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Involvement of PlsX and the acyl-phosphate dependent *sn*-glycerol-3-phosphate acyltransferase PlsY in the initial stage of glycerolipid synthesis in *Bacillus subtilis*

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The gene responsible for the first acylation of *sn*-glycerol-3-phosphate (G3P) in *Bacillus subtilis* has not yet been determined with certainty. The product of this first acylation, lysophosphatidic acid (LPA), is subsequently acylated again to form phosphatidic acid (PA), the primary precursor to membrane glycerolipids. A novel G3P acyltransferase (GPAT), the gene product of *plsY*, which uses acyl-phosphate formed by the *plsX* gene product, has recently been found to synthesize LPA in *Streptococcus pneumoniae*. We found that in *B. subtilis* growth arrests after repression of either a *plsY* homologue or a *plsX* homologue were overcome by expression of *E. coli plsB*, which encodes an acyl-acylcarrier protein (acyl-ACP)-dependent GPAT, although in the case of *plsX* repression a high level of *plsB* expression was required. *B. subtilis* has, therefore, a capability to use the acyl-ACP dependent GPAT of PlsB. Simultaneous expression of *plsY* and *plsX* suppressed the glycerol requirement of a strict glycerol auxotrophic derivative of the *E. coli plsB26* mutant, although either one alone did not. Membrane fractions from *B. subtilis* cells catalyzed palmitoylphosphate-dependent acylation of [^{14}C]-labeled G3P to synthesize [^{14}C]-labeled LPA, whereas those from Δ*plsY* cells did not. The results indicate unequivocally that PlsY is an acyl-phosphate dependent GPAT. Expression of *plsX* corrected the glycerol auxotrophy of a Δ*ygiH* (the deleted allele of an *E. coli* homologue of *plsY*) derivative of BB26-36 (*plsB26 plsX50*), suggesting an essential role of *plsX* other than substrate supply for acyl-phosphate dependent LPA synthesis. Two-hybrid examinations suggested that PlsY is associated with PlsX and that each may exist in multimeric form.

Key words: glycerolipid synthesis, *sn*-glycerol-3-phosphate acyltransferase, PlsX, PlsY, acyl-phosphate, lysophosphatidic acid, LPA, phosphatidic acid, *Bacillus subtilis*

INTRODUCTION

The membrane of *Bacillus subtilis* cells contains many lipids. In addition to the three major glycerophospholipids, phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin, which it shares with *Escherichia coli* membrane, the *B. subtilis* membrane contains lysylphosphatidylglycerol, mono-, di-, and triglucosyldiacylglycerols, and glycerophosphoglucolipid (de Mendoza et al., 2002; Kawai et al., 2004). The study of the biosynthetic pathways of the lipids of *B. subtilis* should help elucidate the physiological roles they play, as in the case of *E. coli* (Shibuya, 1992; Dowhan, 1997; Matsumoto, 2001; Cronan, 2003). Genetic studies coupled with homology search with the sequences of known enzymes for lipid synthesis have revealed many of the genes involved and have led to an outline of the biosynthesis of lipid headgroups in *B. subtilis* (de Mendoza et al., 2002; Nishibori et al., 2005; Matsumoto et al., 2006). However, the first stage in the

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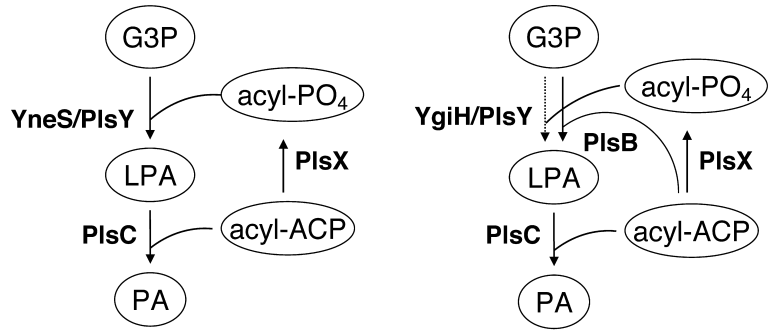

Fig. 1. Biosynthetic pathways for phosphatidic acid in *B. subtilis* and *E. coli*. Results from Paoletti et al. (2007) and Yoshimura et al. (2007) and our confirmatory evidences *in vivo* and *in vitro* are compiled to make up a new view of PA synthesis in *B. subtilis* and *E. coli*. Abbreviations used are: G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; acyl-PO_4, acyl-phosphate; acyl-ACP, acyl acyl-carrier protein. Acyl-CoA is also the substrate for PlsB and PlsC but not for YgiH in *E. coli* (Lu et al., 2006) and it is not for PlsY and PlsC in *B. subtilis* (Paoletti et al., 2007).

biosynthetic pathway of lipid headgroups, the synthesis of the primary molecule, phosphatidic acid (PA), has not yet been fully clarified.

Synthesis of PA proceeds by double acylation of glycerol-3-phosphate (G3P). The first and second acylation reactions, the acylation of G3P to form 1-acyl-*sn*-glycerol-3-phosphate, also termed lysophosphatidic acid (LPA), and the second acylation of LPA to form PA are catalyzed by the gene products of *plsB* and *plsC*, respectively, in *E. coli* (Cronan and Rock, 1996; Cronan, 2003). In the *B. subtilis* genome, however, only one gene involved has been found: *yhdO*, a homologue of *plsC*, appears to be responsible for the second acylation step (Kunst et al., 1997). The product of the gene involved in the first acylation would likely have a motif similar to those of other acyltransferases (Lewin et al., 1999; Yamashita et al., 2007), but no such homologue has been discovered in the *B. subtilis* genome (Kunst et al., 1997; Kobayashi et al., 2003).

