JOURNAL OF BACTERIOLOGY, Dec. 1997, p. 7257–7263 Vol. 179, No. 23
0021-9193/97/$04.00+0

A Gene (*plsD*) from *Clostridium butyricum* That Functionally Substitutes for the *sn*-Glycerol-3-Phosphate Acyltransferase Gene (*plsB*) of *Escherichia coli*

RICHARD J. HEATH,[1] HOWARD GOLDFINE,[2] AND CHARLES O. ROCK[1,3]*

Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee 38101[1]; Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104[2]; and Department of Biochemistry, University of Tennessee, Memphis, Tennessee 38163[3]

Received 24 June 1997/Accepted 8 September 1997

The *sn*-glycerol-3-phosphate acyltransferase (*plsB*) of *Escherichia coli* is a key regulatory enzyme that catalyzes the first committed step in phospholipid biosynthesis. We report the initial characterization of a novel gene (termed *plsD*) from *Clostridium butyricum*, cloned based on its ability to complement the *sn*-glycerol-3-phosphate auxotrophic phenotype of a *plsB* mutant strain of *E. coli*. Unlike the 83-kDa PlsB acyltransferase from *E. coli*, the predicted *plsD* open reading frame encoded a protein of 26.5 kDa. Two regions of strong homology to other lipid acyltransferases, including PlsB and PlsC analogs from mammals, plants, yeast, and bacteria, were identified. PlsD was most closely related to the 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (*plsC*) gene family but did not complement the growth of *plsC*(Ts) mutants. An in vivo metabolic labeling experiment using a *plsB plsX plsC*(Ts) strain of *E. coli* confirmed that the *plsD* expression restored the ability of the cells to synthesize 1-acyl-glycerol-3-phosphate. However, glycerol-3-phosphate acyltransferase activity was not detected in vitro in assays using either acyl-acyl carrier protein or acyl coenzyme A as the substrate.

The individual reactions of fatty acid and phospholipid biosynthesis in the dissociated, type II, systems as typified by *Escherichia coli* are extensively characterized (for reviews, see references 12 and 34). The first step in phospholipid biosynthesis is the transfer of a fatty acyl group from acyl-acyl carrier protein (acyl-ACP) or acyl coenzyme A (acyl-CoA) to the *sn*-1 position of glycerol-3-phosphate (G3P) by the enzyme G3P acyltransferase (PlsB). The phospholipids from *E. coli* contain predominantly saturated fatty acids at the *sn*-1 position and unsaturated fatty acids at the *sn*-2 position when grown at 37°C (12). The determining factor for establishing these ratios appears to be the G3P acyltransferase, since preparations of PlsB have a preference for saturated acyl donors (33, 37). The PlsB enzyme is a target for inhibition by the global regulator of cellular function, guanosine tetraphosphate, thus coordinating phospholipid biosynthesis with macromolecular biosynthesis (19, 30). Fatty acid biosynthesis is thought to be coordinately regulated with phospholipid production via acyl-ACPs, the end products of de novo fatty acid biosynthesis and substrates for PlsB. Acyl-ACPs accumulate following inhibition of PlsB and function to inhibit early steps in fatty acid synthesis (19–22). Overexpression of thioesterases prevents the accumulation of acyl-ACP and leads to uncontrolled fatty acid biosynthesis, underscoring the regulatory importance of acyl-ACP (7, 24, 42).

The structural gene for the PlsB acyltransferase (*plsB*) maps to min 92 on the *E. coli* chromosome and has been cloned and biochemically characterized (26, 28, 29). Membranes from strains harboring the *plsB26* mutation retain a membrane-associated G3P acyltransferase activity with a lower apparent affinity for G3P (2, 3). The presence of a second mutation, *plsX*, located in a cluster of fatty acid biosynthetic genes at min 24.5 (27), is required to observe G3P auxotrophy (27). The wild-type PlsX protein has been proposed to increase the affinity of the mutant PlsB for G3P in vivo, although membranes prepared from either *plsB* or *plsB plsX* strains have the same K_m for G3P (27). It has also been suggested that the high-K_m G3P acyltransferase activity could be due to the inefficient utilization of G3P by a second acyltransferase in the membrane preparations, such as the 1-acyl-G3P acyltransferase (37). 1-Acyl-G3P acyltransferase is encoded by the *plsC* gene located at min 65 on the *E. coli* chromosome and catalyzes the second acyltransferase step in phospholipid biosynthesis to produce phosphatidic acid (8, 9).

The genes and enzymes responsible for the formation of phosphatidic acid in gram-positive bacteria are less well characterized. Clostridial species contain mainly unsaturated fatty acids at the *sn*-1 position and saturated chains at *sn*-2 of the G3P backbone (25), which is the reverse of the orientation found in *E. coli* (12). The G3P acyltransferase from *Clostridium beijerinckii* has been shown to preferentially utilize unsaturated chains in crude membrane preparations (16). The *C. beijerinckii* enzyme appears to utilize acyl-CoAs inefficiently (16, 17), while the PlsB from *E. coli*, either in vivo or in vitro, will utilize both CoA and ACP thioesters with approximately equal efficiency (37, 41). Thus, there may be significant differences between the acyltransferases of gram-positive and gram-negative bacteria. In this study, we isolated and characterized a gene, termed *plsD*, from *C. butyricum* (an organism closely related to *C. beijerinckii*) that complements the defect in *plsB* strains of *E. coli* but encodes a protein significantly different in structure from the PlsB protein.

MATERIALS AND METHODS

Materials. Restriction enzymes, *E. coli* S30 transcription translation extract, and molecular biology supplies were from Promega. New England Nuclear supplied the *sn*-[U-^{14}C]G3P (specific activity, 148 mCi/mmol), American Radiochemical Co. supplied the [U-^{14}C]glycerol (specific activity, 130 mCi/mmol), and ICN provided the [^{35}S]methionine (specific activity, 1,194 Ci/mmol). Scint-A XF scintillation fluid was from Packard. Protein was measured by the method of

* Corresponding author. Mailing address: Department of Biochemistry, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38101. Phone: (901) 495-3491. Fax: (901) 525-8025. E-mail: charles.rock@stjude.org.

