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Biochemistry

Gene cloning for the isolation of enzymes of membrane lipid synthesis: Phosphatidylserine synthase overproduction in *Escherichia coli*

(hybrid ColE1 plasmids/phospholipids/CDP-diglyceride/CDP-diglyceride-serine *O*-phosphatidyltransferase)

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ABSTRACT **We have screened a bank of 2000 *E. coli* strains carrying hybrid ColE1 plasmids [Clarke, L. & Carbon, J. (1976) *Cell* 9, 91–99] for those that correct the temperature sensitivity of a mutant in CDP-1,2-diacyl-*sn*-glycerol:L-serine *O*-phosphatidyltransferase (EC 2.7.8.8, phosphatidylserine synthase). Two hybrid plasmids of this kind (pLC34-44 and pLC34-46) were identified and characterized. Strains carrying these plasmids overproduce the synthase by 6- to 15-fold, as demonstrated by assays of extracts and purification to homogeneity of the overproduced enzyme. The overproduced synthase, like the wild-type enzyme, is found associated predominately with the ribosomal fraction of crude cell extracts. Because the membrane phospholipid composition of these overproducers is not greatly altered, we suggest that the synthase is normally present in excess.**

Enzymes of membrane phospholipid synthesis (see Fig. 1). like other constitutive enzymes, are present in small amounts in *Escherichia coli* (less than 0.1% of the protein) (1–5). Changes in growth conditions do not alter their specific activities, and no one has described methods for their overproduction. Although the phosphatidylserine decarboxylase (EC 4.1.1.65) (1), CDP-1,2-diacyl-*sn*-glycerol:L-serine *O*-phosphatidyltransferase (EC 2.7.8.8, phosphatidylserine synthase) (3), and CDP-1,2-diacyl-*sn*-glycerol:*sn*-glycero-3-*P* phosphatidyltransferase (EC 2.7.8.5, phosphatidylglycero-*P* synthase) (2) have been obtained in near homogeneous forms, the hydrophobic character and low levels of these enzymes have rendered their purification to homogeneity in large quantities exceptionally difficult. Consequently, the chemistry, regulation, and orientation of these and other phospholipid biosynthetic enzymes in biological membranes remain essentially unknown.

Molecular cloning permits the construction of strains carrying multiple copies of specific genes or gene clusters (6–8). Clarke and Carbon (9) have prepared a collection of 2000 *E. coli* strains, each carrying a distinct hybrid ColE1 plasmid into which a fragment of *E. coli* chromosomal DNA has been inserted. The hybrid plasmids (with their inserted DNA) are maintained at 10–20 copies per chromosome, and the average molecular weight of the inserted fragment is 8×10^6 (¼ min on the linkage map). Because the fragments were generated by random shearing of the chromosomal DNA, the collection should contain hybrid plasmids representing most of the *E. coli* genome (9).

In the present work we have identified two hybrid plasmids in the collection that carry the gene for phosphatidylserine synthase, a key enzyme in the biogenesis of membrane phosphoglycerides (Fig. 1, reaction 3). Strains carrying these plasmids overproduce the synthase by as much as 15-fold (presumably on the basis of gene dosage), as demonstrated by purifying the enzyme to homogeneity. The Clarke and Carbon strains should be extremely useful for purifying a variety of other constitutive enzymes, because overproduction (by assay of extracts) has recently been reported for factors involved in DNA synthesis (10, 11) and for two other enzymes involved in membrane lipid synthesis.§

