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Isolation of *Escherichia coli* Mutants Defective in Enzymes of Membrane Lipid Synthesis

(colony autoradiography/enzyme screening/CDP-diglyceride/phosphatidylserine/phosphatidylglycerophosphate)

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ABSTRACT **A new method has been developed which permits the rapid screening of *E. coli* colonies for mutants with defective enzymes of phospholipid metabolism. In this procedure, a disc of filter paper is pressed down on an agar plate containing several hundred colonies of mutagen-treated cells, after which the paper is lifted off. In the process the colonies are transferred to the paper, giving rise to a replica print of the master plate. The few cells from each colony left on the master keep growing in the original pattern. The pattern of colonies is also retained on the filter paper, even after the cells are rendered permeable with lysozyme and EDTA. Colonies treated in this manner remain adsorbed to the paper, where they can convert *sn*-[*U*-^{14}C]glycero-3-*P* to phosphatidyl[*U*-^{14}C]glycerophosphate, dependent on added CDP-diglyceride. Unrelated reactions of *sn*-[*U*-^{14}C]glycero-3-*P* that may obscure the synthesis of phosphatidylglycerophosphate are inhibited by the addition of reagents poisoning energy generation. The radioactive phospholipid that forms around each colony on the paper is precipitated *in situ* with trichloroacetic acid, and unreacted *sn*-[*U*-^{14}C]glycero-3-*P* is washed away. After autoradiography, the colonies on the filter paper are stained with Coomassie blue. When the autoradiogram is superimposed on the stained paper, mutants are identified as blue colonies lacking a black halo. With this method, 20,000 colonies were screened in several days. Four mutants were identified with low levels of CDP-diglyceride:*sn*-glycero-3-*P* phosphatidyl transferase (EC 2.7.8.5, glycerolphosphate phosphatidyltransferase, phosphatidylglycerophosphate synthetase) in extracts. With a similar assay, 10,000 additional colonies were screened for mutants with altered CDP-diglyceride:L-serine *O*-phosphatidyltransferase (EC 2.7.8.8, phosphatidylserine synthetase), and four strains were found in which the enzyme is thermolabile. The screening technique described here is termed replica printing and should be applicable not only to studies of phospholipid metabolism but also to nucleic acid and protein synthesis.**

Enzymes found in cell-free extracts of *Escherichia coli* catalyze the conversion of CDP-diglyceride to phosphatidylserine and phosphatidylglycerophosphate (1, 2). *In vitro*, this biosynthetic branchpoint leads to the formation of the three main phospholipids found in the membrane of this organism, which are phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (Scheme 1). Phosphatidic acid, CDP-diglyceride, and phosphatidylserine are also present in small amounts in living cells, and they are rapidly metabolized (3–5), consistent with the pathways of Scheme 1.

Abbreviation: *sn*-glycero-3-*P*, L-α-glycerophosphate.

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Although genetic techniques have been employed in studies of fatty acid biosynthesis and acylation of glycerophosphate (5), very few mutants have been described in the later steps of phospholipid metabolism (5–8). The present work was undertaken in order to obtain mutants at the CDP-diglyceride branchpoint leading to phosphatidylserine or phosphatidylglycerophosphate (Scheme 1, reactions 1 and 2). Strains with lesions in these enzymes should be useful in studying the regulation of phospholipid synthesis *in vivo* and might also shed some light on the processes by which membrane lipid and membrane protein synthesis are coordinated.

Isolation of mutants in phospholipid enzymes has been particularly difficult, since appropriate selection techniques have not been found (5). The same problem has frequently been encountered with enzymes involved in macromolecular synthesis, such as the DNA polymerases of *E. coli* (9, 10).

