Effects on *Bacillus subtilis* of conditional expression of the *accBC* operon encoding subunits of acetyl coenzyme A carboxylase, the first enzyme of fatty acid synthesis

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A *Bacillus subtilis* strain was constructed in which the operon *accBC*, encoding the biotin carboxyl carrier protein (BCCP) and biotin carboxylase (BC) subunits of acetyl-CoA carboxylase (ACC), was placed under the control of the IPTG-inducible promoter *spac*. A reduction in the levels of BCCP resulted in a decrease of *de novo* fatty acid synthesis and in the total content of membrane fatty acids. This strain was dependent upon the presence of IPTG for a normal growth phenotype. Growth was specifically restored by supplying exogenous long branched-chain fatty acids in the medium, indicating that the inducer-dependent phenotype was specifically related to a conditional block in fatty acid biosynthesis. The strain showed a strong decrease in sporulation frequency when it was induced to sporulate in an IPTG-free medium. Germination and outgrowth were both delayed in spores of the mutant obtained in the absence of IPTG.

Keywords: *Bacillus subtilis*, fatty acid synthesis, acetyl-CoA carboxylase, cell cycle

INTRODUCTION

Acetyl-CoA carboxylase (ACC, EC 6.4.1.2) plays a pivotal role in the biosynthesis of fatty acids. It catalyses the first committed step of fatty acid synthesis, the synthesis of malonyl-CoA. The overall reaction consists of two distinct half-reactions, the carboxylation of biotin with bicarbonate and the transfer of the CO₂ group from carboxy-biotin to acetyl-CoA to form malonyl-CoA. In *Escherichia coli*, the half-reactions are catalysed by two different ACC subunits, biotin carboxylase (BC) and carboxyl transferase (CT). A third subunit, biotin carboxyl carrier protein (BCCP), carries the essential biotin cofactor covalently bound to a lysine residue proximal to its carboxyl terminus (Li & Cronan, 1992a, b; Magnuson *et al.*, 1993).

We have recently cloned and characterized the *Bacillus subtilis* genes encoding two subunits of ACC, BCCP and BC (Marini *et al.*, 1995). The *B. subtilis accB* gene, which encodes BCCP, is part of an operon that includes *accC*, the gene encoding the BC subunit of ACC. We also found that the 17.2 kDa BCCP subunit from *B. subtilis* is able to interact with other subunits of the *E. coli* ACC as

well as the *E. coli* biotin apoprotein ligase that biotinylates BCCP (Marini *et al.*, 1995).

Since ACC catalyses the first committed step of fatty acid synthesis, it has been the subject of intensive study (Volpe & Vagelos, 1976; Wakil *et al.*, 1983). Several mechanisms have been proposed for the regulation of mammalian ACC. These include allosteric regulation by citrate and isocitrate (López-Casilla *et al.*, 1987), feed-back inhibition by the end product, long chain acyl-CoA (Nikawa *et al.*, 1979), and modification by phosphorylation and dephosphorylation (Kim, 1983). Recently, regulation of ACC at the level of gene expression has also been demonstrated in mouse preadipocytes (Luo & Kim, 1990). Unlike its eukaryotic counterpart, almost no information is available on the regulation of ACC in *E. coli* and other prokaryotes (Magnuson *et al.*, 1993; Rock & Cronan, 1996). Physiological studies to determine the rate-limiting step in *E. coli* phospholipid biosynthesis have led to the suggestion that ACC is the controlling step (for a review see Magnuson *et al.*, 1993). Furthermore, recent studies indicate that the *E. coli* ACC is regulated by growth rate (Li & Cronan, 1993).

