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Revisiting the coupling of fatty acid to phospholipid synthesis in bacteria with FapR regulation

Federico Machinandiarena[1] | Leandro Nakamatsu[2] | Gustavo E. Schujman[2,3] | Diego de Mendoza[1] | Daniela Albanesi[1]

[1]Instituto de Biología Molecular y Celular de Rosario (IBR), CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

[2]División de Biología Sintética, Ingeniería Metabólica SA (INMET), Rosario, Argentina

[3]CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

Correspondence
Diego de Mendoza and Daniela Albanesi, Instituto de Biología Molecular y Celular de Rosario (IBR), CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Santa Fe, Argentina.
Email: demendoza@ibr-conicet.gov.ar (D.d.M) and albanesi@ibr-conicet.gov.ar (D. A.)

Abstract

A key aspect in membrane biogenesis is the coordination of fatty acid to phospholipid synthesis rates. In most bacteria, PlsX is the first enzyme of the phosphatidic acid synthesis pathway, the common precursor of all phospholipids. Previously, we proposed that PlsX is a key regulatory point that synchronizes the fatty acid synthase II with phospholipid synthesis in *Bacillus subtilis*. However, understanding the basis of such coordination mechanism remained a challenge in Gram-positive bacteria. Here, we show that the inhibition of fatty acid and phospholipid synthesis caused by PlsX depletion leads to the accumulation of long-chain acyl-ACPs, the end products of the fatty acid synthase II. Hydrolysis of the acyl-ACP pool by heterologous expression of a cytosolic thioesterase relieves the inhibition of fatty acid synthesis, indicating that acyl-ACPs are feedback inhibitors of this metabolic route. Unexpectedly, inactivation of PlsX triggers a large increase of malonyl-CoA leading to induction of the *fap* regulon. This finding discards the hypothesis, proposed for *B. subtilis* and extended to other Gram-positive bacteria, that acyl-ACPs are feedback inhibitors of the acetyl-CoA carboxylase. Finally, we propose that the continuous production of malonyl-CoA during phospholipid synthesis inhibition provides an additional mechanism for fine-tuning the coupling between phospholipid and fatty acid production in bacteria with FapR regulation.

KEYWORDS

acetyl-CoA carboxylase, acyl-ACP, fatty acid kinase, Gram-positive bacteria, membrane biogenesis regulation, PlsX

1 | INTRODUCTION

Phosphatidic acid (PA) is the universal intermediate in the biosynthesis of membrane glycerophospholipids (Figure 1). Most bacteria, including Gram-positives, synthetize PA by the PlsX/PlsY/PlsC pathway (Lu *et al*., 2006; Paoletti *et al*., 2007) (Figure 1). PlsX is a peripheral membrane-associated protein that catalyzes the formation of acyl phosphate (acyl-PO_4) from long-chain acyl-acyl carrier protein (acyl-ACP), the end-products of the de novo fatty acid (FA) synthase II (FASII) (Lu *et al*., 2006). The acyl-PO_4 is then used by the PlsY acyl transferase to acylate the 1-position of glycerol-3-phosphate (G3P) (Lu *et al*., 2006). A second acyl chain is transferred to the position 2 of 1-acyl-glycerol phosphate (lyso-PA) from acyl-ACP by PlsC, producing PA (Lu *et al*., 2006; Paoletti *et al*., 2007).

In Gram-positive bacteria, exogenous and endogenous free FAs (FFAs) can access the PlsX/PlsY/PlsC acyltransferase system after their activation by the fatty acid kinase (FA kinase) (Parsons *et al*., 2014; 2015). FA kinase is composed of two subunits: a kinase domain protein (FakA) and a FA binding protein (FakB) that work together to produce acyl-PO_4 (Parsons *et al*., 2014). The resulting acyl-PO_4s are either incorporated into the 1-position of G3P by PlsY or converted to acyl-ACP by PlsX to be elongated by FASII or utilized by

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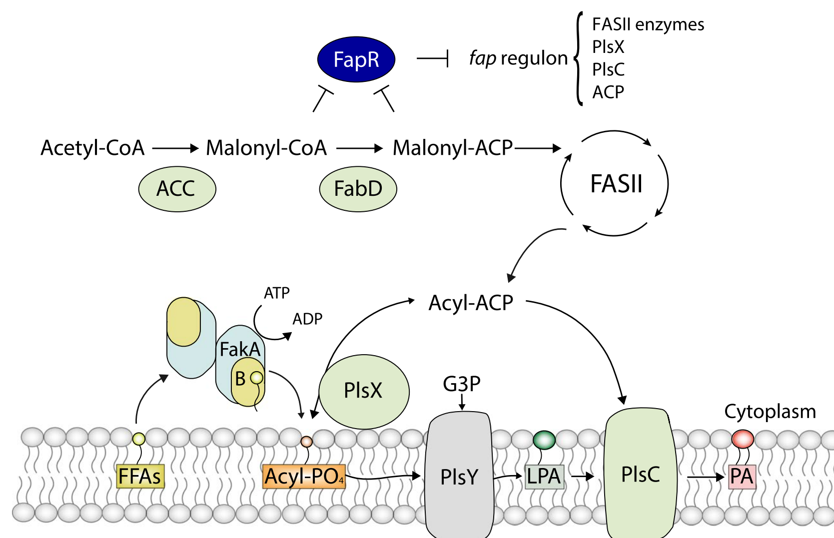

FIGURE 1 Fatty acid and phosphatidic acid synthesis in Bacillales. Malonyl-CoA is generated from acetyl-CoA by acetyl-CoA carboxylase (ACC), and then, is transferred to ACP by malonyl-CoA transacylase (FabD). The malonate group enters the type II fatty acid synthase (FASII), to generate an acyl-ACP two carbons longer than the original acyl-ACP at the end of each cycle. When the acyl chains reach an appropriate length, they become substrates of the acyl-transferase enzymes to generate phosphatidic acid (PA). First, PlsX catalyzes the synthesis of acyl-phosphate (acyl-PO_4) from acyl-ACP; then, PlsY transfers the fatty acid from acyl-PO_4 to the 1-position of glycerol-3-phosphate (G3P). Finally, lyso-PA (LPA) is acylated by PlsC obtaining PA. Free fatty acids (FFAs) can be phosphorylated by the fatty acid kinase FakA/FakB. The resultant acyl-PO_4s can be either incorporated into the 1-position of G3P by PlsY or converted to acyl-ACP by PlsX to be elongated by FASII or utilized by PlsC. Malonyl-CoA and malonyl-ACP inhibit the transcriptional repressor FapR, which controls at least 10 genes (the *fap* regulon) coding for FASII enzymes, PlsX and PlsC [Colour figure can be viewed at wileyonlinelibrary.com]

PlsC (Figure 1) (Parsons *et al*., 2011). Thus, PlsX is a key enzyme that interconverts the two acyltransferase acyl donors.