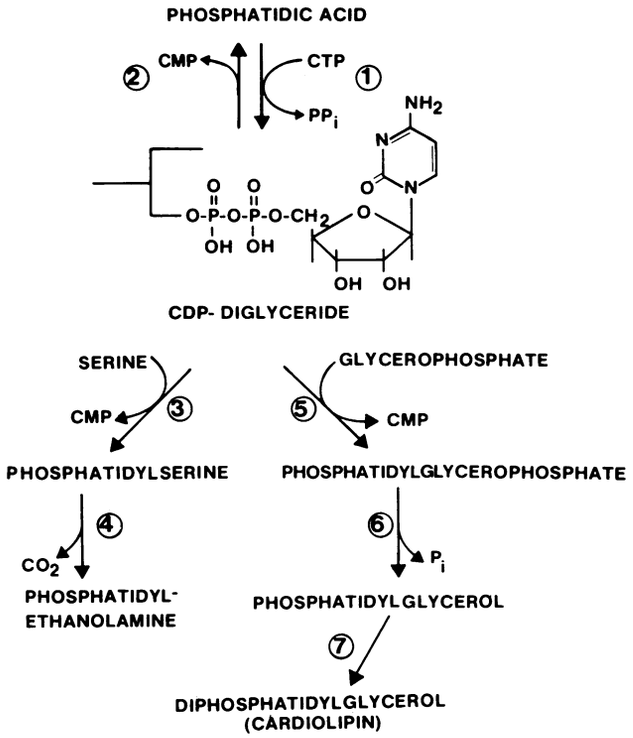

FIG. 1. Biosynthesis of phospholipids in *E. coli* as catalyzed by the following enzymes: 1, CTP-phosphatidic acid cytidylyltransferase; 2, CDP-1,2-diacyl-*sn*-glycerol hydrolase; 3, CDP-1,2-diacyl-*sn*-glycerol:L-serine *O*-phosphatidyltransferase; 4, phosphatidylserine decarboxylase; 5, phosphatidylglycero-*P* synthase; 6, phosphatidylglycero-*P* phosphatase; 7, cardiolipin synthase.

MATERIALS AND METHODS

Materials. DL-[3-^{14}C]Serine, *sn*-[*U*-^{14}C]glycero-3-*P*, and $^{32}P_i$ were products of New England Nuclear, Boston. CDP-1,2-diacyl-*sn*-glycerol (with the fatty acid composition of egg

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§ Phosphatidylserine decarboxylase and *sn*-glycero-3-*P* acyltransferase have recently been obtained in hybrid plasmids and are similarly overproduced (E. Hawrot, M. D. Snider, and E. P. Kennedy, personal communication).

lecithin) was synthesized and stored as described previously (12).

Bacterial Strains and Growth Conditions. Strains of *E. coli* K-12 are listed in Table 1. *E. coli* B (¾ through logarithmic phase of growth) grown on rich medium was purchased as the frozen cell paste from Grain Processing of Muscatine, IA. The isolation and characterization of the phosphatidylserine synthase mutant (*pss-8*) is described elsewhere (13). Organisms from the Clarke and Carbon collection (which carry the hybrid ColE1 plasmids) are designated MV12, followed by an identification number that specifies the hybrid plasmid (9). For instance, MV12/pLC34-44 carries plasmid pLC34-44 (located on plate 34, patch 44 in the collection).

All small cultures were grown aerobically on LB broth (14) at 37°, unless otherwise stated. Cells were grown on a large scale in either LB broth or glucose/yeast extract medium [0.2 M potassium phosphate (pH 7.3)/1% yeast extract/1% glucose]. Organisms harboring hybrid plasmids were generally grown in the presence of crude colicin E1 (10–20% vol/vol, as described further below).

Enzyme Assays, Subcellular Distribution, and Phospholipid Composition. For assay of enzymes from small cultures cells were harvested in late logarithmic phase and disrupted by sonication (12). Assay conditions for phosphatidylserine (12) and phosphatidylglycero-*P* (12) synthases were the same as previously described, except the final DL-serine concentration in the case of the former enzyme was 2 mM. For both enzymes a unit is defined as the amount of enzyme that synthesizes 1 nmol of product per min at 37° unless stated otherwise.

The subcellular distribution of phosphatidylserine synthase in extracts prepared by passage through a French pressure cell was determined by sucrose gradient sedimentation (15). The phospholipid composition was determined by long-term labeling with $^{32}P_i$, followed by thin-layer chromatography (13).

Preparation of Colicin E1. Crude colicin was made by growing JF390 (16), an *E. paracoli* strain carrying a wild-type ColE1 plasmid, overnight on LB broth, preparing a cell-free supernatant by a 15 min centrifugation at 10,000 × *g*, and sterilizing this supernatant by Millipore filtration. This material was mixed with growth medium in the volume ratios given above to kill noncolicinogenic bacteria, which might overgrow the plasmid-bearing cells.

