Transcriptional and functional characterization of the gene encoding acyl carrier protein in *Bacillus subtilis*

Mariano A. Martinez, Diego de Mendoza and Gustavo E. Schujman

Correspondence

Gustavo E. Schujman
schujman@ibr.gov.ar

Instituto de Biología Molecular y Celular de Rosario, and Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

Acyl carrier protein (ACP) is a universal and highly conserved carrier of acyl intermediates during fatty acid biosynthesis. The molecular mechanisms of regulation of the *acpP* structural gene, as well as the function of its gene product, are poorly characterized in *Bacillus subtilis* and other Gram-positive organisms. Here, we report that transcription of *acpP* takes place from two different promoters: *PfapR* and *PacpP*. Expression of *acpP* from *PfapR* is coordinated with a cluster of genes involved in lipid synthesis (the *fapR* operon); the operon consists of *fapR-plsX-fabD-fabG-acpP*. *PacpP* is located immediately upstream of the *acpP* coding sequence, and is necessary and sufficient for normal fatty acid synthesis. We also report that *acpP* is essential for growth and differentiation, and that ACP localizes in the mother-cell compartment of the sporangium during spore formation. These results provide the first detailed characterization of the expression of the ACP-encoding gene in a Gram-positive bacterium, and highlight the importance of this protein in *B. subtilis* physiology.

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INTRODUCTION

Acyl carrier protein (ACP) plays a central role in plant and bacterial fatty acid biosynthesis, where it shuttles the insoluble fatty acid intermediates during their synthesis and subsequent utilization by the different enzymes of the pathway. The best characterized example is *Escherichia coli* ACP, a 77 aa protein that has a 4-phosphopantetheine prosthetic group covalently attached to the amino acid S36 via a phosphodiester linkage (Byers & Gong, 2007; Cronan & Rock, 1996). During fatty acid biosynthesis, and transfer of the complete fatty acids to complex lipid precursors, the growing acyl chain is bound through a thioester linkage to the terminal thiol of the prosthetic group (Cronan & Rock, 1996; de Mendoza *et al.*, 2002).

In *E. coli*, ACP is encoded by the *acpP* gene, which is located within a cluster of genes involved in fatty acid biosynthesis. The gene cluster comprises (in order) *plsX*, *fabH*, *fabD*, *fabG*, *acpP* and *fabF*, which encode phosphate:acyl-ACP acyltransferase, β -ketoacyl-ACP synthase III, malonyl-CoA:ACP transacylase, β -ketoacyl-ACP reductase, ACP, and β -ketoacyl-ACP synthase II, respectively. *acpP* is transcribed from two promoters: one transcript of 0.3 kb starts just upstream of *acpP*, whereas

a longer (1.1 kb) transcript begins upstream of *fabG* (Rawlings & Cronan, 1992). The former promoter is sufficient for the synthesis of the abundant protein ACP (Zhang & Cronan, 1996). As expected, because of the key function of ACP in lipid biosynthesis, expression of *acpP* is essential in *E. coli* (De Lay & Cronan, 2006).

In contrast to the wealth of knowledge available on *E. coli* ACP, information about this protein in Gram-positive bacteria is scarce. In *Streptococcus pneumoniae*, *acpP* is the fourth ORF in the cluster of 13 genes involved in fatty acid biosynthesis (Marrakchi *et al.*, 2002), and it seems to be transcribed from at least two different promoters (Lu & Rock, 2006), although the physiological relevance of this has not yet been established. In *Bacillus subtilis*, the *acpP* gene is included in a cluster of lipid biosynthesis genes (Morbidoni *et al.*, 1996). In this organism, *acpP* expression has been preliminarily characterized, and the results suggest the existence of more than one promoter for this gene (Morbidoni *et al.*, 1996). Since ACP is a critical component of type II fatty acid synthase, and one of the most abundant soluble proteins in *E. coli*, it is of interest to study the mechanism(s) responsible for producing appropriate amounts of this protein in *B. subtilis*, as well the relevance of ACP in the physiology of this organism.

Here, we report that *acpP* is the last gene of the pentacistronic *fapR* operon, which encodes five proteins involved in lipid synthesis, and that *acpP* is transcribed from two promoters: *PfapR* and *PacpP*. We show that *acpP* is co-transcribed with the upstream genes from *PfapR*,

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; RACE, rapid amplification of cDNA ends; SD, Shine-Dalgarno; tsp, transcription start point.

Details of plasmid construction, operon organization and primers used in this study are available with the online version of this paper.

whereas it is specifically expressed as a single gene from the strong promoter *PacpP*. Expression from the latter promoter is sufficient to supply ACP for normal fatty acid biosynthesis. We also demonstrate that, in the absence of *PacpP*, the synthesis of ACP becomes coupled to derepression of the *fap* regulon, which is composed of 10 genes involved in fatty acid and phospholipid synthesis. Finally, we present evidence that *acpP* is an essential gene for growth and differentiation in *B. subtilis*, and that ACP is preferentially confined to the mother cell during sporulation. This study provides the first physiological characterization of ACP in a Gram-positive bacterium, and describes the mechanisms employed by *B. subtilis* to control the synthesis of appropriate amounts of this essential protein.

METHODS

Strains. All bacterial strains are listed in Table 1. The *B. subtilis* strains were grown in Luria–Bertani (LB) broth (Difco), Spizizen minimal medium (SMM: Spizizen, 1958) or Difco sporulation medium (DSM). *E. coli* strains were propagated in LB broth.

Plasmids and DNA manipulation. Plasmids were constructed using standard methods, and amplified in *E. coli* DH5 α and XL1-Blue. PCR fragments were amplified from chromosomal DNA of *B. subtilis* strain JH642. For further details on DNA manipulation and plasmid construction, see Supplementary material. Plasmids and primers used in this study are listed in Supplementary Tables S1 and S2, respectively (available with the online version of this paper).

General methods. Preparation of *B. subtilis* competent cells was carried out by using the two-step method described by Harwood (1990). Antibiotics were supplied by Sigma, and used at the following concentrations: 5 μ g chloramphenicol ml⁻¹, 0.5 μ g erythromycin ml⁻¹ plus 12.5 μ g lincomycin ml⁻¹, 100 μ g spectinomycin ml⁻¹ and 5 μ g kanamycin ml⁻¹. For the β -galactosidase assay in plates, 40 μ g X-Gal ml⁻¹ was used.

