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# Regulation of the synthesis of unsaturated fatty acids by growth temperature in *Bacillus subtilis*

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## Summary

***Bacillus subtilis* synthesizes, almost exclusively, saturated fatty acids, when grown at 37°C. When cultures were transferred from 37°C to 20°C, a chloramphenicol- and rifampicin-sensitive synthesis of a C-16 unsaturated fatty acid was observed. Synthesis of this compound reached a plateau after 5 h at 20°C, reaching levels of 20% of the total fatty acid content. [$^{14}$C]-labelled fatty acids attached as thioesters to acyl-carriers compounds, such as coenzyme A (CoA) or acyl-carrier protein (ACP) synthesized *de novo* by glycerol-requiring auxotrophs deprived of glycerol to arrest phospholipid synthesis, could not be desaturated at 20°C. Desaturation of these fatty acids was readily observed when glycerol was restored to the cultures allowing resumption of transfer of acyl-moieties from acyl-thioesters to phospholipid. It was also observed that depletion of the pools of CoA and ACP by starvation of pantothenate auxotrophs had no effect on the observed synthesis of unsaturated fatty acid at 20°C. The overall results indicate that synthesis of unsaturated fatty acids in *B. subtilis* is a cold-inducible process and that phospholipids are obligate intermediates in this fatty acid desaturation pathway.**

## Introduction

Bacteria, in common with most (if not all) other organisms, synthesize phospholipids with a greater proportion of unsaturated fatty acids (UFAs) when grown at low, rather than high, temperatures (de Mendoza and Cronan, 1983; de Mendoza and Farias; 1988; Russell, 1984). This regulatory mechanism system, called thermal control of fatty acid synthesis, is thought to be designed to ameliorate the effects of temperature change on the physical state of the membrane phospholipids (de Mendoza and Cronan, 1983; de Mendoza and Farias, 1988; Russell, 1984). It is well documented that the proportion of fluid (disordered) lipid to no fluid (ordered) lipid in cell membranes plays a major role in membrane function (Cronan and Rock, 1987; de Mendoza and Cronan, 1983; de Mendoza and Farias, 1988). Increased incorporation of UFAs decreases the melting temperature of the membrane phospholipids, whereas increased incorporation of saturated fatty acid has the opposite effect (Cronan and Rock, 1987; de Mendoza and Cronan, 1983). The thermal regulatory system can thus adapt the membrane lipid for optimal functioning at new growth temperatures.

The mechanism used to alter the unsaturation of bacterial membrane lipids in response to growth temperature depends on the mechanism of UFA synthesis. In bacteria, both anaerobic and aerobic mechanisms are responsible for the synthesis of UFAs (for reviews see de Mendoza and Farias, 1988; de Mendoza *et al.*, 1992). The anaerobic pathway, elucidated in detail using *E. coli*, produces *cis*-UFA by a specific 2,3-dehydrase acting at the C-10 level (for a review see Bloch, 1970). In certain bacteria (usually Gram-positive), and in eukaryotes, the introduction of double bonds into the fatty acids employs a common general mechanism. The reaction is catalysed by an oxygen-dependent desaturation system requiring the participation of a specific electron transport chain (de Mendoza and Farias, 1988; de Mendoza *et al.*, 1992).

The molecular mechanism of thermal control of UFA biosynthesis has been extensively studied in *E. coli* (for a review see de Mendoza and Cronan, 1983). In this organism, the UFA synthesized in greater quantity at low temperature is *cis*-vaccenic acid (de Mendoza *et al.*, 1983). This regulatory response does not require protein synthesis (Cronan and Rock, 1987; de Mendoza and Cronan, 1983) and functions by thermal modulation of the activity of the soluble enzyme 3-ketoacyl-acyl-carrier-protein-synthase II that converts palmitoleic acid to *cis*-vaccenic acid (de Mendoza *et al.*, 1983).