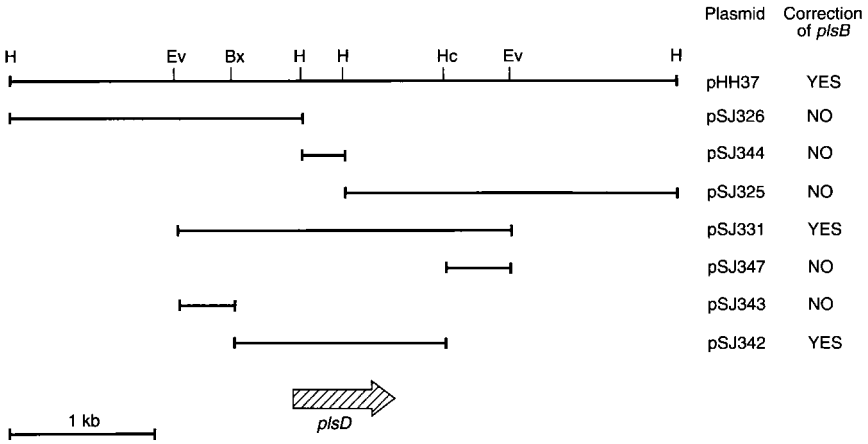

FIG. 1. Complementation of the *plsB* growth phenotype by subclones of pHH37. Plasmid pHH37, originally isolated from a *Clostridium* library, was subcloned into pBluescript KS by using the indicated restriction enzymes, and the individual clones were tested for the ability to complement the G3P auxotrophic phenotype of strain SJ52 (*plsB26 plsX50*). The smallest complementing fragment in plasmid pSJ342 was completely sequenced. Abbreviations for restriction enzymes: H, *Hin*dIII; Ev, *Eco*RV; Bx, *Bst*XI; Hc, *Hin*cII.

Bradford (5). Acyl-ACPs were synthesized enzymatically (23, 35) and analyzed by conformationally sensitive electrophoresis (32). All other chemicals were reagent grade or better.

Subcloning the *plsD* open reading frame. Plasmid pRS25 was isolated from a *C. butyricum* library (15) that complemented the G3P auxotrophy of *E. coli* TL400 (*plsB26 plsX50*) (27). This plasmid contained a 6.7-kbp insert and was subjected to a partial digest with *Hin*dIII, and the fragments were subcloned into plasmid pBR327. A mixture of the clones was transformed into strain TL400, and plasmids that complemented the G3P auxotrophy of strain TL400 were analyzed. One of these plasmids, pHH37, contained a 4.4-kbp insert and had *Hin*dIII sites at both ends and also contained two internal *Hin*dIII sites (Fig. 1). Fragments of the insert in plasmid pHH37 were subcloned into pBluescript II KS(+) (Stratagene, Inc.) and tested for the ability to complement the G3P auxotrophy of *E. coli* SJ52 (*plsB26 plsX50 panD2 glpD3 glpR2 glpKi relA1 spoT1 pit-10 phoA8 ompF627 fluA22 fadL701*), a tetracycline-sensitive derivative of strain SJ22 (38), as described below. The *Hin*dIII-*Hin*dIII, *Bst*XI-*Hin*dIII, and *Hin*dIII-*Hin*cII fragments from plasmid pSJ342 were subcloned into pBluescript II KS(+), and both strands were sequenced. Based on this information, sequencing primers were synthesized to confirm the junctions across the internal *Hin*dIII sites. DNA sequencing was performed by the Center for Biotechnology staff at St. Jude Children's Research Hospital, using an ABI automated sequencer.

In vitro transcription and translation. Expression of PlsD was examined by an in vitro transcription-translation assay using an *E. coli* S30 extract as instructed by the manufacturer (Promega Biotech, Inc.). Briefly, 2 μg of purified plasmid DNA was incubated with [^{35}S]methionine (specific activity, 1,194 Ci/mmol), premix, and S30 extract at 37°C for 1 h. Protein from a 5-μl aliquot of the incubation was then precipitated with 20 μl of acetone, resuspended in sodium dodecyl sulfate (SDS) sample buffer, and electrophoresed through a 15% polyacrylamide-SDS gel. The gel was then stained with Coomassie blue, fixed, dried, and exposed to X-ray film. The size of the protein products was estimated by comparing the band on the developed autoradiogram to the molecular weight markers on the dried gel.

Construction of *plsC* and *plsX* plasmids. A positive control plasmid, pSJ345, bearing the wild-type *plsC* gene with its own promoter was constructed by PCR amplifying the gene from the laboratory strain *E. coli* UB1005. The primers (5′-CGTCATCCGGAAGCTTATTCAACC-3′ and 5′-GAAGCGCTCGAGGA ATGAACTGACGCCGACT-3′) created unique *Hin*dIII and *Xho*I restriction sites, which were used to ligate the fragment into *Hin*dIII and *Ava*I sites of pBR322. The *plsX* and *fabH* genes (40) were cloned into the *Hin*dIII site of pBluescript II KS(+) by PCR amplification of genomic DNA from *E. coli* UB1005 with primers 5′-GCAAGGTCATAAGTAATCACGC-3′ and 5′-CAA CAGATGCTCAACAGC-3′ to create plasmid pSJ10B. A plasmid containing only the wild-type *plsX* was constructed from plasmid pSJ10B by digestion with *Nco*I, blunt ending with mung bean nuclease, digesting with *Sma*I, and religating the plasmid. This procedure excised the *fabH* gene from the parent plasmid. The resultant plasmid, pRJ11, complemented the G3P auxotrophic requirement of strain SJ52 (*plsB plsX*).

Phenotype complementation assays. During the subcloning procedures, plasmids were assessed for complementation of the G3P auxotrophy of *E. coli* SJ52 (*plsB plsX*). Following transformation and recovery, cells were plated onto minimal medium E plates (31) containing β-alanine (1.5 μM), glucose (0.4%), Casamino Acids (0.1%), thiamine (0.001%), and ampicillin (100 μg/ml) either with or without 0.04% G3P. Growth on the plates lacking G3P indicated complementation of the phenotype.