In most experiments cells were induced to sporulate by resuspension in StM medium, as described by Sterlini & Mandelstam (1969). Alternatively, DSM was used (Schaeffer *et al.*, 1965). Spore titre was determined by heat treatment (20 min at 80°C) 18 h after sporulation initiation.

FapR production and purification. Full-length FapR was produced from plasmid pGES229 as an N-terminal His₆-fusion protein in *E. coli*, and it was affinity purified on a Ni-NTA resin, according to the manufacturer's protocol (Qiagen). Protein concentrations were determined by using the Bradford assay (Bradford, 1976), with BSA as a standard.

Western blot analyses, and β -galactosidase assays. Western blot analyses were performed as previously described (Schujman *et al.*, 2001). GFP antibodies were raised in rabbits that had been injected with GFP, and they were used at a dilution of 1:1000. Proteins were detected using alkaline-phosphatase-conjugated anti-rabbit secondary antibodies (Bio-Rad).

β -Galactosidase specific activity [ΔA_{420} min⁻¹ (ml culture)⁻¹ $\times$ 1000/OD₅₂₅] was determined as described by Miller (1972), after pelleting cell debris.

In vitro transcription. *In vitro* transcription assays were performed as described by Opdyke *et al.* (2001). The templates for the reactions

were DNA fragments containing the promoter regions of *fapR* (192 bp) and *acpP* (255 bp); the fragments were amplified by PCR using primer pairs ylpC116U–ylpC116L and *PacpEco*–*PextACP*, respectively (Supplementary Table S2). Purified template DNA was added to the reaction buffer at a final concentration of 13 nM for *PacpP*, and 4.3 nM for *PfapR*, and then incubated in the absence and presence of FapR (213 nM final concentration). Transcription was initiated by adding RNA polymerase purified from *B. subtilis*, NTPs and [α -³²P]UTP (Perkin–Elmer), as previously described (Schujman *et al.*, 2006). The RNA transcripts were precipitated, resuspended in formamide loading buffer, and subjected to electrophoresis in a 6 % polyacrylamide gel containing 7 M urea. Radioactive signals were detected by exposing the gel to a PhosphorImager screen, and they were digitalized by using a Storm 840 scanner (GE Healthcare). Quantification of the corresponding signals was carried out using ImageQuant software (version 5.2) (www.imsupport.com).

Growth and metabolic labelling of mutant strains. BMM94 (*acpP::kan amyE::Pxyl-acpP-cat*) was grown overnight on LB agar containing kanamycin and 0.3 % (w/v) xylose. Cells were resuspended in LB medium without xylose to an OD₅₂₅ of 0.05, and incubated at 37 °C for 2 h in the absence of inducer to dilute the pre-existing protein. Then, the culture was divided into two parts, and *acpP* expression was induced in one half of the culture by the addition of 0.3 % (w/v) xylose. After incubation for 30 min, cells were incubated with 74 kBq [¹⁴C]acetate ml⁻¹ for 60 min ([2-¹⁴C]acetate with a specific activity 2.035 TBq mol⁻¹ was obtained from American Radiolabeled Chemicals). Cells were collected, and the total lipids were extracted by using the Bligh and Dyer method (Bligh & Dyer, 1959). Radioactivity incorporated into the total lipids was quantified by using a scintillation counter, and the results were expressed as c.p.m. (OD₅₂₅ unit)⁻¹. For sporulation studies of strain BMM94 and derivatives, cells were grown in CH medium (Harwood, 1990), in the absence of xylose, until ACP became limiting for growth. The cells were then harvested, and resuspended in Sterlini–Mandelstam sporulation medium (StM) in the presence or absence of the inducer. Sporulation frequency was measured 24 h after initiation of sporulation. BMM105 (Δ *PacpP*) and GS282 were grown overnight in SMM containing the appropriate antibiotic. Cells were inoculated in fresh minimal medium to an OD₅₂₅ of 0.1, and grown at 37 °C. At the indicated time, cells were incubated with 37 kBq [¹⁴C]acetate ml⁻¹ for one generation period. Cells were collected, and total lipids were extracted, as described above.

Temperature-sensitive phenotypes of *B. subtilis* strains bearing mutant forms of ACP were tested at 42 °C, as reported for *E. coli* (De Lay & Cronan, 2006), or at higher temperatures, such as 44 or 50 °C, as *B. subtilis* is able to grow at temperatures of up to 56 °C.

RNA analysis. Total *B. subtilis* RNA was isolated from cells grown in LB medium, as described by Raya *et al.* (1998). For Northern blot analyses, 15 μ g total RNA was separated under denaturing conditions in a 1.2 % agarose gel containing 1.1 % formaldehyde, and transferred to a Hybond-N+ membrane (Amersham). RNA blots were hybridized with ³²P-labelled DNA fragments, using random hexanucleotides as primers for Klenow DNA polymerase. Probe 1 was a 360 bp fragment obtained from the chromosome of strain JH642 by PCR amplification using the oligonucleotides ACPBgIIIF and ACPBamHI, which included the coding region of *acpP*. Probe 2 was a 1000 bp fragment obtained by PCR amplification from the chromosome of strain JH642 using the oligonucleotides FabDupp and FabDLw, which included part of the coding region of *plsX* and *fabD* (Supplementary Table S2). Hybridization of the membranes was carried out at 65 °C in a solution containing 5 $\times$ SSPE [0.9 M NaCl, 0.05 M sodium phosphate (pH 7.7), 5 mM EDTA], 0.5 % SDS, 5 $\times$ Denhardt's solution, and 20 mg salmon sperm DNA l⁻¹. Final washing of the membranes was conducted at 65 °C in 0.1 $\times$ SSPE

