Research article

Involvement of the YneS/YgiH and PlsX proteins in phospholipid biosynthesis in both *Bacillus subtilis* and *Escherichia coli*

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Abstract

Background: Phospholipid biosynthesis commences with the acylation of glycerol-3-phosphate (G3P) to form 1-acyl-G3P. This step is catalyzed by the PlsB protein in *Escherichia coli*. The gene encoding this protein has not been identified, however, in the majority of bacterial genome sequences, including that of *Bacillus subtilis*. Recently, a new two-step pathway catalyzed by PlsX and PlsY proteins for the initiation of phospholipid formation in *Streptococcus pneumoniae* has been reported.

Results: In *B. subtilis*, 271 genes have been reported to be indispensable, when inactivated singly, for growth in LB medium. Among these, 11 genes encode proteins with unknown functions. As part of a genetic study to identify the functions of these genes, we show here that the *B. subtilis* ortholog of *S. pneumoniae* PlsY, YneS, is required for G3P acyltransferase activity, together with PlsX. The *B. subtilis* genome lacks *plsB*, and we show in vivo that the PlsX/Y pathway is indeed essential for the growth of bacteria lacking *plsB*. Interestingly, in addition to *plsB*, *E. coli* possesses *plsX* and the *plsY* ortholog, *ygiH*. We therefore explored the functional relationship between PlsB, PlsX and YgiH in *E. coli*, and found that *plsB* is essential for *E. coli* growth, indicating that PlsB plays an important role in 1-acyl-G3P synthesis in *E. coli*. We also found, however, that the simultaneous inactivation of *plsX* and *ygiH* was impossible, revealing important roles for PlsX and YgiH in *E. coli* growth.

Conclusion: Both *plsX* and *yneS* are essential for 1-acyl-G3P synthesis in *B. subtilis*, in agreement with recent reports on their biochemical functions. In *E. coli*, PlsB plays a principal role in 1-acyl-G3P synthesis and is also essential for bacterial growth. PlsX and YgiH also, however, play important roles in *E. coli* growth, possibly by regulating the intracellular concentration of acyl-ACP. These proteins are therefore important targets for development of new antibacterial agents.

Background

Phospholipids are major components of the cell membrane. Glycerol-3-phosphate (G3P) forms the backbone of all phospholipid molecules [1]. Phospholipid biosynthesis begins with two steps of G3P acylation, leading to the formation of phosphatidic acid (PA). In bacteria, PA is converted to CDP-diacylglycerol, the precursor of the three major phospholipids, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CA) [2] (Figure 1). Earlier biochemical and genetic analyses disclosed that G3P and 1-acylglycerol-phosphate (1-acyl-G3P) acyltransferase are encoded by the *plsB* and *plsC* genes, respectively, in *Escherichia coli* [3-5]. Interestingly, while *plsC* is universally conserved in eubacteria, *plsB* has

been identified in only a limited number of species, mainly belonging to the gamma proteobacterial group. Another gene homologous to *E. coli plsC*, possibly encoding G3P acyltransferase, *plsD*, has been cloned from the *Clostridium butyricum* genome, based on its ability to complement *plsB* defects in *E. coli*. However, the role of *plsD* in *C. butyricum* cells is currently unclear [6].

E. coli PlsB utilizes acyl-ACP (Acyl Carrier Protein) and acyl-CoA as acyl donors to synthesize 1-acyl-G3P [7]. Recently, Lu et al. [8,9] have reported a new two-step pathway that utilizes a novel fatty acid intermediate for the initiation of phospholipid formation in *Streptococcus pneumoniae*. They demonstrated biochemically that PlsX produced a unique activated fatty acid by catalyzing the synthesis of fatty acyl-phosphate from acyl-ACP, and then showed that PlsY transferred the fatty acid moiety from acyl-phosphate (acyl-PO$_4$) to G3P. The *plsX* gene is widely conserved in eubacteria and it had been suggested that this gene was involved in fatty acid and/or phospholipid synthesis in *E. coli*, although the exact role of the gene remained unknown [3]. In *B. subtilis*, PlsX is essential for growth [10] and PlsX expression is controlled by FapR, a protein that regulates fatty acid and phospholipid biosynthesis genes [11]. Although the *plsY* gene, encoding a membrane protein around 200 amino acids long, is also widely conserved in eubacteria [8,9], the *plsY* gene function had been completely unknown.

In the gram-positive spore-forming bacterium,*Bacillus subtilis*, we showed that 271 genes, including *plsC* (*yhdO*) and *plsX*, when inactivated singly, were indispensable for growth in LB medium [10]. Among these genes, 11 encoded proteins with unknown functions, and these proteins are therefore targets for development of new antibacterial agents. As part of a genetic study to identify the functions of these genes, here we show that the *B. subtilis* ortholog of *S. pneumoniae* PlsY, YneS, is required for G3P acyltransferase activity, together with PlsX. The *B. subtilis* genome lacks *plsB*, and our results demonstrate in vivo that the PlsX/Y pathway is essential for the growth of bacteria lacking *plsB*. Interestingly, in addition to *plsB*, *E. coli* possesses *plsX* and the *plsY* ortholog, *γgiH*. Thus, we explored the functional relationship between PlsB, PlsX and YgiH in *E. coli*, and found that, although PlsB plays the principal role in 1-acyl-G3P synthesis, PlsX and YgiH are also important for optimal *E. coli* growth.

Results

Isolation of a B. subtilis yneS-ts *mutant*

To explore the functions of essential genes of unknown function, we adopted a strategy to isolate temperature-sensitive (*ts*) mutants of these genes and then to seek extragenic suppressors of the *ts* genes. To generate a *ts* mutation in *yneS*, we initially introduced a nonfunctional

chloramphenicol-resistant gene (*cat*) downstream of *yneS*, and transformed cells with the *yneS*-functional *cat* fragment mutagenized by PCR in vitro. Chloramphenicol-resistant transformants were selected at 30°C, and colonies that were unable to grow above 42°C were analyzed. In this manner, we isolated a *ts* mutant of *yneS* (strain MY103) that had a one-base substitution altering Val190 to Glu in the C-terminal cytoplasmic region of the protein.

