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# Mechanism of membrane fluidity optimization: isothermal control of the *Bacillus subtilis* acyl-lipid desaturase

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## Summary

**The Des pathway of *Bacillus subtilis* regulates the expression of the acyl-lipid desaturase, Des, thereby controlling the synthesis of unsaturated fatty acids (UFAs) from saturated phospholipid precursors. Previously, we showed that the master switch for the Des pathway is a two-component regulatory system composed of a membrane-associated kinase, DesK, and a soluble transcriptional regulator, DesR, which stringently controls transcription of the *des* gene. Activation of this pathway takes place when cells are shifted to low growth temperature. Here, we report on the mechanism by which isoleucine regulates the Des pathway. We found that exogenous isoleucine sources, as well as its α-keto acid derivative, which is a branched-chain fatty acid precursor, negatively regulate the expression of the *des* gene at 37°C. The DesK–DesR two-component system mediates this response, as both partners are required to sense and transduce the isoleucine signal at 37°C. Fatty acid profiles strongly indicate that isoleucine affects the signalling state of the DesK sensor protein by dramatically increasing the incorporation of the lower-melting-point anteiso-branched-chain fatty acids into membrane phospholipids. We propose that both a decrease in membrane fluidity at constant temperature and a temperature downshift induce *des* by the same mechanism. Thus, the Des pathway would provide a novel mechanism to optimize membrane lipid fluidity at a constant temperature.**

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## Introduction

Lipids in biological membranes are usually maintained in the fluid, liquid-crystalline state, so that the gel to liquid crystalline phase transition temperature is below the environmental temperature (Vigh *et al*., 1998). The correct physical state of membrane lipids is required for optimal membrane structure and function. Temperature markedly affects membrane lipid composition, and changes in lipid composition are thought to occur in order to maintain an appropriate liquid crystalline state. The major way in which bacteria, generally lacking cholesterol, maintain this functional membrane physical state is by changing their fatty acid composition (de Mendoza and Cronan, 1983; Vigh *et al*., 1998). As the growth temperature decreases, the proportion of low-melting-point fatty acids in the membrane lipids increases. *Bacillus* cells respond to a decrease in ambient growth temperature by desaturating the fatty acids of their membrane lipids (for a recent review, see de Mendoza *et al*., 2001) and by increasing the proportion of anteiso-branched fatty acids (Klein *et al*., 1999). Anteiso-branched fatty acids have a lower melting point than iso-branched fatty acids (Kaneda, 1991; Suutari and Laasko, 1994). Therefore, it has been proposed that this adaptive response could be important in providing an appropriate degree of membrane fluidity for growth at low temperatures (Klein *et al*., 1999). The 'primer' carbons for the synthesis of branched-chain fatty acids are α-keto acids derived from valine, leucine and isoleucine (Kaneda, 1977; 1991). While isoleucine is the precursor of anteiso-branched-chain fatty acids, leucine and valine give raise to the primers for iso-branched fatty acids (Kaneda, 1977; 1991). In consequence, it has been hypothesized that the increased synthesis of anteiso-fatty acids at low growth temperatures could result from temperature regulation of primer selection specificity of the FabH condensing enzymes (Choi *et al*., 2001) or to changes in the cellular primer pools for fatty acid synthesis (Klein *et al*., 1999). In contrast to the poorly studied mechanism of temperature-mediated adjustment of branched-chain fatty acids in *Bacillus subtilis*, a novel pathway for the adjustment of unsaturated fatty acid (UFA) synthesis has recently been described in this organism. This pathway, termed the Des pathway, responds to a

decrease in growth temperature by enhancing the expression of the *des* gene coding for an acyl-lipid desaturase (Aguilar *et al*., 2001). The Des pathway is uniquely and stringently regulated by a two-component system composed of a membrane-associated kinase, DesK, and a soluble transcriptional activator, DesR. We suggested that activation of this transduction pathway might be mediated by a decrease in membrane fluidity provoked by a temperature downshift (Aguilar *et al*., 2001). Recent studies have shown that *B. subtilis* produces significant amounts of UFAs at 37°C in cultures grown in the absence of isoleucine (Weber *et al*., 2001). This result suggests that a decrease in the content of isoleucine-derived fatty acids in the membrane at a constant temperature could mimic a drop in growth temperature and that both stimuli could induce UFAs synthesis. Thus, the aim of the present study was to test the hypothesis that the same transmembrane signal pathway regulates induction of *des* at constant temperature or after a cold shock.

In this paper we demonstrate that expression of *des* at 37°C in the absence of exogenous isoleucine is via the DesK–DesR two-component regulatory system. We present evidence that this is caused by restricted membrane fluidity, resulting from the relatively high proportion of iso- to anteiso-branched-chain fatty acids. On the other hand, when membrane fluidity is increased by the provision of exogenous substrates that lead to the formation of primers for anteiso-branched fatty acids (e.g. isoleucine or threonine), *des* is not expressed. We propose that induction of the Des pathway, in response to either temperature downshift or restricted membrane fluidity in the presence of high proportions of iso-branched fatty acids, is brought about via the ability of DesK to sense a decrease in membrane fluidity.