Table 1. Bacterial strains

Strain	Relevant characteristics	Source or reference
<i>B. subtilis</i>		
JH642	<i>trpC pheA1</i>	Laboratory stock
GS69	<i>PacpP-acpP-gfp-spc</i>	This study
GS282	<i>pMutin::fabHB-lacZ-ermC</i>	Schujman <i>et al.</i> (2003)
GS277	<i>amyE::P_{fapR}-lacZ-spc</i>	Schujman <i>et al.</i> (2003)
GS285	<i>pMutin::fabHB-lacZ-ermC fapR::cat</i>	Schujman <i>et al.</i> (2003)
GS367	<i>ΔfapR-cat</i>	Schujman <i>et al.</i> (2006)
GS380	<i>pMutin::fabHB-lacZ-ermC fapR::cat thrC::Pxyl-fapR7-spc</i>	Schujman <i>et al.</i> (2006)
MF56	<i>spoIIIGB-gfp-spc</i>	Fujita & Losick (2002)
MF339	<i>amyE::P_{spac}^c-gfp-spc</i>	Fujita & Losick (2002)
MO1190	<i>ΔspoIIIGA</i>	Guerout-Fleury <i>et al.</i> (1996)
BMM18	<i>thrC::PacpP-lacZ-ermC</i>	This study
BMM44	<i>amyE::PacpP-gfp-spc</i>	This study
BMM62	<i>amyE::P_{spoIIQ}-acpP-gfp-cat</i>	This study
BMM64	<i>spoIIAC::kan amyE::P_{spac}^c-gfp-spc</i>	This study
BMM88	<i>spoIIAC::kan amyE::PacpP-gfp-spc</i>	This study
BMM93	<i>amyE::Pxyl-acpP-cat</i>	This study
BMM94	<i>acpP::kan amyE::Pxyl-acpP-cat</i>	This study
BMM99	<i>ΔPacpP-kan</i>	This study
BMM101	<i>ΔSpoIIIGA amyE::PacpP-gfp-spc</i>	This study
BMM102	<i>ΔSpoIIIGA amyE::P_{spac}^c-gfp-spc</i>	This study
BMM105	<i>ΔPacpP-kan fabHB-lacZ-ermC</i>	This study
BMM106	<i>acpP::kan amyE::Pxyl-acpP-cat spoIIIGB-gfp-spc</i>	This study
BMM109	<i>thrC::Pxyl-acpP-spc</i>	This study
BMM111	<i>thrC::Pxyl-acpP-spc acpP::kan</i>	This study
BMM115	<i>ΔfapR-cat amyE::P_{fapR}-lacZ-spc</i>	This study
BMM118	<i>acpP::kan thrC::Pxyl-acpP-spc amyE::P_{spac}^{hy}-acp E. coli</i>	This study
BMM119	<i>acpP::kan thrC::Pxyl-acpP-spc amyE::P_{spac}^{hy}-acp A68T N73D E. coli</i>	This study
BMM120	<i>acpP::kan thrC::Pxyl-acpP-spc amyE::P_{spac}^{hy}-acp D38V E. coli</i>	This study
BMM122	<i>acpP::kan thrC::Pxyl-acpP-spc amyE::P_{spac}^{hy}-acpP JH642</i>	This study
BMM123	<i>acpP::kan thrC::Pxyl-acpP-spc amyE::P_{spac}^{hy}-acpP D38V B. subtilis</i>	This study
BMM124	<i>ΔPacp-kan fabHB-lacZ-ermC amyE::Pxyl-acpP-spc</i>	This study
BMM125	<i>ΔPacp-kan fabHB-lacZ-ermC thrC::Pxyl-fapRWT-spc</i>	This study
BMM126	<i>ΔPacp-kan fabHB-lacZ-ermC thrC::Pxyl-fapR7-spc</i>	This study
BMM127	<i>ΔPacp-kan fabHB-lacZ-ermC thrC::Pxyl-fapRWT-spc amyE::P_{spac}^{hy}-acpP-cat</i>	This study
BMM128	<i>ΔPacp-kan fabHB-lacZ-ermC thrC::Pxyl-fapR7-spc amyE::P_{spac}^{hy}-acpP-cat</i>	This study
BMM129	<i>thrC::Pxyl-acpP-spc ter-ΔPacpP-kan</i>	This study
BMM131	<i>ΔfapR::cat thrC::PacpP-lacZ-ermC</i>	This study
RG19148	<i>spoIIAC::kan</i>	Arabolaza <i>et al.</i> (2003)
<i>E. coli</i>		
MG1655	Wild-type	Strain collection
M15	Used for expression of <i>fapR</i>	Qiagen
NRD28	<i>acpP(ts) A68T N73D ΔfabF::cat</i>	De Lay & Cronan (2006)
NRD29	<i>acpP(ts) D38V ΔfabF::cat</i>	De Lay & Cronan (2006)

plus 0.1% SDS. Radioactive signals were detected by exposing the nitrocellulose membranes to a PhosphorImager screen. The size of the *acpP* transcript was determined by comparison with RNA molecular mass standards (Promega).

Fluorescence microscopy. Sporulation was induced according to the method of Sterlini & Mandelstam (1969). At appropriate time points during sporulation, 0.5 ml aliquots of the sporulating cultures were removed, centrifuged briefly, and resuspended in 0.05 ml PBS supplemented with the membrane stain FM4-64 (1 μg ml⁻¹; Molecular Probes). The concentrated cell suspension was placed on

a microscope slide that had been covered with a thin layer of 1% agarose.

RT-PCR. For RT-PCR analysis, RQ1-DNase (Promega) was added to each RNA preparation at a final concentration of 0.05 U (μg RNA)⁻¹ μl⁻¹. The preparations were incubated for 30 min at 37 °C, and then inactivated by adding EGTA to a final concentration of 2 mM, and heating the mixture at 65 °C for 10 min. The amount of extracted RNA was determined by measuring A₂₆₀. RT was performed with 2.4 μg RNA, 0.5 μg dN₆ primers, and 8 U M-MLV reverse transcriptase (Promega). PCRs on the RT products were

performed using primer pairs ylpC5Hind and plsXBglII, pplsXUp and fabDmutB, fabGUp and fabGmut, and PacpEco and PacpSph (Supplementary Table S2). A PCR performed using equal amounts of non-retrotranscribed RNA was used as a control for genomic DNA contamination of the RNA samples. To detect the whole operon transcript, primers FapRT137 and PacpSph (Supplementary Table S2) were used, as described in the protocol developed by Agüena & Spira (2003).

Identification of the transcriptional start site using RNA-ligase-mediated rapid amplification of cDNA ends. RNA was extracted from strain JH642, which had been grown to OD₆₀₀ 1.5 at 37 °C in 15 ml LB medium, and the *acpP* transcription start site was mapped using the GeneRacer kit (Invitrogen). All experimental steps were performed according to the manufacturer's instructions. This technique is based on RNA-ligase-mediated and oligocapping rapid amplification of cDNA ends (RACE) methods, and it results in the selective ligation of an RNA oligonucleotide to the 5' ends of decapped mRNA using T4 RNA ligase. Synthesis of first-strand cDNA was performed with the gene-specific reverse primer ACPGFPEco, and direct amplification of tailed cDNA using a nested gene-specific primer ACPD38VR, and an abridged anchor primer. PCR products were cloned into pCR BluntII TOPO (Invitrogen), and five clones were sequenced. The first nucleotide in the sequence that came after the ligated RNA oligonucleotide sequence was taken as the transcriptional start site.