In *B. subtilis*, *Staphylococcus aureus* and other Bacillales, the expression of the enzymes involved in FA and PA syntheses is coordinately regulated by the FapR transcriptional repressor which controls the expression of at least 10 genes (the *fap* regulon) coding for FASII enzymes, PlsX and PlsC (Schujman *et al*., 2003; 2006; Albanesi *et al*., 2013; Parsons and Rock, 2013). The activity of FapR is controlled by malonyl-CoA, the product of acetyl-CoA carboxylase (ACC), the first enzyme of the FA biosynthetic pathway. Binding of this effector promotes the release of the repressor from its operator sites inducing the expression of the *fap* regulon. This property makes FapR an accurate sensor of the intracellular levels of malonyl-CoA (Albanesi and de Mendoza, 2016).

Even though the enzymology (Lu *et al*., 2006; Paoletti *et al*., 2007; Parsons and Rock, 2013) and the spatial organization (Sastre *et al*., 2020) of PA synthesis is well understood, little is known about the role of the PA biosynthetic pathway in the coupling of FA to phospholipid (PL) synthesis. Early experiments performed by our group in *B. subtilis*, through the analysis of conditional knockout mutants, showed that depletion of PlsY or PlsC blocked PL synthesis but not FA synthesis, inducing the intracellular accumulation of FFAs. Nevertheless, depletion of PlsX led to cessation of both FA and PL syntheses, suggesting that PlsX is a key regulatory point that synchronizes both biosynthetic routes. Since PlsX makes acyl-PO_4 from long-chain acyl-ACP, the most straightforward model for the inhibition of FA synthesis upon PlsX depletion is that acyl-ACPs species accumulate and inhibit key FA biosynthetic enzymes (Paoletti *et al*., 2007). This hypothesis is based on a model proposed for *E. coli* in which long-chain acyl-ACPs are feedback regulators of the FA biosynthetic pathway (Jiang and Cronan, 1994; Voelker and Davies, 1994; Cho and Cronan, 1995). The main in vivo evidence for feedback inhibition by acyl-ACPs is that overexpression of either TesA or TesB *E. coli* thioesterases not only eliminated the accumulated long-chain acyl-ACPs upon phospholipid synthesis arrest due to glycerol starvation, but also reactivated FA synthesis, allowing for their continuous synthesis (Jiang and Cronan, 1994). These results indicated that inhibition of FA synthesis may be caused by the high levels of acyl-ACPs species in the cell (Jiang and Cronan, 1994). Furthermore, acyl-ACPs have been reported to inhibit in vitro three *E. coli* FA biosynthetic enzymes: the FabI enoyl reductase (Heath and Rock, 1995), the FabH 3-ketoacyl reductase (Heath and Rock, 1996), and the acetyl-CoA carboxylase (ACC) (Davis and Cronan, 2001; Evans *et al*., 2017). Clearly, the redundant nature of FA synthesis inhibition by acyl-ACPs suggests a coordinated shutdown of the pathway in *E. coli*.

The goal of this study was to determine how PlsX coordinates the production of FAs and membrane PLs in *B. subtilis*. To more precisely investigate this regulation, we constructed a nonpolar markerless Δ*plsX* mutant. Using this strain, we show that inhibition of FA and PL synthesis mediated by acyl-ACPs upon PlsX depletion can be eliminated by heterologous expression of a cytosolic version of the type I thioesterase of *E. coli* (‘TesA). Moreover, we demonstrate that the FakA/B system is essential in *B. subtilis* for the reestablishment of PL synthesis after release of FASII inhibition by ‘TesA. Finally, in contrast to previous observations in *E. coli* and to a hypothesis that

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has been proposed for Gram-positive bacteria, we report in vivo data showing that ACC is not feedback inhibited by acyl-ACPs.

In summary, our results show that in *B. subtilis*, when acyl-PO_4 synthesis rate is lower than FA synthesis rate, the resulting accumulation of acyl-ACPs has no effect on ACC activity, but would feedback inhibit one or more steps of FASII in a coordinated manner. We postulate that the continuous production of malonyl-CoA during PL synthesis inhibition provides a mechanism to relieve dysfunctions of lipid metabolism in bacteria with FapR regulation.

2 | RESULTS

2.1 | A *B. subtilis* Δ*plsX* mutant strain accumulates long-chain acyl-ACPs

Initial experiments showing that depletion of PlsX led to cessation of both FA and PL synthesis were performed with a conditional knockout strain (LP39, Table 1) (Paoletti *et al*., 2007). Nevertheless, LP39 contains a P*xyl-plsX* gene fusion exerting a polar effect on the essential downstream fatty acid biosynthetic genes *fabD*, *fabG*, and *acpA*, complicating further investigations. To circumvent this drawback, we constructed a new *plsX* deletion strain through a markerless mutagenesis strategy (Wenzel and Altenbuchner, 2015) (see "Experimental Procedures"). Being *plsX* an essential gene, a P*xyl-plsX* construct was first placed at the non-essential *manP-manA* locus (Figure 2a). In the resultant strain (FM105, Table 1) the expression of the genes contained in the *fapR* operon are regulated by FapR and malonyl-CoA as in the wild-type strain, with the exception of *plsX* which requires xylose as inducer for its ectopic expression (Figure 2a,b).

As observed in Figure 3a, similarly to strain LP39 (Paoletti *et al*., 2007), the growth of FM105 ceased after the removal of the xylose inducer due to PlsX depletion. Next, we labeled FM105 cultures with [^{14}C]-acetate when PlsX was becoming limiting for growth, as indicated by a red arrow in Figure 3a. As expected, the acetate incorporation rate into the lipid fraction of strain FM105 in the absence of inducer was about 10% of that observed in the presence of xylose (Figure 3b). Previously (Paoletti *et al*., 2007), we suggested that the abrupt decrease in the rate of total lipids synthesis in PlsX-depleted cells could be due to a feedback regulation mechanism similar to that operating in *E. coli*, where long-chain acyl-ACPs are negative regulators of FASII (Davis *et al*., 2000; Davis and Cronan, 2001; Evans *et al*., 2017). To examine the intracellular level of long-chain acyl-ACPs, strain FM105 was cultured in LB medium in the presence or absence of xylose. The ACP pool of both cultures was analyzed by conformationally sensitive polyacrylamide gel electrophoresis (see Experimental Procedures). As shown in Figure 3c, long-chain acyl-ACPs accumulate in the absence of PlsX function. These data suggest that acyl-ACPs could be negative regulators of the initiation of lipid synthesis in *B. subtilis* and part of a mechanism responsible for the coupling between FA and PL syntheses.

