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. ROCK1,3*

Department of Biochemistry, St. Jude Children’s Research Hospital, Memphis, Tennessee 38101; Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104; and Department of Biochemistry, University of Tennessee, Memphis, Tennessee 38163

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 (term plsD) from *Clostridium butyricum*, cloned based on its ability to complement the *sn*-glycerol-3-phosphate auxotrophic phenotype of a *E. coli* 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 *pldB* plsX plsC strain of *E. coli* confirmed that the plsD expression restored the ability of the cells to synthesize *1*-acyl-*sn*-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* synthesis 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 G3P backbone (8, 9). The *E. coli* acyl-G3P acyltransferase (PlsB) catalyzes the first committed step in phospholipid biosynthesis. We report the initial characterization of a novel *C. butyricum* gene, termed plsD, that complements the *E. coli* mutant strain of *E. coli*. The G3P acyltransferase from *C. butyricum* is a key regulatory enzyme that catalyzes the first committed step in phospholipid biosynthesis. We report the initial characterization of a novel gene (term plsD) from *Clostridium butyricum*, cloned based on its ability to complement the *sn*-glycerol-3-phosphate auxotrophic phenotype of a *E. coli* 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 *pldB* plsX plsC strain of *E. coli* confirmed that the plsD expression restored the ability of the cells to synthesize *1*-acyl-*sn*-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 structural gene for the PlsB acyltransferase (plsB) was not detected in vitro in assays using either acyl-acyl carrier protein or acyl coenzyme A as the substrate. 

The *C. butyricum* plsD gene was most closely related to the *C. beijerinckii* 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (37) and was most closely related to the *C. beijerinckii* 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (37). The G3P backbone (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 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 *Km* for G3P (27). It has also been suggested that the high-*Km* 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 the sn-2 position when grown at 37°C (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 *U*-14C]G3P (specific activity, 148 mCi/mmol), American Radiochemical Co. supplied the *U*-14C]glycerol (specific activity, 130 mCi/mmol), and ICN provided the *L*-35S]methionine (specific activity, 1,194 Ci/mmol). Scint-A-XF scintillation fluid was from Packard. Protein was measured by the method of...
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 (pBluesrl pLSX50) (27). This plasmid contained a 6.7-kbp insert and was subjected to a partial digest with HindIII, and the fragments were subcloned into plasmid pBR322. 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, pHS7, contained a 4.4-kbp insert and had HindIII sites at both ends and also contained two internal HindIII sites (Fig. 1). Fragments of the insert in plasmid pHS7 were subcloned into pBluescript II KS (+) (Stratagene, Inc.) and tested for the ability to complement the G3P auxotrophy of E. coli S125 (pBluesrl pLSX50 panD3 gpl28 gpl28 gplK0 relA1 spoT1 pit-10 phoA8 ompF26 fla22 ladl701), a tetracycline-sensitive derivative of strain SJ22 (38), as described below. The HindIII-HindIII, BsrXI-HindIII, and HindIII-HincII fragments from plasmid pHS342 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 HindIII sites. DNA sequencing was performed by the Center for Biotechnology staff at St. Jude Children’s Research Hospital, using an ABI automated sequence.

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 [35S]methionine (specific activity, 1.19 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 plsD and plsX plasmids. A positive control plasmid, pSJ345, bearing the wild-type plsD gene with its own promoter was constructed by PCR amplifying the gene from the laboratory strain E. coli UB1005. The primers (5′-CGCTATCCGAAGCTTATCAACG-3′ and 5′-GAAGCTTCACAGCG-3′) created unique HindIII and XhoI restriction sites, which were used to ligate the fragment into HindIII and AvrII sites of plasmid pBR322. The plsX and fabH genes (40) were cloned into the HindIII site of pBluescript II KS (+) by PCR amplification of genomic DNA from E. coli UB1005 with primers 5′-GCAAGTCTAAATGATTCACGC-3′ and 5′-CAGATGGCTCAACGAC-3′ to create plasmids pSJ101 and pSJ102. Plasmid containing the wild-type plsD was constructed from plasmid pSJ101 by digestion with NcoI, blunt ending with mung bean nuclease, digesting with SmaI, and religating the plasmid. This procedure excised the fabH gene from the parent plasmid. The resultant plasmid, pSJ11, complemented the G3P auxotrophic requirement of strain SJ52 (pBluesrl pLSX).

Phenotype complementation assays. During the subcloning procedures, plasmids were assessed for complementation of the G3P auxotrophy of E. coli SJ52 (pBluesrl pLSX) by using the indicated restriction enzymes, and the individual clones were tested for the ability to complement the G3P auxotrophic phenotype of strain SJ52 (pBluesrl pLSX50). The smallest complementing fragment in plasmid pSJ342 was completely sequenced. Abbreviations for restriction enzymes: H, HindIII; E, EcoRl; B, BsrXI; H, HincII.

