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Revisiting the cell biology of the acyl-ACP:phosphate transacylase PlsX suggests that the phospholipid synthesis and cell division machineries are not coupled in *Bacillus subtilis*

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Summary

PlsX is a central enzyme of phospholipid synthesis in bacteria, converting acyl-ACP to acyl-phosphate on the pathway to phosphatidic acid formation. PlsX has received attention because it plays a key role in the coordination of fatty acid and phospholipid synthesis. Recently, PlsX was also suggested to coordinate membrane synthesis with cell division in *Bacillus subtilis*. Here, we have re-investigated the cell biology of PlsX and determined that the enzyme is uniformly distributed on the membrane of most cells, but occasionally appears as membrane foci as well. Foci and homogenous patterns seem freely interconvertible but the prevalence of the uniform staining suggests that PlsX does not need to localize to specific sites to function correctly. We also investigated the relationship between PlsX and the divisome. In contrast to previous observations, PlsX's foci showed no obvious periodicity of localization and did not colocalize with the divisome. Furthermore, depletion of PlsX did not affect cell division if phospholipid synthesis is maintained by an alternative enzyme. These results suggest that coordination between division and membrane synthesis may not require physical or functional interactions between the divisome and phospholipid synthesis enzymes.

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Introduction

Phospholipids are abundant and essential structural components of the cell membrane in all bacterial species. The enzymology of phospholipid synthesis in bacteria has been well worked out. The universal precursor of these molecules is synthesized by the successive acylation of glycerol-3-phosphate to yield 1,2-diacyl-sn-glycero-3-phosphate, or phosphatidic acid (PtdOH) (Parsons and Rock, 2013). There are two distinct pathways capable of acylation of the glycerol backbone of PtdOH. One of the pathways was initially discovered in *Escherichia coli* and is composed by the PlsB/PlsC acyltransferases. This system is largely limited to Gram-negative bacteria (mostly γ-proteobacteria), but homologs are also present in mammals (Parsons and Rock, 2013). This protein family primarily uses acyl-acyl carrier protein (acyl-ACP) end products of fatty acid synthesis as acyl donors, but may also use acyl-CoA derived from exogenous fatty acids. The PlsB enzyme is responsible for selecting the fatty acids incorporated into the position 1 of phospholipids and is a key point of regulation. The second acyltransferase, PlsC, completes the synthesis of PtdOH, by transfering an acyl group from acyl-ACP (or acyl-CoA) to position 2 of 1-acyl-glycerol-3-phosphate (Parsons and Rock, 2013). Another pathway of three steps (PlsX/PlsY/PlsC) for the formation of PtdOH was recently discovered in Firmicutes, which

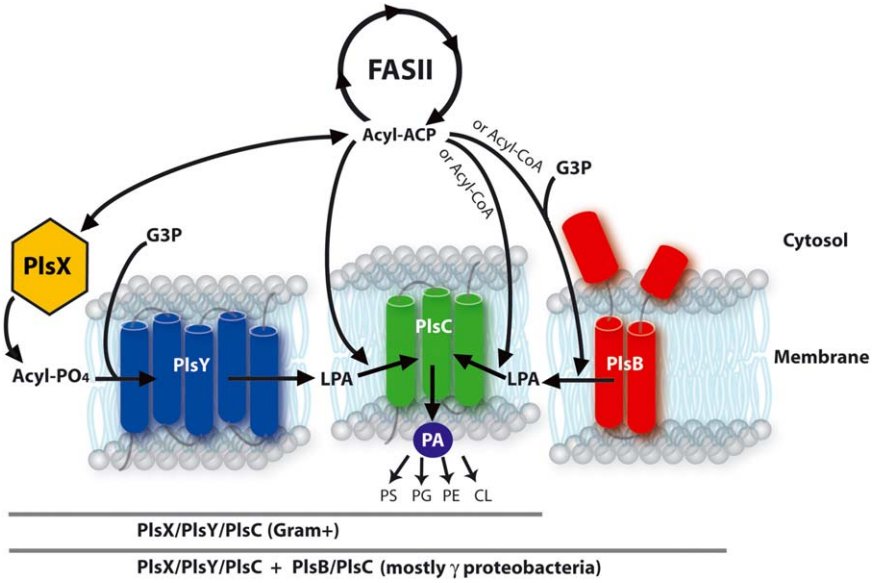

Fig. 1. Two-pathways for the formation of phosphatidic acid in bacteria. The long-chain acyl-ACP end products of FASII are substrates of the acyltransferases. The PlsX protein transforms acyl-ACP into acyl-phosphates and these are the substrates used by PlsY to acylate glycerol-3-phosphate (G3P) to 1-acyl-glycerol-3-phosphate or lysophosphatidic acid (LPA). Subsequently, PlsC transfers a fatty acid from acyl-ACP to the 2-position of LPA to form phosphatidic acid (PA), the common intermediate in the synthesis of all membrane glycerolipids. Some bacteria have an alternative pathway, independent of PlsX, where PlsB acylates glycerol-3-phosphate using either acyl-ACP or acyl-CoA as the acyl donor. phosphatidylglycerol (PG); phosphatidylserine (PS); phosphatidylethanolamine (PE) and cardiolipin (CL). FASII: fatty acid synthase type II.

include most clinically important Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae* (Lu *et al.*, 2006; Schujman and de Mendoza, 2006; Paoletti *et al.*, 2007; Parsons and Rock, 2013). The first step in this pathway is catalyzed by PlsX a phosphotransacylase that converts acyl-ACP in an acylphosphate (acyl-PO_4). The second step is catalyzed by the integral membrane protein PlsY, an acyl-PO_4-dependent acyltransferase which transfers the acyl group of acyl-PO_4 to position 1 of glycerol-3-phosphate. The last step is similar to the one in Gram-negatives, with the difference that whereas *E. coli's* PlsC uses acyl-ACP or acyl-CoA as acyl donors, in *S. pneumoniae* and *Bacillus subtilis* PlsC only uses acyl-ACP (Lu *et al.*, 2006; Paoletti *et al.*, 2007) (Fig. 1). Blocking the PlsX reaction results in the cessation of fatty acid synthesis by FASII. Therefore, PlsX seems to be a key regulatory point that synchronizes FASII with phospholipid synthesis (Paoletti *et al.*, 2007).

An extra dimension to the understanding of biochemical pathways is their spatial organization, and how this contributes to the morphogenesis and compartmentalization of the bacterial cell. Several proteins have now been shown to reside in specific subcellular locations where they must carry out their functions (Rudner and Losick, 2010; Nevo-Dinur *et al.*, 2012; Cornejo *et al.*, 2014). This is particularly evident for proteins in charge of building defined structures, such as polar organelles (flagella, pili, chemoreceptor arrays) or the division septum. In the case of cell division, the tubulin-like protein FtsZ forms a dynamic ring-like structure at midcell and, together with a dozen other cell division proteins, promotes the localized ingrowth of the cell wall and cytoplasmic membrane to create the septum (Gueiros-Filho, 2011; Tsang and Bernhardt, 2015).