A new G3P acyltransferase (GPAT), PlsY, that uses a novel acyl-donor, acyl-phosphate produced by PlsX, has recently been found to be responsible for the synthesis of LPA in *Streptococcus pneumoniae* (Lu et al., 2006). This PlsX/Y pathway is widely distributed in bacteria and a homologue of *plsY*, *yneS*, and a homologue of *plsX* have been found in the *B. subtilis* genome (Kunst et al., 1997; Lu et al., 2006). The role of *plsC*, *plsY*, and *plsX* in the synthesis of glycerolipid in *B. subtilis* has recently been described by Paoletti et al. (2007). A similar study by Yoshimura et al. (2007) stresses the synthetic lethality of *plsY* (*ygiH*) and *plsX* mutations in *E. coli*.

Here we confirm that both *plsX* and *plsY* are involved in the initial acylation stage of glycerolipid synthesis in *B. subtilis* through detailed genetic complementation studies with *E. coli plsB*. We demonstrate further that PlsY catalyzes the novel type of acylation of G3P to produce LPA using acyl-phosphate as acyl donor and perform as unequivocal control an assay of the membrane fraction of Δ*plsY* cells. Furthermore, a possible indispensable function of PlsX in the acyl-acylcarrier protein (acyl-ACP) metabolism, distinct from that providing acyl-phosphate substrate for LPA synthesis, is discussed. Compilation of the results of Paoletti et al. (2007) and Yoshimura et al. (2007) and of our confirmatory evidences *in vivo* and *in vitro* illustrate a new view of the biosynthetic pathways of PA, the initial product for glycerolipid synthesis, in bacteria (Fig. 1).

MATERIALS AND METHODS

Bacterial strains and plasmids *B. subtilis* and *E. coli* strains and plasmids used in this study are listed in Tables 1 and 2. Primers for construction of plasmids are listed in Table 3. The *B. subtilis* strains were constructed by integration of a plasmid or by transformation of chromosomal DNA. BYH12 [*plsX*::pMT3plsX (P*spac-plsX* Em^r) *fabD*::pfabD15(P*repU-neo-fabD-fabG*)] is the P*spac-plsX* strain that suppresses the polar effect of *plsX*, which is localized in an operon in which essential genes *fabD* and *fabG* are localized downstream of *plsX*. BYH12 was constructed by transformation of BYH01 (P*spac-plsX* Em^r) with the pfabD15 (P*repU-neo-fabD* p15A*ori*) plasmid by single cross over at *fabD*. The genes *fabD-fabG* of the resultant transformant is thus under a constitutive promoter P*repU* independent of P*spac-plsX*. The *E. coli* strains were constructed by P1 transduction.

Most of the plasmids in Table 2 were constructed by inserting PCR-amplified DNA fragments using the primer

Table 1. Bacterial strains

Strain	Relevant characteristics	Source or Reference
B. subtilis		
168	*trpC2*	Laboratory Stock
BYH01	168 *plsX*::pMT3plsX [P*spac-plsX* Em^r]	Integration of pMT3plsX
BYH12	BYH01 *fabD*::pfabD15(P*repU-neo-fabD-fabG*)	Integration of pfabD15
BYH13	BYH12 *amyE*::[*xylR* P*xyl-plsX cat*]	Integration of pXplsX
BYH15	BYH12 *amyE*::[*xylR* P*xyl-plsB cat*]	Integration of pXplsB
BYH16	BYH12 *amyE*::[*xylR* P*xyl cat*]	Integration of pX
BYH21	168 *amyE*::[*xylR* P*xyl-plsX cat*]	TF with BYH13 DNA
BYH22	BYH21Δ*plsX*::*neo* P*repU-fabDG*	Integration of pΔplsX
PMYNES	168 *plsY*::pMutin [P*spac-plsY* Em^r]	Kobayashi, K.
BYH05	PMYNES *amyE*::[*xylR* P*xyl-plsB cat*]	Integration of pXplsB
BYH24	168 *amyE*::[*xylR* P*xyl-plsB cat*]	TF with BYH05 DNA
BYH25	BYH24 Δ*plsY*::*spc*	Integration of pΔplsY
BYH28	168 *amyE*::[P*rpsD-plsB-ha cat*]	Integration of pX-PlsB-HA
BYH31	BYH28 Δ*plsY*::*spc*	TF with BYH25 DNA
BYH32	BYH31 Δ*plsX*::*neo* P*repU-fabDG*	TF with BYH22 DNA
BYH33	168 *amyE*::*cat*	Integration of pXΔxyl
TYHDO	168 *yhdO*::pMT2yhdO[P*spac-yhdO* Em^r]	Integration of pMT2yhdO
E. coli		
MC4100	*Δ(argF-lac)U169 araD139 rpsL150 thiA1 relA1 flbB5301 deoC1 ptsF25 rbsR*	Laboratory Stock
JW3031	BW25113 Δ*ygiH*::FRT-*kan*-FRT	KO collection
JW3386	BW25113 Δ*glpR*::FRT-*kan*-FRT	KO collection
BB26-36	*plsB26 plsX50 glpD3 glpR2 glpK^i phoA8 relA1 spoT1*	Larson et al. (1984)
TL84	BB26-36 *zjb-750*::Tn*10*	NBRP
JC201	*plsC1*	Coleman, R.
EYH01	BB26-36 Δ*ygiH*::FRT-*kan*-FRT	P1 TD
EYH04	MC4100 Δ*glpR*::FRT-*kan*-FRT	P1 TD
EYH05	EYH04 *plsB26 zjb-750*::Tn*10*	P1 TD
BTH101	F$^-$ *cya-99 araD139 galE15 galK16 rpsL1*(Str^r) *hsdR2 mcrA1 mcrB1*	Euromedex

TF: transformation; TD: transduction.

pairs given in Table 3. Plasmid pΔplsY (Ap^r *spc yneT yneR* ColE1 *ori*) was constructed by ligation of three fragments, the Ap^r ColE1 *ori* fragment, *yneT*, and *yneR*. Plasmid pfabD15 (P*repU-neo-fabD* p15A *ori*) was constructed by replacing the ColE1 *ori* of plasmid pfabD with p15A *ori* of pPA114; pTSCneo was a derivative of pBEST501 that was constructed by replacing the region of P*repU-neo* with the corresponding PCR fragment of the region lacking the terminator of *neo*; pΔplsX was constructed by insertion of a *fapR* (*ylpC*)-*fabD* region into pTSCneo. See Tables 2 and 3 for the construction of other plasmids.

