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De novo fatty acid synthesis is required for establishment of cell type-specific gene transcription during sporulation in *Bacillus subtilis*

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Summary

A hallmark of sporulation of *Bacillus subtilis* is the formation of two distinct cells by an asymmetric septum. The developmental programme of these two cells involves the compartmentalized activities of σ^E in the larger mother cell and of σ^F in the smaller prespore. A potential role of *de novo* lipid synthesis on development was investigated by treating *B. subtilis* cells with cerulenin, a specific inhibitor of fatty acid biosynthesis. These experiments demonstrated that spore formation requires *de novo* fatty acid synthesis at the onset of sporulation. The transcription of the sporulation genes that are induced before the formation of two cell types or that are under the exclusive control of σ^F occurred in the absence of fatty acid synthesis, as monitored by *spo–lacZ* fusions. However, expression of *lacZ* fusions to genes that required activation of σ^E for transcription was inhibited in the absence of fatty acid synthesis. The block in σ^E-directed gene expression in cerulenin-treated cells was caused by an inability to process pro-σ^E to its active form. Electron microscopy revealed that these fatty acid-starved cells initiate abnormal polar septation, suggesting that *de novo* fatty acid synthesis may be essential to couple the activation of the mother cell transcription factors with the formation of the differentiating cells.

Introduction

A hallmark of the process of sporulation in the Gram-positive bacterium *Bacillus subtilis* is the formation of an asymmetrically positioned (polar) septum that divides the cell into a small forespore compartment and a large cell compartment (Lutkenhaus, 1994). The unequal-sized progeny resulting from the formation of the polar septum have different developmental fates and express different sets of genes (for reviews see Errington, 1993; Losick and Stragier, 1996). The fate of the forespore chamber is determined by the transcription factor σ^F, which is present before the formation of the polar septum but does not become active in directing gene transcription until completion of asymmetric division, when its activity is confined to the smaller compartment of the sporangium (for reviews see Losick and Stragier, 1996)

The activity of σ^F is regulated by a pathway consisting of the proteins SpoIIAB, SpoIIAA and SpoIIE, all of which are produced before the formation of the polar septum (Duncan and Losick, 1993; Min *et al*., 1993; Alper *et al*., 1994; Diederich *et al*., 1994; Arigoni *et al*., 1995; Duncan *et al*., 1995). SpoIIAB is an anti-sigma factor that binds to σ^F and holds it in an inactive complex (Duncan and Losick, 1993; Min *et al*., 1993; Magnin *et al*., 1996). SpoIIAA, on the other hand, is an anti-anti-sigma factor that binds to SpoIIAB, thereby causing the release of free and active σ^F from the SpoIIAB–σ^F complex (Alper *et al*., 1994; Diederich *et al*., 1994). SpoIIAB is also a protein kinase that phosphorylates and thereby inactivates SpoIIAA (Min *et al*., 1993). Finally, SpoIIE is a serine phosphatase that is capable of dephosphorylating SpoIIAA-P, thereby restoring the ability of SpoIIAA to bind SpoIIAB (Duncan *et al*., 1995; Arigoni *et al*., 1996; Feucht *et al*., 1996). Interestingly, SpoIIE has an additional function beyond its role in σ^F activation (Barák and Youngman, 1996; Feucht *et al*., 1996). In its absence, but not in mutants specifically affected in SpoIIE phosphatase activity, the sporulation septum develops an aberrantly thick layer of peptidoglycan and the time of septum formation is delayed (Barák and Youngman, 1996; Feucht *et al*., 1996); the later effect is attributed to an amino acid sequence at the extreme COOH-terminus of SpoIIE (Feucht *et al*., 1996). Thus, combination of these two functions in a single polypeptide may serve to couple the release of the cell-specific transcription factors with the formation of the differentiating cells.

Establishment of forespore specificity through activation of σ^F is soon followed by induction in the mother cell of a large set of genes under the control of the transcription factor σ^E (Losick and Stragier, 1996). σ^E is an unusual