In the present work, a new method is described, called replica printing (because of its similarity to replica plating), which permits rapid screening of thousands of colonies of mutagenized (treated with mutagen) cells per day for defects in specific enzymes of phospholipid synthesis. In this procedure colonies are transferred from an agar plate to a disc of filter paper, termed a replica print. On the paper, the colonies can be treated with lysozyme and assayed for lipid-synthesizing enzymes, using an adaptation of the methods of Olivera and Bonhoeffer (11) for detecting DNA polymerase activity in colonies of *E. coli* on Millipore filters. With replica printing, 20,000 colonies were screened in several days, and four strains were identified with very low levels of phosphatidylglycerophosphate synthetase (EC 2.7.8.5, glycerolphosphate phosphatidyltransferase; CDP-diglyceride: *sn*-glycerol-3-phosphate phosphatidyl transferase) (Scheme 1, reaction 2) in extracts. No mutants of this type have been reported previously. Four other mutants with thermolabile phosphatidylserine synthetase (EC 2.7.8.8, CDP-diglyceride: L-serine *O*-phosphatidyltransferase) (Scheme 1, reaction 1) were also obtained. Replica printing should be applicable not only to enzymes of phospholipid metabolism but also to enzymes of nucleic acid and protein synthesis.

MATERIALS AND METHODS

Materials. DL-[3-^{14}C]Serine, *sn*-[*U*-^{14}C]glycerol-3-*P*, and Triton X-100 were products of the New England Nuclear Corp., Boston. CDP-Diglyceride was prepared and stored as reported previously (4), except that phosphatidic acid was made enzymatically from egg lecithin (12). Egg lecithin was isolated by solvent fractionation and chromatography on

alumina (13). CDP-Diglyceride tritiated in the cytidine moiety was prepared enzymatically (14).

Bacterial Strains and Mutagenesis. E. coli strain DJ 105, kindly provided by Dr. Max Gottesman, was the parent of the mutants obtained in the present work. DJ 105 is a derivative of *E. coli* K-12, is closely related to CSH 57 (15), and is a multiply marked female (*ara, leu, lacY, proC, gal, trp, his, malA, strA, xyl, mtl, ilv, argG, metA, thi, purE*). The specific activities of phosphatidylserine and phosphatidylglycerophosphate synthetases in this strain were nearly the same as those of *E. coli* K-12 and *E. coli* B.

A stock of mutagenized cells was prepared from a culture of DJ 105 under standard conditions (15), using 100 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 37° for 60 min. About 95% of the cells were killed, while 20% of the survivors had new growth requirements and 4% were temperature sensitive. After segregation in broth (15), the mutagenized cells were diluted 10-fold into LB broth (15) containing glycerol (31 g/100 ml) and were stored at −25°. For replica printing, the mutants were plated on nutrient agar, containing 8 g of nutrient broth powder (Difco), 15 g of agar, 5 g of NaCl, and 1 g of dextrose per liter.

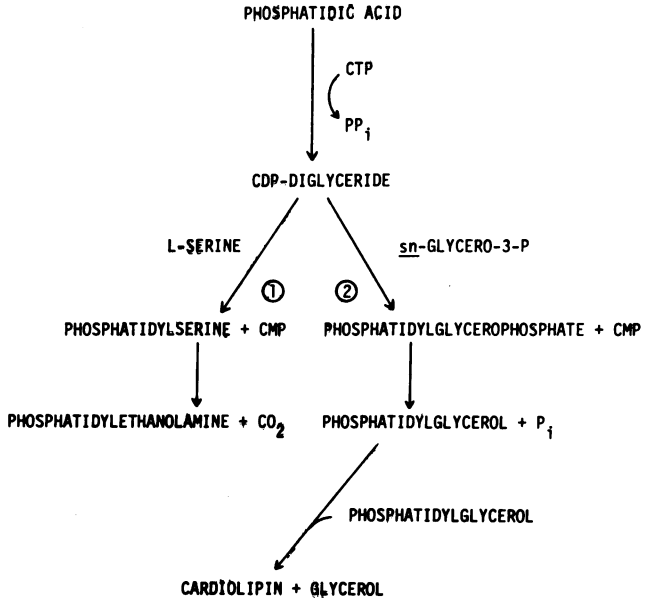
Preparation of Cell Extracts. To prepare extracts for enzyme assays, we grew strains on LB broth (15) in a rotary shaker at 30°. At a density of 3×10^8 cells per ml, 25 ml of culture was centrifuged for 10 min at $5000 \times g$. The pellet was resuspended in 3 ml of cold 20 mM Tris·HCl buffer, pH 8. Cells were disrupted by sonic irradiation (16). Protein concentration was measured by the method of Lowry *et al.* (17).

