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A genetic screen for mutations affecting temperature sensing in *Bacillus subtilis*

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Abstract

Two component systems, composed of a receptor histidine kinase and a cytoplasmic response regulator, regulate pivotal cellular processes in microorganisms. Here we describe a new screening procedure for the identification of amino acids that are crucial for the functioning of DesK, a prototypic thermosensor histidine kinase from *Bacillus subtilis*. This experimental strategy involves random mutagenesis of the membrane sensor domain of the DesK coding sequence, followed by the use of a detection procedure based on changes in the colony morphogenesis that take place during the sporulation programme of *B. subtilis*. This method permitted us the recovery of mutants defective in DesK temperature sensing. This screening approach could be applied to all histidine kinases of *B. subtilis* and also to kinases of other bacteria that are functionally expressed in this organism. Moreover, this reporter assay could be expanded to develop reporter assays for a variety of transcriptionally regulated systems.

INTRODUCTION

Bacteria sense and respond to their environment mainly using two-component signal transduction systems (TCS). These systems are generally composed of a receptor histidine kinase (HK) that detects a specific signal and communicates this input to its cognate response regulator by phosphorylation of a conserved Asp residue. These TCS are widespread throughout the bacterial kingdom: genomes of nearly all sequenced bacteria contain genes encoding these signal transduction proteins, with some species containing hundreds of TCS that respond to an enormous range of signals and stressors (for reviews, see [1–3]). Bacterial TCS play roles in virulence and antimicrobial resistance responses and, because they are not present in mammalian genomes, they are potential targets for the development of new antimicrobial compounds.

Sensor domains of HK are highly diverse. The structure most frequently found consists of an extracellular loop located between two membrane-spanning helices of a homodimeric integral membrane protein. However, we can also find sensor domains composed of a transmembrane (TM) core formed by a bundle of TM helices, or a cytoplasmic input domain either anchored to the membrane or as a soluble protein.

The acyl lipid desaturase of *Bacillus subtilis*, Δ5-Des, is synthesized following an increase in the order of membrane phospholipids. This response is mediated by a TCS, composed by the HK DesK and the response regulator DesR, that controls the expression of the *des* gene [4]. The sensor domain of DesK contains five TM segments devoid of any extracellular domain. This N-terminal domain is connected to a C-terminal cytoplasmic domain, DesKC, that holds the catalytic activity. A kinase-dominant state of DesK predominates when the acyl chains of membrane lipids became ordered after a temperature downshift or when high-melting-point fatty acids are incorporated into the membrane [4–6] (Fig. 1a). Under these conditions, DesK is autophosphorylated in the conserved His188 of the catalytic domain and the phosphoryl group is then transferred to the response regulator DesR [7]. Phospho-DesR (DesR~P) is the active form of the protein that binds DNA, promoting expression of the *des* gene [8]. As a result of *des* transcriptional activation, Δ5-Des is expressed and desaturates the acyl chain of membrane phospholipids (Fig. 1b). This change in membrane fatty acid composition decreases the

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Abbreviations: Amp, ampicillin; Cm, chloramphenicol; DesKC, catalytic core of DesK; DesR~P, Phospho-DesR; EP-PCR, error-prone PCR; HK, histidine kinase; Km, kanamycin; LB, Lysogeny broth; MS-DesK, DesK minimal sensor; MU, Miller units; PKm, kanamycin resistance cassette promoter; SFA, saturated fatty acid; SM, Schaeffer´s sporulation medium; Sp, spectinomycin; TCS, two-component system; TM, transmembrane; UFA, unsaturated fatty acid; 2-HCC, two-helix coiled coil.

Two supplementary figures are available with the online version of this article.

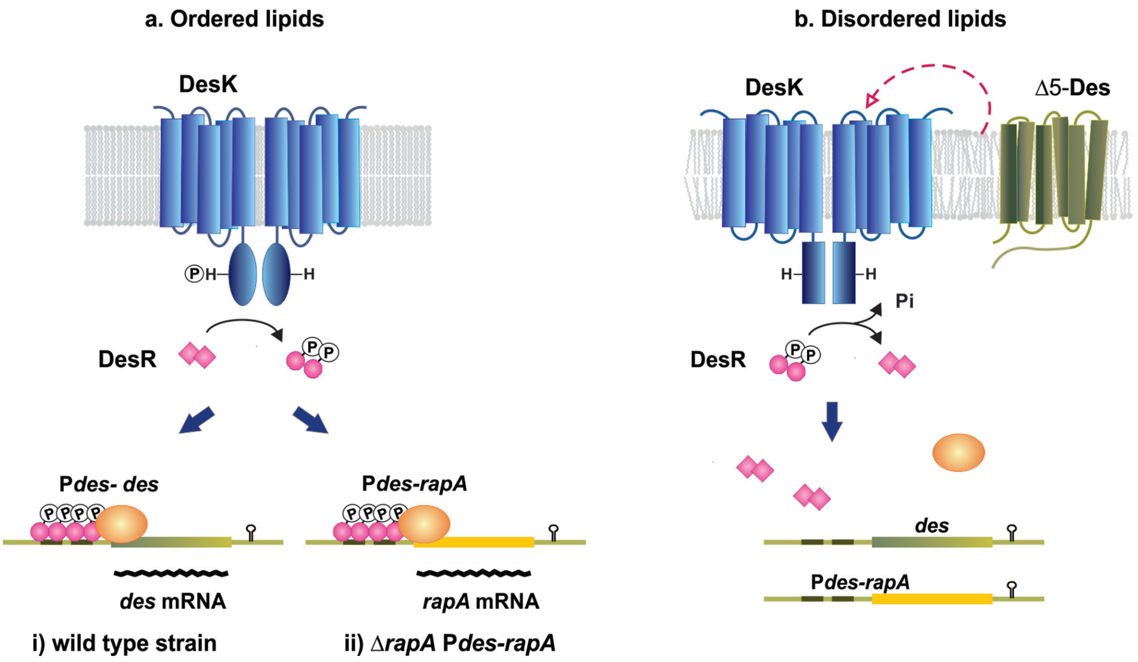

Fig. 1. Model for regulation of fatty acid desaturation in *B. subtilis* and strategy for expressing *rapA* under DesK control. (a) When the temperature drops, the acyl chains of the membrane phospholipids became ordered promoting a kinase-dominant conformation of DesK, which autophosphorylates and transfers the phosphoryl group to the response regulator DesR. DesR~P dimers interact with the *des* promoter, recruiting the RNA polymerase and thus promoting transcription of the gene encoding the desaturase. (b) Δ5-Des is incorporated into the membrane and introduces double bonds into the acyl chains of the membrane phospholipids. These newly synthesized unsaturated fatty acids (UFAs) cause an increase in membrane fluidity favouring a phosphatase-dominant conformation of DesK. DesR is dephosphorylated and *des* transcription turned off. Panels (a) and (b) also show the strategy for expressing *rapA* under the control of DesK.

phase transition temperature of the phospholipids, favouring a phosphatase-dominant conformation of DesK with the consequent hydrolysis of DesR~P. As the concentration of DesR~P reduces, *des* transcription is turned off. Therefore, the switch of DesK conformation between kinase- and phosphatase-competent forms, regulated by changes in the order of lipids, determines the level of phosphorylation of DesR and, subsequently, the synthesis of unsaturated fatty acids (UFA) [4, 7, 8].

