

Spo0A links *de novo* fatty acid synthesis to sporulation and biofilm development in *Bacillus subtilis*

María E. Pedrido,[†] Paula de Oña,[†] Walter Ramirez, Cecilia Leñini, Anibal Goñi and Roberto Grau*

Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, CONICET-Argentina.

Summary

During sporulation in *Bacillus subtilis*, the committed-cell undergoes substantial membrane rearrangements to generate two cells of different sizes and fates: the mother cell and the forespore. Here, we demonstrate that the master transcription factor Spo0A reactivates lipid synthesis during development. Maximal Spo0A-dependent lipid synthesis occurs during the key stages of asymmetric division and forespore engulfment. Spo0A reactivates the *accDA* operon that encodes the carboxylase component of the acetyl-CoA carboxylase enzyme, which catalyses the first and rate-limiting step in *de novo* lipid biosynthesis, malonyl-CoA formation. The disruption of the Spo0A-binding box in the promoter region of *accDA* impairs its transcriptional reactivation and blocks lipid synthesis. The Spo0A-insensitive *accDA*^{0A} cells were proficient in planktonic growth but defective in sporulation (σ^E activation) and biofilm development (cell cluster formation and water repellency). Exogenous fatty acid supplementation to *accDA*^{0A} cells overcomes their inability to synthesize lipids during development and restores sporulation and biofilm proficiencies. The transient exclusion of the lipid synthesis regulon from the forespore and the known compartmentalization of Spo0A and ACP in the mother cell suggest that *de novo* lipid synthesis is confined to the mother cell. The significance of the Spo0A-controlled *de novo* lipid synthesis during *B. subtilis* development is discussed.

Introduction

Forty years have passed since the formulation of the 'fluid mosaic model' to interpret the structure and biochemistry of

a biological membrane (Singer and Nicolson, 1972). However, how cells build a functional membrane remains one of the most captivating mysteries in cellular biology (Engelman, 2005; Lee, 2005). In organisms ranging from bacteria to humans, membranes undergo changes and remodelling as the cell cycle progresses and as differentiated cell types are established (Schneider and Kohlwein, 1997; Emoto and Umeda, 2001; Jahn *et al.*, 2003; Burton *et al.*, 2007). At the microscopic scale, the two main components of a biological membrane, lipids and proteins, must be correctly packed, and they must interact with each other to create a functional membrane (Matsumoto *et al.*, 2006; Bogdanov *et al.*, 2008; Dowhan and Bogdanov, 2009; 2011). However, the role of lipids in membrane homeostasis and development still remains poorly understood (Schneider and Kohlwein, 1997; Jahn *et al.*, 2003; Engelman, 2005; Lee, 2005; Mileykovskaya and Dowhan, 2005; Choad, 2008).

In this context, spore formation in the Gram-positive bacterium *Bacillus subtilis* provides a useful model for investigating the formation of new membranes and their remodelling during development (Stragier and Losick, 1996; Errington, 2003; Hilbert and Piggot, 2004; Higgins and Dworkin, 2012). As illustrated in Fig. 1A, complex and remarkable membrane lipid-associated morphogenetic rearrangements (e.g. the biogenesis, migration, curvature, engulfment, fusion and fission of membranes) accompany the development of a bacterial spore (Errington, 2003; Higgins and Dworkin, 2012). Concurrently with these dynamic membrane remodelling events, different critical cell fate determinants become asymmetrically distributed on the sporulation membranes (Ramamurthi and Losick, 2005; 2009). Sporulation is triggered by starvation-related signals that activate, by phosphorylation, the master regulatory protein Spo0A. During the initial developmental stages, the formation of an asymmetrically positioned septum delimited by two membranes of opposite topology creates a small and specifically localized cell, which is known as the forespore, as well as a larger cell, which is called the mother cell (Fig. 1A, T₀–T_{II}) (Losick and Stragier, 1992; Stragier and Losick, 1996). During sporulation, the polar septum is formed prior to the incorporation of a complete chromosome into the forespore. Approximately, one-third of the forespore-destined chromosome is trapped in the forespore at the time of polar septum forma-

Accepted 13 November, 2012. *For correspondence. E-mail robertograu@fulbrightmail.org; Tel. (+54) 341 4353377; Fax (+54) 341 4804592/3. [†]These authors contributed equally to this work.

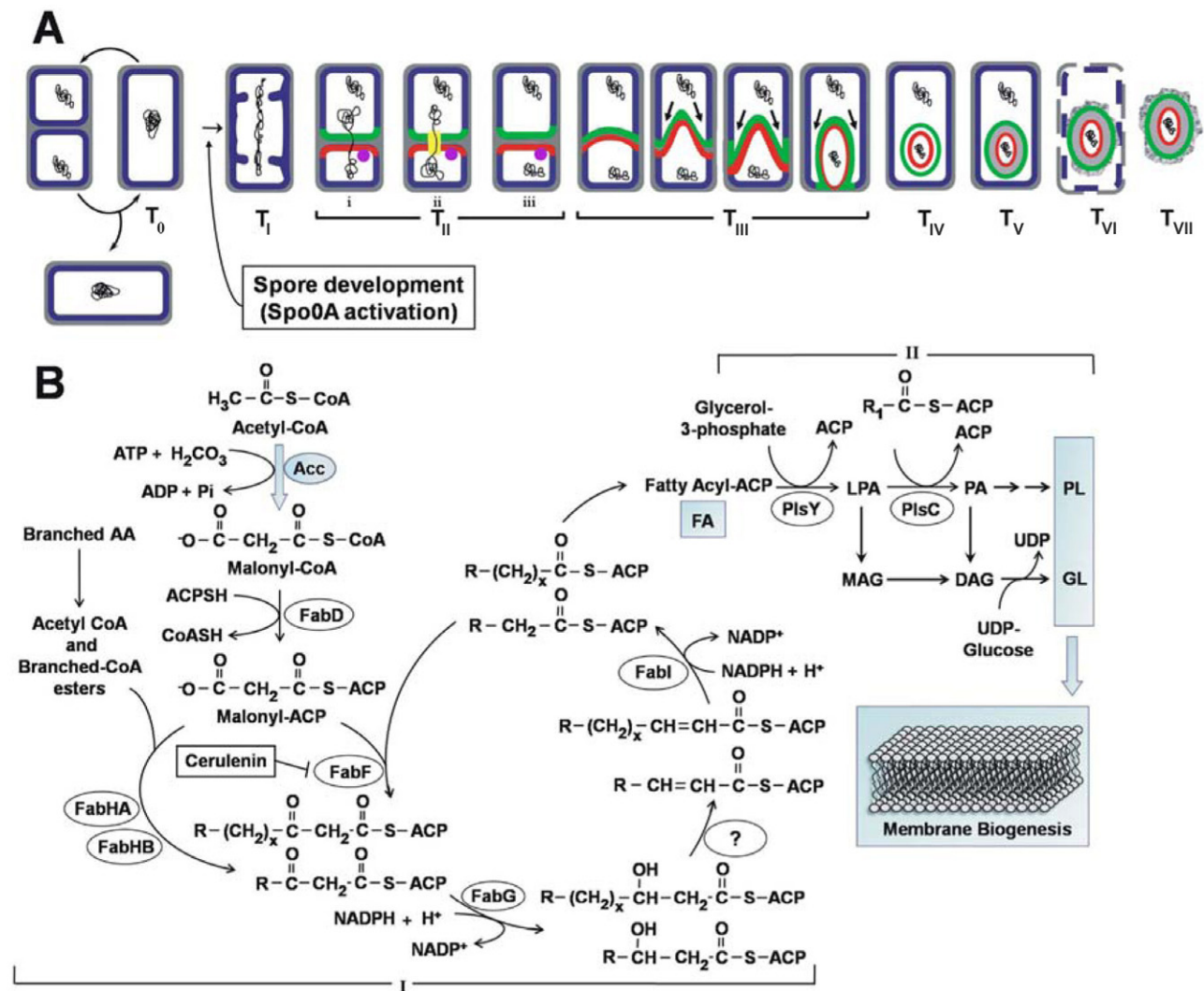


Fig. 1. Lipids and development.

A. Two key membrane events that are hallmarks of spore development are the formation of the asymmetrically positioned sporulation septum (T_{II}), which is surrounded by two double membranes (red and green) of opposite polarity, and forespore engulfment by the mother cell membrane (T_{III}). Septum formation and engulfment progression involve dynamic protein localization events and large-scale rearrangements of the cellular membranes. However, nothing is known about the regulation and homeostasis of new lipid formation during these morphogenetic processes. Yellow cylinder, SpoIIIE; violet circle, active SpoIIIE (see text for details).

B. Lipid synthesis in *B. subtilis* consists of two spatially separated stages: (I) the synthesis of soluble FAs and (II) the synthesis of membrane-bound PLs and GLs. (I) The formation of malonyl-CoA from acetyl-CoA, catalysed by acetyl-CoA carboxylase (ACC), constitutes the first and rate-limiting step in FA synthesis. This compound is converted to malonyl-ACP by malonyl-CoA transacylase (*fabD* gene product). The enzymes FabHA and FabHB then condense malonyl-ACP with acetyl-CoA or branched acyl-CoA esters, which are derived from the branched amino acids, to give rise to linear and branched (iso- and anteiso-) short- and medium-length fatty-acyl moieties with an average carbon size ranging from C4 to C8. The subsequent two-carbon elongations are accomplished by cyclic reactions in which FabHA and FabHB are replaced by the FabF elongation enzyme, whose activity is specifically blocked by the antibiotic cerulenin. (II) Fatty acyl-ACPs of the proper carbon length (C_{14} – C_{17}) are substrates for the membrane-bound enzymes involved in PL and GL synthesis. The acyl transferases PlsY and PlsC catalyse the formation of lyso-phosphatidic acid (LPA) and phosphatidic acid (PA) respectively. PA is then activated (not shown for simplicity) to CDP-diacylglycerol, leading to the formation of the main PL species present in the membranes of *B. subtilis* cells: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), lysyl-PG and cardiolipin (CL). Diacyl- and monoacylglycerides (DAG and MAG) are synthesized by the de-phosphorylation of PA and LPA respectively. The products are then glycosylated by the product of the *ugtP* gene with up to three molecules of glucose from UDP-glucose before giving rise to mature GL (glucosyl-MAG and glucosyl-DAG).

tion (Fig. 1A, T_{III}). This atypical chromosome partitioning creates a transient genetic asymmetry where the mother cell becomes diploid for two-thirds of the chromosomal genes, which are simultaneously absent in the forespore,

after asymmetric division (Fig. 1A, T_{III}). The dissolution of the polar septum peptidoglycan allows for the fusion of the two membranes and the SpoIIIE-dependent DNA translocation (yellow cylinder in Fig. 1A, T_{III}) of the remaining

two-thirds of the forespore-destined chromosome into the smaller compartment (Burton *et al.*, 2007). While chromosome translocation occurs, the mother cell membrane initiates a phagocytosis-like process to engulf the forespore (Fig. 1A, T_{III}–T_{IV}). During engulfment, the mother cell membrane (green in Fig. 1A) migrates around the forespore membrane (red in the figure), fuses to pinch off the forespore so that it becomes a free protoplast surrounded by two lipid bilayers (green and red in the figure) within the mother cell (Fig. 1A, T_{IV}). Engulfment eventually creates three topologically distinct membranes within the sporangium: the cytoplasmic membrane and the mother cell and forespore membranes (blue, green and red colours in Fig. 1A respectively). Following these events, the forespore matures into a dormant and highly resistant spore that is released following lysis of the mother cell (Fig. 1A, T_V–T_{VII}). In parallel with these numerous membrane phenomena, cell type-specific gene expression is triggered immediately after completion of the asymmetric division (T_{II}) by the active and septum-localized key cell fate phosphatase SpoII ϵ (violet circle in Fig. 1A, T_{II}), which releases active σ^F into the forespore cell (not shown in Fig. 1A for simplicity). This activation initiates the forespore-specific lineage of gene expression, which continues with the activation of σ^E by the σ^F -dependent gene-product SpoII Γ in the mother cell compartment (criss-cross regulation) (Losick and Stragier, 1992; Stragier and Losick, 1996; Higgins and Dworkin, 2012). How the activity of SpoII Γ , which is produced in the forespore and secreted into the septal membranes, is restricted to the mother cell site of the sporulating cell is unknown (Errington, 2003; Hilbert and Piggot, 2004; Higgins and Dworkin, 2012). The recently discovered role of lipids as topological determinants and lipochaperones suggests that these non-protein elements of the cell may also be important for the proper sorting, localization and functionality of key membrane determinants (Emoto and Umeda, 2001; Vanounou *et al.*, 2003; Barák *et al.*, 2008; Dowhan and Bogdanov, 2009; 2011). The numerous and dynamic membrane remodelling events that occur during the morphogenesis of the spore (Fig. 1A) also suggest that the metabolism of fatty acids (FAs) and membrane lipids (Fig. 1B) may have an active structural and/or regulatory role during the development in *B. subtilis*.