## Results

### *Isoleucine-dependent control of* des *expression at 37°C*

It has been well established that *B. subtilis* JH642 does not synthesize UFAs at 37°C when it is growing in rich medium such as Luria–Bertani or SMM supplemented with casein hydrolysate (Aguilar *et al*., 1998; 1999). However, Weber *et al*. (2001) reported recently that membranes of *B. subtilis* strain JH642 grown at 37°C in the absence of isoleucine contained significant amounts of UFAs. As the cold-inducible *des* gene encodes the sole desaturase of *B. subtilis* (Aguilar *et al*., 1998), which is responsible for the synthesis of UFAs, we decided to determine whether exogenous isoleucine has some effect on *des* transcription at 37°C. To this end, we used strain AKP3 (Table 1) containing a fusion of the *lacZ* gene to the *des* promoter integrated ectopically at the non-essential *amyE* locus of *B. subtilis*. This strain was grown in either SMM or SMM supplemented with isoleucine at final concentrations ranging from 10 to 300 μM and assayed for β-galactosidase activity. As shown in Fig. 1A, the *des* promoter was active during growth in SMM at 37°C, reaching a peak of 95 Miller units at the end of exponential phase. The expression of the *des–lacZ* fusion was decreased when the concentration of isoleucine was increased in the growth medium (Fig. 1A), reaching a 10-fold repression in the presence of 100 μM amino acid. The effects of the branched-chain amino acids leucine and valine as well as of threonine, which is the precursor of isoleucine in the biosynthetic pathway (Klein *et al*., 1999), were tested by measuring β-galactosidase activity of strain AKP3. Comparison of relative enzyme levels revealed that threonine was as effective as isoleucine in repressing *des* transcription, although treatment with valine and leucine did not

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

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| Strains | | |
| JH642 | *trpC2 pheA1* | Laboratory stock |
| AKP3 | JH642 *amyE*::*Pdes–lacZ* | Aguilar *et al*. (2001) |
| AKP4 | AKP3 *des*::Km$^r$ | Aguilar *et al*. (2001) |
| AKP9 | AKP3 *desR*::Km$^r$ | Aguilar *et al*. (2001) |
| AKP20 | AKP3 *desK*::Km$^r$ *PKm–desR* | Aguilar *et al*. (2001) |
| AKP2047 | AKP20 *thrC*::(*PXyl–desKR*) | Aguilar *et al*. (2001) |
| AKP21 | AKP3 *desKR*::Km$^r$ | Aguilar *et al*. (2001) |
| AKL59 | JH642 *amyE*::(*Pdes*)$^a$–*lacZ* | Aguilar *et al*. (2001) |
| AKL62 | JH642 *amyE*::(*Pdes*)$^b$–*lacZ* | Aguilar *et al*. (2001) |
| AE3 | JH642 *amyE*::(*PdesKR*)–*lacZ* | A. Erazo (personal communication) |
| Plasmids | | |
| pAD1 | 3.2 kb fragment from pAG47 containing *PXyl–desKR* cloned into pHPKS | This study |
| pCM1 | 1.97 kb fragment from pAG52 containing *PXyl–desR* cloned into pHPKS | This study |

**a.** *des* promoter region containing a 22 bp deletion (Aguilar *et al*., 2001).
**b.** *des* promoter region containing a 5 bp substitution (Aguilar *et al*., 2001).

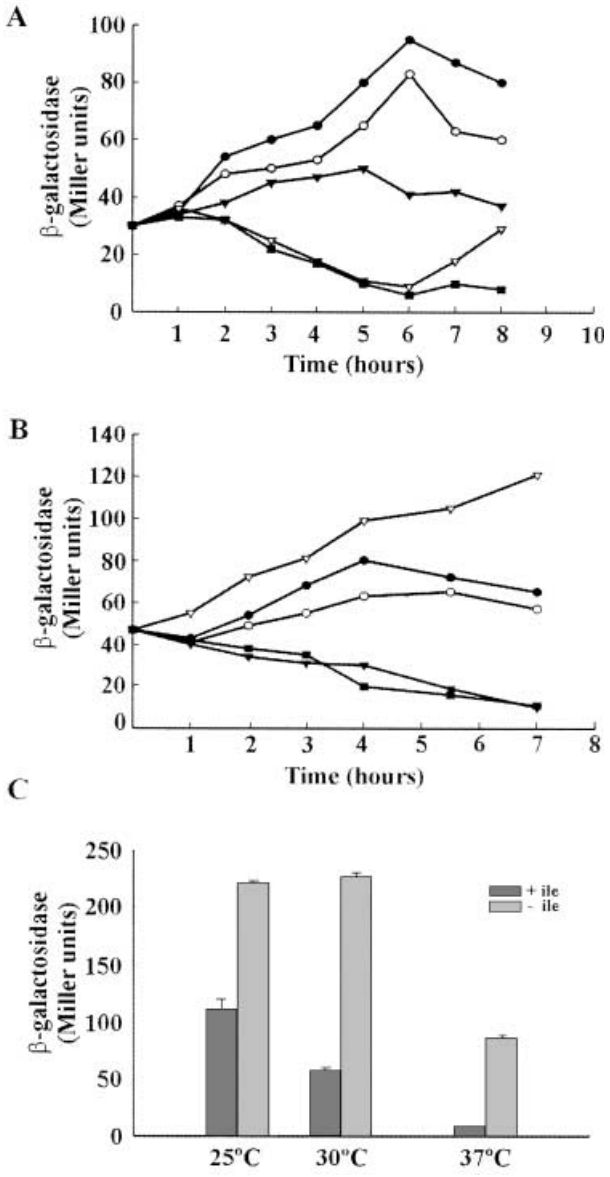


**Fig. 1.** Effect of different amino acids on *des–lacZ* expression.
A. *B. subtilis* AKP3 cells were grown overnight at 37°C in SMM and then diluted in SMM (black circles) or SMM supplemented with 10 μM (white circles), 30 μM (black triangles), 100 μM (white triangles) or 300 μM (black squares) isoleucine. β-Galactosidase specific activities were determined at the indicated intervals after resuspension. Each datum point is the mean of three separate experiments with a mean error of <5%.
B. *B. subtilis* AKP3 cells were grown overnight at 37°C in SMM and diluted in SMM supplemented with 150 μM isoleucine (black squares), leucine (white circles), valine (white triangles), threonine (black triangles) or no addition (black circles). β-Galactosidase specific activities were determined at the indicated intervals. Each datum point is the mean of three separate experiments with a mean error of <5%.
C. *B. subtilis* AKP3 cells were grown overnight at 37°C in SMM and then diluted in SMM with or without the addition of 150 μM isoleucine. At an $OD_{600}$ of 0.3, aliquots of each culture were transferred to 25°C, 30°C or kept at 37°C. β-Galactosidase specific activities were determined 4 h after transferring the cells to the indicated temperatures. The results shown are the average of three independent experiments.

appreciably repress the activity of the reporter (Fig. 1B). In order to study whether isoleucine is able to repress *des* activity at low temperatures, the transcription of *des* in strain AKP3 was monitored at a range of temperature between 25°C and 37°C in the presence or absence of isoleucine. As shown in Fig. 1C, in the presence of exogenous isoleucine, the expression of *des* was reduced 2.6-, 4.0- and 10.0-fold when cells were grown at 25°C, 30°C or 37°C respectively. This experiment shows that repression of *des* transcription by isoleucine increases when growth temperature is increased. Together, these data indicate that, in addition to low temperature, expression of the *des* gene is regulated by the availability of exogenous isoleucine.

