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DNA supercoiling and thermal regulation of unsaturated fatty acid synthesis in *Bacillus subtilis*

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Summary

***Bacillus subtilis* growing at 37°C synthesizes, almost exclusively, saturated fatty acids. However, when a culture growing at 37°C is transferred to 20°C, the synthesis of unsaturated fatty acids is induced. The addition of the DNA gyrase inhibitor novobiocin specifically prevented the induction of unsaturated fatty acid synthesis at 20°C. Furthermore, it was determined that plasmid DNA isolated from cells growing at 20°C was significantly more negatively supercoiled than the equivalent DNA isolated from cells growing at 37°C. The overall results agree with the hypothesis that an increase in DNA supercoiling associated with a temperature downshift could regulate the unsaturated fatty acids synthesis in *B. subtilis*.**

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. This regulatory mechanism system, called thermal control of fatty acids synthesis, is thought to be designed to attenuate the effects of temperature changes on the fluidity of the cell membrane (for a review see de Mendoza and Cronan, 1983). Thus, the ratio between fluid (disordered) lipids and non-fluid (ordered) lipids in cell membranes plays a major role in membrane functions. While increased incorporation of UFAs decreases the melting temperature of the membrane phospholipids, increased incorporation of saturated fatty acids has the opposite effect (de Mendoza and Cronan, 1983). The thermal regulatory system can thus adapt the membrane lipids for optimal behaviour at new growth temperatures. This kind of adjustment of membrane fatty acid composition has been already reported in a large number of *Bacillus* species that synthesize UFAs by an oxygen-dependent desaturation mechanism (Fulco, 1983). In these bacteria there is an induction of a fatty acid desaturating system when the cultures are grown at low temperatures (for reviews see de Mendoza *et al.*, 1993; Fulco, 1983). This adaptive response has been extensively characterized in *Bacillus megaterium* by Fulco and coworkers (Fujii and Fulco, 1977; Fulco, 1983), who demonstrated that a fatty acid desaturation system is transcriptionally induced at low growth temperatures. These authors (Fujii and Fulco, 1977) also found that the levels of desaturation of cultures of *B. 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 35°C to 20°C, Fulco (1983) proposed that transcription of the fatty acid desaturase gene occurs only at low growth temperatures. To account for the unexpectedly large initial degree of unsaturation seen immediately after a downward temperature shift, 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 downshifted 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 thermally regulated synthesis of UFAs clearly requires much further investigation.

To further explore the molecular mechanism(s) of cold-induction of UFA biosynthesis and the mechanism(s) for sensing temperatures changes in bacilli we decided to study these phenomena in the well-studied and easily manipulable bacterium *Bacillus subtilis*. *B. subtilis* growing at 37°C almost exclusively synthesizes saturated fatty acids; however, when a culture growing at 37°C is transferred to 20°C the synthesis of a C-16 monounsaturated fatty acid is induced (Grau and de Mendoza, 1993). As with *B. megaterium*, the desaturation system of *B. subtilis* required *de novo* synthesis of RNA since it was completely abolished by rifampicin added before a downward temperature shift (Grau and de Mendoza, 1993).

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Several independent experiments performed with Gram-negative bacteria showed that DNA supercoiling plays a role in the transduction of environmental signals to the bacterial nucleoid, with important consequences for the regulation of transcription (for reviews see Drlica, 1990; 1992; Higgins *et al.*, 1990; Pruss and Drlica, 1989; Wang and Syvanen, 1992). In particular, changes in growth temperature (Dorman *et al.*, 1990; Goldstein and Drlica, 1984), osmolarity (Higgins *et al.*, 1988; 1990) and oxygen availability (Dorman *et al.*, 1988) are able to change DNA supercoiling in bacteria, with the DNA topoisomerase activities being essential for transduction of the environmental signal. The environmentally induced changes in the pattern of gene expression often correlate with significant changes in plasmid DNA supercoiling (for reviews see Drlica, 1990; 1992; Higgins *et al.*, 1990). In this work we report that the induction of UFA synthesis in *B. subtilis* by low temperatures was blocked by DNA gyrase specific inhibitors. In addition, plasmid DNA isolated from cells grown at 20°C is significantly more supercoiled than that isolated from cells growing at 37°C. Thus, we suggest that the increase in DNA supercoiling levels, brought about by the temperature downshift, could play a role in the regulation of fatty acid desaturation in *B. subtilis*.

Results

DNA gyrase inhibitors block the cold-induction of UFA synthesis

We have previously reported that desaturation of fatty acids in *B. subtilis* shifted from 37°C to 20°C is completely abolished by rifampicin added before the temperature shift (Grau and de Mendoza, 1993). Addition of the antibiotic 30–60 min after the temperature shift did not appreciably affect desaturation when compared with an untreated control (data not shown). These results indicate that synthesis of UFAs is transcriptionally activated by an environmental signal such as a temperature downshift and, as stated above, at least some environmental signals could be transduced by DNA gyrase (Higgins *et al.*, 1990); we assayed the effects of the DNA gyrase inhibitor novobiocin (Gellert *et al.*, 1976) on this induction process. As shown in Fig. 1, A and B, the induction of fatty acids desaturation by shifting a *B. subtilis* (strain 168) culture from 37°C to 20°C was inhibited by novobiocin following a dose-response curve. When added before the temperature shift, 3 $\mu g\,ml^{-1}$ novobiocin was sufficient to block the synthesis of UFAs more than 90%. Similar inhibition of UFA biosynthesis was obtained with the DNA gyrase inhibitors nalidixic acid (10 $\mu g\,ml^{-1}$) and coumermycin (40 $\mu g\,ml^{-1}$) (data not shown). The effect of these specific DNA gyrase inhibitors on UFA biosynthesis seems not to be related to DNA damage since addition of mytomicin C (10 $\mu g\,ml^{-1}$) before the temperature downshift did not inhibit the cold-induction of UFA synthesis (data not shown). We also observed that a culture of strain 168 treated with 5 $\mu g\,ml^{-1}$ novobiocin 1 h after the temperature shift from 37°C to 20°C (when the desaturation system is fully induced; data not shown) synthesized the same amount of UFAs as non-treated cultures (Fig. 2A). Thus, once desaturation was induced by temperature downshift, novobiocin had not inhibited UFA synthesis. In addition, up to 10 $\mu g\,ml^{-1}$ novobiocin did not affect the synthesis of UFAs in the novobiocin-resistant strain RG1 (Fig. 2B) indicating that, under our assay conditions, the only target of novobiocin is DNA gyrase.