Identification of Strains in the Clarke and Carbon Collection Carrying the *pss*$^+$ Gene on Hybrid ColE1 Plasmids. This was done as described previously for mutants that are temperature sensitive in DNA synthesis (10). Briefly, fresh patches (grown at 37°) of the 2000 F$^+$, hybrid ColE1-bearing strains (MV12 background) were replica plated onto thin lawns of RA80 (F$^-$ *pss-8*) grown at 25°, using the procedure of Low (17). Each lawn contained about 10^8 cells, evenly spread over the surface of a minimal Vogel–Bonner plate (18). After 30 min at 37° to allow F$^+$-mediated transfer of the hybrid plasmids, the minimal plates were sprayed with nalidixic acid (2 mg/ml) to ensure complete killing of the donor males. Thereupon the plates were incubated at 44° for 2 days to select for temperature-resistant, nalidixic acid-resistant organisms (the expected phenotype of the plasmid-corrected mutant). In this way two strains (plate 34, patches 44 and 46) were identified as carrying the *pss*$^+$ gene on the hybrid plasmids. After purification to single colonies, both the donors (designated MV12/pLC34-44 and MV12/pLC34-46), as well as the corrected mutants (RA80/pLC34-44 and RA80/pLC34-46), were grown in liquid medium and assayed as described above.

Construction of RA324. The plasmid pLC34-44 was transferred to A324 (F$^-$ *pss*$^+$ *proC lacI strA*) in order to eliminate the *recA* mutation present in the MV12 background. Strain A324 was cross-streaked with MV12/pLC34-44 on a plate of LB agar; after 4 hr to allow mating, colonies resistant to both colicin E1 and streptomycin sulfate were selected by streaking a small portion of the mating mixture onto an appropriate LB plate. The resulting strain (RA324) had the genetic markers of A324; it was used for large scale purification because the specific activity of the phosphatidylserine synthase was somewhat higher in this strain than in MV12/pLC34-44.

Table 1. Strains of *E. coli* K-12

Strain designation	Relevant genotype	Ref. or source
RA800	F$^-$ *pss*$^+$ *nalA*	13
RA80	F$^-$ *pss-8 nalA*	13
A324	F$^-$ *pss*$^+$ *proC lacI strA*	J. Beckwith
MV12/pLC11-9*	F$^+$/Δ*trpE thr leu recA*/ ColE1-*dnaB*$^+$	9
MV12/pLC34-44	F$^+$/Δ*trpE thr leu recA*/ ColE1-*pss*$^+$	9
MV12/pLC34-46	F$^+$/Δ*trpE thr leu recA*/ ColE1-*pss*$^+$	9
RA80/pLC34-44	F$^+$/*pss-8 nalA*/ColE1-*pss*$^+$	This work
RA80/pLC34-46	F$^+$/*pss-8 nalA*/ColE1-*pss*$^+$	This work
RA324	F$^+$/*pss*$^+$ *proC lacI strA*/ ColE1-*pss*$^+$	This work

* This was previously designated MV12/28 (10).

Purification to Homogeneity of Phosphatidylserine Synthase. For large-scale growth of RA324 (14–100 liters) a 6% inoculum was grown overnight to stationary phase in the presence of 10% (vol/vol) crude colicin E1; no additional colicin was present during the large-scale growth. A324 was grown in an identical manner in the absence of colicin. All glassware and fermentation equipment were sterilized before and after use. The synthase was extracted from cells (late logarithmic phase) of either A324 or RA324 and purified by a procedure scaled down from that previously described for frozen cells of *E. coli* B (3), except that only one phosphocellulose column was required.