### *Isoleucine inhibits production of the* des *transcript and UFA synthesis at 37°C*

We performed Northern blot analysis to examine the expression of the *des* gene in wild-type strain JH642 growing at 37°C in SMM or SMM supplemented with isoleucine. This analysis indicates that cells growing at 37°C in SMM minimal medium synthesize a *des* transcript of ≈1.1 kb (Fig. 2A), which is indistinguishable from the transcript synthesized by cells downshifted from 37°C to 20°C in complex medium (Aguilar *et al*., 1999). However, the accumulation of the *des* transcript at 37°C was not observed in strain JH642 growing in SMM medium containing 150 μM isoleucine, demonstrating directly that expression of *des* was greatly reduced in the presence of this branched-chain amino acid (Fig. 2A). It should be noted that, although the levels of the *des* transcript have the highest induction after 2–3 h of growth in SMM (Fig. 2A), the maximum activity of β-galactosidase, coded by a *des–lacZ* fusion, is observed after about 6 h of growth in an isoleucine-free medium. This difference in the time-dependent induction of *des* mRNA synthesis and activity of the *lacZ* reporter gene was also observed upon a downshift in temperature of cells growing in rich medium (Aguilar *et al*., 1998; 1999).

The levels of saturated and unsaturated fatty acids of strain JH642 growing at 37°C in different growth media was compared (Fig. 2B). The fatty acids were labelled by growing the strains in the presence of [$^{14}$C]-acetate, followed by argentation chromatography of the radioactive fatty acids. Strain JH642 grown in SMM or SMM supplemented with either leucine or valine synthesized UFAs (Fig. 2B, lanes 1, 2 and 4), but formed only background levels of UFAs in SMM medium containing isoleucine (Fig. 2B, lane 3). These experiments confirm that the *des* gene is transcribed and that the desaturase is active in *B. subtilis* cultures grown at 37°C in the absence of isoleucine.

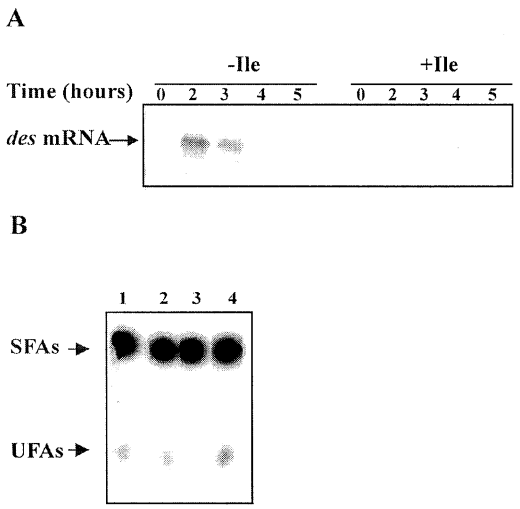


**Fig. 2.** *des* transcription and UFA synthesis are inhibited by exogenous isoleucine.
A. Northern blot analysis using formaldehyde–agarose gel was carried out as described in *Experimental procedures*. Total RNA from strain JH642 grown at 37°C in SMM or in SMM supplemented with 150 μM isoleucine was isolated at the indicated intervals.
B. Fatty acids synthesized by strain JH642 at 37°C. Cultures of strain JH642 were grown to an OD of 1.0 at 37°C in SMM in the presence of 10 μCi of [$^{14}$C]-acetate and supplemented with 150 μM leucine (lane 1), valine (lane 2), isoleucine (lane 3) or no addition (lane 4). Lipids were extracted and transesterified. Methyl esters were separated into saturated (SFAs) and unsaturated (UFAs) fractions by chromatography on 20% silver nitrate-impregnated silica gel thin plates. The plates were developed at –17°C and autoradiographed for 5 days. The samples in lanes 1, 2 and 4 contained 9690, 9680 or 9640 c.p.m. of radioactivity in the SFA fraction and 310, 320 or 360 c.p.m. in the UFA fraction respectively. The sample in lane 3 contained 9980 c.p.m. in the SFA fraction, whereas the UFA fraction contained only background levels of radioactivity.

### *Regulation of* des *expression in response to different precursors of branched-chain fatty acid biosynthesis*

To test whether the effect of isoleucine on *des* transcription was specifically related to its ability to act as a primer of branched-chain anteiso-fatty acids, we added several precursors for the synthesis of anteiso- and iso-branched fatty acids to cultures of strain AKP3 growing in SMM at 37°C and assayed the β-galactosidase activity of the reporter gene, which is under the control of the *des* promoter. The valine derivative, isobutyrate, displayed no effect, whereas the leucine precursor, isovalerate, stimulated the transcription of the *des–lacZ* fusion of strain AKP3 (Fig. 3). The isoleucine-related fatty acids precursor 2-methyl-butyrate showed an inhibitory effect on *des* transcription, comparable with that seen for isoleucine and threonine (Fig. 3). These results strongly suggest that the mechanism by which isoleucine negatively regulates *des* transcription is by serving as the primer of anteiso-branched chain fatty acids and thus by modulating the membrane physical state.

