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Acylation of Glycerol 3-Phosphate Is the Sole Pathway of De Novo Phospholipid Synthesis in *Escherichia coli*

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The inhibition of phospholipid synthesis engendered by starving glycerol 3-phosphate (G3P) auxotrophs of *Escherichia coli* (*plsB* or *gpsA*) for G3P is incomplete; 5 to 10% of the normal rate of phospholipid synthesis remains, even after prolonged starvation. We report that G3P starvation of a strain having lesions in both the *gpsA* and *plsB* genes resulted in essentially complete (>98.5%) inhibition of phospholipid synthesis, indicating that all de novo glycerolipid synthesis in *E. coli* proceeds by acylation of G3P.

The synthesis of phosphatidic acid is the first step taken in the formation of all phospholipid species of *Escherichia coli* (13). It is now clear that the major pathway of phosphatidic acid synthesis involves the acylation of *sn*-glycerol 3-phosphate (G3P) with two fatty acid moieties donated by the acyl-acyl carrier protein pool (15–18). This pathway can be blocked by starvation of G3P auxotrophs (*gpsA* or *plsB*) for G3P. *gpsA* mutants lack the biosynthetic G3P dehydrogenase responsible for the synthesis of G3P from dihydroxyacetone phosphate (8), whereas *plsB* mutants owe their G3P auxotrophy to a defect in G3P acyltransferase that results in an elevated K_m for G3P (1, 2, 9, 11). However, the blockage of phospholipid synthesis engendered by starving these strains for G3P is incomplete; about 5 to 10% of the normal rate of phospholipid synthesis remains, even after prolonged starvation (1, 5, 12). This residual synthesis has been attributed to residual G3P synthesis in *gpsA* strains and slow utilization of endogenously synthesized G3P by the *plsB* acyltransferase (4). However, there are no data supporting this interpretation, and thus the possibility of minor pathways of phospholipid synthesis must be considered. It should be noted that the residual synthesis has been observed by pulse-labeling with [^{14}C]acetate as well as with $^{32}PO_4$ in *plsB* mutants during prolonged G3P starvation long after the stoppage of growth (1). This is important since the diglyceride kinase pathway is expected to account for a portion of the residual $^{32}PO_4$ incorporation into phospholipid but does not influence incorporation of [^{14}C]acetate (13, 19).

We constructed double mutant strains which carry lesions in both the *gpsA* and *plsB* genes to examine the possibility of a minor pathway of phospholipid synthesis. The rationale is that the two mutations should act synergistically to give a complete inhibition of phospholipid synthesis unless other minor phospholipid synthetic pathways are present in *E. coli*. The strains and their derivations are listed in Table 1.

The double mutant was constructed starting with the *plsB plsX* mutant BB26-36 (1, 9–11). A lesion in the *gpsA* gene was introduced into strain BB26-36 by a P1 bacteriophage transduction from strain TR65, which has a Tn*10* insertion located very close to the *gpsA* locus. The resulting strain, TR134, was one of the Tet^r transductants requiring G3P for growth (Table 1).

Since both the phospholipid synthesis lesions in strain TR134 have the same phenotype (G3P auxotrophy), the presence of the *gpsA* lesion was verified both genetically and biochemically. First, the Tn*10* insertion in strain TR134 was transduced into the wild-type strain 6 with phage P1, and the Tet^r transductants were scored for G3P auxotrophy. As expected, about 70% of the Tet^r recombinants required G3P, one of which was designated strain TR136.

Second, the activity of the biosynthetic G3P: NADPH dehydrogenase (the *gpsA* gene product) of strains TR134 and TR136 was assayed and found to be <0.3% of the normal

TABLE 1. Bacterial strains[a]

Strain	Relevant genotype	Relevant phenotype	Reference or mode of construction[b]
6	HfrC *glpT*	Wild type	7
BB26-36	HfrC *plsB26 plsX50*	G3P requirement	9, 10
BB20-14	HfrC *gpsA20*	G3P requirement	4
DM73	HfrC Tn*10 gpsA*$^+$	Tet^r	6
TR65	HfrC Tn*10 gpsA20*	Tet^r, G3P requirement	P1(DM73) × BB20-14
TR134	HfrC Tn*10 gpsA20 plsB26 plsX50*	Tet^r, G3P requirement	P1(TR65) × BB26-36
TR136	HfrC Tn*10 gpsA20*	Tet^r, G3P requirement	P1(TR134) × 6
RZ60	*malB*$^+$ *dgk-6*	Deficient in diglyceride kinase	14, 19

[a] All strains are derivatives of *E. coli* K-12. The Tn*10* insertion in the Tet^r strains is the *zib*::Tn*10* insertion described earlier (6).
[b] Transductions were done with P1 *vir* as previously described (6). The donor strain is given in parentheses, and the selections were for Tet^r.

