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Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis*

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Both prokaryotes and eukaryotes respond to a decrease in temperature with the expression of a specific subset of proteins. Although a large body of information concerning cold shock-induced genes has been gathered, studies on temperature regulation have not clearly identified the key regulatory factor(s) responsible for thermosensing and signal transduction at low temperatures. Here we identified a two-component signal transduction system composed of a sensor kinase, DesK, and a response regulator, DesR, responsible for cold induction of the *des* gene coding for the Δ5-lipid desaturase from *Bacillus subtilis*. We found that DesR binds to a DNA sequence extending from position –28 to –77 relative to the start site of the temperature-regulated *des* gene. We show further that unsaturated fatty acids (UFAs), the products of the Δ5-desaturase, act as negative signalling molecules of *des* transcription. Thus, a regulatory loop composed of the DesK–DesR two-component signal transduction system and UFAs provides a novel mechanism for the control of gene expression at low temperatures.

Keywords: signal transduction/temperature regulation/thermosensing

Introduction

Variability and adaptability are crucial characteristics of organisms possessing the ability to survive and prosper in a wide variety of environmental conditions. In order for bacteria to effectively compete and survive, they have to sense environmental conditions and respond accordingly. Temperature is one of the major stresses that all living organisms face (Phadtare *et al*., 2000). It has been demonstrated that bacteria respond to high growth temperatures by the induction of a group of heat shock proteins, but also to low temperatures by the induction of a group of cold shock proteins (Phadtare *et al*., 2000). In contrast to heat shock proteins, which include chaperones required for protein folding and peptidases (Yura *et al.*, 2000), cold-induced proteins appear to be involved in cellular functions such as general metabolism, transcription, translation and recombination (Phadtare *et al*., 2000). A universally conserved adaptation response observed among bacteria and most (if not all) poikilothermic organisms is the adjustment of membrane lipid composition at low temperatures (Cronan and Rock, 1996; Vigh *et al*., 1998). As the growth temperature is lowered, the proportion of unsaturated fatty acids (UFAs) in the membrane lipids increases. This regulatory mechanism system, called thermal control of fatty acid synthesis, is thought to be designed to ameliorate the effects of temperature changes on the physical state of the membrane phospholipids (for a review see Cronan and Rock, 1996). There are a variety of mechanisms that can alter the membrane phospholipid composition in response to a temperature change. Bacilli cells respond to a decrease in ambient growth temperature by introducing double bonds into pre-existing fatty acids of their membrane phospholipids (Grau and de Mendoza, 1993; Aguilar *et al*., 1998). These double bonds are inserted by specific fatty acid desaturase enzymes (de Mendoza *et al*., 2001). In a previous study we reported the isolation of the *des* gene coding for the Δ5-desaturase of *Bacillus subtilis* (Aguilar *et al*., 1998). Studies of operon fusion as well as transcriptional analysis demonstrated that *des* is tightly regulated during cold shock (Aguilar *et al*., 1998, 1999). While the *des* transcript is barely detected at 37°C, the production of *des* mRNA is transiently induced upon a temperature downshift. Derepression of *des* occurs exclusively at the level of transcription in a promoter-dependent fashion and does not require *de novo* synthesis of protein (Aguilar *et al*., 1999). Nevertheless, the molecular mechanism by which the transcription of the *B.subtilis des* gene as well as the synthesis of membrane UFAs are induced transiently by low temperature remains unsolved. Previous studies suggest that membranes can sense environmental changes and, as a consequence of changes in their phase state and microdomain organization, transmit signals that activate transcription (Vigh *et al*., 1998; Hoppe *et al*., 2000; Suzuki *et al*., 2000). This signalling mechanism was proposed to control the expression of cold-induced desaturases from cyanobacteria and heat shock-induced genes in *Saccharomyces cerevisiae* and cyanobacteria (for a review see Vigh *et al*., 1998). For signal transduction across the cell membrane, bacteria extensively use two-component systems, which have an input-sensing domain (histidine kinase) and an output effector domain (response regulator) (Hoch, 2000). Because sensor kinases are generally integral membrane proteins that respond to environmental signals (Dutta *et al*., 1999), it seems likely that temperature regulation of UFAs in bacilli could be controlled by members of the family of two-component regulatory proteins. In connection with this possibility, Suzuki *et al*. (2000) have recently reported that two histidine kinases and a response regulator

modulate the transcription of low temperature-inducible genes from cyanobacteria.

Here we report the identification of the *desK* and *desR* genes from *B.subtilis*, which are directly responsible for the transcriptional regulation of the *des* gene. These genes encode products with similarity to an autophosphorylatable histidine kinase (DesK) and a DNA binding response regulator (DesR) of the two-component signal transduction system. In addition, we demonstrate that DesR interacts specifically with the regulatory region of the gene it controls and that UFAs act as negative regulators of *des* expression. Thus, a regulatory loop composed of the DesK–DesR two-component signal transduction system and UFAs provides a novel mechanism for the control of gene expression at low temperatures.

Results

Inactivation of the yocF and yocG genes prevents induction by low temperature of β-galactosidase activity in a des–lacZ fusion

A key issue in the regulation of UFAs synthesis in bacilli is to understand how a decrease in growth temperature induces the expression of the *des* gene required for oxygen-dependent desaturation of fatty acids. To gain insight into this regulatory pathway, the *B.subtilis* genome sequence (Kunst *et al*., 1997) was searched for potential two-component regulatory gene pairs involved in *des* environmental regulation. Among the 35 two-component signal transduction systems identified in *B.subtilis*, the two-gene operon formed by the *yocF* and *yocG* genes encodes a two-component system with no known function (Fabret *et al*., 1999). The predicted products of *yocF* and *yocG* exhibit structural similarity to the histidine kinases and response regulators of two-component regulatory systems, respectively (Fabret *et al*., 1999). The *yocFG* operon is located immediately downstream of the *des* gene (Kunst *et al*., 1997). This prompted us to investigate whether this signal transduction system could be involved in the regulation of the desaturase synthesis. To test the induction of desaturase expression upon a temperature shift we used the strain AKP3, which contains a fusion of the *lacZ* gene to the *des* promoter integrated ectopically at the non-essential *amyE* locus of *B.subtilis* JH642. When *B.subtilis* strain AKP3 is grown at 37°C the levels of β-galactosidase (β-gal) are very low (Figure 1A). However, when this strain is grown at 37°C and then shifted to 25°C, β-gal synthesis reaches induction levels ~10-fold higher than the levels found at 37°C (Figure 1A). In addition, the induction of the *des–lacZ* fusion in strain AKP3 can be easily monitored in media containing X-gal, where the colonies turn blue at 25°C (Figure 1B, panel I). To determine whether the *yocF* and *yocG* genes are responsible for the control of *des*, we disrupted the operon with a kanamycin-resistance gene (Km^R) cassette as described in Materials and methods. The *yocFG* gene disruption was introduced by homologous recombination into strain AKP3, giving strain AKP21. In contrast to strain AKP3, strain AKP21 did not form blue colonies at 25°C (Figure 1B, panel I) nor were its β-gal levels increased upon downshift from 37 to 25°C (Figure 1A). This result indicates that a mutation in the *yocFG* operon