In bacteria, fatty acids are primarily precursors of phospholipids rather than storage fuels, and so ACC activity is coordinated with cell growth and division. *B.*

Abbreviations: ACC, acetyl-CoA carboxylase; BCCP, biotin carboxyl carrier protein; BC, biotin carboxylase; CT, carboxyl transferase.

subtilis, in addition, in response to conditions of nutrient limitation enters a developmental process that culminates in the formation of spores (for a review see Errington, 1993). Spore formation in this organism (and also spore germination and outgrowth) constitutes a differentiation process that has long been appreciated as a model for investigation of prokaryotic development (for reviews see Errington, 1993; Foster & Johnstone, 1990; Moir & Smith, 1990). This cellular life cycle involves a variety of biochemical and physiological events, including membrane differentiation accompanied by phospholipid synthesis (Piggot *et al.*, 1994), that must start with the reaction catalysed by ACC. This feature, combined with the fact that ACC has been postulated as a primary site for regulation of lipid synthesis (Magnuson *et al.*, 1993), suggests that its activity could be important during sporulation, providing a link between intracellular lipid levels and the differentiation process. However, the study of the role of ACC in the *B. subtilis* cell cycle is hampered by the fact that the activity of this enzyme is probably essential for growth and that viable mutants of ACC are not yet available. As a first step in the study of the role(s) ACC plays in the cell, the *B. subtilis* *accBC* operon was placed under the control of the IPTG-inducible promoter *Pspac*. This mutant was used to analyse the effect of conditional expression of *accBC* on growth and differentiation.

METHODS

Bacterial strains and plasmids. *B. subtilis* was propagated in DS medium (Schaeffer *et al.*, 1965). The parental strain was JH642 (*trpC2 pheA1*). Strain DL2, carrying the integrated plasmid that introduces the *accBC* operon under the control of the *spac* promoter, was obtained by transformation as described by Spizizen (1958). This strain was grown in medium containing 5 µg chloramphenicol ml⁻¹ (final concentration). Plasmid pDH88, suitable for placing genes under control of the inducible *spac* promoter in *B. subtilis*, was described by Henner (1990). Plasmid pHLacI, containing the *lacI* gene under the control of the *Bacillus licheniformis* penicillinase transcriptional and translational control signals (Henner, 1990) on the multicopy plasmid pHT1030 (Arantes & Lereclus, 1991), was the generous gift of T. Msadek (Unité de Biochimie Microbienne, Institut Pasteur, Paris, France).

DNA methods. Plasmid pDL1 (Fig. 1) was constructed as follows. Firstly the oligomers 5'-AATTTCAAGCTTAGGA-GTGCGATTGAAATGTTAAATATC-3' and 5'-AAGCTT-CATGGATCCTACAATGCAGACAACTGTGTTTTTC-3' were synthesized so that PCR could be used to amplify a chromosomal segment of DNA containing a promoterless 397 bp fragment of *accBC* (coordinates 320–717 in Marini *et al.*, 1995). This fragment contains 26 nucleotides preceding the *accBC* initiation codon, which includes the natural ribosome-binding site (RBS), and extends through the first 124 of the 159 codons of the *accB* gene. Oligomers were synthesized so that the 397 bp product contained a *Hind*III site preceding the *accB* RBS and a *Bam*HI site close to the end of the fragment. The PCR product was cloned into the *Hind*III–*Bgl*II sites of pDH88, giving rise to pDL1 (Fig. 1).

Total DNA was isolated from *B. subtilis* strains grown in LB medium (Sambrook *et al.*, 1989) as described by Gardiol *et al.* (1993). Southern blots were performed applying stringent hybridization and washing conditions (Sambrook *et al.*, 1989).

An internal 300 bp *Eco*RI–*Hind*III fragment of *accB* (see Fig. 1 and Marini *et al.*, 1995) labelled with ³²P by the random priming method (Sambrook *et al.*, 1989) was used as a DNA probe.

Detection of BCCP. Cells were grown in DS medium and labelled with [³H]biotin as previously described (Marini *et al.*, 1995). After harvesting and disrupting the cells, the proteins were separated by SDS-PAGE and biotinylated BCCP was detected by fluorography (Marini *et al.*, 1995).

Fatty acid synthesis *in vivo*. *B. subtilis* cultures were grown to OD₆₀₀ 0.5 in DS medium and then labelled with 2.5 µCi (92.5 kBq) of either [¹⁴C]acetate (56.7 mCi mmol⁻¹; 2.1 GBq mmol⁻¹) or [¹⁴C]isoleucine (342 mCi mmol⁻¹; 12.7 GBq mmol⁻¹) for 1 h as previously described (Grau & de Mendoza, 1993; Grau *et al.*, 1994). Cellular lipids were extracted from whole cells, saponified, and the amount of [¹⁴C]acetate incorporated into long-chain fatty acids was determined as previously described (Grau & de Mendoza, 1993; Grau *et al.*, 1994; de Mendoza *et al.*, 1983).