Enzyme Assays. Phosphatidylserine synthetase was assayed as previously described (16), except that the concentration of DL-serine was 2 mM. Phosphatidylglycerophosphate synthetase was assayed by the method of Chang and Kennedy (2) with a final $MgCl_2$ concentration of 30 mM. Synthesis of radioactive phospholipid was determined both by chloroform extraction (16) and by trichloroacetic acid precipitation (18) with no differences noted. Extracts were assayed at various temperatures, as indicated in the *Results* section. CDP-Diglyceride hydrolase activity was measured by the procedure of Raetz *et al.* (19).

RESULTS

Isolation of Mutants in Phosphatidylglycerophosphate Synthetase by Replica Printing. Nutrient agar plates (9-cm diameter), each spread with about 400 mutagenized cells, were incubated at 30° for 36 hr. A dry filter paper disc (Whatman no. 42, 9-cm diameter) was then gently pressed down on the agar surface, after which the paper was slowly lifted off. This procedure resulted in the transfer of the colonies to the paper disc (referred to as a replica print in the following discussion). The few cells from each colony remaining on the master plate continued to grow at 30° without significant distortion of the pattern. Most colonies became visible again on the master plate after 24 hr, and viable cells could be recovered even after several weeks of storage at 4°. Adsorption of colonies on filter paper has been described by other investigators (15, 20), and paper can be used instead of velveteen in conventional replica plating (15).

The bacteria in the colonies adsorbed to the filter paper were rendered permeable with lysozyme and EDTA. For this purpose 1.5 ml of a solution containing 10 mg/ml of egg lysozyme and 10 mM disodium EDTA, pH 6, were spread over the bottom of a glass petri dish (9-cm diameter), and the replica



SCHEME 1. Enzymatic synthesis of phospholipids in *Escherichia coli*.

print was placed in this solution with the colonies facing upwards. (In all manipulations described below, the replica print was transferred facing upward in order to preserve the pattern of colonies.) After the paper was saturated, it was incubated for 30 min at room temperature. Following this the replica print was placed on a dry paper towel to remove excess liquid and was then transferred to another petri dish containing 1 ml of a solution that poisons energy-generating systems (2 mM 2,4-dinitrophenol, 20 mM sodium azide, 10 mM KF, 1 mM Na_2HAsO_4, and 4 mM Tris·HCl of pH 8). These compounds inhibit unrelated metabolism of the radioactive precursors employed below, which may obscure phospholipid synthesis by the permeable colonies. Phosphatidylglycerophosphate and phosphatidylserine synthetase activities are not affected by these inhibitors in assays of extracts prepared from the parent strain.

After being saturated with energy poisons, the replica print was rapidly frozen by touching the bottom of the petri dish to a bath of *n*-propyl alcohol cooled with dry ice. The colonies were allowed to thaw for 15 min at room temperature, and the cycle was then repeated a second time. Following this, the replica print was blotted on a paper towel, as described above, and incubated in a dry petri dish for 20 min in a water bath at 70°. The phosphatidylglycerophosphate synthetase of the parent strain was stable under these circumstances. However, heating reduced unrelated reactions of *sn*-[*U*-^{14}C]-glycero-3-*P* and increased phosphatidylglycerophosphate synthesis by the colonies on the replica print, possibly by improving cell lysis. The original pattern of colonies was retained even after these treatments.