To test for the ability of *plsD* to complement the growth defect of a temperature-sensitive 1-acyl-G3P acyltransferase strain of *E. coli*, the plasmid containing the minimal fragment required for complementation of G3P auxotrophy, pSJ342, as well as plasmids with wild-type *plsB*, *plsX*, and *plsC* genes were transformed into the *plsC*(Ts) strain SM2-1 [*plsC1 metC162*::Tn*10 thr-1 ara-14* Δ(*gal-att*λ)*99 hisG4 rpsL136 xyl-5 mtl-1 lacY1 tsx-78 eda-50 rfbD1 thi-1*] (8). Transformed cells were plated on rich medium plus ampicillin at 30°C for overnight growth, and then individual colonies were scored for growth at 42°C.

Detection of acyltransferase activity in vivo. To examine the function of the *plsD* gene product, an in vivo assay was used. A strain harboring *plsB*, *plsX*, and *plsC*(Ts) mutations was first engineered by moving the *plsC*(Ts) lesion from strain SM2-1 (*plsC1 metC162*::Tn*10*) (8) into strain SJ52 (*plsB26 plsX50*) by P1*vir*-mediated transduction using standard procedures (31). The resultant strain, SJ361 (*plsB26 plsX50 plsC1 metC162*::Tn*10*), required supplementation with 0.04% G3P for growth at 30°C and did not grow at 42°C. This strain was transformed with pSJ342 (*plsD*), pRJ22 (*plsB*) (19), or pRJ11 (*plsX*), and isolated colonies were grown overnight in minimal E medium containing β-alanine (1.5 μM), glucose (0.4%), Casamino Acids (0.1%), thiamine (0.001%), and ampicillin (100 μg/ml) at 30°C. These overnight cultures were used to inoculate experimental cultures in 10 ml of the same medium and grown to a density of 5×10^8 cells per ml at 30°C. Cultures were then shifted to 42°C, and [^{14}C]glycerol (specific activity, 130 mCi/mmol) was added to a final concentration of 0.1 μCi/ml. Incubation was continued for 1 h, then cells were collected by centrifugation and washed once with fresh medium, and the lipids were extracted by the method of Bligh and Dyer (4). Total incorporation of label was determined by scintillation counting an aliquot of the extracted lipids. The phospholipid classes produced were examined by two-dimensional thin-layer chromatography on Silica Gel 60 plates developed with chloroform-methanol-water (65:25:4) in the first dimension and chloroform-methanol-acetic acid (65:25:10) in the second dimension. The incorporation of label into lysophosphatidic acid and other lipids was detected and quantitated by using a Molecular Dynamics PhosphorImager.

In vitro assays for PlsD. In an attempt to further characterize the reaction catalyzed by PlsD, G3P acyltransferase assays were performed in vitro, using total cellular protein, cytosolic protein, and membrane protein fractions. Fractions were isolated from SJ52 (*plsB plsX*) transformed with plasmids expressing either the *plsB*, *plsC*, *plsD*, or *plsX* gene, and a standard acyltransferase assay was used as a basis for further experimentation (36). The standard reaction mixture contained 50 μM [U-^{14}C]G3P (specific activity, 60 mCi/mmol), 12.5 μM palmitoyl-CoA or palmitoyl-ACP, 0.1 M Tris-HCl (pH 8.0), 1 mg of bovine serum albumin per ml, and 1 to 100 μg of protein. Variations on the reaction conditions included increasing the [^{14}C]G3P concentration to 200 μM, adding ATP (10 mM) and $MgCl_2$ (10 mM), altering the pH between 6.0 and 9.0, adding 1 mM dithiothreitol, including *E. coli* phospholipids, and using *cis*-vaccenoyl-CoA or -ACP in place of the palmitate thioesters. Reactions were stopped by pipetting onto Whatmann 3MM filter paper discs that were then washed with trichloroacetic acid (37). A 1-acyl-G3P acyltransferase assay was also performed (8) with 1-acyl-G3P as the substrate in place of G3P and using saturated and unsaturated acyl-ACPs and acyl-CoAs. Products from these reactions were analyzed by thin-layer chromatography on Silica Gel H plates developed with chloroform-methanol-acetic acid (90:10:10).

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been deposited in the GenBank database with accession no. AF009362.