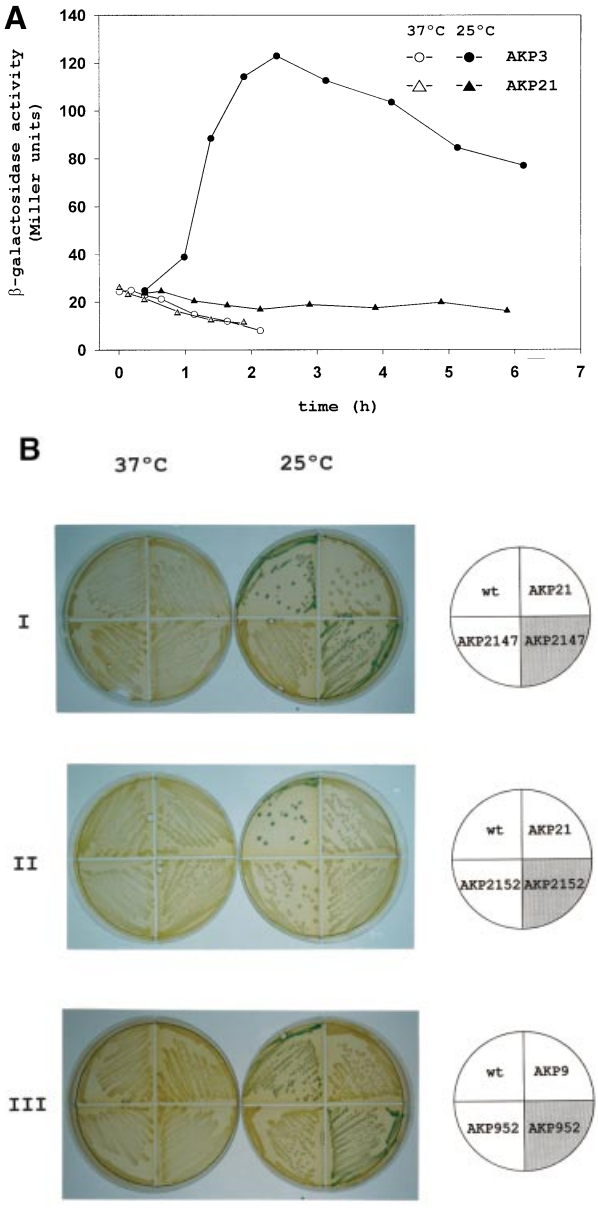

Fig. 1. Pattern of *des–lacZ* expression in wild-type and *yocF*⁻*yocG*⁻ cells before and after temperature downshift. (**A**) *Bacillus subtilis* AKP3 cells (circles) and AKP21 cells (triangles) harbouring a *des–lacZ* transcriptional fusion were grown at 37°C to an optical density of 0.35 (at 525 nm) and then divided into two samples. One sample was transferred to 25°C (filled symbols), and the other was kept at 37°C (open symbols). Specific β-gal activities were determined at the indicated time intervals. (**B**) Bacterial strains [wild-type AKP3 (wt), *yocF*⁻*yocG*⁻ (AKP21), *yocF*⁻*yocG*⁻ p*Xyl:yocFG* (AKP2147), *yocF*⁻*yocG*⁻ p*Xyl:yocG* (AKP2152), *yocG*⁻ (AKP9) and *yocG*⁻ p*Xyl:yocG* (AKP952)] were streaked onto Luria–Bertani (LB) medium containing 30 μg/μl X-Gal, with (shaded quarter) or without (empty quarter) the addition of 0.8% L-xylose. The strains were incubated at 37°C for 12 h (left column) or for 5 h at 37°C, and then transferred to 25°C for 36 h (right column) before photography.

eliminates the low-temperature inducibility of the *des* promoter.

The yocFG mutation can be complemented in trans

To verify that the *yocF* and *yocG* genes were responsible for cold induction of *des*, plasmids containing the *yocFG*

operon or the *yocG* gene alone under the control of the xylose-inducible *Xyl* promoter were integrated into the *thr* locus of strain AKP21 giving strains AKP2147 and AKP2152, respectively. In strain AKP2147, in which the *yocF* and *yocG* genes are provided *in trans*, the cold induction of the *des–lacZ* fusion was dependent upon the addition of xylose to the growth medium (Figure 1B, panel I). The xylose-induced expression of *yocG* alone, however, was unable to restablish the cold-dependent *lac*$^+$ phenotype of strain AKP2152 (Figure 1B, panel II). Nevertheless, expression of this gene in strain AKP952, in which inactivation of *yocG* eliminated the low-temperature inducibility of the *des* promoter (Figure 1B, panel III), resulted in a xylose-dependent induction of the *des–lacZ* fusion at low growth temperatures (Figure 1B, panel III). Therefore, the *yocG* and *yocF* genes are essential for the low-temperature induction of the *des* gene.

Inactivation of yocG inhibits the induction of the des transcript and UFA synthesis at low temperature

We performed northern blot analysis to examine the expression of the *des* gene in the wild-type strain JH642 or in the *yocG*$^-$ strain AKP8, before and after a shift in temperature from 37 to 25°C (Figure 2A). As observed previously in the wild-type cells, the size of the *des* transcript is ~1.1 kb, and this mRNA was only detected when cells were shifted to 25°C (Aguilar *et al*., 1999). However, the accumulation at 25°C of the *des* transcript was not observed in strain AKP8, directly demonstrating that inactivation of the *yocG* gene suppressed the low temperature-induced expression of the desaturase gene (Figure 2A).

The fatty acid profile of the wild-type strain JH642 was compared with that of strain AKP8. The fatty acids were labelled by growth of the strains in [^{14}C]acetate, followed by argentation chromatography of the radioactive fatty acids. While strain JH642 shifted from 37 to 20°C synthesized UFAs, strain AKP8 formed no detectable UFAs after the temperature downshift (Figure 2B). These experiments confirm that the *yocG* is essential for the synthesis of UFAs at low growth temperatures.

Since both the histidine kinase YocF and its cognate regulator YocG are required for *des* induction, we have named the genes coding for these two-component regulatory proteins as *desK* and *desR*, respectively.

h-DesR binds to the des promoter

In most cases, the response partner of the two-component transduction system is a transcriptional activator for genes whose products are specifically utilized to respond to the unique nature of a given input signal (Hoch, 2000). The genetic data presented above suggested that DesR, a response regulator for low-temperature response may bind to the *des* promoter region. The binding of purified His-tagged DesR (h-DesR) to the *des* promoter was tested using the electrophoresis mobility shift assay (EMSA). The h-DesR protein was expressed by isopropyl-β-D-thio-galactopyranoside (IPTG) induction with *Escherichia coli* M15 (pREP4, pAR18) and purified by nickel affinity chromatography (data not shown). A PCR-amplified 367 bp DNA containing the *des* promoter (p*des*DNA) was used as target DNA. Formation of complexes between

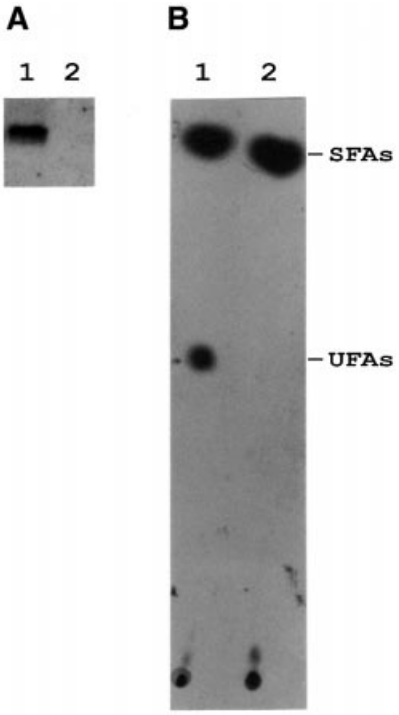

Fig. 2. *des* mRNA and UFA production in wild-type and *yocG*$^-$ strains after a downshift temperature. (**A**) Northern blot analysis using formaldehyde agarose gels was carried out as described in Materials and methods. Total RNA was isolated from strains JH642 (lane 1) or strain AKP8 (*yocG*$^-$, lane 2) grown until mid-exponential phase at 37°C and then shifted to 25°C by 30 min. Each lane contains 10 μg of total RNA. (**B**) Fatty acids synthesized by strains JH642 and AKP8 at 25°C. Cultures of strains JH642 (lane 1) and AKP8 (lane 2) were grown to mid-exponential phase at 37°C, then 2 ml of these cultures were challenged with 10 μCi of [^{14}C]acetate and further shifted to 25°C for 12 h. The lipids were then extracted and transesterified, and the resulting methyl esters were separated into saturated (SFAs) and unsaturated (UFAs) fractions by chromatography on 20% silver nitrate-impregnated silica gel thin-layers plates. The plates were developed at –17°C and autoradiographed by 7 days. The sample in lane 1 contained 15 000 c.p.m. and 2000 c.p.m. in the SFA and UFA fractions, respectively. The sample in lane 2 contained 14 000 c.p.m. in the SFA fraction, while the UFA fraction contained only background levels of radioactivity.