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sigma factor in that it is synthesized as an inactive large precursor, pro-σ^E, the primary product of the promoter distal member (*spoIIGB*) of the two cistron *spoIIG* operon (LaBell *et al*., 1987). Pro-σ^E is converted to its active form by proteolytic removal of its pro-amino acid sequence, an N-terminal extension of 27 residues. The product of the first gene of the operon, *spoIIGA*, is a multispanning membrane protein that is sufficient for activation of pro-σ^E and that is believed to be the processing enzyme (Stragier *et al*., 1988). As both pro-σ^E and SpoIIGA are present before septation, and are presumably segregated into each of the cells after asymmetric division, confinement of σ^E activity to the mother cell implies that SpoIIGA-mediated pro-σ^E processing is delayed until after septation, with the possibility that it occurs only in the mother cell (Losick and Stragier, 1996). The proteolytic activation of σ^E requires the activity of σ^F through the activity of the σ^F-directed gene *spoIIR* (Karow *et al*., 1995; Londoño-Vallejo and Stragier, 1995). It has been suggested by Losick and Stragier (1996) that *spoIIR* would be transcribed by σ^F in the prespore and act as a vectorial cell–cell signal that would activate σ^E only in the mother cell. Consistent with SpoIIR acting as a cell–cell signal, characterization of the *spoIIR* gene indicated that it encoded a protein with a secretion signal, and recent biochemical analysis has shown that SpoIIR is secreted (Hoffmeister *et al*., 1995). Once secreted, SpoIIR could interact with the membrane-bound SpoIIGA that cleaves the pro sequence from σ^E to activate it. Thus, the main function of SpoIIR would be to tie the transcription programme in the mother cell to the transcription programme in the forespore by allowing σ^E to become active only after σ^F has itself been activated and thus ensuring that σ^E does not become active in the predivisional sporangium. Although this model is intriguing, the notion that SpoIIR must act vectorially to activate pro-σ^E processing is not without controversy. Zhang *et al*. (1996) observed that σ^E activation was limited to the mother cell compartment, even when SpoIIR was synthesized before septation and therefore presumed to be present in both compartments. These authors conclude that a σ^E activation factor other than SpoIIR, possibly SpoIIGA in a particular orientation, could be restricted to the mother cell. The identity of the factor(s) responsible for cell compartment-specific activation of σ^E and the details of how the directional processing is accomplished remain to be resolved. This intercellular signal transduction pathway and the mechanism that prevents activation of σ^E in the forespore could be connected to the asymmetrical septum biosynthesis because this event is the key morphological step in the formation of the two cell types and compartmentalization of gene expression (for a review see Duncan *et al*., 1994). In connection with this possibility, it has been suggested that specific fatty acids may act as signals linking the initiation of sporulation to the status of membrane synthesis and septation in *B. subtilis* (Strauch *et al*., 1992). Moreover, it has recently been demonstrated that branched-chain fatty acids play an important role in cell–cell communication and development in *Mixococcus xantus* (Downward and Toal, 1995; Toal *et al*., 1995).

In this report, we demonstrate that *de novo* fatty acid synthesis after the end of exponential phase is required for spore formation and that a reduced level of fatty acid synthesis allows formation of asymmetric septa but prevents activation of σ^E. We speculate that septal membrane lipids play a key role in the establishment of differential gene expression during the morphogenetic programme of sporulation in *B. subtilis*.

Results

The effect of inhibition of the de novo *fatty acid synthesis on sporulation*

To determine the role of *de novo* fatty acid synthesis on sporulation, we used the antibiotic cerulenin. This antibiotic is a potent inhibitor of lipid synthesis known to irreversibly alkylate an essential thiol group of β-ketoacyl-ACP synthase, the enzyme that catalyses the condensation of malonyl-CoA with acyl-ACP (D'Agnolo *et al*., 1973). Thus, cerulenin inhibits the fatty acid chain elongation step and thereby lipid synthesis (Fig. 1). Cerulenin (10 μg ml^{-1}) was added to strain JH642 grown in DS medium at different times: 1.5 h ($t_{-1.5}$) and 0.5 h ($t_{-0.5}$) before cells enter stationary phase, at the onset of stationary phase (t_0) or at several times after t_0 (Fig. 2). Cerulenin was a potent inhibitor of spore formation when added to cells at t_{-1}, t_0 or $t_{0.5}$; however, this inhibitory effect disappeared when the antibiotic was added at more advanced stages of sporulation (t_1 to t_4) (Fig. 2). Typically, 16 h after reaching stationary phase, cells treated with cerulenin at t_0 had a viable count of about 10^8 ml^{-1} and produced about 10^6 spores ml^{-1}. In contrast, in the absence of cerulenin, strain JH642 had a viable count of about 6×10^8 ml^{-1} and produced about 5×10^8 spores ml^{-1}. Monitoring the levels of fatty acid synthesis in the experiments shown in Fig. 2 revealed that *de novo* incorporation of [^{14}C]-acetate into phospholipids of cerulenin-treated cells decreased about 90% when compared with non-treated cells (data not shown). To establish whether the primary action of cerulenin on spore formation is due to its inhibitory effect on *de novo* fatty acid synthesis, we used strain GS77. The growth of this strain is not inhibited by the addition of cerulenin up to 50 μg ml^{-1}, whereas the growth of the isogenic strain JH642 is inhibited at a concentration of 5 μg ml^{-1} (data not shown). We found that extracts of strain GS77 contained a fatty acid synthetase (FAS) activity about sevenfold higher than the isogenic strain JH642 (Table 1). Although the FAS activity of strain GS77 is inhibited