### *The two-component signal transduction system DesK–DesR controls* des *expression at 37°C*

The two-component signal transduction system DesK–DesR is essential for cold induction of the *des* gene (Aguilar *et al.*, 2001). To determine whether the DesK–DesR regulatory system is also responsible for *des* transcription at 37°C, we used strains AKP9 and AKP21 (Table 1), which contain a kanamycin resistance gene (*Km$^R$*) cassette interrupting the *desR* gene or the *desK–desR* operon respectively. These mutations eliminated the activity of the *des* promoter when the strains were grown at 37°C in minimal medium (Table 2). The expression of the *des–lacZ* fusion of strains AKP9 and AKP21 was recovered when they were complemented *in trans* with plasmid pAD1 carrying the *desKR* operon (Table 2). As expected, the β-galactosidase activities of the complemented strains were repressed by isoleucine (Table 2). In addition, the expression of the *des* promoter of strain AKP9 could be complemented *in trans* with plasmid pCM1 expressing the *desR* gene alone. pCM1 was unable to re-establish the *lac*$^+$ phenotype of strain AKP21 (data not shown), thus indicating the essentiality of both partners of the two-component regulatory system for *des* expression at 37°C.

In a previous work (Aguilar *et al.*, 2001), we demonstrated that the DesR transcriptional factor binds specifically to promoter regions of the *des* gene, and that a dyad symmetric element is essential for low-temperature induction of the *des* promoter. In order to investigate whether these regulatory regions were required for DesR transcriptional activity at 37°C, strains AKL59 and AKL62 (Table 1), which contain promoter variants of *des* carrying

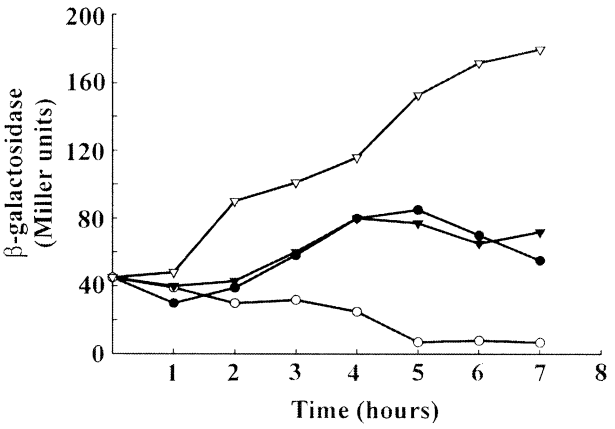


**Fig. 3.** Effect of branched-chain fatty acid precursors on *des–lacZ* expression. *B. subtilis* AKP3 cells were grown overnight at 37°C in SMM and diluted in SMM or SMM supplemented with 50 μM of the following α-keto acids: 2-methylbutyric acid (white circles), isobutyric acid (black triangles), isovaleric acid (white triangles) or no addition (black circles). β-Galactosidase activities were determined at the indicated intervals. The results shown are the average of three independent experiments, with a mean error of <5%.

**Table 2.** The expression of *des* at 37°C is regulated by the DesK–DesR system.

| Strain | β-Galactosidase activity[a] | | |
|---|---|---|---|
| | –Isoleucine | +Isoleucine | Repression[b] |
| AKP9 (*desR*⁻) | 4.3 ±0.2 | 3.6 ±0.2 | 1.2 |
| AKP21 (*desK*⁻ *desR*⁻) | 3.3 ±0.1 | 3.4 ±0.1 | 1.0 |
| AKP9/pAD1 (*PXyl-desKR*) | 51.0 ±3.2 | 9.9 ±0.6 | 5.1 |
| AKP21/pAD1 (*PXyl-desKR*) | 34.8 ±2.2 | 8.2 ±0.4 | 4.2 |

**a.** Cells were grown overnight in SMM at 37°C and then diluted in the same media in the presence or absence of 150 μM isoleucine. Cells were harvested after 4 h of growth at 37°C, and β-galactosidase specific activities were determined as described in *Experimental procedures*.
**b.** Repression is the ratio of expression in the absence versus the presence of isoleucine.

a deletion or a mutation in the DesR binding site, respectively, were assayed for β-galactosidase activity in an isoleucine-free medium. In both strains, transcription from the *des* promoter was undetectable at either 25°C or 37°C (data not shown), suggesting that the response regulator DesR binds to the same regulatory regions at both temperatures to promote activation of *des* transcription.

### *Isoleucine affects the signalling state of DesK*

To test whether *des* expression is downregulated by isoleucine as a result of shut-off of the transcription of *desKR*, coding for DesK and DesR, we assayed the β-galactosidase activity of strain AE3 (Table 1), which contains a fusion of the *lacZ* gene to the *desKR* promoter. This experiment indicated that the addition of isoleucine to the growth medium did not affect the transcription of *desKR* at 37°C (data not shown). Thus, the repression of *des* transcription by isoleucine should result from the inhibition of the pathway sensed and transduced by the DesK–DesR two-component regulatory system.

To investigate whether isoleucine represses *des* transcription by affecting the signalling state of DesK, we used strain AKP20 (Table 1). This strain lacks *desK* and overproduces DesR, resulting in constitutive expression of *des*, presumably because of the existence of a second kinase or another phosphodonor capable of phosphorylating the response regulator at 37°C (Aguilar *et al.*, 2001). We assayed the β-galactosidase activity controlled by the *des* promoter of strain AKP20 growing at 37°C in minimal medium supplemented or not supplemented with isoleucine (Fig. 4). The β-galactosidase levels of strain AKP20 were not affected by isoleucine, indicating that, in the absence of DesK, cells express the desaturase at 37°C regardless the presence of isoleucine (Fig. 4). This assumption was confirmed with strain AKP2047, a derivative of strain AKP20 containing the *desKR* operon under the *PXyl* promoter integrated at the *thrC* locus (Table 1). In this particular experiment, we tested the effect of threonine, instead of isoleucine, on *des* expression, as strain AKP2047 is a threonine auxotroph (Table 1). As shown in Fig. 4, threonine did not inhibit *des* transcription in the absence of *desK* expression. However, the xylose-induced transcription of *desK* in strain AKP2047 resulted in inhibition of *des* transcription by threonine. These results once again support the conclusion that DesK is specifically sensing the 'isoleucine signal' that controls the desaturase expression at 37°C.