Because cell division requires the localized expansion of the cytoplasmic membrane, there has been speculation as to whether the membrane synthetic machinery might be coorganized with the cell division apparatus (or divisome) during the bacterial cell cycle. Classic autoradiography experiments revealed that new phospholipid synthesis occurs diffusely throughout the membrane, even in actively dividing cells (Green and Schaechter, 1972; Mindich and Dales, 1972), thus failing to support the idea of spatial coupling between membrane synthesis and cell division. In contrast, the application of GFP microscopy showed that at least some of the phospholipid synthetases of *B. subtilis* seem to localize preferentially to the septal membrane, suggesting a potential association between these enzymes and the divisome. (Matsumoto *et al.*, 2006). More recently, Takada *et al.* (2014) reported that the key phospholipid synthesizing enzyme PlsX of *B. subtilis* interacts with FtsA, a divisome protein structurally related to actin and which plays an important role in the assembling of the FtsZ ring. This same work reported that PlsX localized preferentially to septal and polar membranes, and as foci at internucleoid spaces that could correspond to future division sites. In addition, inactivation of PlsX perturbed FtsZ and FtsA localization and septum formation. On the basis of these results, Takada *et al.* (2014) concluded that PlsX is important for coordinating membrane synthesis with cell division and that this protein may act upstream of FtsZ in establishing the correct site of cell division.

In this study, we have reinvestigated the subcellular localization of the PlsX and its role in cell division. In contrast to previous observations (Takada *et al.*, 2014), we report that PlsX is mostly homogeneously distributed

on the cell membrane, with only occasional cells exhibiting foci or septal accumulation. PlsX foci do not seem to be related to cell division, as they show little colocalization with Z rings and are randomly distributed along the cell length. Finally, we demonstrate that absence of PlsX in a situation in which phospholipid synthesis is not interrupted does not affect Z-ring formation or cell division. *In toto*, our results indicate a lack of association between PlsX and the cell division apparatus, suggesting that membrane synthesis does not need to be spatially coupled to cell division in bacteria.

Results

Localization of PlsX at the cell membrane is predominantly homogenous and dynamic

Initial immunofluorescence (IFM) localization studies of PlsX showed that this enzyme exhibited a peripheral localization indicative of association with the cell membrane of *B. subtilis* (Paoletti *et al*., 2007). The IFM staining of PlsX displayed a punctate appearance, being less homogenous than the staining of an integral membrane protein (PlsC) studied in the same work. However, the loss of detail caused by chemical treatments of IFM precluded us from concluding if PlsX had a preference for specific sites on the membrane. To revisit this issue, we switched to live cell microscopy using a strain (DS69) containing an N-terminal fusion of GFP to PlsX (GFP-PlsX) integrated at the *amyE* locus and under the control of an IPTG-inducible promoter ($P_{hy\text{-}spank}$). Curiously, imaging of DS69 under the standard conditions used in our laboratory (cells grown in rich medium and sampled at mid log phase) revealed that GFP-PlsX was found uniformly distributed in the periphery of cells, with rare foci or accumulation at septa or poles (Fig. 2A, right). The GFP-PlsX fluorescence was often more intense at septa, but this is because septa contain two juxtaposed membrane bilayers and not because the protein is more concentrated in septal membranes. This can be seen in cells simultaneously labeled with GFP-PlsX and FM4-64, a lipophilic dye that stains membranes nonspecifically, where it is evident that brighter GFP-PlsX fluorescence is always paralleled by brighter FM4-64 fluorescence (Supporting Information Fig. S1).

The observation that PlsX displays a uniform membrane distribution is in contrast with the findings of Takada *et al*. (2014), who recently reported that PlsX localized predominantly at septa and cell poles, and as membrane associated foci at positions along the cell length that could correspond to potential division sites. The discrepancy between our results and the published localization of PlsX cannot be ascribed to a nonfunctional fusion because our GFP-PlsX allele was capable

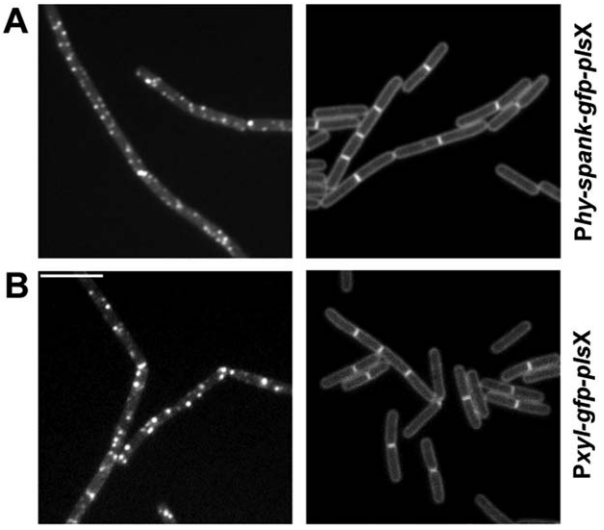

Fig. 2. Localization of PlsX is mostly homogenous but foci can be detected in early log cells. GFP-PlsX was expressed form either an IPTG inducible (A) or a xylose inducible (B) construct.
Fluorescence microscopy images obtained at two different ODs (0.3 – left column, and 0.8 – right column) from cells grown in LB at 37°C are shown. Scale bars for all microscopic images: 5 μm.
A. Strain DS69 (*P_{xyl}-plsX, P_{spac}-fabD, amyE::$P_{hyspank}$-gfp-plsX*) grown in the presence of IPTG 0.1 mM.
B. Strain DS09 (*amyE::P_{xyl}-gfp-plsX*) grown in the presence of xylose 0.5%.

of fully complementing the growth of a PlsX conditional mutant (Supporting Information Fig. S2). We thus screened different growth conditions and found that if cells were grown in LB medium and sampled at early log phase ($OD_{600} = 0.2–0.3$) the localization pattern of GFP-PlsX changed, and a significant proportion of cells now presented the protein as membrane-associated foci and accumulations at septa and cell poles (Fig. 2A, left). To confirm that the localization of GFP-PlsX varied depending on growth stage, we verified the prevalence of the homogenous and focus localization patterns in cells harvested at three different points along their growth curve (typically, at OD_{600} of 0.2, 0.5 and ≥ 0.8). This revealed a trend for the appearance of foci at low OD_{600}, although the frequency of cells with foci was highly variable from experiment to experiment (from about 20% to as high as 80% in one experiment). At $OD_{600} = 0.5$ foci became significantly rarer (10–30%) and at yet later stages of growth ($OD_{600} \geq 0.8$) the foci were no longer visualized and PlsX was found to be homogeneously distributed in all cells. This result indicates that the subcellular localization of PlsX is dynamic and can change depending on the physiological state of the cell. Nevertheless, to rule out that the homogenous membrane localization was artefactual (basically, the loss of foci or septal patterns) several controls were carried out. Peripheral membrane proteins such as PlsX

are known to have particularly labile localizations, being affected by abrupt changes in the physicochemical conditions (pH, osmolality, oxygenation) to which cells are exposed (Gueiros-Filho *et al.*, unpublished, see also Strahl and Hamoen, 2010, which shows the effect of membrane potential). Thus, we imaged our cells in agarose pads made with exactly the same medium in which the cells had been grown. Furthermore, we imaged cells that had been grown for several generations on the agarose pad that was subsequently used for microscopy. These procedures did not change the localization of GFP-PlsX, with the homogenous membrane pattern still predominating (data not shown). Another possible explanation for the homogenous membrane pattern is that overexpression of GFP-PlsX was saturating a limited number of preferential sites for PlsX on the membrane. To discard this possibility, we carried out experiments at varying IPTG concentrations and also constructed a GFP-PlsX fusion under the control of the weaker P_{xyl} promoter. In both cases, lower levels of GFP-PlsX still produced the uniform membrane pattern (Supporting Information Fig. S3). As a final way to demonstrate that the homogenous localization of PlsX is physiological, we carried out time-lapse microscopy. Under time-lapse conditions, cells with foci were rarely observed, even when inoculated at a very low OD on the agarose pads. This may be related to the fact that cells on agarose pads do not achieve the same growth rates as in liquid culture (see below). As can be seen in Supporting Information Movie S1 (Fig. 3A, Supporting Information Movie

