-off state, subordinating DOT3 into adopting a conformation biased toward the kinase state.

Together these results suggest that DOT2 has a predominant role in defining the active conformation of DesK in response to temperature and serves as the master activation switch.

Table I. Sequences of MS-DesK variants. Mutations are highlighted with squares. Positions of DOT1, DOT2 and DOT3 are highlighted with green, orange and blue, respectively. An asterisk means “this work.”



Tuning master DOT2

To test if DOT2 can be tuned, serine residues in the zipper were replaced with asparagines, endowed with higher hydrogen-bonding propensity and able to promote helix association [7]. Variants S23N and S30N were competent to activate the sensor (Fig. 3). Their kinase activity at 25 °C was 2.3 and 1.8 times higher than that of the parental protein, in consonance with the fact that asparagine forms stronger hydrogen bonds [8]. At 37 °C, these mutants showed higher levels than background, suggesting that the stabilized H-bond zipper is not totally broken at 37 °C. A similar result was obtained with the replacement S30E, glutamate being another residue with high hydrogen-bonding propensity [9]. The results support the hypothesis that formation of inter-helical hydrogen bonds between the TMS monomers is required to promote the kinase state, adding a tuning dial to manipulate the sensor response. Variant S33N showed low kinase activity, likely because it is located in a more hydrophilic environment where asparagine gets better hydration than serine, preventing hydrogen bond formation between monomers (see Fig. 1C). This environment differs from that of S23N and S30N, which are located in the membrane core, making it more favorable to form hydrogen bonds to mitigate the dehydration penalty. To test if DOT2 can be tuned in full length DesK, we introduced the following mutations: S143N and S150N, which locate in the C-terminus of the fifth TMS and correspond to positions S23 and S30 in the chimerical MS-DesK. Figure 3B shows an activating effect when DOT2 is reinforced in native DesK, in particular for the mutant S150N, which doubles the performance of the sensor in terms of relative activity at 25 °C *versus* 37 °C, being 128/24 for native DesK and 276/29 for mutant S150N (Fig. 3B), suggesting that differently from the simplified version MS-DesK, the reinforced zipper of native DesK can be completely destabilized at 37 °C. The dimerization zipper interface in native DesK is tuned to be highly destabilized at high temperature. It is likely that TM5, containing the serine zipper, also interacts with some of the other four TMSs, favoring its destabilization at 37 °C. Together these results support the importance of DOT2 as a target to manipulate DesK activity.

Reverse engineering to T-sensitize a transmembrane helix

To validate the overarching hypothesis that the three DOTs are sufficient for a TMS to sense temperature, we were prompted to reverse engineer the natural design into a synthetic sensor. We adopted a valine sequence as scaffold to which the three DOTs were incorporated: the F/K dyad at the N-terminus (DOT1, green), an expanded dimerization zipper (including the hydrogen formers S23, S27, S30 and S33; DOT2, orange) and the linker connecting the synthetic transmembrane helix to the catalytic domain (DOT3, blue). In addition to the DOTs, two PF (proline–phenylalanine) motifs, which mimic the PY and PF motifs in the chimeric MS-DesK construct, were incorporated to the C-terminus and in the middle of the scaffolding helix, respectively (Fig. 4A). Since Proline residues induce kinks in TM helices increasing conformational flexibility, they play a key role in signaling proteins [8]. Accordingly we

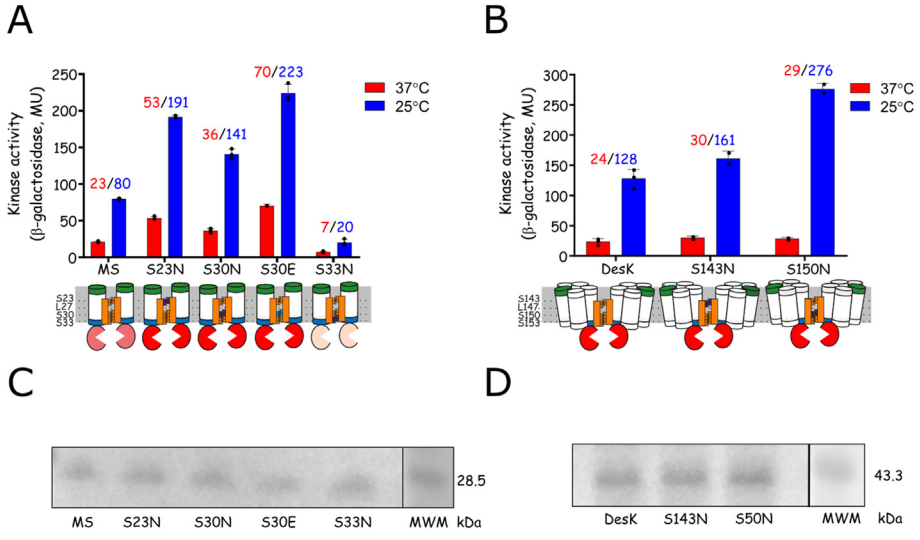


Fig. 3. Re-tuning the mater switch. Mutations that reinforce the zipper have a stimulating effect on the activity of MS-DesK (A) and full length DesK (B). Western blots of the membrane fraction show that the levels of expression and integration of DesK mutants shown in panels A and B are similar (C and D, respectively).