Recently, we proposed a potential mechanism of DesK sensing and transduction based on structure-guided mutagenesis combined with computational modelling and simulation [9], for a recent review, see [10]. We suggested that DesK responds to membrane thickness, modulating its catalytic output by inducing the reversible formation of a two-helix coiled coil (2-HCC) in the fifth TM segment [9]. Indeed, we found that the stabilization of 2-HCC favours the phosphatase state while preventing any autokinase activity, whereas the opposite occurs upon 2-HCC destabilization. From the protein side, the initial cold signal at the TM domain must force rotation and separation of the 2-HCC helices. From the membrane side, the nature of the mechanical stress that acts on the TM domain following temperature drop is much more speculative. The signal sensed by DesK is likely a combination of membrane thickness, permeability to water and fluidity/rigidity, especially when fluid-to-gel phase transitions are caught in the relevant temperature range, hampering a clear assessment of which properties are actually sensed by the protein. In any case, it seems clear that DesK senses membrane status as a proxy for cold, through a largely unknown mechanism. Thus, the most interesting properties of DesK TM helix interactions with the lipid environment may be the least amenable to study by current techniques, and further experimental approaches to solve this fundamental question are required. For example, a reliable screening technique to identify essential amino acid residues for the signal perception *in vivo* is essential. Site-directed mutagenesis studies, in which specific amino acids are replaced by other residues, have provided valuable insight into how DesK functions at the molecular level [9]. However, using this methodology we were unable to find any mutant whose TM segments showed altered sensing properties. When we attempted to apply the widely used *lacZ* reporter system in *B. subtilis* to screen random libraries of DesK mutants in the sensor domain, we detected only variants that had completely lost their kinase activity but we could not easily distinguish among colonies with different levels of expression of the *des* gene after overnight incubation. Thus, to identify mutations in the sensor domain of DesK altered in membrane properties sensing, we needed a screening method that would allow unambiguous detection of up- or down-regulatory mutants. This class of mutants should help to close a major gap in the regulation model of DesK (Fig. 1). Since *B. subtilis* has been demonstrated to be a remarkable model system for the study of colony morphogenesis, due to its well-characterized differentiation programme, we attempted to develop a genetic screen for the isolation of DesK mutants based on the intricate regulatory network that controls sporulation. Thus, we took advantage of the fact that signals that induce endospore formation in *B. subtilis* are primarily integrated by the components of the phospho-relay signal transduction system that culminates in the phosphorylation of Spo0F, an intermediate response regulator, and its output product, Spo0A [11] (Fig. S1, available with the online version of this article). Signals countering sporulation activate aspartyl-phosphate phosphatases, Spo0E or Rap families that specifically dephosphorylate Spo0A~P [12] or Spo0F~P [13], respectively, in order to prevent sporulation [14].

In this work, we report the identification of DesK mutants altered in their sensing ability using a screening procedure based on the placement of the *rapA* gene under the control of the temperature-regulated *des* promoter. These fusions allowed us to visually monitor mutants defective in colony morphogenesis resulting from altered promoter activity.

METHODS

Strains and growth conditions

Escherichia coli DH5α was used for plasmid construction and propagation. *E. coli* strains were grown in Lysogeny broth (LB) [15] supplemented with ampicillin (Amp, $100\,\mu g\,ml^{-1}$), kanamycin (Km, $30\,\mu g\,ml^{-1}$) or chloramphenicol (Cm, $15\,\mu g\,ml^{-1}$). *B. subtilis* strains used in this study were the wild-type JH642 (*trpC*2, *pheA1*) and JH642 derivative strains listed in Table 1. *B. subtilis* strains were grown in LB, Spizizen minimal salts medium [16] supplemented with glucose (0.5 %), vitamin-free casein hydrolysate (0.1 %), tryptophan ($50\,\mu g\,ml^{-1}$) and phenylalanine ($50\,\mu g\,ml^{-1}$) (minimal medium), or Schaeffer's sporulation medium (SM) [17]. Media were supplemented with the appropriate antibiotics at the following concentrations: chloramphenicol $5\,\mu g\,ml^{-1}$; kanamycin $5\,\mu g\,ml^{-1}$; spectinomycin (Sp) $100\,\mu g\,ml^{-1;}$ lincomycin (Ln) $12.5\,\mu g\,ml^{-1;\ \text{and}}$ erythromycin (Em) $1\,\mu g\,ml^{-1}$. Competent cells of *B. subtilis* strains were prepared by the method of Anagnostopoulos and Spizizen [18].

Plasmid construction

All plasmids used in this study are listed in Table 2. To build a convenient delivery expression vector to clone *desK*-Nend mutagenic alleles, the pHPKS vector was used [19]. The *xylose* expression cassette, obtained from pAX01 [20] by digestion with *Sac*I, was cloned into the *Sac*I site of pHPKS rendering plasmid pARD7. Correct orientation was confirmed by the restriction pattern. We PCR-amplified *desK*-Cend, encoding residues 185–370 of DesK, from plasmid pAG47 [4], with oligonucleotides desKSmaUp and desKPDw (Table 3). The PCR fragment was digested with *Sma*I and *Pst*I and cloned into the corresponding sites in pARD7. The resulting plasmid containing P*xyl-desK*-Cend was named pARD8.

To restore a functional *desK* gene, we amplified from plasmid pAG47 [4] the fragment encoding residues 1–185 of the kinase (*desK*-Nend), with the primers desKB33 and desKSmaDw (Table 3). The PCR fragment was digested with *BamH*I and *Sma*I and cloned into the corresponding sites in pARD8. The resulting plasmid containing P*xyl-desK*Nend- Cend was named pARD9.