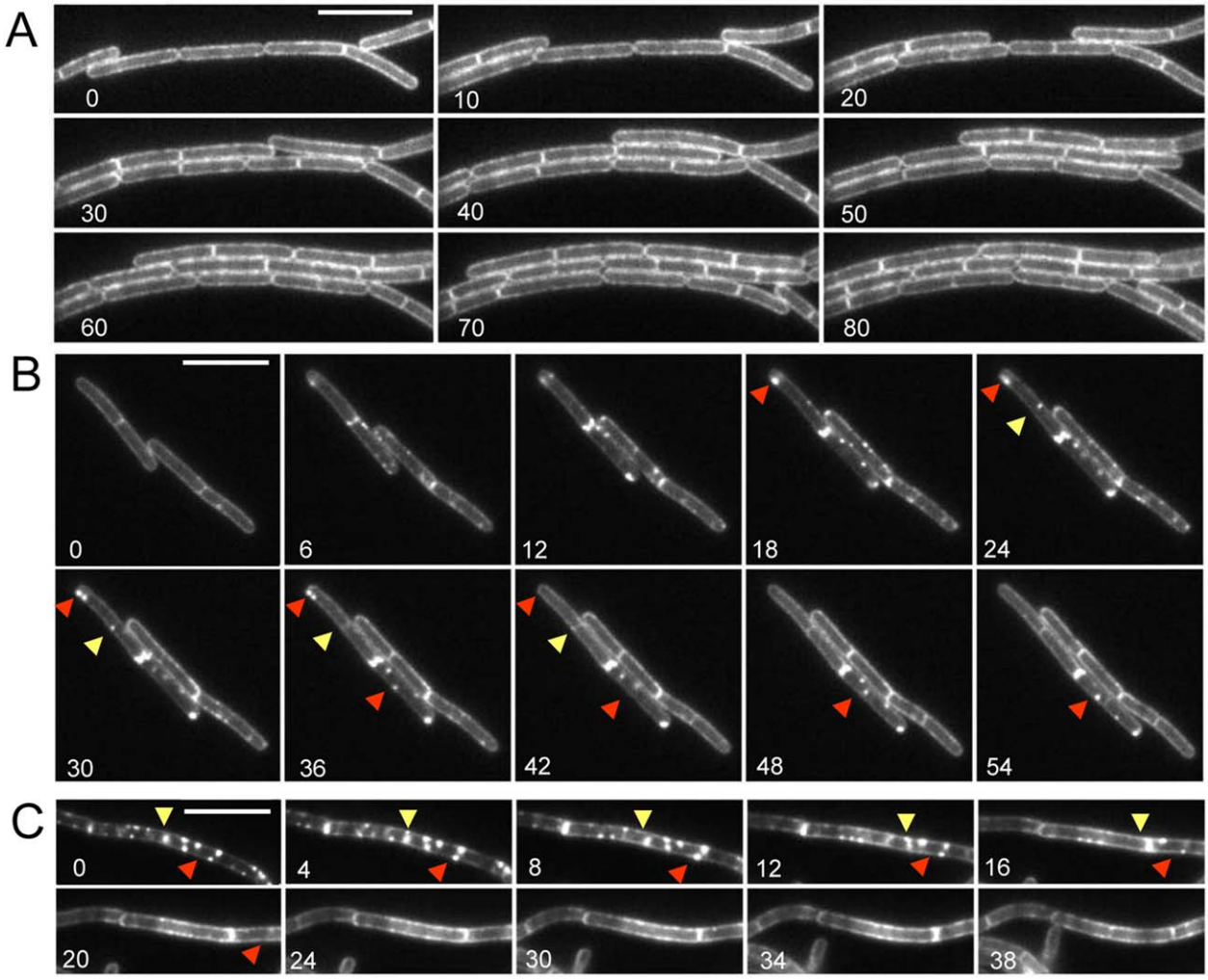

Fig. 3. Time-lapse movies of PlsX localization. Strain DS69 (*P_{xyl}-plsX, P_{spac}-fabD, amyE::$P_{hyspank}$-gfp-plsX*) was grown in the presence of IPTG 0.25 mM and imaged on agarose pads in a HiLo microscopy system as described in the methods.
A. Selected frames of a movie (Supporting Information Movie S1) that displays the typical uniform distribution of PlsX in vigorously dividing cells.
B and C. Selected frames of movies (Supporting Information Movies S2 and S3) that display the dynamic behavior of PlsX foci. Red arrows point to examples of PlsX foci that do not colocalize or become incorporated into septa, and yellow PlsX foci that do become septa. Numbers indicate minutes. Scale bar indicates 5 μm.

S1), cells growing and dividing vigorously exhibit PlsX uniformly distributed in their membranes, demonstrating that the homogenous pattern is not simply due to delocalization of PlsX in unhealthy cells. The movies also revealed that PlsX association with the membrane was quite dynamic, as seen by the fluorescence constantly changing its distribution along the periphery of the cell. We also succeeded in observing a few cells with the foci/septal/polar pattern of PlsX under time-lapse conditions. In some cases the foci/septal/polar accumulation originated in a cell with homogenous PlsX immediately after it underwent septation. This can be seen in the upper cell pair in Fig. 3B (Supporting Information Movie S2), which in the first movie frame has PlsX uniformly distributed in its membrane but soon after develops a bright accumulation of PlsX at the central septum and foci at both cell poles. Interestingly, as these cells continued to grow and approached a new round of division PlsX tended to become homogenous again. Other examples of foci that dissipate into a homogenous membrane pattern are shown in Fig. 3C (Supporting Information Movie S3). Thus, time-lapse microscopy showed that foci/septal accumulation of PlsX seem to be a transient state that readily interconverts with the homogenous pattern exhibited by most cells.

Foci formation by PlsX may depend on growth rate and is independent of flotillins

Even though our results indicate that in most cells and under most growth conditions PlsX is found uniformly on the membrane, we sought to better understand the origin of PlsX foci. The higher prevalence of PlsX foci at low OD_{600} could mean that PlsX localization was responding to cell density or to growth rate, as cells tend to have a significantly faster instantaneous doubling time at early log than mid or late log phases (Jakiela *et al*., 2013). To distinguish between these possibilities we grew the strain expressing GFP-PlsX from the $P_{hyspank}$ promoter (DS69) under conditions that reduced its growth rate and determined the localization of GFP-PlsX when cells reached the OD_{600} of 0.2 or 0.3, normally associated with PlsX foci. Growth of DS69 in TSS minimal medium (Fouet and Sonenshein, 1990) decreased its growth rate about 3 fold (Supporting Information Fig. S4) and resulted in PlsX becoming uniformly localized (Fig. 4A). To rule out a nutritional effect we also did experiments using LB, the medium where we normally see foci formation, but in the presence of a subinhibitory concentration of L-arginine hydroxamate, which slows growth (fivefold, see growth curve in Supporting Information Fig. S4) by triggering the intracellular accumulation of the alarmone (p)ppGpp. As shown in Fig. 4A, in LB containing L-arginine hydroxamate the GFP-PlsX fusion was also found uniformly in the cell membrane. A similar result was obtained and no foci were observed if we slowed growth by inducing the accumulation of (p)ppGpp genetically, using a Δ*rel*A strain (DS51) (Fig. 4A). These data indicate that the formation of PlsX foci is favored by fast growth rates and is not a function of low cell density.