Mapping of the suppressor mutation site in the yneS-ts sup-1 *strain*

Next, we isolated a spontaneous extragenic suppressor mutant of the *ts* phenotype (strain MY105, *yneS-ts sup-1*, Figure 2) by cultivation at the restrictive temperature. Transformation of wild-type strain 168 cells with chromosomal DNA of MY105 led to the *ts* growth of more than 90% of chloramphenicol-resistant transformants, indicating that the *sup-1* mutation was not linked to the *yneS* gene. MY105 cells lost sporulation ability, and the Spo⁻ phenotype was maintained in MY107 cells (Pspac::*yneS sup-1*) in which the *yneS-ts* gene was replaced with wild-type *yneS* under control of the IPTG-dependent Pspac promoter. A DNA fragment (ca. 8 kb) that complemented the Spo⁻ phenotype of MY107 in trans was isolated from a *B. subtilis* genomic DNA library in which an *Mbo*I partial digest was ligated with the phage vector, φCM, as described previously [12]. The 8 kb fragment was cut into 2.5 kb and 5 kb fragments. The 5 kb fragment with the *cat* gene of the phage vector was cloned into pBR322, while the 2.5 kb fragment was ligated into pCA191 containing the *cat* gene [13]. MY107 cells were transformed with these plasmids for integration into the chromosome via single crossovers. The results indicated that the 5 kb fragment allowed recovery of the ability to sporulate, while the 2.5 kb fragment did not. Sequencing of the 5 kb fragment revealed three genes. These were the 3'-terminal region of *glpF*, encoding the glycerol uptake facilitator, and full-length *glpK* and *glpD*, encoding glycerol kinase and glycerol-3-phosphate dehydrogenase, respectively. Because *glpF* and *glpK* constitute an operon, and because the cloned 8 kb fragment did not contain the promoter sequence [14], *glpK* was possibly not expressed from the fragment cloned in φCM. The *glpD* promoter between *glpK* and *glpD* has, however, been identified [15]. These results suggested that the mutation(s) responsible for the Spo⁻ phenotype mapped in the *glpD* gene. To confirm this theory, full-length wild type *glpD* (in pCA191*glpD*-FL), the 5'-terminal half of the gene (in pCA191*glpD*-N), and the 3'-terminal half (in pCA191*glpD*-C), were separately cloned into pCA191. The resulting constructs were integrated into the MY107 chromosome by single crossovers and the sporulation abilities of transformants were examined. The 5'-terminal region of *glpD*, and the full-length gene, conveyed the ability to sporulate, but the 3'-terminal *glpD*
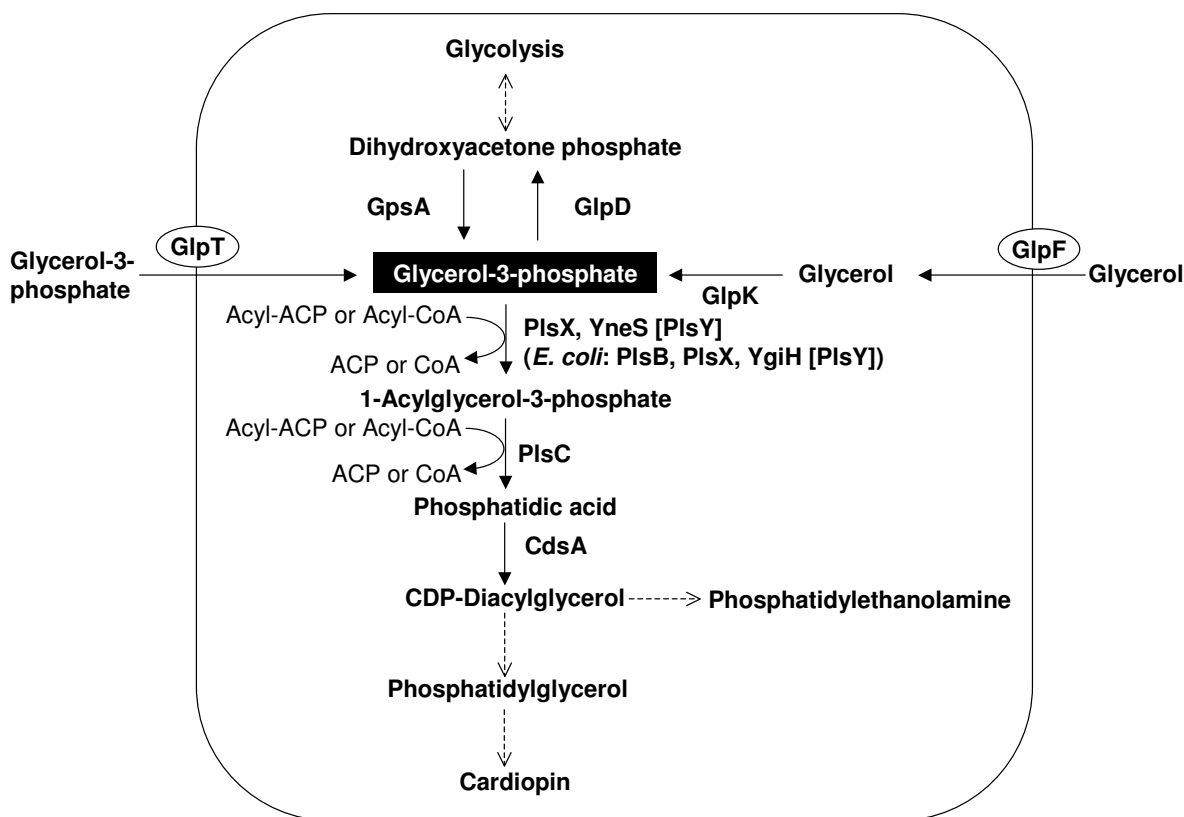
Figure 1
Phospholipid synthesis pathway in *B. subtilis*. Abbreviations: GpsA, NAD(P)H-dependent glycerol-3-phosphate dehydrogenase; GlpD, glycerol-3-phosphate dehydrogenase; GlpT, glycerol-3-phosphate permease; GlpF, glycerol uptake facilitator protein; GlpK, glycerol kinase; PlsC, 1-acylglycerol-3-phosphate O-acyltransferase; CdsA, phosphatidate cytidylyltransferase.

region did not. Sequencing of the 1.2 kb 5'-terminal half of *glpD* in MY103 (*yneS-ts*) revealed a single base (G) insertion between nucleotides C 492 and G 493, which resulted in inactivation of the gene. This result was consistent with the previous finding that *glpD* inactivation impairs sporulation ability [16].

We further confirmed that *glpD* inactivation suppressed the *yneS-ts* phenotype by insertion of a plasmid, pMutinNC [17], carrying an internal segment of the *glpD* gene, into the *glpD* gene of *yneS-ts* cells (strain MY109, *yneS-ts*, Δ*glpD*::pMutin NC, Figure 2).

Increases in intracellular G3P concentration suppress the yneS-ts phenotype
The intracellular G3P concentration is known to be elevated in *glpD* mutants [16]. We speculated that increases in the G3P level might suppress the *yneS-ts* phenotype. Indeed, we found that supplementation of the growth