A different regulatory mechanism of UFA synthesis has been reported in a large number of *Bacillus* species that synthesize UFAs by the oxygen-dependent desaturation mechanism (Fulco, 1983). In these bacteria a desaturating system is induced at low growth temperatures (for reviews see Fulco, 1983; de Mendoza *et al.*, 1992). Thus, the regulatory system of bacilli seems to involve an 'on or

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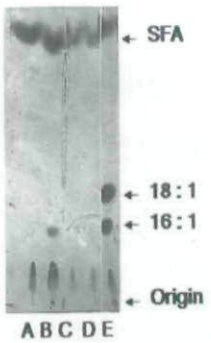


**Fig. 1.** Autoradiographic pattern of synthesis of fatty acids in *B. subtilis* at different temperatures. One-millilitre aliquots from cultures of strain 168 grown to exponential phase at 37°C were shifted to 20°C (lanes B, C and D) for 1 min and then exposed to 10 μCi of [$^{14}$C]-acetate. After 4 h of incubation at 20°C, lipids were extracted, fatty-acid moieties converted to methyl esters and chromatographed in the argentation system described in the *Experimental procedures*. The radioactive compounds were located by autoradiography and then quantified by scintillation counting. About 20 000 c.p.m. of radioactivity was loaded in all lanes.
A. Culture grown at 37°C and labelled with 10 μCi of [$^{14}$C]-acetate at this temperature. The radioactivity detected in the fraction containing saturated fatty acids (SFAs) was 15 000 c.p.m.
B. Culture shifted from 37°C to 20°C. The radioactivity detected in the fraction of SFAs and UFAs was 13 000 and 2500 c.p.m., respectively.
C. Culture shifted from 37°C to 20°C with chloramphenicol (100 μg ml$^{-1}$) added 5 min before the temperature shift.
D. Culture shifted from 37°C to 20°C with rifampicin (100 μg ml$^{-1}$ added as in C).
E. Radioactive fatty acids synthesized by *E. coli* C600 at 30°C. The UFAs synthesized by this strain are: palmitoleic acid (*cis*-$\Delta^9$-hexadecenoic) and *cis*-vaccenic acid (*cis*-$\Delta^{11}$-octadecenoic) (de Mendoza *et al.*, 1982). The final positions of UFAs and SFAs are indicated in the right margin.

off' transcriptional regulatory system rather than modulation of the activity of a lipid biosynthetic enzyme. To further define the molecular mechanism(s) of cold-induction of UFA biosynthesis and the mechanism(s) for sensing temperature changes in bacilli it is essential to characterize the pathway(s) of desaturation of fatty acids in these bacteria. In this context, Guffanti *et al.* (1987) have recently shown that mutants of *Bacillus subtilis* selected for resistance to the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) have decreased contents of mono-unsaturated fatty acids (*c.* 50% of the normal levels). Dunkley *et al.* (1991) found that these mutants lack an NADPH-dependent palmitoyl-CoA desaturase that requires both membrane and cytosolic components. Although the CCCP-resistant mutants completely lack palmitoyl-CoA desaturase activity, the UFA content is decreased by only one-half (Dunkley *et al.*, 1991). It was concluded from these data that *B. subtilis* must contain an alternate synthetic pathway accounting for the remaining UFA content (Dunkley *et al.*, 1991). In this report, we have used *B. subtilis* auxotrophs that allow arrest either at phospholipid or fatty acid synthesis to examine the cold-induced synthesis of UFAs. Our results provide strong evidence to show that a *B. subtilis* pathway of UFA synthesis involves direct desaturation of the fatty-acyl groups of membrane phospholipids

## Results

### In vivo *characterization of the synthesis of UFAs in* B. subtilis.

Since the characteristics of the fatty acid desaturation system in *B. subtilis* have not been reported, we have studied some of its properties. We first analysed the synthesis of radioactive fatty acids by growing *B. subtilis* (strain 168) in the presence of [$^{14}$C]-acetate at 37°C. As shown in Fig. 1 (lane A), only saturated fatty acids are found to be synthesized under these conditions. However, when a culture growing at 37°C was transferred to 20°C and pulsed with [$^{14}$C]-acetate, synthesis of an UFA which closely comigrated with palmitoleic acid (lane B) was observed. The desaturation of fatty acids at 20°C required *de novo* synthesis of RNA and proteins since it was abolished by rifampicin or chloramphenicol added before the temperature shift (Fig. 1, lanes C and D). Addition of chloramphenicol 30 min after the temperature shift did not affect the observed desaturation (data not shown). These results indicate that the dramatic increase in the synthesis of UFAs in cells shifted from 37°C to 20°C is a cold-inducible process.

The proportion of UFAs synthesized by *B. subtilis* after transferring the cultures from 37°C to 20°C increased for about 5 h before reaching a plateau (Fig. 2). It is interesting to note that the exponential growth of *B. subtilis* at 20°C (after shift from 37°C) was not preceded by a lag phase and that the maximum level of UFA synthesis was reached about 1 h before a growth division cycle was completed (Fig. 2). The proportion of UFAs initially found in cultures transferred from 37°C to 20°C far exceeded the levels of synthesis found in cultures growing for several generations at 20°C (Fig. 3). In addition, the extent of induction of the synthesis of UFAs was lower after a shift from 37°C to 30°C than from 37°C to 20°C (Fig. 3).