Foci detected by fluorescence microscopy may represent large oligomeric protein assemblies. As such, focus formation can be affected by intracellular protein concentration. This was shown for the formation of clusters of chemotaxis receptors in *E. coli*, which increased in number, size and brightness as the expression of the receptors increased (Thiem and Sourjik, 2008). To investigate if PlsX levels correlated with focus formation we used Western blots to quantify GFP-PlsX production from the IPTG and xylose driven constructs and determined the frequency of foci under each condition. Plotting foci frequency as a function of GFP-PlsX expression showed no correlation (Fig. 4B, also see Supporting Information Fig. S5), suggesting that the intracellular concentration of PlsX is not important for foci formation. Membrane protein foci were also reported to form in response to certain perturbations of the bacterial envelope. In the case of the LiaIH phage-shock proteins of *B. subtilis* this occurs in response to antibiotics that interfere with the utilization and membrane distribution of lipidII, such as nisin and vancomycin (Dominguez-Escobar *et al*., 2014). We tested the conditions that induced formation of LiaIH foci and they failed to promote formation of PlsX foci (data not shown). Thus, PlsX foci have a different origin than LiaIH foci.

Finally, we tested whether PlsX foci were associated with lipid rafts. The essential structural components of these membrane microdomains are the flotillins, membrane-bound proteins that act as chaperones in recruiting other proteins to lipid rafts to facilitate their interaction and oligomerization. *B. subtilis* contains two flotillin-like proteins, referred to as FloA and FloT, which are organized as discrete foci heterogeneously distributed on the cellular membrane (Donovan and Bramkamp, 2009; Lopez and Kolter, 2010). Thus, the subcellular localization of flotillins is similar to the one presented by PlsX foci, suggesting that PlsX could be part of lipid rafts in *B. subtilis*. To test this hypothesis we introduced the GFP-PlsX fusion in a double mutant Δ*flo*A-Δ*flo*T strain (DS97) and examined the localization of GFP-PlsX fusion at different OD_{600}. It has been reported that membrane lipids become more homogenously distributed in cells lacking both flotillins, suggesting that lipid rafts are poorly structured or do not form in these mutants (Bach and Bramkamp, 2013). Interestingly, however, PlsX foci were still detected in the Δ*flo*A-Δ*flo*T strain, indicating that their formation does not

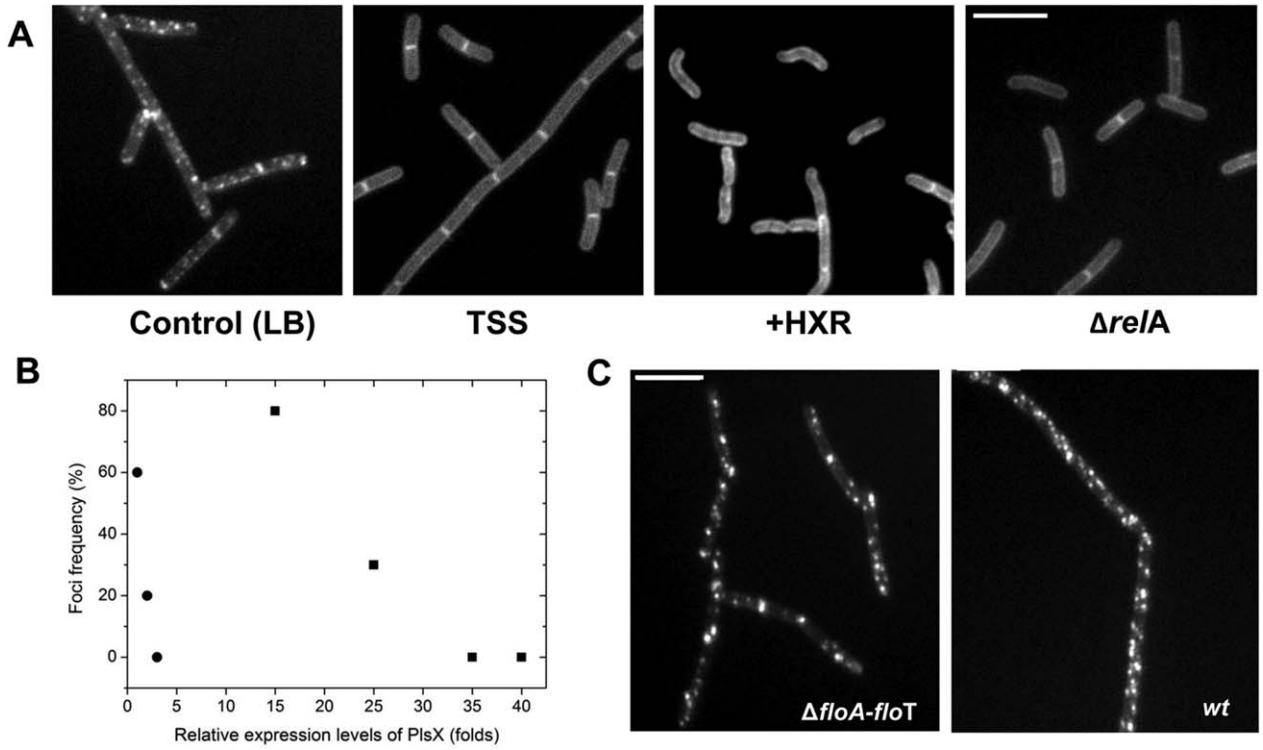

Fig. 4. Foci formation by PlsX depends on growth rate and is independent of flotillins.
A. Localization of GFP-PlsX in LB (LB), minimal medium (TSS), LB with arginine hydroxamate (+HXR) and in a strain that over accumulates ppGpp (Δ*rel*A). All cultures were imaged at a similarly low OD (0.2–0.3). In panels LB, TSS and +HXR the strain is DS69. For panel Δ*rel*A the strain is DS51.
B. Foci frequency as a function of GFP-PlsX expression level. Cells harvested at different times after the induction of GFP-PlsX from either IPTG- or xylose-controlled constructs were analysed by microscopy to quantify foci and by Western blots to determine GFP-PlsX relative levels. P_{xyl}-*gfp-pls*X (filled circles), $P_{hyspank}$-*gfp-pls*X (filled squares).
C. PlsX foci are independent of flotillins: Strains DS97 (top) (Δ*floA*-Δ*floT amyE::*$P_{hyspank}$-*gfp-plsX*) and DS69 (bottom) were grown as before and harvested at OD 0.3. Scale bar indicates 5 μm.

require the flotillins *(Fig.* 4*C)* and may be unrelated to lipid rafts.

PlsX does not colocalize with the division site or the Z-ring

Our observation that PlsX is predominantly localized uniformly in the membrane suggests that the reported interaction between PlsX and the divisome should be weak and/or transient enough as not to affect the localization of PlsX in the majority of cells. However, it remained a possibility that in the rare cells with PlsX foci their localization would coincide with the divisome's. If this were the case, it would be evidence that PlsX and the cell division apparatus are indeed physically associated, even if transiently. We quantified the spatial distribution of PlsX foci within cells whose nucleoid had been stained with DAPI. We found that PlsX foci were randomly distributed in the cell, with no obvious association with future division sites (internucleoid spaces). PlsX foci were frequently (about 80% of foci) localized over nucleoids and off the cell center, positions along the cell length where septa ordinarily do not form. (Fig. 5A). In addition, time lapse microscopy showed that, even though the occasional PlsX focus became incorporated into a nascent septum, most of the time foci dissolved without giving rise to septa at the position they occupied (see arrows in Fig. 3B and C). To further investigate the relationship between PlsX and the divisome we examined the colocalization of PlsX and FtsZ using a strain that expresses GFP-PlsX and FtsZ-m*Cherry* fusion proteins. m*Cherry* was fused to *ftsZ* at *thr*C locus by double cross-over and was controlled by a IPTG inducible promoter, while a copy of P_{xyl}-GFP-*plsX* was introduced into the *amy*E locus by double cross-over. After xylose and IPTG induction of the fusion proteins for two hours, the cells were analyzed by fluorescence microscopy. As shown in Fig. 5B, PlsX foci and FtsZ rings did not colocalize, a finding in accordance with the observation that foci were not enriched at the midcell position (Fig. 5A). Lastly, we investigated the localization of PlsX in a mutant lacking FtsA, the divisome protein reported to interact with PlsX. A similarly random pattern of localization of PlsX foci was observed in FtsA filaments