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 plsD, plsX, and plsC genes were transformed into the pBluescript(Ts) strain SM2-1 (pBluesrl metC162::Tn10 thr-1 ars-14 Apgd-1 sosA99 lacZ44 plr136 xyl5-1 met-1 lacY1 tuc-78 edu-50 rfdD1 thr-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 pldsB, plsX, and pBluescript(Ts) mutations was first engineered by moving the pBluescript(Ts) lesion from strain SM2-1 (pBluesrl metC162::Tn10) (8) into strain SJ52 (pBluesrl pLSX50) by P1vir-mediated transduction using standard procedures (31). The resultant strain, SJ536 (pBluesrl pLSX50 plr136::Tn10), required supplementation with 0.01% G4P for growth at 30°C and did not grow at 42°C. This strain was transformed with pSJ342 (plsD), pRS22 (plsD) (19), or pRS11 (plsD), and isolated colonies were grown overnight in minimal E medium containing [3-14C]glycerol (specific activity, 130 μCi/μmol) plus 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 [14C]glycerol 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 phospholipids 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 (pBluesrl pLSX) transformed with plasmids expressing either the pldsB, plscB, 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-14C]G3P (specific activity, 60 mCi/μmol), 12.5 μM palmityl-CoA or palmityl-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 [14C]G3P concentration to 200 μM, adding ATP (10 mM) and MgCl2 (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. 1. Complementation of the plsB growth phenotype by subclones of pH37. Plasmid pH37, 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 (pBluesrl pLSX50). The smallest complementing fragment in plasmid pSJ342 was completely sequenced. Abbreviations for restriction enzymes: H, HindIII; Ev, EcoRl; B, BsrXI; H, HincII.
RESULTS

Cloning of the \textit{plsD} gene. Plasmid pH37 was isolated from clone pRS25 obtained from a \textit{C. butyricum} library by complementation of the G3P auxotrophy of the \textit{E. coli} TL400 (\textit{plsB} \textit{plsX}) as described in Materials and Methods. The size of the insert in pH37 was approximately 4.4 kbp, and the open reading frame required for complementation was localized to a 1.5-kbp \textit{Bst}XI-\textit{Hin}cII fragment by subcloning restriction fragments into pBluescript as depicted in Fig. 1. Plasmids were transformed into \textit{E. coli} SJ52 (\textit{plsB} \textit{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 \textit{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 \textit{plsD} was the carboxy-terminal portion of a gene homologous to a hypothetical \textit{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 \textit{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 \textit{E. coli} (26.7% identical and 47.4% identical plus similar residues), although two domains of \textit{plsD} also had a high degree of similarity to regions of the PlsB acyltransferase of \textit{E. coli} (Fig. 3). The \textit{plsC} genes from several plant
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 gycerolipid 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 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 Bluescript 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 plasmid 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.

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 [35S]methionine. 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 [35S]glycerol in the phospholipid fraction of strains complemented with the plsd 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 pBlue- script 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 plsd 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-[3H]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...
that encodes a G3P acyltransferase. The predicted PlsD protein sequence showed a high degree of homology to the PlsC1-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 lysophosphatic 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 \( \text{plS} \) and \( \text{pscC} \) homologs but lacks a \( \text{plS}B \) homolog (14). Similarly, \textit{Helicobacter pylori} does not contain a \( \text{plS}B \) homolog but does have a \( \text{plS}C \)-like gene (39). \textit{Saccharomyces cerevisiae} does not contain either a \( \text{plS}B \) or a \( \text{plS}X \) homolog, but does have a gene that is related to \( \text{plS}C \). \textit{Bacillus subtilis} has a \( \text{plS}X \) homolog, but neither a \( \text{plS}B \) nor a \( \text{plS}C \) gene has appeared in the current database release. The \( \text{plS}B26 \) 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 \( \text{plS}B26 \) 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 \( \text{plS}B26 \) mutation and test the idea that the \( \text{plS}B \) gene is dispensable by gene disruption.

**ACKNOWLEDGMENTS**

We thank Suzanne Jackowski and Lensek Kaeu for informative discussions and Jina Wang, Christopher Salvatore, and Joshua Rosenthal for expert technical assistance.

This work was supported by NIH grant GM 34406, Cancer Center (CORE) support grant CA 21765, and the American Lebanese Syrian Associated Charities.

**REFERENCES**

12. Goldfine, H. 1993. Phospholipid biosynthetic enzymes of butyric acid-pro-