Results

Active lipid synthesis in the absence of growth is linked to spore development

The treatment of *B. subtilis* cultures with subinhibitory doses of ethanol does not affect vegetative growth but instead produces a developmental blockage that inhibits Spo0A activation (Gottig *et al.*, 2005). Interestingly, the

same dose of ethanol that inhibits Spo0A activation also inhibits *de novo* lipid synthesis (Gottig *et al.*, 2005). Therefore, we explored the possibility of a previously undetected connection between the activity of the master regulator Spo0A (Fig. 1A) and *de novo* lipid formation (Fig. 1B) during the morphogenesis of the spore.

To test this hypothesis, we measured the levels of *de novo* lipid synthesis through specific incorporation of [¹⁴C]-acetate into lipids in the presence of sublethal doses of alcohol (ethanol) that were added at specific developmental times before and after Spo0A activation (the onset of sporulation). Supplementation of the sporulation-supporting medium [Difco sporulation medium (DSM) broth] with alcohol 30 min before T₀ (T_{-0.5}) reduced the *de novo* synthesis of the three main lipid species present in *B. subtilis* (phospholipids, PLs; neutral glycolipids, GLs; and FAs) more than 90% before the entry into stationary phase (Fig. 2A and B, lanes 1 and 2). In contrast, when alcohol was added to the DSM broth 30 min after T₀, the observed levels of *de novo* lipid synthesis were comparable to those observed in a parallel culture that was not treated with alcohol (Fig. 2A and B, lanes 3 and 4). This substantial difference in *de novo* lipid synthesis levels was dependent on whether the addition of alcohol occurred before (full sensitivity) or after T₀ (full insensitivity). Importantly, the lipid synthesis effect correlated robustly with the onset of the sporulation phase as evidenced by the expression pattern of the Spo0A-controlled early developmental gene *spoII ϵ* (Fig. 2C), which suggests a requirement for Spo0A activity for active lipid synthesis after growth has ended. Supporting this conclusion, when *B. subtilis* was grown in Luria–Bertani (LB) broth, a medium that permits active vegetative growth but does not support active sporulation, lipid synthesis was active during the vegetative phase and significantly decreased at the onset of the stationary phase (Fig. 2D and E, T₀–T₃). As expected from the low levels of active Spo0A in LB broth (Hilbert and Piggot, 2004), *spoII ϵ* was not expressed during the process of bacterial growth (Fig. 2F).

Spo0A reactivates de novo lipid synthesis during spore development

The key role of Spo0A in the formation of new lipids after vegetative growth was confirmed by measuring the *de novo* lipid levels of Spo0A-proficient and Spo0A-deficient *B. subtilis* cultures grown under sporulation conditions (Fig. 3A and B). For both cultures (*spo0A*⁺ and *spo0A*⁻), the rate of *de novo* lipid synthesis per cell (measured as the incorporation of [¹⁴C]-acetate into the lipids) reached a maximum at the mid-logarithmic phase (T₋₂) and then decreased until it reached a minimum at the end of vegetative growth (T₀) (Fig. 3C). However, after the onset of the stationary phase and during active sporulation, only the

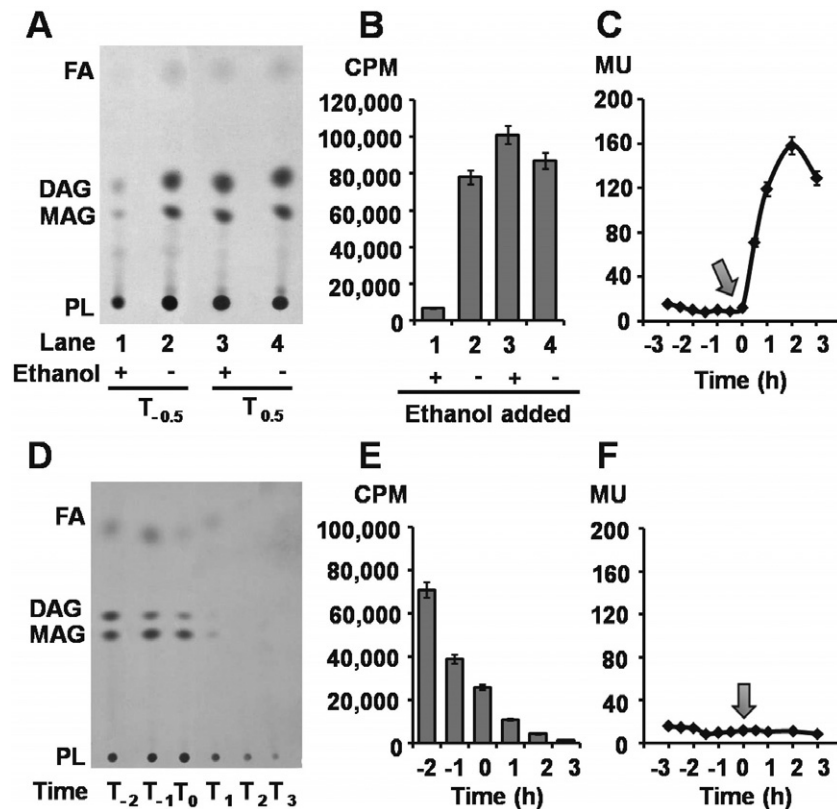


Fig. 2. Lipid synthesis after growth has ended is under developmental control.

A. The autoradiographic pattern of the lipids synthesized under ethanol stress before or after the onset of sporulation. The cells of wild-type strain RG1680 (Table S1) were grown at 37°C in DSM until 30 min prior to the onset of the stationary phase ($T_{-0.5}$). Two 1 ml samples were then transferred separately to fresh, pre-warmed flasks, and 4% ethanol (0.7 M) was added to one flask. This alcohol concentration does not affect the onset of the stationary phase but blocks sporulation (Gottig *et al.*, 2005). Five minutes after ethanol addition, each 1 ml sample (lanes 1 and 2) was exposed to 20 μ Ci of [14 C]-acetate for 2 h at 37°C. When the original culture reached the time point designated as 30 min after the onset of the stationary phase ($T_{0.5}$), two new samples of 1 ml each were separated and treated as indicated. After the 2 h incubation period, the lipids were extracted, analysed by chromatography and autoradiographed as described in the *Experimental procedures*.

B. The total radioactivity of the *de novo*-synthesized lipids (PLs, MAGs, DAGs and FAs) in DSM broth (A) was quantified and expressed as counts min^{-1} (c.p.m.), which corresponds to 1×10^8 UFC ml^{-1} , with the indicated average error.

C. Strain RG1680 was grown at 37°C in DSM, and samples were collected at the indicated time points relative to the initiation of the stationary phase (indicated by the arrow) and assayed for *spoIIIE-lacZ* β -galactosidase activity as described in the *Experimental procedures*.

D. Strain RG1680 was grown at 37°C in LB broth (a non-sporulation medium), and 1 ml samples were transferred at the indicated time points to fresh pre-warmed flasks and exposed to 10 μ Ci of [14 C]-acetate for 1 h at 37°C. After the 1 h incubation period, the lipids were extracted, analysed by chromatography and autoradiographed as described in the *Experimental procedures*.

E. The total radioactivity of the *de novo*-synthesized lipids in LB broth (C) was quantified as indicated.

F. β -Galactosidase activity present in the RG1680 strain grown in LB broth. The growth conditions and sample collections were as indicated in C.

Spo0A-proficient culture was capable of re-establishing active lipid synthesis and was thus able to produce new lipid levels comparable to those observed during active vegetative growth (Fig. 3A and B, T_0 – T_3). Culture aliquots of Spo0A-proficient and Spo0A-deficient cells were taken at different times during growth in DSM broth and labelled with the fluorescent dye FM4-64 (specific for membrane staining) to follow the percentage of cells that went through the processes of asymmetric division and engulfment during the sporulation phases. Interestingly, most Spo0A-dependent lipid synthesis occurred at developmental stages (2–4 h after the onset of the stationary phase of the

Spo0A-proficient culture) that are expected to require high levels of new membrane formation for the biosynthesis of the asymmetric septum and forespore engulfment by the mother cell membrane (Fig. 3C and data not shown). In contrast, the *B. subtilis* culture deficient in Spo0A activity continued to demonstrate decreased levels of new lipid synthesis during the stationary phase in DSM broth (Fig. 3B and C) and its cells did not go through the process of asymmetric division as evidenced by fluorescence microscopy (data not shown). To confirm whether the regulatory impact on *de novo*-synthesized lipids could be attributed to the absence of Spo0A activity, we performed a

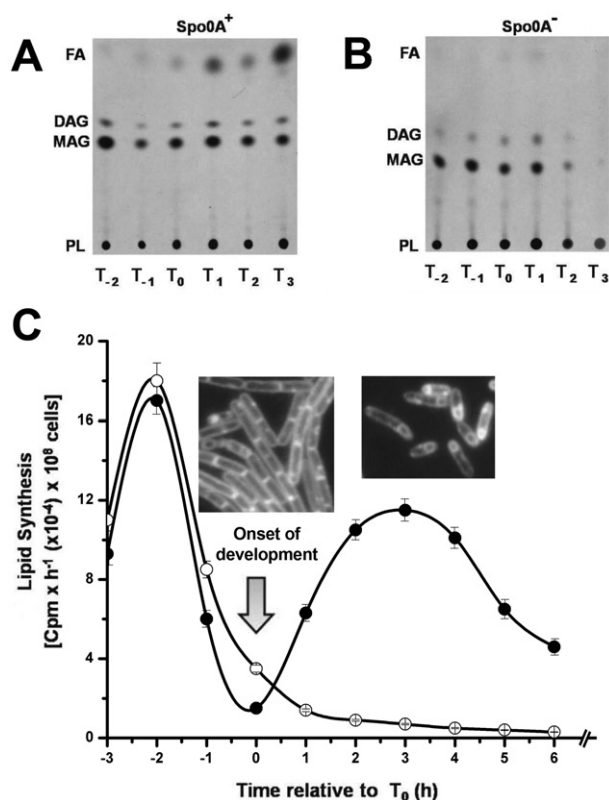


Fig. 3. Spo0A controls *de novo* lipid synthesis during development.

A and B. The autoradiographic pattern of the lipids synthesized by wild-type cells and their isogenic *spo0A* derivatives grown under sporulation conditions at 37°C. At the indicated times, 1 ml samples of each strain were transferred to fresh, pre-warmed flasks and exposed to 10 μ Ci of [14 C]-acetate for 1 h at 37°C. After the incubation period, the lipids were extracted and analysed by chromatography.

C. The total radioactivity of the *de novo*-synthesized lipids (PLs, MAGs, DAGs and FAs) in the wild-type and *spo0A* strains (closed and open circles respectively) grown in DSM broth was quantified as indicated in the *Experimental procedures*. Quantitative data are expressed as counts min^{-1} , which corresponds to 1×10^8 UFC ml^{-1} and correlates with the developmental stage of spore formation. The arrow indicates the onset of the stationary phase, which is equivalent to the initiation of sporulation in the wild-type strain. The inserted photographs represent the predominant sporulation stage that was observed by fluorescence microscopy (after sample staining with FM4-64 fluorescent dye) at the beginning of the stationary phase and after 3 h of growth of the wild-type culture in DSM broth (see text for details).

complementation experiment with a *spo0A* mutant strain expressing an ectopically integrated copy of the *sad* 67-*spo0A* gene under IPTG control (strain RG1491, Table S1) (Arabolaza *et al.*, 2003). Under IPTG supplementation, this strain produces a constitutively active form of Spo0A (Sad67), and it was able to reactivate the *de novo* lipid synthesis under sporulation conditions only when IPTG was added to the DSM broth (data not shown).

Spo0A reactivates the global synthesis of membrane lipids harbouring linear and branched fatty-acyl chains during spore development

[1- 14 C]-acetate is a specific precursor for the synthesis of linear FAs (n-C₁₄ to n-C₁₈). [1- 14 C]-acetate treatment results in membrane lipids that harbour straight fatty-acyl chains (Fig. 1B). However, in membranes of the *Bacillus* genus, branched (iso- and anteiso-) fatty-acyl moieties are also major membrane lipid components (Fig. 1B). Therefore, to confirm that the role of Spo0A in the global reactivation of lipid synthesis was not restricted to linear FA synthesis (i.e. affecting the substrate preference of the FabHA and FabHB condensing enzymes for straight-FAs instead branched-FAs precursors) (Kingston *et al.*, 2011), we measured the incorporation of [1- 14 C]-isoleucine, a specific precursor for the synthesis of branched FAs, into newly formed lipids. As illustrated in Fig. 4A, in the wild-type culture, strong reactivation of *de novo* lipid synthesis at the onset of sporulation was observed using [1- 14 C]-isoleucine. In contrast, the Spo0A-deficient culture was unable to reactivate *de novo* branched-lipid synthesis during the stationary phase (Fig. 4B). The capacity of these two FA precursors ([1- 14 C]-acetate and [1- 14 C]-isoleucine) to account for the bulk of total fatty-acyl moieties present in *B. subtilis* membranes demonstrates that Spo0A induces global reactivation of the synthesis of the membrane lipids (GLs and PLs) that harbour linear and branched fatty-acyl chains during sporulation.