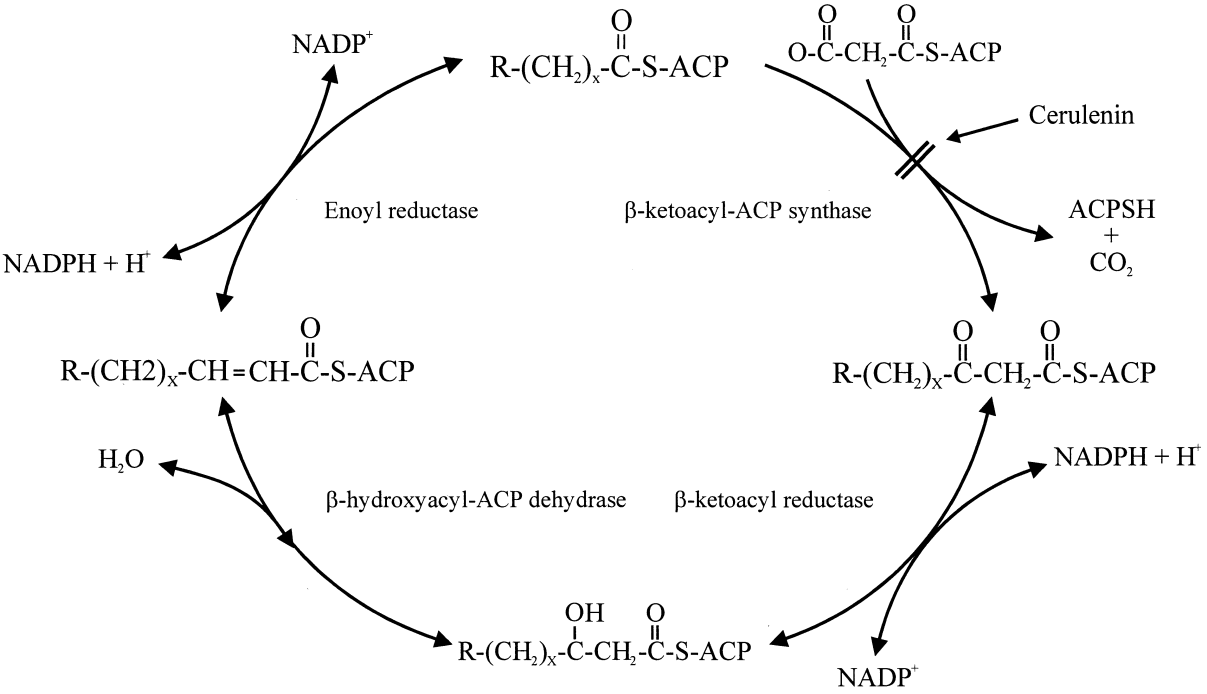

Fig. 1. Elongation of fatty acid biosynthesis. Each elongation cycle is initiated by the condensation of malonyl-acyl carrier protein (ACP) with acyl-ACP carried out by β-ketoacyl-ACP-synthase. This step is blocked by cerulenin (D'Agnolo *et al*., 1973). The next step is the reduction of β-ketoesters by β-ketoacyl reductase. The β-D-hydroxyacyl-ACP is then dehydrated to the *trans*-2 unsaturated acyl-ACP, which is reduced by enoyl reductase to generate an acyl-ACP two carbons longer than the original acyl-ACP. Several lines of evidence indicate that this pathway is used in *B. subtilis* to elongate straight and branched-chain fatty acids (de Mendoza *et al*., 1993; Morbidoni *et al*., 1996). R denotes the terminal group of branched-chain fatty acids derived from ketoacids from valine, leucine or isoleucine (de Mendoza *et al*., 1993).

about 70% by cerulenin (40 μg ml^{-1}), its residual activity is still about twofold higher than the basal FAS activity of the wild-type isogenic strain JH642 (Table 1). Therefore, the resistance to cerulenin belonging to strain GS77 seems to be due to overproduction of FAS activity rather than a decrease in the enzyme affinity by the antibiotic. In agreement with these findings, the frequency of sporulation of GS77 was essentially the same in the presence or in the absence of 10 μg ml^{-1} cerulenin added at t_0 (data not shown). These experiments indicate that, under our assay conditions, the only target of cerulenin is the *B. subtilis* FAS. Thus, *de novo* fatty acid synthesis at the onset of stationary phase is essential for *Bacillus* cell differentiation.

Sporulation gene expression in the absence of fatty acid synthesis

To determine the stage in the developmental gene expression pathway inhibited by the addition of cerulenin, we used a series of strains containing *lacZ* fusions to promoters known to be under the control of specific regulatory proteins and to be expressed at characteristic sporulation stages. Cerulenin was added at the onset of sporulation to strains carrying *lacZ* fusions to each of these genes (Fig. 3). The expression levels of the genes *spoIIA*, *spoIIG* and *spoIIE* [whose expression early in stationary phase depends on the activation of Spo0A by phosphorylation (Hoch, 1993)] were similar in cells treated or untreated with cerulenin, at least within the first and second hour after the addition of the antibiotic; at later times, the level of β-galactosidase

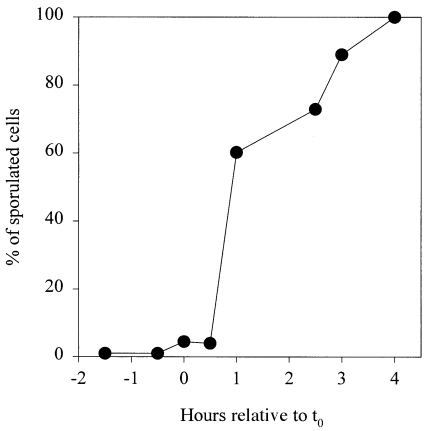

Fig. 2. Effect of cerulenin on the extent of sporulation. Strain JH642 was grown in DS medium at 37°C and cerulenin was added at the indicated times relatives to the end of exponential growth (t_0). The spore titre was measured 24 h after t_0. Values represent the average of at least five independent experiments.