The *rapA* gene was PCR amplified from chromosomal DNA of strain JH642 using oligonucleotides rapAUp and

Table 1. *Bacillus subtilis* strains used in this study

Strain	Relevant characteristics*	Source
JH642	*trp*C2 *phe*A1	Laboratory stock
AKP3	JH642 *amyE::Pdes-lacZ*	[4]
AKP20	JH642 *desK::Km* P*Km-desR amyE::Pdes-lacZ*	[4]
AM100	JH642 *desK::Km* P*Km-desR*	[22]
CM21	JH642 *desKR::Km amyE::Pdes-lacZ thrC::Pxyl-desR*	[6]
LC5	JH642 *des::Km*	[21]
VR2	JH642 *rapA::Sp*	This work
VRA2	VR2 *amyE*::P*des-rapA*	This work
VRA2des-	VRA2 *des::Km*	This work
VRA2desR^{++}	VRA2 *desK::Km* P*Km-desR*	This work
ARDCM	JH642 Δ*des desK::Km* P*xyl-desR*	This work
ARDCM1	ARDCM *rapA::Sp*	This work
ARDCM2	ARDCM1 *amyE*::P*des-rapA*	This work
CM36	AKP3 Δ*des desK::Km* P*xyl-desR*	This work

**Km* and *Sp* denote kanamycin and spectinomycin resistance cassettes, respectively.

Table 2. Plasmids used in this study

Plasmid	Relevant characteristics*	Source or reference
pHPKS	*B. subtilis* replicative low copy number vector	[18]
pBluescript SKII(-)	Cloning vector	Stratagene
pAD4	P*xyl-desK* cloned into pHPKS	[6]
pAX01	*B. subtilis* integrative vector containing the xylose expression cassette	[19]
pDES913KAN	contains *Km* cassette disrupting *des* gene	[4]
pARD7	P*xyl* of pAX01cloned into pHPKS	This work
pARD8	*desK*-Cend (residues 185–370 of DesK) cloned into pARD7 under P*xyl*	This work
pARD9	*desK*-Nend cloned into pARD8 between P*xyl* and *desK*-Cend to generate *desK* wt full size	This work
pDesK1	*desK*$_{F8L,\ T76P}$ cloned under P*xyl*	This work
pDesK2	*desK*$_{S122C}$ cloned under P*xyl*	This work
pAR12	P*des* cloned into pJM116	[4]
pCM7	pDG1731 P*xyl-desR*	[6]
pCM9	P*xyl-desKC* cloned into pHPKS	[6]
pCM18	P*xyl-desR* cloned into pBluescript SKII(-)	This work
pCM19	5′region of *des* followed by *Km* cassette cloned into pCM18	This work
pDesK-ΔABD	Truncated version of DesK lacking ATP-binding domain (residues 1–242) cloned under P*xyl* promoter	[9]
pAG47	*desKR* under P*xyl* promoter cloned into pDG795	[4]
pDesK$_{DEST}$	*desK*$_{DEST}$ (A167R, I171G, L174G) cloned into pHPKS under P*xyl* promoter	[9]
pDesK$_{STA}$	*desK*$_{STA}$ (S150I, S153L, R157I) cloned into pHPKS under P*xyl* promoter	[9]
pVR1	*rapA* with its ribosome binding site cloned into pAR12	This work
pVRAG1	*rapA* with its ribosome binding site cloned into pBluescript SKII(-)	This work
pVRAG12	pVRAG1 *rapA::Sp*	This work

**Km* and *Sp* denote kanamycin and spectinomycin resistance cassettes, respectively.

rapADw (Table 3). The cloned fragment was sequenced to corroborate its identity and correct sequence. The PCR fragment containing *rapA* was digested with *Bam*HI and *Cla*I and ligated into pBluescript SKII (–) giving rise to plasmid pVRAG1. Then, a spectinomycin cassette obtained from digestion with *Eco*RV of plasmid pJM134 [21] was cloned into the unique *Eco*RI site of the vector pVRAG1, previously treated with the Klenow fragment of DNA polymerase I to create blunt ends. The resulting plasmid was named pVRAG12. The *rapA* gene obtained by digestion with *Bam*HI-*Cla*I from pVRAG1 was cloned downstream of the P*des* promoter of pAR12, a derivative from the integrational vector pJM116 [4], to generate plasmid pVR1.

An *Eco*RI-*Bam*HI fragment containing P*xyl-desR* was digested from pCM7 [7] and cloned into vector pBluescript SKII (–) digested with the same enzymes, giving rise to plasmid pCM18. A *Sal*I-*Eco*RI fragment of 1.9 kb from pDES913KAN [4] containing the 5′fragment of the *des* gene and the Km-resistance cassette was cloned into pCM18 digested with the same enzymes, yielding plasmid pCM19.

Strain construction

In order to obtain a *rapA-phrA* null mutant, plasmid pVRAG12 was used to transform strain JH642 by a double recombination event creating strain VR2. To place a P*des-rapA* fusion into the *amyE* locus of the *B. subtilis* VR2 chromosome, plasmid pVR1 was linearized with *Pst*I and used to transform strain VR2, giving rise to strain VRA2. Strains VRA2*des*$^-$ and VRA2*desR*$^{++}$ were obtained by transforming strain VRA2 with chromosomal DNA from strains LC5 [22] and AM100 [23], respectively.

To build a *des* and *desK* null mutant expressing isotopically *desR* under the control of the P*xyl* promoter, strain AKP3 was transformed with pCM19 giving rise to strain CM36. Chromosomal DNA from strain CM36 was used to transform strain JH642. Selected clones Cm^S, Km^R and *amyE*$^+$ were confirmed by PCR and stored as strain ARDCM.

The plasmid pVRAG12 was used to transform strain ARDCM, yielding strain ARDCM1. Plasmid pVR1 was linearized with *Pst*I and used to transform strain ARDCM1, giving rise to strain ARDCM2.

Sporulation assays

These assays were performed using Schaeffer's medium (SM) with salts (MnCl$_2$, 0.01 mM; CaCl$_2$, 0.1 mM and FeSO$_4$, 0.001 mM). In order to delay sporulation initiation, in some experiments the amount of salts was reduced to a quarter of the usual concentration or not added. Sporulation efficiency was tested by growing strains in SM at 37 °C for 5 h and then dividing the samples in two. One sample was transferred to 21 °C and the other was kept at 37 °C, growth being extended by the time indicated in each case. Serial dilutions were plated in duplicate before and after 60 min

Table 3. Oligonucleotide primers

Oligonucleotide	Sequence (5′ – 3′)*
desKSmaUp	ATTGCCCGgGATCTCCATGATAC
desKSmaDw	GGAGATCcCGGGCAATTCGCTGACG
desKB33	AGTAAcATGgAtccCaGAAAATGAGGTAAGATC
desKPDw	GCTGATCTTCTGCAgTAAATATACTAATC
rapAUp	TAgGAtccATCTCGGGTAATTCAAAAGG
rapADw	CAAgGAtCcAtcgAtCTGACATCCATTTAG

*Lowercase letters indicate variations with respect to the wild-type *B. subtilis*. Restriction sites are underlined.

treatment with $CHCl_3$ (10 % of sample volume). Colony counts were averaged, and the percentage of sporulation was calculated as the ratio between the viable count of treated and untreated samples. For experiments involving expression of different *desK* alleles under the control of the inducible promoter P*xyl*, 0.1 % xylose was added to the growth medium.