RESULTS

acpP is transcribed from two independent promoters

It has been previously reported that *acpP*, the gene encoding *B. subtilis* ACP, is contained in a cluster of lipid biosynthesis genes that is located downstream of *plsX*, *fabD* and *fabG* (Morbidoni *et al.*, 1996). In a later study, we found that *fapR*, the gene encoding the global regulator of fatty acid and phospholipid biosynthesis, is localized upstream in the same cluster (Schujman *et al.*, 2003). Several observations have strongly suggested that *fapR*, *plsX*, *fabD* and *fabG* are co-transcribed in an operon (previously named *fapR*): the intergenic distance between each of the genes is less than 22 nt, and expression of the genes is under the transcriptional control of FapR, responding to the same stimuli (Schujman *et al.*, 2003). Moreover, in a strain containing a deletion of *fapR*, which has a polar effect on the transcription of downstream genes, expression of *plsX*, *fabD* and *fabG* is diminished (Schujman *et al.*, 2008). In contrast, *acpP* is separated by 87 nt from the 3' end of *fabG*, and a σ^A -dependent promoter has been proposed to exist in this intergenic sequence (Jarmer *et al.*, 2001; Morbidoni *et al.*, 1996); this suggests that *acpP* is transcribed independently (Fig. 1a). To address this matter, we performed Northern blot analysis using probes against different genes of the cluster. A probe containing the full sequence of *acpP* annealed to an RNA fragment of approximately 300 bp, which correlates with the length of *acpP* mRNA, while no signal was detected for longer RNA fragments (Fig. 1b). Moreover, we did not detect any signal with probes containing the *fabG* sequence (data not shown) or the *plsX-fabD* region (Fig. 1b). These results suggest that *fapR-plsX-*

fabDG mRNA is labile, or that it is present in very low amounts, and the results strongly indicate that *acpP* is transcribed as a single gene, in agreement with previous results (Morbidoni *et al.*, 1996). As described below, confirmation of the existence of an independent promoter for the *acpP* gene came from the analysis of gene reporter fusions to the *fabG-acpP* intergenic region.

To test whether the cluster of lipid genes is transcribed as an operon, and to find out if *acpP* is part of the operon, we performed RT-PCR assays. In these experiments, we determined the existence of mRNA containing the intergenic region between each gene of the cluster, and also mRNA comprising the whole cluster (Fig. 1c). Taken together, our results demonstrate that *fapR*, *plsX*, *fabD*, *fabG* and *acpP* comprise the *fapR* operon, and that *acpP* is transcribed from two different promoters: (1) *PfapR*, which is shared with the rest of the lipid biosynthesis cluster; and (2) *PacpP*, which is specific for the gene (Fig. 1a).

PacpP is a FapR-independent strong promoter

Although *acpP* is transcribed from *PfapR* and *PacpP*, previous results have suggested that *acpP* expression is not significantly regulated by FapR (Schujman *et al.*, 2003). Those observations seem to indicate that expression from *PacpP* is independent of FapR, and that this promoter is stronger than *PfapR* in physiological conditions. To confirm these hypotheses, we constructed four strains bearing *PacpP-lacZ* or *PfapR-lacZ* fusions in a wild-type or a *fapR*[−] background. The strains were grown in LB broth, and assayed for β -galactosidase activity. There were no appreciable differences in the growth curves of the four strains (data not shown). The pattern of expression from the *PacpP* promoter in wild-type and *fapR*[−] strains was similar, confirming that *PacpP* expression is not regulated by FapR (Fig. 2a). In contrast, the levels of β -galactosidase expressed from a *PfapR-lacZ* fusion were 5- to 10-fold higher in the *fapR*[−] strain than in the wild-type strain (Fig. 2a). These results were confirmed by *in vitro* transcription assays: transcription from *PfapR* was inhibited in presence of purified FapR, while transcription from *PacpP* was unaffected by the repressor (Fig. 2b). In wild-type strains, β -galactosidase specific activity from the *PacpP-lacZ* fusion was about 10-fold higher than that from *PfapR-lacZ* (Fig. 2a). This result agrees with the results obtained with Northern blot experiments, in which a band corresponding to *acpP* mRNA was readily observed, while no signal was detected for the *fapR* operon mRNA. Together, these results indicate that while the expression of *fapR* operon is coordinated with that of the *fap* regulon (Schujman *et al.*, 2003), *acpP* is also expressed independently from the strong *PacpP* promoter.

Determination of the transcription start point of *acpP*

In order to characterize the *PacpP* promoter, the transcription start point (tsp) of the *acpP* gene transcript

Fig. 1. Genetic organization of the *fapR-plsX-fabD-fabG-acpP* operon. (a) Schematic representation of the *fapR* operon. Numbered arrows indicate the annealing regions of the oligonucleotide primer pairs utilized for the RT-PCR experiments. The primers were as follows: 1, ylpC5Hind; 2, plsXBglII; 3, pplsXUp; 4, fabDmutB; 5, fabGUp; 6, fabGmut; 7, PacpEco; 8, PacpSph; 9, FapRT137. The predicted sizes of the amplified DNA fragments are indicated. (b) Analysis of the mRNA transcripts derived from the *fapR* operon. RNA from the exponentially growing wild-type strain JH642 was isolated, and the full coding sequence of *acpP*, and a sequence containing the 3' half of *plsX* and the 5' half of *fabD*, were used to prepare the ³²P-labelled probes. Northern blotting was carried out with the individual probes, as described in Methods. Transcripts for *plsX* and *fabD* were below detection limits, and the same result was obtained using RNA from the *fapR*⁻ strain GS367. (c) RT-PCR of *fapR* operon intergenic sequences. cDNA was synthesized using dN₆ primers and RNA of strain JH642 as the template. PCR was performed with the oligonucleotide pairs 1 and 2, 3 and 4, 5 and 6, and 7 and 8, as shown in (a). cDNA for the RT-PCR of the whole *fapR* operon was synthesized, as described in Methods. PCR was performed with the oligonucleotides PacpSph and FapRT137 [8 and 9, respectively, in (a)]. DNA from strain JH642 or GS367 was used as a positive control (DNA), and RNA that had not been treated with reverse transcriptase was used as a negative control (RNA-RT).