Table 1. Effect of cerulenin (40 μg ml^{-1}) on *in vitro* synthesis of fatty acids by cell-free extracts.

Strain	Specific activity[a] (pmol min^{-1} mg^{-1} protein)	
	−Cerulenin	+Cerulenin
JH642	4.1 (1.00)[b]	0.3 (0.07)
GS77	27.7 (6.80)	8.8 (2.10)

a. Activities were determined in cell extracts as described under *Experimental procedures*.
b. Overproduction relative to the isogenic wild-type strain grown in parallel without treatment.

expressed from those fusions was significantly higher after the addition of cerulenin (Fig. 3A and B). These results are significant because they indicate that cerulenin-treated cells were capable of entering sporulation and that the presence of the antibiotic does not inhibit transcription of sporulation genes expressed before septation.

The gene *spoIIQ* is under the exclusive control of σ^F (Londoño-Vallejo *et al*., 1997), whereas the genes *spoIID* and *spoIIID* require σ^E for transcription (Losick and Stragier, 1996). A fusion of *lacZ* to the promoter of *spoIIQ* was expressed in cells treated with cerulenin, although at about 50% of the rate observed in non-treated cells (Fig. 3C and D). In contrast to the expression of *spoIIQ*, the transcription of the σ^E-directed *spoIID–lacZ* and *spoIIID–lacZ* was totally curtailed in the presence of cerulenin (Fig. 3E and F). These results suggest that, in the absence of lipid synthesis, cells can initiate septation exhibiting activation of σ^F but not activation of σ^E.

Cells blocked in lipid synthesis do not process Pro-σ^E

To examine whether the inhibition of lipid synthesis blocks σ^E activation by affecting its synthesis, processing or another activation step, we performed a Western blot analysis of protein extracted from cerulenin-treated cells using

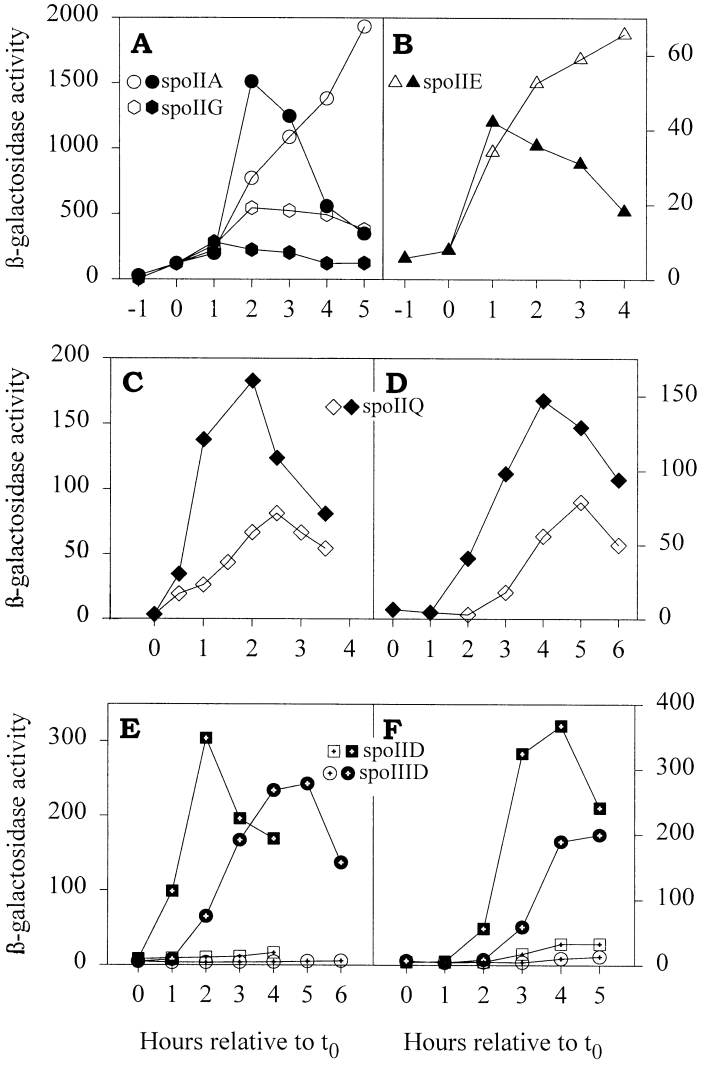

Fig. 3. Effect of cerulenin on β-galactosidase activity expressed from various *spo–lacZ* fusions. In A, B, C and E the strains were grown in DS medium at 37°C. Cerulenin (10 μg ml^{-1}) was added to one half of each culture at the end of exponential growth (t_0). In D and F strains were induced to sporulate by the resuspention method. Cerulenin (5 μg ml^{-1}) was added to one half of each culture at the time of resuspention (t_0). Samples were collected at the indicated times relative to t_0 and assayed for β-galactosidase activity. Control without cerulenin, filled symbols; cerulenin-treated cells, open symbols.