To assess how general the sensitivity of stress-regulated genes to novobiocin might be in *B. subtilis*, we investigated the effect of this antibiotic on the inducible protection by low levels of H_2O_2 to oxidative stress. Pretreatment of *B. subtilis* with 50–100 μM H_2O_2 protects

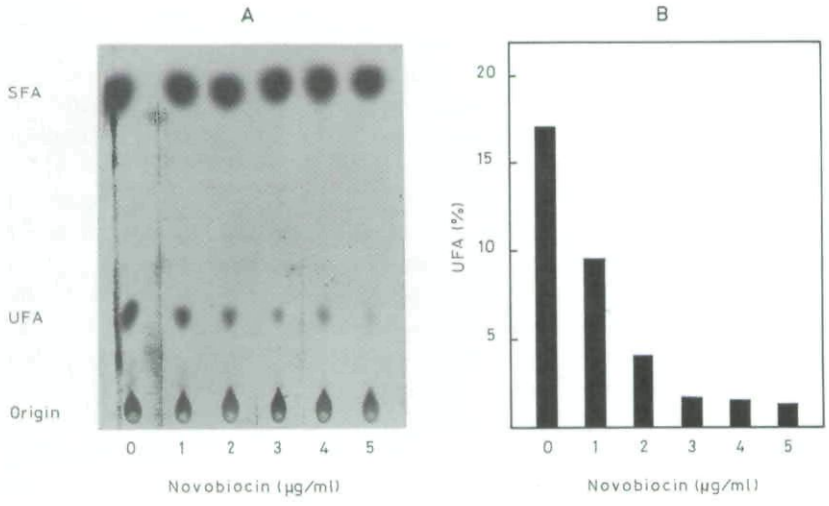

Fig. 1. Sensitivity of UFA synthesis to novobiocin.
A. Autoradiographic pattern of synthesis of [^{14}C]-fatty acids in *B. subtilis* 168 exposed to the indicated concentrations of novobiocin. The experiment was performed as described in the *Experimental procedures*. The final positions of UFAs and saturated fatty acids (SFAs) are indicated in the left margin.
B. Percentage of inhibition of UFA synthesis by novobiocin. The total extracted radioactive methyl esters (about 60 000 c.p.m./sample) were separated into unsaturated and saturated fractions as shown in (A). Results are expressed as the percentages of the total methyl esters recovered at each concentration of novobiocin. Similarly, extents of UFA synthesis inhibition by novobiocin were obtained in several independent experiments. At the concentrations of novobiocin used in these experiments, no inhibition of SFA synthesis was observed.

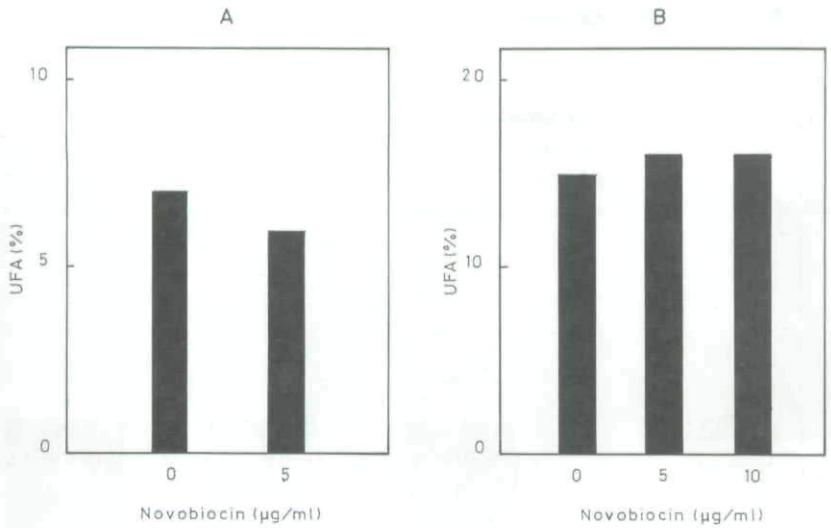

Fig. 2. Addition of novobiocin to strain 168 after temperature downshift or to strain RG1 (*gyrB*) does not affect UFA synthesis.
A. A culture of strain 168 growing exponentially at 37°C was transferred to 20°C for 1 h. After this incubation period two samples (1 ml each) were incubated with 10 μCi of [^{14}C]-acetate in the presence or in the absence of novobiocin (5 μg ml^{-1}) for 3 h at 20°C. The chromatography of labelled methyl esters was performed as described in the *Experimental procedures*. The percentage of UFAs was determined as described in the legend to Fig. 1.
B. A culture of strain RG1 (*gyrB*) was treated with novobiocin as described in Fig. 1. The percentage of UFAs was determined as described in panel A.

cells against the lethal effect of 10 mM H_2O_2 (Hartford and Dowds, 1992). The H_2O_2 pretreatment leads to an induced synthesis of several proteins that seem to play a protective role against high levels of H_2O_2 (Hartford and Dowds, 1992). Chloramphenicol blocks the protection of low concentrations of H_2O_2 (50 μM) against the lethal effect of 10 mM H_2O_2, while the protective response was unaffected by novobiocin (Table 1). Although care must be taken when interpreting the results of inhibitor experiments (Higgins *et al.*, 1990), these results at least suggest that not all of the environmentally induced genes respond in a similar way to novobiocin.