### *Cold-induced desaturation of phospholipids synthesized at 37°C*

To determine if [$^{14}$C]-labelled phospholipids synthesized by *B. subtilis* at 37°C are desaturated after a temperature shift to 20°C, cultures of strain 168 were labelled with [$^{14}$C]-acetate at 37°C. The labelled cells were washed, resuspended in media without labelled acetate, grown at 20°C for 3 h and UFA contents were then determined. We found that about 20% of the labelled fatty acids were UFAs (Table 1). These results suggest that membrane phospholipids may act as substrates for the desaturase since the pools of labelled fatty-acyl thioester intermediates should be small. The possibility that an acyl-thioester

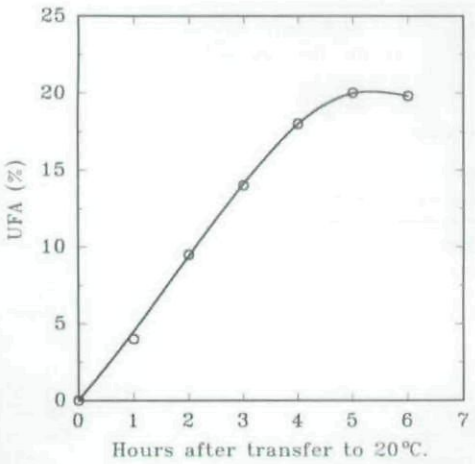


**Fig. 2.** Time course of UFA synthesis by *B. subtilis* after shift from 37°C to 20°C. Cells were grown to exponential phase at 37°C and then 1 ml samples were shifted to 20°C and exposed to 10 μCi of [$^{14}$C]-acetate during the times indicated in the Figure. After these incubation periods, the lipids were extracted and the fatty-acid methyl esters prepared as described in the legend of Fig. 1. The resulting methyl esters (about 20 000 c.p.m. per sample) were separated into unsaturated and saturated fractions and quantified as described in the *Experimental procedures*. Results are expressed as the percentage of the total methyl esters recovered. Data are means of three experiments with the average error in percentage values of UFAs being less than 5%.

could also be a substrate, however, could not be ruled out.

### *UFA biosynthesis in the absence of phospholipid synthesis*

To test directly if a fatty-acyl thioester(s) can be desaturated *in vivo* by the *B. subtilis* desaturase, we uncoupled fatty acid synthesis from phospholipid synthesis by a specific inhibition of the latter process. It has been shown previously that glycerol starvation of glycerol-auxotrophs (*glyc*) of *B. subtilis* results in a profound inhibition of phospholipid synthesis, although the synthesis of fatty acids continues at a normal rate (Mindich, 1972). Because glycerol starvation of *glyc* mutants blocks the synthesis of the acyl acceptor, fatty acids cannot be transferred to phospholipids and, consequently, accumulate as free species (Mindich, 1972). This manipulation allows specific labelling of the pools of thioester intermediates. To determine if fatty-acyl moieties not bound to phospholipids could be desaturated at 20°C, cultures of strain B42 were grown at 37°C to exponential phase, starved for glycerol, and shifted to 20°C to induce desaturation. At this time the cultures were exposed to [$^{14}$C]-acetate, and incubation was continued for 3 h at 20°C before analysis of the lipid fraction. As shown in Fig. 4 (lane A), about 97% of the radioactivity found in these conditions was in the free fatty acid (FFA) fraction. The analysis of this fraction by silver-nitrate-impregnated plates showed that the FFAs accumulating at 20°C did not contain UFAs (Fig. 4, lane C). In order to distinguish whether the acyl-thioesters synthesized in the absence of glycerol are not substrates of the desaturase or constitute a pool that is not metabolically active, we starved B42 for glycerol at 37°C in the presence of [$^{14}$C]-acetate until labelled FFAs accumulated. We then restored glycerol to the culture and simultaneously removed the [$^{14}$C]-acetate. Following glycerol restoration at 37°C, the label in the FFA fraction decreased with an equivalent increase in the label found in glycerolipids (Fig. 4, lane B). These labelled cultures were then shifted to 20°C for 2 h, the phospholipids extracted, purified by thin-layer chromatography, converted to methyl esters and analysed by argentation chromatography. As shown in Fig. 4 (lane D), the acyl chains synthesized in the absence of glycerol were incorporated into phospholipids and readily desaturated at 20°C. These results demonstrate that the FFAs accumulating in the absence of glycerol are metabolically active, and following re-acylation into phospholipids become substrates of the desaturase. Accordingly, these findings suggest that the desaturase cannot desaturate fatty acids not bound to phospholipids. The possibility that an inhibition of phospholipid synthesis may be responsible for the absence of desaturation of acyl-thioesters synthesized at 20°C in glycerol-deprived cells (e.g. blocking induction of the desaturation system) was ruled out as follows. Lipids of glycerol-supplemented cultures of strain B42 were labelled with [$^{14}$C]-acetate at 37°C. These cells were subsequently starved for glycerol, chased with cold acetate,