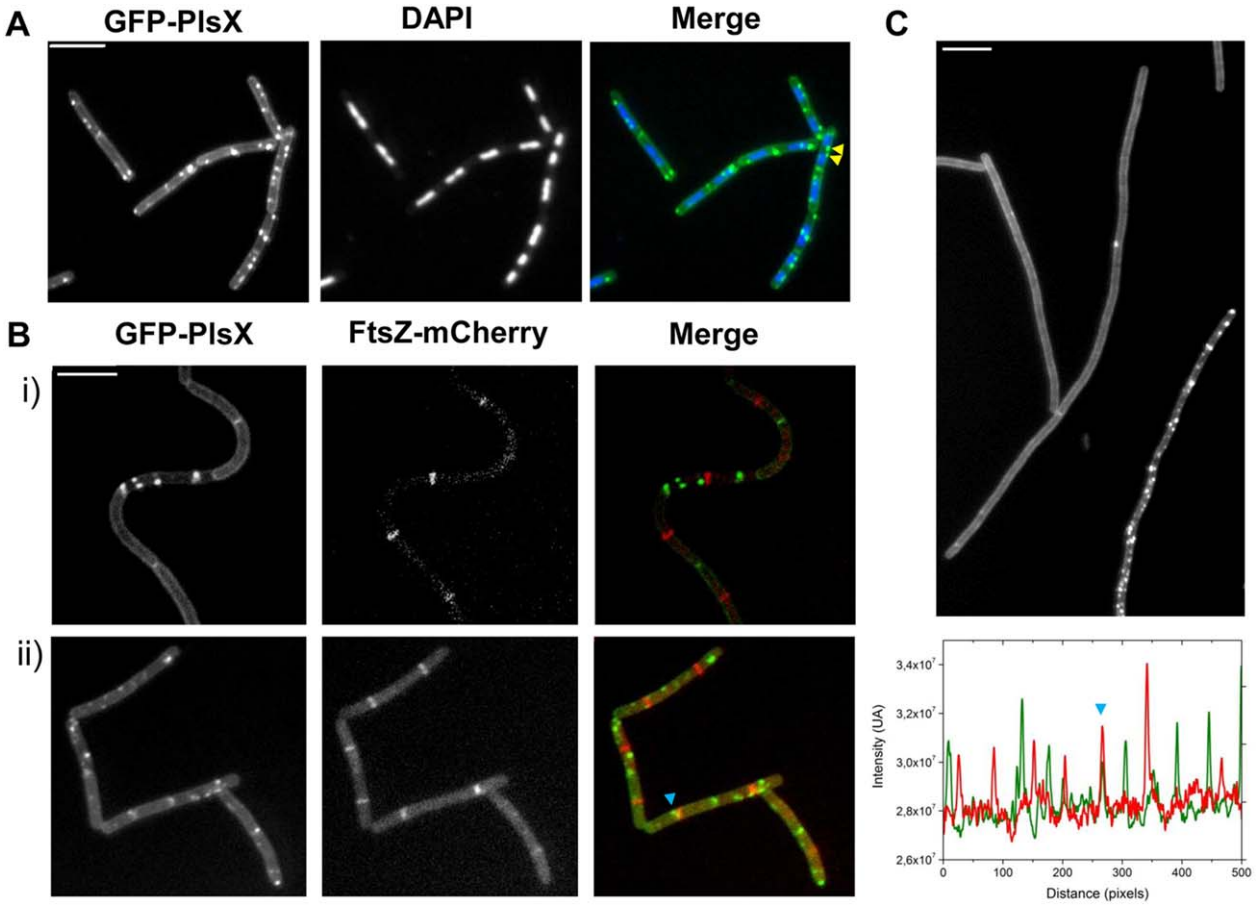

Fig. 5. PlsX foci do not colocalize with the division site or the Z-ring.
A. Distribution of GFP-PlsX foci relative to cell length and nucleoids. Two examples of foci that overlap with nucleoids are marked with yellow arrowheads, but note that other foci in the same image also display overlap.
B. Colocalization of GFP-PlsX and FtsZ-mCherry. Strain DS50 (*thr*C::P_{spac}-*ftsZ*-m*Cherry*; *amy*E::P_{xyl}-*gfp-plsX*) was grown in LB medium with 0.5 mM IPTG and 0.5% xylose at 37°C and harvested at OD_{600} 0.3.
C. Intensity profile of GFP-PlsX and FtsZ-mCherry in a cell from image (B) ii (marked by blue arrowhead). Note the general absence of colocalization.
D. Effect of *fts*A mutation on the localization of GFP-PlsX. Strain DS40 (Δ*ftsA amy*E:$P_{hyspank}$-*gfp-plsX*) was grown in LB medium with 0.1 mM IPTG at 37°C and harvested at at OD_{600} 0.3. Typical filaments are shown. Scale bar indicates 5 μm.

(Fig. 5C). Taken together, our results strongly suggest that PlsX localization neither coincides with, nor depends on that of the divisome.

PlsX is not necessary for cell division

Previous work reported that inactivation of PlsX perturbed Z-ring formation and cell division (Takada *et al.*, 2014). Nevertheless, this study did not exclude the possibility that the cause of abnormal cell division was the inhibition of lipid synthesis resulting from PlsX inactivation, and not the absence of the PlsX protein per se. In fact, PlsX is an essential protein in *B. subtilis* and its prolonged inactivation or depletion will lead to loss of viability, unless this bacterium is supplemented with a mixture of branched-chain fatty acids (Parsons *et al.*, 2014a). To clarify this issue, we designed a system in which the requirement of PlsX for lipid synthesis in *B. subtilis* was bypassed by the heterologous expression of PlsB, an acyltransferase of *E. coli* unrelated in sequence and structure to PlsX. PlsB is a glycerol phosphate acyltransferase that introduces an acyl group (from acyl-ACP or acyl-CoA) in the sn-1 position of glycerol-3-phosphate. It has been shown that PlsB complements the essential function of PlsX in phospholipid synthesis in *B. subtilis* (Hara *et al.*, 2008) and we confirmed this by showing that the growth defect of PlsX depletion strain DS28 in the absence of xylose was suppressed by the expression of PlsB (Supporting Information Fig. S6). To test whether PlsX is necessary for Z-ring formation we used as a marker ZapA-GFP, which can function as a surrogate for FtsZ localization (Gueiros-Filho and Losick, 2002) and was introduced by