To further examine the regulatory role of Spo0A in lipid synthesis during sporulation, we took advantage of the fact that, unlike mammals, bacteria possess a FA synthase (FASII) complex that is composed of monofunctional proteins (Fujita *et al.*, 2007; Zhang and Rock, 2008). Each FASII protein catalyses a step in the biosynthetic pathway of lipid formation that begins with the key metabolite malonyl-CoA (Fig. 1B). Therefore, using [2- 14 C]-malonyl-CoA as a specific substrate, we compared the *in vitro* lipid synthesis activities of protein extracts prepared from isogenic Spo0A-proficient and Spo0A-deficient *B. subtilis* cultures grown in sporulation-supporting broth. The *in vitro* FA synthesis activities of the Spo0A-proficient (Fig. 4C) and the Spo0A-deficient protein extracts (Fig. 4D) were similar during the vegetative phase (T₋₂–T₀). However, during spore development, only protein extracts prepared from stationary-phase cultures of the Spo0A-proficient cells demonstrated high lipid synthesis activity when [2- 14 C]-malonyl-CoA was used as a specific precursor for *in vitro* FA synthesis (compare the levels of [14 C]-FAs synthesized by both types of protein extracts during T₁–T₄ in Fig. 4C and D).

In summary, these results (Figs 2–4) demonstrate, for the first time, that new lipid formation (FA and membrane lipid synthesis) during the stationary phase remains active

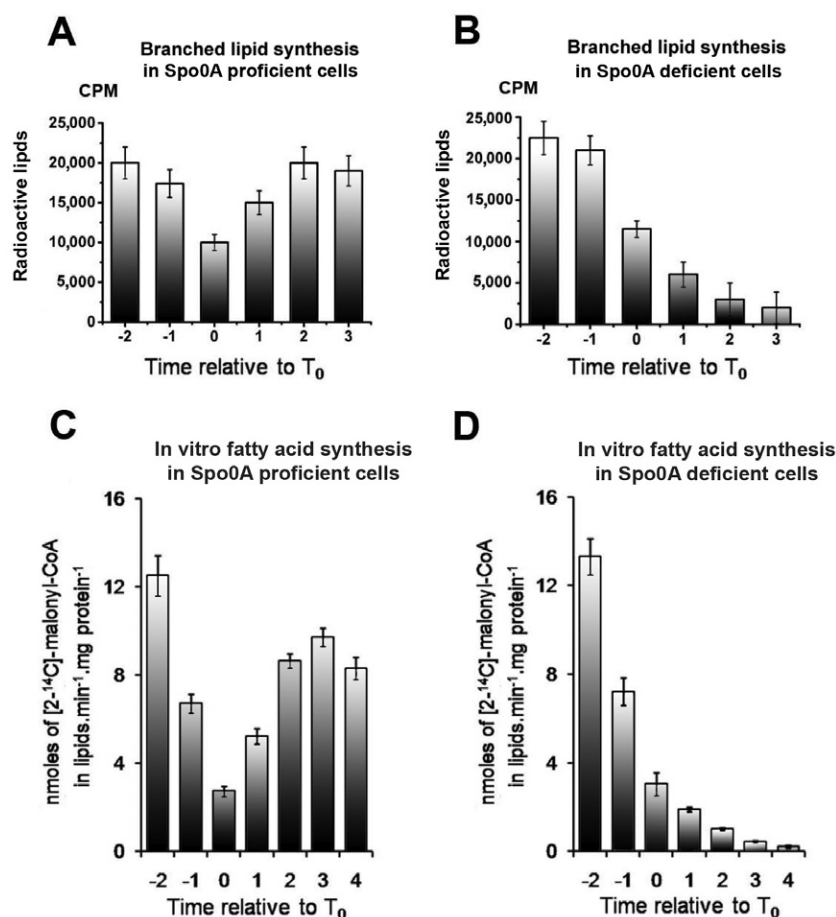


Fig. 4. Spo0A controls global lipid synthesis during development.

A and B. The total radioactivity of the *de novo*-synthesized lipids of the wild-type strain 168 (A) and its isogenic *spo0A* derivative (B) using [¹⁴C]-isoleucine as the precursor of the branched-chain FAs in the DSM broth. At the indicated time points, 1 ml samples of each strain were incubated for 1 h at 37°C in the presence of 10 µCi of [¹⁴C]-isoleucine. After this incubation period, the lipids were extracted, analysed by chromatography and quantified as previously described. The results are expressed as counts min⁻¹ (c.p.m.), which corresponds to 1 × 10⁸ UFC ml⁻¹. C and D. The *in vitro* FA synthase activity of *B. subtilis* cultures grown under sporulation conditions. The cell-free protein extracts, which were prepared at different developmental stages, from the *B. subtilis* cultures that were either proficient (C) or deficient (D) in Spo0A activity were assayed for their *in vitro* FASII activity (see text for details).

only under sporulation conditions. In addition, the data indicate a novel role for Spo0A in controlling the activity of the central metabolic pathway of lipid synthesis, and thus new membrane formation and remodelling (see Fig. 1A), during the morphogenesis of the spore cell.

Spo0A reactivates soluble fatty acid synthesis, the first stage of de novo lipid formation

A key checkpoint in coupling FA synthesis to the synthesis of membrane lipids (GLs and PLs) is the formation of long-chain acyl-ACP intermediates that connect stages I and II of lipid synthesis (Fig. 1B and Fujita *et al.*, 2007; Zhang and Rock, 2008). To investigate the stage (I or II) during which Spo0A-dependent reactivation of lipid synthesis occurs, we uncoupled FA synthesis from membrane lipid synthesis by specifically inhibiting the latter process (Mindich, 1970; Grau and de Mendoza, 1993). Although glycerol starvation of *B. subtilis* glycerol-auxotrophs (*glyc*) results in the profound inhibition of membrane PL and GL synthesis, the synthesis of soluble FAs (long-chain [¹⁴C]-FAs) continues at a normal rate, and *de novo* long-chain [¹⁴C]-FAs accumulate as free species (Mindich, 1970; Grau

and De Mendoza, 1993). We took advantage of the fact that sporulation can also be induced by resuspension of a growing culture in a poor medium (Sterlini-Mandelstam sporulation medium). Cultures of *glyc* strains proficient or deficient in Spo0A activity (strains B42 or RGB42 respectively, Table S1) were grown in glycerol-supplemented growth medium (Schujman *et al.*, 1998, a medium that allows for the physiological manipulation of the glycerol auxotroph without affecting its proficiency in sporulation) at 37°C until the mid-logarithmic phase was reached. We then resuspended the cultures in Sterlini-Mandelstam poor medium in the absence or presence of glycerol to induce sporulation. Both cultures were then exposed to [¹⁴C]-acetate and cultured for 3 h at 37°C prior to lipid synthesis analysis. Upon glycerol starvation, the Spo0A-proficient *glyc* mutant strain, B42, halted new membrane lipid formation and accumulated more than 90% of the radioactivity in the soluble fraction of the *de novo* long-chain [¹⁴C]-FAs (compare lanes 1 and 2 in Fig. 5A). In contrast, the *glyc spo0A* double-mutant strain, RGB42, was unable to synthesize soluble FAs when resuspended in sporulation medium without glycerol supplementation (Fig. 5A, lane 3). Because soluble FAs did not accumulate in strain

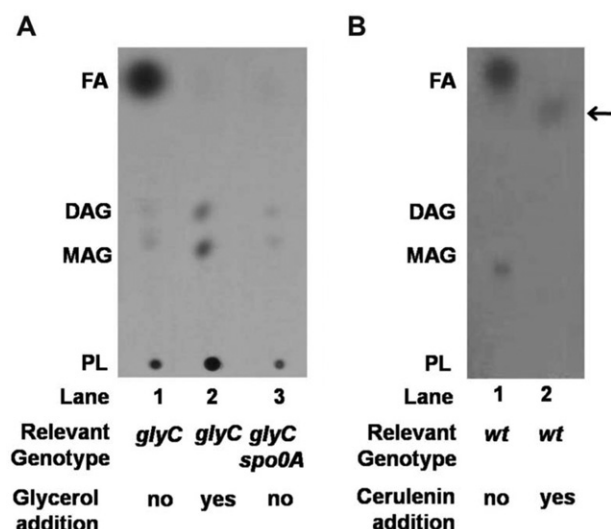


Fig. 5. Spo0A reactivates stage I of lipid synthesis: the formation of soluble fatty-acyl moieties.

A. The autoradiographic pattern of the lipids synthesized by strains B42 and RGB42 (Table S1) grown in the presence or absence of glycerol under sporulation conditions. Strain B42 (*glyC*), which grows exponentially at 37°C in glycerol-supplemented growth medium, was deprived of this nutrient as previously described (Grau and De Mendoza, 1993). The culture was then resuspended (Sterlini-Mandelstam resuspension broth) in the absence or presence of 50 µg ml⁻¹ of glycerol (lanes 1 and 2 respectively). Aliquots (1 ml) of each culture were exposed to 10 µCi of [¹⁴C]-acetate for 3 h at 37°C prior to lipid extraction and chromatographic separation. A parallel culture of strain RGB42 (*glyC spo0A*) grown in the presence of glycerol at 37°C up to the mid-logarithmic phase was deprived of glycerol and resuspended in glycerol-free Sterlini-Mandelstam resuspension broth. The culture was then labelled with 10 µCi of [¹⁴C]-acetate for 3 h at 37°C prior to lipid extraction and analysis (lane 3). The sample in lane 1 contained 21 500 and 800 c.p.m. of radioactivity in the FA and membrane lipid fractions respectively. The sample in lane 2 contained 17 600 and 200 c.p.m. of radioactivity in the membrane lipid and FA fractions respectively. The sample in lane 3 contained 300 c.p.m. of radioactivity in the membrane lipid fraction and no detectable radioactivity in the FA fraction. Similar results were observed in several independent experiments.

B. The autoradiographic pattern of the lipids synthesized *in vitro* using the cell-free protein extracts from the wild-type 168 cells grown in DSM broth until 1 h after the onset of sporulation. [²⁻¹⁴C]-malonyl CoA was used as a specific substrate for *in vitro de novo* lipid synthesis in the absence (lane 1) or presence (lane 2) of 10 µg ml⁻¹ cerulenin. The arrow indicates the medium-chain FAs synthesized in the presence of cerulenin (inhibition of the FabF enzyme).

RGB42 after glycerol removal (Fig. 5A, lane 3) and because the only difference between strains B42 and RGB42 was proficiency in Spo0A activity, we conclude that Spo0A specifically reactivates soluble FA synthesis, the first stage in the formation of new lipids (Figs 1B and 5A).

Spo0A regulates the first and rate-limiting step of de novo fatty acid synthesis: malonyl-CoA formation

The lack of medium-chain [¹⁴C]-FAs in the glycerol-starved *spo0A*-mutant RGB42 cells (lane 3 in Fig. 5A)

compared with the long-chain [¹⁴C]-FAs synthesized in the glycerol-starved BR42 cells (lane 1 in Fig. 5A) strongly suggests that Spo0A participates in the reactivation of soluble FA synthesis during a critical step upstream of the enzymatic activity of the FabHA and FabHB condensing enzymes, which are required for the initial condensation and two-carbon elongation of malonyl-ACP with acetyl-CoA or branched-CoA esters (formation of medium-chain FAs, see Fig. 1B). To test this hypothesis, we treated cell-free protein extracts prepared from wild-type (*Spo0A*-proficient) *B. subtilis* cultures with cerulenin, a specific inhibitor of FabF, the condensing enzyme that controls the formation of long-chain FAs (Fujita *et al.*, 2007; Zhang and Rock, 2008). As expected, this treatment blocked the formation of radioactive long-chain FAs but permitted the accumulation of medium-chain [¹⁴C]-FAs (Fig. 5B). These medium-chain FAs, absent in glycerol-starved *glyC-spo0A* cells (see Fig. 5A lane 3), are formed via the activities of FabHA and FabHB, which are cerulenin-insensitive condensing enzymes. This result confirms that Spo0A regulates a crucial step in FA synthesis that occurs prior to the enzymatic activities of FabHA and FabHB (compare the absence of *de novo*-formed FAs in Fig. 5A lane 3 with the presence of *de novo*-synthesized FAs in Fig. 5B lane 2).

