

## A Missense Mutation Accounts for the Defect in the Glycerol-3-Phosphate Acyltransferase Expressed in the *plsB26* Mutant

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**The *sn*-glycerol-3-phosphate acyltransferase (*plsB*) catalyzes the first step in membrane phospholipid formation. A conditional *Escherichia coli* mutant (*plsB26*) has a single missense mutation (G1045A) predicting the expression of an acyltransferase with an Ala349Thr substitution. The PlsB26 protein had a significantly reduced glycerol-3-phosphate acyltransferase specific activity coupled with an elevated  $K_m$  for glycerol-3-phosphate.**

The *sn*-glycerol-3-phosphate (G3P) acyltransferase catalyzes the first committed step in membrane phospholipid formation and acylates the 1-position of G3P with either acyl-acyl carrier protein (ACP) or acyl-coenzyme A (CoA) thioesters. The G3P acyltransferase in *Escherichia coli* has been extensively studied and is the membrane-bound product of the *plsB* gene (5, 15). Consistent with its position at the start of the phospholipid biosynthetic pathway, the PlsB protein functions as a sensor that monitors the metabolic state of the cell through its allosteric interactions with ATP and guanosine-3',5'-tetraphosphate to coordinate the rate of G3P acylation with macromolecular biosynthesis and cell growth (5, 7, 11). Mutants defective in PlsB (*plsB26*) activity were isolated as G3P auxotrophs (1), a growth phenotype attributed to the expression of an acyltransferase with an elevated  $K_m$  for G3P (1, 2). The identity of the mutation in *plsB26* strains is unknown. Thus, the biochemical properties of the mutant protein are unknown, and it is not even clear if an active protein is expressed from the *plsB26* allele since the possibility remains that the high  $K_m$  activity detected in mutant membranes was due to a novel acyltransferase revealed by the absence of the normal PlsB protein. The structural gene for the PlsB acyltransferase is located at 92 min on the linkage map (10); however, moving this region of the chromosome from the *plsB26* mutant into another genetic background did not transfer the G3P auxotrophic phenotype. This led to the discovery that a second mutation, called *plsX*, is required for expression of the G3P auxotrophic phenotype (9). The role of the PlsX protein in phospholipid biosynthesis is unknown, although it probably is not itself an acyltransferase (6). In light of the critical function of the acyltransferase reaction in phospholipid biosynthesis and the central role that the *plsB26* mutation has played in studying this pathway (5, 15), we have determined the molecular and biochemical basis for the acyltransferase defect in *plsB26* strains.

**Identification of the mutation in the *plsB26* allele.** The structure of the *plsB26* allele in strain BB26 (*plsB26 plsX50 glpD3 glpR2 phoA8 Str<sup>s</sup> relA1 spoT1 tonA22 T2R pit-10 HfrC*) (1) was

determined by using a PCR-based approach to sequence the entire gene plus about 100 bases of upstream DNA. Oligonucleotides (Table 1) were used in pairs to amplify overlapping 500-bp fragments, which were sequenced with the same primers by the Center for Biotechnology at St Jude Children's Research Hospital. This procedure gave complete coverage of the 2.5-kbp gene and led to the identification of a single base pair change of G1045A compared to the sequence of the wild-type gene. This missense mutation lies in the first position of codon 349 and changes the predicted amino acid at this position from an Ala to a Thr. The wild-type *plsB* gene in the parental strain 8 (*glpD3 glpR2 phoA8 Str<sup>s</sup> relA1 spoT1 tonA22 T2R pit-10 HfrC*) was also sequenced in the same manner to verify that it was identical to the previously published sequence (10).