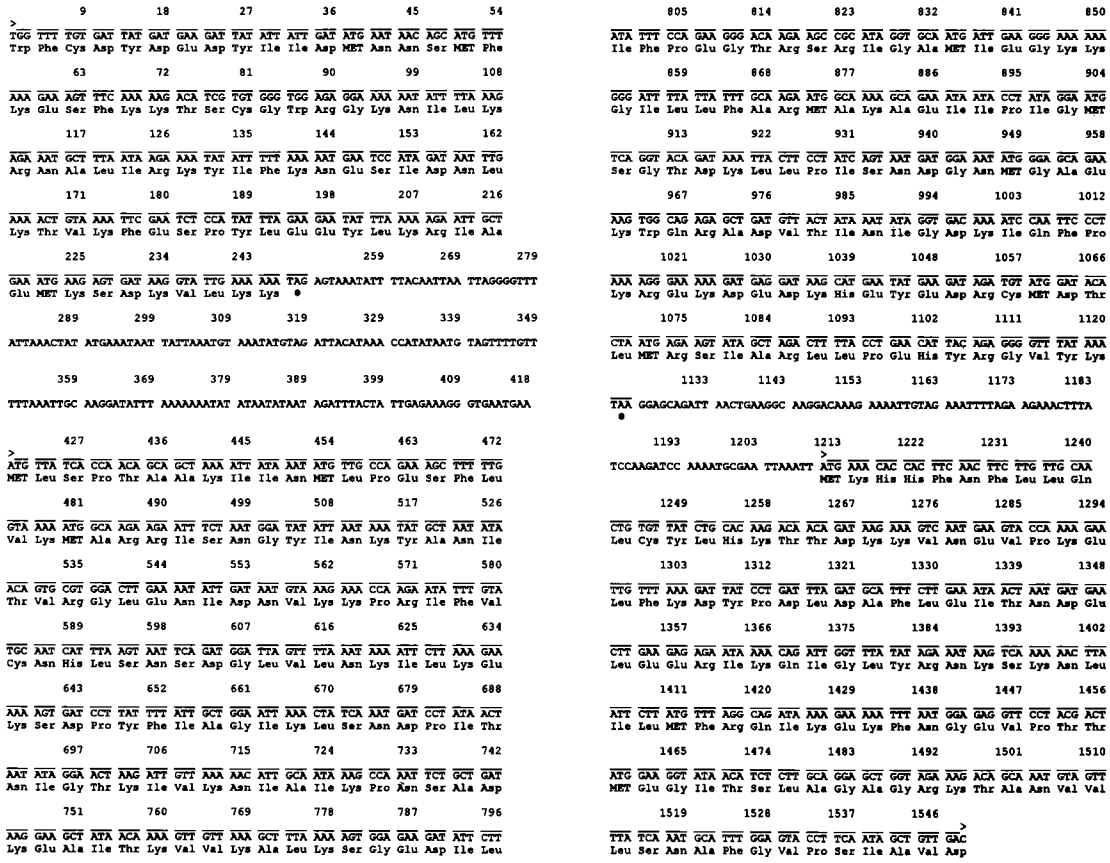

FIG. 2. DNA sequence of the plasmid pSJ342 and definition of the *plsD* gene. Both strands of plasmid pSJ342 were sequenced, and analysis revealed a single intact open reading frame flanked by the partial sequences of two other genes. The initiator methionine codon at position 419 is preceded by a potential ribosome binding site (GAA) at position 414 and a TATAAT element at position 386. The *plsD* gene begins at position 419 and ends at position 1123.

RESULTS

Cloning of the *plsD* gene. Plasmid pHH37 was isolated from clone pRS25 obtained from a *C. butyricum* library by complementation of the G3P auxotrophy of the *E. coli* TL400 (*plsB plsX*) as described in Materials and Methods. The size of the insert in pHH37 was approximately 4.4 kbp, and the open reading frame required for complementation was localized to a 1.5-kbp *Bst*XI-*Hin*cII fragment by subcloning restriction fragments into pBluescript as depicted in Fig. 1. Plasmids were transformed into *E. coli* SJ52 (*plsB plsX*) and scored for complementation of the growth phenotype on plates with and without G3P as described in Materials and Methods. The sequence of the smallest complementing fragment, contained in plasmid pSJ342, was determined (Fig. 2). A single complete open reading frame was predicted, starting at position 419 and terminating at position 1123. This open reading frame, designated *plsD*, was predicted to encode a 26,577-Da protein with an isoelectric point of 10.25. Two other partial open reading frames were also present on this insert. Upstream of *plsD* was the carboxy-terminal portion of a gene homologous to a hypothetical *Bacillus* protein terminating at position 249, while a gene encoding an endonuclease III homolog was predicted to start at bp 1211 of the insert (Fig. 2).

A BLAST search (1) of the translated open reading frame of *plsD* against the nonredundant database revealed two distinct regions of homology with glycerolipid acyltransferases, which we have designated domains A and B (Fig. 3). In both size and sequence, the predicted PlsD protein most closely resembled the 27.5-kDa PlsC of *E. coli* (26.7% identical and 47.4% identical plus similar residues), although two domains of *plsD* also had a high degree of similarity to regions of the PlsB acyltransferase of *E. coli* (Fig. 3). The *plsC* genes from several plant

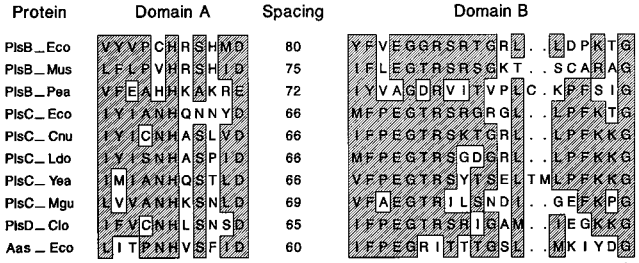

FIG. 3. Acyltransferase sequence similarities. The predicted protein sequence encoded by the *plsD* gene is aligned with acyltransferases from bacteria, yeast, plants, and mammals. Genes from related species that are virtually identical, such as *plsC* from *E. coli* and *Salmonella typhimurium*, are not duplicated in the figure. Origins of the sequenced acyltransferases: PlsB_Eco, *E. coli* (accession no. K00127); PlsB_Mus, mouse (accession no. M77003); PlsB_Pea, *Picus sativum* (accession no. X59041); PlsC_Eco, *E. coli* (accession no. M63491); PlsC_Cnu, *Cocos nucifera* (accession no. U29657); PlsC_Ldo, *Limnanthes douglasii* (accession no. X83266); PlsC_Yea, *Saccharomyces cerevisiae* (accession no. Z74100); PlsC_Mgu, *Mycoplasma genitalium* (accession no. U39698); PlsD_Clo, *Clostridium butyricum* (this study); Aas_Eco, 2-acyl-GPE synthetase/acyl-ACP synthetase from *E. coli* (accession no. L14681). Similar amino acid groups were defined as follows: P,A,G,S,T; Q,N,E,D; H,K,R,C; V,L,I,M; F,Y,W.

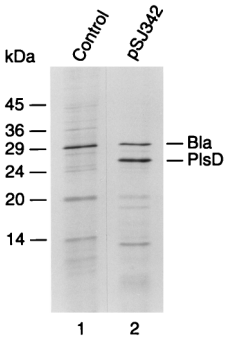

FIG. 4. Expression of the *plsD* gene in vitro. Plasmids pSJ342 and pBluescript KS (control) were incubated in an *E. coli* transcription-translation mixture with [^{35}S]methionine as described in Materials and Methods. The labeled proteins produced in the incubations were separated by SDS-gel electrophoresis on 12% polyacrylamide gels. The gels were stained to located the indicated molecular weight markers, and the ^{35}S-labeled proteins were visualized by autoradiography. Bla denotes the position of the β-lactamase gene (Amp) expressed by both plasmids. The new protein produced in incubations with pSJ342 (PlsD) corresponded to a molecular mass of 26 kDa.