medium (LB) with 0.1% (w/v) G3P, or glycerol, complemented the *yneS-ts* phenotype (Figure 2). Growth of MY105 (*yneS-ts sup-1*) and MY109 (*yneS-ts*, Δ*glpD*::pMutinNC) cells was impaired compared to *glpD⁺* cells (wild type and *yneS-ts* cells), when glycerol or G3P was supplemented in the growth medium. This phenotype was also observed in MY108 (Δ*glpD*::pMutinNC) cells (data not shown). Given that accumulation of G3P is known to result in abnormal septation and inhibition of sporulation [18,19], an increase in the intracellular concentration of G3P would explain the growth impairment of cells without *glpD*. It should be noted that G3P and glycerol did not complement the growth defect resulting from YneS depletion in the IPTG-dependent *yneS* mutant cells, strain YNESp [10] (data not shown). The YneS-ts mutant protein will retain a reduced activity at high temperature, although the activity is insufficient to support cell growth. Residual activity would be enhanced by increases in the intracellular concentrations of G3P or glycerol.
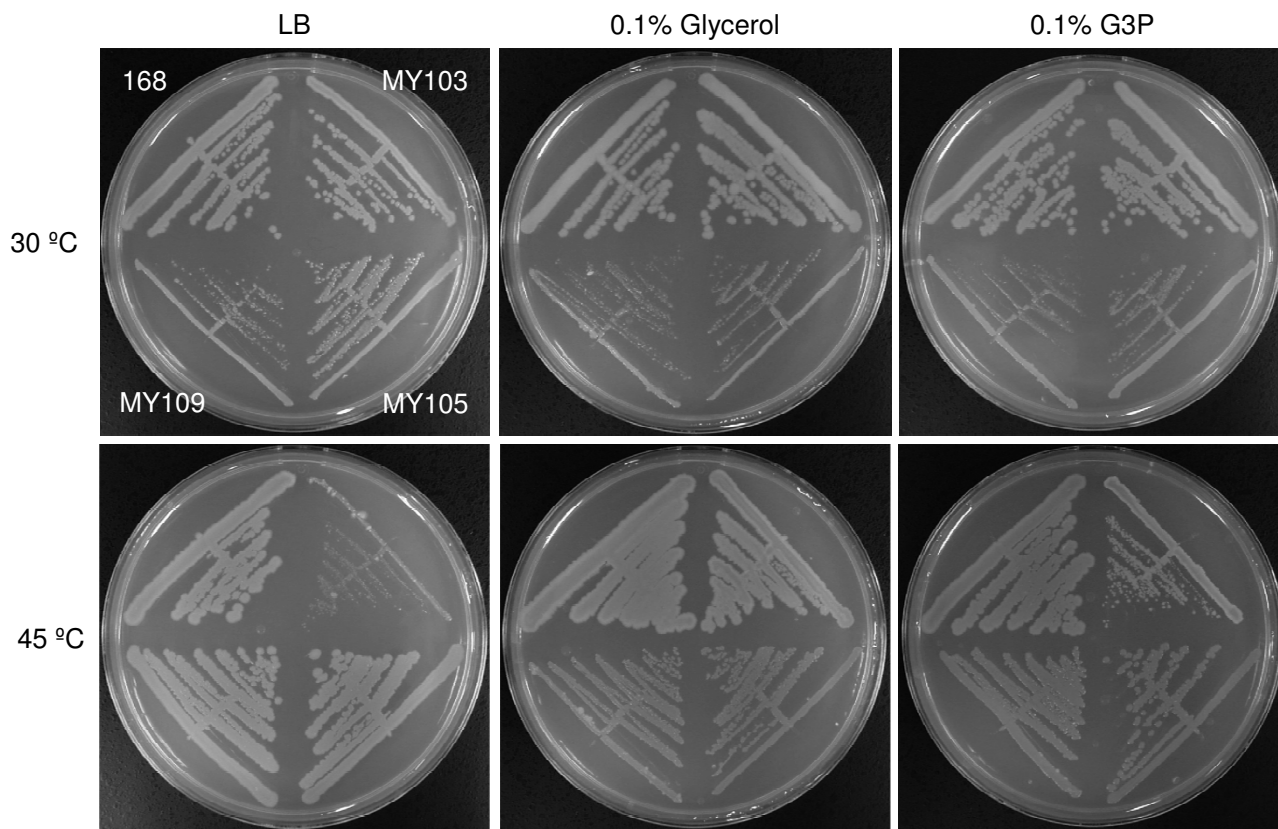
Figure 2
Growth properties of *B. subtilis yneS-ts* and suppressor mutants. *B. subtilis* wild-type (168), *yneS-ts* (strain MY103), *yneS-ts sup-1* (strain MY105), and *yneS-ts ΔglpD*::pMutinNC (strain MY109) cells were grown in LB, LB with 0.1% (v/v) glycerol, and LB with 0.1% (w/v) glycerol-3-phosphate, at 30°C and 45°C.

Phospholipid synthesis in* B. subtilis plsC-ts, yneS-ts, *and IPTG-dependent* plsX *mutants, under restrictive conditions

G3P is a substrate for G3P acyltransferase, and inhibition of G3P acyltransferase activity as a result of simultaneous mutations in *plsB* and *plsX* in *E. coli* led to G3P auxotrophy [3]. Accordingly, we examined the involvement of YneS in 1-acyl-G3P synthesis by analyzing phospholipid synthesis in *yneS-ts* cells at the restrictive temperature. We additionally constructed mutations in genes possibly involved in phospholipid synthesis. These included a *ts* mutation in *plsC*, in which 7 C-terminal residues were deleted (strain MY112), and an IPTG-dependent *plsX* mutation in strain MY111.

To determine phospholipid biosynthesis rates, *B. subtilis* cells were pulse-labeled with ^{32}[Pi] for 5 min. Phospholi-

pids were extracted using the method of Bligh and Dyer [20], and separated by two-dimensional thin layer chromatography [5]. We detected PA, PG, PE, and CL in wild-type cells growing at either 30°C or 42°C (Figure 3A). When *plsC-ts* cells were labeled at 30°C, a pattern similar to that of wild-type cells was obtained. At the restrictive temperature, however, 1-acyl-G3P accumulation was observed, and phospholipid synthesis did not take place (Figure 3B). This is consistent with the known 1-acyl-G3P acyltransferase activity of PlsC. In contract, no phospholipids were detectable in *yneS-ts* cells following a shift to the restrictive temperature (Figure 3C). Our data indicate that YneS inactivation results in blockage of the first step in phospholipid synthesis in *B. subtilis* cells. Furthermore, PlsX depletion in IPTG-dependent mutant cells induces a similar change in phospholipid composition (Figure 3D). The results are consistent with the two-step mechanism of

1-acyl-G3P synthesis reported by Lu *et al*. [8], and show that the PlsX/Y pathway is essential for growth of *B. subtilis* lacking *plsB*.

Functional relationship between plsB, plsX *and* yneS/ygiH *in* E. coli

It is generally believed that *E. coli plsB* is essential for bacterial growth, although experimental evidence supporting this hypothesis has yet to be obtained, and transposon mutagenesis experiments suggested that *plsX* is dispensable in *E. coli* [21]. The ortholog of *S. pneumoniae plsY* and *B. subtilis yneS* in *E. coli*, *ygiH*, is yet another dispensable gene [21]. Accordingly, we determined the functional relationship between *plsB*, *plsX*, and *ygiH*, in *E. coli*.