What enzymatic step of *de novo* FA synthesis (see Fig. 1B), preceding the activities of the three condensing enzymes (FabHA, FabHB and FabF), is under Spo0A control? Interestingly, through *in silico* bioinformatic analysis, *in vitro* chromatin immunoprecipitation and gene microarray analysis, two independent research groups have proposed that the genes comprising the bicistronic operon *accDA*, which encodes the β and α subunits (carboxyltransferase component) of the acetyl-CoA carboxylase (ACC) enzyme, belong to a new group of genes that are directly and positively controlled by Spo0A (Liu *et al.*, 2003; Molle *et al.*, 2003). ACC catalyses the first and rate-limiting step of *de novo* FA synthesis, which entails the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate (see Fig. 1B and Fujita *et al.*, 2007; Zhang and Rock, 2008 for references). In addition to its central role in new lipid synthesis, malonyl-CoA negatively controls the activity of the master repressor of *de novo* lipid synthesis in *B. subtilis*, the transcription factor FapR (Fujita *et al.*, 2007; Zhang and Rock, 2008). Taking into account the reports of Liu *et al.* (2003) and Molle *et al.* (2003) and based upon our *in vivo* and *in vitro* results, we hypothesized that the *de novo* formation of malonyl-CoA constitutes the key step of *de novo* lipid synthesis under developmental Spo0A control.

Therefore, we analysed the role of Spo0A in the *in vivo* expression of the genes that encode the ACC enzyme carboxyltransferase component, which is responsible for malonyl-CoA formation. As illustrated in Fig. 6A, the

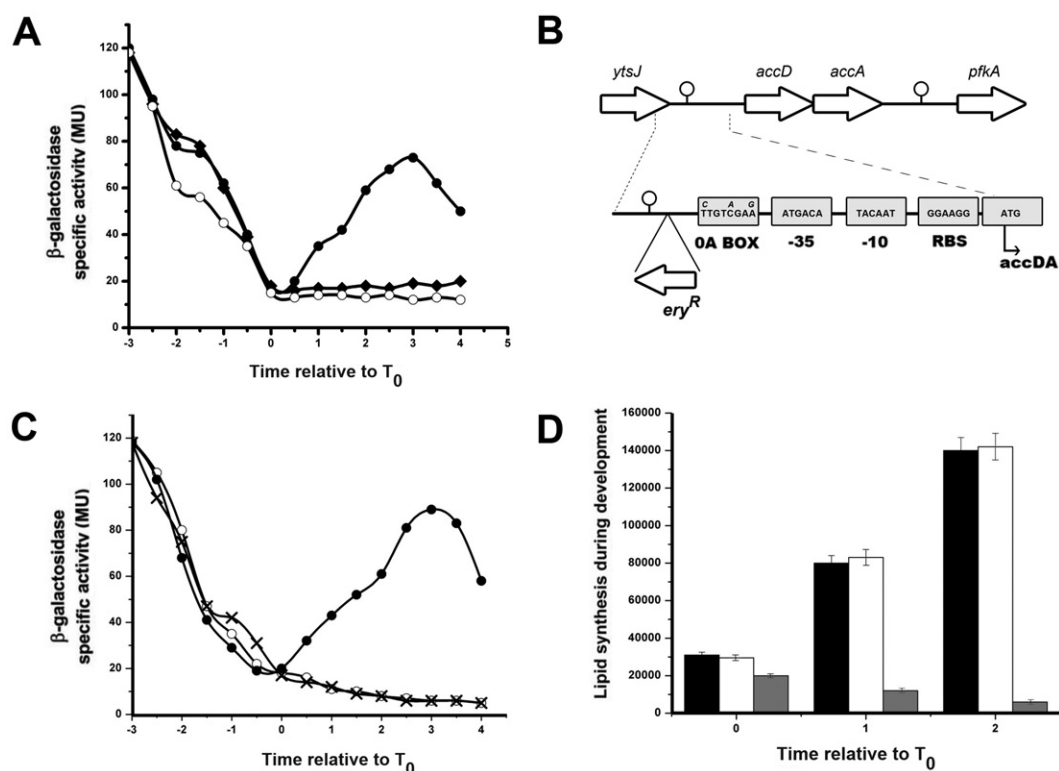


Fig. 6. Spo0A reactivates malonyl-CoA formation during development.

A. β-Galactosidase activity of the reporter fusion *PaccDA^{wt}-lacZ::amyE* in Spo0A-proficient (closed symbols, strain RG1606) and Spo0A-deficient (open symbols, strain RG1607) cells grown in sporulation-supporting DSM (—●—, strain RG1606; —○—, strain RG1607) and non-sporulation-supporting LB (—○—, strain RG1606) media. The cultures were incubated at 37°C, and the samples were collected at the indicated times relative to the start of the stationary phase (time zero) and assayed for β-galactosidase activity.

B. A diagram of the *accDA* promoter region. The Spo0A-binding sequence (OA box), the position of the *ery^R* cassette, the -10 and -35 consensus sequences for σ^A -dependent promoters and the Shine–Dalgarno sequence are indicated. The mutated nucleotides of the OA-box are indicated in bold and italic. See *Experimental procedures* for details.

C. β-Galactosidase activity of Spo0A-proficient cells harbouring the reporter fusions *Pery Ω accDA^{OA}-lacZ::amyE* (strain RG1158) and *Pery Ω accDA^{wt}-lacZ::amyE* (strain RG1160) grown in sporulation-supporting (DSM) and non-supporting (LB) media (—○— and —x—, strain RG1158; —●—, strain RG1160). The pattern of β-galactosidase activity of strain RG1160 grown in DSM (this panel) was indistinguishable from that exhibited by strain RG1606 in DSM (panel A). The results depicted are the averages of five independent experiments.

D. The total radioactivity of the *de novo*-synthesized lipids in the DSM broth of wild-type cells (black) and the Spo0A-proficient mutant strains RG1130 (*ery Ω accDA^{OA}*, grey) and RG1340 (*ery Ω accDA^{wt}*, white) using [$1\text{-}^{14}\text{C}$]-acetate as the precursor for newly formed lipids. At the indicated time points, 1 ml samples of each strain were incubated for 1 h at 37°C in the presence of 10 μCi of [$1\text{-}^{14}\text{C}$]-acetate. After this incubation period, the lipids (FAs, GLs and PLs) were extracted, analysed by chromatography and quantified as previously described. The results are expressed as counts min^{-1} , which corresponds to 1×10^8 UFC ml^{-1} .

expression of the *accDA* operon was similar between the Spo0A-proficient and Spo0A-deficient cultures (strains RG1606 and RG1607 respectively, Table S1) during vegetative growth in DSM broth. In both cultures, the expression of the *accDA* operon, measured as β-galactosidase activity driven by the *accDA* promoter, was high at low cellular densities and decreased as the cultures reached the end of the vegetative phase. This behaviour suggests that *accDA* expression could be growth-rate regulated during the vegetative growth as previously suggested for the expression of *accBC* that codes for the biotin carboxyl-carrier component of ACC (Marini *et al.*, 2001). Interestingly, at the end of the vegetative phase, a significant difference in *accDA* operon expression was observed between the two isogenic cultures. While *accDA* expres-

sion in the Spo0A-deficient culture continued to decrease until reaching an undetectable level of β-galactosidase activity, strong reactivation (starting at T_0) of β-galactosidase expression was detected in the Spo0A-proficient cells. The reactivation of *accDA* promoter-driven expression reached a maximum at T_3 . This pattern correlated well with the patterns of both *in vivo* (Figs 3C and 4A) and *in vitro* (Fig. 4C) reactivation of *de novo* lipid biosynthesis in the wild-type cultures of *B. subtilis* grown in sporulation-supporting medium (DSM). As expected, the reactivation of carboxyltransferase expression at the end of vegetative growth was not observed when the Spo0A-proficient and Spo0A-deficient cultures were grown under non-sporulating conditions (growth in LB broth, Fig. 6A and data not shown). These *in vivo* results are in complete

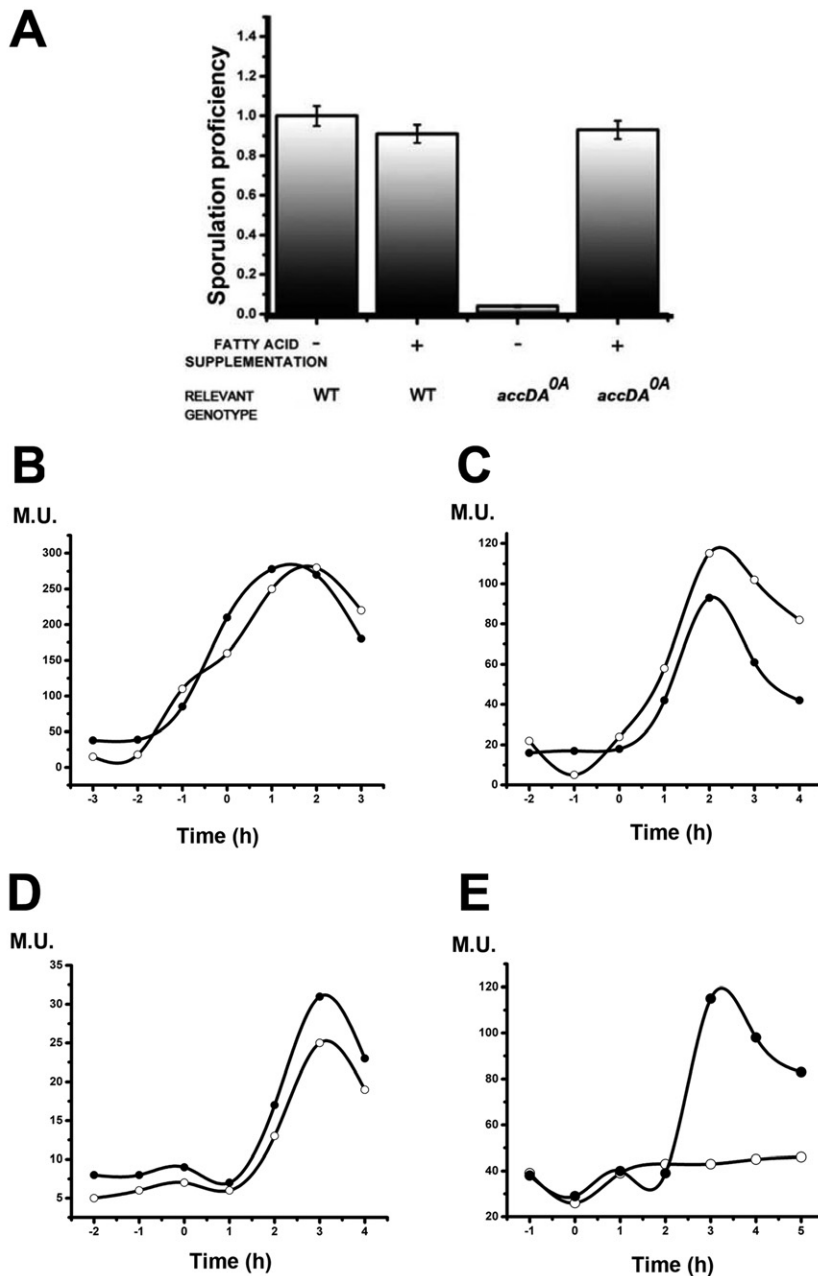
agreement with the *in vitro* results of Molle *et al.* (2003), who through the use of gel shift binding assays and chromatin immunoprecipitation experiments, demonstrated the specific binding of Spo0A to *accDA*. *In toto*, the present *in vivo* (Fig. 6A) and former *in vitro* results (Liu *et al.*, 2003; Molle *et al.*, 2003) suggest the physiological dependency of *accDA* expression on Spo0A activity.