Media and growth conditions *B. subtilis* and *E. coli* strains were grown in LB broth containing 1% tryptone (Difco), 0.5% yeast extract (Difco) and 1% NaCl. When required, 0.1% glycerol was added. E medium (1% K_2HPO_4, 0.35% $NaNH_4HPO_4 \cdot 4H_2O$, 0.2% citrate$\cdot H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$)(Bell, 1975) containing 0.4% glucose, 0.1% casamino acid, and 0.001% thiamine, was used for the cultivation of *E. coli* cells. MacConkey agar medium (Nissui) was used for the *E. coli* two-hybrid experiment. When required, the following supplements were added to the media (per liter): 100 mg of ampicillin (Sigma), 15 mg of neomycin (Wako Pure Chem.), 100 and 50 mg of spectinomycin (Sigma) for *B. subtilis* and *E. coli*, respectively,

Table 2. Plasmids

name	Relevant characteristics	Source or Reference
pSK6	*bla* P_{araBAD} pSC101 *ori*	Suzuki et al. (2002)
pSK6plsB	*bla* P_{araBAD}-*plsB* pSC101 *ori*	This work
pSK6plsC (*E.coli*)	*bla* P_{araBAD}-*plsC*(*E.coli*) pSC101 *ori*	This work
pSK6yhdO	*bla* P_{araBAD}-*yhdO* pSC101 *ori*	This work
pPA114	*Cat nahR* P_{nahG}-*tsr* p15A *ori*	Ames et al. (2002)
pPAplsX	*Cat nahR* P_{nahG}-*plsX* p15A *ori*	This work
pX	*bla cat xylR 5'-amyE 3'-amyE* P_{xyl}	Kim et al. (1996)
pXplsB	*bla cat xylR 5'-amyE 3'-amyE* P*xyl-plsB*	This work
pXplsX	*bla cat xylR 5'-amyE 3'-amyE* P*xyl-plsX*	This work
pBEST501	*bla* P_{repU}-*neo*	Itaya et al (1989)
pTSCneo	*bla* P_{repU}-*neo*, pBEST501 derivative	This work
pfabD	*bla* P_{repU}-*neo-fabD*, pTSCneo derivative	This work
pfabD15	P_{repU}-*neo-fabD* p15A *ori*	This work
pΔplsX	*bla fapR*(*ylpC*) P_{repU}-*neo-fabD*	This work
pCL1921	*spc* P_{lac} pSC101 *ori*	Lerner and Inouye (1990)
pCL1921plsY	*spc* P_{lac}-*plsY*	This work
pBAD24	*bla* P_{araBAD}	Guzman et al. (1995)
pnyneR	*bla spc* P_{araBAD} *yneR*	This work
pyneT	*bla* P_{araBAD} *yneT*	This work
pΔplsY	*bla spc yneT yneR*	This work
pMutinT3	*bla ery* P*spac lacZ lacI*	Vagner et al. (1998)
pMT3plsX	*bla ery* P*spac-plsX' lacZ lacI*	This work
pMT2yhdO	*bla ery* P*spac-yhdO' lacZ lacI*	This work
pKT25	*kan* P_{lac}-*T25'* p15A *ori*	Euromedex
pKNT25	*kan* P_{lac}-*'T25* p15A *ori*	Euromedex
pUT18	*bla* P_{lac}-*'T18* ColE1 *ori*	Euromedex
pMSK01	*kan* P_{lac}-*T25-plsX* p15A *ori*	This work
pMSK04	*bla* P_{lac}-*plsX-T18* ColE1 *ori*	This work
pMSK05	*bla* P_{lac}-*plsY-T18* ColE1 *ori*	This work
pMSK10	*kan* P_{lac}-*plsY-T25* p15A *ori*	This work
pX-rpsD	*bla cat 5'-amyE 3'-amyE PrpsD-plsX-ha*	This work
pX-rpsDII	*bla cat 5'-amyE 3'-amyE* (*0.5 kb*) *PrpsD-plsX-ha*	This work
pX-PlsB-HA	*bla cat 5'-amyE 3'-amyE* (*0.5 kb*) *PrpsD-plsB-ha*	This work
pXΔxyl	*bla cat 5'-amyE 3'-amyE*, pX lacking P*xyl xylR*	This work

[a] Gene symbols followed by *-ha* indicate that the gene is modified with the sequence for HA tag.

50 mg of kanamycin (Kyowa) or 0.3 mg of erythromycin (Sigma). Growth of bacteria was monitored by measuring turbidity with a Klett-Summerson photoelectric colorimeter (No. 54 filter). For solid media, 1.5% agar (Difco) was included.

Assay of acyl-phosphate dependent acyltransferase activity The enzyme assay was performed as previously described (Lu et al., 2006) with minor modifications. Essentially, the membrane fractions (200 μg protein) from wild type and the mutant strain were incubated with 100 μM [^{14}C] L-α-glycerophosphate (G3P) in the presence or absence of 200 μM palmitoyl-PO_4 for 30 min at 30°C and palmitoyl-PO_4-dependent [^{14}C] LPA formation was determined. The typical assay mixture contained 0.1 M Tris-HCl (pH 8.8), 0.1 M sodium phosphate, 1 mg/ml bovine serum albumin, 2 mM $MgCl_2$, 5 mM Na_3VO_4 (phosphatase inhibitor), 0.1 μCi [U-^{14}C]G3P (150 mCi/mmol, American Radiolabeled Chemicals Inc.), 100 μM G3P, and 200 μM palmitoyl-PO_4. Reactions were started by adding the