### *UFAs regulate the expression of the* des *gene at 37°C*

Previous work has shown that the transcriptional activity of the *des* promoter of *B. subtilis* cells growing at low temperatures in rich medium is inhibited by either endogenously synthesized or exogenously added UFAs (Aguilar *et al.*, 2001). To test whether UFAs also regulate the Des pathway in *B. subtilis* cells growing at 37°C in the absence of isoleucine, we used strain AKP4 (Table 1), which allows monitoring of the induction of a *des–lacZ* fusion in the absence of UFA synthesis (Aguilar *et al.*, 2001). The β-

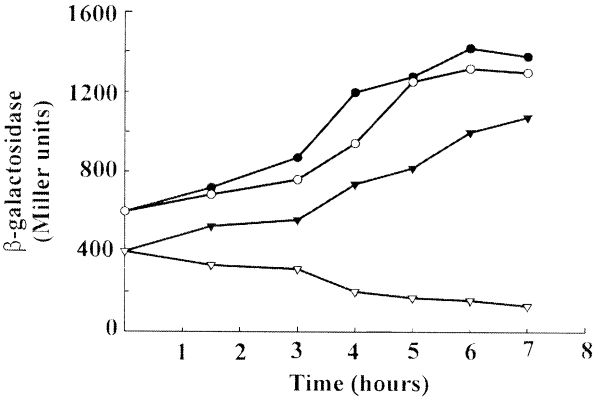


**Fig. 4.** Constitutive expression of *des* is not repressed by isoleucine. *B. subtilis* AKP20 cells were grown overnight at 37°C in SMM. Cells were collected and diluted either in the presence (black circles) or in the absence (white circles) of isoleucine. AKP2047 cells were grown at 37°C in SMM supplemented with threonine, in the presence (white triangles) or in the absence (black triangles) of 0.8% xylose. β-Galactosidase activities were determined at the indicated intervals. Each datum point is the mean of three separate experiments with a mean error of <5%.

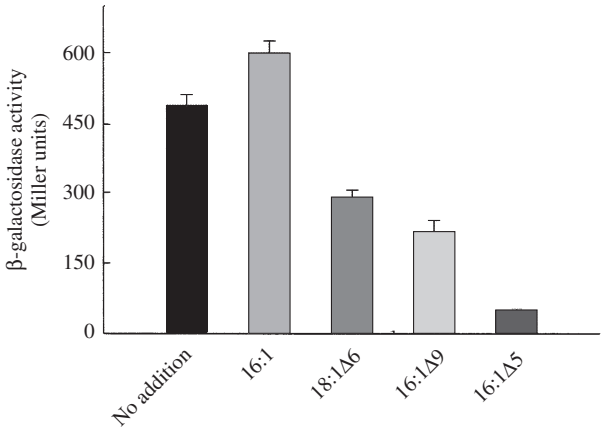


**Fig. 5.** UFAs inhibit *des* transcription at 37°C. *B. subtilis* strain AKP4 (*des*⁻), harbouring a *des–lacZ* fusion located in the *amyE* locus, was grown at 37°C to an O.D of 0.3 in SMM and then treated with 5 μM of the indicated fatty acids. After 4 h, the cells were harvested, and β-galactosidase activities were determined. The results shown are the average of three independent experiments.

galactosidase activity of this strain was repressed by UFAs at 37°C, although saturated fatty acids did not repress the activity of the reporter (Fig. 5). Thus, the regulatory loop composed of the DesK–DesR two-component signal transduction system and UFAs also operates at 37°C in the absence of an isoleucine supply.

### *Changes in the fatty acid profile of* B. subtilis *in response to exogenous branched-chain amino acids*

To verify our model that isoleucine produces a change in the fatty acid branching pattern, we performed fatty acid analyses of cells grown at 37°C in SMM supplemented with different branched-chain amino acids. The data presented in Fig. 6 show that the ratio of anteiso- to iso-branched-chain fatty acids is 4.0 in cells grown in the presence of isoleucine. This ratio decreases to 0.88, 0.45 and 0.61 in untreated cells or cells grown in the presence of either leucine or valine respectively. The major changes observed in lipids from cells grown in the presence of isoleucine were the decrease in iso-C15:0 and iso-C17:0 and the concomitant increase in the respective anteiso-branched forms (Fig. 6). These last results agree with those of Klein *et al*. (1999) and highlight the importance of an exogenous source of isoleucine for the increase in low-melting-point anteiso-branched fatty acids in *B. subtilis* membranes.

To study whether *B. subtilis* cells containing membranes enriched with anteiso-branched fatty acids are able to repress the activity of the *des* promoter in the absence of isoleucine in the growth medium, strain AKP3 was grown at 37°C in SMM supplemented with isoleucine to an OD of 0.3. Cells were then washed free of isoleucine by filtration, resuspended in SMM, and the β-galactosidase activity was assayed at different time intervals. Under these growth conditions, *des* induction did not occur (data not shown), whereas parallel control experiments with cells grown in SMM and then washed and resuspended in an isoleucine-free SMM medium expressed the *des–lacZ* fusion normally (data not shown). This experiment demonstrates that the continuous presence of isoleucine in the growth medium is not necessary to repress transcription of the *des–lacZ* fusion at 37°C. In addition, these data