Fatty acid supplementation studies and fatty acid analysis. Fatty acid supplementation studies and fatty acid analysis were performed with cells grown in the mineral salts medium of Spizizen (1958) supplemented with glucose (0.5%), casein hydrolysate (0.1%) and tryptophan (40 µg ml⁻¹). For fatty acid supplementation studies the growth media contained 0.075% Brij 58. The different fatty acids were added at a final concentration of 16 µg ml⁻¹. For fatty acid analysis cell pellets were lyophilized, resuspended in 1 ml 5% (v/v) HCl in methanol and heated for 2.5 h at 85 °C; the resulting fatty acid methyl esters were extracted as described elsewhere (Grau & de Mendoza, 1993; Grau *et al.*, 1994). Fatty acid methyl esters were analysed by gas chromatography using a Konik KNK-3000 gas chromatograph. Fatty acid methyl esters were separated isothermally at 180 °C using an HP-FFAP column (25 m × 0.2 mm internal diameter; Hewlett Packard). The injection port and detector temperature were operated at 250 and 300 °C respectively. Fatty acid methyl esters were identified by comparing their retention times with those of standards of authentic branched-chain and straight-chain fatty acids (Sigma). Total fatty acids were quantified by comparison with a known amount of decanoic acid added prior to methanolysis.

Spore formation and germination. The GMP synthetase inhibitor decoyinine was added to a final concentration of 1.0 mg ml⁻¹ to initiate sporulation in S7 medium (Nicholson & Setlow, 1990). Spore titres were determined by heating dilutions of the culture to 80 °C for 20 min and plating on LB medium supplemented with 1 mM IPTG. For assays of spore germination, *B. subtilis* strains JH642 and DL2 were induced to sporulate in DS medium and the resultant spores washed as described by Nicholson & Setlow (1990). Immediately prior to germination, spore suspensions were heat-activated at 70 °C for 60 min and cooled on ice (Nicholson & Setlow, 1990; Venkatasubramanian & Johnstone, 1993). Germination of spores was induced in two systems as follows: (i) the ALA system, in which the spores were germinated for 80 min at 37 °C in 50 mM Tris/HCl, pH 8.0, by the addition of 1 mM (final concentration) L-alanine; (ii) the GFAK system, in which the spores were germinated for 80 min at 37 °C in 50 mM Tris/HCl, pH 8.0, by the addition of 5.6 mM glucose, 5.6 mM fructose, 30 mM L-asparagine and 67 mM KCl. The extent of germination in spore suspensions was determined as the percentage of the control loss of OD₆₀₀ and the rate of germination was measured as the maximum rate of loss of OD₆₀₀ min⁻¹ divided by the initial OD₆₀₀, as described by

Venkatasubramanian & Johnstone (1993). Outgrowth studies were carried out in enriched media supplemented with glucose and L-alanine (Venkatasubramanian & Johnstone, 1993).

RESULTS

Construction of a strain with the *accBC* operon under control of the *spac* promoter

We have recently reported that the *accB* and *accC* genes encoding the BCCP and BC subunits of ACC form part of a single-copy operon located 220° downstream of *spoIIIA* on the *B. subtilis* chromosome (Marini *et al.*, 1995). Many attempts to interrupt either the *accB* or the *accC* gene by a simple crossover recombination event using integrative plasmids containing internal fragments of each gene were unsuccessful. Our failure to interrupt *accBC* in the chromosome of *B. subtilis*, combined with the fact that ACC catalyses the first committed step in fatty acid (and hence lipid) synthesis, suggests an essential role for these gene products. In order to test this possibility, the single chromosomal copy of the *accBC* operon (Marini *et al.*, 1995) was placed under *spac* promoter control. To do this, a fragment containing the 5' end of the *accB* gene (Fig. 1) was cloned along with the *spac* promoter and the *lacI* gene into an integrational plasmid vector to give pDL1. Transformation of *B. subtilis* JH642 with this plasmid only yielded transformants in the presence of IPTG. One of these transformants was designated DL2. The integration of plasmid pDL1 by Campbell insertion into DL2 placed the single copy of *accBC* downstream from the *spac* promoter (Fig. 1). The integration of pDL1 by this event was confirmed by Southern blot hybridization experiments. As shown in Fig. 2, in an *EcoRI* digest of wild-type DNA, *accB* and part of *accC* are located in a single 1.32 kbp fragment. In strain DL2, the fragment was split into a 6.5 kb fragment carrying the truncated gene of *accB* and a 1.3 kb fragment that was joined to the *spac* promoter (Fig. 2).