How does Spo0A regulate *accDA* expression? As shown in Fig. 6B, the promoter region of the *accDA* operon contains conserved -35 and -10 sequences, separated by an ideal spacer of 17 bp, with homology to the housekeeping σ^A -dependent promoters. Upstream of the -35 region is a perfect Spo0A-binding box (5'-TTGTCGAA-3') that could be necessary for maintaining active expression of *accDA* during the stationary phase of growth in Spo0A-proficient cultures under sporulation conditions (Fig. 6A, strain RG1606). Therefore, the expression of *accDA* during the vegetative phase of growth (in LB or DSM) would be driven by the housekeeping σ^A -containing RNA polymerase irrespective and independent of Spo0A activity. Otherwise, to maintain active expression of *accDA* during the stationary phase of growth (i.e. under sporulation conditions) the presence of an active Spo0A, capable of binding to the Spo0A-box present in the promoter region of the operon (as previously shown by Liu *et al.*, 2003), would be essential to reactivate *accDA* expression. To demonstrate the essentiality of Spo0A for the reactivation of *accDA* expression during sporulation, we constructed a Spo0A-proficient mutant strain that harboured a mutation in the Spo0A-binding box present in the promoter region of the *accDA* operon (strain RG1130, see *Experimental procedures* and Table S1). In addition to the *accDA^{OA}* mutation, the strain RG1130 harboured an erythromycin resistance cassette (*ery^r*), used for the selection of the construction, upstream of the regulatory region of *accDA* (see Fig. 6B). During growth in sporulation-supporting medium (DSM broth, 200 r.p.m. at 37°C), there were no significant differences in the rate of growth or the cellular yield reached at the beginning of the stationary phase between *ery^rΩaccDA^{OA}* and wild-type cells (data not shown), indicating that the *ery^rΩaccDA^{OA}* construct did not affect the vegetative growth of RG1130 cells. To confirm that Spo0A governs *accDA* expression during sporulation, we measured *accDA* expression (Fig. 6C) and *de novo* FA synthesis (Fig. 6D) in cultures that harboured the mutation in the Spo0A-binding box of *accDA* (*ery^rΩaccDA^{OA}* mutant cells) during growth in LB and DSM broths. As illustrated in Fig. 6C, during the vegetative phase in DSM or LB broth, cultures of the Spo0A-proficient cells harbouring the reporter fusion driven by the mutated promoter (*P_{ery^rΩaccDA^{OA}}-lacZ::amyE*, strain RG1158, see Table S1) exhibited a pattern of β-galactosidase expression similar to that of the cells harbouring the wild-type reporter fusion (*P_{accDA^{wt}}-lacZ::amyE*, strain RG1606, see Fig. 6A).

However, during the stationary phase (after T_0), the expression of the mutated promoter *P_{ery^rΩaccDA^{OA}}* was not reactivated in the Spo0A-proficient culture and remained downregulated throughout the stationary phase (Fig. 6C). The absence of transcriptional reactivation of β-galactosidase expression driven by cells that harboured the *P_{ery^rΩaccDA^{OA}}* promoter during the stationary phase of growth in DSM broth was not due to the presence of the erythromycin resistance cassette (*ery^r*), as β-galactosidase expression was normally reactivated by cells in which the *accDA* promoter harboured (in the same position as in strain RG1158) an erythromycin resistance cassette but a fully functional Spo0A-binding box (*P_{ery^rΩaccDA^{wt}}*, strain RG1160, Table S1) (see Fig. 6C). In accord with the arrest of sporulation-linked *accDA* expression when the Spo0A-binding box present in the promoter region of *accDA* was disrupted (strain RG1158, Fig. 6C), *de novo* lipid synthesis in the Spo0A-insensitive *ery^rΩaccDA^{OA}* cells (strain RG1130) was not restored under sporulation conditions (Fig. 6D). As expected, the reactivation of *de novo* lipid synthesis during sporulation in DSM broth was not affected in the *ery^rΩaccDA^{wt}* strain RG1160 (Fig. 6D). Overall, these results confirm that Spo0A regulates the reactivation of malonyl-CoA synthesis and becomes the master regulator of *de novo* FA synthesis and membrane lipid homeostasis during sporulation.

Spo0A-dependent de novo fatty acid synthesis is required for spore development

The strict Spo0A dependency of the formation of new malonyl-CoA (see Fig. 6A and C) and the inability of the Spo0A-proficient *accDA^{OA}* cells to synthesize new lipids under sporulation conditions (see Fig. 6D) strongly suggest that *de novo* FA synthesis could have a structural and/or regulatory role during development in *B. subtilis*. As shown in Fig. 7A, the Spo0A-proficient *accDA^{OA}* cells (strain RG1130) failed to make spores in DSM broth unless they were supplemented and grown in the presence of exogenous FAs (palmitic and oleic acids, nC16:0 and nC18:1 respectively). The exogenous FAs completely restored the sporulation proficiency of the Spo0A-insensitive *accDA^{OA}* cells to that of the parental wild-type strain (Fig. 7A). This result confirms that the sporulation deficiency of the Spo0A-proficient *accDA^{OA}* cells was due to a deficiency in the reactivation of *de novo* lipid synthesis at the onset of the sporulation phase because the supplementation with exogenous FAs permitted the *accDA^{OA}* cells to bypass their inability to synthesize new FAs under Spo0A control. Our data correlate the oligosporic phenotype of the Spo0A-insensitive *accDA^{OA}* cells (strain RG1130, Fig. 7A) with its inability to restore *accDA* expression (Fig. 6C), and hence form new lipids and membranes (Fig. 6D), under Spo0A control.



To test whether the inability to make new lipids in the *accDA^{OA}* cells blocked *spo0A* expression and/or Spo0A activity (and hence downregulated spore formation), we measured the expression pattern of key sporulation genes that are under temporal and spatial regulation during the morphogenesis of the spore. Neither *spo0A* expression (Fig. 7B) nor Spo0A activity (Fig. 7C) was affected by the inactivation of the Spo0A-binding box in *accDA*. These results confirm that the downregulation of the sporulation proficiency of *accDA^{OA}* cells was not due to the inability of these cells to trigger the onset of the sporulation programme. In addition, the expression of the σ^F -controlled

regulatory gene *spoIIIR* was not affected in *accDA^{OA}* cell cultures (Fig. 7D), but the expression of the σ^E -controlled *spoIID* gene was severely downregulated (Fig. 7E). These results are in agreement with a previous report that demonstrated that the inhibition of *de novo* FA synthesis in wild-type cells with cerulenin, an antibiotic that does not affect malonyl-CoA formation but inhibits the activity of FabF (Schujman *et al.*, 1998 and Fig. 1B), blocked sporulation at the developmental stage of pro- σ^E processing. Together, these results suggest that *de novo* FA synthesis has a regulatory role in the specific activation of σ^E in the mother cell compartment (see Discussion).

Fig. 7. Spo0A-dependent *de novo* fatty acid synthesis is required for σ^E activation during spore development.

A. The sporulation proficiency of wild-type and *accDA^{OA}* (RG1130) cells after 36 h of incubation in DSM broth at 37°C with or without fatty acid supplementation. The supplemented FAs were palmitic acid (nC16:0) and oleic acid (nC18:1) at final concentrations of 10 μ M and 1 μ M respectively. After the incubation period, the number of viable cells per millilitre and heat-resistant spores were determined as previously described (Arabolaza *et al.*, 2003). The efficiency of spore formation is expressed as the relative frequency of sporulation compared to the sporulation level of the wild-type strain. The proficiency of spore formation of strain RG1340 (*ery^raccDA^{wt}*, Table S1) was indistinguishable from that exhibited by the wild-type strains 168 and JH642 (data not shown). There were no significant differences between the final numbers of viable cells reached by the three studied strains (JH642, RG1130 and RG1340). The number of viable cells (measured as colony-forming units, cfu) among different experiments ranged from 3×10^8 to 7×10^8 cfu ml⁻¹. In the case of the strain RG1130 (*accDA^{OA}*), grown in the absence of FA supplementation, the number of cfu ml⁻¹ and heat-resistant spores was of 4.0×10^8 and 3.0×10^6 respectively. Similar reductions in spore formation (100 to 1000 folds) were observed in several independent experiments when the strain RG1130 was grown in the absence of FA supplementation. B–E. The β -galactosidase activity of wild-type (—●—) and *accDA^{OA}* (—○—) strains harbouring different reporter *lacZ* fusions to the sporulation genes *spo0A* (strains JH19005 and RG19036) (B), *spoIIA* (strains JH16302 and RG19888) (C), *spoIIIR* (strains SL6418 and RG19890) (D) and *spoIID* (strains RG851 and RG865) (E). The strains were grown at 37°C in DSM, and samples were collected at the indicated time points relative to the initiation of stationary phase (T_0) and were assayed for β -galactosidase activity as described in the *Experimental procedures*. The showed result in each panel (B–E) is representative of five independent experiments.

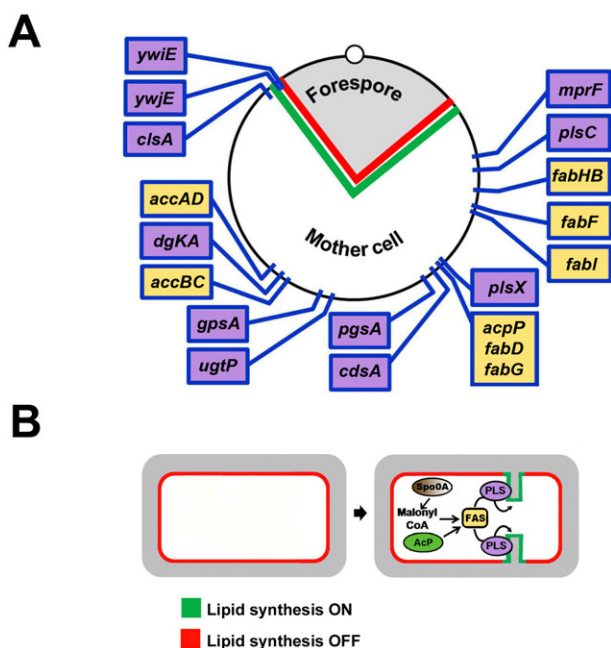
The asymmetric nature of de novo lipid synthesis during spore development

Beyond its primary role of supplying structural PLs and GLs for new membrane formation, is it possible that the robust *de novo* lipid synthesis machinery that we discovered here plays an unknown role during development? Interestingly, most, if not all, of the enzymes involved in PL and GL formation were located in the membrane of the asymmetric septum during sporulation (Nishibori *et al.*, 2005; Matsumoto *et al.*, 2006). In addition, Spo0A functions as a cell type-specific transcription factor in the mother cell compartment for several hours after asymmetric division and does not function in the forespore chamber (Arabolaza *et al.*, 2003; Fujita and Losick, 2003). When *spo0A* is ectopically integrated into the chromosome to restrict Spo0A expression and activity to the smaller compartment, the emerged sporulation phenotype is defective, indicating that the mother cell-compartmentalized expression of Spo0A is essential for the success of the sporulation programme (Fujita and Losick, 2003). More recently, a key protein of the metabolism of FAs, acyl carrier protein (ACP), which is in charge of the transport and solubilization in the cytoplasm of the fatty-acyl groups during FA and PL synthesis (see Fig. 1B), was shown to be asymmetrically compartmentalized in the mother cell compartment during sporulation (Martínez *et al.*, 2010). Taken together, our results and previous studies (Arabolaza *et al.*, 2003; Fujita and Losick, 2003; Martínez *et al.*, 2010) indicate that Spo0A-activated *de novo* lipid synthesis occurs only in the mother cell compartment of sporulating cells to provide soluble fatty acyl-ACP substrates to the septum-localized FL synthases (Nishibori *et al.*, 2005; Matsumoto *et al.*, 2006) during sporulation. In addition to the mother cell compartmentalization of Spo0A and ACP (Fujita and Losick, 2003; Martínez *et al.*, 2010), and supporting our hypothesis, we found that all the genes encoding the enzymes involved in the synthesis of FAs and most, if not all, the genes encoding enzymes involved in the synthesis of the lipid species present in *B. subtilis* membranes (PG, PE, CL, lysyl-PG, glucosyl-MAG and glucosyl-DAG) were located outside of the chromosomal region that is initially trapped in the forespore compartment immediately after polar septum formation (lipid gene asymmetry, Fig. 8A). It has been proposed that transient genetic asymmetry would allow the transient asymmetry of gene expression in favour of one of the two cell compartments of the sporangium (Arigori *et al.*, 1999; Frandsen *et al.*, 1999; King *et al.*, 1999; Losick and Dworkin, 1999). The proposed genetic asymmetry might be responsible, at least in part, for the establishment of σ^F -dependent cell type-specific gene expression during sporulation (Dworkin and Losick, 2001; Dworkin, 2003; Hilbert and Piggot, 2004; Higgins and Dworkin, 2012). Among the complete set of genes (more

than 20) encoding the enzymes involved in *de novo* lipid synthesis, which are transiently trapped in the mother cell compartment, are the ACP-coding gene, *acpP*, which is essential for the synthesis of soluble fatty-acyl moieties (stage I of the pathway of lipid synthesis, Fig. 1B), and three key genes (*gpsA*, *ugtP* and *pgsA*) involved in membrane lipid synthesis (stage II of the pathway of lipid synthesis, Fig. 1B) that are located far from the *oriC* and are thus among the last genes to be translocated by SpoIIIE (Burton *et al.*, 2007) to the forespore compartment after asymmetric cell division (see Fig. 8A). Thus, our data suggest that the temporary exclusion or asymmetry of the complete set of genes involved in FA and membrane lipid synthesis (lipid gene asymmetry) from the forespore (Fig. 8A), the compartmentalization of ACP into the mother cell (Martínez *et al.*, 2010), and the mother cell-compartmentalized activity of Spo0A (Arabolaza *et al.*, 2003; Fujita and Losick, 2003), which is responsible for the reactivation of *de novo* FA synthesis during sporulation (this work), produce an imbalance in which the newly synthesized lipids are preferentially formed in the mother cell (asymmetric lipid synthesis) (Fig. 8B and *Discussion*).