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TABLE 2. Biosynthetic G3P dehydrogenase activity of strains TR134, BB26-36, TR136, and 6

Strain (relevant genotype)	Sp act (U/mg of protein)[a] in extract	
	Crude	Partly purified
6 (wild type)	10.6	18.7
BB26-36 (*plsB26*)	15.5	19.8
TR134 (*gpsA20 plsB26*)	<0.1	<0.1
TR136 (*gpsA20*)	<0.1	<0.1

[a] For specific activity, 1 U is the dihydroxyacetone phosphate-dependent oxidation of 1 nmol of NADPH per min at 25°C. Crude and partially purified enzyme extracts were prepared and assayed for enzyme activity by the method of Kito and Pizer (8).

level (Table 2). Moreover, the apparent K_m of the G3P acyltransferase for G3P of strain TR134 (ca. 950 μM) was similar to that of BB26-36 (10-fold above the normal concentration), whereas the strain TR136 enzyme was identical to that of the wild-type strain 6 with regard to the K_m value (ca. 100 μM) for G3P, confirming the presence of the *gpsA* and the *plsB* lesions in strain TR134.

Cells of strain TR134 were grown in a low-phosphate–glucose–casein hydrolysate–G3P (or glycerol) medium (1) and then starved for glycerol. The growth of strain TR134 stopped abruptly shortly after G3P starvation (Fig. 1). This result is in marked contrast to those obtained with strains carrying single lesions in either *plsB* (1) or *gpsA* (5); these strains cease growth slowly and gradually following G3P

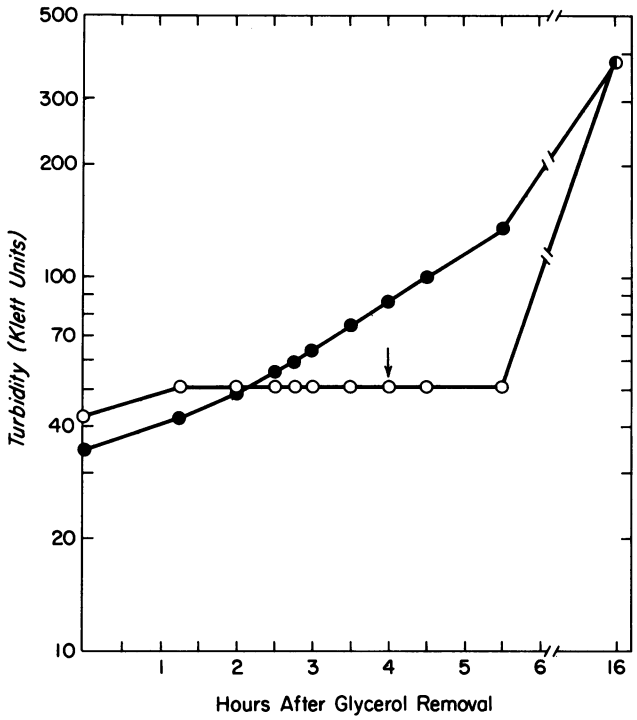

FIG. 1. Effect of glycerol deprivation on the growth of the mutant strain TR134 (*gpsA20 plsB26*) requiring glycerol or G3P for growth. Cultures of TR134 were grown in medium supplemented with glycerol to early phase, filtered, washed with prewarmed minimal medium to completely remove glycerol, suspended in a prewarmed minimal medium containing succinate (0.4%) and vitamin-free casein hydrolysate (0.05%), and incubated with shaking at 37°C in the absence (○) or presence (●) of glycerol (0.2%) starting at zero time. The starved cells were resupplemented with 0.2% glycerol at the time indicated by the arrow.

TABLE 3. Rate of phospholipid synthesis in TR134 (*plsB26 gpsA20*) during glycerol starvation and supplementation

Starvation time (min)[a]	Growth (Klett units)		$^{32}PO_4$ incorporation into phospholipids (10^4 cpm)[b]	
	+ Glycerol	− Glycerol	+ Glycerol	− Glycerol
0	34	41		
60	42	50	19	0.25
120	49	50	56	0.24
180	62	50	93	0.25

[a] Time after suspension in medium 56 LP (1)–succinate (0.4%)–vitamin-free casein hydrolysate (0.05%) in either the presence or absence of glycerol (0.2%).

[b] Cells were labeled with 5 μCi of $^{32}PO_4$ per ml of cell culture for 5 min, followed by extraction of total lipid from 1 ml of labeled culture by the method of Bligh and Dyer (3). The total lipids in the washed chloroform phase were applied on a silica gel G plate, and the plate was developed in $CHCl_3$–CH_3OH–14 N NH_4OH (70:25:1). The silica gel containing total phospholipid fraction (which migrated from the origin) was scraped and counted. The blank tube contained 0.95 ml of cell culture suspension (the same amount extracted in the experimental samples), and 3.75 ml of $CHCl_3$–CH_3OH (1:2) was added before the $^{32}PO_4$ was added. The phospholipid fraction from the blank tube mixture contained about 600 cpm, which was subtracted from the counts per minute of the experimental tubes.

starvation. In some of our starvation experiments, strain TR134 cultures accumulated particulate matter after a few hours of starvation. We attribute this material to cell clumping. Cellular lysis was ruled out, since no β-galactosidase activity was detected in the supernatant of starved cultures of strain TR134 previously induced by the addition of isopropyl β-D-thio-galactoside (data not shown). Moreover, the starved cells retained phospholipid synthetic capacity, since the growth resumed upon G3P or glycerol supplementation in the cultures, even after 16 h of starvation.