Growth temperature affects plasmid DNA supercoiling

As already shown (Grau and de Mendoza, 1993), *B. subtilis* cells shifted from 37°C to 20°C for 12 h synthesize about 20% of UFAs, while only 0.2% of UFAs are synthesized when the culture is maintained at 37°C (Fig. 3A). On the other hand, the observed effects of DNA gyrase inhibitors on cold-induction of UFA biosynthesis are consistent with an effect mediated by changes in DNA topology. Therefore we used the plasmid pUB110 and agarose gel electrophoresis as tools to measure the changes in the topological state of *B. subtilis* DNA. In order to resolve the different topoisomers we ran the gels in the presence of 3 μg ml^{-1} chloroquine (see below). Figure 3B shows the plasmid DNA isolated from strain 168 growing at 37°C or shifted from 37°C to 20°C for 12 h. While the plasmid isolated at 20°C migrated as fully supercoiled, the one isolated at 37°C migrated as a distribution of more relaxed adjacent topoisomers differing by one linking number (Pruss, 1985). Densitometric analysis of the agarose gels (Fig. 3C) displayed a broad distribution of about 13 topoisomers for the plasmid isolated at 37°C. In addition, this analysis showed that about 50% of the plasmid isolated at 20°C and 20% of the one isolated at 37°C were nicked (form II) by extraction procedures. Since the topoisomers in these two plasmid preparations were still negatively supercoiled at 3 μg ml^{-1} chloroquine (see below) and since mobility in these gels increases with increasing number of supercoils (Pruss, 1985), the results in Fig. 3, B and C indicate that most of the pUB110 DNA isolated from cells shifted to 20°C has a lower linking number (higher absolute value of a negative linking number) than the same plasmid when isolated from cells growing at 37°C.

Table 1. Effect of novobiocin on survival of *B. subtilis* 168 after H_2O_2 treatments.[a]

Sample	Treatment	Percentage survival
1	Untreated[b]	100
2	50 μM H_2O_2	100
3	10 mM H_2O_2	0.8
4	50 μM H_2O_2, then 10 mM H_2O_2	16
5	Cm 100 μg ml^{-1}, then 50 μM H_2O_2, then 10 mM H_2O_2[c]	0.8
6	Nov 10 μg ml^{-1}, then 50 μM H_2O_2, then 10 mM H_2O_2[d]	20

a. Samples subjected to two or three treatments were treated sequentially. Each value is the average of three determinations.
b. With no treatment there was 100% survival (2.5 × 10^7 cfu ml^{-1}).
c. 100 % survival was taken as 1.2 × 10^7 cfu ml^{-1} and corresponds to cells treated with chloramphenicol (Cm) alone.
d. 100 % survival after novobiocin (Nov) treatment was indistinguishable from untreated cells (2.5 × 10^7 cfu ml^{-1}).

Thermal downshift produces an increase in plasmid DNA negative supercoiling

To quantify the topological changes induced by the temperature downshift, we ran the electrophoresis of the

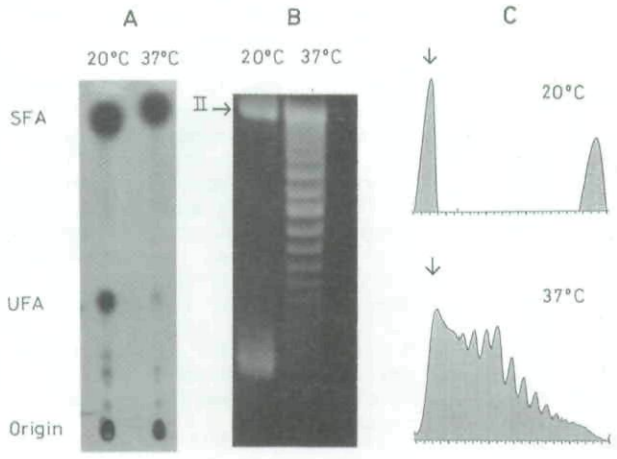

Fig. 3. Temperature-dependent synthesis of UFAs correlates with the level of DNA supercoiling.
A. Autoradiographic pattern of synthesis of fatty acids in strain 168 (pUB110) grown overnight at 20°C or 37°C. Cells were grown to exponential phase at 37°C. A 1 ml sample was transferred to 20°C and exposed to 10 μCi of [^{14}C]-acetate overnight. A second sample from the same culture was maintained overnight at 37°C in the presence of 10 μCi of [^{14}C]-acetate. The phospholipids were extracted and the fatty acids converted to methyl esters and chromatographed in the argentation system. The percentage of UFAs synthesized at 20°C or 37°C was 21% and 0.2%, respectively.
B. Chloroquine–agarose gel electrophoresis of pUB110 isolated for cells grown overnight at 20°C or 37°C. Plasmid DNA was isolated and analysed on a 1.2% agarose gel with 3 μg ml^{-1} chloroquine as described in the *Experimental procedures.* Migration is from top to bottom. II: relaxed/nicked circular DNA.
C. Densitometric tracing of the banding patterns pictured in (B). Migration is from left to right, and the arrow indicates the position of form II DNA.