2.2 | Expression of a cytosolic thioesterase in the *B. subtilis* Δ*plsX* mutant relieves FASII inhibition

It has been reported that hydrolysis of the acyl-ACPs, by heterologous expression of the TesS thioesterase from *Streptococcus pneumoniae*, rescued the growth of a *S. aureus* Δ*plsX* strain (Parsons *et al*., 2015). Thus, to test whether hydrolysis of the supraphysiological levels of long-chain acyl-ACPs accumulating in the absence of PlsX relieves FASII inhibition in *B. subtilis*, we constructed strain FM341 (Table 1). This strain is a markerless Δ*plsX* conditional mutant that expresses a cytosolic form of the type I thioesterase of *E. coli* ('TesA) in an IPTG-dependent manner from the non-essential *amyE* locus. FM341 was grown overnight in LB medium in the presence of 0.4% xylose to allow *plsX* expression. Subsequently, cells were centrifuged and three sequential washes were performed with fresh LB in order to remove the inducer. Washed cells were resuspended in LB to an $OD_{600} = 0.04$ and the culture was divided into four fractions. Each fraction was differentially treated with no inducers or either xylose, IPTG or both inducers to induce the expression of *plsX*, '*tesA* or the two genes simultaneously (Figure 4a). The growth of strain FM341 was unaffected when both, 'TesA and PlsX are expressed. As expected, in the absence of xylose and IPTG, FM341 growth is arrested due to PlsX depletion (Figure 4a). However, the induction of 'TesA expression rescued the growth of strain FM341 deprived of

TABLE 1 List of relevant *B. subtilis* strains used in this work

Strain	Genotype[a]	Reference
JH642	*trpC2 pheA1*	Dean *et al*. (1977)
LP39	JH642 *plsX*::P*xyl-plsX*, *cm*^{r}; *fabD*::P*spac-fabD*, *mls*^{r}	Paoletti *et al*. (2007)
FM105	JH642 Δ*plsX*; Δ*manPA*::P*xyl-plsX*, *kn*^{r}; Δ*amyE*::P*fabHB-lacZ*, *sp*^{r}	This work
FM341	JH642 Δ*plsX*; Δ*manPA*::P*xyl-plsX*, *kn*^{r}; Δ*amyE*::P*spank*^{hy}-'*tesA*, *cm*^{r}	This work
FM347	JH642 Δ*plsX*; Δ*manPA*::P*xyl-plsX*, *kn*^{r}; Δ*fakA*::*mls*^{r}; Δ*amyE*::P*spank*^{hy}-'*tesA*, *cm*^{r}	This work

[a]*cm*^{r}, *km*^{r}, *sp*^{r}, and *mls*^{r} denote resistance to chloramphenicol, kanamycin, spectinomycin and macrolides, respectively.

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PlsX (Figure 4a), suggesting that the free fatty acids (FFAs) liberated from the hydrolysis of acyl-ACPs by 'TesA (Figure S1) bypassed the PlsX catalyzed step to be incorporated into phospholipids.

A possible pathway that could be involved in the bypass of PlsX in strain FM341 is the FA kinase system FakA/B for the incorporation of FFAs (Parsons *et al.*, 2015; Yao and Rock, 2017). In this pathway, FFAs bind to the fatty acid binding protein FakB to be subsequently phosphorylated by the FA kinase FakA. FakB can deliver the resulting acyl-PO_4 to the membrane-bound acyltransferase PlsY to acylate G3P in the first step of phosphatidic acid synthesis (Figure 1). Therefore, to investigate whether the FakA/B system is required to bypass PlsX when FA are released by 'TesA, we interrupted *fakA* with an antibiotic cassette (*mls*^{r}) in FM341, obtaining strain FM347 (Table 1). In contrast to FM341, the Δ*fakA* FM347 strain is unable to grow in the absence of PlsX when 'TesA is expressed (Figure 4b). This result suggests that the FFAs released by 'TesA are being reincorporated as acyl-PO_4 by the action of the FakA/B system, bypassing the PlsX catalyzed step (Figure 4c). Notably, our results indicate that in *B. subtilis* the FakA/B system is able to activate endogenous FAs released from acyl-ACPs for their incorporation into PLs.

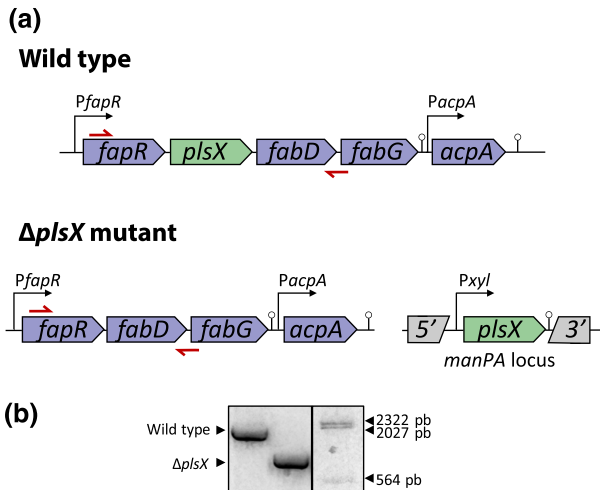

FIGURE 2 Genotyping of the markerless Δ*plsX* mutant strain. (a) Organization of the *fapR* operon in wild-type and markerless Δ*plsX* mutant strains. Ectopic expression of *plsX* from the xylose-inducible P*xyl* promoter in the mutant strain is shown. (b) PCR genotyping of strains JH642 (wild-type) and FM105 (markerless Δ*plsX*) using primers flanking the gene of interest (semi arrows of panel A and B), confirming the deletion of the *plsX* gene in the knockout FM105 strain [Colour figure can be viewed at wileyonlinelibrary.com]

To directly test if 'TesA hydrolyzes the acyl-ACPs accumulated in the absence of PlsX in strain FM347, samples were taken when the culture without inducers entered the transition from the log to the stationary phase (indicated by an arrow in Figure 4b) and the composition of the ACP pool was analyzed as described in Experimental Procedures. As shown in Figure 5a, the long-chain acyl-ACPs accumulated in cells deprived of PlsX (lane 3) were not detected when 'TesA is expressed (lane 4). Moreover, the analysis of the lipid fractions by TLC shows that expression of 'TesA in FM347 produces FFAs (Figure 5b), confirming the hydrolysis of the accumulated long-chain-acyl-ACPs. Furthermore, the analysis of the de novo FA synthesis rate showed that upon hydrolysis of the long-chain acyl-ACPs by 'TesA, FASII activity was reestablished (Figure 5c), demonstrating that in *B. subtilis* the acyl-ACPs are indeed feedback inhibitors of FA synthesis. It should be noted that even though the expression of 'TesA relieves the inhibition of FASII activity (Figure 5c), the growth of FM347 remains arrested (Figure 4b) because in the absence of *fakA*, the formation of acyl-PO_4 is impaired (Figure 4c).