Growth characteristics of DL2 and effects of BCCP depletion on lipid synthesis

As shown in Fig. 3, normal growth of strain DL2 depended on the presence of IPTG. In the absence of IPTG, cell growth was strongly affected. Growth returned to the normal level upon addition of 1 mM IPTG, which derepresses the expression of *accB*. However, when cultures were inoculated at initial OD₆₀₀ 0.1–0.2, growth of strain DL2 reached stationary phase in DS medium without IPTG after about 24 h at 37 °C. This growth was probably due to leakiness of the control system (Beall & Lutkenhaus, 1991; Micka *et al.*, 1991) since strain DL2 transformed with pH*lacI*, a high-copy-number plasmid containing the *lacI* gene, was unable to grow in the absence of IPTG (Fig. 3). The addition of 1 mM IPTG reestablished the growth of this strain to normal levels (data not shown).

To determine the effect of IPTG on the BCCP content, we exploited the fact that this 17.2 kDa biotinylated

subunit of ACC can be easily detected in crude cell extracts previously labelled with [³H]biotin (Marini *et al.*, 1995). To this end, strain DL2 was labelled with biotin in medium supplemented or unsupplemented with IPTG and crude cell extracts were examined by SDS-PAGE and autoradiography. The levels of BCCP of strain DL2 growing exponentially in the presence of 1 mM IPTG (Fig. 4a, lane 1) were comparable with the wild-type levels (Fig. 4a, lane 3). However, in the absence of the inducer the BCCP content of strain DL2 decreased to about 15% of the levels observed in IPTG-supplemented cells (Fig. 4a, lane 2; Fig. 4b). In addition to BCCP, *B. subtilis* contains an 85 kDa biotinylated protein (Marini *et al.*, 1995). The biotinylation level of this protein was comparable in strain DL2 growing in media with or without IPTG (Fig. 4a, lanes 1–3), showing that the lack of IPTG specifically affects the synthesis of BCCP rather than the transport of biotin or the activity of the biotin apoprotein ligase.

To determine the effect of BCCP depletion on total fatty acid synthesis, we analysed the synthesis of radioactive fatty acids in cultures of strain DL2 growing exponentially in the presence of [¹⁴C]acetate in media supplemented or unsupplemented with 1 mM IPTG. In the absence of IPTG we observed about 90% inhibition of the incorporation of [¹⁴C]acetate into the phospholipids of strain DL2 (8628 c.p.m. in the presence of IPTG, 489 c.p.m. in its absence). Similar results were obtained when cells were labelled with [¹⁴C]isoleucine (21372 c.p.m. in the presence of IPTG, 853 c.p.m. in its absence); isoleucine is the precursor of 12-methyltetradecanoic acid (anteiso-15), one of the major branched-chain fatty acids of *B. subtilis* (de Mendoza *et al.*, 1993). All these results suggest an essential role of the *accBC* operon gene products for growth in *B. subtilis*. They also show that the fully induced *spac* promoter could provide sufficient BCCP and BC for fatty acid synthesis and normal growth.

The growth defect of DL2 is corrected in the presence of long branched-chain fatty acids

The above results indicated that the mutant strain DL2 has a conditional decreased synthesis of the BCCP and BC subunits of ACC. Since ACC catalyses the synthesis of malonyl-CoA, the primer for the elongation step of fatty acids by the condensing enzyme(s) (de Mendoza *et al.*, 1993; Magnuson *et al.*, 1993; Rock & Cronan, 1996), we investigated whether this defect could be corrected by growing DL2 in the presence of long branched-chain fatty acids. Strain DL2 grew poorly in an IPTG-free medium, reaching only OD₆₀₀ 0.5 after 7 h growth (Fig. 3). However, when the medium was supplemented with different long branched-chain fatty acids, growth was restored to the level of IPTG-supplemented cultures (Fig. 3). The growth of the mutant was slightly stimulated by the straight-chain fatty acids decanoic acid or palmitic acid, but the addition of palmitoleic acid, a straight-chain unsaturated fatty acid resulted in cell lysis (Fig. 3).