During starvation, the rate of $^{32}PO_4$ incorporation into phospholipids declined to less than 1.5% of that of the glycerol-supplemented culture (Table 3). This level of incorporation was roughly that expected (see below) from phosphorylation of the diglyceride resulting from donation of the *sn*-glycerol 1-phosphate moiety of phosphatidylglycerol in the synthesis of the membrane-derived oligosaccharide (14, 19). Indeed, when strain TR134 was labeled with [1-^{14}C]acetate in the absence of glycerol or G3P, no detectable incorporation into phospholipids was observed (<0.04% of the normal rate) (Table 4). Therefore, it seemed likely that the residual $^{32}PO_4$ incorporation into phospholipids of strain TR134 in the absence of glycerol was due to recycling of diglyceride rather than to de novo glycerolipid synthesis. To strengthen this conclusion, we estimated the level of diglyceride available for phosphorylation during a brief labeling. This was done by briefly labeling the *dgk-6* strain RZ60 (which lacks diglyceride kinase) with [1-^{14}C]acetate (Table 4). The amount of diglyceride accumulated during the pulse period was about 2.5% of the total glycerolipids synthesized. Thus, phosphorylation of diglyceride by diglyceride kinase should account for no more than 2.5% of the amount of $^{32}PO_4$ incorporated in pulse-labeling experiments, and therefore, the residual $^{32}PO_4$ incorporation (about 1.3% of the normal rate) seen in strain TR134 in the absence of G3P can be attributed to diglyceride phosphorylation rather than to de novo phospholipid synthesis. We attempted to transduce the *dgk-6* lesion into a *plsB26* strain to allow a more direct comparison. However, we were unable to construct *dgk-6 plsB26* strains, presumably owing to the fact that these two genes are separated by only 166 base pairs (11).

A complication to our results is the presence of the *plsX*

TABLE 4. Incorporation of [1-^{14}C]acetate into phospholipids and diglycerides in strains RZ60 and TR134 during pulse-labeling of the culture[a]

Strain (relevant genotype)	Growth condition	Incorporation of [1-^{14}C]acetate into:		
		Total phospholipids (10^4 cpm)	Diglycerides	
			10^4 cpm	% Total lipid incorporation
RZ60 (*dgk-6*)	+ NaCl	11.5	0.3	2.5
TR134 (*gpsA20 plsB26*)	+ Glycerol	27	ND[b]	
TR134 (*gpsA20 plsB26*)	Glycerol starved	0.01	ND	

[a] Cells of RZ60 and TR134 (medium supplemented with glycerol) were grown at 37°C to about 40 Klett units. TR134 cells were washed free of glycerol and suspended in prewarmed medium with or without glycerol as indicated. RZ60 and TR134 cells were labeled with 10 μCi of [1-^{14}C]acetate per ml of cell culture for 5 min. After labeling, total lipids were extracted from 1 ml of culture by the method of Bligh and Dyer (3). The neutral lipids were separated from total phospholipids by thin-layer chromatography on silica gel H plates by using a solvent system consisting of hexane-diethyl ether-acetic acid (30/70/1 [vol/vol]) (18). The areas of silica gel portions containing total phospholipids (at the origin) and individual neutral lipids were scraped and counted in a liquid scintillation counter by using PCS (Amersham Corp.) as scintillation fluid. The media were as described in Table 3, footnote *a*, with the exception that strain RZ60 was grown on glucose (0.4%) rather than on succinate in the presence of 0.1 M NaCl (14). The blank values were obtained as described in Table 3, footnote *b* (except for the differing isotopes).

[b] ND, Not detected.

mutation in strain TR134 owing to its derivation from strain BB2636. Efficient supplementation of *plsB* strains (but not *gpsA* strains) requires the *plsX* mutation (10). The nature of the *plsX* lesion is unknown, as is the role of the *plsX* gene product in the function of the *plsB*-encoded acyltransferase.

In conclusion, our results indicate that all de novo *E. coli* glycerolipid synthesis proceeds by acylation of G3P. Labeled precursors can be incorporated into phospholipids by recycling reactions such as diglyceride kinase or lysophosphatidylethanolamine acyltransferase, which salvage the portion of phospholipids remaining after donation of lipid moieties. However, this incorporation is not a measure of de novo synthesis but measures only the flux of the recycling reaction.

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