was determined. We performed five independent 5' RACE experiments, and all sequences were mapped to the same nucleotide: the adenine nucleotide 22 nt upstream of the translation start (Fig. 3); we propose that this nucleotide is the tsp. Consensus -10 and -35 sequences of the σ^A -dependent *B. subtilis* promoter are present upstream of the *acpP* transcriptional start, and a consensus Shine-Dalgarno (SD) sequence is located downstream of the tsp, separated by nine bases from the translation start codon (Fig. 3). In agreement with these results, *in vitro* transcription assays using purified *B. subtilis* σ^A -RNA polymerase, and the complete *fabG-acpP* intergenic region as a template, produced mRNA of a length compatible with that expected

for the identified tsp (data not shown). The genetic elements described, and operon organization, are not restricted to *B. subtilis*. Analysis of the genomes of all bacteria belonging to the phylum *Firmicutes*, and containing a *fapR* homologue, indicates that they all share the pentacistronic operon structure, the same *PacpP* promoter, and a perfect consensus for the SD sequence (Supplementary Fig. S1).

ACP is essential for growth in *B. subtilis*

In *E. coli*, *acpP* is an essential gene because its product is the only ACP present in this organism, and it is indispensable

Fig. 2. *PacpP* is a strong promoter not regulated by *FapR*. (a) *B. subtilis* strains BMM18 (*fapR*⁺, ●) and BMM131 (*fapR*⁻, ◆) harbouring a *PacpP-lacZ* fusion, and strains GS277 (*fapR*⁺, ■) and BMM115 (*fapR*⁻, ▼) containing a *PfapR-lacZ* fusion, were grown in LB medium at 37 °C. At the indicated times, samples of each culture were collected, and assayed for β-galactosidase activity (filled symbols). Growth was monitored by measuring OD₅₂₅, and a representative curve is shown (○). A single result representative of at least three independent experiments is shown. Replicate values varied by not more than 15% about the mean. (b) *In vitro* transcription assays were performed with the *fabG-acpP* intergenic region or *PfapR* promoter as the template, in the absence and presence of *FapR*. The radioactive signal from each transcript, from two independent experiments, was quantified as described in Methods. The ratios of signal intensities of the *in vitro* transcription performed in the presence versus the absence of *FapR* were 0.85 ± 0.05 for *PacpP*, and 0.04 ± 0.01 for *PfapR*.

for fatty acid biosynthesis (De Lay & Cronan, 2006). In the genome of *B. subtilis*, several sequences encode ACP homologues, although most of the homologues are involved in secondary metabolism, such as polyketide and non-ribosomal peptide synthesis (Mootz *et al.*, 2001). To determine if *acpP* is essential in *B. subtilis*, we constructed the strain BMM94, in which *acpP* expression is controlled by the xylose-inducible Pxyl promoter. This strain required xylose for growth, indicating that the *acpP* gene is essential in *B. subtilis* (Fig. 4). We confirmed that the growth defect was due to reduction of fatty acid biosynthesis, by labelling cultures of BMM94 with [2-¹⁴C]acetate, in the presence and absence of xylose. Radioactivity incorporated into lipids of the non-induced cells was less than 10 % of that detected when *acpP* expression was induced (Fig. 4, inset). In contrast, incorporation of [2-¹⁴C]acetate into non-lipid (essentially protein) cell components of ACP-depleted cells was above 50 % of that detected in cells expressing *acpP* (data not shown); this confirmed that the cells were metabolically active, but that they were specifically blocked in lipid

biosynthesis. These results indicate that the only acyl carrier protein of *B. subtilis* that is able to interact with the fatty acid synthase to produce new fatty acids is the product of the *acpP* gene.

As described above, strain BMM94 allowed us to examine the effects of depletion of the *acpP* product on cell growth and lipid synthesis. However, this strategy is not suitable for rapid inactivation of ACP function, as depletion of both mRNA and previously synthesized protein has to take place after elimination of the inducer. Therefore, we tried to construct temperature-sensitive ACP mutants. A recent report has shown that amino acid substitutions D38V and A68T,N73D in *E. coli* ACP result in proteins that confer a temperature-sensitive phenotype (De Lay & Cronan, 2006). Because the D38 residue is conserved in *B. subtilis* ACP, we generated strain BMM123, which is a BMM94 derivative that expresses ACP[D38V] under the control of the IPTG-inducible *Pspac* promoter. As expected, strain BMM123 did not grow in the absence of inducers, but when xylose (which induces ACP gene expression) or IPTG (which

Fig. 3. Determination of the *PacpP* transcription start site. Sequence of the *PacpP* promoter region. The -10 and -35 consensus sequences for σ^A -RNA polymerase are underlined; rbs, Shine–Dalgarno sequence; $+1$, transcription initiation site.

Fig. 4. *acpP* is essential in *B. subtilis*. Growth curves in LB medium at 37 °C of strain JH642 (wild-type, ●), and strain BMM94 (*acpP::kan amyE::Pxyl-acpP-cat*) in the presence (▼) and absence (■) of xylose. Inset: fatty acid biosynthesis is precluded in the absence of ACP. Strain BMM94 was cultured in LB medium in the absence of xylose (Xyl) until ACP became limiting to growth, then xylose (0.3 %, w/v) was added to half of the culture, and after incubation for 30 min both cultures were labelled with [2-¹⁴C]acetate for 30 min. Cells were collected, and radioactivity incorporated into the lipids was measured and normalized, as described in Methods. A single result representative of at least three independent experiments is shown. Replicate values varied by not more than 15% about the mean.

induces ACP[D38V] gene expression) were added to the culture medium, the bacteria grew normally at 30 °C (data not shown). However, growth of strain BMM123 in the presence of IPTG was indistinguishable from that of the wild-type strain at 42, 44 and 50 °C, indicating that, in contrast to *E. coli*, the substitution D38V in *B. subtilis* ACP does not result in a temperature-sensitive phenotype. Then, we constructed strains BMM120 and BMM119, which express *E. coli* ACP[D38V] and ACP[A68T,N73D], respectively, under *Pspac* control in the BMM94 genetic background. These strains grew normally at 44 °C when IPTG was the only inducer added (data not shown). These results indicate that although *E. coli* ACP can fully replace the *B. subtilis* protein *in vivo*, the thermolability of *E. coli* ACP does not preclude *B. subtilis* growth. This highlights the different roles of the protein, other than fatty acid biosynthesis, in Gram-negative and Gram-positive bacteria.