Fig. 1. Construction of IPTG-dependent *B. subtilis* strain DL2, with the *accBC* operon under *spac* promoter control. Plasmid pDL1, which contains the RBS and the first 124 codons of the *accBC* gene downstream of the *spac* promoter, was used to transform wild-type strain JH642 to Cm^R in the presence of 1.0 mM IPTG to give strain DL2. Campbell insertion of this plasmid places the *accBC* operon under *spac* promoter control. The black bar above the chromosomal copy of *accB* denotes the restriction fragment used to confirm the integration of pDL1 by Southern blot hybridization, as shown in Fig. 2. Only the position of the *EcoRI* sites in the *accBC* operon is indicated.

Fig. 2. Characterization of *B. subtilis* DL2 by Southern hybridization. Chromosomal DNA of *B. subtilis* wild-type (lane 1) and DL2 (lane 2) was digested with *EcoRI*. The cleaved product was separated on a 0.8% agarose gel, transferred to nitrocellulose and hybridized with a probe specific for *accB* (Fig. 1). The positions of the *accBC* genes in the wild-type (1.3 kb) and in strain DL2, containing the truncated copy (6.5 kb) and the *spac accBC* fusion (1.3 kb), are indicated.

Fatty acid content of DL2

Table 1 shows the fatty acid composition of strain DL2 grown without supplements or in the presence of either IPTG or 13-methyltetradecanoic acid (iso-15:0). The most abundant fatty acid in strain DL2 grown with

Fig. 3. Effects of IPTG and various fatty acids on growth of *B. subtilis* DL2 bearing the *Pspac-accBC* fusion. Cultures of strain DL2 were grown with 5 µg chloramphenicol ml⁻¹ in non-supplemented medium (●) or medium supplemented with IPTG (△), decanoic acid (▽), palmitic acid (×), palmitoleic acid (■), iso-15 acid (◆), anteiso-15 acid (○) or iso-17 acid (+). ◇, DL2 transformed with the plasmid *pHlaI* (see Methods) grown in a non-supplemented medium.

IPTG was iso-15:0, which constitutes 38% of total fatty acids (Table 1). The mutant strain growing without IPTG had a reduced level of iso-15:0 and increased levels of 14-methylpentadecanoic acid (iso-16:0) and 15-methylhexadecanoic acid (iso-17). The different fatty acid composition obtained in cells with decreased intracellular levels of malonyl-CoA could be due to the relative affinities of the condensing enzyme(s) for the different branched-chain primers and malonylacyl-carrier protein, the chain extender (de Mendoza *et al.*, 1993). In lipids isolated from the mutant strain supple-

Fig. 4. Characterization of *B. subtilis* DL2 bearing the *Pspac-accBC* fusion. (a) Effect of IPTG on synthesis of biotinylated BCCP. Strain DL2 growing with IPTG (lane 1) or without IPTG (lane 2), or the wild-type strain (lane 3), was grown for several generations at 37 °C in D5 medium in the presence of [3 H]biotin as described in Methods. The proteins were separated by SDS-PAGE (15%, w/v, acrylamide) and the bands were detected by fluorography. (b) Densitometric tracing of the BCCP radioactive bands pictured in lanes 1 and 2, respectively, of (a).

mented with iso-15:0, the fraction of this fatty acid increased about sixfold (Table 1). This result indicates that iso-15:0, a fatty acid that restores normal growth of strain DL2 (Fig. 3), is incorporated into membrane

phospholipids of the mutant strain. It should be noted that the total content of fatty acids of strain DL2 growing in an IPTG-free medium was about 30% of the total fatty acid content of cells grown in a medium supplemented with IPTG or with iso-15:0. In Table 1 it is shown that the ratio of iso to anteiso acyl chains is increased in strain DL2 (growing with or without IPTG) when compared to the wild-type strain JH642. The different fatty acid composition observed in DL2 could be due to the fact that in this strain the transcription of *accBC* is unregulated. In *E. coli* the transcription of the *accBC* operon is tightly regulated, possibly by a positive activator (Li & Cronan, 1993).