Error-prone PCR method

The plasmid pAG47 containing *B. subtilis desK* gene was used as a template for error-prone PCR (EP-PCR) with Taq polymerase. PCR was performed with the primers desKB33 and desKSmaDw (Table 3), which generate *desK*-Nend flanked by 5′ and 3°C terminal restriction sites *Bam*HI and *Sma*I, respectively. The cycling parameters were 94 °C for 3 min, 19 cycles of 94 °C for 30 s, 57 °C for 45 s and 72 °C for 1 min, and an additional 72 °C for a 5 min extension step. The PCR mixture contained unbalanced dNTP (dATP, dGTP, dTTP 200 µM each and dCTP 1 mM) and commercial buffer, with additional $MgCl_2$ 7 mM and $MnCl_2$ 0.15 mM.

Amplified DNA obtained by EP-PCR was purified, digested with *Bam*HI and *Sma*I, and cloned into vector pARD8. Ligation mixes were transformed in DH5α and total plasmid DNA preparations were used to transform *B. subtilis* strain ARDCM2.

β-Galactosidase activity assays

Bacillus subtilis DesK⁻ cells harbouring a P*des-lacZ* chromosomal fusion (strain CM21 [6]) were transformed with pHPKS expressing different DesK variants under the inducible P*xyl* promoter. The resulting strains were grown in LB, at 37 °C in the presence of 0.1 % xylose. At DO_{525}=0.3 the cultures were divided into two fractions, one being transferred to 25 °C and the other one kept at 37 °C. Culture samples were taken at 1 h intervals after the temperature downshift and assayed for β-galactosidase activity, as described previously [24]. To assay the phosphatase activity of DesK variants, AKP20 cells [4] transformed with pHPKS expressing different DesK variants under the inducible P*xyl* promoter were grown at 37 °C in LB, either in the presence or absence of the inductor, and grown at 37 °C with shaking. β-Galactosidase activities were determined after 4 h of growth, as previously described [24].

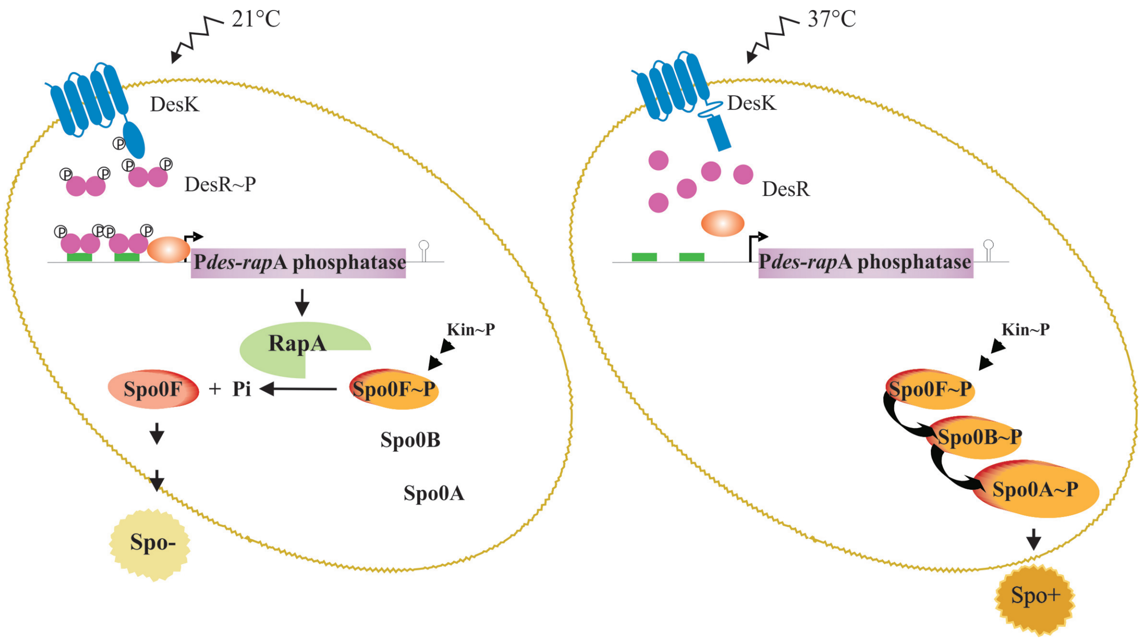

Fig. 2. Diagram of the sporulation phenotype of *rapA* mutants bearing a P*des*-*rapA* fusion in response to a temperature shift. Following cold shock of the reporter cells growing in SM medium, the RapA phosphatase (in the absence of its inhibitor, PhrA) is expressed from P*des* and dephosphorylates Spo0F~P, thereby preventing sporulation (a). At 37 °C P*des* is switch off, RapA is not expressed and the sporulation phospho-relay cascade is activated (b).

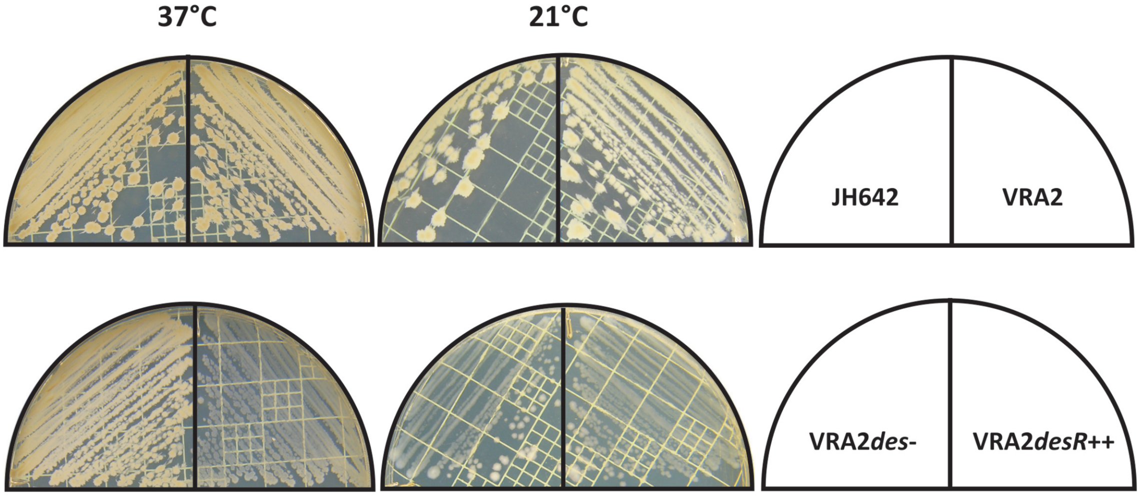

Fig. 3. Sporulation phenotype of VRA2 and its derivatives VRA2*des*$^-$ and VRA2*desR*$^{++}$. Strains JH642, VRA2 (*rapA::Sp amyE*::P*des-rapA*), VRA2*des*$^-$ (*rapA::Sp amyE*::P*des-rapA* Δ*des*) and VRA2*desR*$^{++}$ (*rapA::Sp amyE*::P*des-rapA desK::Km* P*Km-desR*) were plated onto SM medium and grown at 37 °C for 48 h, or for 5 h at 37 °C and then transferred to 21 °C for 72 h. Spo$^-$ phenotype: clear to transparent colonies. Spo$^+$ phenotype: opaque to dark colonies.