h-DesR and labelled p*des*DNA was tested by EMSA in the presence of an excess of competitor poly(dI–dC) (Figure 3A). The results show that the p*des*DNA exhibited changes in mobility in the presence of 21 nM h-DesR (Figure 3A, lane 1). A more dramatic shift in the mobility of this 367 bp fragment was observed with increasing concentrations of h-DesR, and at the highest concentration of protein tested (336 nM), >90% of the input DNA was complexed with h-DesR (Figure 3A, lane 5). Two bands with shifted mobilities were usually observed, indicating that two different complexes are formed. Addition of a 3-fold excess of an unlabelled DNA fragment containing the *des* promoter diminishes the labelled complex formation (Figure 3B, lane 2), and a 15-fold excess of unlabelled p*des*DNA abolished the labelled complex formation (Figure 3B, lane 3), suggesting specific interaction. The binding specificity was confirmed by determining that h-DesR did not shift an unrelated 290 bp fragment belonging to the *repAB* promoter of plasmid pLS1 (del Solar *et al*., 1990; data not shown).

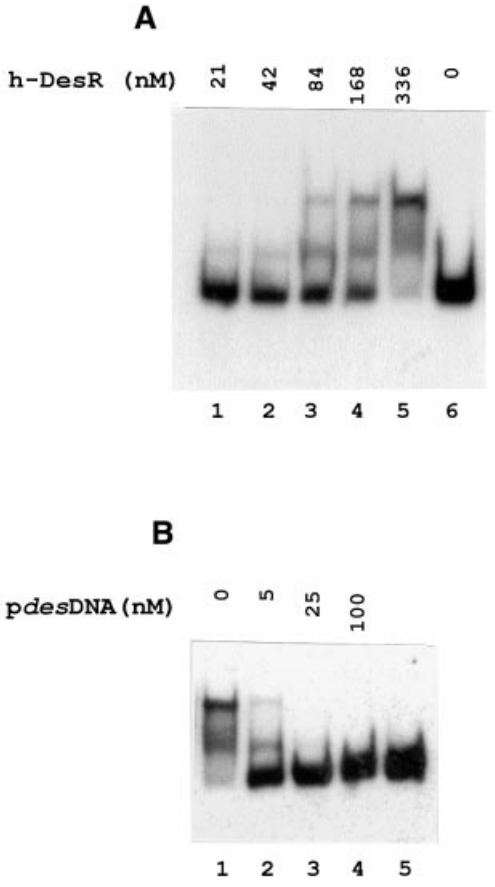

Fig. 3. Gel shift assay showing the binding of DesR to the *des* promoter region. (**A**) The 367 bp *des* promoter fragment (p*des*DNA) was prepared by [α-^{32}P]dATP PCR labelling as described in Materials and methods. The [^{32}P]p*des*DNA concentration in the binding mixtures was 1.7 nM in all cases. The concentration of h-DesR used in each binding reaction is indicated above the respective line. (**B**) Specific competition in binding reactions using 1.7 nM [^{32}P]p*des*DNA and 336 nM h-DesR. Lane 1 shows the retarded species in the absence of unlabelled homologous DNA. Lanes 2, 3 and 4 show the dissociation of the labelled complex in the presence of 3, 15 and 60-fold molar excess of unlabelled p*des*DNA, respectively, added to the binding mixtures before the addition of h-DesR. Lane 5 shows [^{32}P]p*des*DNA without the addition of h-DesR.

h-DesR protects extended DNA segments upstream from its target promoter

To identify h-DesR binding sites in p*des*DNA, DNase I protection analysis was performed on both strands of the *des* promoter. As shown in Figure 4A, binding of h-DesR resulted in the protection of DNA sequences extending from the –28 to –77 position, relative to the start of transcription. In addition to the protected regions, several hypersensitive bonds (indicative of local deformation and presumably caused by bending of the helix) were detected (Figure 4A). DNase I treatment of the h-DesR–DNA complex revealed five protected regions for both strands; these upper and lower strand protections are offset from each other towards the 3′ end of both strands. In the protected region two inverted repeats (5′-TCAT-3′) separated by nine nucleotides were found (Figure 4A). The axis of this dyad symmetry exactly matches the centre of the protected region determined by the footprinting analysis. The finding of a dyad symmetry in the centre of the protected region together with the existence of two complexes of h-DesR–DNA suggests that more than one subunit of h-DesR associates with the *des* promoter at one face of the DNA helix.

To determine whether this dyad symmetric element is directly involved in *des* transcriptional regulation, promoter variants carrying a deletion or a mutation in the DesR binding site were cloned into plasmid pJM116 to create transcriptional fusions, which were then integrated at the *amyE* locus of the *B.subtilis* chromosome. Twenty-two base pairs out of the 49 bp protected region, including both inverted repeats, were deleted, yielding strain AKL59 (Figure 4B). Under cold shock conditions this deletion almost completely abolished promoter activity (Figure 4B). This result strongly suggests that the dyad symmetric element is essential for promoter activity at low temperature. Confirming this interpretation, no promoter activity was detected when the symmetry of the dyad element was disrupted by site-directed mutagenesis of the right inverted repeat (strain AKL62, Figure 4B).

DesK acts to control the activation and deactivation of DesR in response to growth temperature

Previous work has shown that the overexpression of response regulators in the absence of their cognate kinases could result in constitutive expression of the gene(s) they control (Powell and Kado, 1990). This suggests that high concentrations of unphosphorylated response regulator could bind *in vivo* to the target promoter and cause unregulated transcription. To determine whether an excess of DesR, without the assistance of DesK, could activate transcription of *des* at 37°C, we constructed the *desK*$^{-}$ strains AKP2152 and AKP20, expressing the wild-type *desR* from the p*Xyl* or the p*Kan* promoters, respectively. Antibody to DesR was generated and immunoblot assays were performed with the wild-type strain JH642 or cells overexpressing *desR*. As shown in Figure 5A (lanes 1–3), DesR was not detected in whole-cell lysates of strain JH642 growing at either 37 or 25°C, indicating that this protein is produced at very low levels at both temperatures. However, we found that strain AKP2152, expressing *desR* from the p*Xyl* promoter, showed a significant level of DesR synthesis after induction with 0.8% xylose (Figure 5A, lane 5). Although this strain overproduced DesR in the presence of the inducer, expression of *des* was still repressed at either 37 or 25°C (Figure 1B, panel II). Nonetheless, we found that strain AKP20, expressing *desR* from the constitutive p*Kan* promoter, showed a DesR production 5-fold greater than strain AKP2152 (Figure 5B, lane 6) and was able to express the *des–lacZ* fusion at 37°C (Figure 5C). This experiment demonstrates that high production of DesR promotes constitutive expression of the desaturase gene without assistance from DesK. This result, therefore, agrees with the observation that unphosphorylated response regulators can activate transcription when they are overexpressed (Powell and Kado, 1990). A somewhat surprising result was that introduction of the *desKR* operon, under the control of p*Xyl* promoter, into strain AKP20 (giving strain AKP2047) restored the cold-inducible expression of *des* (Figure 5C). This effect could not be attributed to a reduction in the synthesis of DesR, since strain AKP2047 also overproduced the DesR protein