Table 3. Primer pairs for PCR and plasmid constructed

primer pair	sequence [a]	plasmid constructed (vector) [b]
plscBsnhe1	gaca***gctagc***atcattgattacg	pSK6plsB (-pSK6)
plscBsasecor1	agg***gatatc***tctcagaacgcctta	
plsbececor1	at***gaattc***ctgctatccttgccgc	pSK6plsC (-pSK6)
plsbasecor5	at***gatatc***tgattacccttcgccctg	
plscececor1	acgt***gaattc***ccagtctctcag	pSK6yhdO (-pSK6)
plscececor5	atgcctgaatgaact***gatatc***gact	
plsxbshind3	gtatcgttcaa***aagctt***cataaa	pMT3plsX (-pMutinT3)
plsxbsasbamh1	tttatcttc***ggatcc***gacatt	
plsxbsnde1	gtatcgttcaa***catatg***cataaag	pPAplsXbs (-pPA114)
plsxbsassac1	ctcat***gagctc***aaaacctccaga	
plsbbgl2	aa***agatct***atgactttctgctatcct	pXplsB (-pX)
plsBbgl22	cggc***agatct***cccgggttacccttcg	
pBEST501Xba1	gac***tctaga***gcttgggatcgatgtcg	pTSCneo (-pBEST501)
pBEST501assal1	agag***gtcgac***gttcaaaatggtatg	
fabDsal1	gaaa***gtcgac***aaaacagatgag	pfabD (-pTSCneo)
fabDasHind3	cggg***aagctt***cagtgacaatag	
yneSbgl2	aagg***agatct***gaaaaaggagaaatg	pCL1921yneS (-pCL1921)
yneSasecoR1	aga***gaattc***ttttaagcttat	
ynersal1	atg***gtcgac***agcttaaaaaacttc	pnyneR (-pBAD24)
ynerassph1	gaagt***gcatgc***ttttttattgg	
yneTNhe1	gca***gctagc***atctacatttctcctt	pyneT (-pBAD24)
yneTasEcoR1	ttg***gaattc***tgcacctattttgttac	
plsXxba1	agg***tctaga***atgcatgagaatagctgta	pMSK01 (-pKT25) and pMSK04 (-pUT18)
plsXassma1	acc***cccggg***agtactcatctgtttttc	
yneSxba1	aga***tctaga***gatgttaattgcttt	pMSK05 (-pUT18) and pMSK10 (-pKNT25)
yneSassma1	ttt***cccggg***ataaccattttactttag	

[a] Bold face letters in nucleotide sequence indicate the sequence for restriction endonuclease recognition.
[b] The name of the plasmid constructed. The vector plasmid used for this construction is also noted in parenthesis.

membrane fraction and were terminated by addition of three volumes of chloroform:methanol (1:2, v/v). After addition of unlabeled LPA as a carrier, total lipids were extracted by the method of Bligh and Dyer (Ames, 1968) under acidic conditions (0.6 N HCl). Total lipids were separated by TLC in chloroform:acetone:methanol:acetic acid:water (5:2:1:1:0.5, v/v). Radioactivity was determined with an imaging analyzer (Fuji-BAS 1500; Fuji Film).

Synthesis of palmitoyl-phosphate The synthesis of palmitoyl-phosphate was achieved using the method of Lehninger (1946) with a minor modification. Silver phosphate (376 mg, 0.90 mmol) was added to anhydrous phosphoric acid (199 mg, 2.03 mmol) in diethyl ether (10 ml), and the mixture was stirred at room temperature for 16 h. Palmitoyl chloride (588 mg, 2.03 mmol; Wako) was dissolved in diethyl ether (5 ml) and added dropwise to the resulting mixture. After the addition was complete, the mixture was stirred for an additional hour. The resulting mixture was centrifuged, and the retained solid was washed with diethyl ether (2×5 ml). The supernatant and washings were combined and concentrated in vacuo. The palmitoyl-phosphate was dissolved in dimethysulphoxide (4 mg/ml) to serve for the assay.

Adenylate cyclase two-hybrid assay The method used for the adenylate cyclase two-hybrid assay was essentially that of Karimova et al. (2005). PlsX and PlsY were fused with the T25 fragment (*cya*'-gene fusion) of adenylate cyclase by cloning into the pKT25 and pKNT25 plasmid (Euromedex), respectively. On the other hand, T18 fragment ('*cya*-gene fusion) fusions were constructed by cloning the genes for PlsX and PlsY into pUT18 plasmid (Euromedex). The plasmids constructed and the primer pairs for gene cloning are given in Tables 2 and 3,

respectively. BTH101 host cells were cotransformed with a test pair of T18- and T25-fused plasmids and transformed colonies were directly selected on MacConkey indicator plates containing 1% maltose, 0.5 mM IPTG, 50 μg/ml kanamycin, and 100 μg/ml ampicillin as described in the protocol of the supplier. The indicator plates were incubated at 30°C for 72 h.

RESULTS

The first and second acylation steps in PA synthesis are catalyzed by the products of *plsB* and *plsC*, respectively, in *E. coli*. In *B. subtilis*, *yhdO*, a homologue of *E. coli plsC*, has recently been shown to be responsible for the second acylation step and thus designated as *plsC* (Paoletti et al., 2007). However, no *plsB* homologue has been found in the *B. subtilis* genome. Since the consensus sequences for the acyltransferases are partly similar (Lewin et al., 1999), we wondered if the product of *yhdO* might also have GPAT activity. To reconfirm the results of Paoletti et al. (2007) and check for a possible contribution of *yhdO* to the first acylation step, *yhdO* (pSK6yhdO) and its control plasmids were introduced into the temperature-sensitive *plsC1* mutant strain JC201 and the glycerol auxotrophic *plsB26* mutant strain BB26-36 of *E. coli*. The introduced *yhdO* corrected the temperature-sensitivity of JC201, but the glycerol auxotrophy of the *plsB26* mutant persisted (data not shown). When the expression of *yhdO* was repressed in *B. subtilis* P*spac-yhdO* cells an accumulation, though rather small in amount, of the lipid spot corresponding to LPA was observed on a two-dimensional thin layer chromatogram (data not shown). Concomitant accumulation of a large amount of fatty acids was also observed in the *yhdO*-repressed cells. The results indicate that YhdO is a LPA acyltransferase, a homolog of *E. coli* PlsC, without GPAT activity. Consequently, we follow the recommendation by Paoletti et al. (2007) and refer to the former *yhdO* as *plsC*.