We initially attempted to systematically inactivate *E. coli* genes either singly or in combination. For this purpose, we replaced the *plsX* and *ygiH* genes with a kanamycin-resistance gene (*kan*) using the method of Datsenko and Wanner [22]. Inactivation of *plsB* was performed in the presence of plasmid-borne *plsB*. Phage P1 transduction frequencies of each allele into cells of various genetic backgrounds were measured to avoid possible effects of secondary mutations arising during gene inactivation (the *kan* cassette was removed, using FLP recombinase, in recipient cells for P1 transduction). The results, summarized in Table 1, show that *plsB* is essential for *E. coli* growth, while single deletions of either *plsX* or *ygiH* do not affect cell growth. These results suggested that PlsB plays a principal role in G3P acyltransferase activity in *E. coli*. Additionally, to examine whether *plsB* function might be complemented by overexpression of other genes, we introduced derivatives of plasmid pSTV28 or pSTV29 (12–15 copies per cell) harboring *E. coli plsX* and *ygiH*, and *B. subtilis plsX* and *yneS* into recipient strains for P1 transduction, without restoration of *plsB* function. Unexpectedly, although single deletions of *plsX* or *ygiH* did not affect cell growth, we found that the simultaneous inactivation of *plsX* and *ygiH* was impossible (Table 2), indicating that *plsX* and *ygiH* also have important roles in *E. coli* growth. Next, we expressed *B. subtilis plsX* and *yneS* genes in *E. coli*, and also the corresponding *E. coli* genes (as controls). The lethal phenotype of the *plsX* and *ygiH* double deletion was suppressed by *B. subtilis plsX* or *yneS*, indicating that these genes were able to complement the *plsX* and *ygiH E. coli* gene activities. In addition, a *plsX* and *ygiH* double deletion was possible when *plsB* was overexpressed from pSTV29*plsB*. To obtain further insights into the functional relationships between *plsB*, *plsX* and *ygiH*, we next introduced a plasmid harboring a mutated *plsB* gene (*plsB26*) into *E. coli*, and then attempted to inactivate *plsB*, *plsX* or *ygiH* (Table 3). The *plsB26* gene has a point mutation that changes Gly1045 to Ala, reducing the affinity of PlsB26 for G3P [23]. In the presence of plasmid-borne *plsB26*, genomic *plsB* could be inactivated, indicating that PlsB26

protein expressed from the plasmid-borne gene could support *E. coli* growth. The plasmid-borne gene could not, however, complement the lethality of the *plsX* and *ygiH* double deletion. Furthermore, while native *plsB* could be inactivated in the presence of pSVT29*plsB26* in cells lacking functional *ygiH*, *plsB* inactivation became impossible in cells with inactivated *plsX*, indicating that PlsX activity becomes indispensable for the growth of cells when the *plsB* activity is supplied by a plasmid copy of *plsB26*.

The essential nature of plsB, and the lethality of the plsX and ygiH double deletion, were further confirmed using inducible forms of these genes. To this end, we placed the coding sequences of plsB, plsX and ygiH under the control of an IAA (3β-indoleacrylic acid)-inducible trp promoter (Pw), integrated the genes into the chromosome as described in Methods, and then deleted the native copies of either plsB alone, or both plsX and ygiH. As expected, strains MEC201 (Pw-plsB, ΔplsB), MEC306 (Pw-plsX, ΔplsX, ΔygiH) and MEC307 (Pw-ygiH, ΔplsX, ΔygiH) cells displayed IAA-dependent growth (Figure 4A and 4B).

The PlsB and PlsX enzymes utilize acyl-ACP as acyl-donor, and abnormal accumulation of acyl-ACP has been shown to result in growth impairment [24]. To examine acyl-ACP accumulation in cells with inactivated *plsX* and *ygiH* genes, we introduced a derivative of pUC18, harboring the *E. coli tesA* gene encoding thioesterase I [25], into cells of strains MEC306 and MEC307. Interestingly, the growth defects of these strains in the absence of IAA were suppressed by the additional supply of thioesterase I (TesA hydrolyses acyl-ACP) (Figure 4C). We have not yet succeeded in demonstrating the expected TesA-dependent suppression of the inability to achieve *plsX* inactivation when *plsB* activity is supplied by a plasmid encoding *plsB26*. This is because it is difficult to construct an appropriate strain in which to examine the hypothesis.

Complementation of the PlsB activity by a combination of PlsX and YneS/YgiH

As we found that the combination of PlsX and YneS conferred the ability to synthesize 1-acyl-G3P upon *B. subtilis*, we expected that simultaneous overexpression of these two proteins would complement a *plsB* mutation in *E. coli*. To test this hypothesis, we introduced various combinations of plasmids harboring *plsX* and *yneS/ygiH* into *E. coli* cells. In these experiments, we employed derivatives of plasmid pMW118 (ca. 5 copies per cell), whose replication is compatible with that of pSTV28/29 derivatives, when more than one cloned gene was to be expressed. We successfully complemented the *E. coli plsB* mutation by expression of *B. subtilis plsX* and *yneS* (Table 4). The complementation of the *E. coli* PlsB activity by the *B. subtilis* PlsX and YneS combination was also demonstrated using an IAA-inducible *plsB* gene (Figure 4A). Other gene com-
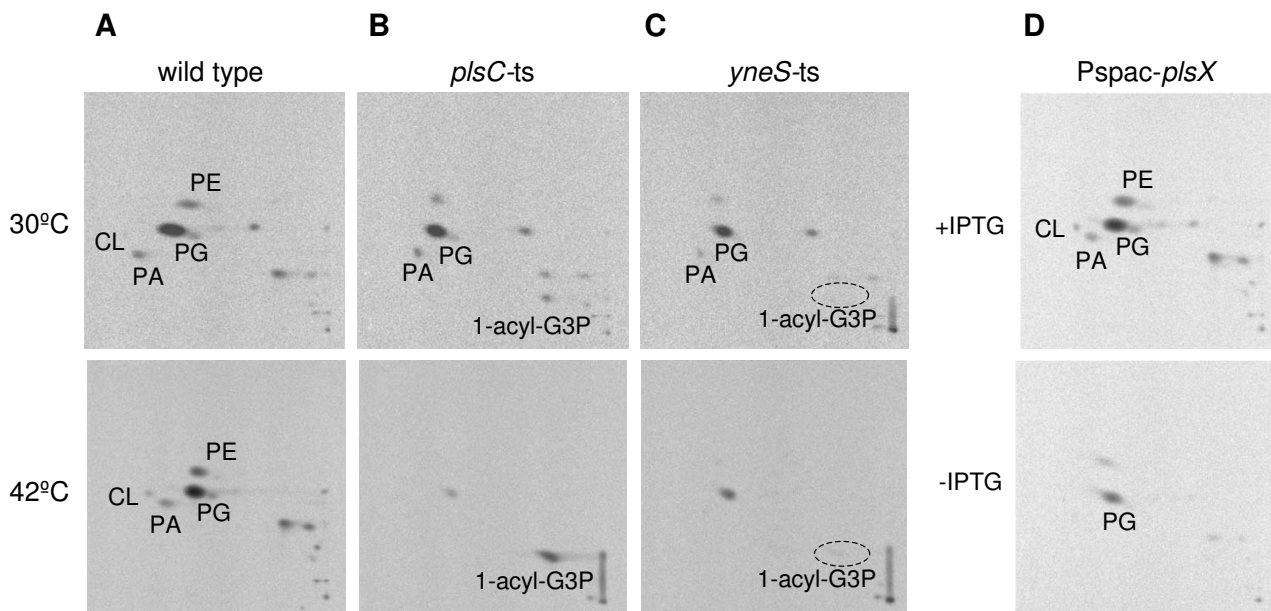
Figure 3
Phospholipid synthesis in *B. subtilis yneS-ts*, *plsC-ts*, and IPTG-dependent *plsX* mutants, under restrictive conditions. Labeling with ³²[Pi] for 5 min, lipid isolation, lipid separation using two-dimensional TLC, and phospholipid detection, are described in Methods. (A) Wild-type cells labeled at 30°C and 42°C. (B) *plsC-ts* cells (strain MY112) labeled at 30°C and 42°C. (C) *yneS-ts* cells (strain MY103) labeled at 30°C and 42°C. (D) Pspac-*plsX* cells (strain MY111) labeled in the presence and absence of IPTG. Abbreviations: 1-acyl-G3P, 1-acylglycerol-phosphate; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CA, cardiolipin.

binations, including those with *E. coli* genes, did not complement *plsB*. Plasmid pMW118*ygiH* complemented the growth defect of MEC306 and MEC307 cells in the absence of IAA (data not shown), indicating that functional YgiH is expressed from pMW118*ygiH*. Although we could not determine the expression levels of the various proteins due to the lack of appropriate antibodies, the specific activities of *E. coli* PlsX and YgiH may be lower than those of their *B. subtilis* counterparts.