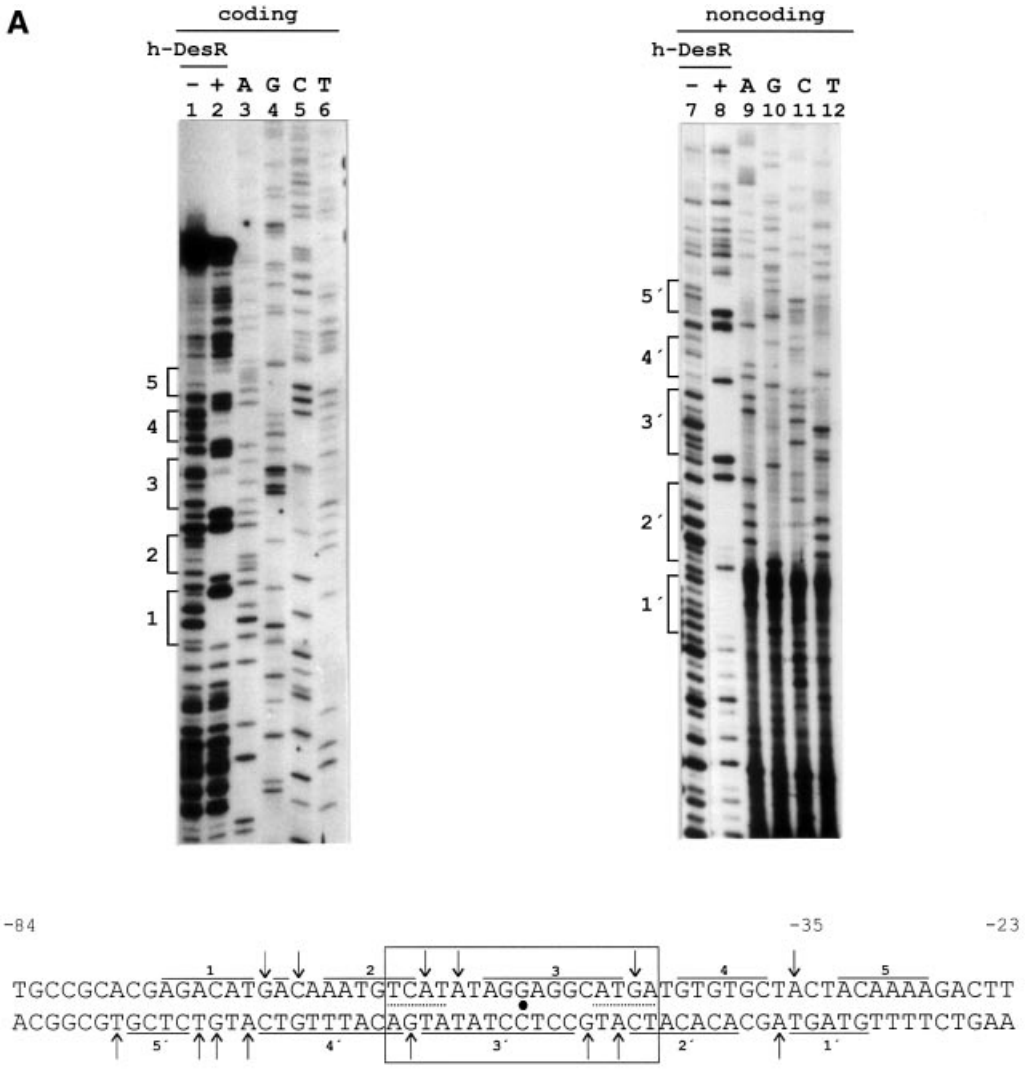

Fig. 4. DNase I footprinting assay of the *des* promoter region and *in vivo* characterization of the *des* promoter protected region. (**A**) DNase I footprinting of h-DesR protein on both strands of a 178 bp DNA fragment containing the *des* promoter (see Materials and methods). Sequencing reactions were performed on the same DNA fragment labelled at the coding (lanes 3 to 6) and non-coding (lanes 9 to 12) strands. Lanes 1, 2, 7 and 8 show the DNase I digestion products of p*des*DNA in the presence (+) or absence (–) of h-DesR. Brackets mark the protected regions in each strand. The putative 17 bp symmetric region of the protected region is boxed, with the dyad axis of symmetry indicated by a dot. The inverted repeats are underlined with dots. DNase I footprints on both strands are shown. Arrows indicate hypersensitive bonds. (**B**) Promoter mutations. The sequence changes in the promoter variants are depicted along the protected region of the *des* promoter. The deleted region is indicated by dots, and the nucleotide changes to introduce mutations in the left inverted repeat are shown in bold characters. The inverted repeat sequences are underlined. The strains were grown at 37°C to an OD of 0.30 and then subjected to a downshift to 25°C. After 3 h of growth at 25°C, the cells were harvested and β-gal activities were determined. The average value of β-gal activity of strain AKP3 (bearing the wild-type promoter) was taken as 100% of promoter activity. The results shown are the average of three independent experiments.

to the same extent as AKP20 (Figure 5B, lanes 4 and 5). It should be noted that the p*Xyl* promoter is ~5-fold weaker than the p*Kan* promoter (as estimated from the levels of production of DesR from *desR* fusions to these promoters; Figure 5A and B). It is more likely, therefore, that the cellular levels of DesK are much lower than the levels of DesR in strain AKP2047. The fact that in strain AKP2047 the xylose-induced expression of *desK* results in deactivation of DesR at 37°C suggests that DesK acts as a phosphatase that dephosphorylates DesR in response to an increase in growth temperature. A downshift in temperature, however, would suppress the phosphatase activity of DesK, favouring the phosphorylation of DesR by DesK, resulting in cold-induced transcriptional activation of the

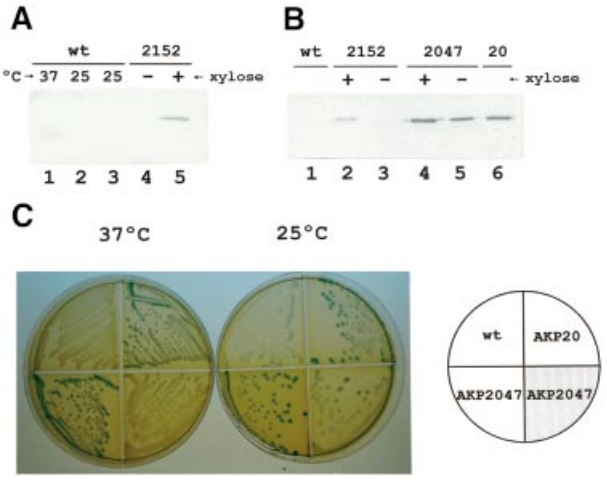

Fig. 5. Overexpression of DesR leads to constitutive expression of *des*. (**A**) JH642 cells (wild type) were cultured at 37°C until mid-exponential phase (lane 1) and then shifted to 25°C for 30 min (lane 2) or 3 h (lane 3). Samples were taken and cell extracts were analysed for the presence of DesR with DesR antiserum. AKP2152 (2152) cells were cultured at 37°C in the absence (lane 4) or presence of 0.8% L-xylose (lane 5) to mid-exponential phase and then shifted to 25°C by 2 h. Samples were taken and analysed for the presence of DesR as described above. (**B**) Strains JH642 (wt, lane 1), AKP2152 (2152) (lanes 2 and 3), AKP2047 (2047) (lanes 4 and 5) and AKP20 (20) (lane 6) were cultured at 37°C until mid-exponential phase and then shifted to 25°C by 2 h. AKP2152 and AKP2047 cultures were supplemented (+) or not (–) with 0.8% xylose. Samples were taken and proteins from the same quantity of cells were analysed for the presence of DesR. (**C**) Bacterial strains AKP3 (wt), AKP20 (*desK*$^{-}$,p*kan-desR*) and AKP2047 (AKP20 p*Xyl-desKdesR::thr*) were streaked onto LB medium containing 30 μg/μl X-Gal, with (shaded quarter) or without (empty quarter) the addition of 0.8% L-xylose. The strains were incubated at 37°C for 12 h (left) or for 5 h at 37°C, and then transferred to 25°C for 36 h (right) before photography.

desaturase gene. The constitutive expression of *desR* in strain AKP20 could be explained by the existence of a second kinase or another phosphodonor, such as acetyl-phosphate (Lukat *et al*., 1992), capable of phosphorylating this response regulator irrespective of the growth temperature. This putative phosphate donor would have low affinity for DesR and would require a high concentration of this response regulator to phosphorylate it.