isolated plasmids in the presence of increasing concentrations of chloroquine: 0, 3, 6, 9, 12 and 18 μg ml^{-1}. As shown in Fig. 4A, in the absence of chloroquine the plasmids isolated in both conditions migrated as fully supercoiled and were undistinguishable. Increasing concentrations of chloroquine provoked the gradual relaxation of both plasmid DNA but at different rates. While plasmid DNA from cells growing at 37°C was resolved into multiple topoisomers at 3 μg ml^{-1} chloroquine, the plasmid DNA of cells shifted to 20°C for 1 h remained unresolved. This plasmid DNA started to relax at 6 μg ml^{-1} chloroquine and at concentrations ranging from 9 to 12 μg ml^{-1} of the drug it was almost completely resolved into topoisomers. At 18 μg ml^{-1} chloroquine, most of the plasmid DNA isolated from cells shifted to 20°C was relaxed, whereas that isolated from cells grown at 37°C started to be positively supercoiled. Thus, this experiment confirmed that cells shifted from 37°C to 20°C for 1 h contained a much higher supercoiled population of pUB110 than cells kept at 37°C. By counting the different topoisomers resolved by 9 and 12 μg ml^{-1} chloroquine and taking the maxima of the topoisomer distribution, we calculated that the difference in linking number (ΔL) between both isolated plasmids ($L_{20°C}$–$L_{37°C}$) is −9 (Table 2). When we took into account the change in DNA winding owing to decreased temperature, by using the formula of 0.012 helical degrees per base pair per degree centigrade (Depew and Wang, 1975), we calculated an increase of about 3.2 in ΔL obtaining a

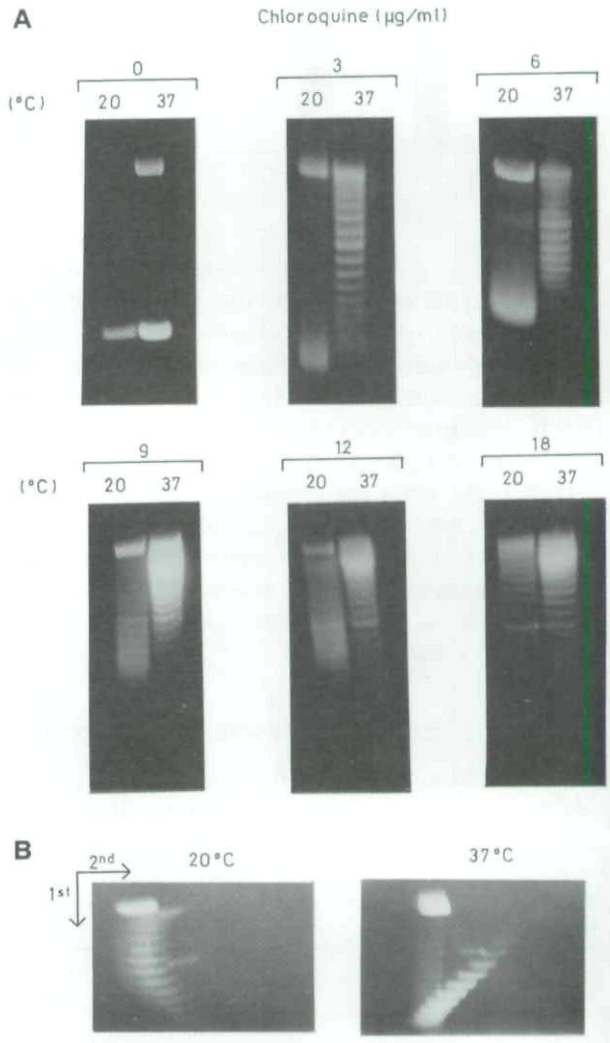

Fig. 4. Temperature downshift increases plasmid DNA negative supercoiling.
A. Chloroquine-agarose gel electrophoresis analysis of pUB110 isolated from strain 168 growing exponentially at 37°C or shifted from 37°C to 20°C for 1 h. The gels depicted in the different panels contained chloroquine at the concentrations indicated at the top of each gel. Plasmid DNA isolated from cells growing at 37°C started to relax at 1.5 μg ml^{-1} chloroquine but was not totally resolved into topoisomers (data not shown).
B. Two-dimensional gel electrophoresis of pUB110 isolated from cells grown as described in (A). The first dimension (vertical) was run with 10 μg ml^{-1} or 2 μg ml^{-1} chloroquine for plasmids isolated from cells shifted from 37°C to 20°C or maintained at 37°C, respectively. The second dimension (horizontal) was run with 24 μg ml^{-1} chloroquine.

Table 2. Effects of some different stresses on DNA supercoiling.

Strain	Plasmid	Size (kb)	Stress	ΔL[a]	Effect	Source
E. coli DM4100	pBR322	4.36	Temperature downshift	+2	Relaxation	Goldstein and Drlica (1984)
B. subtilis 168	pUB110	4.55	Temperature downshift	−9	Supercoiling	This study
B. subtilis 168	pUB110	4.55	Nutrient depletion (sporulation)	−13	Supercoiling	Nicholson and Setlow (1990)

a ΔL = difference between L after stress and L before stress (Drlica, 1984).

corrected value of about −12. Two-dimensional gel electrophoresis (Fig. 4B) allowed the separation of the linear form from all the different closed circular forms of plasmid DNA. Both culture temperatures produced a plasmid population with a monomodal distribution of about 13 topoisomers whose linking numbers differ from that of an adjacent one by one.