***PacpP* is required for normal lipid homeostasis**

To understand the evolutionary advantage of transcribing *acpP* from two different promoters, we generated strain BMM105, a GS282 derivative, in which the *fabG-acpP* intergenic region is deleted (see Methods). We expected that growth of this strain would be severely compromised due to a diminution in the ACP content, but, surprisingly,

it grew at the same rate as the wild-type strain (Fig. 5a). Moreover, *de novo* fatty acid biosynthesis was not reduced in strain BMM105 compared with the wild-type strain (Fig. 5a, inset). These observations indicate that, at least in laboratory conditions, *PacpP* is not necessary for growth or for proper fatty acid biosynthesis. A possible explanation for these results is that *PfapR*, in concert with the *fap* regulon promoters, is de-repressed in the deletion strain, thus compensating for the lack of *acpP* expression from *PacpP*. To test this hypothesis, we constructed strain BMM124, which is a BMM105 derivative containing a *Pxyl-acpP* fusion at the non-essential *amyE* locus, and a *PfabHB-lacZ* fusion. *fabHB* expression, which is under *FapR* control, was turned off under normal growth conditions, but was greatly increased in a *fapR*⁻ strain (Fig. 5b); expression of *fabHB* is also enhanced when intracellular malonyl-CoA levels increase to induce the release of *FapR* from its operator sequences (Schujman *et al.*, 2003). In strain BMM124, we found that expression of *PfabHB* was augmented 20-fold compared with the wild-type strain (Fig. 5b). Inducing the expression of *acpP* by addition of xylose to cultures of strain BMM124 reduced *PfabHB* expression to its basal level (Fig. 5b). Based on these results, we propose a model in which the absence of *PacpP* reduces *acpP* expression, slowing down fatty acid biosynthesis, and causing malonyl-CoA accumulation; this is promptly compensated by overexpression of the *fap* regulon, and this, in turn, increases ACP levels, to reach a new steady state in which fatty acid biosynthesis proceeds at a normal rate, but malonyl-CoA levels and *fap* regulon expression are increased. To further challenge this model, we used the *fapR7* allele, which encodes the superrepressor *FapR*[G107V,L128W], in which the *FapR*–malonyl-CoA interaction is disrupted (Schujman *et al.*, 2006). Expression of the *fapR7* allele in multicopy plasmids results in a lethal phenotype in *B. subtilis* cells because of transcriptional inhibition of the *fap* essential genes (Schujman *et al.*, 2006). However, although expression of *fapR7* ectopically integrated into the chromosome of wild-type cells decreased the transcription of the *fap* regulon (data not shown), the levels of enzymes required for lipid synthesis were sufficient to support normal growth (Fig. 5c, strain GS380). In contrast, the growth of Δ *PacpP* cells expressing a chromosomal copy of the *fapR7* allele (strain BMM126) was impaired (Fig. 5c), suggesting that this strain is unable to produce sufficient ACP to sustain fatty acid biosynthesis. To confirm this, the *acpP* gene, under the control of the IPTG-inducible *hyperspac* promoter, was integrated ectopically into the *amyE* locus of strain BMM126 to generate strain BMM128. IPTG-induced production of ACP relieved the deleterious effect of *fapR7* on Δ *PacpP* cells, while, in the absence of IPTG, growth of these cells was severely affected (Fig. 5c). These data demonstrate that malonyl-CoA-mediated overexpression of *acpP* is essential to overcome the defect in lipid synthesis generated by the absence of the *PacpP*-specific promoter. Altogether, our results indicate that, although *PacpP* is not essential for *B. subtilis* growth, it contributes to maintain lipid homeostasis in this organism.

Fig. 5. *PacpP* deletion alters fatty acid biosynthesis regulation. (a) *B. subtilis* strains BMM105 ($\Delta PacpP$, ●) and GS282 (wild-type *PacpP*, ▼) were grown in SMM at 37 °C. Growth was monitored by measuring OD₆₂₅. [2-¹⁴C]Acetate was added at the indicated time (arrow). After one generation, cultures were collected, and radioactivity incorporated into the lipids was determined, as described in Methods (see inset). (b) *B. subtilis* strains GS282 (wild-type), GS285 ($\Delta fapR$) and BMM124 ($\Delta PacpP$), harbouring a *PfabHB-lacZ* fusion at the *amyE* locus, were grown in LB medium, with and without xylose (0.3%, w/v) at 37 °C. At the indicated times, samples of each culture were collected, and assayed for β -galactosidase activity. ■, GS282; ◇, GS285; ●, BMM124 cultured without xylose; ▽, BMM124 cultured with xylose. In (a) and (b), a single result representative of at least three independent experiments is shown. Replicate values varied by not more than 15% about the mean. (c) Derivatives of strain BMM105 ($\Delta PacpP$) containing wild-type or superrepressor (*fapR7*) alleles of *fapR* under the control of the xylose-inducible *Pxyl* promoter, and a copy of the *acpP* gene fused to the IPTG-inducible *Pspac* promoter were generated. Strains were grown on LB agar containing no inducers (top-right plate); LB agar plus 0.3% (w/v) xylose (Xyl) (bottom-left plate), and LB agar plus 0.3% (w/v) xylose and 1 mM IPTG (bottom-right plate), for 24 h at 37 °C. The names and genotypes of each strain are indicated in the top-left diagram.

tion. This process is characterized by an asymmetrical division in which two compartments are created. The larger compartment, called the mother cell, engulfs and nurtures the smaller compartment, which is known as the forespore, and eventually lyses to release a dormant environmentally resistant spore. In a previous work, we reported that inhibition of FabF (the condensing enzyme involved in fatty acid elongation) impairs sporulation, thus preventing activation of σ^E , which directs mother-cell-specific gene transcription. The factor σ^E is synthesized as a pre-protein, and is cleaved by the membrane protease SpoIIIGA in the mother cell only (Schujman *et al.*, 1998). To test if ACP is also necessary for *B. subtilis* differentiation, we compared the sporulation frequencies of strain BMM94 induced to sporulate in the absence and presence of xylose. We found that in the absence of ACP, sporulation efficiency was less than 0.005% of that of the corresponding strain induced to sporulate in the presence of the inducer, as judged by the production of heat-resistant c.f.u. (spores). As shown in Fig. 6(a), ACP-depleted cells produced pro- σ^E in normal amounts, but they did not process it; this confirmed that *de novo* fatty acid biosynthesis was essential for σ^E -directed gene expression.