2.3 | *B. subtilis* cells deprived of PlsX accumulate malonyl-CoA

The reactivation of FASII by hydrolysis of the accumulated acyl-ACPs (Figure 5c) was an indication of a regulatory mechanism that sensed the

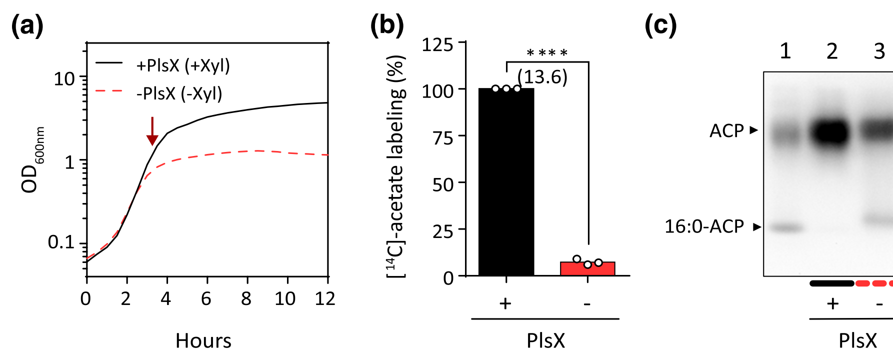

FIGURE 3 *B. subtilis* accumulates long-chain acyl-ACPs and inhibits FA synthesis in the absence of PlsX. (a) Growth curves of FM105 (Δ*plsX*; Δ*manPA*::P*xyl-plsX*; Δ*amyE*::P*fabHB-lacZ*) in LB in the presence (black line) or absence (red line) of xylose. (b) Lipid synthesis rate in FM105. Both cultures were labeled with 2 μCi/ml [^{14}C]-acetate for 30 min starting at the transition from log to stationary phase of the one without xylose (dark red arrow in Figure 2a). Cells were harvested and total lipids were quantified in a scintillation counter. The fold change of [^{14}C]-acetate incorporation in the absence of xylose is shown between brackets (Student's t-test, ****$p < .0001$). (c) ACP pool analysis in FM105 by conformationally sensitive polyacrylamide gel (20% polyacrylamide, 2.5 M urea) electrophoresis and subsequent Western Blot (WB) using rabbit anti-ACP_{Bs} antibodies. Lane 1: mixture of purified ACP and straight-chain C16:0-ACP; Lanes 2 and 3:20 μg of total protein samples from strain FM105 cultured in presence or absence of xylose, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

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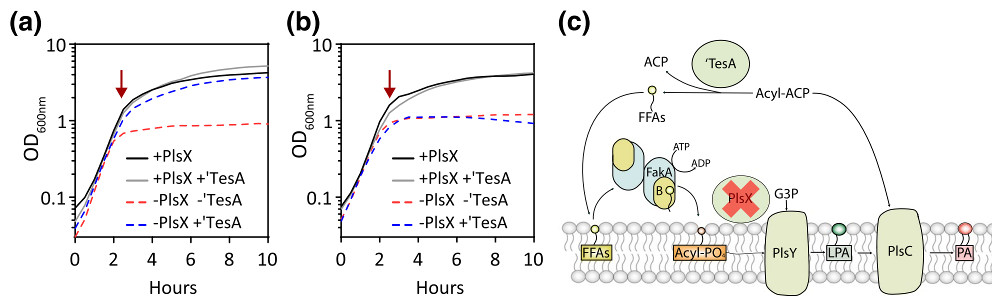

FIGURE 4 Growth curves of strains (a) FM341 (Δ*plsX*; P*xyl*-*plsX*; P*spank*^{hy}-*'tesA*) and (b) FM347 (Δ*plsX*; P*xyl*-*plsX*; P*spank*^{hy}-*'tesA*; Δ*fakA*::*mls*^{r}) in LB in the presence or absence of inducers: + xylose, -IPTG (continuous black line); + xylose, + IPTG (continuous gray line); – xylose, -IPTG (dashed red line) and – xylose, + IPTG (dashed blue line). The arrows indicate the transition to the stationary phase due to PlsX depletion. (c) Model for the bypass of the PlsX catalyzed step by the action of the FakA/B system when 'TesA is expressed. The hydrolysis of the accumulated long-chain acyl-ACP by 'TesA generates free fatty acids (FFAs) that are converted to acyl-PO_4 by the FakA/B system. The acyl-PO_4 can be taken by PlsY to acylate position 1 of G3P to generate lyso-phosphatidic acid (LPA) and proceed with the synthesis of phosphatidic acid (PA) through PlsC, reestablishing cell growth (blue dashed line in panel A). In contrast, when 'TesA is expressed but FakA is absent *B. subtilis* is incapable of producing acyl-PO_4 after PlsX is depleted and phospholipids cannot be synthetized impairing cell growth (blue dashed line in panel B) [Colour figure can be viewed at wileyonlinelibrary.com]

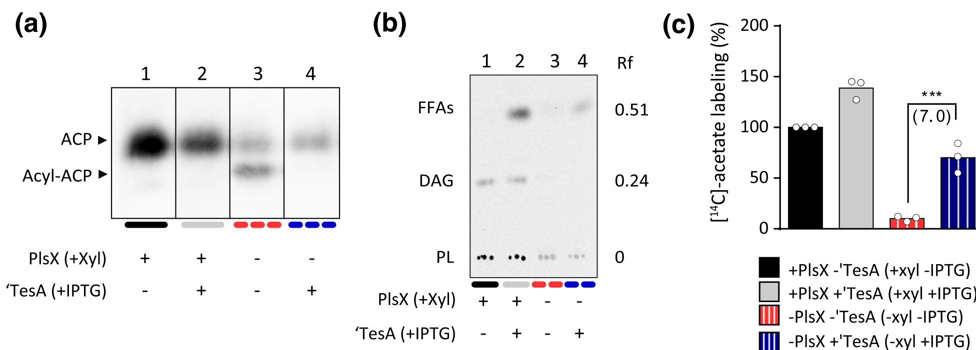

FIGURE 5 Hydrolysis of the long-chain acyl-ACPs that accumulate in the absence of PlsX suppresses FASII inhibition. (a) ACP pool analysis from *B. subtilis* FM347 (Δ*plsX*; P*xyl*-*plsX*; P*spank*^{hy}-*'tesA*; Δ*fakA*::*mls*^{r}) by conformationally sensitive polyacrylamide gel electrophoresis (20% polyacrylamide, 2.5 M urea) and subsequent Western Blot using anti-ACP_{Bs} antibodies. Cells were collected during the transition from the log to the stationary phase due to PlsX depletion, indicated by a red arrow in Figure 4b. Lanes 1 to 4:20 µg of total protein extracts from FM347 grown in the presence or absence of inducers, as indicated. (b) In parallel, all the cultures were labeled with 2 µCi/ml [^{14}C]-acetate for 30 min. Cells were harvested, total lipids were extracted, quantified in a scintillation counter and analyzed by TLC on preadsorbent Silica Gel G layers (Merck), developed with hexane-ethyl ether-acetic acid (60/40/1, vol/vol/vol). 500 cpm of labeled lipids were loaded on each lane. The different lipid species were identified by their co-migration with standards or the corresponding mobility reported in the literature. FFAs, free fatty acids; DAG, diacylglycerol; PL, phospholipid. The results are representative of three independent experiments. (c) [^{14}C]-acetate incorporation rate in the total lipid fraction of strain FM347, normalized to the + xylose (+PlsX) growth condition. The fold change of acetate incorporation when 'TesA is expressed in absence of PlsX is indicated between brackets (ANOVA test, ***$p < .001$). In all panels, the color and line patterns correlate with the growth curves shown in Figure 4b [Colour figure can be viewed at wileyonlinelibrary.com]