tested its functional role in DesK sensing by constructing the mutant P28A. Figure 4C shows that this variant is inactive, suggesting that proline has a possible structural/or functional role in inducing a hinge in the TM helix, which may be required for proper interaction with its homodimer [10]. Proline having an adjacent aromatic residue enhances the stability of the turn conformation through a stacking CH/π interaction, in which the partial positive charge on a proline hydrogen(s) interacts with the π face of the aromatic ring [11,12]. Since proline 28 is followed by an aromatic residue, F29, we tested its role in activity. Figure 4 shows that the replacement F29A resulted in a protein with reduced activity supporting the role of the dyad P28–F29 in breaking the helix to generate a functional kink. To further validate this finding, we constructed MS-DesK variant I25P–P28A. In this mutant, proline is moved up one turn of helix and broken apart from F29. Figure 4C shows that this variant is a kinase-off protein suggesting that P28 plays a key structural role, and that moving it along the helix could affect the resulting effective hydrophobic thickness of the TMS. Together, these results prompt us to include the P28–F29 motif in the synthetic construction (boxed in Fig. 4A). A similar motif (proline-aromatic residue: P16–Y17) is located in the middle of the TMS of the MS-DesK and DesK (proline-aromatic residue: P135–F136). We deduced that this motif could also have a structural/or functional role in the thermosensor helix. Accordingly, we included it in the poly-valine scaffold (boxed in Fig. 4A). The resulting synthetic helix, Syn1, containing the three DOTs and the structural PF motifs, was kinase inactive (Fig. 4C). Taking into account that DOT2 has a dominant role in thermosensing, we reinforced the synthetic zipper by including a threonine at position 20, since there is a threonine in this position in the sensor MS-DesK. This replacement introduces an H-bond-forming residue in the same face of the helix where the zipper is located. This synthetic helix, Syn2, is active driving reversible activation of the catalytic cytoplasmic domain in response to temperature, triggering the appropriate cellular response (Fig. 4C). This result suggests that the hydroxyl group of T20 belongs to the zipper dimerization interface and that it is required for activation.

Discussion

DesK contributes with thermogenetics

Bacterial membrane homeostasis is essential to maintaining the function and integrity of the lipid bilayer as a physical–chemical barrier and as a functional matrix in which embedded signaling proteins, receptors and transporters work. The membrane sensor DesK, in charge of controlling lipid fluidity in the model bacteria *B. subtilis*, is regarded as a prototype for this type of homeostasis. Genetic and