Spo0A-dependent de novo fatty acid synthesis is required for biofilm development

In addition to sporulation, Spo0A regulates other adaptive and developmental pathways that are thought to be important for the expression of the different social behaviours of *B. subtilis* (Kearns and Losick, 2005; Aguilar *et al.*, 2007; Abee *et al.*, 2011; Shank and Kolter, 2011). One of these pathways is the capacity of *B. subtilis* to form floating biofilms, called pellicles, in standing cultures (Branda *et al.*, 2001; Hamon and Lazazzera, 2001; Lombardía *et al.*, 2006) and architecturally complex colonies on solid media (Branda *et al.*, 2001; Lombardía *et al.*, 2006). Although non-domesticated strains of *B. subtilis*, such as the Marburg-related strain NCIB3610 (Branda *et al.*, 2001; 2006) and the natto strain (Lombardía *et al.*, 2006; Vidyalaxme *et al.*, 2012), make robust, highly structured and long-lasting biofilms, the domesticated strain JH642 is also capable of making floating biofilms (Hamon and Lazazzera, 2001; Hamon *et al.*, 2004; Lazazzera, 2005) (Fig. S1). Therefore, we wanted to know if the *de novo* lipid synthesis that is under Spo0A control and is essential for spore development is also necessary for biofilm development. To explore this possibility, we used a modified version of LB broth (LB_Y medium, see *Experimental procedures*) that enhanced biofilm formation in several *B. subtilis* strains that we tested, including the domesticated strains 168 and JH642 (Fig. S1 and A. Rovetto and R. Grau, unpubl. results). As shown in Fig. 9A, the *accDA*^{OA} cells (strain RG1130) were severely impaired in their capacity to make a biofilm (floating pellicle) in comparison



with the biofilm proficiency of wild-type cells of the JH642 strain inoculated in the same LB medium after 36 h of incubation at 30°C (Fig. 9A). The quantification of the rate of biofilm formation (see *Experimental procedures*) confirmed the inability of the *accDA*^{OA} cells to construct a biofilm (Fig. 9B). To confirm that the deficiency in biofilm development of the Spo0A-insensitive *accDA*^{OA} cells was due to a deficiency in *de novo* FA synthesis, we supplemented the LB broth with exogenous FAs (nC16:0 and nC18:1) and followed the kinetics of biofilm formation. The supplementation with exogenous FAs completely restored the proficiency of biofilm formation in the *accDA*^{OA} cells (Fig. 9A and B).

Why would *de novo* lipid synthesis be required for biofilm development? The proficiency of the *accDA*^{OA} cells to grow planktonically but not to develop spores raises the possibility that *de novo* FAs synthesized under conditions that allow the formation of the biofilm would have an unnoticed function in biofilm development. We hypothesized that the requirement of active and Spo0A-dependent *de novo* lipid synthesis during the development of the biofilm would not be restricted to the structural role of lipids as components of the cell membranes (Flemming and Wingender, 2010). Therefore, how do *B. subtilis* cells develop a biofilm? The extra cellular matrix (ECM) of bacterial biofilms contains several extracellular polymeric substances mainly formed by polysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender, 2010; Abee et al., 2011). In addition to the highly hydrated extracellular polymeric substances (polysaccharides and proteins), other secreted molecules (lipids) have hydrophobic properties that would enhance the repellency properties of the biofilm (Fig. S2

Fig. 8. Mother cell compartmentalization of *de novo* lipid synthesis during sporulation.

A. The genes for the *de novo* lipid synthesis machinery are asymmetrically distributed. The cartoon depicts the chromosomal positions (in a 360° array) of the genes required for soluble FA (*fabHA*, *fabHB*, *fabF*, *fabI*, *acpA*, *fabD*, *fabG*, *accBC* and *accDA*, yellow in the figure) and membrane-bound PL and GL synthesis (*pssA*, *psd*, *mpfF*, *plsC*, *plsX*, *pgsA*, *cdsA*, *ugtP*, *gpsA*, *dgkA*, *clsA*, *ywjE* and *ywiE*, violet in the figure) in *B. subtilis* (<http://genolist.pasteur.fr/SubtilList>). The only genes that are not depicted in the cartoon are *pssA* and *psd* because, although initially trapped in the forespore, they are involved in the synthesis of phosphatidylserine, which is absent from the membranes of *B. subtilis* (Nishibori et al., 2005; Matsumoto et al., 2006). The open circle represents the location of *oriC* (0°/360°). The double lines represent the mother cell and forespore membranes (green and red respectively) that delimit both compartments of the sporangium. Note that all of the genes that encode the enzymes involved in the formation of FAs (yellow colours) and most, if not all, of the genes involved in the synthesis of the membrane lipids (violet colours) normally found in the membranes of *B. subtilis* are located at positions that are trapped in the mother cell compartment at the time of asymmetric division. The genes *ywiE* and *ywjE* are depicted between the two membranes because their chromosomal locations are close to the location of the forespore DNA region that is trapped within the septum at the time of asymmetric division. Apart from the genes involved in FA synthesis (yellow), three key genes involved in the synthesis of membrane lipids that are far from the *oriC* (and are thus the last genes to be translocated to the forespore) are: *gpsA*, which product Glycerol 3-phosphate dehydrogenase (GpsA) catalyses the synthesis of glycerol 3-phosphate, a key precursor of membrane lipid synthesis; *pgsA*, which product Phosphatidyl-glycerophosphate synthase (PgsA) catalyses the committed step in the synthesis of the acidic PLs, and *ugtP*, which product the Glucosyltransferase UgtP acts in the maturation of the membrane GLs (see text for details).

B. At the end of the exponential phase of growth, the activity of the *de novo* lipid synthesis machinery is minimal (left cartoon). Then, after the activation by phosphorylation of Spo0A, this transcription factor triggers the asymmetric division of the sporulation-committed cell and the reactivation of *de novo* lipid synthesis in the larger compartment (right cartoon). Asymmetric lipid synthesis in the mother cell compartment results from four mechanisms: (i) the Spo0A-driven reactivation of malonyl-CoA synthesis in the mother cell (this work) but not in the forespore due to the absence of Spo0A activity in the smaller compartment (Arbolaza et al., 2003; Fujita and Losick, 2003), (ii) the compartmentalization of ACP and its absence from the forespore compartment (Martínez et al., 2010), (iii) the positioning of all the enzymes involved in PL and GL synthesis in the membrane of the asymmetric septum during sporulation (Nishibori et al., 2005; Matsumoto et al., 2006), and (iv) a temporary asymmetry in gene distribution (see A in this figure) in which two copies and zero copies of each gene involved in new FA formation are present in the mother cell and forespore compartments respectively. These four events result in an imbalance in new lipid formation that favours the mother cell site. FAS: soluble FA synthase complex; PLS: membrane-bound PL and GL synthases.

and Conrad et al., 2003). In several Gram-positive bacteria, including *B. subtilis*, membrane glycolipids have been implicated in biofilm formation, but an explanation of the mechanism by which these lipids affect biofilm formation is lacking (Lazarevic et al., 2005; Theilacker et al., 2009). In addition to the presence of lipids, the hydrophobic characteristics of the biofilm have been attributed to the covalent modification of extracellular polymeric molecules with

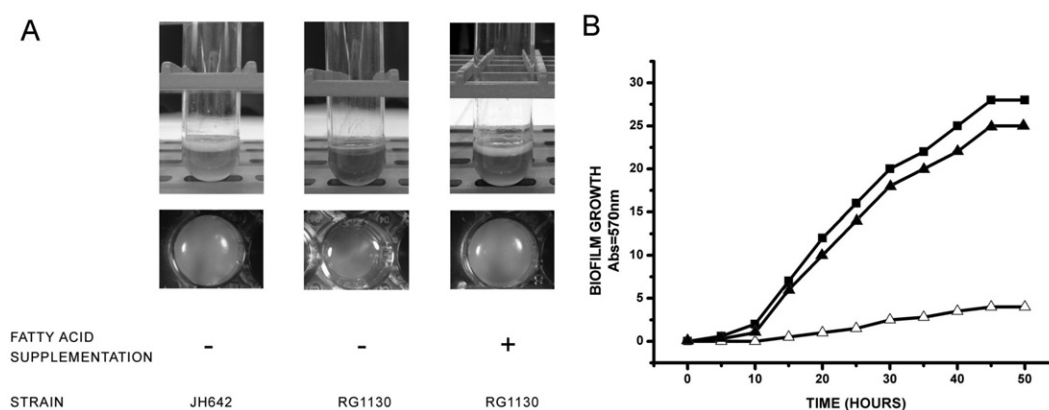


Fig. 9. Spo0A-dependent *de novo* fatty acid synthesis is required for biofilm development.

A. Biofilm development (floating pellicles) of standing cultures of wild-type (left) and *accDA*^{0A} cells (middle and right pictures) grown in biofilm-stimulating medium (LBV) with or without supplementation with exogenous fatty acids (nC16:0 and nC18:1). Biofilms were developed in glass tubes (side views) and microtitre wells (top-down images) at 30°C, and pictures were taken after 36 h of incubation. Similar results were observed in several independent experiments.

B. Rate of biofilm formation (see *Experimental procedures*) of wild-type and *accDA*^{0A} cells grown in LBV broth using 96-well microplates with or without exogenous fatty acid supplementation (nC16:0 and nC18:1). Symbols: (■) wild type cells and (Δ) or (▲) *accDA*^{0A} cells grown in the absence or presence of fatty acid supplementation respectively.

FA-related molecules (Neu and Poralla, 1988; Neu *et al.*, 1992) and the secretion of biosurfactants to the ECM of the biofilm (Davey *et al.*, 2003; López and Kolter, 2010; Epstein *et al.*, 2011). The ECM of the biofilm made by *B. subtilis* contains an exopolysaccharide (EPS) of an incompletely known structure (EPS) and the proteins BslA (formerly named YuaB; Kobayashi and Iwano, 2012; Kovács *et al.*, 2012) and TasA (an amyloid protein with antimicrobial properties, Branda *et al.*, 2001; 2006). TasA and the EPS are secreted to the ECM in the early stages of biofilm development to allow the cells in the biofilm (pellicles) to be held together and to form clusters resembling bundled chains of cells (Branda *et al.*, 2001; 2006; Romero *et al.*, 2010). Biofilm-deficient deletion-mutants strains unable to synthesize TasA and/or EPS do not form cell clusters (Branda *et al.*, 2001; 2006; Kobayashi and Iwano, 2012), while *bslA* (*yuaB*)-mutant cells do not form pellicles after 24 h of incubation but form submerged cell clusters that resemble the cell clusters found in wild-type floating pellicles (Kobayashi and Iwano, 2012). The amphiphilic nature of BslA, its participation in biofilm formation after the cell clusters are allowed to be formed by EPS and TasA and its topographical distribution encasing the biofilm suggest that this protein (BslA) is the major contributor to the surface repellency and hydrophobicity of *B. subtilis* biofilms (Kobayashi and Iwano, 2012; Kovács *et al.*, 2012).

To determine at which stage of biofilm development the active Spo0A-dependent *de novo* lipid synthesis machinery is required, we compared the phenotype of *accDA*^{0A} mutant cells grown under biofilm-supporting conditions (growth in LBV broth) with the biofilm phenotype of different *B. subtilis* mutant strains deficient in the synthesis of the ECM components TasA (*tasA*), EPS (*epsG*) and BslA

(*bslA*). As shown in Fig. 10, the biofilm phenotype of *accDA*^{0A} cells was similar to the biofilm phenotype of *tasA* and *epsG* cells (Fig. 10A and B) in that they all failed to form bundled chains (clusters) of cells (Fig. 10C). In contrast, although, the BslA-deficient mutant cells also failed to make floating pellicles, they were capable to form cluster of cells similar to the ones formed by wild-type cells (Fig. 10C). One important property of *B. subtilis* biofilms, apart from the capacity to form cell clusters, is their water repellency (Epstein *et al.*, 2011; Kobayashi and Iwano, 2012 and Fig. S2). It is proposed that the robustness of the *B. subtilis* ECM is reached by BslA acting synergistically with TasA and EPS in a way to increase the surface repellency and impermeability of the biofilm (Epstein *et al.*, 2011; Kobayashi and Iwano, 2012; Kovács *et al.*, 2012). Therefore, we investigated whether *de novo*-formed FAs are also required to achieve the hydrophobicity (water repellency) of the *B. subtilis* biofilm (Fig. S2). As shown in Fig. 10D, in contrast to wild-type JH642 cells, *accDA*^{0A} cells (along with the *eps*, *bslA* and *tasA* cells) completely failed to repel the entrance of water into the biofilm. This result indicates a novel role of *de novo* FA synthesis, under Spo0A control, in the capacity of *B. subtilis* cells to abandon the vegetative lifestyle and develop into a biofilm.