acyl-ACPs and inhibited FA synthesis in *B. subtilis*. We and others suggested that in this organism and in *S. aureus* the acetyl-CoA carboxylase (ACC) could be an important acyl-ACP target (Paoletti *et al*., 2007; Parsons and Rock, 2013; Yao and Rock, 2015). Inhibition of ACC should deprive the pathway of the malonyl-CoA required to continue the extension of acyl-chains. This question was explored by measuring by mass spectrometry the intracellular levels of malonyl-CoA and acetyl-CoA, the product and substrate of ACC, respectively, by switching on or off the expression of PlsX in strain FM105 (Figure 6). These data indicated that the malonyl-CoA levels increased about 20-fold following PlsX depletion (Figure 6a), while the levels of acetyl-CoA only increased 1.5-fold under the same conditions (Figure 6b). This observation was consistent with the inhibition of FASII in cells deprived of PlsX, but at the same time illustrated that the ratio (%) of malonyl-CoA/acetyl-CoA is about 15 (Figure 6c). This ratio is not compatible with inhibition of ACC by acyl-ACPs because the cells accumulated a high amount of malonyl-CoA that was not consumed in PlsX-depleted cells. The higher levels of malonyl-CoA also significantly increased the expression of genes controlled by FapR, as illustrated by the 20-fold induced transcription from the *fabHB* gene promoter (P*fabHB*) (Figure 7a) and the increased synthesis of the FabF condensing enzyme (Figure 7b,c). Altogether, these data demonstrate that accumulation of acyl-ACPs does not feedback on malonyl-CoA production to arrest FA production.

In toto, our results provide strong evidence that the general belief that acyl-ACPs are negative regulators of ACC cannot be extended to the *B. subtilis* model.

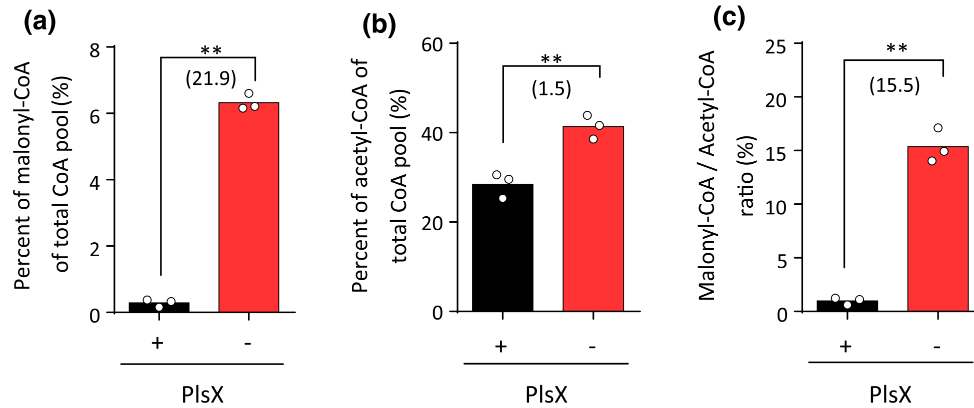

FIGURE 6 *B. subtilis* accumulates malonyl-CoA in the absence of PlsX. Cultures of FM105 (Δ*plsX*; Δ*manPA*::P*xyl-plsX*) were grown in the presence or absence of xylose and collected at the transition from log to stationary phase of the PlsX-depleted cells. Subsequently, total CoAs were extracted as described in Materials and Methods, and analyzed by HPLC-MS/MS. The amounts of (a) malonyl-CoA and (b) acetyl-CoA are enunciated relative to the total pool of CoAs. (c) Relative quantity of malonyl-CoA with respect to the acetyl-CoA pool. The fold changes of malonyl-CoA, acetyl-CoA, or malonyl/acetyl-CoA ratio, when PlsX is not expressed, are shown in brackets (Student's t-test, $^{**}p < .01$) [Colour figure can be viewed at wileyonlinelibrary.com]

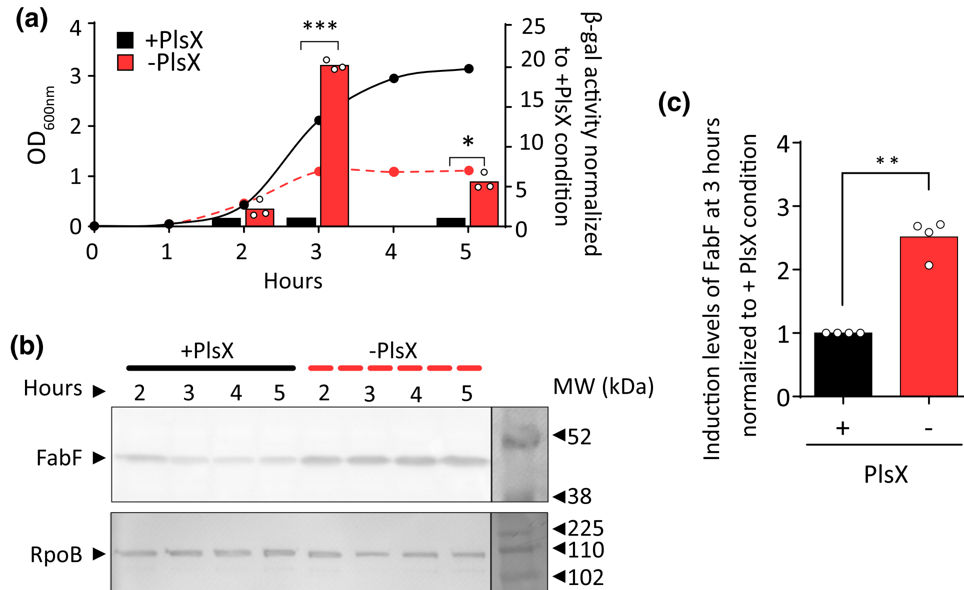

FIGURE 7 *B. subtilis* derepress the *fap* regulon in the absence of PlsX. (a) Growth curves (continuous lines) and P*fabHB-lacZ* expression analysis through β-galactosidase activity assay (bars), in FM105 (Δ*plsX*; Δ*manPA*::P*xyl-plsX*; Δ*amyE*::P*fabHB-lacZ*) cultured in LB in the presence (+PlsX, black line, and black bars) or absence (-PlsX, red line and red bars) of xylose. (b) SDS-PAGE of 20 μg of total protein extracts from FM105 strain cultured in presence or absence of xylose, followed by Western blot using antibodies against FabF and RpoB (load control, β subunit of RNA Polymerase). MW: Molecular weight marker. (c) FabF/RpoB ratio band densitometry at 3 hr after xylose removal, normalized to the +PlsX condition. The bands were quantified using ImageJ (Schneider et al., 2012). (Student's t-test, $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