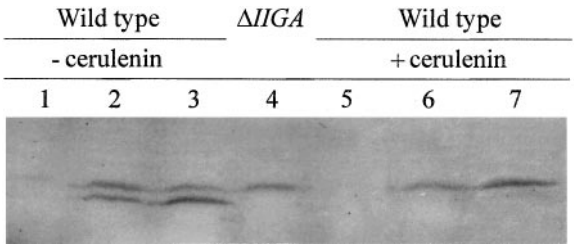

Fig. 4. Effect of cerulenin on pro-σ^E processing. Crude protein extracts were processed and analysed as described in *Experimental procedures*. Lanes 1–3, extracts from JH642 cells harvested 1.0, 2.2 and 3.5 h after t_0; lane 4, extracts from strain MO1190 (*ΔspoIIGA*) cells harvested 3 h after t_0. Lanes 5–7, extracts from JH642 cells treated with 10 μg ml^{-1} of cerulenin at t_0 and harvested 1.0, 2.2 and 3.5 h after the addition of the antibiotic.

anti-σ^E antibodies. As shown in Fig. 4, cells that do not synthesize lipids at the onset of sporulation produced pro-σ^E normally but did not process it, indicating that *de novo* lipid synthesis is required for pro-σ^E processing.

Morphological analysis

Examination of more than 150 complete longitudinal sections from randomly selected fields of cerulenin-treated cells by electron microscopy showed that the septation frequency was reduced after 6 h in stationary phase in DS medium and revealed septa with different abnormalities (Figs 5 and 6). We have chosen to distinguish between cells on the basis of septum thickness, number and position, according to the key given by Barák and Youngman (1996) (Fig. 6). At least 40% of the scored cells displayed complete septal structures, and some of the scored cells contained two polar asymmetric septa or double asymmetric septa (Fig. 6). These results indicate that cells with a limited supply of fatty acids at the onset of sporulation are still able to form septa. About 70% of the septated cells (Fig. 6) make thick, morphological abnormal spore septa (Fig. 5A and D) similar to those shown by strains containing *spoIIE* null mutations (Barák and Youngman, 1996; Feucht *et al*., 1996). Our results are quantitatively similar to those reported by Barák and Youngman (1996) for *spoIIE* null mutants, except for the presence in our studies of ≈30% of cells with thin asymmetric septa (class 5 in Fig. 6) among the cells that produce septa in the presence of cerulenin.

To obtain more precise information about the effect of cerulenin on morphological development, we investigated the effect of the antibiotic inducing sporulation by resuspension of exponentially growing cells in a nutrient-limited medium, essentially by the method of Sterlini and Mandelstam (1969). Cells treated with cerulenin at the time of resuspention (t_0) were collected 3 and 16 h later and analysed by electron microscopy. This experiment showed basically the same septum morphologies as those obtained

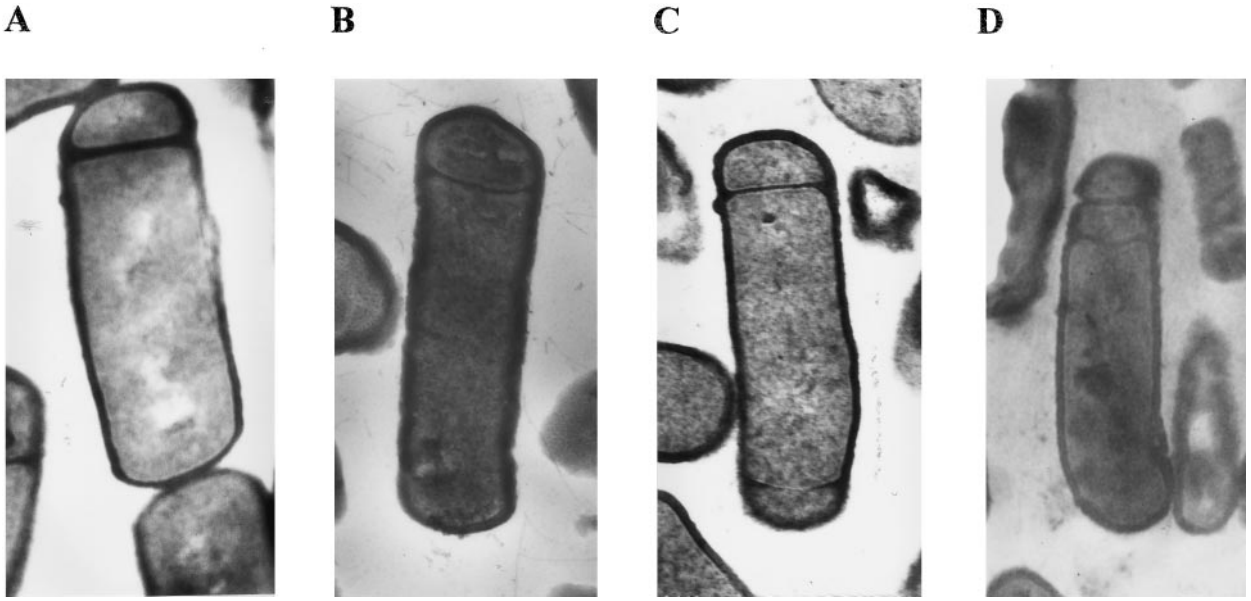

Fig. 5. Representative examples of septum morphologies scored in strain JH642 treated with cerulenin. At the end of exponential growth cerulenin was added at a concentration of 10 μg ml^{-1} and the incubation continued until t_6. Cells were harvested and processed for electron microscopy as described in *Experimental procedures*.
A.Cells with thick septa at one polar position.
B. Cells with thin septa at one polar position.
C. Cells with thin septa at two polar positions.
D. Cells with double asymmetric septum at one pole.