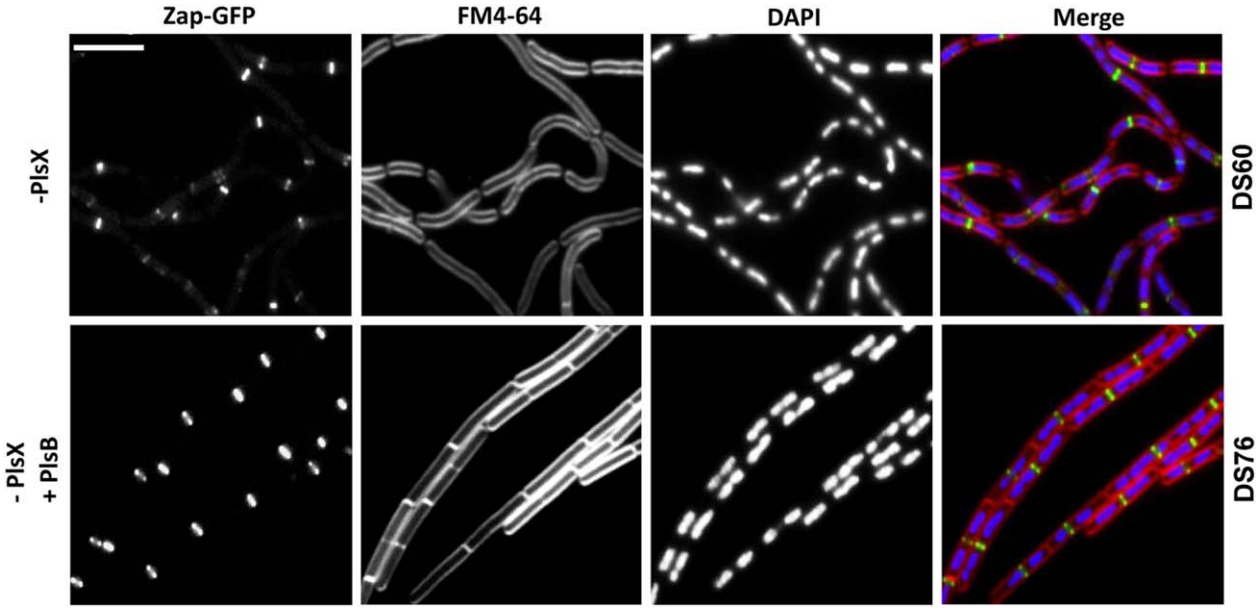

Fig. 6. Depletion of PlsX does not affect septum formation. Fluorescence microscopy images after 3 h of PlsX depletion (without xylose). Top, strain DS60 (P_{xyl}-*plsX*, P_{spac}-*fabD*, *zapA*-GFP); bottom, strain DS76 (P_{xyl}-*plsX*, P_{spac}-*fabD*, *zapA-gfp*, $P_{hyspank}$-*pls*B), which expresses PlsB and produces fatty acids in the presence of IPTG. ZapA-GFP fusion was used as a Z-ring marker. PlsB was induced with IPTG 0.1 mM. Scale bar indicates 5 μm.

single-crossover in the endogenous locus of strain DS28, generating strain DS60. Growth of strain DS60 for 4 h in the absence of inducer led to a peculiar phenotype in which cells became curved, thinner and exhibited gaps between cells in a chain. Interestingly, however, septation was only mildly affected in the absence of PlsX, and many cells still displayed ZapA rings that were properly placed between segregated nucleoids (Fig. 6, top). After 4 h of depletion, there was an increase in cells with abnormal or irregular septation (e.g. septum at the cell poles, bisection of the chromosome and several nucleoids per cell) but since this was accompanied by a drastic decrease of cell viability we decided to use 4 h as the depletion limit. Similar depletion experiments carried out with strain DS76, containing an IPTG inducible copy of the *pls*B gene, showed that cells maintained the wild-type morphology and exhibited a normal pattern of division and robust and perfectly positioned ZapA-GFP rings (Fig. 6, bottom). Another way to bypass the biochemical consequences of the *plsX* mutation is to supplement the medium with exogenous fatty acids (Parsons *et al.*, 2014a, 2014b). We have repeated these experiments and confirmed that *B. subtilis* cells continue to divide normally in the absence of PlsX if fatty acids are provided (Supporting Information Fig. S7). These results clearly demonstrate that if lipid synthesis is not curtailed, Z-ring formation and cell division are independent of PlsX. Thus, we conclude that PlsX is not necessary for Z-ring formation and correct divisome assembly.

PlsX association with the membrane is independent of PlsY, and inhibition of phospholipid synthesis perturbs PlsX localization

We sought to determine if the localization of PlsX depends on PlsY, the integral membrane acyltransferase that acts downstream of PlsX in phosphatidic acid synthesis. Previous observation that PlsX interacted with PlsY in two-hybrid experiments (Hara *et al.*, 2008), suggested the possibility that PlsX requires PlsY to associate with the membrane. We constructed strain DS62 (P_{spac}-*plsY*; *amyE*::P_{xyl}-*gfp-plsX*) to analyze GFP-PlsX localization in PlsY-depleted cells. PlsY was depleted for 2-3 h and then the expression of GFP-PlsX was induced by the addition of xylose. Depletion led to the same abnormal cell morphology (a large number of curved, narrower cells) observed upon PlsX depletion (Fig. 7A, left), indicating the cessation of phospholipid synthesis. In addition, in PlsY-depleted cells, PlsX was clearly delocalized, being found predominantly in the cytosol (Fig. 7A, left, -IPTG). To determine if delocalization was due to the absence of PlsY or an indirect effect of inhibition of phospholipid synthesis, we imaged PlsX in PlsY-depleted cells that were complemented with PlsB, which can also bypass the biochemical requirement for PlsY, and was expressed under the control of the constitutive *Pveg* promoter (DS105). Expression of PlsB restored the membrane localization of PlsX in PlsY-depleted cells (Fig. 7A, right), thus indicating that the association of PlsX with the membrane does not require a specific

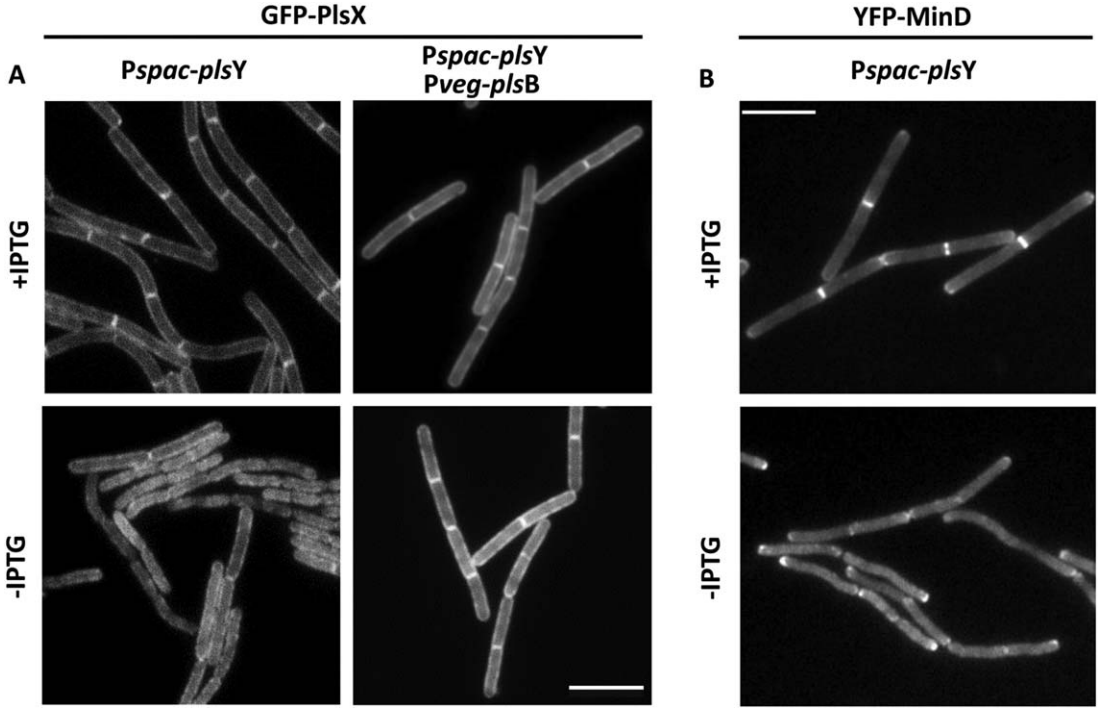

Fig. 7. PlsX membrane localization is independent of PlsY if phospholipid synthesis is maintained.
A. Effect of PlsY depletion on GFP-PlsX localization. Left panel. Strain DS62 (P_{spac}-*plsY*, *amyE*::P_{xyl}-*gfp-plsX*) was cultured in LB containing 0.5 mM IPTG and 0.5% xylose at 37°C. PlsY was depleted by growth without IPTG for 3.5 h. Right panel. GFP-PlsX localization in a strain that constitutively expresses PlsB (DS105 – P_{spac}-*plsY*, *amyE*::P_{xyl}-*gfp-plsX*, *thrC*::P_{veg}-*plsB*) and thus continues to produce phospholipids even after depletion of PlsY.
B. YFP-MinD localization in PlsY depletion strain (DS112 – P_{spac}-*plsY*, *amyE*::P_{xyl}-*yfp-minD*) in the presence or absence of IPTG 0.5 mM. Scale bar indicates 5 μm.