Fatty acid profile analysis

For the measurement of fatty acid synthesis, JH642 and ARDCM2 cells were grown overnight in minimal medium at 37 °C. Cells were diluted in fresh medium and grown until exponential phase. Then, cells were labelled with 1 µCi ml^{-1} of [^{3}H]-acetate and split in two aliquots, one being kept at 37 °C and the other shifted to 25 °C for 3 h. Lipids were extracted, converted to methyl esters and separated into unsaturated and saturated fractions by chromatography on 20 % silver nitrate-impregnated silica gel thin-layer plates [25]. The radioactivity of the spots in thin-layer chromatography plates was visualized using a Typhoon 9200 PhosphorImager screen (STORM840, GE Healthcare) and quantified using ImageQuant software (version 5.2).

RESULTS

Reporter assay using the *rapA* gene

Although the DesK kinase is well studied, a central issue remains uncertain: how does the DesK TM region sense the physical state of membrane lipids? To assess the role of essential TM residues in DesK membrane structure perception, we performed random mutagenesis on this domain of the protein. To identify DesK mutants altered in its sensing ability we devised a screening procedure based on the use of the gene *B. subtilis rapA*. This gene specifies an aspartyl-phosphate phosphatase that targets the Spo0F~P response regulator of the phospho-relay pathway that controls the initiation of sporulation (Fig. S1) [25]. RapA is paired with the specific pentapeptide, PhrA, which inhibits its phosphatase activity by binding to RapA at a different domain from its substrate, Spo0F~P (Fig. S1) [26–28]. As sporulation is impaired when RapA is expressed in the absence of its inhibitor, PhrA, we hypothesized that, by expressing *rap*A under the control of a promoter positively governed by a specific TCS (DesKR) in a *rapA-phrA* null mutant, the sporulation programme would be controlled by the flux of phosphate from the particular autophosphorylated HK to the cognate response regulator (Fig. 2). Since sporulating clones can readily be distinguished from non-sporulating ones, this experimental strategy could provide a simple visual procedure to screen a large collection of mutations inactivating or upregulating the autokinase activity of the DesK sensor in response to temperature shifts.

We constructed a *rapA-phrA* null mutant strain, named VRA2, which carries the P*des* promoter fused to the *rapA* gene ectopically integrated at the non-essential *amyE* locus (Table 1). Since DesK-mediated activation of P*des* takes

Table 4. Sporulation deficiency of *rapA-phrA* mutants expressing P*des-rapA*

Strain	37 °C*,†	21 °C*,†
JH642	66±4	57±1
VR2 (*rapA::Sp*)	97±1	91.5±1.5
VRA2*des*$^-$ (*rapA::Sp amyE*::P*des-rapA Δdes*)	35.5±0.5	0.8±0.1
VRA2*desR*$^{++}$ (*rapA::Sp amyE*::P*des-rapA desK::Km,* P*Km-desR*)	7±1	2.2±0.2

*Cells were grown at 37 °C for 5 h and then divided into two samples; one sample was transferred to 21 °C and the other was kept at 37 °C, with growth extended for 48 h. Sporulation efficiency was determined as described in Methods.

†Data expressed as percentages are representative of two independent experiments.

Table 5. Sporulation efficiency of *B. subtilis* reporter strain ARDCM2 expressing wild-type DesK or mutant versions

Plasmid	37 °C*,†	21 °C*,†
No plasmid	44±6,4	35±4,2
pARD 8 (empty vector)	46±5,7	33±2,1
pARD9 (P*xyl-desK* wt)	43±4,9	7±2,8
pDesK-ΔABD	62±6,4	71±6,4
pCM9 (P*xyl-desKC*)	15±4,2	8±2,1
pDesK1 (P*xyl-desK*$_{F8L,\ T76P}$)	43±3,5	32±2,1
pDesK2 (P*xyl-desK*$_{S122C}$)	11±2,1	6±0,7

*ARDCM2 cells (Δ*des desK::Km* P*xyl-desR rapA::Sp amyE::*P*des-rapA*) transformed with the indicated plasmids were grown in SM at 37 °C for 5 h and then divided into two samples. One sample was transfered to 21 °C and the other was kept at 37 °C for 72 h. Sporulation efficiency was determined as described in Methods.

†Data expressed in percentages are representative of two independent experiments.

place when cells are shifted from 37 °C to 20–25 °C, we expected that sporulation would be inhibited after a downshift in temperature (Fig. 2). Nevertheless, we found that strain VRA2 formed spores on SM both at 37 °C and after a downshift to 21 °C (Fig. 3). This finding suggests that RapA activity is insufficient to inhibit sporulation at low temperatures, which might be caused by feedback repression of DesK by the UFAs synthetized by Δ5-Des (Fig. 1) [4]. Thus, we reasoned that if we abolish the synthesis of UFAs in VRA2, P*des-rapA* transcription would not be shut off. To test this idea, we designed two approaches: (1) we built a *des* null mutant derivative strain, VRA2*des*$^{-}$ (Table 1), that does not produce UFAs, and (2) we constructed a strain, VRA2-*des*R^{++} that overproduces DesR in the absence of DesK, thus avoiding feedback inhibition of *des* expression by UFAs. It should be noted that in this latter strain, DesR is phosphorylated without DesK assistance and therefore it generates constitutive high transcription of P*des* [4]. As predicted, when VRA2*des*$^{-}$ was streaked onto SM plates sporulating colonies formed at 37 °C, while sporulation was inhibited after temperature downshift (Fig. 3). In addition, strain VRA2*desR*$^{++}$ showed a Spo– phenotype at both 37 and 21 °C (Fig. 3), confirming the constitutive, temperature-independent expression of P*des*.