Following the incubation at 70°, the filter paper was transferred to another petri dish in a water bath at 42°, and containing 1.5 ml of a reaction mixture used for detecting phosphatidylglycerophosphate synthetase in extracts. The composition of this solution, which contains CDP-diglyceride and *sn*[*U*-^{14}C]glycero-3-*P*, is shown in the legend to Fig. 1. It is almost identical to that employed by Chang and Kennedy (2) to assay the synthetase in conventional cell-free preparations, in which the incorporation of *sn*-[*U*-^{14}C]glycero-3-*P* into

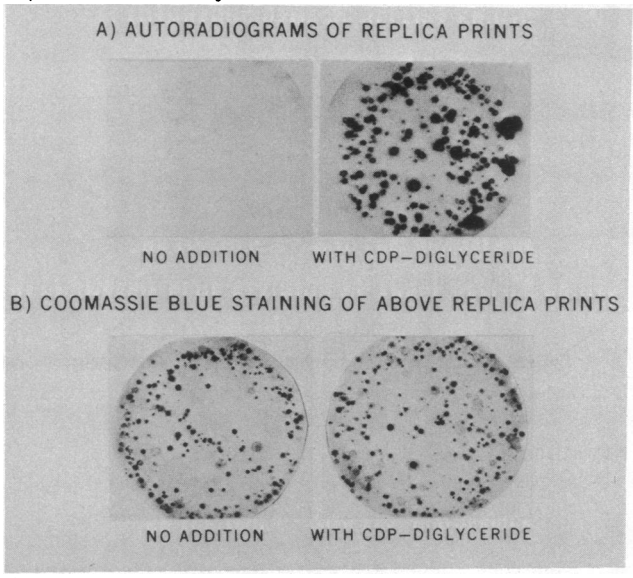


FIG. 1. Autoradiographic assay of phosphatidylglycerophosphate synthetase in colonies on replica prints. The assay mixture contained 0.8 mM *sn*-[*U*-^{14}C]glycero-3-*P* (10^4 cpm/nmol), 30 mM $MgCl_2$, 0.25 M Tris·HCl, pH 8, 5 mM 2-mercaptoethanol, 2 mg of Triton X-100 per ml, 2 mM 2,4-dinitrophenol, 10 mM KF, 1 mM Na_2HAsO_4, 20 mM sodium azide, and 0.1 M glycerol. The replica print in the right-hand side of the figure was also incubated in the presence of 0.13 mM CDP-diglyceride (which was omitted for the replica print on the left). In general, autoradiograms were developed for 6 days at −80° (to prevent chemical reactions between residual trichloroacetic acid and the film), using Kodak medical x-ray film (RP/R54). Following autoradiography, replica prints were stained for 10 min at 25° with 0.5 mg/ml of Coomassie brilliant blue dissolved in 10% (v/v) acetic acid. The prints were then destained for 48 hr in methanol:water:acetic acid (45:45:10 v/v). Stained prints corresponding to the autoradiograms in panel A are shown in the lower half of the figure (panel B). The original diameter of the discs was 9 cm. There is a slight difference in magnification, however, and the damaged edges of the discs shown in panel B were cut off after staining.

phosphatidylglycerophosphate is dependent on added CDP-diglyceride.

After 30 min at 42°, phosphatidylglycerophosphate synthesis was stopped by transferring the replica print to a petri dish containing 2 ml of 0.2 g/ml trichloroacetic acid solution, chilled to 4°. DL-Glycero-3-*P* (20 mM) was included in the trichloroacetic acid solution as carrier to prevent nonspecific binding of labeled *sn*-glycero-3-*P* to the paper. Exposure to trichloroacetic acid resulted in the precipitation of phosphatidyl[*U*-^{14}C]glycerophosphate as a halo surrounding each colony on the replica print.† After 10 min, unreacted *sn*-[*U*-^{14}C]glycero-3-*P* was washed away with five portions (50 ml each) of cold trichloroacetic acid (0.1 g/ml). This was performed on a Büchner funnel under gentle suction with the colonies facing upwards. The washed replica print was dried in an oven at 120° for 60 min, after which the colonies appeared as pale, yellow spots on the paper.

† In crude extracts some of the phosphatidylglycerophosphate formed is rapidly converted to phosphatidylglycerol and inorganic phosphate. The product distribution of the radioactive phospholipid precipitated on the filter paper was not determined.