A novel GPAT, PlsY, that uses PlsX produced acylphosphate as acyl-residue donor, has recently been found to be responsible for LPA synthesis in *S. pneumoniae* and respective homologues of *plsY* (formerly *yneS*) and *plsX* have been found in the *B. subtilis* genome (Lu et al., 2006; Paoletti et al., 2007; Yoshimura et al., 2007). In order to study the roles of *plsY* and *plsX* in *B. subtilis*, we first examined the growth of strains in which the expression of *plsY* and *plsX*, respectively, is under the control of the P*spac* promoter. The growth of P*spac-plsY* strain PMYNES depended on IPTG. Two hours after depletion of IPTG growth was arrested (see Fig. 2A).

To examine the effect of the repression of P*spac-plsX*, we constructed strain BYH12 (P*spac-plsX* P*repU-fabD-fabG*), in which the essential genes *fabD* and *fabG* (downstream from *plsX* and assumed to be in the same operon)

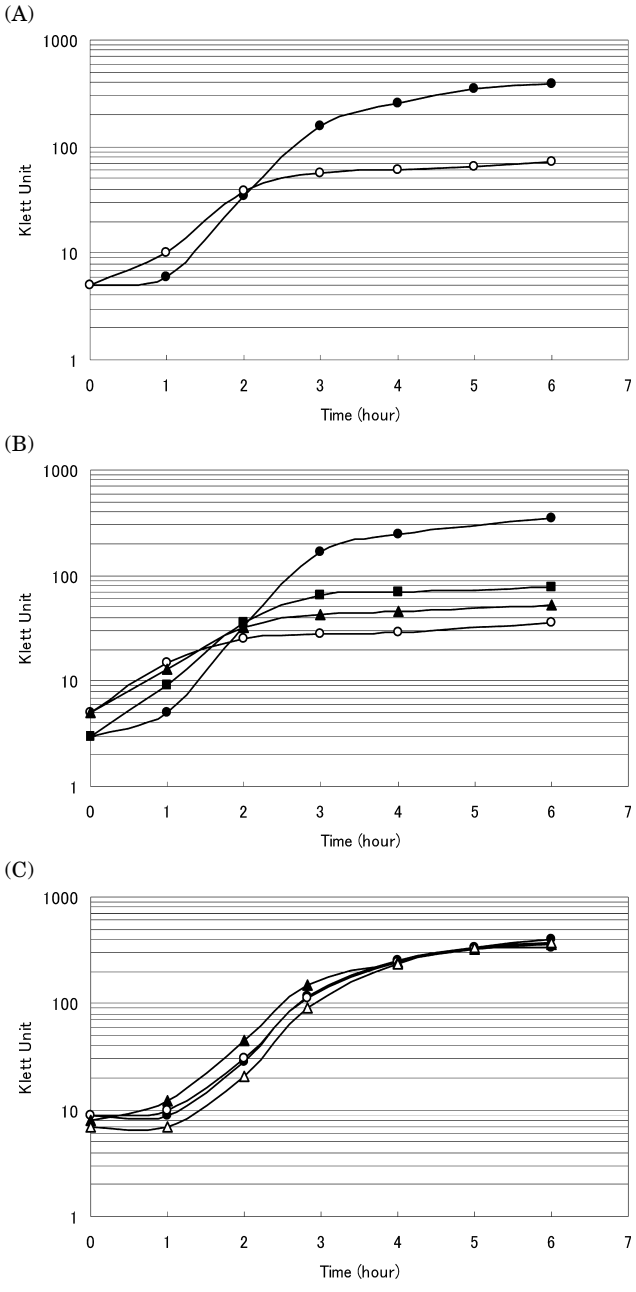

Fig. 2. Effect of *plsB* expression on the arrested growth of P*spac-plsY* and P*spac-plsX* cells. (A) Cells of strain BYH05 (P*spac-plsY* P*xyl-plsB*) were incubated overnight in LB medium supplemented with 100 μM IPTG. The cells of the overnight cultures were washed once with LB medium and cultivated for one hour in LB medium. These cultures were then inoculated at 1/100 volume into fresh LB medium supplemented with 0.2% xylose (●), or 0% xylose (○) and Klett units were measured at 37°C. (B) Cells of strain BYH15 (P*spac-plsX* P*xyl-plsB*) were cultivated as above except that the supplement was 300 μM IPTG, and the growth was monitored in LB medium supplemented with 300 μM IPTG (●), 1% xylose (■), 0.2% xylose (▲), or 0% xylose (○). (C) Effect of *plsB* expression on the arrested growth of Δ*plsY* cells and Δ*plsX* Δ*plsY* double mutant cells. The cells of the overnight cultures were inoculated at 1/100 volume into fresh LB medium and Klett units were measured at 37°C. Strains BYH28 (*amyE*::P*rpsD-plsB-ha cat*) (●), BYH31 (*amyE*::P*rpsD-plsB-ha* Δ*plsY*) (○), BYH32 (*amyE*::P*rpsD-plsB-ha* Δ*plsY* Δ*plsX*) (▲), and BYH33 (*amyE*::*cat*) (Δ).

were placed under the control of an independent constitutive promoter P*repU*. The growth of BYH12 cells was arrested two hours after repression of *plsX* by IPTG depletion (see Fig. 2B). The growth arrest of the strains after IPTG depletion is consonant with the previous suggestion that *plsY* and *plsX* are essential (Kobayashi et al., 2003).