We then compared the sporulation efficiencies of strains VRA*des*$^{-}$ and VRA*des*R^{++} to those of the wild-type strain JH642 and the *rapA* deficient strain VR2 (Table 4). As expected, strain VR2 lacking the phosphatase targeting Spo0F~P, which controls the initiation of sporulation (Fig. 2), showed a 50 % higher rate of sporulation than the wild-type strain at both 37 and 21 °C. VRA2*des*$^{-}$ formed spores at 37 °C but displayed marked sporulation deficiency following cold shock (Table 4). Finally, the sporulation efficiency of VRA2*desR*$^{++}$ was much lower than the wild-type strain at both 37 and 21 °C (Table 4). These findings are in agreement with the observed colony phenotypes on SM-agar plates, indicating that RapA expression without its inhibitor, PhrA, could indeed be used as a reporter gene to screen for DesK mutants.

Phenotypic screening of the reporter strain expressing different *desK* alleles

To select mutations in DesK TM segments using the sporulation phenotypic screen, we constructed a *desK* mutant strain derived from the *des* null mutant VRA2*des*$^{-}$, named ARDCM2. This strain contains a Kanamycin resistance cassette disrupting *desK* and isotopically expresses *desR* from a xylose-inducible promoter (P*xyl*) (Table 1). ARDCM2 displays a Spo^{+} phenotype at both 37 and 21 °C (Table 5) and is unable to produce UFAs, as confirmed by both [^{3}H]-acetate labelling and analysis of the methyl esters of fatty acids by silver nitrate thin-layer chromatography (Fig. S2). Since this strain lacks *desK*, it can be used to produce and select DesK variants in the sensor domain. To express different *desK* alleles in ARDCM2 we built an inducible expression vector, pARD8, carrying the *desK*-C end domain under the control of P*xyl* (Table 2). This vector is suitable for cloning of *desK*-Nend alleles upstream of the wild-type *desK*-Cend domain to regenerate full-length *desK*.

To test the suitability of the reporter strain ARDCM2 as a host for screening sensor domain mutants of DesK, we first performed a sporulation efficiency assay with well-characterized *desK* alleles expressed under the P*xyl* promoter from plasmid pHPKS (Table 5). To this end we used pARD9 carrying a *desK*-Nend wild type cloned into pARD8, thus restoring a functional *desK* gene, pDesK-ΔABD, which codes for a DesK mutant lacking the ATP binding domain, and plasmid pCM9 carrying the catalytic core of DesK (DesKC) (Table 2). As expected, the sporulation efficiency of ARDCM2 carrying pARD9, which contains a full-length wild-type DesK, decreased after a temperature downshift whereas temperature did not affect the sporulation efficiency of either ARCDM2 or ARCDM2/pARD8, which do not express DesK (Table 5 and Fig. 4). On the other hand, ARDCM2 expressing DesK-ΔABD displayed high sporulation efficiency both at 37 and 21 °C, in agreement with the inability of this mutant protein to be autophosphorylated (Table 5 and Fig. 4). Finally, when we expressed DesKC into ARDCM2, the sporulation efficiency of this strain was severely curtailed at both 37 and at 21 °C, consistent with the constitutive kinase activity of DesKC (Table 5 and Fig. 4).

We also tested the sporulation ability of ARDCM2 expressing DesK mutants that weaken (DesK$_{\mathrm{DEST}}$) or favour (DesK$_{\mathrm{STA}}$) the formation of the 2-HCC, which connects the DesK TM bundle to its cytosolic catalytic region [9]. As shown in Fig. 4, ARDCM2 expressing DesK$_{\mathrm{STA}}$ gave rise to the Spo^{+} phenotype after a cold shock stimulus, whereas the expression of DesK$_{\mathrm{DEST}}$ generated a Spo^{-} phenotype at 37 °C. These findings are in full agreement with previous *in vivo* and *in vitro* experiments showing that DesK$_{\mathrm{DEST}}$ exhibits a favoured kinase state, while DesK$_{\mathrm{STA}}$ produces a kinase-OFF state [9]. Taking these findings together, we demonstrate that the switch from a Spo^{+} to a Spo^{-}