3 | DISCUSSION

A crucial aspect in membrane biogenesis is the need of precise coordination of the rate of FA synthesis to the rate of PL synthesis. Since phosphatidic acid is the last common precursor of all glycerophospholipids, the glycerol-phosphate acyltransferases, which are responsible for intercepting the FASII products and transferring the acyl chains to either the *sn*-1 or the *sn*-2 carbons of G3P, are positioned at the apex of a pivotal metabolic bifurcation point.

It has been suggested that the glycerol phosphate acyltransferase PlsB, which catalyzes the first step in membrane bilayer formation in *E. coli* plays a major role in the coordination of FA with PL synthesis in this bacterium (Heath *et al*., 1994). While PlsB is almost restricted to γ-proteobacteria, most bacteria, including *B. subtilis* and *S. aureus*, lack PlsB and use the PlsX/PlsY pathway for the acylation of the *sn*-1 carbon of G3P (Figure 1) (Lu *et al*., 2006).

Here, we tested the hypothesis proposing that long-chain acyl-ACPs, end products of FASII, are key regulators of membrane

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glycerophospholipid biosynthesis in *B. subtilis* (Paoletti *et al.*, 2007). By use of a *B. subtilis* non-polar conditional *plsX* mutant strain, we showed that upon blocking PL synthesis by PlsX depletion cell growth ceased, long-chain acyl-ACPs accumulated and FA biosynthesis was downregulated. Expression of the 'TesA thioesterase resulted in relief of the inhibition of FA synthesis engendered by PlsX depletion. Since 'TesA hydrolyzed the accumulated acyl-ACPs and FASII activity was restored, it follows that the inhibition of FA synthesis is indeed caused by accumulation of acyl-ACPs.

Other interesting findings of our study were that expression of 'TesA not only reactivates FASII, but also allows *B. subtilis* PlsX-depleted cells to grow and that FakA, part of the FA kinase system, was required to bypass PlsX. These results indicate that the FakA/B system activates the endogenous FAs released from acyl-ACPs, which are used as acyl donors by PlsY in the synthesis of lyso-PA, rescuing the growth of *B. subtilis* deprived of PlsX. It should be noted that when the TesS thioesterase of *S. pneumoniae* is expressed in a Δ*plsX* strain of *S. aureus* growth is poorly restored unless a FA supplement is provided (Parsons *et al.*, 2015). This difference in the growth requirement of FA observed in *B. subtilis* and *S. aureus* upon PlsX depletion is unknown.

Our findings suggest that FA kinase may have a physiological role in *B. subtilis* recycling FAs into the PL biosynthetic pathway in the transition from exponential to stationary phase. Although little is understood about the asymmetric septum formation in cells committed to sporulate, it has been established that this process requires the synthesis of a great deal of new lipids (Schujman *et al.*, 1998; Diez *et al.*, 2012; Pedrido *et al.*, 2013). Metabolically labeled cells with [^{14}C]-acetate revealed that at the onset of sporulation a high amount of [^{14}C]-FFAs is detected (Pedrido *et al.*, 2013). These FFAs could be recycled into plasma membrane and septum membrane phospholipids of sporulating cells by the concerted action of FA kinase and PlsY.

One puzzling aspect of the regulation of the PA pathway is that long-chain acyl-ACPs accumulate in the absence of PlsY function (Figure S2), although we consistently observed that in this condition the overall rate of FA synthesis is not affected and FFAs are generated (Paoletti *et al.*, 2007; Sastre *et al.*, 2020). A similar uncoupling of FA and PL synthesis with the accumulation of long-chain acyl-ACPs and FFAs was reported using a glycerol-3-phosphate auxotroph of *S. aureus* by switching-off the PlsY activity upon deprivation of the required glycerol supplement (Parsons *et al.*, 2013). The origin of the FFAs after PlsY depletion in both, *B. subtilis* and *S. aureus*, is unknown. One possibility is that long-chain FFAs accumulate due to the hydrolysis of the unstable acyl-PO_4 formed from acyl-ACP by PlsX (Parsons *et al.*, 2013). However, long-chain acyl-PO_4 has a half-life of 12 hr at pH 7.4 and 37°C (Lehninger, 1946) being clearly sufficiently stable to be converted to acyl-ACP for the readily reversible PlsX reaction (Lu *et al.*, 2006). Alternatively, acyl-PO_4 could act as a signal to activate a yet unknown regulatory pathway that allows bypassing the inhibition of FASII by acyl-ACPs. In relation to this possibility, it has been suggested that a regulatory function for acyl-PO_4 may explain the retention of PlsX in bacteria that possesses PlsB (Lu *et al.*, 2006; Zhang and Rock, 2008). Nevertheless, the role of acyl-PO_4 in either the accumulation of FFAs or FA synthesis remains speculative.

It is clear from our work that the observed inhibition of FA synthesis can be attributed to feedback inhibition of FA biosynthetic enzymes by accumulated acyl-ACPs. In a previous work, we suggested that ACC, the enzyme that catalyzes the conversion of acetyl-CoA to malonyl-CoA, was a likely candidate for downregulation of FA synthesis following the accumulation of acyl-ACP in PlsX deprived *B. subtilis* cells (Paoletti *et al.*, 2007). This hypothesis was then extended to other Gram-positive bacteria (Zhang and Rock, 2008; Parsons and Rock, 2013; Parsons *et al.*, 2015). However, we show here that in *B. subtilis* PlsX depletion triggered a 20-fold increase of malonyl-CoA, while the levels of acetyl-CoA increased 1.5-fold under the same conditions. Clearly, these data are not compatible with feedback inhibition of ACC by acyl-ACP after arrest of both, FA and PL syntheses. Thus, we propose that in *B. subtilis* acyl-ACPs inhibit one or more enzymes of the FASII biosynthetic pathway, rather than targeting the conversion of acetyl-CoA into malonyl-CoA by ACC. Our evidence showing that ACC is not inhibited by acyl-ACPs appears to be physiologically unreasonable because this enzyme consumes a great deal of ATP and acetyl-CoA, a key metabolic intermediate (James and Cronan, 2004). Nevertheless, the continued production of malonyl-CoA by ACC during phospholipid synthesis inhibition warrants the release of FapR from its DNA binding sites, leading to overexpression of PlsX and PlsC. Thus, if the PL synthesis falls below the rate of FA synthesis, the resulting accumulation of acyl-ACPs would inhibit FASII until the excess acyl-ACP is consumed by incorporation of acyl chains into PLs by the coordinated activity of overexpressed PlsX and PlsC (Figure 8). These data provide the basis for understanding why in bacteria with FapR regulation the expression of the acyl-ACP consuming PlsX and PlsC enzymes is coordinated with the expression of most of the genes involved in FA synthesis. Perhaps PlsY is not transcriptionally regulated by FapR (Schujman *et al.*, 2003) because this enzyme uses exclusively acyl-PO_4 rather than acyl-ACP to acylate G3P (Figure 1) (Lu *et al.*, 2006).