species have been cloned by their ability to complement the *plsC*(Ts) phenotype in *E. coli*, and these also show regions of similarity to each other, to *plsB*, and to the *plsC* genes of yeast and *E. coli* (6, 18). Our analysis has extended these studies to include the PlsB analog from mouse mitochondria (43) and the 2-acyl-glycerol phosphoethanolamine acyltransferase/acyl-ACP synthetase (Aas) (23) from *E. coli*. Removal of a 78-amino-acid stretch of the mouse PlsB, covering domain B, produced an inactive enzyme (43), although these authors note that this could be due to an improperly folded enzyme and not just removal of an active site. Our alignment (Fig. 3) suggests that the two domains, and the spacing between them, were highly conserved in glycerolipid acyltransferases. We postulate that the conserved histidine in the center of domain A is critical for the catalytic activity of these enzymes, and we are testing this idea by site-directed mutagenesis. A conserved histidine has been identified to play an essential role in catalysis in the myristoyl-ACP acyltransferases of the luminescence biosynthetic pathway (13).

Expression of the *plsD* gene in vitro. To characterize the protein expressed from pSJ342, an in vitro coupled transcription-translation assay was performed as described in Materials and Methods. A major product of approximately 26 kDa was observed in incubations which contained plasmid pSJ342 but not in those with a pBluescript control plasmid (Fig. 4). The size of this protein corresponded to the predicted size of the translated *plsD* open reading frame in pSJ342 (26.577 kDa).

Biological activity of the PlsD protein in vivo. In light of the similarities between *plsD* and *plsC*, the ability of pSJ342 (*plsD*) to complement a strain of *E. coli* with a temperature-sensitive *plsC* gene product {strain SM2-1 [*plsC*(Ts)]} was investigated (Table 1). Expression of *plsD* from pSJ342 complemented the G3P growth requirement of strain SJ52 (*plsB plsX*) and gave colonies similar in size to those formed by cells transformed with the *plsB* clone. Neither *plsB* or *plsD* complemented the temperature-sensitive growth phenotype of strain SM2-1 [*plsC*(Ts)]. A control plasmid carrying a wild-type *plsC* gene complemented the *plsC*(Ts), but not the *plsB*, phenotype. The *plsX* gene, which was isolated by its ability to complement the *plsB* phenotype (27), displayed the same pattern of complementation as the *plsB* and *plsD* clones (Table 1). These results demonstrate that the PlsD protein complemented the defect in the G3P acyltransferase and not the 1-acyl-G3P acyltransferase.

TABLE 1. Complementation pattern of *plsD*

Plasmid	Complementation[a] of:	
	plsB	*plsC*
pSJ342 (*plsD*)	Yes	No
pRJ22 (*plsB*)	Yes	No
pPlsC (*plsC*)	No	Yes
pRJ11 (*plsX*)	Yes	No

[a] Complementation of the G3P growth requirement of SJ52 (*plsB plsX*) or the temperature-sensitive growth phenotype of strain SM2-1 [*plsC*(Ts)] was assessed as described in Materials and Methods.

To determine whether PlsD expression restored G3P acyltransferase activity in vivo, strain SJ361 was constructed with mutations in *plsC*(Ts), *plsB*, and *plsX* (see Materials and Methods). By shifting this strain from growth at 30°C to the nonpermissive temperature (42°C), PlsC activity was inhibited, and we could examine the ability of the strains harboring our panel of clones to produce lysophosphatidic acid. Cultures of strain SJ361 [*plsB plsX plsC*(Ts)] harboring *plsD*, *plsB*, or *plsX* clones were shifted to the nonpermissive temperature and labeled with [^{14}C]glycerol. Analysis of the labeled phospholipid fraction by two-dimensional thin-layer chromatography and quantitation of the lysophosphatidic acid produced demonstrated that strains with either *plsB* (24 pmol/ml) or *plsD* (16 pmol/ml) clones continued to produce lysophosphatidic acid at the nonpermissive temperature (Fig. 5). Thus, *plsD* expression restored the ability of strain SJ361 to produce lysophosphatidic acid. Strain SJ361 containing a *plsX* clone did not produce significant amounts of lysophosphatidic acid (1.2 pmol/ml) (Fig. 5), demonstrating that PlsX did not functionally restore G3P acyltransferase in vivo. The total accumulation of [^{14}C]glycerol in the phospholipid fraction of strains complemented with the *plsX* plasmid was only 20% of the incorporation of label into the strains complemented with either *plsB* or *plsD* clones. A control *plsB plsX plsC*(Ts) strain containing pBluescript was grown in the presence of G3P. At the same time as the temperature was shifted to 42°C, the G3P was removed and the label was added. This strain stopped growing and incorporated only 0.4% of the label into the lipid extract as strain SJ342 complemented with the *plsB* or *plsD* clones and did not produce any detectable lysophosphatidic acid based on thin-layer chromatographic analysis (Fig. 5).

These data strongly indicated that the product of the *plsD* gene was a G3P acyltransferase. We attempted to verify this conclusion by assaying crude cell extracts, soluble fractions, and membrane preparations for G3P acyltransferase activity. We were unable to detect G3P acyltransferase activity in extracts from strains harboring plasmid pSJ342 (*plsD*) above the background activity in the parent strain SJ52 (*plsB plsX*). Neither saturated or unsaturated acyl-ACPs or acyl-CoAs were acyl donors, and neither G3P or 1-acyl-G3P acted as an acyl acceptor. Also, we detected no reaction when 1-[^{14}C]acyl-G3P was used as the acyl donor and ACP was used as the acyl acceptor. Thus, we were unable to verify that PlsD was a G3P acyltransferase using an in vitro biochemical assay.