UFAs regulate the expression of the des gene

Previous work has shown that a downshift of *B.subtilis* cells to low temperature produces a transient increase in *des* mRNA due to shutoff of transcription rather than to the instability of *des* mRNA (Aguilar *et al*., 1999). Since DesK and DesR are necessary for induction of *des* mRNA, it is possible that *des* expression is downregulated by shutoff of *desKR* transcription at low temperatures. To test this hypothesis, total cellular RNA was isolated from cultures of strain JH642 grown at 37°C and then shifted to 25°C for various times. Northern blot analysis indicated that the 1.9 kb *desKR* mRNA is constitutively expressed both at 37 and 25°C, although its level is slightly increased at 25°C, probably due to increased stability of the transcript at the low temperature (Figure 6). This result shows that downregulation of *des* transcription cannot be attributed to shutoff of *desKR* transcription at low temperature. Therefore, the transient induction of *des*

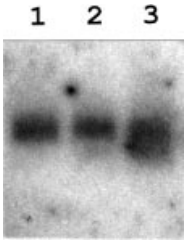

Fig. 6. *desKR* mRNA levels before and after a downshift temperature. Northern blot analysis using formaldehyde agarose gels was carried out as described in Materials and methods. Total RNA was isolated from strain JH642 grown until mid-exponential phase at 37°C (lane 1) and then shifted to 25°C by 30 min (lane 2) or 180 min (lane 3). Each lane contains 15 μg of total RNA.

should be due to inhibition of the pathway that senses or transduces low-temperature signals. Since UFAs are sophisticated signalling molecules that can mediate a myriad of cellular processes (Dowhan, 1997), including gene expression (Choi *et al*., 1996; Hoppe *et al*., 2000), we reasoned that the UFAs formed at low temperatures could act as negative regulators of the low-temperature signalling transduction system that induces the synthesis of the Δ5-desaturase. To test this hypothesis we inserted a *Km*^{R} cassette into the *des* gene of strain AKP3, giving strain AKP4. This strain allows monitoring of the low-temperature inducibility of a *des–lacZ* fusion in the absence of UFAs synthesis. We assayed the β-gal activity of strains AKP3 and AKP4 upon a temperature downshift. While the β-gal levels of the *des*$^{+}$ strain AKP3 began to decrease after 6 h at 25°C, the β-gal activity of the *des–lacZ* fusion contained into the *des*$^{-}$ strain AKP4 continued increasing during this incubation period, reaching levels 10-fold higher than those of strain AKP3 (Figure 7A). This experiment shows that in the absence of UFA synthesis the transcription of the desaturase promoter is increased and is not downregulated by prolonged incubation of cells at 25°C. Figure 7B shows the effects of a series of saturated fatty acids and UFAs on β-gal activity of strain AKP4. In these experiments cells were shifted from 37 to 25°C, and the fatty acids were added to the culture. Comparison of relative enzyme levels revealed that β-gal activity was repressed in all cultures containing UFAs at a concentration of 5 μM, although treatment with saturated fatty acids at this concentration did not appreciably repress the activity of the reporter (Figure 7B). Control experiments showed that UFAs do not affect the expression of *lacZ* fusions to promoters of other genes involved in lipid synthesis in *B.subtilis* (data not shown). When the regulation of the reporter was studied over a range of concentrations of several different UFAs, we found that the most effective repressor of expression of the *des* promoter was 16:1 Δ5 (Figure 7B). The difference in potency among 16:1 Δ5 and the other fatty acids tested strongly suggests that fatty acids with a double bond at the Δ5 position act as specific signals regulating the DesK–DesR signal transduction pathway.

Discussion

Although a large body of information concerning the cold shock response and cold shock proteins has been accumulated (Phadtare *et al*., 2000), several questions still

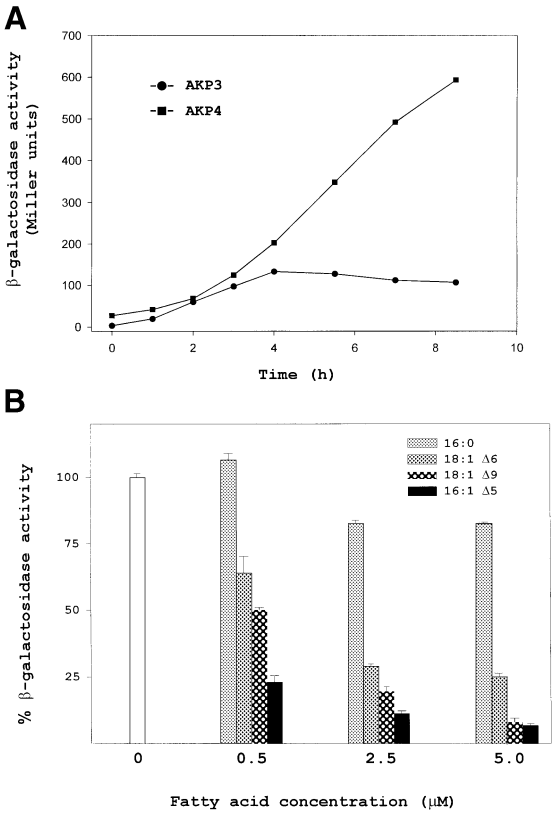

Fig. 7. The expression of the *des* gene is regulated by UFAs. (**A**) *Bacillus subtilis* strains AKP3 (*des*$^+$) (circles) and AKP4 (*des*$^-$) (squares) harbouring a *des–lacZ* fusion located in the *amyE* locus were grown at 37°C to an OD of 0.35 and then transferred to 25°C. Aliquots were taken and specific β-gal activities were determined at the indicated time intervals. (**B**) *Bacillus subtilis* AKP4 cells were grown at 37°C to an OD of 0.27 at 525 nm and were then subjected to 0, 0.5, 2.5 or 5 μM of the indicated fatty acids before being transferred to 25°C. After 4 h of growth at 25°C, the cells were harvested and β-gal activities were determined. The average value of β-gal activity without supplement of fatty acid was taken as 100% of activity. The results shown are the average of three independent experiments.

remain unanswered. Unlike the heat shock response, a specific transcription factor governing low temperature-inducible genes has not been identified (Phadtare *et al*., 2000). The identification of such cellular thermosensors is an essential step in understanding the cold shock response and adaptation. However, the molecular mechanism(s) of thermosensing is far from understood, because the wide spectrum effects of temperature make it difficult to study these mechanisms directly. In contrast to other poikilothermic organisms, such as cyanobacteria (Sakamoto and Bryant, 1997) and plants (Los and Murata, 1998), *B.subtilis* contains a unique desaturase that is encoded by a single gene, which is tightly regulated by temperature at the transcriptional level (Aguilar *et al*., 1998, 1999). Due to its simplicity, the synthesis of UFAs in *B.subtilis* is an exceptionally well-suited system to study the sensing and the transduction of low temperature as a biological signal. We have identified a pair of two-component regulatory proteins, DesK and DesR, which recognize low-temperature signals and govern the expression of the gene coding for the desaturase of *B.subtilis*. Although kinase–response regulator pairs of this type were frequently reported as governors of a wide variety of pathways in response to a myriad of signals (Dutta *et al*., 1999; Hoch, 2000), the requirement of this system for low-temperature adaptation has only recently been suggested in cyanobacteria (Suzuki *et al*., 2000). In this organism it was found that inactivation of two histidine kinases moderates the low-temperature induction of the genes coding for Δ6- and ω3-desaturases. However, the transcriptional regulators of these genes were not identified (Suzuki *et al*., 2000). The genetic studies shown in this work indicate that the DesR protein is a transcriptional regulator controlling the low-temperature induction of the *des* gene. Confirming this prediction we found that the DesR protein binds specifically to the promoter region of the gene it controls, and that the dyad symmetric element found in the centre of the protected DNA region is essential for *des* induction. Therefore, we demonstrate here that DesR is a transcription factor directly involved in the transcriptional activation of the *des* gene at low temperature. Another transcription factor that has been found to stimulate transcription of two cold shock genes from *E.coli*, *hns* (La Teana *et al*., 1991) and *gyrA* (Jones *et al*., 1992), is CspA. Although the signal that triggers the transcriptional activity of both CspA and DesR is low temperature, the mechanism by which these transcription factors are activated is very different. While CspA mRNA is dramatically stabilized after cold shock (Phadtare *et al*., 2000), we suggest here that DesR is activated by a transmembrane signal transduction pathway.