Sporulation and germination of DL2

We determined that fatty acid synthesis is required for spore formation when sporulation is induced with decoyinine in the early exponential phase of growth. Typically, the sporulation efficiency was between 50% and 70% (usually corresponding to approximately 3×10^7 spores ml^{-1}) for the wild-type strain JH642 or for DL2 growing in the presence of IPTG. The sporulation frequency of strain DL2 was typically reduced 100-fold, with no effect on cell viability, when IPTG was omitted from the sporulation medium. These results show that under these assay conditions inhibition of fatty acid synthesis dramatically reduces spore formation.

Spores of *B. subtilis* can be induced to germinate by incubation in L-alanine (the ALA pathway) or in a combination of glucose, fructose, L-asparagine and K^+ (the GFAK pathway) (Foster & Johnstone, 1989, 1990). As shown in Fig. 5, when germination was measured by monitoring the decrease in OD_{600} of a spore suspension upon the addition of 10 mM L-alanine, spores of strain DL2 obtained without IPTG responded to the germinant. However, the OD_{600} values of the spore suspension decreased more slowly compared either with spores of DL2 obtained with IPTG or with the wild-type strain, as shown in Fig. 5. Similar results were obtained when germination was measured in the GFAK pathway

Table 1. Effect of growth supplement on the fatty acid content of *B. subtilis* strain DL2

Cells were grown in the media described in Methods at 37 °C and harvested at OD_{600} 0.5.

Strain	Supplement	Percentage (w/w) of fatty acid type*						Relative amount of fatty acids (%)†	Ratio iso:anteiso
		iso-15	anteiso-15	iso-16	n-16	iso-17	anteiso-17		
DL2	IPTG	38	18	15	14	2	10	112	1.96
DL2	—	4	24	33	14	14	9	39	1.54
DL2	Iso-15	25	17	27	14	2	11	118	1.92
JH642	—	18	40	14	14	11	2	100	1.0

* Data are the means of at least three experiments with the mean error in percentage values of fatty acids being less than 5%. The values are expressed as percentage of total fatty acid methyl esters analysed.

† Total cellular fatty acid was measured as described in Methods, using decanoic acid as an internal standard. Amounts are related to that in wild-type strain JH642 (taken as 100%).

Fig. 5. Germination of spores of DL2 obtained in the presence or in the absence of IPTG in the ALA system. JH642 spores (□) or DL2 spores obtained with IPTG (○) or without IPTG (●) were treated with L-alanine and the percentage of initial OD₆₀₀ determined as described in Methods.

Fig. 6. Outgrowth of spores of DL2 obtained in the presence or in the absence of IPTG. □, ■, Spores obtained with 1 mM IPTG; ○, ●, spores obtained without IPTG. Spores were germinated either in medium supplemented with 1 mM IPTG (■, ●) or in medium devoid of IPTG (□, ○).

(data not shown). Lineweaver–Burk plots of L-alanine concentration against germination rate (Venkatasubramanian & Johnstone, 1993) indicated that spores of strain DL2 obtained without IPTG have a K_m of 91 μ M for the germinant, while the K_m of spores obtained with IPTG was 56 μ M (data not shown). This suggests that spores produced during conditions of limited lipid synthesis possess a receptor responding to L-alanine in the ALA pathway with a decreased affinity for the ligand that triggers spore germination.

Outgrowth studies were carried out in medium supplemented with glucose and L-alanine. In this medium, spores of strain DL2 obtained in media supplemented

with IPTG showed the expected drop in OD₆₀₀ for the first hour of incubation (Fig. 6). After about 2 h at 37 °C, the OD₆₀₀ of DL2 cultures began to rise; further growth was dependent on the presence of IPTG (Fig. 6). On the other hand, the rise in OD₆₀₀ that followed the germination of spores obtained in an IPTG-free medium was significantly delayed when compared with spores formed in the presence of the inducer, irrespective of whether the growth medium was supplemented with IPTG (Fig. 6).