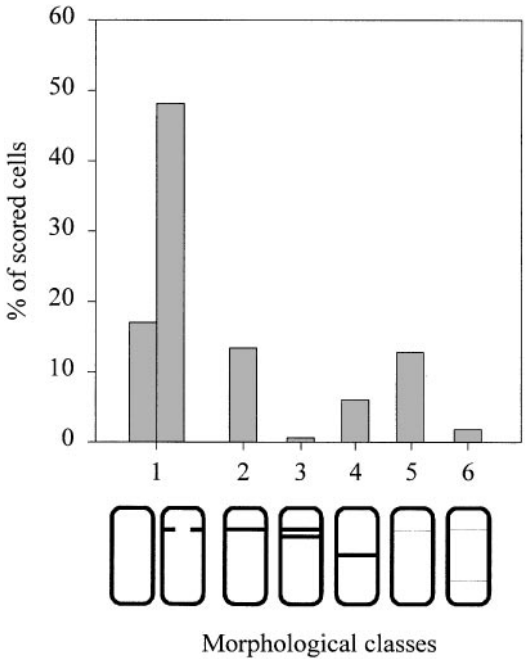

Fig. 6. Quantification of morphological classes in populations of JH642 cells treated with cerulenin. Cells were treated with cerulenin and harvested for examination by electron microscopy as described in the legend of Fig. 5. Class 1, no septa or incomplete septum; Class 2, thick septum at polar position; Class 3, double asymmetric thick septum at polar position; Class 4, thick septum at the mid-cell position; Class 5, thin septum at polar position; Class 6, double asymmetric thin septum.

in DS medium. However, all the septa formed by cerulenin-treated cells in the resuspension medium were abnormally thick (data not shown). In addition, as observed in DS medium, the forespore sigma σ^F was active, as judged from *spoIIQ–lacZ* expression (Fig. 3D), whereas the mother cell sigma factor σ^E was completely inactive, as judged by *spoIID–lacZ* and *spoIIID–lacZ* expression (Fig. 3F) and Western blot experiments (data not shown) in cells induced to sporulate by the resuspension method in the presence of cerulenin.

The results of the resuspension experiments suggest that some cells in DS medium are able to form a thin sporulation septum before sporulation is arrested by lipid starvation. The different results obtained by the addition of cerulenin to cells induced to sporulate with the nutrient or resuspension method could be explained by the fact that the last procedure provides a fixed point of the initiation of sporulation from which subsequent events can be accurately timed (Errington, 1993).

Discussion

To form spores, *B. subtilis* needs to expend energy and synthesize specialized structures in an environment in which nutrients are scarce. Thus, the ability to sporulate depends on the availability of sufficient metabolic reserves to complete the developmental process. Morphological and physiological studies have pointed to the importance of membrane synthesis during sporulation for asymmetric septation and prespore engulfment (for a review see Piggot *et al*., 1994). Freese and Oh (1974) reported that growing glycerol auxotrophs without glycerol prevented sporulation and septum formation. However, glycerol starvation also produced membrane collapse, and much of the cellular ATP was released in the medium. In addition, these manipulations did not stop lipid synthesis at the end of exponential growth because to observe effects in sporulation efficiency the auxotrophs were deprived of glycerol during the exponential phase of growth (Freese and Oh, 1974).

In this work, we show that sporulation was blocked by the addition of the antibiotic cerulenin, which is a specific inhibitor of fatty acid synthesis, at the end of exponential growth. Furthermore, we demonstrated that the inhibition of spore formation is not due to a side-effect of cerulenin because no effect of the antibiotic was observed in a mutant that overproduces FAS activity. The inhibition of fatty acid synthesis at t_0 allowed the expression of σ^F-directed genes but not σ^E-directed genes. Lipid-starved cells produced pro-σ^E but were unable to process it, indicating that *de novo* lipid synthesis at the onset of sporulation plays a role in pro-σ^E processing. In addition, lipid-starved cells form primarily thick septal structures. The thick septum phenotype is apparently caused by the incorporation of excess peptidoglycan into the space between the septum membranes (Barák and Youngman, 1996). It has been proposed that the SpoIIE protein could be involved in determining the extent to which peptidoglycan becomes inserted between the lipid bilayers that constitute the sporulation septum (Barák and Youngman, 1996). This proposal is based on the finding that SpoIIE is a membrane-bound protein that accumulates at sites of asymmetric septum synthesis and that *spoIIE* null mutants form a thick septum (Arigoni *et al*., 1995; Barák and Youngman, 1996; Barák *et al*., 1996). Thus, inhibition of phospholipid synthesis could interfere with the role of SpoIIE in septum assembly, causing a thick phenotype. If this is the case, new phospholipid synthesis at the initiation of sporulation would be essential for the morphogenetic activity of SpoIIE, but not for its phosphatase activity needed to release the first cell-specific sigma factor σ^F in the prespore. Alternatively, the rate of phospholipid and murein synthesis could be tightly coupled during septum formation. The increased peptidoglycan deposition between the septum membranes may thus be a consequence of an unbalanced synthesis of membrane and murein.