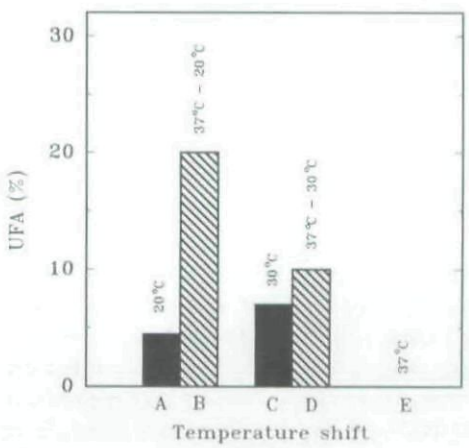


**Fig. 3.** UFA synthesis in *B. subtilis* growing at a constant temperature and after temperature shifts. In all cases the lipids of strain 168 growing exponentially were labelled with 10 μCi of [$^{14}$C]-acetate for a time equivalent to one generation at the indicated temperature. The percentage of UFAs was determined as described in the legend to Fig. 2. Data are means of three determinations with the average error in percentage values of UFAs being less than 5%. The temperature shifts from 37°C to 20°C (B) or 30°C (D), respectively, were performed as described in Fig. 1. (A), (C) and (E) show results obtained with cultures growing at 20, 30 and 37°C, respectively.

**Table 1.** Desaturation of pre-labelled phospholipids by strains 168[a], B42[b], and UR1[c].

| Strain | Glycerol in culture | Pantoate in culture | Radioactivity in SFA[d] | Radioactivity in UFA[d] | Total radioactivity[d] |
|---|---|---|---|---|---|
| 168 | – | – | 9315 | 2185 | 11 500 |
| B42 | Absent | – | 4106 | 919 | 5025 |
| B42 | Present | – | 2759 | 593 | 3352 |
| UR1 | – | Absent | 7464 | 653 | 8117 |
| UR1 | – | Present | 4581 | 293 | 4874 |

**a.** A 1 ml aliquot from a culture of strain 168 grown to exponential phase at 37°C was incubated with 10 μCi of [$^{14}$C]-acetate for 1 h. The culture was then washed and transferred to growth medium containing 0.03 M of non-labelled acetate and shifted to 20°C for 3 h. The phospholipids were extracted and the fatty-acid moieties converted to methyl esters and chromatographed in the argentation system. The radioactive compounds were located by autoradiography and quantified by scintillation counting.

**b.** Strain B42 was grown at 37°C to exponential phase in glycerol-supplemented medium. Two samples (1 ml each) were incubated with 10 μCi of [$^{14}$C]-acetate for 1 h at 37°C. The cultures were then transferred to a medium lacking glycerol but containing non-labelled acetate. One sample was supplemented with glycerol, and the other remained unsupplemented. Both cultures were then shifted to 20°C and incubated for 3 h. The chromatography of labelled methyl esters was performed as described above.

**c.** Two samples (1 ml each) of a culture of strain UR1 grown to exponential phase at 37°C were incubated with 10 μCi of [$^{14}$C]-acetate for 1 h. The cultures were deprived of pantoate for 30 min and shifted to 20°C exactly as described for strain B42. The chromatography of labelled methyl esters was also performed as described above.

**d.** The radioactivity is expressed in counts min$^{-1}$. Data are means of three determinations with the average error in percentage values of UFAs being less than 5%.

and shifted to 20°C. The result of this experiment (Table 1) indicates that desaturation of prelabelled fatty acids from glycerol-starved or glycerol-supplemented cells is almost identical (in both cultures about 20% of UFAs were synthesized). This demonstrates that the desaturation system is induced and is active in the absence of phospholipid synthesis.