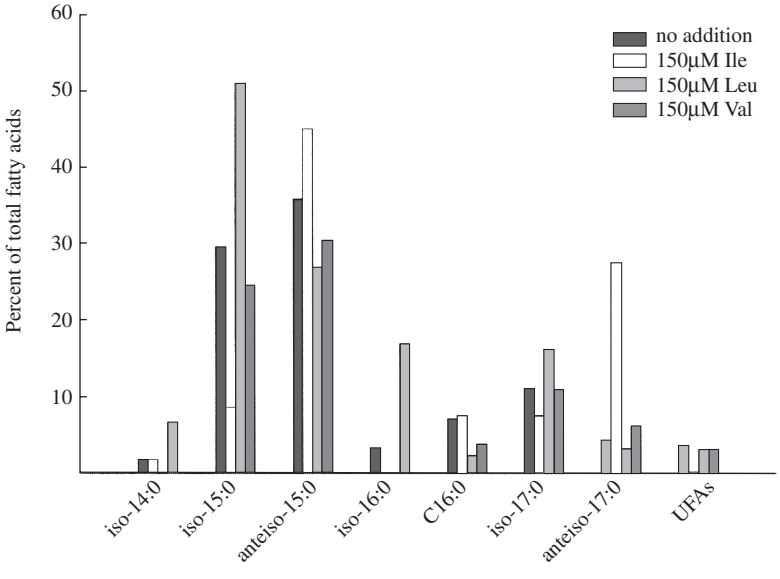


**Fig. 6.** Fatty acids profiles of *B. subtilis* grown at 37°C supplemented with different amino acids. Strain JH642 was grown at 37°C to an OD of 1.0 in SMM or in SMM supplemented with 150 μM isoleucine, leucine or valine. Cells were harvested, and the methyl ester–fatty acid derivatives were analysed in a TurboMass Perkin-Elmer gas chromatographer-mass spectrophotometer, as described in *Experimental pro*cedures.

reinforce the hypothesis that *des* transcription shut-off by isoleucine at 37°C is caused by an increase in membrane fluidity provoked by an isoleucine-dependent switch from iso- to anteiso-branched fatty acids.

## Discussion

Transcription of the *des* gene coding for the *B. subtilis* desaturase is tightly regulated by temperature. Although the *des* transcript is barely detected in cells growing in rich medium at 37°C, production of *des* mRNA is dramatically induced upon temperature downshift (Aguilar *et al.*, 1998; 1999). However, we demonstrate here, by means of operon transcriptional fusions as well as Northern blot analysis, that *des* expression takes place at 37°C in cultures grown in minimal medium. Our results also show that transcription of *des* is specifically repressed by isoleucine, whereas other branched amino acids such as leucine and valine do not significantly affect *des* transcription. Therefore, in addition to growth temperature, transcription of *des* is controlled by the availability of isoleucine in the growth medium. Our results agree with those of Weber *et al*. (2001), who were able to detect significant amounts of UFAs in membranes of *B. subtilis* grown at 37°C in an isoleucine-free medium.

### *How could the transcription of* des *be regulated by isoleucine at 37°C?*

Previous work has revealed that a two-component system composed of a membrane-bound kinase, DesK, and a soluble response regulator, DesR, controls *des* transcription (Aguilar *et al.*, 2001). We proposed that DesK is a bifunctional enzyme with both kinase and phosphatase activities that could assume different signalling states under varying growth temperatures (Aguilar *et al.*, 2001). 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 within the membrane that is sufficient to alter its activity significantly. 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 (de Mendoza and Cronan, 1983; Cronan and Rock, 1996; Vigh *et al.*, 1998). This change from a fluid (disordered) to a non-fluid (ordered) state might cause activation of the autokinase activity, resulting in autophosphorylation of a conserved histidine (His-188) contained in the transmitter domain of DesK. The phosphoryl group of His-188 could be transferred directly to DesR, thus activating transcription of *des*. Our data reveal a refinement of the function of the DesK–DesR system, namely to sense a change in membrane lipid fluidity at a constant temperature and to transmit this stimulus to the *des* gene. The following lines of evidence lead us to propose that DesK is necessary to sense changes in membrane fluidity and to control *des* expression at 37°C: (i) the DesK histidine kinase is essential for *des* transcription at 37°C; (ii) in the presence of sufficient exogenous isoleucine, the transcription of *des* is repressed; and (iii) when DesR is overexpressed in the absence of DesK, isoleucine is unable to repress *des* transcription. Strong support for this model is provided by experiments showing that (i) supplementation of JH642 with the isoleucine derivative 2-methyl-butyrate regulates *des* transcription in the same way that isoleucine does (Fig. 3); and (ii) the ratio of anteiso- to iso-branched-chain fatty acids is greatly increased at 37°C by the addition of isoleucine (Fig. 6). This shift in the fatty acid branching pattern should result in a reduction in the phase transition temperature of membrane lipids, based on the significantly larger cross-sectional area occupied by the anteiso-fatty acids compared with that occupied by the respective iso-species. Thus, anteiso-fatty acids disrupt the close packing of phospholipid acyl chains and provide a greater flexibility to the membrane favouring a phosphatase-dominant state of DesK. In the absence of isoleucine, the proportion of ordered iso-fatty acids into membrane lipids is increased (Fig. 6), favouring the phosphorylation of DesR by DesK and resulting in transcriptional activation of *des* at 37°C. Activation of *des* results in synthesis of Des, which in turn desaturates the acyl chain of membrane lipids. These newly synthesized UFAs decrease the phase transition temperature of the phospholipids and inhibit the transcriptional activity of the *des* promoter, presumably by decreasing the kinase activity of DesK. A model illustrating our current view of the control of *des* transcription by a transmembrane signal transduction pathway is shown in Fig. 7.