Acyl-ACPs have been reported to inhibit two FASII biosynthetic enzymes in *E. coli*, the enoyl reductase FabI (Heath and Rock, 1995) and the FabH condensing enzyme (Heath and Rock, 1996). Nevertheless, *B. subtilis* codes for two homologs of FabH, FabHA and FabHB with different substrate preferences for straight- or branched-chain primers (Choi *et al.*, 2000; Kingston *et al.*, 2011) and two enoyl reductases, FabI and FabL (Heath *et al.*, 2000). Due to the redundant nature of these enzymes, it seems unlikely that, similarly to the *E. coli* model, they are specifically targeted by acyl-ACPs. It remains possible that in *B. subtilis* FA inhibition by acyl-ACPs is due to shutdown of either the malonyl transacylase FabD, the condensing enzyme FabF, the FabG reductase or the FabZ dehydratase, or a coordinate inhibition of these enzymes. It is worth noting that most of these enzymes will be overexpressed upon the accumulation of malonyl-CoA. This implies that acyl-ACPs should exert a very effective biochemical downregulation of their activities. Although striking, this stringent biochemical inhibition has already been reported for *S.*

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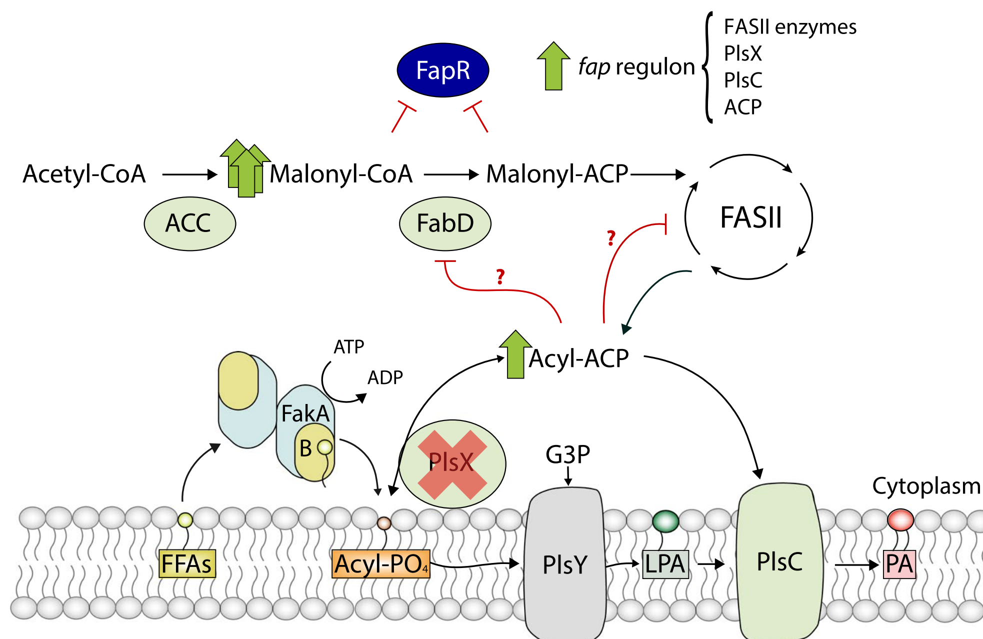

FIGURE 8 Coupling of fatty acid to phospholipid biosynthesis in *B. subtilis*. Obstruction of phospholipid synthesis at the PlsX step promotes the accumulation of long-chain acyl-ACPs and the cessation of fatty acid synthesis by inhibition of a yet unknown step of the FASII cycle and/or FabD. Reduction of FASII activity triggers a large increase in malonyl-CoA, releasing FapR-mediated repression of the *fap* regulon. Upregulation of PlsX and PlsC consume the excess of acyl-ACP reestablishing the control of fatty acid and phospholipid synthesis. FFAs: free fatty acids, G3P: glycerol-3-phosphate, LPA: lysophosphatidic acid, PA: phosphatidic acid [Colour figure can be viewed at wileyonlinelibrary.com]

pneumoniae. These bacteria strongly repress FA biosynthesis when cultured in presence of exogenous FAs, even in FabT-mutant strains that possess constitutively high levels of FASII enzymes (Parsons *et al*., 2011). The identity of the FA biosynthetic enzyme(s) modulated by long-chain acyl-ACPs in *B. subtilis* is unknown. It seems likely that the identification of the inhibited enzyme(s) will require an in vitro system that accurately reflects in vivo metabolism.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and growth conditions

All *B. subtilis* strains used in this study derived from JH642 (wild-type) and are listed in Tables 1 and S1. *E. coli* and *B. subtilis* strains were routinely grown in Lysogeny-Broth (LB) (Bertani, 1951; 2004). Antibiotics were used at the following final concentrations: 5 μg/ml chloramphenicol (Cm), 1 μg/ml erythromycin + 25 μg/ml lincomycin (macrolides, MLS); 100 μg/ml spectinomycin (Sp), 10 μg/ml kanamycin (Kn) and 100 μg/ml for ampicillin (Ap). For the experiments involving expression of PlsX under the control of the inducible P*xyl* promoter the growth medium was supplemented with 0.4% xylose. For those involving expression of ‘TesA under the control of the inducible P*spank*^{hy} promoter 1 mM of Isopropyl-β- D-thiogalactopyranoside (IPTG) was used. The Δ*amyE* phenotype was assayed by growing candidate colonies for 48 hr in LB agar plates supplemented with 0.5% starch and subsequent flooding of the plates with 1% I_2, KI solution. The Δ*manP-manA* phenotype was assayed in minimal medium composed of Spizizen (SPI) salts (Spizizen, 1958), 0.5% mannose (as sole carbon source), 0.01% casamino acids, 0.01% tryptophan and 0.01% phenylalanine.