interaction with PlsY, as previously suggested (Paoletti *et al.*, 2007). We postulate that inhibition of lipid synthesis when PlsY is depleted leads to disruption of membrane potential and this, in turn, disrupts PlsX localization. This hypothesis is based on the following observations: (i) the localization of PlsX is sensitive to changes in membrane potential caused by the ionophore CCCP (Takada *et al.*, 2014), and (ii) in a conditional *plsY* knockout there is copious accumulation of free fatty acids (Paoletti *et al.*, 2007), which are known inhibitors of membrane potential (Desbois and Smith, 2010). To give support to our hypothesis, we examined if PlsY depletion affected the localization of MinD, a cell division protein whose interaction with the lipid bilayer depends on membrane potential (Strahl and Hamoen, 2010). We found that in the presence of PlsY YFP-MinD displayed its usual membrane localization, being enriched at the septum and cell poles (Fig. 7B, top). In contrast, in PlsY-depleted cells the YFP-MinD fluorescence signal became more diffuse and cytoplasmic (Fig. 7B, bottom), an indication that PlsY depletion compromises membrane potential, as predicted. We also reproduced the previous observation that perturbation of membrane potential by treatment with CCCP leads to PlsX delocalization (Supporting Information Fig. S8). The sensitivity of PlsX localization to membrane potential and its independence of PlsY suggest that PlsX interacts directly with membranes. In a series of experiments to be published elsewhere, we have gathered evidence that this is indeed the case (D. E. Sastre, D. de Mendoza and F. J. Gueiros-Filho, unpubl. obs.).

Discussion

PlsX is not spatially or functionally coupled to cell division

The subcellular localization of proteins is now recognized as an important determinant of their function (Rudner and Losick, 2010; Kuwada *et al.*, 2015). Here we have addressed the question of whether the synthesis of phospholipids in *B. subtilis* is spatially and functionally coupled to cell division. We studied the subcellular localization of PlsX, a central enzyme in phospholipid synthesis and found that it has a dynamic localization pattern, being homogenously distributed in the cytoplasmic membrane under most growth conditions and only occasionally appearing as membrane-associated foci. Importantly,

time-lapse analysis revealed that cells with homogenous PlsX grew and divided vigorously, suggesting that this is the physiological localization of PlsX, and that the function of this enzyme does not require its assembly into foci. Even though foci were rare and not required for cell division we also investigated if they were associated with the divisome. We observed a random distribution of PlsX foci along the cell length and, importantly, no colocalization was found between PlsX foci and FtsZ. Finally, we reinvestigated the proposed role of PlsX in cell division. By using a strain in which we could compensate for the role of PlsX in phospholipid synthesis we failed to see any effect of the absence of PlsX in the efficiency and precision of cell division. *In toto*, our results indicate that there is no strict physical or functional association between PlsX and the cell division apparatus, suggesting that membrane synthesis is not targeted to the specific cellular site where the bacterial envelope is expanding.

Our results are in contrast to recently published observations (Takada *et al*., 2014). We can envision straightforward explanations for this discrepancy in some cases. For example, the defects in FtsZ localization and cell division resulting from PlsX inactivation reported by Takada *et al*. (2014) are in all likelihood a secondary effect of phospholipid starvation, rather than a direct requirement for PlsX. However, in the case of the localization of PlsX the origin of the discrepancy is less clear. It seems unlikely that the problem would be due to different fusion proteins (both use the same GFP *mut2* allele but have different linkers: EAGGR in our construct, and LELPGPELPGPE in the Takada *et al*. construct) because both proteins seem to fully complement a PlsX mutant. One possible explanation for the predominance of foci and septal and polar accumulations of PlsX in Takada *et al*. is the imaging of stressed or unhealthy cells. In this regard, it is noteworthy that these authors describe having used polylysine, which is known to be toxic and perturb protein localization (Colville *et al*., 2010; Strahl and Hamoen, 2010), as a means to immobilize cells for microscopy. One evidence indicating that the cells shown in Takada *et al*. could indeed be stressed is the localization of FtsA, used as a divisome marker in their paper. In healthy, vigorously dividing cells, FtsA is found as rings at future division sites significantly before there is any sign of septation (by membrane staining) at these sites. Furthermore, FtsA should be almost completely absent from completed septa and cell poles. In contrast, throughout the work of Takada *et al*. FtsA appears to colocalize with septa and cell poles, almost never being detected as rings at future division sites.

Another apparent discrepancy worth discussing is that we did not detect colocalization between PlsX and the divisome, despite the fact that Takada *et al*. detected a physical interaction between PlsX and FtsA by two-hybrid and pull-down experiments. Two-hybrid and pull-down experiments are notorious at producing false positive results and, thus, not every interaction detected by these methods will prove to be physiologically relevant (Huang *et al*., 2007; Wodak *et al*., 2009). It is also possible that the interaction between PlsX and FtsA does occur *in vivo* but is too transient, or restricted to a stage of the cell cycle in which it would not be detected as the colocalization of PlsX and the divisome. One example of proteins that interact *in vivo* but that do not display colocalization are the *B. subtilis* ParAB orthologs Soj and Spo0J (Marston and Errington, 1999; Quisel *et al*., 1999).

Is spatial coupling of phospholipid synthesis and cell division necessary?

Cell division requires that the cytoplasmic membrane and peptidoglycan (PG) cell wall expand in concerted fashion to create the new septum. Insertion of new material into a covalently-connected polymeric mesh such as PG has to occur at the site where this structure is supposed to grow. Such localized growth of the PG mesh has been initially observed by autoradiography (Pooley *et al*., 1978) and confirmed with methods that either reveal PG turnover (D-cys) (De Pedro *et al*., 1997) or that detect specific features of newly synthesized PG (FL-Vancomycin) (Daniel and Errington, 2003; Tiyanont *et al*., 2006). Localized PG synthesis at the site of septation results from the recruitment of several PG biosynthetic enzymes, such as PBPs and lipidII flippase by the divisome. In *Caulobacter* and *E. coli*, the last enzyme (MurG) for the synthesis of the lipidII PG precursor is also recruited to the division site, suggesting that efficient septal PG synthesis requires a localized supply of the PG substrate in addition to the targeting of the enzymes (Aaron *et al*., 2007; Mohammadi *et al*., 2007).

The spatial coupling of cell division with PG synthesis may have suggested to some that membrane synthesis ought to also be physically connected to septal growth. However, in contrast to the covalent and somewhat static nature of the PG mesh, the membrane is a highly dynamic structure maintained by weak intermolecular interactions and whose constituents are constantly diffusing and intermixing (van Meer *et al*., 2008). The measured diffusion coefficient of lipids in bacterial membranes is around 1 $\mu m^2 . s^{-1}$ (Nenninger *et al*., 2014). This means that a lipid molecule will travel 1.4 μm, almost the length of a bacterium, in 1 s. Thus, in principle, the expansion of such a plastic structure should be indifferent to the site of incorporation of newly synthesized phospholipid molecules. Indeed, pulse labeling

experiments showed that phospholipid synthesis occurs throughout the periphery of the cell, with no evidence for a hotspot of synthesis at septa or nascent division sites, leading to the conclusion that the membrane grows by a dispersive mechanism (Lin *et al.*, 1971; Green and Schaechter, 1972; Mindich and Dales, 1972).