DISCUSSION

PlsD as a G3P acyltransferase. The clostridial clone pHH37 was isolated by complementation of the *plsB* defect in mutant strains of *E. coli*, suggesting that this plasmid expresses a gene

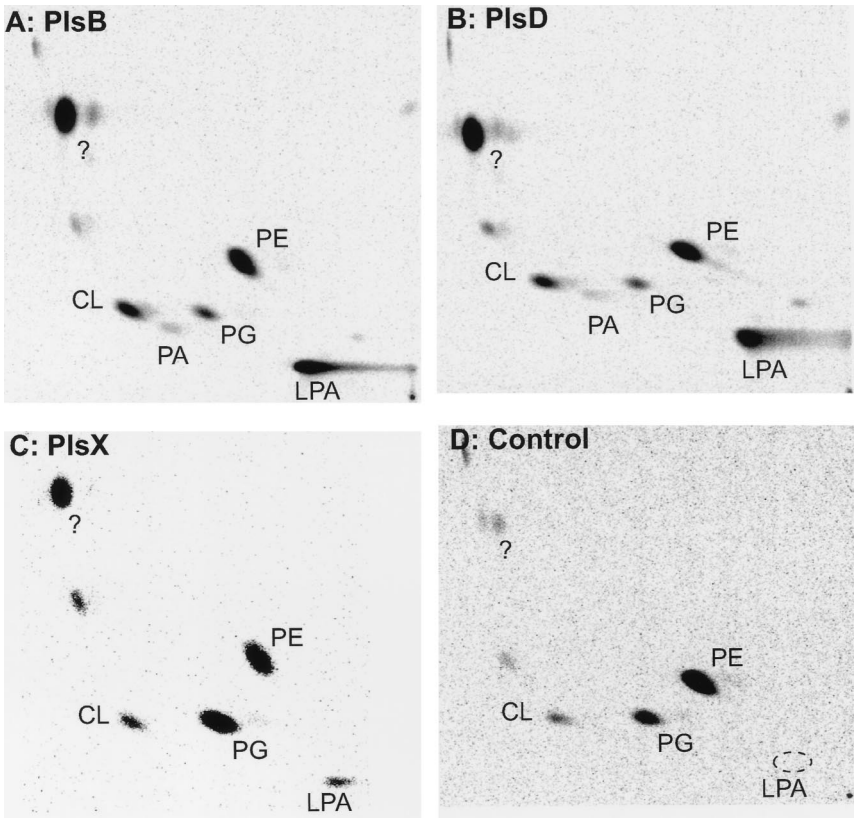

FIG. 5. PlsD restores G3P acyltransferase activity in vivo. Strain SJ361 [*plsB plsX plsC*(Ts)] harboring the indicated plasmid was shifted to the nonpermissive temperature and labeled with [^{14}C]glycerol as described in Materials and Methods. Phospholipids were extracted after 1 h and chromatographed on Silica Gel 60 plates developed with chloroform-methanol-water (65:25:4, vol/vol/vol) in the first dimension and chloroform-methanol-acetic acid (65:25:10, vol/vol/vol) in the second. Incorporation of label into lysophosphatidic acid was detected and quantitated with a Molecular Dynamics PhosphorImager. (A) Strain SJ361/pRJ22 (*plsB*); (B) strain SJ361/pSJ342 (*plsD*); (C) strain SJ361/pRJ11 (*plsX*); (D) strain SJ361/pPlsC (*plsC*). LPA, lysophosphatidic acid; CL, cardiolipin; PA, phosphatidic acid; PE phosphatidylethanolamine; PG, phosphatidylglycerol; ?, unidentified phospholipid.

that encodes a G3P acyltransferase. The predicted PlsD protein sequence showed a high degree of homology to the PlsC 1-acyl-G3P acyltransferase; however, plasmid pSJ342 (*plsD*) did not complement the 1-acyl-G3P acyltransferase mutation in strain SM2-1 [*plsC*(Ts)] (Table 1), indicating that *plsD* does not encode a 1-acyl-G3P acyltransferase. Expression of PlsD in strain SJ361 [*plsB plsX plsC*(Ts)] led to the accumulation of lysophosphatidic acid following the shift to the nonpermissive temperature to block phosphatidic acid production (Fig. 5). Thus, the PlsD protein is able to restore G3P acyltransferase activity in *plsB* mutants. We attempted to assay the function of PlsD in vitro, using a standard PlsB acyltransferase assay (36) or many modifications, including both saturated and unsaturated acyl-ACP and acyl-CoA-acyl donors and either G3P or 1-acyl-G3P as the acyl acceptor. The reason for our inability to detect acyltransferase activity in vitro is unclear. It is possible that we are missing an essential cofactor or activator from the reactions. However, the data in Table 1 and Fig. 5 clearly demonstrate that PlsD complements PlsB, but not PlsC, within the cell, and thus the PlsD protein should be grouped with the G3P family of acyltransferases and not 1-acyl-G3P acyltransferase. This conclusion would not be reached based on the comparison of the *plsD* sequence to known acyltransferases, which leads to the conclusion that *plsD* is a 1-acyl-G3P acyltransferase. This conclusion is clearly incorrect and illustrates that functional analysis will be required to assign the role of *plsC*-related genes in phospholipid biosynthesis.

The role of PlsX. The *plsX* mutation was identified by Larson et al. (27) as essential for *plsB* strains to exhibit an auxotrophic requirement for G3P (2, 10, 11). Complementation of *plsB plsX* double mutants with either of these loci circumvents the G3P requirement for growth. Importantly, *plsB* expression restores G3P acyltransferase activity in vitro, whereas *plsX* expression does not (27). The most direct conclusion is that PlsX acts by enhancing the activity of the mutant PlsB in vivo, but our data open the possibility of an alternative interpretation. At the nonpermissive temperature, the mutant PlsC protein in strain SM2-1 [*plsC*(Ts)] is rapidly inactivated, and lysophophatidic acid accumulates in strains with a functional G3P acyltransferase (encoded by either *plsB* or *plsD*) (Fig. 5). If PlsX functions to enable the mutant PlsB to catalyze the acylation of G3P, then strains bearing the *plsX* plasmid would be predicted to produce lysophosphatidic acid. However, this result was not obtained and may suggest instead that PlsX interacts with another protein, perhaps PlsC, to produce phosphatidic acid in vivo. The biochemical mechanism that accounts for the ability of PlsX plus PlsC, or PlsD alone, to complement the *plsB26* mutation in the production of phosphatidic acid in vivo, and yet not exhibit G3P acyltransferase activity in vitro, remains unknown. Perhaps PlsD interacts to stabilize and activate the mutant PlsB protein in vivo, but complementation by this interaction cannot be detected in vitro. The inference from the in vivo genetic experiments is that PlsB may not be essential for the formation of phosphatidic acid. Examination of the data-