RESULTS

Identification of Hybrid ColE1 Plasmids Carrying the *pss*$^+$ Gene. Amongst the 2000 strains of the collection we found two hybrid ColE1 plasmids that restore near-normal growth to RA80 at 44°. These plasmids (pLC34-44 and pLC34-46) do not correct temperature sensitivities resulting from mutations in distant chromosomal genes, such as *dnaB, dnaC, dnaZ,* and *dnaE* (10). Furthermore, these plasmids do not carry *tyrA*$^+$ (52% cotransducible with *pss-8*) or *purI*$^+$ (9% cotransducible with *pss-8*), as judged by their inability to correct mutations at these loci. However, both plasmids carry *nadB*$^+$ (33% cotransducible with *pss-8*) (13), because they render *nadB7* mutants prototrophic. The gene order (13) in the vicinity of *pss*$^+$ is *purI, nadB, pss, tyrA* (clockwise direction). Our data demonstrate that the host DNA inserted into both of these plasmids carries the *nadB-pss* interval (about ½ min), in good agreement with the average molecular weight (8×10^6) of the sheared, inserted DNAs used to construct the hybrid plasmids (9).

Specific Activity of Phosphatidylserine Synthase in Strains Carrying pLC34-44 and pLC34-46. Because hybrid ColE1 plasmids are maintained at 10–20 copies per chromosome (9),

Table 2. Overproduction of phosphatidylserine synthase in strains carrying specific hybrid ColE1 plasmids

Strain	Hybrid plasmid	Phosphatidylserine synthase, units/mg
RA800*	None	15
RA800†	None	10
A324	None	14
E. coli B‡	None	10
RA80†	None	0.2
MV-12	pLC11-9	17
MV-12	pLC34-44	140
MV-12	pLC34-46	110
RA80	pLC34-44	210
RA80	pLC34-46	170
RA324	pLC34-44	210

* Grown at 37°.
† Grown at 25°.
‡ See ref. 19.

we examined the activity of the synthase in organisms carrying pLC34–44 and pLC34–46. As shown in Table 2, ordinary *pss*$^+$ bacteria (RA800, A324, and *E. coli* B) have a specific activity of 10–15 units/mg of protein at 37°. An organism from the Clarke and Carbon collection, which contains the *dnaB* region (far removed from *pss*$^+$) on its hybrid plasmid (pLC11-9), has a specific activity similar to wild type. In contrast, MV12/pLC34–44 and MV12/pLC34–46 have specific activities which are six to eight times higher, presumably as a result of the increased gene dosage. High levels of phosphatidylserine synthase were also observed in the plasmid-corrected temperature-resistant mutant (i.e., in RA80/pLC34–44 and RA80/pLC34–46). Slightly higher specific activities were attained (about 15-fold above the wild type) when pLC34-44 was transferred into a different genetic background (A324). The resulting strain designated RA324 was used for large scale preparations. Strains that overproduce the phosphatidylserine synthase have normal amounts of phosphatidylglycero-*P* synthase (data not shown).

Purification to Homogeneity of Phosphatidylserine Synthase from Wild-Type and Overproducing Strains. To exclude the possibility of enzyme activation in these plasmid-bearing strains, we purified the synthase to homogeneity from RA324 and compared the enzyme to that purified from the parent strain A324. As expected for overproduction (rather than activation) the polypeptide previously identified as the synthase (3) required approximately a 300-fold purification from RA324, rather than 5000-fold from either A324 (Table 3) or *E. coli* B (3). Furthermore, the specific activity of the homogeneous enzyme was essentially the same as that of either the parent strain A324 or *E. coli* B (3). Enzymes from both sources behaved in a similar manner throughout the purifications, with proportional increases in specific activity except for the major purification step, i.e., the phosphocellulose affinity chromatography step. The synthases from both A324 and RA324 migrated as single protein species upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and had an apparent molecular weight of 54,000, as previously reported for the synthase from *E. coli* B (3). All final enzyme preparations were at least 98% homogeneous as judged by Coomassie blue staining.

Association of the Overproduced Synthase with Ribosomes. Phosphatidylserine synthase activity is present in extracts of most Gram-negative organisms. In *E. coli*, as in several other Gram-negative bacteria (A. Dutt and W. Dowhan, unpublished data), the enzyme is tightly bound to ribosomes in such extracts. It is not a major ribosomal protein, however, because there are only about 800 synthase molecules per cell as compared with 75,000 ribosomes (3). Although the significance of the ribosomal association of the synthase is unknown, it differs strikingly from all other enzymes of phospholipid biosynthesis, which are associated with the cytoplasmic membrane (20, 21).

