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 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.

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; Suttari 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 rise to the primers for iso-branched fatty acids (Kaneda, 1977; 1991). 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 this 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.

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JH642</td>
<td>trpC2 pheA1</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>AKP3</td>
<td>JH642 amyE::Pdes–lacZ</td>
<td>Aguilar et al. (2001)</td>
</tr>
<tr>
<td>AKP4</td>
<td>AKP3 des::Km'</td>
<td>Aguilar et al. (2001)</td>
</tr>
<tr>
<td>AKP9</td>
<td>AKP3 des::R::Km'</td>
<td>Aguilar et al. (2001)</td>
</tr>
<tr>
<td>AKP20</td>
<td>AKP3 desK::Km' PKm::desR</td>
<td>Aguilar et al. (2001)</td>
</tr>
<tr>
<td>AKP2047</td>
<td>AKP20 thrC::(PXYl–desKR)</td>
<td>Aguilar et al. (2001)</td>
</tr>
<tr>
<td>AKP21</td>
<td>AKP3 desKR::Km'</td>
<td>Aguilar et al. (2001)</td>
</tr>
<tr>
<td>AKL59</td>
<td>JH642 amyE::(Pdes)−lacZ</td>
<td>Aguilar et al. (2001)</td>
</tr>
<tr>
<td>AKL62</td>
<td>JH642 amyE::(Pdes)−lacZ</td>
<td>Aguilar et al. (2001)</td>
</tr>
<tr>
<td>AE3</td>
<td>JH642 amyE::(PdesKR)−lacZ</td>
<td>A. Erazo (personal communication)</td>
</tr>
<tr>
<td>Plasmids</td>
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<tr>
<td>pAD1</td>
<td>3.2 kb fragment from pAG47 containing PXYl–desKR cloned into pHPKS</td>
<td>This study</td>
</tr>
<tr>
<td>pCM1</td>
<td>1.97 kb fragment from pAG52 containing PXYl–desR cloned into pHPKS</td>
<td>This study</td>
</tr>
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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).

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
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 [14C]-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.
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 anteisoo 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-methylbutyrate 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<sup>R</sup>) 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<sup>+</sup> 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...
Table 2. The expression of des at 37°C is regulated by the DesK–DesR system.

<table>
<thead>
<tr>
<th>Strain</th>
<th>–Isoleucine</th>
<th>+Isoleucine</th>
<th>Repression</th>
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<tbody>
<tr>
<td>AKP9 (desR)</td>
<td>4.3 ±0.2</td>
<td>3.6 ±0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>AKP21 (desK–desR)</td>
<td>3.3 ±0.1</td>
<td>3.4 ±0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>AKP9/pAD1 (PXyl-desKR)</td>
<td>51.0 ±3.2</td>
<td>8.9 ±0.6</td>
<td>5.1</td>
</tr>
<tr>
<td>AKP21/pAD1 (PXyl-desKR)</td>
<td>34.8 ±2.2</td>
<td>8.2 ±0.4</td>
<td>4.2</td>
</tr>
</tbody>
</table>

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.

Isoleucine affects the signalling state of DesK

To test whether des expression is downregulated by isoleucine as a result of shut-off of the translation 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 over-produces 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).

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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%.
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...
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
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. 16:1 ∆5 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 BamHI and a 3.2 kb DNA fragment, containing the desKR operon under the PxyI 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 BamHI and EcoRI to isolate a 1.97 kb fragment containing desR under the PxyI promoter. This fragment was cloned in pHPKS (Johansson and Hederstedt, 1999), yielding pCM1.

A 0.4 kb EcoRI–BamHI 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 Scal 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 CACTGAAACCGAGACAGA-3′) and desECO2207 (5′-GCCA GAATTCCACCCTCAACATAAAA-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 [1-14C]-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 OD600 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 chromatograph–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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