Previous experiments that have assessed the expression of FASII genes during growth have shown that their transcription is maximal during mid-exponential growth, and is almost turned off after the cells reach stationary phase, and begin to sporulate (Schujman *et al.*, 2001). However, we found a different expression pattern of *PacpP* in cells growing in LB medium, where the highest transcriptional activity was observed at the end of exponential growth (Fig. 2a). To analyse *acpP* expression

ACP is required for, and is compartmentalized during *B. subtilis* differentiation

Under nutrient limitation and high cell density, *B. subtilis* initiates a differentiation process that is termed sporula-

Fig. 6. ACP is necessary for sporulation, and it localizes in the mother cell. (a) Strain BMM94 was induced to sporulate, as described in Methods, and harvested 6 h after initiation of sporulation. Pro- σ^E and σ^E content was determined by immunoblotting, as described in Methods. Similar results were obtained for the untreated culture when samples were collected 18 h after resuspension in sporulation medium. (b) Subcellular localization of ACP-GFP during sporulation. Strain GS69, carrying the *PacpP-acpP-gfp* fusion, was induced to sporulate by resuspension in StM, and cells were examined 2 h later by using fluorescence microscopy. The sporangia were treated with 4,6-diamidino-2-phenylindole (DAPI) dye to visualize DNA. Left, fluorescence from DAPI; right, fluorescence from ACP-GFP. The arrows indicate the forespore (condensed DNA in the DAPI image). (c) Effect of the σ^E -dependent *PspolIQ* promoter on the localization of ACP-GFP in strain BMM62 (*amyE::PspolIQ-acpP-gfp*). Cells were induced to sporulate by resuspension in StM, and examined 3 h later. (d) Distribution of GFP (top panels) generated from the *PacpP* promoter. Two hours after induction of sporulation by resuspension in StM, the sporangia were treated with the vital membrane stain FM4-64 to visualize membranes (bottom panels). Columns: 1, BMM44 (*amyE::PacpP-gfp*); 2, BMM88 (*sigF⁻ spollAC::kan amyE::PacpP-gfp*); 3, BMM101 (unable to process σ^E ; Δ *spolIGA amyE::PacpP-gfp*). (e) Distribution of GFP generated from the constitutive σ^A -dependent *Pspac^c* promoter. Columns: 1, MF339 (*amyE::Pspac^c-gfp*); 2, BMM64 (*spolIAC::kan amyE::Pspac^c-gfp*); 3, BMM102 (Δ *spolIGA amyE::Pspac^c-gfp*). Cells were treated as described in (d).

during sporulation, we turned to DSM medium, in which *B. subtilis* starts to sporulate at the beginning of stationary phase, after nutrients have been exhausted. In this condition, *acpP* transcription was maximal at the end of exponential growth, and continued for at least 3 h after the initiation of sporulation (data not shown). The reporter-fusion results were confirmed by fluorescence microscopy experiments, which analysed the signal of the GFP fused to the carboxyl end of ACP, or to the carboxyl end of FabF,

which was used as a control. Both tagged proteins were evenly distributed in the cytoplasm during the exponential phase of growth. However, while the signal for FabF-GFP was below the level of detection after sporulation initiation (data not shown), ACP-GFP was readily observed in sporulating cells (Fig. 6b). Strikingly, the distribution of the signal of ACP-GFP in septated cells was not uniform, and it was only barely detectable in the smaller compartment (Fig. 6b). To determine whether this localization pattern

was due to a differential expression of *acpP* in both of the compartments, or to the degradation of the ACP–GFP chimera in the forespore, we utilized two different strategies. First, we fused the gene encoding ACP–GFP to a promoter (*PspoIIQ*) that is recognized by the forespore-specific transcription factor σ^F immediately upon septation. By using this fusion, we determined that fluorescence was localized in the forespore (Fig. 6c). This result indicates that, when targeted to the forespore, ACP–GFP is stable, and accumulates in the small chamber of the sporangium. Second, we analysed the localization of GFP when expressed directly from the *PacpP* promoter. The fluorescent probe was found predominantly in the larger compartment (Fig. 6d). Together, these results strongly suggest that localization of the ACP–GFP chimera in the mother cell is due to transcriptional events, rather than to translational or post-translational events.

Next, we sought to examine whether a negative regulator synthesized specifically in the forespore was repressing *PacpP*, or whether a transcriptional activator of this promoter was specifically acting in the mother cell. To address these questions, we analysed *PacpP-gfp* expression in mutants blocked in cell-specific activation of σ^F (which is responsible of forespore-specific gene expression) or σ^E (which directs mother-cell-specific gene transcription). As shown in Fig. 6(d), expression from *PacpP* did not change in the mutant cells, suggesting that transcription from *PacpP* is not regulated by a compartment-specific regulator during sporulation. When GFP was transcribed from the σ^A -dependent constitutive promoter *Pspac^E*, we observed only a slight decrease in the fluorescent signal from the forespore after septum formation (Fig. 6e). This suggested that preferential expression of ACP in the mother cell is not common to all σ^A -dependent promoters, but that it especially increased for its native promoter, *PacpP*.

DISCUSSION

In this work, we provide evidence to demonstrate that *acpP* is part of the pentacistronic operon *fapR-plsX-fabD-fabG-acpP*. Although mRNA of the expected size for the entire operon was not detected by Northern blots, we demonstrated its existence by RT-PCR (Fig. 1c). We also provide evidence that each gene of the cluster is transcribed concatenated with the adjacent gene (Fig. 1c), and expression of the four initial genes is controlled by the same transcriptional regulator FapR, which interacts with only one operator located at the promoter of the operon (Schuijman *et al.*, 2003). Finally, when the intergenic region *fabG-acpP* was deleted, expression of *acpP* became regulated by FapR, indicating that it is transcribed exclusively from *PfapR*, together with the other four genes of the operon (Fig. 5). It should be noted that de Hoon *et al.* (2005) predicted, by using bioinformatics, the existence of a transcriptional terminator in the *fabG-acpP* intergenic region. To discard this hypothesis, we constructed a Δ *PacpP* *B. subtilis* strain that conserves the

putative transcriptional terminator (BMM129, see Methods), and determined that *acpP* is efficiently transcribed from *PfapR* (data not shown), demonstrating that the predicted sequence does not encode a functional transcriptional terminator.