DISCUSSION

Although *B. subtilis* is genetically the best-characterized Gram-positive organism, it has not been fully exploited for lipid metabolism and membrane research, and only two classes of mutants defective in branched-chain fatty acids have been reported (for a review see de Mendoza *et al.*, 1993). One mutant (Willecke & Pardee, 1971) lacks the ability to synthesize branched-chain fatty acids because of a deficiency of the branched-chain 2-ketoacid dehydrogenase (*bfmA*). This mutant can grow on a wide variety of low-molecular-mass branched and cyclic fatty acids, but it does not sporulate because it is also deficient in pyruvate dehydrogenase activity (Willecke & Pardee, 1971). To investigate the effects of membrane changes on growth and sporulation in a strain having normal pyruvate dehydrogenase activity, Boudreaux *et al.* (1981) isolated a *bfmB* mutant that results in an acyl-CoA:ACP transacylase with low affinity for branched acyl-CoA precursors. The consequence of this mutation is a growth requirement for a branched-chain fatty acid precursor (Boudreaux *et al.*, 1981). However, in our hands the *bfmB* mutant did not show the reported phenotype and precluded any manipulation to study lipid synthesis during growth and sporulation. Thus, studies aimed at identifying specific functions of branched-chain fatty acids have been difficult because of a lack of experimental models that allow the manipulation of fatty acids during the cell cycle. With the aim of studying the role of fatty acid synthesis in growth and differentiation in *B. subtilis*, we constructed a strain bearing a copy of the *accBC* operon under the control of the *spac* promoter. Cells containing the *Pspac-accBC* fusion had reduced BCCP levels and grew much more slowly than the same cells in the presence of 1 mM IPTG. The reduction in the levels of BCCP drastically reduced *de novo* fatty acid synthesis and the total content of cellular fatty acids (Table 1). The growth of DL2 was restored by supplying exogenous long-chain fatty acids in the medium, indicating that the inducer-dependent phenotype was specifically related to a conditional block in fatty acid biosynthesis. In agreement with the fact that iso and anteiso branched-chain fatty acids are the dominant acyl components of the lipids of *B. subtilis* (de Mendoza *et al.*, 1993), the growth of DL2 was specifically stimulated by these fatty acids (Fig. 3). While the growth of the mutant was slightly stimulated by decanoic or palmitic acid, which are straight-chain saturated fatty acids, the addition of palmitoleic acid, a straight-chain unsaturated fatty acid,

resulted in cell lysis (Fig. 3). These results suggest that straight-chain fatty acids are not incorporated efficiently into *B. subtilis* membrane phospholipids or that branched-chain fatty acids may play some specific role that can not be replaced by straight-chain fatty acids.

There are several lines of evidence indicating that iso and anteiso acyl chains play the biological role of straight saturated and unsaturated acyl chains, respectively, in a membrane containing large amounts of these compounds (de Mendoza *et al.*, 1993). However, although the ratio of iso to anteiso acyl chains in DL2 (growing with IPTG) is significantly higher than the wild-type strain JH642, these strains do not show a difference in growth rate. Thus it is evident that *B. subtilis* can grow normally despite the fact that more than 50% of the fatty acids of the membrane lipids are iso fatty acids.

DL2 cells growing without IPTG showed a marked decrease in iso-15 fatty acid content, with a concomitant increase in iso-16 and iso-17 fatty acids, while the percentage of anteiso fatty acids remained almost unchanged. Since the ratio of iso to anteiso acyl chains only decreases about 25% in the absence of IPTG (Table 1) it seems that the major consequence of reducing the ACC levels is to decrease the total amount of membrane fatty acids rather than to change the physical state of the membrane phospholipid acyl chains. It is worth noting that although most cells maintain a constant ratio of proteins (both whole cell and membrane), the synthesis of membrane proteins is not coupled to phospholipid synthesis (for a review see Cronan & Rock, 1987). For example, by using *E. coli* mutants blocked in the first step of phospholipid synthesis, McIntyre & Bell (1975) found that both the inner membrane and the outer membrane became enriched with protein. This work demonstrates that the membrane is not normally saturated with protein and that the synthesis of membrane phospholipids is not required for the synthesis and insertion of bulk membrane proteins. In addition the protein-enriched membranes were not lethal to the cell, and the enrichment was quickly normalized upon the restoration of phospholipid synthesis (McIntyre *et al.*, 1977). Taking into account these considerations, *B. subtilis* cells growing with a limited supply of fatty acids (and hence phospholipids) should have membranes with an increased ratio of protein to phospholipids.