How might the deprivation of fatty acid synthesis at t_0 prevent the compartment-specific activation of σ^E? It is well established that the mother cell transcription factor σ^E is derived by proteolytic cleavage from pro-σ^E (LaBell,

1987; Stragier *et al*., 1988). Much experimental evidence suggests that SpoIIGA, a multispanning membrane protein, is the processing enzyme (for a review see Losick and Stragier, 1996). Hofmeister *et al*. (1995) proposed that SpoIIGA may be a two-domain protein that resides within the forespore septum with one domain serving as the pro-σ^E protease and the second serving as a signal transduction domain that responds to transmembrane activation by SpoIIR. Thus, it is possible that lipids might be essential for the catalytic activity, assembly or SpoIIR-mediated activation of SpoIIGA into the septal membrane. A large body of evidence attests that lipids have a strong influence on the properties of membrane-bound enzymes, and many solubilized or reconstituted enzymes are specifically stimulated by the addition of particular lipids (for a review see Gennis, 1989). Moreover, recent experiments established that a phospholipid acts as a chaperone in the assembly of a membrane transport protein (Bogdanov *et al*., 1996). If the amphipatic environment of the septal membrane is an important determinant in the assembly or activity of SpoIIGA, the addition of cerulenin could inhibit the accumulation of lipids synthesized at t_0 necessary for SpoIIGA activation. If this hypothesis is correct, lipid-mediated activation of SpoIIGA could be relevant for the compartment-specific activation of σ^E. A topological model for SpoIIGA proposes that multiple membrane-spanning segments in the N-terminus of the protein anchor it to the membrane and present the C-terminus to the interior of the cell (Stragier *et al*., 1988). If SpoIIGA is inserted in this manner into both septum membranes, it might be available to interact with pro-σ^E in both the forespore and the mother cell compartments. Localized activation of σ^E in the mother cell might then occur as a result of an asymmetric lipid composition in the sporangium septal membranes. The particular phospholipid composition of the mother cell could be essential for the activation of the protease domain to cleave pro-σ^E into mature σ^E. In this regard, it is important to mention that phospholipids in growing and sporulating cells of *B. subtilis* can vary qualitatively and quantitatively and are greatly affected by *spo* mutations (Rigomier *et al*., 1974; Rigomier *et al*., 1978). It has also been demonstrated that membranes of *B. megaterium* show extremely fast lipid translocation, presumably mediated by specific translocases (Rothman and Kennedy, 1977). This transmembrane traffic activity could also be important determining a differential phospholipid composition or lipid microdomains in the forespore and mother cell septal membranes. Moreover, evidence consistent with the existence of a variety of types of membrane domains within prokaryotic cells has been obtained using many different techniques (for a review see Norris and Madsen, 1995).

It is worth noting that Zhang *et al*. (1996) explored the possibility that the septum morphology of *spoIIE* mutants could affect the ability of SpoIIR to activate SpoIIGA. To this end they constructed a strain in which *spoIIR* expression was independent of σ^F and that also contained the *spoIIE20* mutation (Zhang *et al*., 1996). However, the resultant strain was unable to form septa and was prevented from testing the role of septum morphology in compartmentalized activation of σ^E (Zhang *et al*., 1996). We report here that cerulenin treatment produces cells that synthesize thick asymmetric septa expressing *spoIIR* (data not shown) but unable to activate σ^E. Thus, our results are also compatible with the hypothesis that the *spoIIE*-like sporulation septum found in cells treated with cerulenin could affect the ability of SpoIIR to act as a cell-to-cell signal for σ^E activation.

Although little is understood about septum formation, it is accepted that its assembly requires the synthesis of a great deal of new lipid (Piggot *et al*., 1994; Feucht *et al*., 1996). Our results show that cells with a deficiency in fatty acid synthesis can still form an asymmetric septum. However, the lipid composition of this asymmetric septum must be determined by 'old lipids' synthesized in the predivisional cell. Under these conditions, the polar septum is not suitable to activate σ^E-directed gene expression. A key challenge now will be to directly determine whether lipids affect the activity, assembly or localization of the SpoIIGA protein, or if they are important for the transmission of the cell–cell signalling pathway mediated by SpoIIR.

Experimental procedures

Bacterial strains

The *B. subtilis* strains used in this study are listed in Table 2. The strain carrying the original resistance to cerulenin (*Cer20*) was a gift from Donald Zeigler (BGSC).

General methods

Difco sporulation medium (DS) was used to induce sporulation in *B. subtilis* in liquid media (Shaeffer *et al*., 1965). The method used for induction of sporulation by resuspension in poor medium was essentially that of Sterlini and Mandelstan (1969). The amount of sporulated cells was determined by heat treatment (20 min at 80°C).

Competent cells were prepared and transformed as previously described (Mansilla and de Mendoza, 1997). Selection for resistance to cerulenin was performed on LB medium (Sambrook *et al*., 1989) containing 10 μg of cerulenin per ml. Cerulenin was stored as a stock solution at 10 mg ml^{-1} in ethanol at −20°C. Aliquots of the solution were added to a shaking culture in order to rapidly disperse the antibiotic.