Two lines of evidence lead us to propose that DesK acts to activate or deactivate DesR at 25 or 37°C, respectively: (i) the DesK histidine kinase is essential for *des* transcription; and (ii) DesK is required to deactivate the constitutive *des* transcription generated by overexpression of DesR. On the basis that no response regulator has yet been found to be active in the unphosphorylated form (Hoch, 2000), the role of DesK would then be to act as a phosphatase that dephosphorylates DesR selectively at 37°C. However, after a temperature downshift DesK would function as a specific kinase phosphorylating DesR, the cognate response regulator, which promotes *des* transcription. The results presented in this report strongly suggest that the sensor protein DesK is a bifunctional enzyme having both kinase and phosphatase activities. These two opposite activities of the sensor protein partner have been demonstrated in different two-component systems (for a review see Dutta *et al*., 1999). We have shown that the transcriptional activity of the *des* promoter is inhibited by either endogenously synthesized or exogenously added UFAs. Similar results have been obtained elsewhere for the *OLE1* gene from *S.cerevisiae* coding for the Δ9-desaturase (Choi *et al*., 1996; Hoppe *et al*., 2000). The data presented here are consistent with either of two models for the control of *des* transcription by UFAs. One is that repression is caused by UFA-mediated dissociation of DesR. This mode of regulation has been reported for the expression of the *Bacillus megaterium* cytochrome P450 gene, which is controlled by the BM3R1 transcriptional repressor (Palmer *et al*., 1998). Binding of this negative regulator to DNA is inhibited by UFAs. A second model is that repression is mediated by inhibition of the histidine kinase activity of DesK by UFAs. Associated with the first model, we were unable to detect,

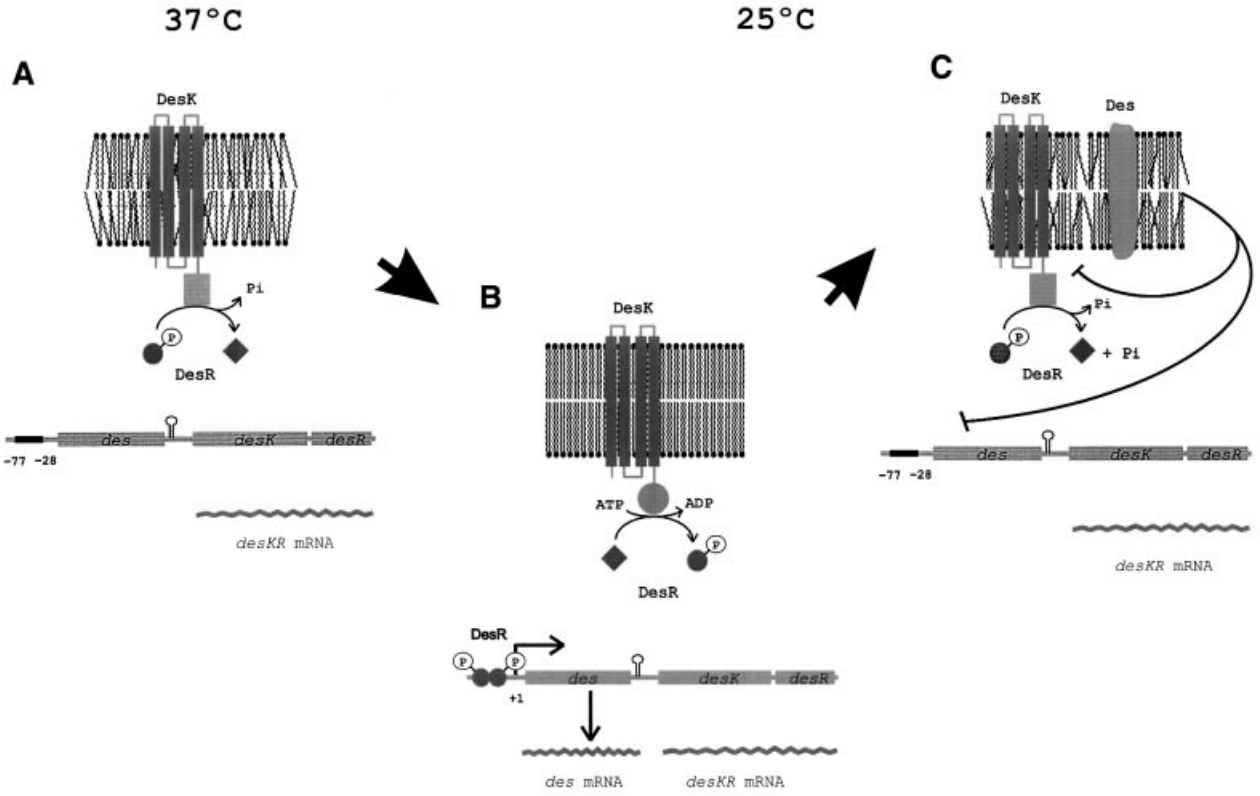

Fig. 8. Model of *des* transcriptional control by two-component temperature signal transduction proteins. It is proposed that DesK assumes different signalling states in response to a temperature-induced change in membrane fluidity. This is accomplished by regulating the ratio of kinase to phosphatase activity such that a phosphatase-dominant state is present at 37°C, when membrane lipids are disordered (**A**), whereas a kinase-dominant state predominates upon an increase in the proportion of ordered membrane lipids after a temperature downshift to 25°C (**B**). DesK-mediated phosphorylation of DesR results in transcriptional activation of *des* (B). Activation of *des* results in synthesis of Des, which desaturates the acyl chains of membrane phospholipids (**C**). These newly synthesized UFAs inhibit *des* transcription either by favouring DesK dephosphorylation of DesR-P or by causing dissociation of DesR-P from its binding site (C) (see text for further details).

in band-shift experiments, any specific displacement of DesR from its operator in the presence of UFAs (data not shown). In addition, we found that UFAs did not repress *des* transcription when DesR was overproduced in the absence of DesK (data not shown). Thus, UFAs do not appear to inactivate DesR directly. However, it remains possible that repression is mediated by an as yet unidentified protein that could displace DesR-P from its target DNA in the presence of UFAs.