Discussion
Our genetic studies have shown that PlsX and YneS (now renamed PlsY) are essential for G3P acyltransferase activity in *B. subtilis* cells, and that the combination of the two *B. subtilis* proteins complements PlsB activity in *E. coli* cells. Our findings are consistent with the two-step mech-

anism of 1-acyl-G3P synthesis reported by Lu *et al.* [8], and we have shown that the PlsX/Y pathway is essential for the growth of *B. subtilis* lacking *plsB*. The combination of *plsX* and *plsY* is widely conserved in eubacteria, but most eubacteria lack *plsB* [8], strongly suggesting that *plsX/Y* will be essential genes in these bacteria.

A number of bacteria, belonging mainly to the gamma group of proteobacteria, possess both *plsB* and *plsX/plsY*, however. Another important finding reported here is that, in such cases, the relationship between the two 1-acyl-G3P synthesis pathways may be variable and complex. Although *plsB* has been reported to be dispensable in *Pseudomonas aeruginosa* [26], we found that the gene is indispensable in *E. coli*, indicating that PlsB is principally responsible for 1-acyl-G3P synthesis in *E. coli*. On the

Table 1: Frequency of P1 transduction of *plsB::kan*, *plsX::kan*, and *ygiH::kan* mutations into W3110 cells harboring various plasmids

| | Recipient strain | | | | | | | | Relative transduction frequency* | | |
| | Chromosomal gene | | Harboring plasmids | | | | | | P1 donor strain | | |
Strain	plsX	ygiH	pSTV29	pSTV29 plsB	pSTV28 EcplsX	pSTV29 ygiH	pSTV28 BsplsX	pSTV29 yneS	ΔplsB::kan	ΔplsX::kan	ΔygiH::kan
W3110	+	+	-	-	-	-	-	-	0	100	100
MEC104	+	+	+	-	-	-	-	-	0	50	64.3
MEC105	+	+	-	+	-	-	-	-	100	65.5	58.9
MEC106	+	+	-	-	+	-	-	-	0	80	50.9
MEC107	+	+	-	-	-	+	-	-	0	100	78
MEC108	+	+	-	-	-	-	+	-	0	63.6	100
MEC109	+	+	-	-	-	-	-	+	0	81.8	70.6

*Transduction frequencies are expressed as percentages of the highest number of Km^r colonies recorded in each experiment, and are depicted as blocks.

other hand, we found that simultaneous inactivation of both PlsX and PlsY is impossible, and that this lethality was complemented by overexpression of PlsB. As single deletions in either *plsX* or *ygiH* did not affect cell growth, the lethality of the *plsX* and *ygiH* double deletion cannot be because of inactivation of a minor pathway (using acyl-PO$_4$) for 1-acyl-G3P synthesis.

Interestingly, PlsX activity becomes indispensable for growth of *E. coli* cells in which PlsB activity is supplied from a plasmid copy of *plsB26*. Growth, in minimal medium, of *E. coli* SJ22 cells harboring the *plsB* mutation on the chromosome is dependent on a supply of exoge-

nous G3P. Depletion of G3P in the growth medium has been found to result in accumulation of acyl-ACP due to the blockage of 1-acyl-G3P synthesis, leading to a severe reduction in fatty acid biosynthesis because accumulated acyl-ACP causes feedback inhibition of enzymes in the biosynthetic pathway [27,28]. G3P-acyltransferase activity expressed from a plasmid-borne copy of *plsB26* may be less than that in wild type cells, and acyl-ACP may therefore accumulate in such cells. PlsX may be able to suppress the deleterious accumulation of acyl-ACP by converting acyl-ACP to acyl-PO$_4$. PlsY may also be involved in control of the intracellular acyl-ACP concentration. *E. coli* PlsB has been shown to utilize both acyl-ACP and acyl-

Table 2: Frequency of P1 transduction of *plsB::kan*, *plsX::kan*, and *ygiH::kan* mutations into strains MEC102 and MEC103 harboring various plasmids

| | Recipient strain | | | | | | | | Relative transduction frequency* | | |
| | Chromosomal gene | | Harboring plasmids | | | | | | P1 donor strain | | |
Strain	plsX	ygiH	pSTV29	pSTV29 plsB	pSTV28 EcplsX	pSTV29 ygiH	pSTV28 BsplsX	pSTV29 yneS	ΔplsB::kan	ΔplsX::kan	ΔygiH::kan
MEC102	-	+	-	-	-	-	-	-	0	100	0
MEC103	+	-	-	-	-	-	-	-	0	0	100
MEC110	-	+	+	-	-	-	-	-	0	55.9	0
MEC111	-	+	-	+	-	-	-	-	100	53.5	59.8
MEC112	-	+	-	-	+	-	-	-	0	56.3	75.3
MEC113	-	+	-	-	-	+	-	-	0	83.1	100
MEC114	-	+	-	-	-	-	+	-	0	45.1	84.2
MEC115	-	+	-	-	-	-	-	+	0	100	92.7
MEC116	+	-	+	-	-	-	-	-	0	0	69.8
MEC117	+	-	-	+	-	-	-	-	100	63.7	85.9
MEC118	+	-	-	-	+	-	-	-	0	99.5	100
MEC119	+	-	-	-	-	+	-	-	0	85.1	73.8
MEC120	+	-	-	-	-	-	+	-	0	36.3	60.5
MEC121	+	-	-	-	-	-	-	+	0	100	96

*Transduction frequencies are expressed as percentages of the highest number of Km^r colonies recorded in each experiment, and are depicted as blocks.

Table 3: Frequency of P1 transduction of *plsB::kan*, *plsX::kan*, and *ygiH::kan* mutations into W3110, strain MEC102, and strain MEC103, harboring pSTV29 -*plsB* and *plsB26*

	Recipient strain					Relative transduction frequency*		
	Chromosomal gene		Harboring plasmids			P1 donor strain		
Strain	*plsX*	*ygiH*	pSTV29	pSTV29 *plsB*	pSTV29 *plsB26*	Δ*plsB::kan*	Δ*plsX::kan*	Δ*ygiH::kan*
MEC104	+	+	+	-	-	0	66.4	76.9
MEC105	+	+	-	+	-	100	100	100
MEC288	+	+	-	-	+	61.9	67.7	55.1
MEC110	-	+	+	-	-	0	98.2	0
MEC111	-	+	-	+	-	100	75	100
MEC289	-	+	-	-	+	0	100	0
MEC116	+	-	+	-	-	0	0	100
MEC117	+	-	+	-	-	100	100	89
MEC290	+	-	-	-	+	59.3	0	61.7

*Transduction frequencies are expressed as percentages of the highest number of Km^r colonies recorded in each experiment, and are depicted as blocks.