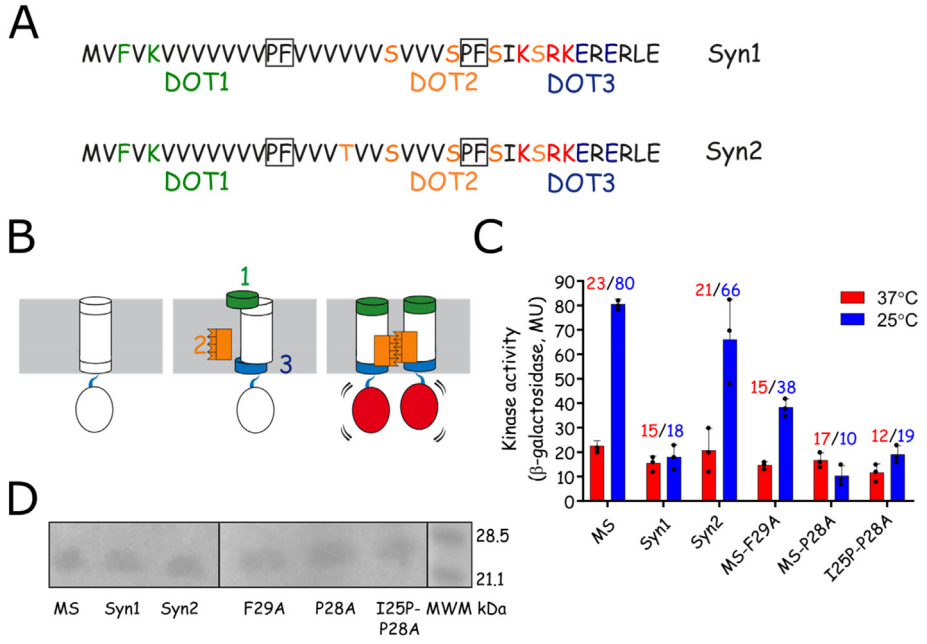


Fig. 4. (A) Sequence of the synthetic transmembrane helix Syn1 and Syn2. Amino acids highlighted with green, orange or red/blue correspond to DOT1, DOT2 and DOT3, respectively. The proline–phenylalanine dyads are boxed. (B) Schematics of the synthetic design. (B) *In vivo* kinase activity of the minimal sensor (MS) [2], Syn1, Syn2, and MS variants with the replacements P28A, F29A and I25P–P28A. (D) Western blots of the membrane fraction show that the levels of expression of DesK variants shown in panel C are similar.

biophysical analysis enabled the identification of three modular structural motifs that we have named DOTs, which are required for signaling. Here we combine and tune these DOTs motifs to determine their contribution to activity and sensing using a chimeric, single-pass sensor derived from DesK.

According to our study, DOT1 seems to play an important role in sensing temperature variations, since as long as DOT1 is functional (DOT1 or DOT1+), differences in activity between 25 °C and 37 °C, are observed, while, no difference is observed for DOT1– (Fig. 2). Since DOT1 and DOT3 are found on both ends of the transmembrane helix, it is likely that DOT1 and DOT3 are sensing motifs, which jointly sense membrane thickness and/or membrane hydration at the interphase to respond to environmental cues. On the other hand, the transmembrane zipper DOT2 is an activating motif that stabilizes the signaling-active conformation, which would be tuned by the input signal from DOT1 and DOT3. Nevertheless, DOT2+ can overrule the signaling from DOT1 and DOT3, as the energetic contribution of a serine residue inside the membrane strongly stabilizes the signaling-active conformation. This finding suggests that a hierarchy of motif dominance is established whereby DOT2 is dominant.

Here we present a reverse engineering path that allowed us to construct a transmembrane helix with thermosensor capacity based on a repetitive hydrophobic scaffold, which can turn on the kinase activity of the sensor in response to low temperature. We have set the basis to engineer a repetitive hydrophobic scaffold, which can be easily incorporated in other more complexed biological structures as a modular temperature sensor. The synthetic transmembrane sensor is unique in that it allows fine-tuned spatial and temporal control of cell states with minimal invasiveness regarding amino acid composition, which makes it more modular for bioengineering purposes. Reverse engineering is expected to impact thermogenetics, enabling the use of temperature to control biological processes in organisms via genetically encoded thermosensing proteins.

It is remarkable that the activity of MS-DesK sensor can be completely hampered or, conversely, enhanced by modifying the physical–chemical properties of individual residues at the dimer interface, as demonstrated in this work. Mutations in a single residue in transmembrane helices have been shown to modulate signaling in other systems, such as the bacterial chemoreceptor Tar [13]. Elimination of Gln 193, an H-bond-forming residue in the TM region, affects the functionally determinant interaction within the homodimer, in consonance with our findings for the DesK system. In other illustrations of this type of analysis, repositioning of aromatic residues in TMS allows to activate EnvZ without any stimulus or to change the information flux in Tar receptors [14,15].

Membrane packing sensors

In a broader context, transmembrane sensors that regulate lipid properties (homeoviscous adaptation) use different motifs that locate either in the core of the lipid bilayer or at the water–lipid interphase to sense acyl chain packing or membrane thickness, respectively [15]. Mga2 and Spt23 are eukaryotic sensors for lipid saturation in the endoplasmic reticulum membrane. They use a conserved tryptophan residue located deep in the transmembrane helix as a sensor. In the presence of unsaturated fatty acids, the bulky Trp accommodates in the voids generated by unsaturated fatty acids, while in a more packed bilayer, it rotates to hide in the dimer interface [16]. The membrane sensing mechanisms of bacterial DesK seem to share some key features with eukaryotic Mga2: they both use rotational motions in response to small perturbations in lipid composition, and both sensory TMSs need proline residues to increase the conformational flexibility of the backbone, which may be required to maintain the interfacial area between two rotating monomers [15] (Fig. 4). A key difference between the sensors is that eukaryotic Mga2 is not sensitive to membrane thickness, likely because the membrane of the endoplasmic reticulum, where Mga2 resides, should be versatile and tuned to accept proteins with TMS of different length and properties. These proteins will be sorted out by organelles with different membrane thickness and lipid composition along the secretory pathway [17]. Other transmembrane sensors of lipid properties are bacterial Hik33, which controls desaturase expression in Synechocystis, and CorS, which controls expression of the phytotoxin coronatine by the phytopathogenic bacteria *Pseudomonas syringae* [18–20]. Both sensors are activated at low temperature; nevertheless, the mechanism of sensing of these kinases is not well understood at present. Curiously, the sequences of the TM helices of Hik33 and CorS include several serine, threonine and aspartate residues, which could potentially form an H-bond zipper, similar to DOT2, involved in sensing membrane properties (Table 2).