Despite their high plasticity and fluidity, it is well established that membranes have a non-homogenous distribution of lipids and proteins (van Meer *et al.*, 2008). In bacteria, acidic phospholipids such as cardiolipin (CL) and phosphatidylglycerol (PG) are enriched at polar and septal membranes (Oliver *et al.*, 2014). Phosphatidylethanolamine (PE) was also reported to accumulate in septal membranes, but not at cell poles (Nishibori *et al.*, 2005). More recently, lipid raft like domains were demonstrated in the *B. subtilis* membrane (Donovan and Bramkamp, 2009; Lopez and Kolter, 2010). The observation that bacterial membranes are organized into microdomains with distinctive lipid composition may also suggest localized synthesis and insertion of lipids. This notion, however, is easily refuted by computational and experimental results that demonstrate that lipids can segregate from each other in response to physical cues, such as the geometry of the membrane, and the structure and charge of the lipid molecules themselves (Huang *et al.*, 2006; Renner and Weibel, 2011). Thus, membrane heterogeneity is achieved basically by self-assembling processes akin to phase separation and would not require localized synthesis of specific phospholipid species.

What is the meaning of PlsX foci?

Our data suggests that the physiological localization of PlsX is the uniform membrane pattern. Nevertheless, we attempted to understand the origin of PlsX foci as this could reveal new principles behind protein organization and localization in bacteria. Membrane-associated foci is a common localization pattern for bacterial proteins, although their origin and meaning is poorly understood. A genome-wide study of *E. coli* found that 99 out of approximately 4200 proteins (2.5%) displayed membrane associated foci (Kitagawa *et al.*, 2005; Janakiraman *et al.*, 2009). A smaller scale study in *B. subtilis* also identified that 11 out 99 proteins localized as foci in the cell's periphery, a pattern called 'membrane proximal' by the authors (Meile *et al.*, 2006). Fluorescent protein tagging can promote artifactual focus formation and it is likely that several of the reported membrane foci are due to the tag (Landgraf *et al.*, 2012). However, the controls we carried out and the behavior of PlsX foci indicate that they are not artifacts. Foci were not favored by higher PlsX expression and thus are unlikely to represent non-physiological aggregates of this protein. Furthermore, PlsX foci tended to form at higher growth rates but were transient and readily redistributed themselves into the uniform pattern. We do not have an explanation for why foci are more frequent at high growth rates, but their transient nature suggests that they could represent a physiological oligomeric state related to the regulation of PlsX activity. Several metabolic enzymes have been reported to exist as discrete subcellular bodies (Wilson and Gitai, 2013; O'Connell *et al.*, 2014), including the bacterial enzymes CTP synthase (Ingerson-Mahar *et al.*, 2010) and tryptophanase (Li and Young, 2012). Oligomerization is a well know way to regulate enzyme activity [interestingly, first described for another lipid synthesis enzyme, acetyl-CoA carboxylase (Beaty and Lane, 1983)] and, at least in the case of *E. coli* tryptophanase, the assembly of the enzyme into a cytoplasmic body or focus correlates with downregulation of its activity (Li and Young, 2012). A similar idea was put forth in the case of MurG, which seems to be stored in an inactive state at the poles of *Caulobacter crecentus* cells when overexpressed (Michaelis and Gitai, 2010). Thus, PlsX foci may represent a way to regulate this enzyme's activity. However, in the case of PlsX it is unlikely that foci reflect storage of inactive or surplus enzyme, since there was no correlation between the appearance of foci and the expression level of PlsX.

Experimental procedures

Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study were listed in Supporting Information Table S1 and primers were summarized in Supporting Information Table S2. Drug concentrations for selection were: erythromycin, 1 μg ml^{-1}; lincomycin 25 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; chloramphenicol, 5 μg ml^{-1}; tetracycline, 15 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}; ampicillin 100 μg ml^{-1}. In general, *B. subtilis* and *E. coli* were grown in Luria–Bertani (LB) broth medium or on plates at 37°C with aeration. Required amino acids (tryptophan and phenylalanine) were added at 50 μg ml^{-1}. *B. subtilis* strains used in this study are derivatives of the laboratory wild type strain PY79.

General molecular techniques

Molecular cloning techniques were performed by standard procedures. DNA fragments were obtained by PCR using Phusion DNA polymerase (NEB). Oligonucleotide primers were purchased from Exxtend SRL (Campinas, SP, Brazil). PCR products of the expected sizes were purified from gels using GeneJET TM Gel Extraction Kit (Thermo Scientific, USA), ligated into the pTZ57R/T cloning vector (Fermentas, USA) and transformed into *E. coli* DH5α (Supporting Information Table S1). Plasmid DNA was prepared using a

GeneJET Plasmid Miniprep kit (Thermo Scientific, USA). Sequencing was carried out using the Big Dye terminator cycle sequencing kit (Applied Biosystems) by the sequencing service of the Departamento de Bioquímica, IQ-USP (SP, Brazil). Transformation of *B. subtilis* was carried out by the method of Dubnau and Davidoff-Abelson (1971). The amylase phenotype was assayed for colonies grown for 24 h in LB-starch plates by flooding the plates with 1% I_2-KI solution. All plasmids and primers used in this study are listed in Supporting Information Tables S1 and S2 respectively.

Fluorescence microscopy

For fluorescence microscopy, overnight pre-cultures of *B. subtilis* were grown in LB medium containing antibiotics were diluted in fresh medium without antibiotics to an optical density at 600 nm of 0.05 and grown at 37°C. At appropriate time intervals, 5 μl of living cells was applied directly on glass slides on a thin film of 1.5% LB-agarose and examined using a 100x oil immersion objective on a Nikon Eclipse Ti microscope, equipped with GFP BrightLineR and mCherry BrightLineR Filter Sets (Semrock).

Membranes were stained with FM4-64 (Molecular Probes, Eugene, Oregon, USA). Nucleoids were stained with DAPI (Sigma, St Louis, Missouri, USA). Images were acquired on a system with the Nikon digital camera (Nikon, Tokyo, Japan). All images were captured using NIS software version 3.07 (Nikon) and fluorescence signals were analyzed and quantified using Image J software (http://rsb.info.nih.gov/ij/).

HiLo time-lapse series were acquired on a DeltaVision API-DV Elite System equipped with a 60× TIRF objective and Hamamatsu digital camera model ORCA-ER. Images were acquired every 30 s, with 300 ms of exposure time. HiLo configuration was defined by optimizing the highest TIRF angle before cytoplasmic signal was visible, and kept constant for all experiments.

Quantitative immunoblotting

Samples were prepared as described (Weart *et al*., 2007). Briefly, harvested cells were resuspended in 50 mM Tris pH 8.0, NaCl 300 mM, 1 mM PMSF, 2 mg ml^{-1} lysozyme. Cells were incubated at 37°C for 20 min and lysed with sodium dodecyl sulphate (SDS). Cell lysates were normalized by OD_{600} at cell harvest and subjected to SDS-polyacrylamide gel electrophoresis. GFP-PlsX was detected using a polyclonal rabbit anti-GFP antibody (Rudner and Losick, 2002). SigA was detected using a polyclonal anti-SigA antibody (a kind gift from R. Losick).

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