In attempts to determine the reversibility of the changes in the superhelical state related to shifts in growth temperature, we subjected the cell cultures to two different protocols. First, plasmid DNA was extracted from strain 168 at various times after cultures were shifted from 37°C to 20°C (Fig. 5). Plasmid DNA became more supercoiled 10 min after cells were transferred to 20°C (Fig. 5, lane 2). This topoisomer distribution did not change for longer incubations at 20°C (Fig. 5, lanes 3–4; see also Fig. 3B). Later, the expected changes in DNA supercoiling were also observed in the reciprocal experiment in which cells were shifted from 20°C to 37°C. About 10 min after the shift, the topoisomer distribution and supercoiling were similar to those of plasmids extracted from cells grown for many generations at 37°C (Fig. 5, lane 5).

To obtain direct evidence that DNA gyrase activity was involved in the increase of plasmid DNA supercoiling after thermal downshift, we isolated plasmid DNA from strains 168 or RG1 shifted from 37°C to 20°C in the

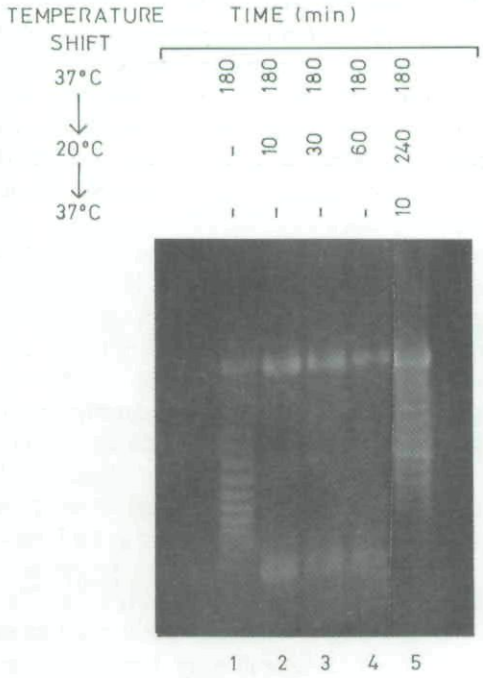

Fig. 5. The changes of the superhelical state of plasmid DNA mediated by growth temperature shifts are reversible. Chloroquine–agarose gel electrophoresis of pUB110 from a culture of strain 168 growing exponentially at 37°C (lane 1), shifted to 20°C for 10 min (lane 2), 30 min (lane 3) and 60 min (lane 4) prior to plasmid isolation. Lane 5 shows pUB110 extracted from strain 168 shifted from 37°C to 20°C for 4 h and then transferred to 37°C for 10 min prior to plasmid isolation. The gel contained 3 μg ml^{-1} chloroquine. Details of plasmid preparation in this experiment are given in the *Experimental procedures*.

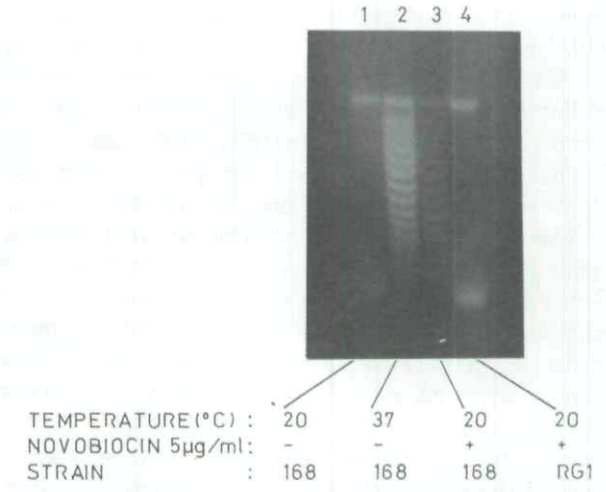

Fig. 6. Novobiocin blocks the cold-induced change in plasmid DNA supercoiling in strain 168 but not in strain RG1 (*gyrB*). pUB110 isolated from a culture of strain 168: lane 1, growing exponentially at 37°C and then shifted to 20°C for 4 h; lane 2, growing at 37°C; lane 3, growing at 37°C and shifted to 20°C for 4 h in the presence of 5 μg ml^{-1} novobiocin; lane 4, pUB110 isolated from strain RG1 treated as described in lane 3. The agarose gel contained 3 μg ml^{-1} chloroquine.

presence of novobiocin. Figure 6 shows that the cold-induced change in DNA supercoiling was blocked by novobiocin in strain 168 (lane 3) but not in the isogenic *gyrB* strain, RG1 (lane 4). Thus, DNA gyrase activity seems to be essential for the temperature-mediated increase in plasmid DNA supercoiling.

