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PHOSPHOLIPASE A-DEFICIENT MUTANTS OF *ESCHERICHIA COLI* B

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SUMMARY Phospholipase A-deficient mutants were isolated from *Escherichia coli* B/SM as follows. Replica plates were incubated to allow formation of colonies and then overlayed with soft agar containing washed sheep erythrocytes, lecithin Ca^{++}, colistin, lysozyme and streptomycin. The mutant colonies were detected as colonies without hemolytic zones. Two or three cycles of treatment with mutagen and selection were necessary for their isolation. The mutants obtained could grow in a synthetic medium with glucose as the sole carbon source, and their phospholipid compositions were similar to that of the parent. They also gave the same agglutination titer as the parent with rabbit antiserum against the parent strain.

They supported the growth of all members of the T-series of bacteriophages as effectively as the parent. Some hemolytic substance was produced from either lecithin or bacterial constituents when the mutants were infected with T even phages, but not with T odd phages.

When the parent strain was infected with either T1 or T4, free fatty acids (FFA) were produced in the cell debris. Only a trace of FFA was formed in the debris of one of these mutants on infection with T4 and no FFA was formed on infection with T1.

INTRODUCTION

In 1970, we isolated phospholipase A-deficient mutants of *Vibrio parahaemolyticus* to analyze the hemolysins and phospholipid-degrading enzymes of this organism (Yanagase et al., 1970). During studies on alteration of phospholipid in the surface structure of *Escherichia coli* in the immune bactericidal reaction (Inoue et al., 1974a, b), a similar mutant was required. *E. coli* is known to possess membrane-bound phospholipase A enzyme which is not released extracellularly, while *V. parahaemolyticus* produces extracellular phospholipase A, which can easily be detected. Recently Ohki et al. (1972) described a phospholipase A-deficient mutant of *E. coli* K12. To isolate this mutant they treated a detergent-sensitive mutant of *E. coli* with mutagen and then selected mutant colonies which could not support growth of a fatty acid auxotroph. However, this mutant seemed unsuitable for

our purposes because its detergent-sensitive character, derived from the parent, might produce some inherent modification of the cell envelope and so complicate interpretation of results.

The principle of our selection procedure is based on the lytic effect of polymyxin antibiotic plus lysozyme on Gram-negative bacteria (Warren et al., 1957) and on the hemolytic activity of lyso-compounds produced by phospholipase A from phospholipid (Fig. 1). The treatment with mutagen and selection had to be repeated several times probably because this organism has more than one kind of phospholipase A.

MATERIALS AND METHODS

1. *Bacteria*

E. coli B/SM, stain 1–1 was obtained by selecting spontaneous mutants resistant to streptomycin from *E. coli* B, strain Hershey. It can grow in medium containing up to 500 μg/ml of streptomycin. *E. coli* K12, W3110(λ) and W3110 were used as the source of the phage and as the indicator, respectively, in studies on the sensitivity of the mutants to λ phage.

2. *Bacteriophages*

Bacteriophages T1, T2L, T3, T4D, T5, T6 and T7 were obtained by courtesy of Dr. A. Matsushiro, Department of Microbial Genetics, Research Institute for Microbial Diseases, Osaka University.

3. *Culture Media*

Tripticase soy broth (TSB) (BBL, Cockeysville, Md.) and modified Tris glucose medium (Echols et al., 1961) containing 0.1% glucose were used. Bacteria were labelled with ^{14}C, by growing them in Tris medium containing 0.03% unlabelled glucose plus 0.5 μCi/ml of ^{14}C-[U]-glucose (specific activity =5.0 mCi/m mole).

4. *Extraction of lipids and thin-layer chromatography (TLC)*

Lipids were extracted by the method of Bligh and Dyer (1959). The lipid fractions were applied to silica gel on an aluminum sheet (5553, E. Merck AG, Darmstadt, Germany). The gel was developed with chloroform-methanol-water (65: 25: 4, by vol.). For two dimensional analysis chloroform-methanol-7M ammonia water (60: 35: 5, by vol.) was used as the first solvent system and chloroform-methanol-acetic acid (65: 25: 8, by vol.) as the second solvent system.

Autoradiography was carried out by exposing the sheet to Fuji X-ray film, medical, Kx (Fuji Photo Film Co., Tokyo) for 5 to 7 days.

5. *Materials*

^{14}C-Phosphatidylethanolamine (^{14}C-PE) was prepared from ^{14}C-labelled *E. coli* B/SM. The lipid fraction extracted from the labelled bacteria was separated on TLC. The silica gel in the area corresponding to the spot of PE on the autoradiogram was scraped off. The PE was extracted with chloroform-methanol mixture (1: 1, v/v) and purified by rechromatography.