Table 2. TMS of Hik33 from Synechocystis and CorS from *P. syringae*

Receptor	Transmembrane sequence
Hik33	10-RLMAAATLVVSLLMTGLTFWAVN-32
	175-TRDVTIAVFISIWVMVILGAVFN-197
CorS	22-SLVKTTLLATSLILTFSLIDGFQ-44
	68-LLLWYVAVVSLRVGQVIYARQVL-90
	102-LLLWYVAVVSLRVGQVIYARQVL-124
	181-LHRILGITVLLYGLGLCRLARSG-204

The first and last residues of each TMS, according to SOUSUI prediction (www.expasy.org/tools) are indicated. Hydrogen bond-forming residues are highlighted.

To conclude, our study shows that the three functional motifs located in the transmembrane domain, hereby named DOTs, are critical for signaling and activity of a kinase sensor that responds to membrane properties and can contribute to understand the sensing mechanism in a broad context of transmembrane signal transductions.

Materials and Methods

Plasmid and strain constructions

MS-DesK and its gene variants were PCR amplified from plasmid pHPKS-TM1/5DesKC, a *Bacillus* low-copy-number plasmid that places the coding regions under the control of the PXyl promoter [2]. Site-directed mutagenesis was performed to introduce mutations in MS-DesK using QuikChange mutagenesis method (Stratagene). The engineered synthetic sensors were built using megaprimers containing the DNA region-coding for the synthetic design Syn1 and Syn2 (MVFVKVVVVVVPFVVVVVVSVVVSPFSIKSRKERER and MVFVKVVVVVVPFVVVTVVSVVVSPFSIKSRKERER). Megaprimers were used to PCR-amplify the cytoplasmic region of DesK. The resulting plasmids were used to transform CM21 *B. subtilis* [1,21]. This strain is a desk and contains a transcriptional fusion between the reporter gene β-galactosidase and the promoter of the desaturase (gene upregulated by DesK–DesR at low temperature), which allows for monitoring kinase activity. All mutations were confirmed by DNA sequence analysis. Strains, full sequences and detailed construction methods are available upon request.

Bacterial strains and growth conditions

B. subtilis JH642-CM21 cells complemented with plasmids encoding MS-DesK and TM5 DesK variants were grown with 250-rpm gyration in Spizizen salts supplemented with 0.1% glycerol, 50 μg/ml tryptophan and 50 μg/ml phenylalanine, 0.05% casamino acids, and trace elements. When cells reached an OD = 0.3, the culture was divided into two flasks. One was maintained at 37 °C and the other shifted to 25 °C. Samples were taken every hour for β-galactosidase measurements. The results shown are the average of three independent assays and correspond to 3 h after the shift from 37 °C to 25 °C. The data presented are average values and standard deviations from at least three independent experiments done in two technical replicates. Significance was determined using GraphPad Software, employing the function Dunnett's multiple comparisons tests, one-way ANOVA, with P values < 0.05 for the variants compared.

Western blot analysis of DesK mutants

Western blots were also performed to confirm the cellular expression and integration levels of each DesK variant. *B. subtilis* desK-CM21 cells transformed with plasmids expressing each of the DesK mutants were grown at 37 °C in the presence of 0.2% xylose to an OD of 1. Membrane and cytoplasmic fractions were separated by ultracentrifugation at 45,000*g* and analyzed by Western blot with aDesKC antibody. Molecular weights of the proteins are indicated.

CRediT authorship contribution statement

María Eugenia Inda: Methodology, Data curation, Conceptualization, Investigation. **Daniela B. Vazquez:** Methodology, Data curation, Investigation. **Ariel Fernández:** Conceptualization, Writing - review & editing. **Larisa E. Cybulski:** Conceptualization, Investigation, Funding acquisition, Supervision, Writing - review & editing.

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Author Contributions: M.E.I. and DBV performed experiments and analyzed data. L.E.C. and M.E.I. designed experiments. L.E.C. envisioned and coordinated the project. L.E.C. and A.F. wrote the manuscript.

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TMS, transmembrane segment; DOTs, determinants of thermodetection.

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