Recently, it has been reported that a *des* deletion mutant of *B. subtilis* underlies a dramatic reduction in viability during cold shock in the absence of exogenous isoleucine sources (Weber *et al.*, 2001). These results demonstrated that, during cold shock adaptation, *des* expression has a crucial role in maintaining an appropriate membrane fluidity. The most straightforward interpretation of the experiments shown here and those reported by Weber *et al*. (2001) is that a functional *B. subtilis* membrane requires that the composition of the membrane phospholipids be within the limits of the phase transition. Many experiments performed with *Escherichia coli* UFA

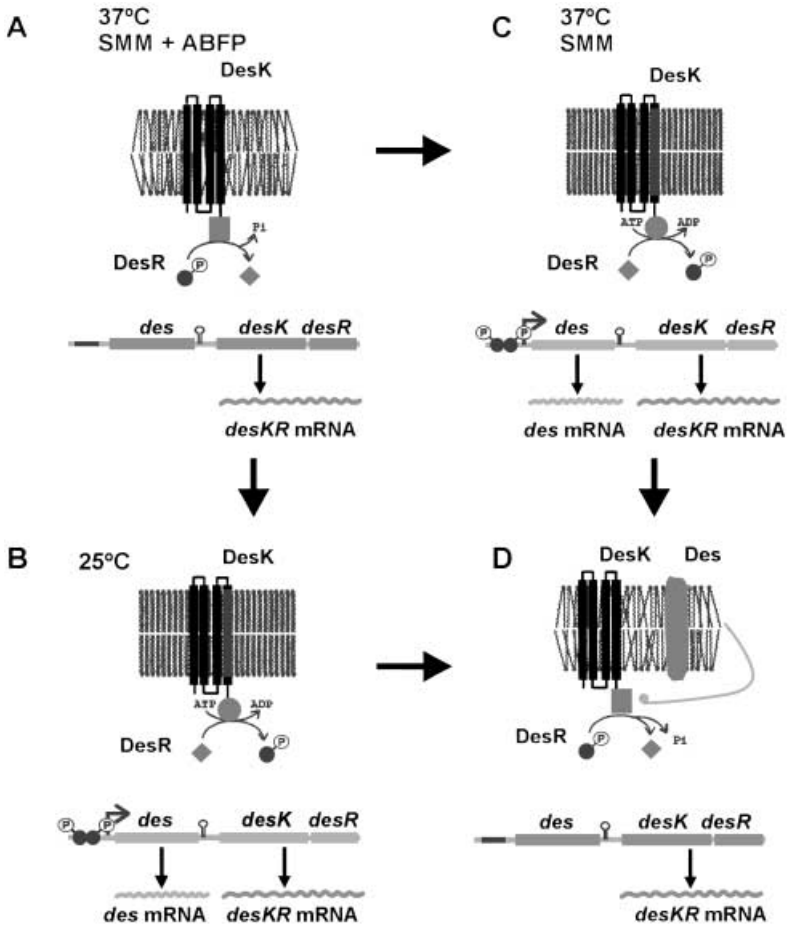


**Fig. 7.** Signal transduction pathway leading to membrane fluidity optimization in *B. subtilis*. It is proposed that DesK could assume different signalling states in response to changes in membrane fluidity (Aguilar *et al*., 2001). This is accomplished by regulating the ratio of kinase to phosphatase activity, such that a phosphatase-dominant state is present at 37°C when cells are growing in media containing anteiso-branched-chain fatty acid precursors (ABFP) and membrane lipids are disordered (A). A kinase-dominant state of DesK predominates upon an increase in the proportion of ordered membrane lipids (B and C). This could be accomplished either after a sudden drop in temperature (B), which results in a drastic reduction in membrane fluidity regardless of the availability of ABFP, or at 37°C (C), when cells are growing in a medium devoid of ABFP. DesK-mediated phosphorylation of DesR results in transcriptional activation of *des* (B) and (C). Activation of *des* results in synthesis of Des, which desaturates the acyl chains of membrane phospholipids (D). These newly synthesized UFAs inhibit *des* transcription by favouring the dephosphorylation of DesR (D) (see text for details).

auxotrophs have demonstrated that, if all the phospholipids are in either the ordered state or the disordered state, the membrane is not functional (for a review, see Cronan and Rock, 1987). Moreover, quite wide variations in fluidity are tolerated; that is, the cells do not have to maintain a precise ratio of fluid to non-fluid lipids to have functional membranes (Cronan and Rock, 1987). However, there does seem to be an optimal fluidity, at which cell growth is more advantageous. Thus, the Des pathway seems to be designed to optimize the fluidity within the tolerated range, rather than to extend the range.

## Experimental procedures

### *Bacterial strains and growth conditions*

Bacterial strains used in this study are listed in Table 1. *B. subtilis* was propagated at 37°C, with 250 r.p.m. gyration, in Spizizen salts (Spizizen, 1958) supplemented with 0.2% glucose, 50 μg ml$^{-1}$ each tryptophan and phenylalanine, and trace elements (Harwood and Cutting, 1990). This medium was designated SMM. Amino acids were added at a final concentration of 150 μM or as indicated in each experiment. Antibiotics were added to media at the following concentrations: chloramphenicol, 5 μg ml$^{-1}$; kanamycin, 5 μg ml$^{-1}$; erythromycin, 1 μg ml$^{-1}$; lincomycin, 12.5 μg ml$^{-1}$. Fatty acids and α-keto acids were purchased from Sigma. 16:1Δ5 was prepared as described earlier (Aguilar *et al*., 2001).

### *Plasmids and strains construction*

To complement *desKR* and *desR* mutant strains, plasmids pAD1 and pCM1 were constructed. To this end, plasmid pAG47 (Aguilar *et al*., 2001) was digested with *Bam*HI, and a 3.2 kb DNA fragment, containing the *desKR* operon under the *PXyl* promoter, was cloned into the replicative vector pHPKS (Johansson and Hederstedt, 1999), yielding plasmid pAD1. To construct plasmid pCM1, pAG52 (Aguilar *et al*., 2001) was digested with *Bam*HI and *Eco*RI to isolate a 1.97 kb fragment containing *desR* under the *PXyl* promoter. This fragment was cloned in pHPKS (Johansson and Hederstedt, 1999), yielding pCM1.