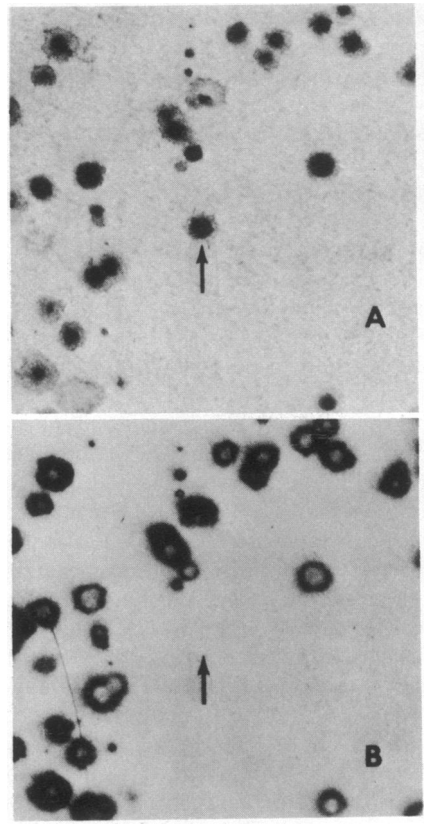


FIG. 2. Identification of a mutant in phosphatidylglycerophosphate synthetase by comparison of a stained replica print with the corresponding autoradiogram. Panel A shows an area of a stained replica print (about 3 cm × 3 cm in the original) in which a mutant was found. This was done by comparing it to the corresponding autoradiogram shown in panel B. The arrow indicates the position of a colony that did not synthesize any radioactive phosphatidylglycerophosphate under these conditions. This strain (RH 10) was subsequently found to lack phosphatidylglycerophosphate synthetase when cell-free extracts were assayed by conventional methods. In practice, mutants can be identified more rapidly by superimposing the autoradiogram (panel B) on the blue replica print (panel A).

The amount of radioactive lipid synthesized in the presence of CDP-diglyceride around each colony was detected as a halo by autoradiography, as shown in the right-hand panel of Fig. 1A. The pattern of colonies is well preserved in the autoradiogram, since even small and closely adjacent colonies can be discerned. In contrast, almost no radioactive phospholipid was formed in the absence of CDP-diglyceride, as shown in the autoradiogram of the replica print in the left-hand panel of Fig. 1A. Omission of other factors required for phosphatidylglycerophosphate synthesis in cell extracts, such as Mg^{2+} and Triton X-100, resulted in similar nonradioactive colonies.

Interpretation of the autoradiograms was facilitated by staining the colonies on the replica prints with Coomassie blue after autoradiography. This served to locate all colonies, including mutants. The stained replica prints, corresponding to the autoradiograms of Fig. 1A, are shown in Fig. 1B. A few lightly colored colonies on the stained replica prints did not reproduce very well during photography.

A mutant in phosphatidylglycerophosphate synthetase can be identified by inspection of an autoradiogram superimposed on its Coomassie-blue-stained replica print. This reveals a

TABLE 1. *Specific activity of phosphatidylglycerophosphate synthetase in extracts of mutants obtained by replica printing*

Strain	Specific activity, units/mg of protein
Exp. 1	
Parent	0.53
RH 2	0.058
RH 4	0.019
RH 9	0.018
RH 10	0.028
Exp. 2	
Parent	0.50
RH 2	0.060
RH 4	0.005
RH 9	0.005
RH 10	0.008

Extracts were assayed at 25° without preincubation. The assay mixture used in Exp. 2 was the same as that of Exp. 1, except that it also contained 1.5 mM 2,4-dinitrophenol and 15 mM sodium azide. A unit of activity is that amount of enzyme which catalyzes the formation of a nanomole of phospholipid in 1 min.

mutant as a blue colony, lacking a black halo. Alternatively, the print and the autoradiogram can be compared side by side. Panel A of Fig. 2 shows an area (3 cm × 3 cm in the original) of a stained replica print in which a mutant was found by comparison to the corresponding autoradiogram shown beneath it in panel B. This colony (strain RH 10), identified by the arrow, made no radioactive phosphatidylglycerophosphate under the conditions of the incubation.