Discussion

Spo0A, lipid homeostasis and development

Biological membranes are more complex than was appreciated when the 'fluid mosaic model' was proposed forty years ago (Singer and Nicolson, 1972; Engelman, 2005; Lee, 2005). Biological membranes form the interface

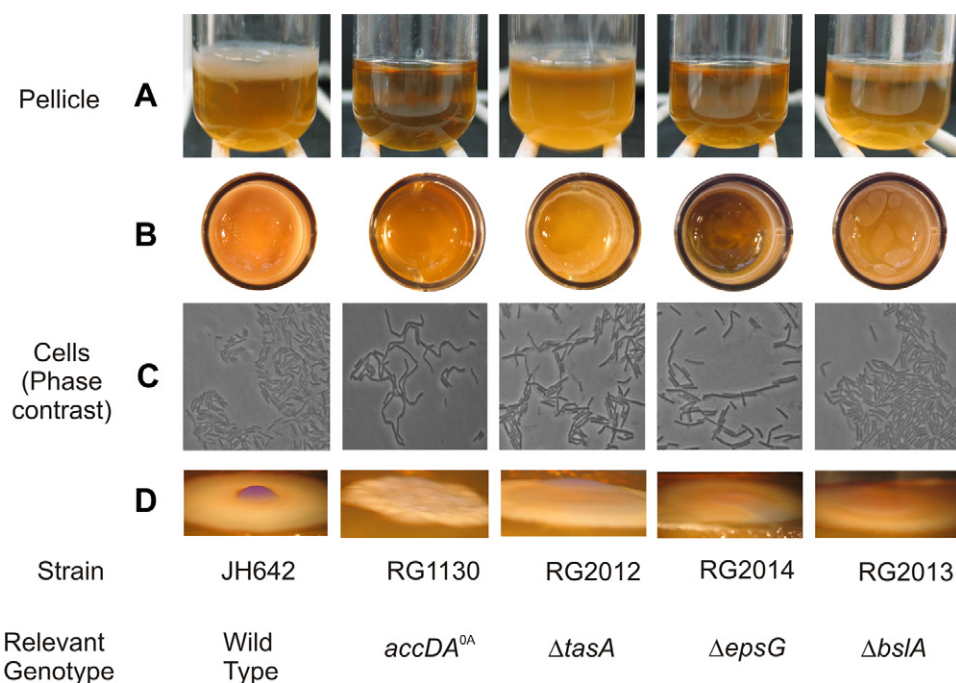


Fig. 10. The Spo0A-dependent *de novo* fatty acid synthesis is required for cell cluster formation and full surface repellency of *B. subtilis* biofilms.

A and B. The biofilms of standing cultures of the wild-type (JH642) and mutant cells (*accDA*^{0A}, *eps*, *tasA*, *bslA*) were developed in glass tubes (A, side views) and microtitre wells (B, top-down images) at 30°C, and pictures were taken after 36 h of incubation.

C. Cells in pellicles from A–B were observed by phase contrast microscopy after 36 h of incubation (D) The surface water repellency capacity of solid biofilms of each strain grown at 30°C for 48 h on solid (1.5% agar) LB medium. Ten-microlitre coloured water drops were placed on top of each colony. Top-side views of the water drops on the biofilms are shown. Similar results were observed in several independent experiments.

between the cell and its environment and are key players in cellular homeostasis and signal transduction (Jahn *et al.*, 2003; Choard, 2008; Fairn and Grinstein, 2008). At the simplest level, to make a functional membrane, two major components (the membrane proteins and their lipid partners) must be correctly synthesized and packed (Dowhan and Bogdanov, 2009; 2011). But how is the biogenesis of a membrane controlled and linked to the correct execution of a genetic programme? During development in *B. subtilis*, Spo0A is the key transcription factor that triggers the developmental pathways of spore and biofilm formation. Here, we demonstrated for the first time that Spo0A also controls the biosynthesis of the second major component of the membrane: the lipids (Fig. 1). We demonstrate the existence of active and robust *de novo* FA and membrane lipid synthesis during sporulation (Figs 2–4). We also demonstrate that the reactivation of the *de novo* lipid synthesis during development is due to the Spo0A-dependent reactivation of the synthesis of malonyl-CoA, the central metabolite of FA synthesis and lipid homeostasis in the cell (Figs 5 and 6). Among the many genes whose products participate in the *de novo* lipid synthesis machinery (Fig. 1B), only one genetic unit, the *accDA* operon, appears to be controlled by Spo0A.

Why does Spo0A only regulate the activity of the operon involved in malonyl-CoA synthesis? And, how does Spo0A control the global synthesis of FAs and membrane lipids by controlling only *accDA* expression? In addition to its essential role as a precursor of FA synthesis, malonyl-CoA is a direct and specific negative modulator of FapR, which is a conserved transcriptional repressor of several genes involved in FA and membrane lipid synthesis (the *fap* regulon) in Gram-positive bacteria (see Fujita *et al.*, 2007; Zhang and Rock, 2008 for reviews). The binding of malonyl-CoA to FapR prevents the binding of FapR to (and/or promotes the release of FapR from) its target DNA sequences, an event that derepress *de novo* lipid synthesis (Fujita *et al.*, 2007). Therefore, Spo0A functions as the key regulator of *de novo* lipid synthesis during development by providing appropriate levels of malonyl-CoA for new lipid formation and linking the levels of this central metabolite to the downregulation of the activity of the lipid synthesis repressor FapR (Fujita *et al.*, 2007). Reinforcing the view of the central role of Spo0A in lipid synthesis homeostasis during development is the fact that the only genes involved in FA synthesis that are not controlled by FapR are those encoding the ACC enzyme responsible for malonyl-CoA formation (Fujita *et al.*, 2007; Zhang and

Rock, 2008), and these *acc* genes are the only genes involved in lipid synthesis that are controlled by Spo0A (Liu *et al.*, 2003; Fujita and Losick, 2003) (Fig. S3).

Lipid asymmetry and development

Although the roles of lipids during development remain poorly understood, FAs have been proposed to play key regulatory roles during the establishment of protein asymmetry in a variety of model organisms, such as the nematode *Caenorhabditis elegans* (Rappleye *et al.*, 2003), the plant *Arabidopsis thaliana* (Baud *et al.*, 2003) and the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Schneider and Kohlwein, 1997; Iwamoto *et al.*, 2004). It was found that depletion of FAs or loss of acetyl-CoA carboxylase activity in *C. elegans* through the *pod-2* mutation causes a loss of asymmetry and mislocalization of the PAR-3/PAR-2 cell fate determinants during the first developmental division, supporting a regulatory rather than structural role of *de novo*-synthesized FAs (Rappleye *et al.*, 2003; Golstein and Macara, 2007). Here, we demonstrated that the Spo0A-controlled *de novo* FA synthesis was required for sporulation proficiency (Fig. 7) and biofilm development (Figs 9 and 10). In addition, our results in combination with previous reports (Arabolaza *et al.*, 2003; Fujita and Losick, 2003; Nishibori *et al.*, 2005; Matsumoto *et al.*, 2006; Martínez *et al.*, 2010) strongly suggest the asymmetric nature of the *de novo* lipid synthesis machinery (confined to the mother cell compartment) during sporulation (Fig. 8). Why should *de novo* lipid synthesis be asymmetric during the development of the spore?

We demonstrated that maximal Spo0A-dependent *de novo* lipid synthesis occurred during the key developmental stages of polar septum formation and engulfment of the forespore by the mother cell membrane (see Figs 3 and 4). The formation of an asymmetrically positioned septum and the phagocytosis-like process of forespore engulfment by the mother cell membrane constitute hallmark events during which active membrane remodelling (membrane curvature, fusion, migration and fission) accompanies the correct positioning and activation of key cell fate determinants to specific membrane sites (Hartwell and Weinert, 1989; Cutting *et al.*, 1990; Ireton and Grossman, 1992; Stragier *et al.*, 1998; Abanes-De Mello *et al.*, 2002; Ramamurthi and Losick, 2005; 2009; Broder and Pogliano, 2006; Campo *et al.*, 2008; Meyer *et al.*, 2010).

Fujita and Losick (2003) proposed that one reason for the high and prolonged activity of Spo0A in the mother cell compartment was to ensure a high expression level of the operon that governs the synthesis of the mother cell transcription factor σ^E (also named SpoIIGB). σ^E is synthesized as an inactive precursor, pro- σ^E , which is processed to its active form, σ^E , by the developmental membrane protease

SpoIIIGA, after the formation of the asymmetric septum (Imamura *et al.*, 2008). Both proteins, which are encoded by the *spoilG* operon, are synthesized under the direct, positive transcriptional control of Spo0A (Hilbert and Piggot, 2004). SpoIIIGA initially resides at the cytoplasmic membrane, but soon after asymmetric division, it is thought to become concentrated (by a diffusion-and-capture mechanism) on both membranes of the sporulation septum (Fawcett *et al.*, 1998; Stragier *et al.*, 1998; Fujita and Losick, 2002; Rudner *et al.*, 2002; Chary *et al.*, 2010). Under physiological conditions, pro- σ^E , present in both chambers, is processed to active σ^E only in the mother cell compartment of the sporulating cell. The action of SpoIIIR is essential for this cell type-specific activation (Karow *et al.*, 1995; Londoño-Vallejo and Stragier, 1995). This regulatory protein is produced in the forespore, under the control of σ^F , and is secreted into the intermembrane space of the sporulation septum. Once in the space between the two septal membranes, SpoIIIR exclusively activates SpoIIIGA localized on the mother cell membrane but not the SpoIIIGA located in the forespore membrane (Karow *et al.*, 1995; Londoño-Vallejo and Stragier, 1995; Fawcett *et al.*, 1998). How σ^E becomes activated only in the mother cell compartment is an unsolved question. SpoIIIR is produced in low amounts, and it has been proposed that the SpoGA molecules inserted into both septal membranes compete for the limited amount of SpoIIIR that is secreted into the intermembrane space (Chary *et al.*, 2010). Ordinarily, the amount of SpoIIIGA molecules localized at the mother cell membrane far exceeds the amount of SpoIIIGA localized at the forespore membrane because of the continuous and robust mother cell-restricted *spoilG* expression driven by Spo0A (Fujita and Losick, 2003), and the mother cell site would win the competition to activate σ^E (Chary *et al.*, 2010). However, under physiological conditions, how does 100% of the SpoIIIR secreted into the intermembrane space interact exclusively with the mother cell membrane SpoIIIGA and not with SpoIIIGA localized at the forespore membrane? In a previous work, we demonstrated that the early addition (before T_0) of the antibiotic cerulenin to *B. subtilis* cells grown in sporulation broth (DSM) does not inhibit the onset of sporulation but blocks the processing of pro- σ^E to active σ^E (Schujman *et al.*, 1998). In the present work, we also showed that the absence of *de novo* FA synthesis in *accDA^{0A}* cells grown under sporulation conditions impaired the expression of σ^E -dependent genes (Fig. 7). Therefore, both sets of results (Schujman *et al.*, 1998 and this work) suggest that *de novo* FA synthesis plays a regulatory role in the activation of SpoIIIR and/or SpoIIIGA to stimulate mother cell-compartmentalized σ^E activity.