Discussion

The synthesis of UFAs in *Bacillus* is an attractive system for the study of thermal gene regulation. In contrast to the constitutive mechanism used by *Escherichia coli* to increase the amount of UFAs in their phospholipids at low temperatures (de Mendoza and Cronan, 1983; de Mendoza *et al.*, 1983), expression of the bacillary desaturation system is a cold-inducible process (de Mendoza *et al.*, 1993; Fulco, 1983; Grau and de Mendoza, 1993). In this work we show that the UFA biosynthesis triggered in *B. subtilis* cells shifted from 37°C to 20°C was blocked by the DNA gyrase inhibitor, novobiocin (Fig. 1). Furthermore, we demonstrated that novobiocin by itself is not responsible for such an effect, because no inhibition of UFA synthesis was observed in a *gyrB* strain (Fig. 2B). In addition, 5 $\mu g\,ml^{-1}$ novobiocin 1 h after the temperature downshift did not affect the observed desaturation (Fig. 2A). Thus, the DNA gyrase activity that partially defines the degree of supercoiling seems to be a key element in the cold-induced synthesis of UFAs in *B. subtilis*. Consequently, we measured plasmid DNA supercoiling of cells shifted from 37°C to 20°C. Plasmid DNA isolated from cells shifted to 20°C was significantly more negatively supercoiled than the equivalent DNA isolated from cells growing at 37°C (Figs 3 and 4, Table 2). This change in DNA conformation was dependent on DNA gyrase activity since novobiocin inhibited the increase of negative supercoiling induced by the temperature downshift in strain 168 but not in an isogenic *gyrB* strain (Fig. 6). We assume that the large temperature-induced changes in superhelical density of isolated plasmids observed *in vitro* reflect an *in vivo* alteration of plasmid and chromosomal structure, although this has not yet been demonstrated. By analogy with the situation of *E. coli*, it seems likely that in *Bacillus* species the supercoiling state of cellular DNA is maintained by a balance between the activities of DNA gyrase, which introduces negative supercoils, and DNA topoisomerase I, which relaxes them (Drlica, 1984). Indeed, both enzymes have been found in *Bacillus* species (Burrington and Morgan, 1978; Sugino and Bott, 1980). Consequently, an increase in negative supercoiling in cells shifted from 37°C to 20°C could be the result of either an increase in the activity of DNA gyrase or a decrease in DNA topoisomerase I activity, or both. It is worth mentioning that Mizushima *et al.* (1992) reported that phospholipid vesicles containing UFAs (but not saturated fatty acids) were strong inhibitors of the relaxation activity of *E. coli* DNA topoisomerase I. Thus, the UFAs accumulated in *B. subtilis* cells shifted to 20°C could inhibit topoisomerase I activity with a consequent increase in DNA supercoiling. On the other hand, Jones *et al.* (1992) have recently shown that the synthesis of the A and B subunits of DNA gyrase is increased during the cold-shock response in exponentially growing cultures of *E. coli*. These authors also presented evidence suggesting that the *E. coli* major cold-shock protein, CS7.4, acts as a transcriptional activator of the A subunit of DNA gyrase (Jones *et al.*, 1992). CspB, a cold-shock protein with 61% homology with the *E. coli* protein CS7.4, has been identified recently in *B. subtilis* (Willimsky *et al.*, 1992). Thus, it could be possible that, as is the case in *E. coli*, *gyrA* and *gyrB* may be transcriptionally activated in *B. subtilis* during the shift from 37°C to 20°C. However, as shown in Table 2, although in *E. coli* there is a sustained synthesis of DNA gyrase following temperature downshift from 37°C to 17°C, the level of plasmid DNA supercoiling is decreased at the latter temperature (Drlica, 1992; Goldstein and Drlica, 1984). Thus, in addition to DNA gyrase, other protein(s) could be involved in the increased negative supercoiling observed in *B. subtilis* cells shifted from 37°C to 20°C, as it occurs during sporulation in *B. subtilis* (Nicholson and Setlow, 1990). Plasmid DNA isolated from the forespore compartment exhibits an increase in negative supercoiling (Table 2) at about the same time that several small, acid-soluble DNA-binding proteins (SASPs) are synthesized (Nicholson and Setlow, 1990). Mutations in the genes encoding SASPs partly block the increase in supercoiling, indicating that they are involved in the change in DNA topology (Tovar-Rojo and Setlow, 1991).

The understanding of the molecular basis of the temperature-induced control by which UFAs are synthesized at low temperatures in bacilli is still limited (de Mendoza *et al.*, 1993). Although indirectly, our results provide evidence that the cold-induced synthesis of UFAs in *B. subtilis* is sensitive to modifications of DNA supercoiling. These observations suggest the possibility that transcription of a gene(s) involved in the synthesis of UFAs is controlled by a promoter(s) whose activity is modulated by changes in the topology of the template DNA. The fact that novobiocin specifically blocks both the cold-induced desaturation of fatty acids and the increase in plasmid DNA supercoiling during the temperature downshift provides support for this model. However, the molecular mechanism(s) that would link these two events remain to be determined. Cloning and characterization of the gene(s) involved in UFA biosynthesis will clarify whether the thermally induced topological change is causally linked with desaturation of fatty acids at low growth temperatures.

Experimental procedures

Bacterial strains and growth conditions

The *B. subtilis* strains used in this study were: strain 168 (*trpC2*) and strain RG1, which is *trpC2 gyrB1*. The *gyrB1* mutation (also termed *novA1*), which maps in the *gyrB* locus of the genetic map of *B. subtilis* (Anagnostopoulos *et al.*, 1993), codes for a gyrase subunit resistant to novobiocin (Sugino and Bott, 1980; Lampe and Bott, 1985). Strain RG1 was constructed by transformation of strain 168 with DNA obtained from strain 1A160 (*gyrB1 sacA321*) and selection by resistance to 3 $\mu g\,ml^{-1}$ novobiocin. Plasmid pUB110 was introduced by transformation into competent cells of *B. subtilis* as described (Gardiol *et al.*, 1993). The strains were grown in the mineral salts medium of Spizizen (1958) supplemented with glucose (0.5%), vitamin-free casein hydrolysate (0.1%) and tryptophan (40 $\mu g\,ml^{-1}$).

Inhibition by novobiocin of UFA synthesis in vivo

One-millilitre aliquots of strain 168 grown to exponential phase at 37°C were exposed to different concentrations of novobiocin for 1 min (Fig. 1) and then transferred to 20°C and labelled for 4 h with [^{14}C]-acetate (59.0 mCi $mmol^{-1}$) before lipid extraction (Grau and de Mendoza, 1993). After these incubation periods lipids were extracted from whole cells as previously described (Grau and de Mendoza, 1993). Fatty acids from glycerolipids were converted to their methyl esters with sodium methoxide and separated into unsaturated and saturated fractions by chromatography on 20% silver nitrate-impregnated silica-gel thin-layer plates (Grau and de Mendoza, 1993). The plates were developed in toluene at −17°C, autoradiographed, and the appropriate areas of silica gel were scraped into vials containing scintillation solution to determine their radioactivity content (Grau and de Mendoza, 1993).