bases lends support to this idea but fails to clarify the point. Analysis of the *Mycoplasma genitalium* genome reveals that this organism contains *plsX* and *plsC* homologs but lacks a *plsB* homolog (14). Similarly, *Helicobacter pylori* does not contain a *plsB* homolog but does have a *plsC*-like gene (39). *Saccharomyces cerevisiae* does not contain either a *plsB* or a *plsX* homolog, but does have a gene that is related to *plsC*. *Bacillus subtilis* has a *plsX* homolog, but neither a *plsB* nor a *plsC* gene has appeared in the current database release. The *plsB26* mutation has not been molecularly characterized, but the conclusion that it is a point mutation is reasonable, based on the in vitro assay of membrane fractions derived from *plsB26* mutants. Clearly, much more needs to be learned about the early steps in bacterial phospholipid synthesis. It will be important to determine the nature of the *plsB26* mutation and test the idea that the *plsB* gene is dispensable by gene disruption.

ACKNOWLEDGMENTS

We thank Suzanne Jackowski and Leszek Kauc for informative discussions and Jina Wang, Chistopher Salvatore, and Joshua Rosenthal for expert technical assistance.

This work was supported by NIH grant GM 34496, Cancer Center (CORE) support grant CA 21765, and the American Lebanese Syrian Associated Charities.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Bell, R. M.** 1974. Mutants of *Escherichia coli* defective in membrane phospholipid synthesis: macromolecular synthesis in an *sn*-glycerol 3-phosphate acyltransferase Km mutant. J. Bacteriol. **117:**1065–1076.
3. **Bell, R. M.** 1975. Mutants of *Escherichia coli* defective in membrane phospholipid synthesis: properties of wild type and Km defective *sn*-glycerol-3-phosphate acyltransferase activities. J. Biol. Chem. **250:**7147–7152.
4. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brown, A. P., J. Coleman, A. M. Tommey, M. D. Watson, and A. R. Slabas.** 1994. Isolation and characterisation of a maize cDNA that complements a 1-acyl *sn*-glycerol-3-phosphate acyltransferase mutant of *Escherichia coli* and encodes a protein which has similarities to other acyltransferases. Plant Mol. Biol. **26:**211–223.
7. **Cho, H., and J. E. Cronan, Jr.** 1995. Defective export of a periplasmic enzyme disrupts regulation of bacterial fatty acid synthesis. J. Biol. Chem. **270:**4216–4219.
8. **Coleman, J.** 1990. Characterization of *Escherichia coli* cells deficient in 1-acyl-*sn*-glycerol-3-phosphate acyltransferase activity. J. Biol. Chem. **265:** 17215–17221.
9. **Coleman, J.** 1992. Characterization of the *Escherichia coli* gene for 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (*plsC*). Mol. Gen. Genet. **232:**295–303.
10. **Cronan, J. E., Jr., and R. M. Bell.** 1974. Mutants of *Escherichia coli* defective in membrane phospholipid synthesis: mapping of *sn*-glycerol 3-phosphate acyltransferase Km mutants. J. Bacteriol. **120:**227–233.
11. **Cronan, J. E., Jr., and R. M. Bell.** 1974. Mutants of *Escherichia coli* defective in membrane phospholipid synthesis: mapping of the structural gene for L-glycerol 3-phosphate dehydrogenase. J. Bacteriol. **118:**598–605.
12. **Cronan, J. E., Jr., and C. O. Rock.** 1996. Biosynthesis of membrane lipids, p. 612–636. *In* F. C. Neidhardt, R. Curtiss III, C. A. Gross, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
13. **Ferri, S. R., and E. A. Meighen.** 1994. An essential histidine residue required for fatty acylation and acyl transfer by myristoyltransferase from luminescent bacteria. J. Biol. Chem. **269:**6683–6688.
14. **Fraser, C. M., J. D. Gocayne, O. White, M. D. Adams, R. A. Clayton, R. D. Fleischmann, C. J. Bult, A. R. Kerlavage, G. Sutton, J. M. Kelley, J. L. Fritchman, J. F. Weidman, K. V. Small, M. Sandusky, J. Fuhrmann, D. Ngiyen, T. R. Utterback, D. M. Saudek, C. A. Phillips, J. M. Merrick, J. Tomb, B. A. Dougherty, K. F. Bott, P. Hu, T. S. Lucier, S. N. Peterson, H. O. Smith, C. A. Hutchison III, and J. C. Venter.** 1995. The minimal gene complement of *Mycoplasma genitalium*. Science **270:**397–403.
15. **Goldfine, H.** 1993. Phospholipid biosynthetic enzymes of butyric acid-producing clostridia, p. 354–362. *In* M. Sebald (ed.), Genetics and molecular biology of anaerobic bacteria. Springer-Verlag, New York, N.Y.
16. **Goldfine, H., and G. P. Ailhaud.** 1971. Fatty acyl-acyl carrier protein and fatty acyl-CoA as acyl donors in the biosynthesis of phosphatidic acid in *Clostridium butyricum*. Biochem. Biophys. Res. Commun. **45:**1127–1133.
17. **Goldfine, H., G. P. Ailhaud, and P. R. Vagelos.** 1967. Involvement of acyl carrier protein in acylation of glycerol 3-phosphate in *Clostridium butyricum*. II. Evidence for the participation of acyl thioesters of acyl carrier protein. J. Biol. Chem. **242:**4466–4475.
18. **Hanke, C., F. P. Wolter, J. Coleman, G. Peterek, and M. Frentzen.** 1995. A plant acyltransferase involved in triacylglycerol biosynthesis complements an *Escherichia coli sn*-1-acylglycerol-3-phosphate acyltransferase mutant. Eur. J. Biochem. **232:**806–810.