### *UFA biosynthesis in cells depleted of CoA*

The experiments performed with glycerol auxotrophs strongly suggest that fatty acids must be incorporated into phospholipids in order to be desaturated at 20°C. Accordingly, these results are compatible with lipid-linked desaturation in which the fatty acid remains esterified to the membrane lipid while being desaturated. Nevertheless, we could not firmly exclude the presence in *B. subtilis* of a pathway which involves deacylation of the membrane lipid, esterification of the fatty acid to a coenzyme, desaturation of the fatty-acyl thioester, and reacylation of the lipid with the resultant UFAs. In order to determine if this pathway is operative in *B. subtilis*, we used the *panE* strain UR1, a strain with an inactive ketopantoic acid reductase (Baigori *et al.*, 1991). UR1 cannot synthesize pantoate, the precursor of pantothenate. Pantothenate is utilized primarily for the biosynthesis of CoA and ACP (Rock and Jackowski, 1982). Strain UR1 is phenotypically recognized as a pantoate or pantothenate auxotroph and optimal growth is achieved at about 1 mM pantothenate (Baigori *et al.*, 1991). The generation times at 37°C of UR1 growing in media supplemented with pantothenate or pantoate are about 30 min and 90 min, respectively (R. Grau and D. de Mendoza, unpublished). When strain UR1 is deprived of pantoate growth stops almost immediately, presumably because of depletion of the CoA pool (see below and Jackowski and Rock, 1986). As shown in Fig. 5, pantoate-deprivation of UR1 results in a complete inhibition of the incorporation of β-alanine into CoA and ACP. This result indicates that pantoate starvation very efficiently blocks the synthesis of both acyl-carrier compounds. Moreover, the intracellular content of CoA and ACP dropped precipitously (about 96%), when compared with pantoate-supplemented cultures, in the first 15 min following the removal of pantoate (data not shown). As expected, pantoate-deprivation of strain UR1 results in 97% inhibition of the incorporation of [$^{14}$C]-acetate into phospholipids (data not shown). To determine if UFA synthesis takes place in the absence of acyl-carriers and fatty acid synthesis, cultures of UR1 were labelled with [$^{14}$C]-acetate at 37°C in the presence of pantoic acid. The labelled cells were then washed, chased with cold acetate, starved for pantoic acid and shifted to 20°C. Table 1 shows that the desaturation levels of pre-labelled phospholipids from pantoate-starved or pantoate-supplemented cells are almost identical. The data show that the cellular levels of acyl carriers can be reduced to <4% of the levels found in pantoate-supplemented cells without a significant decrease in desaturation. Thus, these results

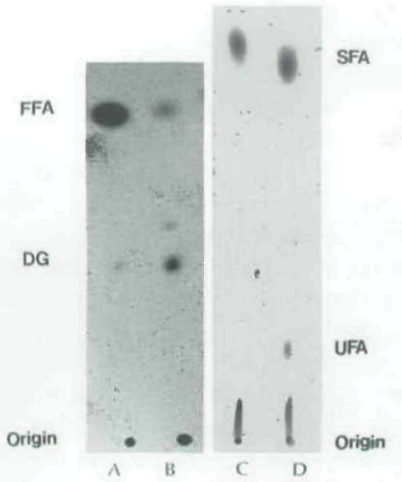


**Fig. 4.** Autoradiographic pattern of lipid synthesized by strain B42 growing in the presence or absence of glycerol. Strain B42 growing exponentially at 37°C in glycerol-supplemented medium was deprived of this substrate as described in the *Experimental procedures*. A 1 ml sample was transferred to 20°C and exposed to 10 μCi of [$^{14}$C]-acetate for 3 h at 20°C (lane A). A second sample from the same culture was maintained at 37°C in the presence of [$^{14}$C]-acetate for 30 min to allow synthesis of radioactive FFAs. This culture was then washed free of [$^{14}$C]-acetate and resuspended in a medium containing glycerol and non-radioactive acetate (0.03 M) and incubated for another 30 min at 37°C (lane B). After these incubation periods the radioactivity present in FFAs and glycerolipids of both cultures (lanes A and B) were quantified as described in the *Experimental procedures*. The sample in lane A contained 10 000 and 500 c.p.m. of radioactivity in the FFA and phospholipid fractions, respectively. The sample in lane B contained 5000, 4000 and 700 c.p.m. of radioactivity in phospholipids, diacylglycerol (DG) and FFA, respectively. Similar results were observed in several independent experiments. The FFA fraction shown in lane A was converted to methyl esters with diazomethane and chromatographed in the argentation system (lane C). A 2 ml sample of a culture treated as described in lane B was transferred to 20°C for 2 h. After incubation, the lipids were extracted and chromatographed as in lane B. The fraction corresponding to phospholipids was eluted from the silica gel and the fatty acids converted to methyl esters with sodium methoxide and resolved into SFAs and UFAs (lane D). In lane C 9000 c.p.m. was detected in the SFA fraction, whereas the sample of lane D contained 9000 and 1500 c.p.m. in the SFA and UFA fractions, respectively. Essentially the same results were observed in several independent experiments.