CoA as acyl donors in the synthesis of 1-acyl-G3P [7], but does not use these donors to make acyl-PO$_4$ [8]. On the other hand, *S. pneumoniae* PlsY has been shown to synthesize 1-acyl-G3P using both acyl-ACP and acyl-PO$_4$. These results suggest that *E. coli* PlsY, in the absence of PlsX, may synthesize 1-acyl-G3P using acyl-ACP, and this activity would then contribute to consumption of acyl-ACP. It is therefore possible that both PlsX and PlsY contribute independently to the maintenance of an appropriate intracellular acyl-ACP concentration, and simultaneous inactivation of both proteins therefore results in deleterious accumulation of acyl-ACP. Overexpression of PlsB increases 1-acyl-G3P synthesis activity and may suppress the accumulation of acyl-ACP resulting from the *plsX* and *ygiH* double deletion. Suppression of growth defects due to the *plsX* and *ygiH* double deletion by overexpression of thioesterase I strongly supports our hypothesis.

Acyl-ACP has been shown to be not only a central cofactor for fatty acid and phospholipid synthesis, but also a regulatory molecule, coordinating the synthesis of these lipids with cell growth. Our results strongly suggest that PlsX, PlsY and PlsB form a complex network functioning to supply appropriate levels of lipid biosynthetic precursors, especially acyl-ACP. In turn, this controls the synthesis of 1-acyl-G3P in *E. coli* cells, resulting in appropriate levels of 1-acyl-G3P under various growth conditions. Further studies are needed to reveal the precise roles of PlsX, PlsY and PlsB.

Conclusion

Both *plsX* and *yneS* are essential for 1-acyl-G3P synthesis in *B. subtilis* cells lacking *plsB*, in agreement with recent reports on the biochemical functions of these genes. Both genes will be essential for bacterial growth lacking *plsB*. In

Table 4: Frequency of P1 transduction of *plsB::kan*, *plsX::kan*, and *ygiH::kan* mutations into W3110 cells harboring various pairs of plasmids.

	Recipient strain								Relative transduction frequency*		
	Chromosomal gene		Harboring plasmids						P1 donor strain		
Strain	*plsX*	*ygiH*	pSTV29	pSTV28 *EcplsX*	pSTV28 *BsplsX*	pMW118	pMW118 *ygiH*	pMW118 *yneS*	Δ*plsB::kan*	Δ*plsX::kan*	Δ*ygiH::kan*
MEC130	+	+	+	-	-	+	-	-	0	56.1	39.4
MEC134	+	+	+	-	-	-	+	-	0	53.7	41.3
MEC136	+	+	+	-	-	-	-	+	0	100	81.7
MEC137	+	+	-	+	-	+	-	-	0	87.8	69.2
MEC139	+	+	-	+	-	-	+	-	0	73.2	100
MEC140	+	+	-	-	+	+	-	-	0	90.2	46.2
MEC142	+	+	-	-	+	-	-	+	100	97.6	90.4

*Transduction frequencies are expressed as percentages of the highest number of Km^r colonies recorded in each experiment, and are depicted as blocks.
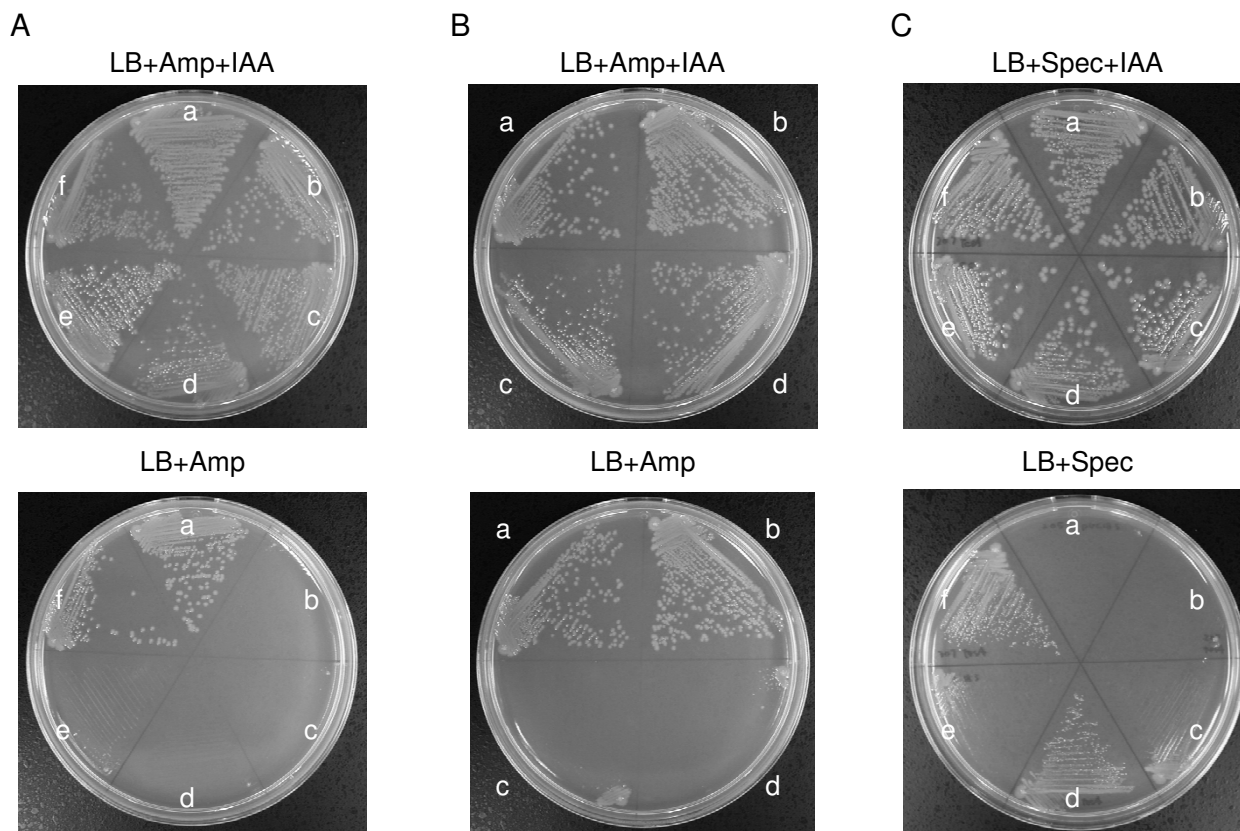
Figure 4
Growth properties of *E. coli* strains harboring IAA-inducible genes. *E. coli* strains harboring IAA-inducible genes were grown in LB medium containing 50 µg/ml ampicillin, with or without IAA (100 µg/ml), at 37°C. (A) a; MEC199 (Pw-*plsB*), b; MEC201 (Pw-*plsB* Δ*plsB*), c; MEC212 (Pw-*plsB* Δ*plsB* pSTV29 pMW118), d; MEC214 (Pw-*plsB* Δ*plsB* pSTV29 pMW118*yneS*), e; MEC218 (Pw-*plsB* Δ*plsB* pSTV29*BsplsX* pMW118), and f; MEC220 (Pw-*plsB* Δ*plsB* pSTV29*BsplsX* pMW118*yneS*). (B) a; MEC308 (Pw-*EcplsX*), b; MEC309 (Pw-*ygiH*), c; MEC306 (Pw-*EcplsX* Δ*plsX* Δ*ygiH*), and d; MEC307 (Pw-*ygiH* Δ*plsX* Δ*ygiH*). (C) *E. coli* strains harboring pUC18S or pUC18S *tesA* were grown in LB medium containing 50 µg/ml spectinomycin, with or without IAA (50 µg/ml), at 30°C. a; MEC323 (Pw-*plsB* Δ*plsB* Δ*ygiH* pUC18S), b; MEC324 (Pw-*plsB* Δ*plsB* Δ*ygiH* pUC18S *tesA*), c; MEC325 (Pw-*EcplsX* Δ*plsX* Δ*ygiH* pUC18S), d; MEC326 (Pw-*EcplsX* Δ*plsX* Δ*ygiH* pUC18S *tesA*), e; MEC327 (Pw-*ygiH* Δ*plsX* Δ*ygiH* pUC18S), e; MEC328 (Pw-*ygiH* Δ*plsX* Δ*ygiH* pUC18S *tesA*).