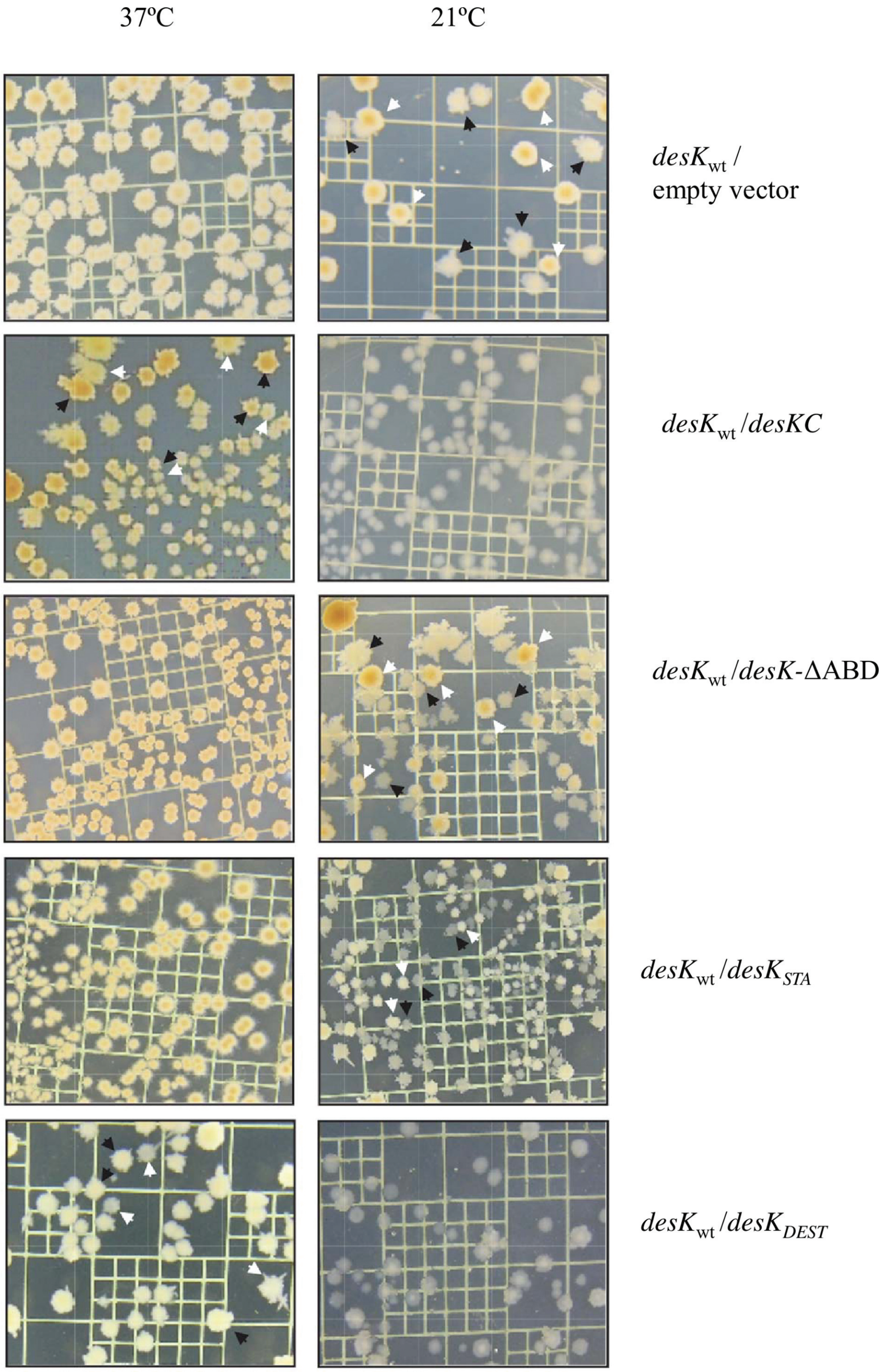

Fig. 4. Phenotypic analysis of the reporter strain ARDCM2 expressing different *des*K alleles. ARDCM2 cells (Δ*des desK::Km* P*xyl-desR rapA::Sp amyE*::P*des-rapA*) expressing *des*Kwt were mixed with ARDCM2 cells bearing plasmids expressing different *desK* alleles, as indicated in the Figure. A suitable dilution of the different mixtures was plated on SM and cultured at 37 °C for 48 h, or for 5 h at 37 °C and then transferred to 21 °C for 72 h. ARDCM2 cells expressing DesKwt are indicated by black arrowheads, and ARDCM2 cells carrying the empty vector (pARD8) or plasmids expressing DesKC, DesK-ΔABD, DesK$_{DEST}$ or DesK$_{STA}$ are indicated by white arrowheads. Spo^{-} phenotype: clear to transparent colonies.

phenotype, as shown by distinct *desK* alleles, is directly associated with DesK activity. Thus, our approach, based on colony morphology, allows clear identification of DesK variants with increased or decreased kinase activity.

Screening for random mutations in the DesK sensor domain

Having demonstrated that sporulation colony morphology screening can efficiently distinguish between DesK variants that downregulate or upregulate kinase activity in response to temperature, we queried whether this technique could be used to isolate random mutations in the DesK sensor helix bundle. To this end, DNA corresponding to the TM domain of DesK was subjected to random mutagenesis using EP-PCR (see Methods). The libraries of *desK*-Nend alleles thus obtained were cloned under the control of P*xyl*, upstream of the *desK*-Cend domain contained in plasmid pARD8. The resulting plasmids were used to transform strain ARDCM2. The first selection was performed on LB agar plates supplemented with erythromycin and lincomycin. Then, transformants were transferred via replica-plating onto plates containing SM medium [29]. The plates were incubated at 37 °C for 48 h or for 5 h at 37 °C and then shifted to 21 °C for at least 4 days. Screening of the libraries was carried out for those *desK*-Nend alleles causing a Spo^{-} phenotype at 37 °C or Spo^{+} at 21 °C. In the first screening, six clones with the Spo^{+} phenotype at 21 °C and one clone with the Spo^{-} phenotype at 37 °C were identified. Plasmid DNA was prepared from each transformant for DNA sequencing. One variant, *desK*$_{\text{F8L, T76P}}$, which causes amino acid substitutions in TM1 and TM3, displayed a Spo^{+} phenotype at 21 °C, indicative of a kinase-OFF state. Another variant, *desK*$_{\text{S122C}}$, causing a single amino acid replacement in TM4, showed a Spo^{-} phenotype at 37 °C, indicative of a kinase-constitutive state. The remaining selected mutants carried premature stop codons generating truncated proteins (three

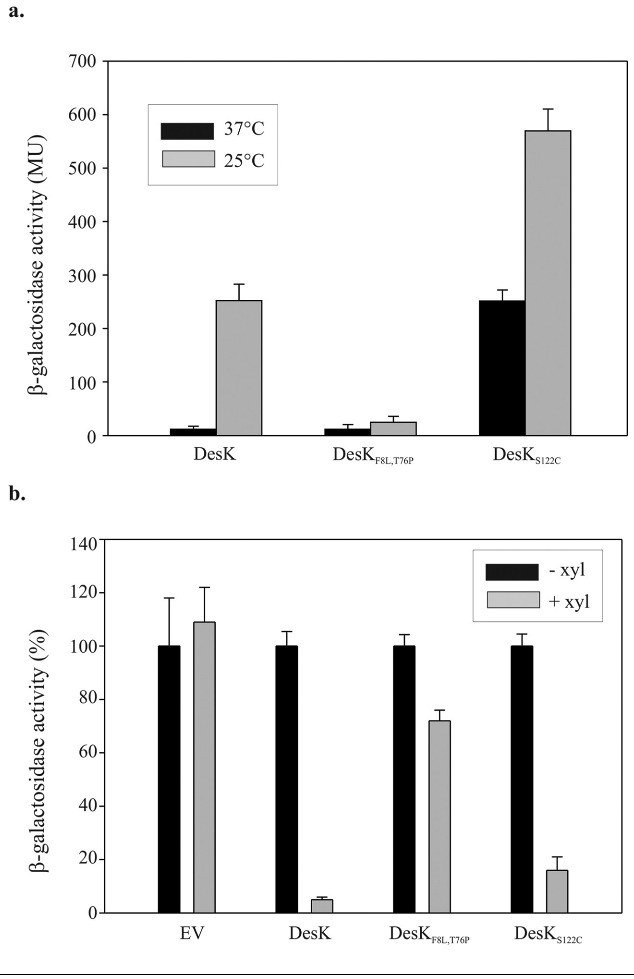

Fig. 5. Altered sensing properties of isolated *desK* mutants (a). Strain CM21 (*desKR::Km amyE::Pdes-lacZ thrC::Pxyl-desR*) transformed with either pAD4 (DesK), pDesK1 (DesK$_{F8L,\ T76P}$) or pDesK2 (DesK$_{S122C}$) was grown overnight in LB at 37 °C. Cells were collected and diluted in the same medium in the presence of xylose. At an OD$_{525}$ of 0.35, cultures were split and incubated at 25 (grey bars) or 37 °C (black bars). β-Galactosidase-specific activities were determined 4 h after the temperature shift. The results shown are the averages from three independent experiments. MU, Miller units. (b). Phosphatase activity assay of AKP20 cells expressing the indicated DesK variants. Cells were grown in LB at 37 °C in the absence (black bars) or presence (grey bars) of the inducer. Values shown correspond to the β-galactosidase activity of aliquots taken after 4 h of growth, expressed as a percentage of activity in the absence of xylose. The results shown are the average from three independent experiments.

clones) or contained a replacement of the N-terminal Met by Leu preventing translation initiation (two clones). Therefore, we did not further characterize these mutants.