strongly suggest that the fatty-acyl moieties of the phospholipids can be desaturated without previously being acylated to a coenzyme such CoA or ACP.

## Discussion

Our understanding of the molecular basis of the temperature-induced control by which UFAs are synthesized by bacilli only at low growth temperatures is severely limited. Initial efforts to understand the molecular mechanism of induction of the desaturation system were hampered by the inability to use *Bacillus* species suitable for genetic manipulation. In this article we report experiments showing that *B. subtilis*, a bacterium widely used for genetic analysis, contains a fatty acid desaturation system that is induced by low temperatures (Fig. 1). We also found that the proportion of UFAs synthesized during the first growth division cycle at 20°C was much higher than those of cultures growing for several generations at 20°C (Fig. 3). These results agree with those of Fulco and coworkers (Fujii and Fulco, 1977; Fulco, 1983). They reported that the levels of desaturation of exogenously supplied palmitic acid in cultures of *Bacillus megaterium* transferred from 35°C to 20°C far exceeded the levels of desaturation of cultures growing at 20°C. To explain the dramatic change in lipid composition of bacilli shifted from 37°C to 20°C, Fujii and Fulco (1977) proposed that transcription of the fatty acid desaturase gene occurs only at low temperatures. To account for the unexpectedly large

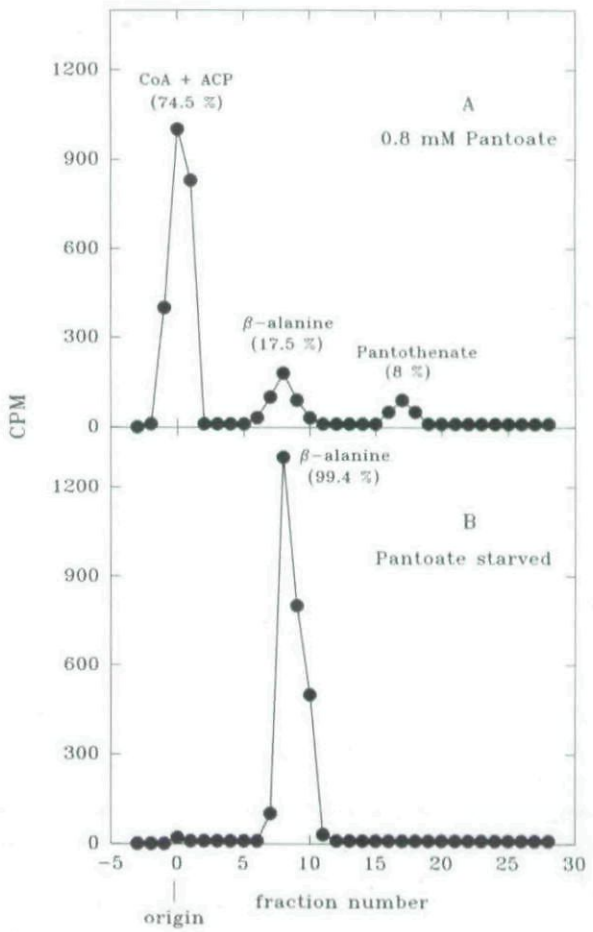


**Fig. 5.** Blocking of the synthesis of β-alanine-derived intracellular metabolites in strain UR1 after starvation for pantoate. Strain UR1 (*panE*) was grown on 4 ml of media supplemented with 0.8 mM pantoate to a density of $1 \times 10^8$ cells ml$^{-1}$. At this point the cells were deprived of pantoate as described in the *Experimental procedures*. The cells were then resuspended in 4 ml of the same media without pantoate. Half of this culture was supplemented with 0.8 mM pantoate, while the other half remained unsupplemented. Both cultures were then labelled with 1 μCi of β-[3-$^3$H]-alanine at 37°C for 3 h. At the end of the experiment, cells were harvested, washed and lysed. Samples were chromatographed on silica gel H layers as described in the *Experimental procedures*.