Induction of protection against oxidative stress

B. subtilis was grown to the mid-log phase in the above-described media. Aliquots (1 ml) were placed in six test tubes and treated as shown in Table 1. Treatment with H_2O_2 at a final concentration of 50 μM (inducing concentrations) or 10 mM (killing concentrations) was for 10 min or 15 min, respectively (Hartford and Dowds, 1992). When required, chloramphenicol (100 $\mu g\,ml^{-1}$) or novobiocin (10 $\mu g\,ml^{-1}$) was added 5 min before the addition of 50 μM H_2O_2 (see Table 1).

Plasmid DNA isolation

B. subtilis cells bearing pUB110 from an overnight culture were diluted 100-fold into two Erlenmeyer 2-litre flasks each containing 500 ml of fresh medium. Cells were grown at 37°C with vigorous aeration to mid-exponential phase; one of the cultures was then shifted to 20°C for the times indicated in the figures (before DNA plasmid extraction) while the other culture was processed immediately. Cells were harvested by centrifugation (10 min, 8000 × *g* in rotor GSA), the pellet was washed with buffer A (20% sucrose, 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM NaCl) and concentrated 50-fold in buffer A containing lysozyme at a final concentration of 4 $mg\,ml^{-1}$. All these procedures were performed at the same temperatures used for cell culture (20°C or 37°C) and the treatment with lysozyme was for 15 min at 37°C or for 1 h at 20°C. The cells were lysed by alkaline–SDS, and cold potassium acetate pH 4.8 was added to the viscous lysate, incubated for 15 min at 0°C and then centrifuged for 15 min at 27 000 × *g* in an SS34 rotor. Plasmid DNA in the supernatant was precipitated with 0.6 volumes of isopropanol at room temperature. The pellet was resuspended in TE Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and further purified with successive RNase A (50 $\mu g\,ml^{-1}$), proteinase K (100 $\mu g\,ml^{-1}$) and 2 M LiCl treatments. The samples were centrifuged and the plasmid DNA was precipitated from the supernatant fluid by addition of 0.3 M potassium acetate and cold ethanol, followed by 1 h at −20°C. Plasmid DNA was suspended in TE and purified by extractions with phenol/chloroform, and chloroform and concentrated by additional precipitation with cold ethanol at −20°C. Plasmid DNA was finally washed with 70% ethanol, resuspended in sterile water and stored at −20°C. For the experiment described in Fig. 5, cells bearing pUB110 from an overnight culture were diluted 100-fold in 200 ml of fresh medium and grown at 37°C with vigorous aeration to mid-exponential phase. A 20 ml fraction was removed from the culture and processed immediately. The remaining culture was transferred to 20°C and 20 ml fractions were removed at the times indicated in the figure for the isolation of plasmid; the rest of the culture was transferred to 37°C for 10 min and a 20 ml fraction was also processed. Plasmid DNA was isolated as described above except that after addition of lysozyme (2 $mg\,ml^{-1}$) they were rapidly chilled at −70°C for 15 min and then incubated at 20°C or 37°C for 15 min before cell lysis.

With these procedures the yield of plasmid DNA obtained from cells treated with lysozyme at 37°C was higher than the yield obtained from cells treated with lysozyme at 20°C.

Agarose gel electrophoresis

Electrophoresis was performed in horizontal slabs gels (5.0 cm × 15.0 cm × 0.4 cm) for one-dimensional electrophoresis and (15.0 cm × 15.0 cm × 0.4 cm) for two-dimensional electrophoresis in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). All of the gels consisted of 1.2% (w/v) ultrapure agarose BRL (Bethesda Research Laboratories) containing chloroquine phosphate (Sigma) at the same concentration as the running buffer. Electrophoresis was performed in the cold room (4°C) without recirculation of buffer. One-dimensional gels and the first dimension of two-dimensional gels were run at 2.0 $V\,cm^{-1}$ for 20–25 h depending on the chloroquine concentration (shorter times for lower concentrations of chloroquine). The second dimensions of two-dimensional gels were run at 2.4 $V\,cm^{-1}$ for 30 h. Electrophoresis in two-dimensions was performed as follows: after the DNA was electrophoresed in the first dimension as described above, the electrophoresis buffer was discarded and the gel was rotated 90° in the electrophoresis cube and soaked in fresh electrophoresis buffer containing chloroquine at the new concentration for about 2 h in the cold room. After running, all of the gels were destained

to remove chloroquine by soaking for 4 h in eight volumes of distilled water changed hourly. For two-dimensional gels one wash overnight was performed before the routine hourly washes. DNA was stained for about 1–3 h at room temperature in 0.5 μg ml^{-1} etihidium bromide and, when convenient, staining was performed overnight in the cold room. The gels were destained for about 1–3 d in sterile water containing 2.5 mM EDTA changed daily before being photographed under ultraviolet light on Polaroid Type 667 film. Densitometric scans of the banding patterns were made from photographic negatives using an Elena Biolaboratories scanning densitometer.

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References

Anagnostopoulos, C., Piggot, P.J., and Hoch, J.A. (1993) The genetic map of *Bacillus subtilis*. In *Bacillus subtilis and Other Gram Positive Bacteria: Physiology, Biochemistry and Molecular Biology*. Losick, R., Sonenshein, A.L., and Hoch, J.A. (eds). Washington, D.C.: American Society for Microbiology, pp. 425–463.

Burrington, M.G., and Morgan, A.R. (1978) The purification and characterization of a DNA nicking-closing enzyme from *Bacillus megaterium*. *Can J Biochem* **56:** 123–128.