The growth arrested after repression of *plsY* (P*spac-plsY*) was restored when PlsB was induced from P*xyl-plsB* in the strain designated BYH05 with 0.2% xylose (Fig. 2A), indicating that the supply of LPA produced from PlsB bypasses the growth defect caused by the lack of PlsY. *B. subtilis* has, therefore, a capability to utilize the acyl-ACP dependent GPAT of PlsB. The growth arrested through repression of *plsX*, however, recovered only very little when PlsB was induced with 0.2% xylose from P*xyl-plsB* (strain BYH15; Fig. 2B). With 1% xylose the growth recovered to a somewhat larger extent than with 0.2%, suggesting that the growth recovery of *plsX*-repressed cells required a higher level of PlsB than the recovery of PlsY-depleted cells. The control strain with an empty vector BYH16 did not grow even with high concentrations of the inducer: the growth recovery depends on PlsB. When *plsB* is placed under the strong constitutive *rpsD* promoter (Jester et al., 2003) to supply a high level of PlsB, in cells with disrupted *plsX* and *plsY* alleles (designated BYH32), a much higher level of growth recovery, close to wild type level growth, was observed (Fig. 2C). The results suggest that a high level of PlsB is required for recovery from arrested growth after repression of *plsX*.

As we anticipated that both PlsY and PlsX are involved in forming LPA in *B. subtilis* (as they are in *S. pneumoniae*), we examined their behavior in *E. coli* cells. Since the glycerol auxotrophic phenotype of BB26-36 (*plsB26 plsX50*) depends on the mutant allele *plsX50* (Larson et al., 1984) and introduction of the wild type *E. coli* or *B. subtilis plsX* allele abolishes its glycerol auxotrophy, BB26-36 is not suitable for examination of the cooperative action of PlsY and PlsX. We therefore constructed a strict glycerol auxotrophic strain EYH05 (MC4100 *plsB26 glpR*) by P1 transduction of *plsB26* into a strain harboring a disrupted allele of *sn*-glycerol-3-phosphate regulatory gene (Δ*glpR*::FRT-*kan*-FRT) which causes constitutive *sn*-glycerol-3-phosphate dehyolrogenase and glycerol kinase production (Larson et al. 1984; McIntyre and Bell, 1975). Although independent expression of either *plsY* or *plsX* in EYH05 cells was ineffective, co-expression of *plsY* and *plsX* suppressed the glycerol auxotrophy of EYH05 cells successfully (Fig. 3). This result indicates that the co-presence of PlsY and PlsX bypasses the function of PlsB, suggesting that PlsY is a GPAT that uses acyl-phosphate produced by PlsX as an acyl donor. This is consistent with observation in *S. pneumoniae*.

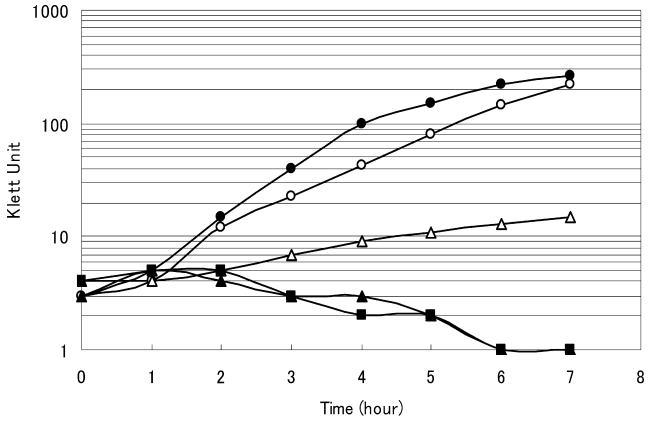

Fig. 3. Effect of *plsY* and *plsX* on the arrested growth of *E. coli plsB26*Δ*glpR* cells after glycerol depletion. Cells of the strain EYH05 (*plsB26*Δ*glpR*) were transformed with either one of pSK6plsB (●), pCL1921plsY (▲), pPAplsX (Δ), or both pCL1921plsY and pPAplsX (○). Empty vectors pCL1921 and pPA114 were also introduced (■). These transformants were cultivated overnight in LB medium supplemented with 0.1% glycerol. The cells of the overnight cultures were washed once with LB medium and cultivated in two volumes of fresh LB medium for one hour. The cultures were inoculated 1/100 volume into fresh E medium and Klett units were measured at 37°C.

It is interesting that PlsX (note that "*plsX*" and "PlsX" are of *B. subtilis*) weakly supported the growth of the glycerol-auxotrophic *E. coli* strain EYH05 (MC4100 *plsB26* Δ*glpR*), whereas PlsY was totally ineffective (Fig. 3). This growth support by PlsX (plasmid pPAplsX) was obviously effective in the Δ*ygiH* derivative (strain EYH01) of BB26-36 (*plsB26 plsX50*) background (data not shown). It has been suggested that the *E. coli* membrane has some activity, though quite small, synthesizing LPA dependent on acyl-phosphate and that this activity is lost in the membrane fraction of Δ*ygiH* mutant cells (Lu et al., 2006). Thus, the growth support by PlsX in the Δ*ygiH plsB26* host indicates that PlsX or the product of the enzyme reaction, acyl-phosphate, has a certain important function other than providing the substrate for acyl-phosphate dependent GPAT. In *B. subtilis* cells under repressed *plsX* condition, strong expression of PlsB, which supplies LPA, was required for growth (Fig. 2B), thus the observed role of PlsX in *E. coli* Δ*ygiH plsB26* cells may have some relation to that required in *B. subtilis* cells.