Electron microscopy of sporulating cells sampled 6 h after the end of exponential phase was performed as described previously (Chatter *et al*., 1989).

β-Galactosidase assays

To test the effect of cerulenin on the expression of *lacZ*

Table 2. *Bacillus subtilis* strains used in this study.

Strain	Relevant characteristics	Source or reference
PY79	Prototrophic	Youngman *et al*. (1984)
PL85	PY79, *spoIIDΩ spoIID–lacZ*	Levin and Losick (1994)
RL905	PY79, SPβ[*spoIIAB–lacZ*]	Levin and Losick (1994)
RL1306	PY79, *usd-spoIIID–lacZ*	R. Losick
JH642	*trpC2 phe1*	J. Hoch
JH16840	JH642, *amyE*::*spoIIE–lacZ*	J. Hoch
JH16182	JH642, *amyE*::*spoIIG–lacZ*	J. Hoch
MO2051	JH642, *amyE*::*spoIIQ–lacZ*	Londoño-Vallejo *et al*. (1997)
BR151	Lys-3 metB10 trpC2	Young *et al*. (1969)
SL6418	BR151, *amyE*::*spoIIR–lacZ*	P. Piggot
1A577	*trpC2 Cer20*	BGSC[b]
GS77[a]	*trpC2 phe1 Cer20*	This work

a. The *cer20* mutation was introduced into JH642 by transformation with DNA of strain 1A577 and selection for Cer^R.
b. BGSC denote strain obtained from *Bacillus* Genetics Stock Center.

fusions to sporulation genes, β-galactosidase levels in sporulating cultures were determined as described (Mansilla and de Mendoza, 1997).

Enzyme preparation

Strains were grown to mid-exponential phase of growth in LB media (250 ml of cell culture); the cells were harvested by centrifugation and washed twice with 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl. Subsequent operations were carried out at 4°C. The washed cell pellets were resuspended in twice their wet weight in 100 mM potassium phosphate buffer pH 7.0 containing 1 mM EDTA, 1 mM β-mercaptoethanol and 10 mM phenylmethylsulphonyl fluoride and lysed in a French pressure cell at 18 000 psi. The lysate was centrifuged for 1 h at 100 000 × *g* using a 80 Ti rotor in a Beckman L8-70 ultracentrifuge. The supernatant fluid was removed, fractionated with ammonium sulphate, and the precipitate formed between 40% and 70% saturation was collected. The precipitated protein was removed by centrifugation. The protein pellet was dissolved in 1 ml of lysis buffer and dialysed against the same buffer.

Fatty acid synthase assays

Fatty acid synthase was measured *in vitro* by a modification of the assay described by Butterworth and Bloch (1970). Under the conditions described, product formation was linear with respect to time and protein concentration. The reaction mixture consisted of 0.5 mM NADP, 5 mM glucose-6-phosphate, 0.25 units of glucose-6-phosphate dehydrogenase, 15 μM of isobutyril-CoA, 20 μM malonyl-CoA, 0.4 μCi of [2-^{14}C]-malonyl-CoA (56.7 mCi mmol^{-1}), 10 μM *Escherichia coli* ACP, 100 mM sodium phosphate and 1.25 or 0.75 mg ml^{-1} protein from free-cell extracts of strains JH642 or GS77 respectively. Cerulenin was added to the assay tube in ethanol solution, preincubated at room temperature for 10 min, and the other assay components were added to the tube. The [2-^{14}C]-malonyl CoA was added last to start incubation at 37°C for 20 min followed by hydrolysis with 0.2 ml of 60% KOH (w/v) at 100°C for 30 min. After acidification the products were extracted into hexane and counted into vials containing scintillation solution to determine their radioactivity content. Specific activity is defined as pmoles [2-^{14}C]-malonyl-CoA incorporated into hexane-extractable fatty acids per mg of protein per min.

Metabolic labelling

To determine *de novo* fatty acid synthesis *in vivo*, strain JH642 was grown in SM medium at 37°C and labelled at the times indicated in Fig. 2 with 10 μCi of [^{14}C]-acetate (56.7 mCi mmol^{-1}) for 1 h. Cellular lipids were extracted (Grau and de Mendoza, 1993), saponified and the amount of [^{14}C]-acetate incorporated into long-chain fatty acids was determined as previously described (de Mendoza *et al*., 1983).

Immunoblot analysis

Strain JH642 was grown in DS medium grown at 37°C. At the end of exponential growth, the culture was split and cerulenin was added at a concentration of 10 μg ml^{-1} to one half of the culture. At the times indicated in Fig. 4 cells (20 ml) were harvested, washed with 5 ml of a buffer containing 100 mM Tris-HCl pH 7.5 and 150 mM NaCl and resuspended in 1 ml of breaking buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1 mM PMSF and 0.1 mM DTT). Cells were then disrupted by passage through a French pressure and the lysates centrifuged for 45 min at 190 000 × *g*. Each sample (100 μg of protein) was subjected to SDS–PAGE in a 12.5% acrylamide gel. Proteins were electroeluted to a nitrocellulose membrane and revealed using anti-σ^E monoclonal antibody and a secondary antibody conjugated to alkaline phosphatase.

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