Properties of Phosphatidylglycerophosphate Synthetase in Extracts of Mutants Obtained by Replica Printing. Fifty plates (or about 20,000 colonies) were screened by the method described above. Six potential mutants were identified, and single colonies were purified from the original master plates. Four of these strains proved to have low levels of phosphatidyl glycerophosphate synthetase by the conventional assay (2) in extracts prepared from exponentially growing cells (Table 1, Exp. 1). The specific activity of the synthetase was about 10% of normal in strain RH 2, while it was only about 4% of normal in the three other strains of Table 1. The residual activity in these three strains (RH 4, RH 9, and RH 10) differed from that of the parent, however, in that it was inhibited by 2,4-dinitrophenol and sodium azide (Table 1, Exp. 2).

The strains shown in Table 1 grew on nutrient agar at temperatures ranging from 20° to 42°. Cells grown in liquid medium at 30° were deficient in phosphatidylglycerophosphate synthetase activity when extracts were assayed at either high or low temperatures. The same low activities were observed whether CDP-diglyceride (derived from egg lecithin), CDP-dipalmitin, or dCDP-dipalmitin were employed as substrates. Assays of mixed extracts indicated that the mutants did not contain an inhibitor of the wild-type enzyme. All strains isolated in the course of the present work were shown to be derivatives of the parent, DJ 105, by their inability to ferment galactose and lactose, by their requirement for histidine, and by their resistance to streptomycin.

Thin-layer chromatography of phospholipids extracted from strains RH 2, RH 4, RH 9, and RH 10 (grown at 30°) was performed as described by Ames (21). Preliminary qualitative analyses of these chromatograms by iodine staining (21) indicate that an appreciable amount of phosphatidylglycerol is still present *in vivo*, in spite of the low levels of phosphatidylglycerophosphate synthetase observed *in vitro*.

TABLE 2. *Thermolability of phosphatidylserine synthetase in extracts of parent and mutant strains*

Strain	No preincubation, units/mg of protein	Preincubated at 42°, units/mg of protein
Parent	6.88	6.06
RA 3	2.20	<0.1
RA 5	4.44	0.22
RA 8	0.81	0.30
RA 20	0.91	0.46

Cell extracts were preincubated, where indicated, at 42° for 60 min. Assays of enzymatic activity were performed at 25°. A unit of activity is defined in Table 1.

Isolation of Mutants Defective in Phosphatidylserine Synthetase. A minor modification of the method described above was used to obtain phosphatidylserine synthetase mutants. First, the replica prints were preincubated for 20 min at 42°, instead of 70°, since the enzyme activity was unstable to 70°. Second, the reaction mixture differed from that described in Fig. 1 in that it contained 0.3 mM CDP-diglyceride, 2 mM, DL-[3-^{14}C]serine (10^4 cpm/nmol), 0.1 M potassium phosphate pH 7.4, 0.1% Triton X-100, 2 mM 2,4-dinitrophenol, 20 mM sodium azide, 10 mM KF, and 1 mM Na_2HAsO_4. Although residual incorporation of radioactive serine into trichloroacetic-acid-precipitable material was not completely eliminated in the absence of CDP-diglyceride, mutant candidates could still be identified. However, three-quarters of these were false positives.

Table 2 shows the specific activity of phosphatidylserine synthetase in four strains which were found after a total of 10,000 colonies were screened. Two of these strains (RA 3 and RA 5) had considerable activity when extracts were assayed at 25° without a preincubation at 42°. In contrast to the parent, all mutant extracts, particularly that of strain RA 3, were reduced in activity by preincubation at 42° (Table 2). Strains RA 3 and RA 8 were found to be temperature sensitive for growth at 42° when tested on nutrient agar plates.

Enzymes of CDP-Diglyceride Metabolism in Mutant Strains. Mutants defective in phosphatidylglycerophosphate synthetase were found to have normal phosphatidylserine synthetase and CDP-diglyceride hydrolase activities (Table 3, Exp. 1). Conversely, mutants with a defective phosphatidylserine synthetase generally had normal amounts (within a factor of 2) of phosphatidylglycerophosphate synthetase and CDP-diglyceride hydrolase (Table 3, Exp. 2). The only exception to this was the low level of CDP-diglyceride hydrolase observed in extracts of strain RA 8, although this may be a separate mutation. CDP-Diglyceride hydrolase is a pyrophosphatase associated with the inner membrane of *E. coli*, which catalyzes the conversion of CDP-diglyceride to phosphatidic acid and CMP (19).