initial degree of unsaturation seen immediately after a downward temperature shift, Fulco *et al.* (Fujii and Fulco, 1977; Fulco, 1983) postulated the existence of a modulator protein whose synthesis also proceeds at lower temperatures, but only following a brief delay. Thus, the rapid desaturation taking place in freshly down-shifted cells would soon be moderated to a rate yielding the steady-state level of fatty acid unsaturation characteristic of that temperature. However, a molecular understanding of this cold-induced hyperinduction of UFA clearly requires much further investigation.

In *Bacillus*, it is a matter of dispute whether acyl-CoA or phospholipids constitute substrates for the desaturating enzyme. Fulco *et al.* (Lombardi and Fulco, 1980; Fulco, 1983) suggested that the substrates for desaturation are the acyl groups present in intact membrane phospholipids, whereas Dunkley *et al.* (1991) recently reported that extracts of *B. subtilis* desaturate palmitoyl-CoA but phosphatidylethanolamine is not a substrate *in vitro*. In an attempt to elucidate the nature of the *in vivo* substrate(s) of the *B. subtilis* desaturase, we have used auxotrophs that allow arresting of phospholipid synthesis. The *glyc* auxotrophs are blocked at the level of the acyl transfer caused by the lack of acyl acceptor, whereas *panE* mutants cannot synthesize fatty acids by depletion of acyl carriers. For direct testing of whether a fatty-acyl thioester(s) such as acyl-ACP or acyl-CoA produced in the synthetic fatty acid pathway can be desaturated *in vivo*, we have used a system that allows us to uncouple fatty acid synthesis from phospholipid synthesis. Glycerol starvation of *B. subtilis* glycerol auxotrophs inhibits phospholipid synthesis, resulting in a significant accumulation of FFAs (see Fig. 4, lane A, and Mindich, 1972). Synthesis of UFAs from these *de novo*-produced fatty acids would be expected in these cells after a temperature shift if fatty-acyl thioesters are used as desaturation substrates. Moreover, these UFAs should be found in the FFA fraction, since it is accepted that free acyl moieties accumulated during glycerol starvation arise by hydrolysis of fatty-acyl thioesters by thioesterase(s) (Rock and Jackowski, 1982). Our results (Fig. 4, lane C) show that free UFAs are not formed after glycerol deprivation. The inability of glycerol-starved cells to desaturate fatty-acyl moieties not bound to phospholipids cannot be attributed to the production of a non-metabolizable pool of fatty acids. After addition of glycerol, these fatty acids are readily incorporated into the phospholipids and subsequently desaturated at 20°C (Fig. 4, lane D). These data support the conclusion that an acyl-ACP derived from fatty acid biosynthesis or an acyl-CoA, that may be formed from acyl-ACP or from FFAs, are not desaturated *in vivo* by *B. subtilis*. Therefore, our data strongly suggest that, *in vivo*, membrane phospholipids are obligate intermediates in the synthesis of UFAs during cold-adaptation.

The results obtained with starved *glyc* auxotrophs are compatible with lipid-linked desaturation in which the fatty acid remains esterified to the membrane lipid while being desaturated. This latter desaturation mechanism has been proposed for the cyanobacterium *Anabaena variabilis* (Sato *et al.*, 1986) and for the psychrophilic bacterium *Micrococcus cryophilus* (Foot *et al.*, 1983). Nevertheless, we needed to firmly exclude the presence in *B. subtilis* of a pathway proposed for *Tetrahymena* (Yasunaga *et al.*, 1984) which involves deacylation of the membrane lipid, esterification of the fatty acid to a coenzyme, desaturation of the fatty-acyl thioester, and reacylation of the lipid with the resultant UFAs. In this case, the substrate for desaturation could be acyl-CoA since Dunkley *et al.*(1991) have recently reported that, like some other bacteria that synthesize UFAs by the oxygen-dependent pathway, extracts of *B. subtilis* can desaturate palmitoyl CoA *in vitro*. However, the data shown in Table 1 indicate that labelled phospholipids from pantoate-starved *panE* auxotrophs are readily desaturated. We determined that pantoate starvation of *panE* mutants results in inhibition of the synthesis of CoA (Fig. 5) and depletion of the CoA pool. This result indicates that the fatty-acid moieties of the phospholipids are desaturated without any necessity for this coenzyme. Nevertheless, with the experiments reported here, we cannot exclude the possibility that a palmitoyl-CoA desaturase becomes inhibited when phospholipid synthesis is arrested by starvation of *glyc* or *pan* auxotrophs. If this is the case, *B. subtilis* would contain two different pathways for the biosynthesis of UFAs: a palmitoyl-CoA desaturase coupled to phospholipid synthesis and a desaturation mechanism, reported here, that is independent of phospholipid synthesis, which uses as substrates the fatty-acyl groups of intact phospholipids. Finally, that membrane phospholipids may be directly desaturated could explain the observation that *B. subtilis* mutants lacking palmitoyl-CoA desaturase can still synthesize UFAs at a significant rate (Dunkley *et al.*, 1991).