The region of *plsB26* containing the mutation, between primers 5 and 6 (Table 1), was amplified by PCR from the chromosome of strain BB26 (*plsB26*), digested with *Sfi*I and *Mlu*I, and then ligated into the corresponding sites in plasmid pRJ54 (PlsB), which contains the wild-type *plsB* gene with a COOH-terminal Flag-tag epitope (8). This construct, pJH3 (PlsB26), expressed the Flag-tag version of the PlsB26 protein. DNA sequencing of the plasmid confirmed that only the A349T mutation was present in the PlsB26 construct.

**Expression and activities of the PlsB and PlsB26 proteins.** Expression vectors containing Flag-PlsB (pRJ54), Flag-PlsB26 (pJH3), or an empty vector (pACYC177) were transformed into strain SJ22 (*plsB26 plsX50 panD2 zac-220::Tn10 glpD3 glpR2 glpKi relA1 spoT1 pit-10 phoA8 ompF627 fluA22*) (14). There were the same amounts of Flag-PlsB and Flag-PlsB26 proteins in the membrane preparations assayed by immunoblotting with the M2 anti-Flag antibody (Kodak IBI, Inc) (Fig. 1A). These data demonstrated that both the wild-type and mutant proteins were expressed at the same levels and were assembled into the membrane. Thus, the differences in specific activity and catalytic properties described below were attributed to inherent differences in the biochemical properties of the proteins and not to differences in the level of expression.

The specific activities of the G3P acyltransferases were compared in membranes isolated from the transformants (Fig. 1B). Membranes from strain SJ22/pACYC177 had a low specific activity (0.004 nmol/min/mg), whereas membranes from strain SJ22/pRJ54 (PlsB) had significantly elevated levels of acyltransferase activity (7.2 nmol/min/mg). The specific activity

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TABLE 1. Primers used to amplify and sequence *plsB26*

Primer <sup>a</sup>	Sequence (5'-3')	bp <sup>b</sup>
1 (+)	TTAGCGCCGCGGAAACAT	-102
2 (-)	AACATCACCGACACTGGCA	386
3 (+)	AGCTGTTCCAGACTATC	308
4 (-)	AGTCGGTTCAGGTGAAG	833
5 (+)	ATGGAAGAGATTGCGGCGAA	754
6 (-)	TCGGAATCAGCGTAATCGGA	1264
7 (+)	ATTCGGTCGAGTACTTCGTG	1133
8 (-)	CACGTTGCGCATCAGATCCA	1677
9 (+)	GCATCACGTCAGCGTCACT	1600
10 (-)	CGCATCAATAACGTCGGCA	2004
11 (+)	CCGCGACGTATTGATGGA	1902
12 (-)	GTTCTCGAGCCGGCAATTCT	2450

<sup>a</sup> Orientation of primer with respect to *plsB* gene sequence is indicated in parenthesis.

<sup>b</sup> Position of 5' base of primer. A of ATG initiation codon is position 1.

from strain SJ22/pJH3 (PlsB26) was 0.1 nmol/min/mg. This specific activity was 25-fold higher than the background rate in the empty vector control but was 72-fold lower than that of membranes derived from cells transformed with the wild-type PlsB. These experiments were performed with palmitoyl-CoA as the acyl donor; however, the specific activities were also tested with palmitoyl-acyl carrier protein and the same ratio of activities were obtained (not shown).

The transformation of strain SJ22 (*plsB26*) with pJH3 (PlsB26) eliminated the G3P auxotrophic growth phenotype. This result suggested that the elevated expression of PlsB26 was sufficient to complement the defect in phospholipid synthesis. We compared the specific activity of the G3P acyltransferase in the wild-type parent strain 8 to that in strain SJ22/pJH3 (PlsB26) at 200  $\mu$ M G3P (Fig. 1B). The specific activity of membranes from strain 8 was 0.33 nmol/min/mg compared to 0.1 nmol/min/mg in strain SJ22/pJH3. The overexpression of PlsB26 in strain SJ22/pJH3 yielded a total G3P acyltransferase activity that was 30% of the activity found in wild-type cells. Thus, the elevation of the PlsB26 G3P acyltransferase level was sufficient to override the G3P auxotrophic phenotype.