IPTG starvation with the *Pspac-accBC* fusion strongly suggested that the *accBC* operon was essential during exponential growth, although the mutant could reach stationary phase after about 24 h incubation at 37 °C. The reduced growth in the absence of IPTG is probably due to leakiness of the inducible *spac* promoter since supplying the LacI repressor *in trans* totally abolished the growth of strain DL2 in the absence of the inducer (Fig. 3).

The major changes associated with membrane differentiation during spore formation in *B. subtilis* are (i) the formation of an asymmetric sporulation septum that gives rise to a sporangium containing unequal progeny

called the prespore and the mother cell and (ii) the process of engulfment in which these two cell types collaborate in the conversion of the postseparation sporangium to a cell-within-a-cell structure (for recent reviews see Errington, 1993; Stragier & Losick, 1996). Although little is understood about the molecular mechanism of these morphological changes (Stragier & Losick, 1996) it is assumed that these differentiation processes require abundant *de novo* lipid synthesis and membrane components (Feucht *et al.*, 1996; Piggot *et al.*, 1994; Stragier & Losick, 1996). To determine the effect of a deficiency in fatty acid supply on sporulation we used the drug decoyinine, which induces spore formation by inhibiting GTP biosynthesis (Mitani *et al.*, 1977). In a defined medium with decoyinine as inducer of sporulation, the mutant strain sporulated poorly without IPTG. These results are consistent with a requirement of active lipid synthesis for spore formation. Clearly, it remains to be determined which stage of sporulation is blocked when there is a reduced rate of fatty acid synthesis.

Transformation of a dormant resistant bacterial endospore into a metabolically active vegetative cell involves a complex series of interrelated biochemical events (for reviews see Foster & Johnstone, 1989, 1990; Moir & Smith, 1990). This germination response is triggered in spores by germinants. However, the mechanism by which the germinants interact with spore components to initiate germination is not known (Foster & Johnstone, 1989, 1990; Moir & Smith, 1990). It has been proposed that alanine acts as a germinant by penetrating the spore as far as the outer surface of the inner membrane, where it interacts with a receptor complex; this interaction results in the transduction of the germination stimulus to initiate a cascade of subsequent hydrolytic reactions (Moir & Smith, 1990). Several lines of evidence indicate that the putative receptor is in a hydrophobic environment and it has been suggested that spore germination could be dependent on membrane lipid composition (Foster & Johnstone, 1989). We show here that spores formed by the mutant strain DL2 in the presence of IPTG germinated just as well as the wild-type in the ALA and the GFAK systems. However, the germination of spores obtained in a medium devoid of IPTG was significantly delayed in these two germination systems, supporting the proposal that part of the commitment process appears to be membrane-associated (Foster & Johnstone, 1989; Nicholson & Setlow, 1990). In addition, we found that the L-alanine receptor has decreased affinity for its ligand under conditions of limited fatty acid synthesis. However, this result does not explain the slower germination rate of spores obtained under conditions of lipid starvation, since spore germination was assayed at saturating germinant concentrations. Thus, a shortage of fatty acids should affect other components of the cascade events comprising the germination response (Foster & Johnstone, 1990).

We also show here that spores formed without IPTG initiated outgrowth much more slowly than spores

formed in the presence of IPTG, irrespective of the presence of IPTG in the spore germination medium (Fig. 6). These results suggest that membrane lipids could play an important role in the initiation of outgrowth of germinating spores.

The decreased synthesis of fatty acids might modify the chemical nature of the membrane, thereby producing the observed phenotypes in the *B. subtilis* cell cycle. Alternatively, the phenotypes may be an indirect and possibly pleiotropic consequence of a sick cell that fails to assemble important membrane components necessary for differentiation. Therefore, ACC activity seems to be an essential component of spore formation and is also required for the germination process, as well as to initiate outgrowth of germinating spores.

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