## Experimental procedures

### *Bacterial strains and growth conditions*

The following *B. subtilis* strains were used: strain 168 (*trpC2*), strain B42 (derived from strain 168), which is *his*, *trpC2*, *glyc*, and *glpD* (Mindich, 1970), and strain UR1 (*trpC2*, *thr5*, *panE*) (Baigori *et al.*, 1991). In all experiments, the strains were grown in Spizizen minimal salts medium (Spizizen, 1958) supplemented with glucose (0.5%), vitamin-free casein hydrolysate (0.1%) and tryptophan (40 $\mu g\,ml^{-1}$). In addition, glycerol (25 $\mu g\,ml^{-1}$) or pantoic acid (0.02%) were included to satisfy growth requirements of strain B42 (*glyc*) or UR1 (*panE*), respectively.

### *Glycerol or pantoic acid starvation*

At the mid-exponential phase of growth, the growing culture was subjected to three cycles of centrifugation in media without glycerol or pantoic acid. Growth of strain B42 continued for about one generation following removal of glycerol (Mindich, 1970), while the growth of strain UR1 ceased after pantoic acid was removed (see the *Results*).

### *Analysis of radioactive lipids*

For measurements of fatty acid synthesis, *B. subtilis* strains were grown in the culture medium described above with aeration to exponential phase. Aliquots of these cells (1 ml) were exposed to 10 μCi of sodium [1-$^{14}$C]-acetate (59.0 mCi mmol$^{-1}$) for different times at the temperatures described in the figure legends. After these incubation periods, lipids were extracted from whole cells as previously described (de Mendoza *et al.*, 1982).

FFAs and glycerolipid fractions were separated by thin-layer chromatography on silica gel plates developed in petroleum ether/ether/acetic acid (70:30:2). In this system the FFAs migrate near the solvent front, whereas phospholipids remain in the origin of the thin-layer plate (de Mendoza *et al.*, 1982; Mindich, 1972). The FFA fraction was converted to methyl esthers with diazomethane (Cronan *et al.*, 1975). Radioactive fatty acid methyl esters were then separated into unsaturated and saturated fractions by chromatography on 20% silver-nitrate-impregnated silica gel thin-layer plates (de Mendoza *et al.*, 1982). The plates were developed in toluene at –17°C, autoradiographed, and the appropiate areas of silica gel were scraped into vials containing scintillation solution (de Mendoza *et al.*, 1982) to determine their radioactivity content.

Fatty acids from glycerolipids were converted to their methyl esters with sodium methoxide (de Mendoza *et al.*, 1982). The esters were then extracted in petroleum ether/ether (1:1), concentrated under a nitrogen atmosphere and chromatographed on silver-nitrate-impregnated plates as described above. The radioactive compounds were located by autoradiography and quantified by scintillation counting.

### *Chromatography of β-alanine-derived metabolites*

The *B. subtilis* strain UR1 was labelled with β-[3-$^{3}$H]-alanine as described in the figure legends. Extraction of β-alanine-derived metabolites was accomplished as described by Jackowski and Rock (1981). Samples were analysed by thin-layer chromatography on silica gel H layers developed with ethanol/28% ammonium hydroxide, 4:1, (v/v) to 14 cm from the origin. In this solvent system coenzyme A (CoA), acyl carrier protein (ACP) and β-alanine-derived phosphorylated metabolites remain in the origin of the thin-layer plate, while β-alanine and pantothenate migrate with a defined $R_F$ (Jackowski and Rock, 1981; Baigori *et al.*, 1991). The distribution of radioactivity on the thin-layer plate was determined by scraping 0.5 cm sections of the silica gel layer into 3 ml of scintillation solution and ascertaining the radioactive content.

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