**Biochemical properties of the PlsB26 protein.** A characteristic feature of the G3P acyltransferase activity in membranes derived from *plsB26* mutants is an elevated  $K_m$  for G3P (1-4,

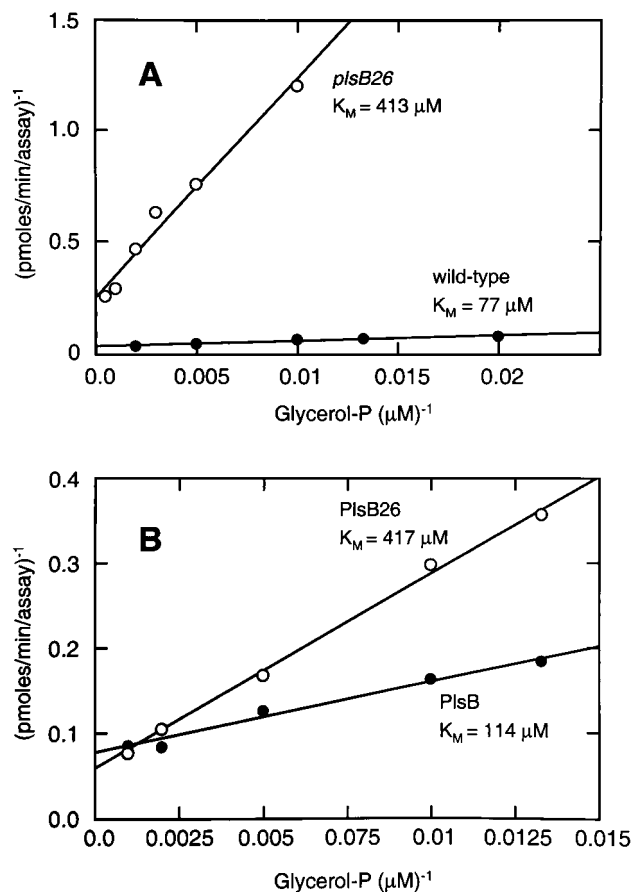


FIG. 2. Kinetic analysis of wild-type and mutant G3P acyltransferase. (A) Membranes were prepared from strain SJ22 (*plsB26*) (○) and the parental wild type, strain 8 (●), and assayed for activity at 50  $\mu$ M palmitoyl-CoA between 50 and 1,000  $\mu$ M G3P. Assays contained 400  $\mu$ g of protein from SJ22 and 50  $\mu$ g of protein from strain 8. (B) Membranes were prepared from strain SJ22 (*plsB26*) harboring either pRJ54 (Flag-PlsB) (2  $\mu$ g/assay) (●) or pJH3 (Flag-PlsB26) (100  $\mu$ g/assay) (○) and were assayed for G3P acyltransferase activity at 50  $\mu$ M palmitoyl-CoA between 50 and 1,000  $\mu$ M G3P.