Next, we examined the expected acyl-phosphate-dependent GPAT activity of PlsY according to the method described in Lu et al. (2006). For a no-PlsY control we prepared the membrane fraction of a Δ*plsY* strain BYH31 whose growth was fully supported by PlsB expressed from *plsB* under the constitutive strong P*rpsD* promoter. Incubation of the membrane fraction from the wild type cells with [^{14}C]-labeled G3P produced a thick band of acylated G3P, LPA, on a thin layer chromatogram plate in

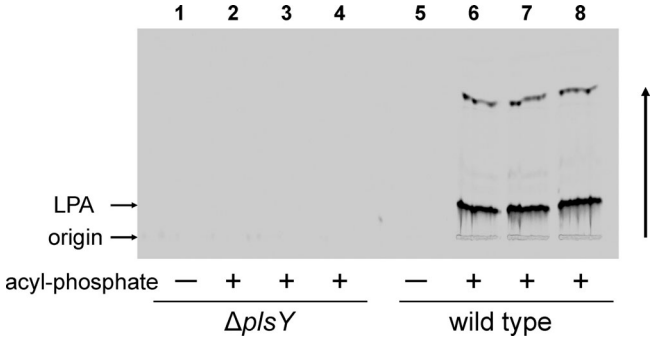

Fig. 4. Acyl-phosphate dependent GPAT activity of *B. subtilis* PlsY. The membrane fractions (200 μg protein) from the wild type and the Δ*plsY* strain (in triplicate assay) were incubated with 100 μM [^{14}C]G3P in the presence or absence of 200 μM palmitoyl-phosphate for 30 min at 30°C. After the reactions were stopped, total lipids were extracted and separated by TLC in chloroform:acetone:methanol:acetic acid:water (5:2:1:1:0.5, v/v). The image of radioactivity in the TLC plate was analyzed with a Fuji-BAS 1500. The origin and the band corresponding to LPA are indicated by arrows.

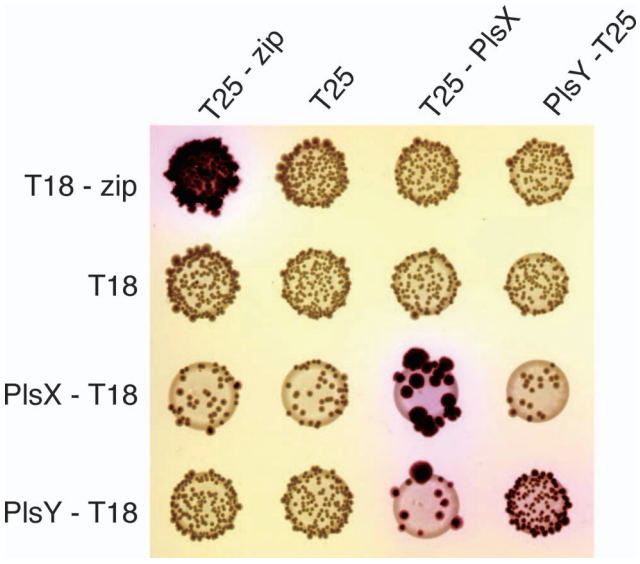

Fig. 5. Bacterial adenyl cyclase two-hybrid assay of PlsY and PlsX. BTH101 host cells cotransformed with a test pair of adenyl cyclase domain T18- and T25-fused plasmids were directly selected on MacConkey indicator plates and incubated at 30°C for 72 h. Empty plasmids with the domains T18 and T25 and the fusions with leucine zipper (T18-zip and T25-zip) are the negative and positive controls, respectively.

the co-presence of palmitoylphosphate, an acyl-phosphate (Fig. 4, lanes 6–8, assays in triplicate). In the absence of palmitoyl-phosphate the LPA band was not detected (lane 5). The membrane fraction from Δ*plsY* cells did not support the synthesis of LPA (lanes 1–4). In addition, the fraction from another Δ*plsY* strain (BYH25), whose growth was supported by PlsB induced from P*xyl-plsB*, did not show LPA synthesis (data not shown). We thus agree that PlsY is a novel acyl-phosphate-dependent GPAT as described (Lu et al., 2006; Paoletti et al., 2007). The faint band which appeared close to the top of the thin layer plate (lanes 6–8), was dependent on LPA synthesis and is probably monoacylglycerol produced after dephosphorylation of LPA by a phosphatase included in the membrane fraction. LPA phosphatase activity that produces monoacylglycerol has been described in PlsC-depleted *B. subtilis* cells *in vivo* (Paoletti et al., 2007).

We examined possible interactions between PlsY and PlsX with the bacterial adenylate cyclase two-hybrid system (Karimova et al., 2005). In this two-hybrid system an interaction of target proteins, resulting in an association of the adenylate cyclase T18 and T25 domains, causes production of cAMP and a resultant increase in β-galactosidase activity in *E. coli* cells. Full-length PlsY and PlsX proteins were fused to T18 and T25 domains. Colonies transformed with a combination of the plasmids for PlsY-T18 and T25-PlsX showed dark red color on MacConkey plates, indicating interaction (Fig. 5), although the opposite combination of the plasmids for PlsY-T25 and PlsX-T18 formed pale colonies (no interaction signal). Both self-pairs of PlsX and PlsY formed dark red colonies indicating self-interaction. These results indicate that the novel GPAT, PlsY, probably interacts with the enzyme PlsX for acyl-phosphate production, and that each could be in a multimeric form, to achieve an efficient LPA synthesis in *B. subtilis* cells. The immunofluorescent image of membrane localization of PlsX, a soluble protein, is not affected by a maximal depletion of PlsY, an integral membrane protein (Paoletti et al., 2007). Hence, the membrane localization of PlsX may be independent of the suggested interaction with PlsY. Detailed examination of the localization of PlsX/Y is required.

DISCUSSION

A novel G3P acyltransferase (GPAT), PlsY, that utilizes acyl-phosphate formed by PlsX, has been found to synthesize LPA in *S. pneumoniae* and it has been suggested that the PlsX/Y system is the most widely distributed pathway for glycerophospholipid synthesis in bacteria (Lu et al., 2006). Our studies show that the membrane fraction from *B. subtilis* cells catalyze an acyl-phosphate dependent acylation of G3P to produce LPA and further that PlsY is unequivocally the acyl-phosphate dependent GPAT in question, as the membrane fraction from Δ*plsY* cells is completely inactive (Fig. 4). Growth arrest of *B. subtilis* cells after repression of either *plsY* or *plsX* was bypassed by expression of *E. coli plsB*, which encodes an acyl-CoA- or acyl-ACP-utilizing GPAT that supplies LPA. Simultaneous expression of *plsY* and *plsX* suppressed the glycerol auxotrophy of a Δ*glpR* derivative of the *E. coli plsB26* mutant, although expression of either one alone did not (Fig. 3). These results indicate that

plsX and *plsY* in *B. subtilis* and *S. pneumoniae* have comparable roles.