Table 3. Purification of phosphatidylserine synthase

Step	A324* Specific activity, units/mg	A324* Yield, %	RA324† Specific activity, units/mg	RA324† Yield, %
1. Broken cells	7.1 (11)‡	100	120 (170)‡	100
2. Cell supernatant	7.3	85	140	97
3. Streptomycin sulfate	27	88	400	83
4. Polymer partitioning	21	42	320	50
5. Ammonium sulfate	47	33	820	42
6. Phosphocellulose	—	20	—	19
7. DEAE-Sephadex	34,000 ±15%§	16	39,000 ±15%§	13
Protein yield	1.9 mg		8.5 mg	

* Started with 320 g wet weight of cells.
† Started with 150 g wet weight of cells.
‡ The activity in parentheses was determined at 37° as described for assays of small cultures (Table 2). For the actual purification of the synthase, the enzymatic activity was determined at 30° under the conditions described by Larson and Dowhan (3). All assays of the homogeneous enzyme must be carried out at 30° because of the apparent instability of the homogeneous enzyme at 37°. When dilutions of the enzyme were required prior to assay, 0.1 M potassium phosphate (pH 7.2) containing bovine serum albumin at 0.5 mg/ml and 0.1% (wt/vol) Triton X-100 was used.
§ Standard deviation of four determinations of the enzymatic activity.

Fig. 2 shows the subcellular distribution of the synthase in extracts of the overproducer MV12/pLC34–44. Most of the activity sediments with the major peak of absorbance near the middle of the gradient, previously demonstrated to represent ribosomes in wild-type strains (15). This profile is very similar to that of wild-type strains (15), indicating that ribosomes have a large excess capacity to bind the enzyme. This renders unlikely the possibility of a special subclass of ribosomes, capable of binding the enzyme. However, these results do not shed any light on a possible physiological role for the ribosomal association.

Phospholipid Composition of the Phosphatidylserine Synthase Overproducers. Table 4 shows that the phospholipid composition of two synthase overproducers (MV12/pLC34–44 and RA324) is not strikingly different from that of the wild type. Although the combined amounts of phosphatidylglycerol and cardiolipin are slightly less than in normal strains, the polyglycerophosphatides are not diluted out by a factor of 10–15, as would be expected if the level of phosphatidylserine synthase alone were rate-limiting for phosphatidylethanolamine synthesis *in vivo*.

DISCUSSION

While molecular cloning techniques have been particularly useful for the isolation of specific nucleic acid sequences (6–8),

Table 4. Phospholipid composition of phosphatidylserine synthase overproducers

Strain	Plasmid	Phosphatidylserine synthase, units/mg	Distribution of lipid ^{32}P, % of total		
			PE	PG	CL
Exp. 1					
A324	None	14	86.0	8.1	5.9
RA324	pLC34-44	209	89.2	7.0	3.8
Exp. 2					
MV12	pLC11-9	17	85.3	10.1	4.6
MV12	pLC34-44	136	88.6	8.3	3.1

Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

the present results demonstrate that hybrid ColE1 plasmids are also very useful for the overproduction and large scale preparation of constitutive biosynthetic enzymes. The availability of large amounts of phosphatidylserine synthase will be particularly valuable for further enzymological and chemical studies. For instance, kinetic data (3, 19) strongly suggest the participation of a novel, covalent phosphatidyl-enzyme intermediate in catalysis, which may provide a mechanism for a transient association of the enzyme with the membrane (3). This can now be investigated directly. Similarly, a separate region of the protein responsible for binding to ribosomes has also been postulated (3, 19).

The present studies strongly support the notion that the region around *nadB* contains the structural gene for the phosphatidylserine synthase. The finding that pLC34-44 and pLC34-46 carry *nadB*$^+$ in addition to *pss*$^+$ provides independent confirmation for the previous genetic mapping of mutants (such as *pss-8*) that are defective in enzyme activity (13).

We have attempted (but have not succeeded) to obtain even higher levels of the synthase by treating growing cultures of RA324 with mitomycin C, which greatly enhances colicin production in strains carrying the wild-type ColE1 plasmid (22). It may be possible to isolate mutants of *E. coli* that maintain the hybrid plasmids at a higher number of copies per chromosome than normal. Alternatively, it may be possible to construct hybrid plasmids with the recently described mini-ColE1 variant, which is maintained at 50 or more copies per host genome (23).