4.2 | Growth of PlsX conditional mutant strains

All the PlsX conditional mutant strains used in this study were grown overnight on LB agar plates supplemented with 0.4% of xylose at 30°C. Colonies were collected, resuspended, and washed three times with fresh LB medium. In all cases, the strains were inoculated in LB to an OD_{600} = 0.04, the cultures were divided into different fractions and grown in the presence or absence of inducers as indicated for each experiment. The inoculation density was empirically determined to provide cell cultures making the transition from log to stationary phase in the absence of inducer (due to dilution of the preexisting PlsX protein) in 3 to 4 hr with an OD_{600} between 0.6 and 1.

4.3 | Metabolic labeling and lipid extraction

FM105 (Δ*plsX*), FM341 (Δ*plsX*, P*spank*^{hy}-‘*tesA*), and FM347 (Δ*plsX*, Δ*fakA*::*mls*^{r}, P*spank*^{hy}-‘*tesA*) cells were cultured as described above (see “Growth of PlsX conditional mutant strains”) and labeled with 2 μCi/ml [^{14}C]-acetate for 30 min during the transition from log to stationary phase of the culture without inducers, as indicated with an arrow in the figures corresponding to each experiment. Cells

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were collected and total lipids were extracted by the Bligh & Dyer method with minor modifications (Bligh and Dyer, 1959). Briefly, the cells were resuspended in 100 µl of water, subsequently 750 µl of chloroform-methanol (1/2, vol/vol) were added and the samples were incubated ON at −20°C. Then, the samples were centrifuged for 2 min at 14,000*g*. The supernatants were recovered and transferred to a new tube containing 250 µl of chloroform and 250 µl of 1 M KCl. The samples were vortexed and centrifuged to promote phase separation. Each organic phase was transferred to a new tube. The aqueous phase was re-extracted with 250 µl of chloroform and combined with the first extraction. The organic phases were evaporated under nitrogen and resuspended with chloroform. Radioactivity was measured on a scintillation counter. Lipids were analyzed using preadsorbent Silica Gel G layers (Merck) developed with hexane-ethyl ether-acetic acid (60/40/1, vol/vol/vol) to separate the neutral lipids. Plates were exposed to a radioactive storage screen and analyzed using an Amersham Biosciences Molecular Dynamics Typhoon™ FLA 7000 scanner. Lipid species were identified by comparison with the migrated fatty acid standard and bibliographic data (Paoletti *et al*., 2007).

4.4 | Measurement of coenzyme A (CoA) species

FM105 (Δ*plsX*) cells were cultured as described above (see "Growth of PlsX conditional mutant strains") and 50 ml of each bacterial culture were collected by centrifugation (5,000*g*, 4°C, 10 min) during the transition from log to stationary phase of the PlsX-depleted culture (without inducers). Then, cells were resuspended in 3 ml of LB and treated with a final concentration of 6% trichloroacetic acid, vortexed, and incubated for 10 min on ice. Then, samples were centrifuged at 20,000*g* and 4°C for 10 min and supernatants were transferred to a new tube. Cells debris were resuspended in 1% of TCA and incubated for 10 min on ice and recentrifuged (20,000*g*, 4°C, 10 min). Supernatants were collected and mixed with the previous ones. These supernatant samples were analyzed by LC-mass at InMet (Rosario, Argentina). Briefly, CoAs species were first extracted using an Empore C18-SD (Supelc-3M™) column (Schujman *et al*., 2008). Then, 10 µl of the sample were injected into a HPLC-MS/MS equipment. To analyze the relative composition of CoAs species (malonyl-CoA, succinyl-CoA, CoASH, lactoyl-CoA, acetyl-CoA, propionyl-CoA, isobutyryl-CoA, and isovaleryl-CoA) a chromatographic run based on acetonitrile gradients was performed (Neubauer *et al*., 2015), coupled to a triple quadrupole mass spectrometer. The amounts of the species of CoAs are enunciated relative to the pool of analyzed CoAs.

4.5 | Gel electrophoresis and immunoblotting

A 5 ml-aliquot of each culture under study (indicated along the manuscript) was resuspended in 90 µl of lysis buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl, 1 mM PMSF and 0.1 mg/ml lysozyme) per OD_{600} unit and incubated at 37°C for 15 min. Then, cells were disrupted using a Bioruptor™-UCD200 (Diagenode). Cell debris were removed by centrifugation at 20,000*g* and 4°C for 10 min and the supernatants recovered. For ACP pool composition analysis, lysates were subjected to conformationally-sensitive electrophoresis in 20% polyacrylamide, 2.5 M urea gels (Cronan and Thomas, 2009). For FabF and RpoB analysis, each sample was subjected to sodium dodecyl sulfate (SDS)—15% of polyacrylamide gel electrophoresis. After the run, proteins were transferred to a 0.2 µm polyvinylidene difluoride (PVDF) membrane by electroblotting. ACP was detected using polyclonal rabbit anti-ACP antibodies (at 1:500 dilution) generated using *Bacillus subtilis* purified ACP as immunogen at the Instituto de Salud y Ambiente del Litoral (ISAL), Facultad de Bioquímica y Cs. Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina. FabF was detected using polyclonal rabbit anti-$FabF_{Bs}$ antibodies (Schujman *et al*., 2001; 2008). RpoB was identified using polyclonal mouse anti-RpoB antibodies at a 1:20,000 dilution (Cabruja *et al*., 2017). Anti-rabbit IgG conjugated with horseradish peroxidase (HRP) or anti-mouse IgG conjugated with alkaline phosphatase (AP), were used as secondary antibody. ECL (GE Healthcare Life Sciences) was used as HRP substrate, and the luminescent signal was recorded using BioRad ChemiDoc XRS system. BCIP/NBT (Bio-Rad) was used as the AP substrate.

4.6 | β-Galactosidase activity assays

FM105 (Δ*plsX*; P*fabHB-lacZ*) was grown as described above (see "Growth of PlsX conditional mutant strains") and aliquots of each culture were collected at different time points as indicated in Figure 7a. Samples were assayed for β-galactosidase activity as previously described (Mansilla and de Mendoza, 1997). Intensity of yellow from o-nitrophenyl (ONP) formation was measured colorimetrically at 420 nm. The results are expressed in Miller Units (Miller, 1972), relative to the activity of the P*fabHB* promoter in the +PlsX (+xylose) condition that was considered as 1.

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AUTHOR CONTRIBUTIONS

F.M., G.E.S., D.d.M., and D.A. designed the study; F.M. and L.N. conducted the experiments; F.M., L.N., G.E.S., D.d.M., and D.A. analyzed the data; F.M., D.d.M., and D.A. wrote the manuscript.

ORCID

Daniela Albanesi https://orcid.org/0000-0003-4380-9152

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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