A 0.4 kb *Eco*RI–*Bam*HI DNA fragment containing the *desKR* promoter was cloned into the integrational vector pJM116 (Dartois *et al*., 1998), generating plasmid pAE3. 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 AE3 (A. Erazo, personal communication).

### *β-Galactosidase assays*

*Bacillus subtilis* strains harbouring *Pdes–lacZ* and *PdesKR–lacZ* chromosomal fusions were grown overnight at 37°C in SMM. Cells were collected by centrifugation and diluted in SMM supplemented with the appropriate amino acids or their derivatives. Samples were taken at defined time intervals after resuspension and assayed for β-galactosidase activity, which was expressed in Miller units (MU), as described previously (Aguilar *et al*., 1998). The results shown are the average of three independent experiments.

### *RNA analysis*

*Bacillus subtilis* JH642 was grown overnight in SMM at 37°C. Cells were collected and diluted in SMM or SMM supplemented with 150 μM isoleucine, and the RNA was isolated as described previously (Aguilar *et al*., 2001). Northern blot analysis was performed with 1.2% formaldehyde–agarose gels. The *des* probe was synthesized using Promega Prime-a-Gene labelling kit on a PCR *des* fragment as a template. Oligonucleotides used were: desBAM1 (5′-CATTAGGATC CACTGAACCGAGACAGA-3′) and desECO2207 (5′-GCCA GAATTCACCCTCCAACAATAAAA-3′). The size of the transcript was determined by comparison with RNA molecular weight standards (Promega).

### *Fatty acids analysis*

For measurement of fatty acid synthesis, JH642 cells were grown overnight in SMM at 37°C. Cells were collected and diluted in SMM or SMM supplemented with 150 μM isoleucine, leucine or valine. Fatty acids were labelled with 10 μCi of [1-$^{14}$C]-acetate. 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 (Aguilar *et al*., 1998).

To analyse the fatty acid profile, *B. subtilis* cells were grown at 37°C in SMM or SMM supplemented with 150 μM isoleucine, leucine or valine to an $OD_{600}$ of 1. Lipids were extracted and fatty acids converted to their methyl esters with sodium methoxide (Aguilar *et al*., 1998). The methyl esters were run in a Perkin-Elmer Turbo mass gas chromatographer-mass spectrometer, equipped with a PEG column, and Perkin-Elmer software. Each fatty acid was identified by comparing its mass spectrum with those obtained from methyl esters of Sigma fatty acid standards.

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## References

Aguilar, P.S., Cronan, J.E., Jr, and de Mendoza, D. (1998) A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase. *J Bacteriol* **180:** 2194–2200.

Aguilar, P.S., López, P., and de Mendoza, D. (1999) Transcriptional control of the low-temperature-inducible *des* gene, encoding the delta 5 desaturase of *Bacillus subtilis*. *J Bacteriol* **181:** 7028–7033.

Aguilar, P.S., Hernandez-Arriaga, A.M., Cybulski, L.E., Erazo, A.C., and de Mendoza, D. (2001) Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis*. *EMBO J* **20:** 1681–1691.

Choi, K.H., Heath, R.J., and Rock, C.O. (2001) Beta-ketoacyl-acyl carrier protein synthase III (FabH) is a determining factor in branched-chain fatty acid biosynthesis. *J Bacteriol* **182:** 365–370.

Cronan, J.E., Jr, and Rock, C.O. (1987) Biosynthesis of membrane lipids. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*. Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., Umbarger, H.E. (eds). Washington, DC: American Society for Microbiology Press, pp. 474–497.

Cronan, J.E., Jr, and Rock, C.O. (1996) Biosynthesis of membrane lipids. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*. Neidhardt, F.C., Curtiss, R., III, Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., *et al.* (eds). Washington, DC: American Society for Microbiology Press, pp. 612–636.

Dartois, V., Dérbarbouillé, F., Kunst, F., and Rapaport, G. (1998) Characterization of a novel member of the DegS–DegU regulon affected by salt stress in *Bacillus subtilis*. *J Bacteriol* **180:** 1855–1861.

de Mendoza, D., and Cronan, J.E., Jr (1983) Thermal regulation of membrane lipid fluidity in bacteria. *Trends Biochem Sci* **8:** 49–52.

de Mendoza, D., Schujman, G.S., and Aguilar, P.S. (2001) Biosynthesis and function of membrane lipids. In *Bacillus subtilis and its Relatives: from Genes to Cells.* Sonenshein, A.L., Hoch, J.A., and Losick, R. (eds). Washington, DC: American Society for Microbiology Press, pp. 43–55.

Harwood, C.R., and Cutting, S.M. (1990) *Molecular Biological Methods for Bacillus*. Chichester: John Wiley & Sons.

Johansson, P., and Hederstedt, L. (1999) Organization of genes for tetrapyrrole biosynthesis in Gram-positive bacteria. *Microbiology* **145:** 529–538.

Kaneda, T. (1977) Fatty acids of the genus *Bacillus*: an example of branched-chain preference. *Bacteriol Rev* **41:** 391–418.

Kaneda, T. (1991) Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol Rev* **55:** 288–302.

Klein, W., Weber, M.H., and Marahiel, M.A. (1999) Cold shock response of *Bacillus subtilis*: isoleucine-dependent switch in the fatty acid branching pattern for membrane adaptation to low temperatures. *J Bacteriol* **181:** 5341–5349.

Spizizen, J. (1958) Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc Natl Acad Sci USA* **44:** 1072–1078.

Suutari, M., and Laasko, S. (1994) Microbial fatty acids and thermal adaptation. *Crit Rev Microbiol* **20:** 285–328.

Vigh, L., Maresca, B., and Harwood, J.L. (1998) Does the membrane's physical state control the expression of heat shock and other genes? *Trends Biochem Sci* **23:** 369–374.

Weber, M.H.W., Klein, W., Muller, L., Niess, U.M., and Marahiel, M. (2001) Role of the *Bacillus subtilis* fatty acid desaturase in membrane adaptation during cold shock. *Mol Microbiol* **39:** 1321–1329.