In vivo characterization of DesK $_{F8L, T76P}$ and DesK$_{S122C}$

We carried out a spore count assay of strain ARDCM2 carrying plasmids pDesK1 or pDesK2 expressing *desK*$_{F8L, T76P}$ or *desK*$_{S122C}$ alleles, respectively. In agreement with the colony morphology observed on SM agar plates, strain ARDCM2/pDesK1 was proficient in sporulation at both 37 and at 21 °C (Table 5), while the ability of ARDCM2/pDesK2 to form spores was significantly decreased at both temperatures (Table 5). To confirm that the mutants identified by sporulation phenotypic assay affected the sensing ability of DesK, we introduced plasmids pDesK1 or pDesK2 into the *desK*$^{-}$ strain CM21. This strain expresses *desR* from a P*xyl* promoter and contains a P*des-lacZ* reporter fusion, and hence is suited to probing changes in DesK kinase activity *in vivo* through β-galactosidase reporter assay [7]. Fig. 5a compares the autokinase activities of the mutant variants to wild-type activities. On the one hand, CM21 cells expressing *desK*$_{S122C}$ exhibited, at 37 °C, β-galactosidase activities similar to those obtained with wild-type DesK at 25 °C. However, when these cells were incubated at 25 °C, they showed β-galactosidase activity levels ~2.2-fold higher than wild-type DesK at the same temperature. These findings show that although DesK$_{S122C}$ kinase activity is upregulated at both 37 and 21 °C, this mutant protein is still able to regulate its activity by sensing a decrease in temperature so that it is not locked into a kinase-dominant conformation. On the other hand, we found that the β-galactosidase levels of CM21 cells expressing *desK*$_{F8L, T76P}$ were low, at both 21 and at 37 °C, probably indicating either that this mutant protein is unpaired in cold-sensing or that it displays a favoured phosphatase state.

To assay the phosphatase activity of DesK variants, we used strain AKP20 [4]. This strain exhibits constitutive expression of P*des*, and hence high β-galactosidase activity levels, due to DesR overexpression from the strong constitutive kanamycin resistance cassette promoter (P*Km*), and its phosphorylation from small phosphor donors or other kinases (EV Fig. 5b; [4]). Expression in this strain of wild-type DesK or a DesK variant, which exhibits DesR~P phosphatase activity, led to a decrease in β-galactosidase activity levels (DesK Fig. 5b, 4, 7]). As shown in this Figure, both DesK$_{F8L, T76P}$ and DesK$_{S122C}$ variants show phosphatase activity since a reduction in β-galactosidase levels is observed following induction of protein expression. However, DesK$_{F8L, T76P}$ dephosphorylates DesR~P less efficiently than DesK$_{S122C}$ and the wild-type kinase. These findings indicate that DesK$_{F8L, T76P}$ is not locked into a phosphatase-dominant conformation. The low phosphatase activity, as well as the lack of autokinase activity of DesK$_{F8L, T76P}$, suggest that these mutations would cause misfolding of the TM helices or suboptimal positioning of the protein in the bilayer, interfering with its signalling function. Together, these data show that the screening strategy described here is suitable for identification of regulatory mutant alleles in the sensory domain of DesK.

DISCUSSION

Various selection methods have been applied to bacteria in the search for key amino acid residues located in the sensor and effector domains of a given protein. Most of these strategies have been designed for the premier model bacterium *E. coli*. Nevertheless, in Gram-positive bacteria tools for selecting mutations are much less developed. Here, we report a method for phenotypic selection in *B. subtilis* based on the initiation of the sporulation programme in this bacterium. We demonstrate here that using the negative regulator of sporulation, *rap*A, as a reporter system is a sensitive method for the detection of different phenotypes in individual colonies. Although the *lacZ* system has been the premier reporter system for the study of transcriptional regulation in *B. subtilis*, the *rap* screening assay was particularly useful in the detection of increases or decreases in activity of the DesK/DesR-controlled *des* promoter. Of course, for any given situation it would be best to use both, the *lac* and *rap* systems, to determine which shows the difference most distinctly. Since sporulation is a development system that characterizes Gram-positive bacteria of the genera *Bacillus* and *Clostridium* [30, 31], the *rap* system might be extended to other species of these genera.

Our initial findings only hint at the collections of mutants that remain to be uncovered by systematic exploration of more DesK variants selected using this screening method. It is worth noting that the mutations obtained in this study are distributed in different TM helices of DesK, indicating the unbiased nature of the procedure. The DesK$_{F8L, T76P}$ double mutant contains a mutation in the first TM1 (F8L), near the lipid–water interface, and a second mutation (T76P) in the TM3 segment. Proline residues are well-known disruptors of secondary structures, by inducing regions of alpha helix distortions like kinks, tight turns or unwinding. DesK contains four Pro residues within the TM helices (Pro16 and Pro26 belong to TM1, and Pro135 and Pro148 to TM5), while the other TMS lack this amino acid. We hypothesize that the kinase-OFF and weak phosphatase activity of DesK$_{F8L, T76P}$ is mainly due to the perturbation introduced in TM3, which would generate arrhythmic motions with neighbouring TMs. This effect would be more marked in a rigid membrane, when DesK is in a kinase-ON conformation, and masked when the membrane is fluid and DesK is in a phosphatase state. The DesKS122C mutant contains a subtle single amino acid substitution (S122C) in TM4, favouring a kinase-ON state of the sensor regardless of the temperature signal. These experiments provide some insight into understanding the helix–helix and helix–lipid interactions required to sense the membrane status. Clearly, much more experimental research is required to gain insight into the sensing mechanism of DesK.

Our findings are particularly interesting since the role of key amino acids in the sensor domain of DesK was initially studied in the so-called minimal sensor (MS-DesK). In this chimeric construction, sensing and signalling are captured in a single TM segment composed of the N-terminal domain of TM1 and the C-terminal domain of TM5 joined to DesKC [32]. Thus, the isolation of a DesK hypersensitive mutant by substitution of S122 by cysteine in TM4 shows that the artificial construct MS-DesK does not retain all the properties of full-length DesK. It follows that the delicate balance of protein conformation and communication between DesK subdomains can only be studied in the full-length protein. Exploiting the strategy described here will enable us to identify other residues located in the TM domains of DesK that are critical for its sensing ability *in vivo*. These data will help to elucidate the crucial question of how the DesK TM domain senses the lipid environment. Taken together, our analysis offers a straightforward method for investigation of *in vivo* TM signalling in a variety of signalling proteins from *B. subtilis* and, potentially, for a diverse range of membrane-associated regulatory proteins that are functionally expressed in this bacterium. The DesK mutants that we generated illustrate the power of the sporulation-screening assay to provide new information on the function of a sensor protein through selection of unbiased mutations.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

This article does not contain any studies with human participants or animals performed by the author.

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