Several sequences similar to that of the *acpP* gene are found in the genome of *B. subtilis* (Calderone *et al.*, 2006; Mootz *et al.*, 2001). Most of the sequences encode carrier protein domains that are present in the context of larger proteins, but others, such as *acpK*, encode free-standing ACPs that are able to receive from malonyl-CoA a malonate group that is processed by other enzymes. These ACP homologues are key components in the production of secondary metabolites, such as polyketides and non-ribosomal peptides (Calderone *et al.*, 2006; Mootz *et al.*, 2001). Here, we showed that, in the absence of *acpP* expression, growth was inhibited as a consequence of lack of *de novo* fatty acid biosynthesis (Fig. 4). These experiments demonstrate that *acpP* is an essential gene in *B. subtilis*, and confirm that its homologues, such as *acpK*, are not involved in primary metabolism. De Lay & Cronan (2006) have shown that ACP is essential in *E. coli* by the use of thermosensitive versions of the protein. Intriguingly, *B. subtilis* strains containing the same construction as the sole functional ACP were able to grow normally, even at higher temperatures. It is possible that a key enzyme in fatty acid biosynthesis in *E. coli* is unable to interact with ACP[D38V] and ACP[A68T,N73D] at 42 °C, but that its counterpart in *B. subtilis* recognizes the mutant proteins at non-permissive temperatures. A second possibility is that the intracellular milieu of *B. subtilis* differs from that of *E. coli*. For example, differences in chaperone proteins, and in protein degradation rates, can suppress temperature-sensitive mutants (Van Dyk *et al.*, 1989). A third plausible explanation is that residual activity of the mutant version of ACP is sufficient for fatty acid biosynthesis in *B. subtilis*, but not in *E. coli*. Another possible explanation is that the growth inhibition in *E. coli* temperature-sensitive mutants at high temperature is, in part, due to an essential function of ACP that is unrelated to lipid biosynthesis, and is not necessary in *B. subtilis*. For example, ACP is needed for the transfer of acyl intermediates in lipid A synthesis, which is essential for *E. coli* growth, but is not synthesized in Gram-positive bacteria. Although ACP is clearly essential in both Gram-negative and Gram-positive bacteria, and the proteins from the different organisms are readily interchangeable (De Lay & Cronan, 2007), our results imply that the physiology and roles of ACP are not entirely conserved in different bacteria. In agreement with this hypothesis, recent work has shown that, while *E. coli* has evolved a specific stringent response involving ACP/Spot interaction, ACP does not interact with the Spot homologue in *B. subtilis* (Battesti & Bouveret, 2008).

From the few micro-organisms for which transcription of the ACP-encoding gene has been analysed (Lu & Rock, 2006; Rawlings & Cronan, 1992), a pattern has emerged, in which *acpP* is contained in a cluster of lipid biosynthesis

genes, and is expressed from two or more promoters, one of which is strong and exclusive for this gene. Physical association of genes related to the same metabolic route is a common theme in bacteria, and is an advantage for regulating the synthesis of enzymes that act sequentially. Strong promoters are usually linked to the expression of abundant proteins, such as those involved in ribosome synthesis, energy metabolism and transport. In agreement with this, *E. coli* ACP is one of the most abundant proteins in *E. coli*, and constitutes about 0.25 % of the total soluble protein (Cronan & Rock, 1996). Although growth of the $\Delta PacpP$ strain was not affected in the conditions tested, we showed *in vivo* that leaving the *acpP* gene under the sole control of the FapR-regulated *PfapR* promoter results in overexpression of *fabHB* (Fig. 5b), which is co-ordinated with the rest of the *fap* regulon (Schujman *et al.*, 2003). This compensatory mechanism to increase *acpP* expression has the adverse consequence of forcing overexpression of the entire *fap* regulon, and this could be energetically expensive. These results highlight the importance of a dedicated promoter for the *acpP* gene, and constitute a new example that emphasizes the role of FapR in bacterial lipid homeostasis.

High intracellular amounts of ACP might be needed because of its role in fatty acid biosynthesis, as the protein has to deliver the acyl intermediates to several different soluble enzymes. Also, ACP interacts with many other proteins that are unrelated to fatty acid biosynthesis (Gully *et al.*, 2003), and it transfers acyl intermediates to other proteins and building blocks (Byers & Gong, 2007). We demonstrate here that ACP plays a crucial role in the differentiation of *B. subtilis*. It is not clear yet whether its activity is necessary for fatty acid biosynthesis only, as we have previously demonstrated that this biosynthesis pathway is necessary for sporulation (Schujman *et al.*, 1998), or whether ACP also delivers an acyl intermediate to a key sporulation enzyme to modulate its function. Pro- σ^E is present in both of the compartments after asymmetrical septum formation, but it is cleaved by SpoIIIGA protease specifically in the mother cell. The mechanism responsible for this compartment-specific activation of σ^E remains elusive. We present evidence that ACP is needed for σ^E processing (Fig. 6a), and that ACP localizes preferentially in the mother cell (Fig. 6b). Thus, it is tempting to speculate that an acyl-ACP participates in the activation of the σ^E cleavage machinery, and restrains its activity to the larger compartment.

The basis for compartmentalization of ACP during sporulation is unclear. It has been reported recently that during passage through the asymmetrical septum, forespore DNA becomes depleted of its associated proteins, including σ^A . This would diminish σ^A -dependent transcription in the small compartment (Marquis *et al.*, 2008). Fujita & Losick (2002), and our own results (Fig. 6e) indicate that this is not the case for the constitutive σ^A -dependent *Pspac^c* promoter. It is possible that the differential expression of σ^A -dependent promoters in each

compartment depends on the strength of the promoter, and is noticeable for only the strong promoters, such as *PacpP*. Other σ^A -dependent promoters that are known to be active during sporulation for a longer period in the mother cell than in the forespore are the Spo0A-dependent promoters (Fujita & Losick, 2003). Spo0A is the master regulator for entry into sporulation, and activates the expression of more than 100 sporulation genes. *PacpP* is not known to be controlled by Spo0A (Molle *et al.*, 2003), and in a Spo0A mutant strain, we found that expression from *PacpP* was slightly induced rather than repressed, indicating that Spo0A does not activate its transcription (data not shown). It is worth noting that we tried to purify a *B. subtilis* protein able to bind to the *PacpP* region, but our initial attempts were unsuccessful (data not shown). Identification of the mechanisms that influence transcription of this essential protein in *B. subtilis* and related bacteria should be of central importance to increase our knowledge of membrane lipid homeostasis, and the role of lipids in sporulation.

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