DISCUSSION

The replica printing technique described here offers a simple, rapid method for obtaining many types of mutants in enzymes of phospholipid biosynthesis. The method requires that the reaction under study involves the conversion of a water-

TABLE 3. *Specific activity of phospholipid enzymes in extracts of various mutant strains*

Strain	Phosphatidylglycerophosphate synthetase, units/mg of protein	Phosphatidylserine synthetase, units/mg of protein	CDP-Diglyceride hydrolase, units/mg of protein
Exp. 1			
Parent	0.53	5.15	0.95
RH 2	0.058	6.18	1.24
RH 4	0.019	4.91	0.47
RH 9	0.018	5.30	1.15
RH 10	0.028	5.66	0.97
Exp. 2			
Parent	0.53	6.06	0.90
RA 3	0.26	<0.1	0.87
RA 5	0.32	0.22	0.70
RA 8	0.24	0.30	<0.1
RA 20	0.28	0.46	0.40

The assays in Exp. 1 were performed at 25° without preincubation. Assays in Exp. 2 were also carried out at 25°, except that the extracts were preincubated for 60 min at 42° just prior to assay. All three enzymes were stable at 42° in the parent strain. A unit of activity is defined in Table 1.

soluble substrate to a product that is precipitable with trichloroacetic acid. Since most phospholipids share this property with polynucleotides and proteins, replica printing should be applicable to many systems. Because replica printing does not involve manipulations of individual colonies, it is much more rapid than the screening technique recently described by Weiss and Milcarek (22). However, reactions in crude extracts not related to the enzyme under study may interfere and must be inhibited.

Several other applications of this technique merit further consideration. First, it should be very useful in genetic mapping, since the same method used to isolate the original mutants can be used to analyze enzymatic activities in recombinants of matings and transductions. Furthermore, replica printing should facilitate the biochemical characterization of survivors of specific selection techniques, such as glycerophosphate or serine suicide (23, 24). Finally, the method should be applicable to other organisms, such as *Saccharomyces cerevisiae*, which, like *E. coli*, adhere tightly to filter paper.

Mutants lacking phosphatidylglycerophosphate synthetase have not been reported previously. The results of Table 1 (Exp. 1) demonstrate that these mutants had no more than one-tenth of the specific activity of the parent in crude extracts. Furthermore, the residual activity in three of these strains (RH 4, RH 9, and RH 10) differed from the enzyme of the parent in its sensitivity to energy poisons (Table 1, Exp. 2). In the presence of these inhibitors, extracts of RH 4, RH 9, and RH 10 had about 1% of the activity of the wild type.

Mutants in which the activity of phosphatidylserine synthetase is thermolabile in extracts have also not been described prior to the present work. However, Kito and coworkers (7) have recently employed the serine suicide technique of Cronan (24) to obtain one strain that lacks phosphatidylserine synthetase entirely. This organism, which was temperature sensitive for growth, was found after several hundred survivors of the suicide selection were examined individually.

Surprisingly, few of the mutants isolated in the present study had growth defects that might be related to the enzymatic lesions observed in extracts. Only two of the four phosphatidylserine synthetase mutants (and none of the four phosphatidylglycerophosphate synthetase mutants) were temperature sensitive for growth on nutrient agar. These observations raise the possibility that some of the enzymatic defects noted *in vitro* are not expressed in living cells. The isolation of phosphatidylglycerol from the four strains lacking phosphatidylglycerophosphate synthetase in extracts supports this notion. Perhaps the phosphatidylglycerophosphate synthetase is stabilized by the internal environment of the cell or is present in a large excess in the wild type. However, a second pathway for phosphatidylglycerophosphate synthesis cannot be excluded. Further enzymological studies, in addition to genetic mapping, which can be done using the replica printing technique, should clarify these alternatives.

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