Overproduction of constitutive protein gene products has recently been described in several other strains of the Clarke and Carbon collection (10, 11). For instance, several factors involved in DNA replication (*dnaB*, *dnaC*, and *dnaZ*), which can be assayed by complementation *in vitro*, are also over-

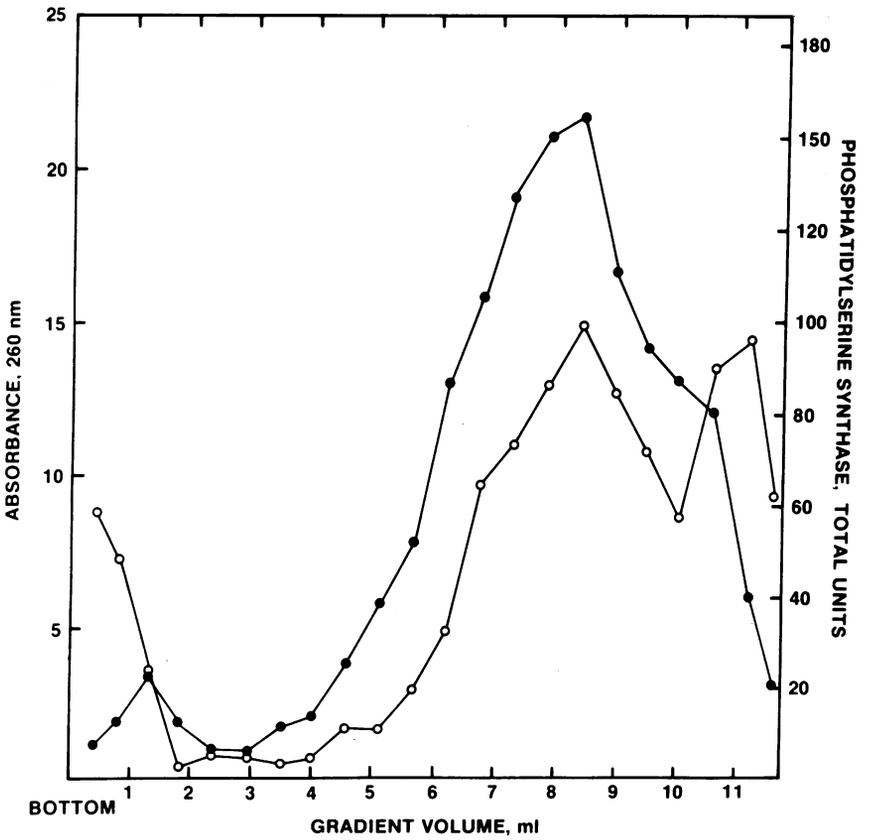

FIG. 2. Subcellular distribution of phosphatidylserine synthase. A sample of cell-free extract from MV12/pLC34-44 was centrifuged through a 5–20% sucrose gradient with a 1 ml layer of 70% sucrose at the bottom at 40,000 rpm for 90 min at 4° in a Beckman SW 41 rotor (15). The buffer used throughout was 10 mM Tris·HCl (pH 8.0) containing 2 mM $MgCl_2$. Absorbance (○) and enzymatic activity (●) were determined after fractionating the gradient.

produced in strains carrying the appropriate hybrid plasmids (10, 11). Furthermore, several hybrid plasmids have been found carrying genes for two other enzymes involved in phospholipid biosynthesis, and these are also presumably overproduced.[§] As yet, however, the purification to homogeneity of these factors has not been reported, so that the possibility of activation (rather than true overproduction) cannot be eliminated.

The ready availability of overproducers provides a new approach to the study of the regulation of this and other pathways. The relatively normal membrane lipid composition of RA324 and MV12/pLC34-44 suggests that the synthase polypeptide is normally present in excess; therefore, the regulation of the ratio of phosphatidylethanolamine to polyglycerophosphatides must involve other factors, such as the intracellular concentration of precursors or the accessibility of the enzyme to its membrane-bound substrate.

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