19. **Heath, R. J., S. Jackowski, and C. O. Rock.** 1994. Guanosine tetraphosphate inhibition of fatty acid and phospholipid synthesis in *Escherichia coli* is relieved by overexpression of glycerol-3-phosphate acyltransferase (*plsB*). J. Biol. Chem. **269:**26584–26590.
20. **Heath, R. J., and C. O. Rock.** 1995. Regulation of malonyl-CoA metabolism by acyl-acyl carrier protein and β-ketoacyl-acyl carrier protein synthases in *Escherichia coli*. J. Biol. Chem. **270:**15531–15538.
21. **Heath, R. J., and C. O. Rock.** 1996. Regulation of fatty acid elongation and initiation by acyl-acyl carrier protein in *Escherichia coli*. J. Biol. Chem. **271:**1833–1836.
22. **Heath, R. J., and C. O. Rock.** 1996. Inhibition of β-ketoacyl-acyl carrier protein synthase III (FabH) by acyl-acyl carrier protein in *Escherichia coli*. J. Biol. Chem. **271:**10996–11000.
23. **Jackowski, S., P. D. Jackson, and C. O. Rock.** 1994. Sequence and function of the *aas* gene in *Escherichia coli*. J. Biol. Chem. **269:**2921–2928.
24. **Jiang, P., and J. E. Cronan, Jr.** 1994. Inhibition of fatty acid synthesis in *Escherichia coli* in the absence of phospholipid synthesis and release of inhibition by thioesterase action. J. Bacteriol. **176:**2814–2821.
25. **Khuller, G. K., and H. Goldfine.** 1974. Phospholipids of *Clostridium butyricum*. V. Effects of growth temperature on fatty acids, alk-1-enyl group, and phospholipid composition. J. Lipid Res. **15:**500–507.
26. **Larson, T. J., V. A. Lightner, P. R. Green, P. Modrich, and R. M. Bell.** 1980. Membrane phospholipid synthesis in *Escherichia coli*: identification of the *sn*-glycerol-3-phosphate acyltransferase polypeptide as the *plsB* gene product. J. Biol. Chem. **255:**9421–9426.
27. **Larson, T. J., D. N. Ludtke, and R. M. Bell.** 1984. *sn*-Glycerol-3-phosphate auxotrophy of *plsB* strains of *Escherichia coli*: evidence that a second mutation, *plsX*, is required. J. Bacteriol. **160:**711–717.
28. **Lightner, V. A., R. M. Bell, and P. Modrich.** 1983. The DNA sequences encoding *plsB* and *dgk* loci of *Escherichia coli*. J. Biol. Chem. **258:**10856–10861.
29. **Lightner, V. A., T. J. Larson, P. Tailleur, G. D. Kantor, C. R. H. Raetz, R. M. Bell, and P. Modrich.** 1980. Membrane phospholipid synthesis in *Escherichia coli*: cloning of a structural gene (*plsB*) of the *sn*-glycerol-3-phosphate acyltransferase. J. Biol. Chem. **255:**9413–9420.
30. **Lueking, D. R., and H. Goldfine.** 1975. The involvement of guanosine 5′-diphosphate-3′-diphosphate in the regulation of phospholipid biosynthesis in *Escherichia coli*. Lack of ppGpp inhibition of acyltransfer from acyl-ACP to *sn*-glycerol-3-phosphate. J. Biol. Chem. **250:**4911–4917.
31. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, N.Y.
32. **Post-Beittenmiller, D., J. G. Jaworski, and J. B. Ohlrogge.** 1991. *In vivo* pools of free and acylated acyl carrier proteins in spinach. J. Biol. Chem. **266:**1858–1865.
33. **Ray, T. K., J. E. Cronan, Jr., R. D. Mavis, and P. R. Vagelos.** 1970. The specific acylation of glycerol 3-phosphate to monoacylglycerol 3-phosphate in *Escherichia coli*. Evidence for a single enzyme conferring this specificity. J. Biol. Chem. **245:**6442–6448.
34. **Rock, C. O., and J. E. Cronan, Jr.** 1996. *Escherichia coli* as a model for the regulation of dissociable (type II) fatty acid biosynthesis. Biochim. Biophys. Acta **1302:**1–16.
35. **Rock, C. O., and J. L. Garwin.** 1979. Preparative enzymatic synthesis and hydrophobic chromatography of acyl-acyl carrier protein. J. Biol. Chem. **254:**7123–7128.
36. **Rock, C. O., S. E. Goelz, and J. E. Cronan, Jr.** 1981. ATP stimulation of the *sn*-glycerol-3-phosphate acyltransferase of *Escherichia coli*. Arch. Biochem. Biophys. **211:**113–118.
37. **Rock, C. O., S. E. Goelz, and J. E. Cronan, Jr.** 1981. Phospholipid synthesis in *Escherichia coli*. Characteristics of fatty acid transfer from acyl-acyl carrier protein to *sn*-glycerol-3-phosphate. J. Biol. Chem. **256:**736–742.
38. **Rock, C. O., and S. Jackowski.** 1982. Regulation of phospholipid synthesis in *Escherichia coli*. Composition of the acyl-acyl carrier protein pool *in vivo*. J. Biol. Chem. **257:**10759–10765.
39. **Tomb, J.-F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gil, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman,**

C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature (London) **388:**539–547.

40. **Tsay, J.-T., W. Oh, T. J. Larson, S. Jackowski, and C. O. Rock.** 1992. Isolation and characterization of the β-ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. J. Biol. Chem. **267:**6807–6814.
41. **van den Bosch, H., and P. R. Vaglos.** 1970. Fatty acid-CoA and fatty acyl-acyl carrier protein as acyl donors in the synthesis of lysophosphatidate and phosphatidate in *Escherichia coli*. Biochim. Biophys. Acta **218:** 233–248.
42. **Voelker, T. A., and H. M. Davies.** 1994. Alteration of the specificity and regulation of fatty acid synthesis of *Escherichia coli* by expression of a plant medium-chain acyl-acyl carrier protein thioesterase. J. Bacteriol. **176:**7320–7327.
43. **Yet, S., Y. K. Moon, and H. S. Sul.** 1995. Purification and reconstitution of murine mitochondrial glycerol-3-phosphate acyltransferase. Functional expression in baculovirus-infected insect cells. Biochemistry **34:**7303–7310.