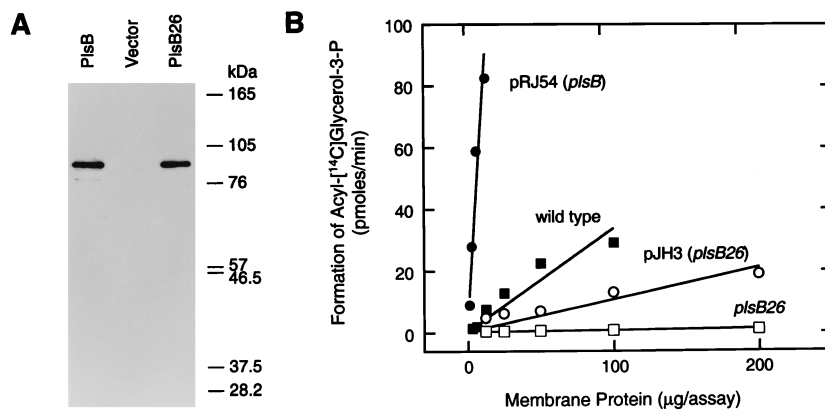


FIG. 1. Expression and activity of the PlsB26 mutant protein. (A) Membranes were prepared from strain SJ22 (*plsB26*) harboring either plasmid pRJ54 (*plsB*) or pJH3 (*plsB26*) or the empty vector (pACYC177), and the proteins were separated by sodium dodecyl sulfate-gel electrophoresis on 10% polyacrylamide gels. The samples were transferred to nitrocellulose membranes and immunoblotted with the M2 anti-Flag antibody to detect the expression of the epitope-tagged acyltransferases. (B) Membranes were prepared from strain 8 (wild type) (■) or strain SJ22 (*plsB26*) transformed with either the empty vector pACYC177 (□), pRJ54 (*plsB*) (●), or pJH3 (*plsB26*) (○). G3P acyltransferase assays were performed with the indicated amounts of membrane protein. The assays contained 0.1 M Tris-HCl (pH 8.6)-1-mg/ml bovine serum albumin-200  $\mu$ M [<sup>14</sup>C]G3P (specific activity, 13 Ci/mmol)-50  $\mu$ M palmitoyl-CoA and were incubated at 37°C for 15 min (12, 13).

9). We first confirmed this original observation under our assay conditions (Fig. 2A). Indeed, the apparent  $K_m$  for G3P in these experiments was about fivefold higher in membranes isolated from *plsB26* mutants (425  $\mu$ M) than in membranes isolated from wild-type cells (75  $\mu$ M). The  $V_{max}$  for the wild-type protein was 0.625 nmol/min/mg, whereas the mutant  $V_{max}$  was reduced 60-fold to 0.01 nmol/min/mg. Both membrane preparations had approximately the same  $K_m$  for palmitoyl-CoA (100  $\mu$ M) when assayed at 200 mM G3P (not shown).

The G3P  $K_m$  in membranes derived from cells that overexpressed either the wild-type or PlsB26 mutant protein was determined to establish whether the PlsB26 protein had an elevated  $K_m$  as well as reduced specific activity. The G3P  $K_m$  values calculated in these experiments were 114  $\mu$ M for PlsB and 417  $\mu$ M for PlsB26 (Fig. 2B). These data confirmed that the PlsB26 protein had an elevated  $K_m$  for G3P compared to that of the wild-type protein. The  $V_{max}$  was 8.77 nmol/min/mg for the overexpressed wild-type protein, and the  $V_{max}$  was 0.27 nmol/min/mg for PlsB26. This difference of 33-fold was very similar to the difference observed between the wild-type strain 8 and strain SJ22 (*plsB26*) and indicated that the PlsB26 protein was the high  $K_m$  acyltransferase previously described in membranes isolated from *plsB26* mutants.

**Conclusions.** The mutant PlsB(A349T) protein expressed from the *plsB26* allele is catalytically defective with a decreased  $V_{max}$  and increased  $K_m$  for G3P compared to those of the wild-type PlsB. These findings are consistent with the work of previous investigators who observed an increased  $K_m$  for G3P in membranes from *plsB26* strains and attributed this activity to a defective PlsB protein (1–4, 9). The function that Ala349 plays in the wild-type protein, and why substitution with Thr should lead to a defective enzyme, is not clear. His306 and Asp311 are involved in catalysis (8), and A349 is relatively close to these residues in the primary sequence. However, the location of A349 cannot be determined in the absence of a three-dimensional structure. Thus, A349 may lie in or very close to the G3P binding site on the enzyme, or it may exert a more subtle effect on the overall shape of the active site by perturbing the protein structure.

**Nucleotide sequence accession number.** The nucleotide sequence of *plsB26* determined in this study has been deposited in the GenBank database under accession no. AF106625.

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