A provisional model accounting for our results is shown in Figure 8. We envisage that DesK could assume different signalling states under varying growth temperatures. This could be accomplished by regulating the ratio of kinase to phosphatase activities, such that a phosphatase-dominant state is present at high growth temperature whereas a kinase-dominant state predominates at low growth temperature. DesK possesses four transmembrane domains, and, therefore, one or more of these domains would function to propagate a conformational change across the membrane that is sufficient to significantly alter its activity. This conformational change could be governed by the physical state of the membrane lipid bilayer. Membranes are normally in a liquid crystalline form and will undergo a transition to a gel phase state when the temperature drops (Cronan and Rock, 1996; Vigh *et al*., 1998). This change from a fluid (disordered) to a non-fluid state (ordered) might cause activation of the autokinase activity, resulting in autophosphorylation of a conserved histidine (His188) contained in the transmitter domain of DesK. The phosphoryl group of His188 could be directly transferred to DesR, which activates transcription of *des.* Derepression of *des* results in synthesis of the desaturase enzyme that specifically introduces a double bond at the Δ5-position of the acyl chains of membrane phospholipids. This metabolic pathway, therefore, generates a regulatory loop where Δ5 UFAs inhibit *des* transcription by favouring DesR dephosphorylation or by interacting with a UFA-responsive DNA binding protein that displaces DesR from its binding site.

In summary, we provide evidence for the first time that a transcription factor responds directly to a temperature-regulated histidine kinase and acts as a molecular switch for the regulated transcription of a cold-induced gene.

Materials and methods

Bacterial strains and media

The bacterial strains used in this work are listed in Table I. *Bacillus subtilis* was propagated in Spizizen minimal salts medium (Spizizen, 1958) supplemented with glucose (0.5%), vitamin-free casein hydrolysate (0.1%), tryptophan (50 μg/ml) and phenylalanine (50 μg/ml). The parental bacterial strain was JH642 (*trpC2 pheA1*). All the strains derived from JH642 were obtained by transformation as described (Aguilar *et al*., 1998). Antibiotics were added to media at the following concentrations:

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

Strain	Relevant characteristics	Source
JH642	*trpC2 pheA1*	laboratory stock
AKP3	JH642 *amyE*::[p*des*(–269 to +31)[a]-*lacZ*]	this work
AKP4	AKP3 *des*::*Km*[r]	this work
AKP8	JH642 *yocG*::*Km*[r]	this work
AKP9	AKP3 *yocG*::*Km*[r]	this work
AKP952	AKP9 *thrC*::(p*Xyl-yocG*)	this work
AKP20	AKP3 *yocF*::*Km*[r] p*Km-yocG*	this work
AKP2047	AKP20 *thrC*::(p*Xyl-yocFG*)	this work
AKP21	AKP3 *yocF*G::*Km*[r]	this work
AKP2147	AKP21 *thrC*::(p*Xyl-yocFG*)	this work
AKP2152	AKP21 *thrC*::(p*Xyl-yocG*)	this work
AKL59	JH642 *amyE*::[p*des*(–269 to +31)[b]-*lacZ*]	this work
AKL62	JH642 *amyE*::[p*des*(–269 to +31)[c]-*lacZ*]	this work

[a]Nucleotide numbers correspond to the *des* promoter region relative to the *des* transcription start point.
[b]*des* promoter region containing a 22 bp deletion (see Figure 4B).
[c]*des* promoter region containing a 5 bp substitution (see Figure 4B).

5 μg/ml chloramphenicol and 3 μg/ml kanamycin. Fatty acids were added at the concentrations indicated in Figure 7B. β-gal was assayed as described previously (Aguilar *et al*., 1998) and the specific activity was expressed in Miller units.

Plasmid and strain constructions

In all cases DNA fragments were obtained by PCR using the oligonucleotides (restriction sites are underlined) described in the text. Chromosomal DNA from strain JH642 was used as the template. To construct a transcriptional fusion between *des* and *lacZ*, a 301 bp DNA fragment containing the *des* promoter was obtained using oligonucleotides I (AAAATGAATTCTCCGGCATCCCGATCATCGC) and II (TAGTATGGATCCTCTCATTGTGTGTCTCGGTTC). This fragment was digested with *Eco*RI and *Bam*HI, and cloned into the integrational vector pJM116 (Dartois *et al*., 1998) generating plasmid pAR11. This plasmid was linearized with *Sca*I and introduced by a double cross-over event at the *amyE* locus of the JH642 chromosome, yielding strain AKP3. To obtain the *des* null mutant and the different *yocFG* mutants, DNA coding regions were obtained in each case and cloned in pBluescript SKII. Then, a kanamycin-resistance gene cassette obtained from pJM114 (Perego, 1993) was inserted in each of these regions, and the resulting plasmids were linearized and used to transform different *B.subtilis* strains by a double recombination event. To obtain the *des* null mutant strain a 913 bp DNA fragment was obtained using the oligonucleotides III (TTAGCGTCGACTGAACCGAGACACACAATG) and IV (ACTTCGAGCTCATAGTTGAGCACCTTTGG), and cloned in pBluescript SKII, yielding plasmid pDES913. In this plasmid a *Km*[r] cassette was inserted between the *Hin*dIII and *Bcl*I sites, yielding pDES913KAN. This plasmid was used to transform the AKP3 strain, yielding strain AKP4. To construct the *yocFG* null mutant a 2378 bp DNA fragment was obtained using the oligonucleotides V (AACATGAGCTCCGGAAGAATGCCTGATG) and VI (AGTGGGTACCTTTTTCTTTATGTGCGATTC) and then cloned in pBluescript SKII, yielding plasmid pFG2378. In this plasmid the *Eco*RI and *Hin*dIII restriction sites were used to introduce the *Km*[r] cassette, yielding plasmid pFG2378KAN. This plasmid was used to transform AKP3, yielding AKP21 strain. To disrupt the *yocG* gene, a 1466 bp DNA fragment obtained using the oligonucleotides VII (CCCGCGAGCTCCATGATACGCTTGGGCAAAAG) and VIII (TTAGTCTCGAGCAGTTGGGCATGGCAGCTTCG) was cloned in pBluescript SKII. Then, in this new construction a *Km*[r] cassette was inserted in the *Cla*I–*Xba*I restriction sites, yielding plasmid pG1466KAN. This plasmid was used to transform the JH642 and AKP3 strains, yielding strains AKP8 and AKP9, respectively. A *desK* null mutant strain containing *desR* under the p*Km* promoter was constructed by first cloning a 1765 bp DNA fragment obtained with oligonucleotides V and XII (TGCTGGGTACCTGAGCGATTTCTTTTGTG) in pBluescript SKII. The resulting plasmid was digested with *Eco*RI and *Hin*dIII, and a *Km*[r] cassette without its transcriptional terminator was inserted, yielding plasmid pFG1765KAN. This construction was used to transform strain AKP3, giving rise to strain AKP20. To place the *yocFG* operon and the *yocG* genes under the p*Xyl* promoter into the *thrC* locus of the *B.subtilis* chromosome, *yocFG* and *yocG* were first amplified by PCR and then cloned under p*Xyl* in plasmid pGS40 (G.Schujman, personal communication). Then the DNA fragments containing the fusion p*Xyl–yocFG* or p*Xyl–yocG* were cloned into the integrative plasmid pDG795 (Guerout-Fleury *et al*., 1995) and recombined into the *thrC* locus of the appropriate strain. A 1814 bp DNA fragment containing the *yocFG* genes obtained using the oligonucleotides IX (AGTAAGTCGACAAGCTGAAAATGAGGTAAGATC) and X (TTCAGGGTACCAAAAAGGATCCTGGCAGATG) was first cloned into the *Sal*I–*Kpn*I sites of plasmid pGS40 to obtain plasmid pGSFGXyl. This plasmid was digested with *Bam*HI, and the DNA fragment containing *yocFG* under the p*Xyl* promoter was cloned into the plasmid pDG795, yielding plasmid pAG47. This plasmid was used to transform strains AKP21 and AKP20 to give strains AKP2147 and AKP2047, respectively. A 677 bp DNA fragment containing the *yocG* gene was obtained using the oligonucleotides XI (TCAAAATCGATATAAAGGATGGCTTATATG) and X and then cloned into pGS40 to give plasmid pGSGXyl. This plasmid was digested with *Bam*HI and *Eco*RI, and the DNA fragment containing *yocG* under the p*Xyl* was cloned into plasmid pDG795, yielding plasmid pAG52. This plasmid was used to transform AKP9 and AKP21 strains, yielding strains AKP952 and AKP2152, respectively.