However, *plsB* counteracted the growth arrest due to *plsX* repression much less efficiently than the arrest due to *plsY* depletion (Fig. 2B). Further, counteracting the impaired growth by *plsX* disruption required a high level of *plsB* expression from the strong promoter P*rpsD* (Fig. 2C). Since the bypass that supplies LPA by *plsB* expression does not require acyl-phosphate, we suggest that PlsX has an essential role other than supplying the substrate for PlsY. In addition, PlsX suppressed the glycerol auxotrophy of a Δ*ygiH* derivative of the *E. coli plsB26 plsX50* mutant. Since this strain should not require acyl-phosphate for the GPAT at all, the question arises: what other function does PlsX perform?

Lethality of the *plsX* and *ygiH* double deletion mutation in *E. coli* has been reported recently (Yoshimura et al., 2007). This synthetic lethality can be corrected by supplying additional copies of the *tesA* gene (the product TesA hydrolyzes acyl-ACP). This effect was explained as a result of the hydrolase activity of the supplementary TesA on the abnormally accumulated acyl-ACP, which causes growth impairment (Jiang and Cronan, 1994). Similarly, the glycerol auxotrophy of the *E. coli* Δ*ygiH plsB26 plsX50* mutant in our experiment may be explained by an abnormal accumulation of acyl-ACP. The correction by the introduction of *B. subtilis plsX* could then be explained as caused by the reduction of the accumulated acyl-ACP by producing acyl-phosphate (see Fig. 1). The mutant PlsB of *plsB26*, a missense mutation that results in an elevated Km value (Bell, 1975), may support LPA synthesis in the absence of the acyl-phosphate dependent GPAT of YgiH. The slight growth support provided by *plsX*, but not by *plsY*, to the glycerol-auxotrophic *E. coli* strain (MC4100 *plsB26* Δ*glpR*) (Fig. 3), which presumably accumulates acyl-ACP, may therefore be explained as a consequence of the reduction of the accumulated acyl-ACP as well.

The inefficient suppression with *plsB* of the growth arrest by *plsX* repression in comparison with the arrest by *plsY* repression in *B. subtilis* and the required high level expression of *plsB* for the suppression of the *plsX* repression (Fig. 2) may be explained in a similar way. *B. subtilis* cells depleted of PlsX are doubly hampered: by the lack of LPA (due to lack of acyl-phosphate) and by an accumulation of acyl-ACP, which apparently leads to cessation of fatty acid synthesis (Paoletti et al., 2007). Hence the depletion of PlsX cannot be overcome by simply supplying LPA. Recovery from PlsX-depletion requires the supply of LPA and a reduction of the accumulated acyl-ACP to allow resumption of fatty acid synthesis. The high level of PlsB is probably required to accomplish the latter mission of reducing the accumulated acyl-ACP. We conclude that the gene *plsX* is dispensable, but only under the condition that *plsB* is highly expressed. It would seem to have no essential function other than those in LPA synthesis and acyl-ACP metabolism. This dispensability may, however, be limited to the conditions of cultivation examined here and the possibility of an essential function still remains under other conditions or in other phases of growth.

The classic question of how the *plsX50* mutation in BB26-36 (*plsB26 plsX50*) cells causes glycerol auxotrophy (Bell, 1975; Larson et al., 1984) perhaps now is resolved. BB26-36 cells are hampered by reduction of LPA synthesis (from the low affinity to G3P of mutant PlsB) and the resulting accumulation of acyl-ACP. The accumulated acyl-ACP would usually be decomposed by PlsX, to produce acyl-phosphate that is then transformed into LPA by YgiH, but lack of sound PlsX makes this impossible (see Fig. 1). To compensate for this disadvantage the cells require supplementary glycerol for efficient LPA synthesis and the ensuing reduction of the accumulated acyl-ACP by PlsC action to form PA.

If one examines the distribution of the PlsX/Y combination and of its counterpart PlsB in the first acylation step of G3P in bacteria among the updated 520 bacterial genomes (blastp, http://blast.ncbi.nlm.nih.gov/Blast), one finds that the PlsX/Y pathway is very common in bacteria (402 of the sequenced genomes). Ninety two species have both PlsX/Y and PlsB pathways (like *E. coli*), while 33 species have only the PlsB pathway. Certain bacteria (26 species), including a group of *Clostridia*, have a PlsX homologue with no apparent PlsY. The PlsX homologue of these bacteria may have a unique function other than what has been described so far. Many (310) of the sequenced species have a PlsX/Y pathway and lack PlsB, as is the case with *B. subtilis*. PlsB is capable of using either acyl-ACP or acyl-CoA as the acyl donor (Lu et al., 2006). Gram-negative bacteria, including *E. coli*, are capable of incorporating exogenous fatty acids and converting them to acyl-CoA (Black and DiRusso, 2003). The presence of PlsB in these bacteria is thus advantageous as it allows production of membrane phospholipids from exogenously incorporated fatty acids in place of the energy-expensive acyl-ACP of *de novo* synthesis (Cronan and Rock, 1996). The large number of bacteria without PlsB pathway should have an inferior capability of incorporating exogenous fatty acids and converting them to acyl-CoA and further to membrane lipids. This apparent weakness, which makes the bacteria dependent on the energy-expensive acyl-ACP and acyl-phosphate, may somehow be compensated by an at present unknown advantage in metabolism provided in part by PlsX. Acyl-CoA inhibits the activity of PlsY both in *S. pneumonia* and *B. subtilis*, whereas PlsB is capable of using acyl-CoA (Lu et al., 2006; Paoletti et al., 2007). Moreover, PlsC of these bacteria cannot use acyl-CoA as the acyl donor (Paoletti et al., 2007), in contrast to the *E. coli* enzyme (Cronan and Rock, 1996). It seems likely that the difference in the

lipid metabolism of the two types of bacteria with variant acyltransferase pathways centers on the different behavior of the enzymes [PlsB/C and PlsX/Y] towards acyl-CoA.

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