E. coli, PlsB plays a principal role in 1-acyl-G3P synthesis and is also essential for bacterial growth. PlsX and YgiH also, however, play important roles in *E. coli* growth, probably by regulating the intracellular concentrations of acyl-ACP at appropriate levels. These proteins are therefore important targets for the development of new antibacterial reagents.

Methods
Materials and strains
The *B. subtilis* and *E. coli* strains used in this study are listed in Tables S1 [see Additional file 1] and S2 [see Additional file 2], respectively. Primers and plasmids are specified in Tables S3 [see Additional file 3] and S4 [see Additional file 4], respectively. *B. subtilis* cells were transformed as described previously [29]. *E. coli* strains DH5α (Takara) and JM105 (Takara), were used throughout as cloning hosts.

Full-length sequences of E. coli plsB, plsX (EcplsX), and ygiH, and B. subtilis plsX (BsplsX) and yneS, were amplified and cloned into plasmids pSTV28 or pSTV29 (Takara, 12–15 copies per cell) as summarized in Table S3 [see Additional file 3]. A DNA fragment containing a mutated plsB sequence, plsB26, was PCR-amplified from genomic DNA of E. coli strain TL84 [3], and cloned into pSTV29.

Inserts of pSTV29ygiH and pSTV29yneS were transferred into pMW118 (Nippon Gene, ca. 5 copies per cell), because the replication of pMW118 is compatible with that of pSTV28 and pSTV29.

The IAA (3β-indoleacrylic acid, Sigma)-inducible *trp* promoter fragment was PCR amplified from W3110 chromosomal DNA. The PCR product was digested with the restriction enzymes *Mun*I and *Eco*RI, and cloned into the *Eco*RI site of pMC1403 [30], generating pMC1403Pw. Full-length sequences of *E. coli plsB*, *plsX*, and *ygiH* were amplified and cloned into the *Eco*RI/*Bam*HI, *Bam*HI, or *Eco*RI/*Bam*HI sites of pMC1403-Pw, respectively, to obtain pMC1403Pw-*plsB*, pMC1403Pw-*plsX* and pMC1403Pw-*ygiH*.

The full-length sequence of *E. coli tesA* was amplified and cloned into plasmid pUC18S. Plasmid pUC18S was created by replacing the *Aat*II/*Eam*1105I fragment (containing the ampicillin-resistance gene) of pUC18 (Takara) with an *Aat*II/*Eam*1105I fragment (containing the spectinomycin-resistance gene) amplified, by PCR, from pAPNC213 [17].

B. subtilis strains were grown in Luria-Bertani (LB) medium, or DSM, supplemented with chloramphenicol (5 μg/ml), kanamycin (5 μg/ml), or erythromycin (0.5 μg/ml), as appropriate. *E. coli* strains were grown in LB or M9 minimal media, supplemented with ampicillin (50 μg/ml), chloramphenicol (5 μg/ml), spectinomycin (50 μg/ml), or kanamycin (25 μg/ml), as appropriate.

Isolation of the *B. subtilis yneS-ts* mutant
The *yneS-ts* mutant was isolated according to the procedure of F. Kawamura (personal communication). The *B. subtilis* strain MY101, in which the sequence 45–103 bp downstream of the termination codon of *yneS* (in the coding sequence of *yneR*, which is downstream of *yneS*) was replaced with a *cat* gene, was generated as follows. The regions upstream and downstream of the insertion site were amplified by PCR using a yneS-F1 and yneS-R1 primer pair, and a yneS-F2 and yneS-R2 primer pair, respectively. The *cat* gene of pCBB31 [31] was amplified using primers cat-F and cat-R. The primer pairs yneS-R1 and cat-R, and cat-F and yneS-F2, contain overlapping sequences, and the three fragments obtained were ligated by recombinant PCR using yneS-F1 and yneS-R2 primers. *B. subtilis* strain 168 was transformed with the resulting fragment, and chloramphenicol-resistant transformants were selected (MY101, ΔyneR::cat⁺). Next, the pCH11 plasmid containing an inactive *cat* gene (a nonsense mutation is located in the gene), a kanamycin-resistance gene, and a *ts* origin of replication, was introduced into MY101. We selected for kanamycin resistance and chloramphenicol sensitivity in order to isolate cells with the inactive *cat*

gene on the chromosome, which occurred when gene conversion from the plasmid copy was successful [32]. The introduced plasmid was removed by cultivation at the temperature restrictive for pCH11 replication, to generate strain MY102 (ΔyneR::cat⁻). PCR mutagenesis of the *yneS-cat* fragment was performed using r*Taq* polymerase (Takara) with MY101 chromosomal DNA as template. The mutated *yneS-cat* fragment was used to transform strain MY102. Chloramphenicol-resistant transformants were selected at 30°C, and colonies that were unable to grow above 42°C were screened to isolate strain MY103 (*yneS-ts*).

Construction of the *B. subtilis plsC-ts* mutant
As the growth of cells in which *gfp* (encoding green fluorescent protein) was translationally fused to the C-terminus of PlsC became temperature-sensitive (data not shown), we predicted that the required *plsC-ts* mutant could be obtained by modification of the C-terminal region of the *plsC* gene. We therefore created a series of mutants with deletions of different numbers of amino acids from the C-terminus of PlsC and found that deletion of the 7 end residues resulted in a *ts* phenotype (strain MY112). To generate strain MY112, the 3' end of the *plsC* coding region (with the 7 codons deleted) was amplified, and a termination codon was added by PCR using the primers plsC-F1 and plsC-CΔ7-R1. The region downstream of *plsC* was amplified with the oligonucleotides plsC-F2 and plsC-R2. The two fragments were ligated to the 5' and 3' ends of the *cat* gene (amplified from pCBB31 using plsC-cat-F and plsC-cat-R primers) by recombinant PCR with overlapping sequences contained within the plsC-CΔ7-R1 and plsC-cat-F primer pair, and the plsC-F2 and plsC-cat-R primer pair. The resulting fragment was used to transform *B. subtilis* strain 168 with selection for chloramphenicol resistance.