To delete 22 out of the 49 bp protected in the DNase I footprinting assay, leaving the –35 plus 7 bp upstream of the *des* gene intact (Figure 4B), oligonucleotides XXII (GTTTGGAATTCACCCCTCAAGTGAGTGGAGC), XXIII (CATTTGTCATGTCTCGTGCGGCATGCATAG), XXIV (GAGGCATGATGTGTGCTACTACAAAAGAC) and XXV (AAATCCGCGGGAGAATAAACATGATAAC) were used to generate two PCR products flanking the region to be deleted. These fragments were purified, blunt ligated, and the ligation reaction product was used as substrate for a PCR reaction using oligonucleotides XXVI (AAAATGAATTCTCCGGCATCCCGATCATCGC) and XXVII (AGTATGGATCCTCTCATTGTGTGTCTCGGTTC). This 279 nt PCR product was cloned into the integrative plasmid pJM116 to generate pAKL59.

To mutate the 3′ inverted repeat of the protected region (Figure 4B), two DNA fragments were amplified using oligonucleotides XXVIII (GTAGCACACTCTAGACCTCCTATATGACATTTGTC), XXII, XXIX (TATAGGAGGTCTAGAGTGTGCTACTACAAAAGAC) and XXV. A mix of these PCR products was used as DNA template for another PCR using oligonucleotides XXVI and XXVII. The 301 bp amplification product was cloned into plasmid pJM116 to generate pAKL62. Plasmids pAKL59 and pAKL62 were introduced into the *amyE* locus of strain JH642 giving strains AKL59 and AKL62, respectively.

RNA analysis

Bacillus subtilis strains were grown in supplemented Spizizen minimal salts medium and the RNA was isolated as described previously (Aguilar *et al*., 1999). Northern blot analysis and hybridization with a single-stranded *des* DNA probe were performed as previously described (Aguilar *et al*., 1999). The single-stranded *yocFG* DNA probe was synthesized with T4 DNA polymerase, [α-^{32}P]dATP and the antisense oligonucleotide XIII (TTGGCCGGTTGTACCTTTGC) as primer of a DNA fragment obtained by PCR amplification from chromosome of strain JH642 using the oligonucleotides XIV (CAAGGAGGCCTAGCGAATGGCCCGCGATCTCCATG) and XV (AGATCTTTGGCCGGTTGTACCTTTGC). The size of the *yocFG* transcript was determined by comparison with Promega RNA molecular weight standards (data not shown).

Construction of expression vector and purification of His-tagged DesR

The *yocG* gene was amplified from genomic DNA isolated from *B.subtilis*. Primers XVI (GGATGGGATCCATGATTAGTATATTTATTGCAG) and XVII (AAAAAGCATGCTGGCAGATGCCAAGATC) were used. The PCR product was digested, purified and ligated into plasmid pQE30, yielding plasmid pAR18. This new construction places a His_6 tag at the N-terminus of the DesR protein and was used to transform the *E.coli* strain M15[pREP4]. Overexpression and purification of h-DesR were performed by standard procedures (Qiagen, Inc.).

Gel shift assays

A DNA fragment including the *des* promoter region was prepared by PCR with plasmid pDM10 (Aguilar *et al*., 1998) as template. The primers used were XVIII (ATGCAGGATTCAAGCTATTTCGGGTACATC) and XIX (TCGAGGCTGAGATAAGCAAGAAACCATAGGC). The 367 bp

DNA fragment was labelled by incorporation of [α-^{32}P]dATP in the PCR amplification, and purified from 5% polyacrylamide gels at the specific activity of 2.5 × 10^6 c.p.m./μg. Binding of h-DesR to DNA fragments was carried out in a 25 μl reaction mix containing 25 mM Tris–HCl pH 8, 1 mM dithiothreitol (DTT), 0.25 mM EDTA, 4 mM $MgCl_2$, 200 ng poly(dI–dC) and 5% glycerol. After 30 min at room temperature, 2.8 μl of 50% glycerol were added and the samples were applied to a 5% polyacrylamide gel, which had been pre-run for 2 h in 45 mM Tris-borate pH 8, 1 mM EDTA. Gels were dried and autoradiographed.

DNase I footprinting assays

DNA for this assay was obtained by PCR using DNA from pDM10 as template and primers XX (CGGGTACATCACGAATATGG) and XXI (TGTGTGTCTCGGTTCAGTATACGC). Both primers were previously labelled at their 5′ ends using [γ-^{32}P]ATP and T4 polynucleotide kinase. The PCR product, a 178 bp fragment, was eluted, purified, and recovered at the specific activity of 8 × 10^6 c.p.m./μg. Reactions were performed in a total volume of 50 μl of a buffer containing 25 mM Tris pH 8.0, 1 mM DTT, 0.25 mM EDTA, 22 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 5% glycerol. The ^{32}P-end-labelled DNA fragments, at a final concentration of 2.6 nM, were incubated with purified h-DesR protein (700 nM) and poly(dI–dC) (4 ng/μl) at room temperature for 30 min. DNase I (0.001 U) was added, and the incubation was continued for 5 min at the same temperature. The reactions were stopped by the addition of 25 μl of a solution containing 2 M ammonium acetate, 0.15 mM EDTA, 0.8 M sodium acetate, 100 μg/ml calf thymus and 400 μg/ml tRNA. The DNA samples were ethanol precipitated and dissolved in 8 μl of sequencing loading buffer (80% deionized formamide, 10 mM sodium hydroxide, 0.1% bromophenol blue, 0.1% xylene cyanol, 1 mM EDTA). Samples (3 μl, 75 000 c.p.m.) were loaded onto 8% polyacrylamide sequencing gels and run together with sequencing reactions obtained by the dideoxy-mediated chain-termination method (T7 DNA polymerase sequencing kit, Pharmacia).

Fatty acid analysis and purification of 16:1 Δ5

Measurements of fatty acid synthesis by *B.subtilis* cells were performed as previously described (Aguilar *et al*., 1998). The 16:1 Δ5 fatty acid was prepared using the *E.coli* strain AK7/pDM10 (Aguilar *et al*., 1998). This strain was grown at 30°C in LB medium to exponential phase, and labelled with [1-^{14}C]palmitate. Lipids were extracted and fatty acids converted to their methyl esters with sodium methoxide. The saturated and unsaturated fractions were separated into two peaks by chromatography on a 20% silver nitrate-impregnated silica gel column. The concentration of the UFA fraction was determined by gas chromatography. To release the free fatty acids, the solution containing the methyl ester was made 0.5 M in KOH and heated for 2 h at 65°C. After washing twice with water and drying under a steam of nitrogen, the residue was resuspended in dimethylsulfoxide (DMSO). The C18:1 Δ6 and C16:1 Δ9 fatty acids were purchased from Sigma. To quantify these fatty acids, both were converted to methyl esters in a 5% HCl–methanol solution followed by heating for 3 h at 80°C. The concentrations were determined by gas chromatography, and the free fatty acids were obtained as described for C16:1 Δ5.

Immunoblot analysis

Bacillus subtilis strains were grown as indicated in each case. Aliquots of 1 ml of each culture were harvested, centrifuged and frozen. The pellets were resuspended in lysis buffer (50 mM Tris–HCl pH 8.0, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 0.1 mM DTT), adding 180 μl of buffer per OD_{525} unit. Twenty microlitres of cell resuspension were disrupted by incubating with lysozyme (500 μg/ml) for 15 min at 37°C, followed by 5 min of boiling in the presence of loading buffer. Each sample was subjected to SDS–PAGE in a 12% acrylamide gel. Proteins were electroeluted to a nitrocellulose membrane and revealed using anti-h-DesR rabbit antibody and a secondary antibody conjugated to alkaline phosphatase.

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