Depew, R.E., and Wang, J.C. (1975) Conformational fluctuations of DNA helix. *Proc Natl Acad Sci USA* **72:** 4275–4279.

Dorman, C.J., Barr, G.C., Ni Bhriain, N., and Higgins, C.F. (1988) DNA supercoiling and the anaerobic and growth phase regulation of *tonB* gene expression. *J Bacteriol* **170:** 2816–2826.

Dorman, C.J., Ni Bhriain, N., and Higgins, C.F. (1990) DNA supercoiling and environmental regulation in virulence gene expression in *Shigella flexneri*. *Nature* **344:** 789–792.

Drlica, K. (1984) Biology of bacterial deoxyribonucleic acid topoisomerases. *Microbiol Rev* **48:** 273–289.

Drlica, K. (1990) Bacterial topoisomerases and the control of DNA supercoiling. *Trends Genet* **12:** 433–437.

Drlica, K. (1992) Control of bacterial DNA supercoiling. *Mol Microbiol* **6:** 425–433.

Fujii, D.K., and Fulco, A.J. (1977) Biosynthesis of unsaturated fatty acids in Bacilli: hyperinduction and modulation of desaturase synthesis. *J Biol Chem* **252:** 3660–3670.

Fulco, A.J. (1983) Fatty acid metabolism in bacteria. *Progr Lipid Res* **22:** 133–160.

Gardiol, D., Gramajo, H., Hirschbein, L., and de Mendoza, D. (1993) Application of the mini-Mu phage for the isolation of *lac* transcriptional fusions in *Bacillus subtilis* genes. *Gene* **123:** 39–44.

Gellert, M., O'Dea, M.H., Itoh, T., and Tomizawa, J. (1976) Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. *Proc Natl Acad Sci USA* **73:** 4474–4478.

Goldstein, E., and Drlica, K. (1984) Regulation of bacterial DNA supercoiling: plasmid linking vary with growth temperature. *Proc Natl Acad Sci USA* **81:** 4046–4050.

Grau, R., and de Mendoza, D. (1993) Regulation of the synthesis of unsaturated fatty acids by growth temperature in *Bacillus subtilis*. *Mol Microbiol* **8:** 535–542.

Hartford, O.M., and Dowds, B.C.A. (1992) Cloning and characterization of genes induced by hydrogen peroxide in *Bacillus subtilis*. *J Gen Microbiol* **138:** 2061–2068.

Higgins, C.F., Dorman, C.J., Stirling, D.A., Waddell, L., Booth, I.R., May, G., and Bremer, E. (1988) A physiological role of DNA supercoiling in the osmotic regulation of gene expression in *Salmonella typhimurium* and *Escherichia coli*. *Cell.* **52:** 569–584.

Higgins, C.F., Dorman, C.J., and Ni Bhriain, N. (1990) Environmental influences on DNA supercoiling: a novel mechanism for the regulation of gene expression. In *The Bacterial Chromosome*. Drlica, K., and Riley, M. (eds). Washington, D.C.: American Society for Microbiology, pp. 421–432.

Jones, P.G., Krah, R., Tafuri, S.R., and Wolffe, A. (1992) DNA gyrase, CS7.4, and the cold shock response in *Escherichia coli*. *J Bacteriol* **174:** 5798–5802.

Lampe, M.F., and Bott, K.F. (1985) Genetic and organization of the cloned *gyrA* and *gyrB* genes of *Bacillus subtilis*. *J Bacteriol* **162:** 78–84.

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

de Mendoza, D., Grau, R., and Cronan, Jr, J.E. (1993) Biosynthesis and function of membrane lipids. In *Bacillus subtilis and Other Gram Positive Bacteria: Physiology, Biochemistry and Molecular Biology*. Losick, R., Sonenshein, A.L., and Hoch, J.A. (eds). Washington, D.C.: American Society for Microbiology, pp. 411–425.

de Mendoza, D., Klages-Ulrich, A., and Cronan, Jr, J.E. (1983) Thermal regulation of membrane fluidity in *Escherichia coli*. Effects of overproduction of β-keto-acyl-acyl-carrier protein synthase I. *J Biol Chem* **258:** 2098–2101.

Mizushima, T., Natori, S., and Sekimizu, K. (1992) Inhibition of *Escherichia coli* DNA topoisomerase I activity by phospholipids. *Biochem J* **285:** 503–506.

Nicholson, W.L., and Setlow, P. (1990) Dramatic increase in negative superhelicity of plasmid DNA in the forespore compartment of sporulating cells of *Bacillus subtilis*. *J Bacteriol* **172:** 7–14.

Pruss, G.J. (1985) DNA topoisomerase I mutants: increased heterogeneity in linking number and other replicon-dependent changes in DNA supercoiling. *J Mol Biol* **185:** 51–63.

Pruss, G.I., and Drlica, K. (1989) DNA supercoiling and prokaryotic transcription. *Cell* **56:** 521–523.

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

Sugino, A., and Bott, K.F. (1980) *Bacillus subtilis* deoxyribonucleic acid gyrase. *J Bacteriol* **141:** 1331–1339.

Tovar-Rojo, F., and Setlow, P. (1991) Effects of mutant small, acid-soluble spore proteins from *Bacillus subtilis* on DNA *in vivo* and *in vitro*. *J Bacteriol* **173:** 4827–4835.

Wang, J.Y., and Syvanen, M. (1992) DNA twist as a transcriptional sensor for environmental changes. *Mol Microbiol* **6:** 1861–1866.

Willimsky, G., Bang, H., Fischer, G., and Marahiel, M.A. (1992) Characterization of *cspB*, a *Bacillus subtilis* inducible cold shock gene affecting cell viability at low temperatures. *J Bacteriol* **174:** 6326–6335.