Construction of an IPTG-dependent mutant of *B. subtilis plsX*
The *plsX* gene is located in the fatty acid biosynthesis operon composed of *fapR*, *plsX*, *fabD*, *fabG*, and *acpA*. To generate cells in which *plsX* expression was under the control of the IPTG-inducible Pspac promoter, we initially integrated Pspac-*plsX* into the *aprE* locus of the bacterial chromosome. The *plsX* gene was amplified by PCR using the pAP-plsX-F and pAP-plsX-R primers, and inserted between the Pspac promoter and the kanamycin-resistance gene (*kan*) on pAPNCK, that contains sequences upstream and downstream of *aprE*, flanking Pspac and *kan*. Plasmid pAPNCK was created by replacing the *Hind*III fragment (containing the spectinomycin-resistance gene) of pAPNC213 [17] with the *Hind*III fragment (containing the *kan* gene) of pDG780 [33]. The resulting plasmid was transformed into *B. subtilis* cells and selection of a double crossover using kanamycin resistance yielded

strain MY110 (Δ*aprE*::Pspac-*plsX-kan*). Next, the native *plsX* gene was replaced with the *cat* gene, without the promoter and terminator sequences, to ensure the correct expression of downstream essential genes. To achieve this, upstream and downstream regions of *plsX* were amplified using the plsX-F1and plsX-R1 primer pair, and the plsX-F2 and plsX-R2 primer pair, respectively. Fragments were ligated to the 5' and 3' ends of the *cat* gene (amplified from pCBB31 using primers cat-F-p and cat-R-t) by recombinant PCR using overlapping sequences in the plsX-R1 and cat-F-p primer pair, and the cat-R-t and plsX-F2 primer pair. The resulting fragment was used to transform strain MY110, and chloramphenicol-resistant transformants were selected to generate strain MY111 (Δ*aprE*:: Pspac-*plsX-kan* Δ*plsX*::*cat*-p-t).

Analysis of phospholipid composition
Cells of *B. subtilis* strains 168, *yneS-ts* (MY103), and *plsC-ts* (MY112) were cultured in LB medium at 30°C until an OD_{600} of 0.4 was attained, and the temperature was then shifted to 42°C. Cells were pulse-labeled with ^{32}[Pi] (50 μCi/ml, Amersham Biosciences) for 5 min after 1 h of cultivation at 42°C. Incorporation of the label was terminated by the addition of 0.8 ml of culture to 3.0 ml of chloroform:methanol (1:2, v/v). Lipids were extracted using the method of Bligh and Dyer [20]. The phospholipids produced were examined by two-dimensional thin-layer chromatography on Silica Gel 60 plates (Merck) developed with chloroform:methanol:water (65:25:4) in the first dimension and chloroform:methanol:acetic acid (65:25:10) in the second dimension [5]. The incorporation of label into lysophosphatidic acid and other lipids was determined and quantitated using the BAS2500 image analyzer (Fuji).

Pspac-*plsX* (MY111) cells were cultured in LB medium containing 50 μM IPTG at 37°C until an OD_{600} value of 0.4 was attained. Cells were harvested, washed twice with LB medium, and suspended in 5 mL LB medium with or without 1 mM IPTG at final densities (OD_{600} values) of 0.06 or 0.1, respectively. Next, cells were pulse-labeled with ^{32}[Pi] (50 μCi/ml) for 5 min after 1 h of cultivation at 37°C. Lipid extraction and analysis were performed as described above.

Gene disruption in **E. coli**
Deletions of the *plsB*, *plsX*, and *ygiH* genes were achieved using a one-step chromosomal gene inactivation method involving the phage λ Red recombination system, as developed by Datsenko and Wanner [22,34]. The *E. coli* strain, BW25113, was used. The primers used for target gene disruptions are listed in Table S4 [see Additional file 4]. The 5' end of each primer contained 60–70 bp of sequences upstream or downstream of the target gene, while the 3' end contained specific 20-nucleotide

sequences from the template, pKD13. The primer pairs plsBdelup and plsBdeldown, plsXdelup and plsXdeldown, and ygiHdelup and ygiHdeldown, were used to generate Δ*plsB*::*kan* (in strain MEC001), Δ*plsX*::*kan* (in strain MEC002), and Δ*ygiH*::*kan* (in strain MEC003) mutations, respectively. Because *E. coli plsB* was essential for growth, inactivation of *plsB* was performed in the presence of a plasmid harboring the gene (pSTV28*plsB-p*). Deletion mutations were confirmed using colony PCR with a *kan*-specific primer (k1 or k2) [22] and the locus-specific primers listed in Table S4 [see Additional file 4]. The deletion mutations, Δ*plsX*::*kan* and Δ*ygiH*::*kan*, were transferred into prototrophic strain W3110 by P1*vir* transduction to obtain strains MEC005 and MEC006, respectively. The *kan* cassettes in strains MEC005 and MEC006 were removed with FLP recombinase, expressed by plasmid pCP20, as described previously [22], to obtain strains MEC102 and MEC103, respectively.

Construction of **E. coli** *strains harboring IAA-inducible genes*
To introduce *plsB*,*plsX* and *ygiH* genes, under the control of IAA-inducible *trp* promoters, into the *E. coli* chromosome, cloned gene fragments in pMC1403-based plasmids were transferred into the λRZ5 phage by *recA*-mediated homologous recombination as previously described [35]. The technique involves double crossovers between common upstream ampicillin-resistance genes and downstream *lac* operon sequences. The resulting λPw-*plsB*, λPw-*plsX*, and λPw-*ygiH* phages were lysogenized into the W3110 chromosome, to obtain strains MEC199, MEC308, and MEC309, respectively. The Δ*plsB*::*kan* mutation was transferred into strain MEC199 by P1*vir* transduction, and the *kan* cassette was then removed with FLP recombinase, expressed from plasmid pCP20, to obtain strain MEC201. The λPw-*plsX* and λPw-*ygiH* phages were also lysogenized into the strain MEC102 chromosome, to generate strains MEC303 and MEC304, respectively. The Δ*ygiH*::*kan* mutation was then transferred into strains MEC303 and MEC304 by P1*vir* transduction.

Abbreviations
G3P, Glycerol-3-phosphate; 1-acyl-G3P, 1-acyl-glycerol-phosphate; PA, phosphatidic acid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CL, cardiolipin; IAA, 3β-indoleacrylic acid.

Authors' contributions
All experimental work was carried out by MY under the supervision of TO and NO. TO provided the *E. coli* transduction system. MY and TO wrote the draft manuscript. NO finalized the analysis and completed the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

B. subtilis *strains used in this study, and their genotypes. List of* B. sub-tilis *strains used in this study.*
Click here for file
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Additional file 2

E. coli *strains used in this study, and their genotypes. List of* E. coli *strains used in this study.*
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Additional file 3

Plasmids used in this study. List of plasmids used in this study.
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Additional file 4

Oligonucleotides used in this study. List of oligonucleotides used in this study.
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[http://www.biomedcentral.com/content/supplementary/1471-2180-7-69-S4.pdf]

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