Interestingly, in a recent work, Diez *et al.* (2012) proposed that SpoIIIR-dependent σ^E activation is linked to *de novo* FA synthesis. They presented evidence for a model

in which SpoIIIGA (localized on either the forespore or mother cell membrane) interacts with SpoIIIR, which allows for its vectorial concentration in the sporulation septum. There, on both septal membranes, SpoIIIR is susceptible to covalent modification (acylation) with the fatty-acyl molecules that originated from *de novo* FA synthesis but not from *de novo* PL synthesis or previously synthesized FAs ('aged FAs') (Diez *et al.*, 2012). Based on our results, we propose that, under physiological conditions, the *de novo*-synthesized fatty-acyl molecules needed for the processing and activation of SpoIIIR (see *Supporting Information*) originate from the mother cell compartment, where *de novo* lipid acid synthesis is active (green membrane in Fig. S4). In this scenario, only the SpoIIIR molecules that interact with SpoIIIGA at the mother cell membrane (Fig. S4) would be acylated with *de novo*-synthesized FAs (Diez *et al.*, 2012) to allow for SpoIIIR processing and activation at the mother cell site of the sporulating cell. Because *de novo* FA synthesis is absent in the forespore compartment (once it receives its complete chromosome with the full set of genes for lipid synthesis, 15–20 min after completion of the asymmetric division, the activity of the lipid synthesis activator Spo0A is turned off, and the ACP molecules are restricted to the mother cell compartment, see Arabolaza *et al.*, 2003; Fujita and Losick, 2003; Martínez *et al.*, 2010), SpoIIIR molecules interacting with SpoIIIGA positioned on the forespore membrane of the sporulation septum (Diez *et al.*, 2012) would not be activated, restricting σ^E activation exclusively to the larger compartment (Fig. S4).

Lipid synthesis and biofilm development

The production, secretion and assembly of the diverse components of the ECM are crucial for the capacity of *B. subtilis* (and bacteria in general) to develop into a mature biofilm (López and Kolter, 2010; Abee *et al.*, 2011; Epstein *et al.*, 2011; Kobayashi and Iwano, 2012). We envision that the requirement of *de novo* FA synthesis during the development of the biofilm in *B. subtilis* (Fig. 9) is not restricted to the supply of new lipids for cell membrane formation. This hypothesis is based on two experimental observations. First, we demonstrated that the Spo0A-proficient *accDA^{OA}* cells were capable of healthy growth during the vegetative phase in DSM broth, with a generation time of symmetric (vegetative) division and a final cellular yield similar to those exhibited by wild-type cells, but failed to activate a specific developmental stage (activation of σ^E -dependent genes) during the stationary (sporulation) phase. These results suggest a regulatory, rather than structural, role for FAs during sporulation (see Figs 8 and S4). Second, the complete loss of water repellency of the *accDA^{OA}* cells developed under biofilm-supporting conditions (Fig. 10D) strongly indicates that the

new FAs formed under Spo0A control play an important role in the development of the floating capacity, impermeability and hydrophobic interactions of the cells within the biofilm (Figs 9 and 10). All of these properties are major determinants in biofilm formation. From these results, we infer that the deficiency in biofilm formation of *accDA^{OA}* cells (Fig. 9) could have been due to a specific blockage during the development of the biofilm rather than a structural role of *de novo*-synthesized lipids as components of the cell membranes in the biofilm. When comparing the biofilm phenotype of *accDA^{OA}* cells with the phenotype displayed by different biofilm-deficient mutant strains of *B. subtilis* (mutant strains *tasA*, *epsG* and *bslA*), we demonstrated that standing cultures of *accDA^{OA}* cells developed weak pellicles similar to the ones formed by *epsG* and *tasA* cells (Fig. 10). The *accDA^{OA}* cells failed to form structured cell clusters and floating pellicles unless exogenous FAs were added to the growth medium, suggesting that the biofilm-defective phenotype was due to the absence of *de novo* FA synthesis under conditions that supported the formation of the biofilm (Figs 9 and 10). These results indicate the requirement of *de novo* FA synthesis at an early stage of biofilm development, before cell cluster formation. What would be the role of *de novo*-synthesized lipids at an early stage of the development of the biofilm in *B. subtilis*? In addition to the presence of lipids (Conrad *et al.*, 2003; Lazarevic *et al.*, 2005; Theilacker *et al.*, 2009; Abee *et al.*, 2011), the hydrophobic characteristics of the biofilm have been attributed to the covalent modification of extracellular polymeric molecules with FA-related molecules (Neu and Poralla, 1988; Neu *et al.*, 1992) and the secretion of biosurfactants to the ECM of the biofilm (Davey *et al.*, 2003; López and Kolter, 2010; Epstein *et al.*, 2011). Because the strain used in this study (JH642) does not form surfactin (the main biosurfactant required for the formation of robust and long-lasting biofilms in undomesticated strains of *B. subtilis*, Branda *et al.*, 2001), it is tempting to speculate that ECM components of the *B. subtilis* biofilm (i.e. TasA, BslA and/or the exopolysaccharide) might be modified with FA-related molecules to achieve the extreme repellency and floating capacity of *B. subtilis* biofilms (Figs S2 and 10) (Epstein *et al.*, 2011; Kobayashi and Iwano, 2012; Kovács *et al.*, 2012). Although, to date, a post-translational modification of any ECM component of the biofilm of *B. subtilis* has not been reported (Abee *et al.*, 2011; Kovács *et al.*, 2012), this possibility should not be completely ruled out.

Experimental procedures

Strains, media and growth conditions

The *B. subtilis* strains used in this study are described in Table S1. *B. subtilis* was propagated in LB broth for experiments performed under non-sporulating conditions. For

sporulation, *B. subtilis* was either grown in DSM, which triggered sporulation via nutrient exhaustion (Arabolaza *et al.*, 2003), or was induced to sporulate via a resuspension method in Sterlini-Mandelstam medium (Schujman *et al.*, 1998). β -Galactosidase activity was assayed as previously described, and the specific activity is expressed in Miller units (Arabolaza *et al.*, 2003).

Strain constructions

A procedure consisting of consecutive PCR steps (Reder *et al.*, 2008) was used to obtain a *B. subtilis* strain carrying point mutations in the OA-box (ttgtcgaa) present in the promoter region of the *accDA* operon. In the first step, an overlapping extension protocol was used to inactivate the OA-box present in the promoter region of the *accDA* operon by introducing site-specific mutations in that OA-box (ctgtagag); the mutated nucleotides are shown in bold. To this end, two sets of primers were used in two separate amplification reactions using 168 chromosomal DNA as template. One pair of primers was used to amplify a DNA fragment of 1059 bp that consisted of the OA-box together with upstream sequences; the forward primer UP-F (5'-aagatattgcagcgccaaac-3') and the reverse primer UP-R (5'-ctcaactctacagctttttatcgatg-3'). The second pair of primers was used to amplify a DNA fragment of 922 bp that consisted of the OA-box together with the downstream sequences; the forward primer DW-F (5'-aaaagctgtagaggttgaggatgac-3') and the reverse primer DW-R (5'-atgaataaccgcatcaagctg-3'). Once purified, these two PCR products, presenting a small overlapping sequence containing the mutated OA-box, were mixed together and extended through the short homologous region in a third PCR using only the flanking primers (underlined). In this way, a linear DNA fragment of 1962 bp, which enclosed the complete promoter region of the *accDA* operon containing a mutated OA-box, was obtained.

This 1962 bp DNA fragment was used as template for a modified two-step fusion protocol (Wach, 1996) designed to insert an erythromycin resistance marker upstream of the mutated OA-box. The antibiotic resistance cassette was obtained through a standard PCR protocol using pMUTIN4 (Vagner *et al.*, 1998) as template and the primers ER-F (5'-gtgccacctgacgtctaag-3') and ER-R (5'-agtctggactgggctgtgtag-3') as described (Reder *et al.*, 2008). Using the 1962 bp DNA fragment as template and two sets of primers in separate amplification reactions, two DNA fragments with almost no overlapping sequence were obtained. One pair of primers was used to amplify an upstream DNA fragment of 1005 bp; the forward primer UP-F (5'-aagatattgcagcgccaaac-3') and the reverse primer UP-R_{II} (5'-cttagacgtcaggtggcacacaaggtgacagcgcatc-3'). The second pair of primers was used to amplify a downstream DNA fragment of 736 bp; the forward primer DW-F_{II} (5'-ctacacagcccagtcagactctgtcgtttcaacacctt atc-3') and the reverse primer DW-R_{II} (5'-aacttaatacgccttctgcatc-3'). The erythromycin resistance marker is indicated with italics. The three DNA fragments were mixed and fused in a stepwise manner through PCR using the flanking primers UP-F and DW-R_{II}. A similar procedure was performed using 168 chromosomal DNA as a template to obtain a wild-type linear DNA fragment containing the promoter region of the *accDA* operon with an erythromycin resistance

marker upstream of the wild-type OA-box. Both linear DNA fragments containing the erythromycin resistance marker upstream of the mutated and the wild-type OA-boxes in *accDA* were used to transform competent wild-type *B. subtilis* cells to produce the strains RG1130 (*ery^raccDA^{OA}*) and RG1340 (*ery^raccDA^{wt}*) respectively. Transformed cells, into which we inserted the erythromycin resistance cassette by a double cross-over event, were selected on LB plates containing 1 μ g ml⁻¹ erythromycin, and chromosomal DNAs of these strains were sequenced to verify the correct resistance marker insertion, the presence of the desired mutation in the OA-box and the absence of any other mutation in the strains.

To construct the β -galactosidase reporter strains RG1606 (*accDA-lacZ cat* at the *amyE* locus), RG1158 (*ery^raccDA^{OA}-lacZ cat* at the *amyE* locus) and RG1160 (*ery^raccDA^{wt}-lacZ cat* at the *amyE* locus) we PCR-amplified DNA fragments containing the promoter regions of *accDA*, *ery^raccDA^{OA}* or *ery^raccDA^{wt}* (see above) from the chromosomal DNAs of the wild-type strain 168 and strains RG1130 and RG1340 respectively. The oligonucleotides 5'-AAACAAGAAAAGGTA CCATTATTGG-3' (*KpnI* site underlined) and 5'-CATACC CGAAGCTTTTATCCAATC-3' (*HindIII* site underlined) were used as primers. The amplified PCR fragments were cloned into PJM116. After linearization, the plasmids were introduced separately via transformation into competent 168 (or JH642) cells to generate strains RG1606, RG1158 and RG1160, as previously described (Arabolaza *et al.*, 2003).

Analysis of radioactive lipids

To measure *in vivo* FA synthesis, *B. subtilis* strains were grown in their corresponding culture media with aeration for the indicated periods of time. Aliquots (1 ml) of the cultures were exposed to 10 μ Ci of sodium [1-¹⁴C]-acetate or [1-¹⁴C]-isoleucine at the time points indicated in the figure legends. After the incubation periods, the lipids were extracted as previously described (Grau and De Mendoza, 1993). FAs, PLs and GLs were separated by thin-layer chromatography on silica gel plates developed in petroleum ether/ether/acetic acid (70:30:10) and autoradiographed. *In vitro* FA synthase activity was measured as previously described (Schujman *et al.*, 1998). Cerulenin was added at a final concentration of 10 μ g ml⁻¹, and [2-¹⁴C]-malonyl-CoA was added last to initiate the reactions at 37°C for 30 min. This was followed by lipid extraction, chromatography in petroleum ether/ether/acetic acid (70:30:10) and autoradiography. In all cases, appropriate areas of the silica gel were scraped into vials containing scintillation solution to determine their radioactive content, as previously described (Grau and de Mendoza, 1993; Schujman *et al.*, 1998).

Biofilm experiments

For biofilm (pellicle) formation, overnight *B. subtilis* cultures were grown in LB medium to the stationary phase. Then, 50 μ l of each culture was diluted in 2 ml of fresh LB medium with (LB_Y) or without (LB) supplementation with 6.0 % yeast extract (biofilm growth medium). All the tubes (or microplates when indicated) were statically incubated at 30°C for 48 h.

Microtitre plate assay for *B. subtilis* biofilm formation rate

This assay measured the amount of cells adhered to the surface of the microtitre plate wells (Hamon and Lazazzera, 2001). *B. subtilis* cells were grown in 96-well polyvinylchloride (PVC) microtitre plates at 37°C in LBY biofilm medium. The bacterial inoculum for the microtitre plates was obtained by growing the cells in LB to mid-exponential phase and then diluting the cells to an OD₆₀₀ of 0.08 in biofilm growth medium. Samples of 100 µl of the diluted cells were then aliquoted to each well of a 96-well PVS microtitre plate, including 12 wells with biofilm growth medium only (negative control). At various time points during the incubation of *B. subtilis* cells in the microtitre plate wells, the biofilm formation was monitored by staining with crystal violet (CV). Growth medium and non-adherent cells were carefully removed from the microtitre plate wells. The microtitre plate wells was incubated at 60°C for 30 min. Biofilms were stained with 300 µl of 0.3 % CV in 30% methanol at room temperature for 30 min. Excess CV was then removed, and the wells were rinsed with water. The CV that had stained the biofilms was then solubilized in 200 µl of 80% ethanol/20% acetone. Biofilms formation was quantified by measuring the Abs_{570nm} for each well using a Beckman Coulter Multimode Detector (DTX 880). Each data point is an average of 12 wells, and error bars indicate the standard error ($P < 0.05$). Representative data from one of at least four independent experiments are shown.

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Supporting information

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