^{14}C-[U]-Glucose (specific activity=5.0 mCi/m mole) was obtained from Daiichi Pure Chemicals, Tokyo. Colistin (polymyxin E) was purchased from Banyu Pharmaceutical Co., Tokyo. Streptomycin sulfate was obtained from Takeda Chemical Industries, Osaka. Egg-white lysozyme (3×crystallized) was purchased from Sigma Chemical Co., St. Louis, Mo. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from K & K Laboratory, New York, N.Y. Egg yolk lecithin was obtained from Sumitomo Chemical Co., Osaka. Purified phospholipase A from " Habu " (*Trimeresurus flavoviridis*) venom was provided through courtesy of Dr. R. Murata, National Institute of Health of Japan, Tokyo. Rabbit antiserum against *E. coli* B was obtained by immunizing rabbits with a suspension of bacteria killed by heating it at 56 C for 60 min. The antiserum was inactivated by heating it at 56 C for 60 min and stored in the frozen state at −20 C without addition of any preservative.

RESULTS

1. *Isolation of mutants deficient in phospholipase A*

Fig. 1 shows an outline of the procedure for isolation of mutants.

An overnight culture of the parent strain, *E. coli* B/SM, strain 1–1, grown in trypticase soy broth (TSB) at 37 C was diluted 1/20 with fresh TSB. After incubation at 37 C for 2 to 3 hr with mechanical shaking, the bacteria were harvested by centrifugation, and washed twice with 0.1 M Tris-maleate buffer, pH 6.0. They

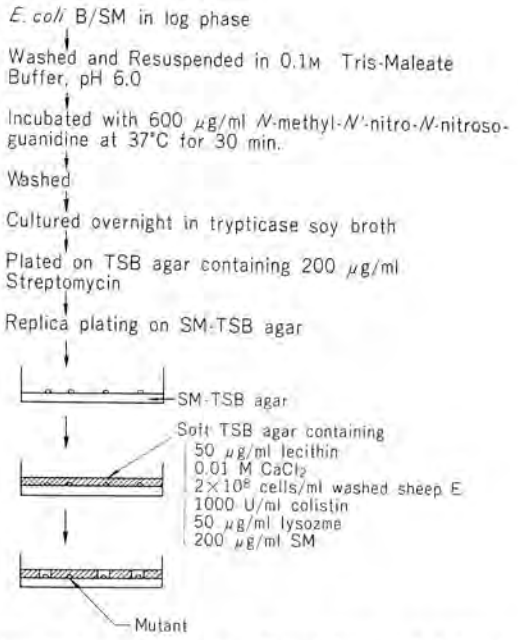

FIGURE 1. *Isolation of Phospholipase A-deficient Mutants of E. coli B.*

were suspended in the same buffer and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added at a final concentration of 600 μg/ml (Adelberg et al., 1965). After incubation at 37 C for 30 min, the bacteria were collected by centrifugation and washed twice with Tris-maleate buffer, pH 6.0. They were suspended in TSB, diluted with a large volume of TSB, and incubated overnight at 30 C. The bacteria were seeded on plates of trypticase soy agar containing 200 μg/ml streptomycin (SM-TSB agar). The plates (master plates) were incubated for several hours. A replica of each master plate was then transferred to a new SM-TSB agar plate, which was incubated for several hours to allow replicate colonies to grow a visible size. Each of the replica plates was then overlayed with 7.5 ml of soft TSB agar containing 50 μg/ml of egg yolk lecithin, 0.01 M $CaCl_2$, 1,000 units/ml of colistin, 50 μg/ml of lysozyme, 200 μg/ml of streptomycin and 2×10^8 washed sheep erythrocytes/ml. The plates were further incubated at 37 C.

Bacteria in each colony are lyzed with colistin and lysozyme (Warren et al., 1957), with release of their membrane-bound phospholipase A into the surrounding soft agar. The enzyme hydrolyzes lecithin to fatty acids and lysolecithin, which in turn lyzes the erythrocytes. In this way hemolytic zones appear around the colonies. Colonies without hemolytic zones are selected.

It was impossible, in practice, to isolate the mutant in a single step. Colonies showing delayed hemolysis were isolated. They were again treated with mutagen and the procedure described above was repeated. Finally 3 strains were isolated, which gave clonies with no hemolytic zone on prolonged incubation. They were designated as B6PLAL, C3PLAL and D4PLAL, respectively. C3PLAL was obtained after two treatments with mutagen, and B6PLAL and D4PLAL after three treatments. Fig. 2 shows mutant colonies, which are colored by the covering erythrocytes and which have no hemolytic zone, among the whitish parent colonies with hemolytic zones. The sensitivity of these mutant strains to colistin was shown to be the same as that of the parent.

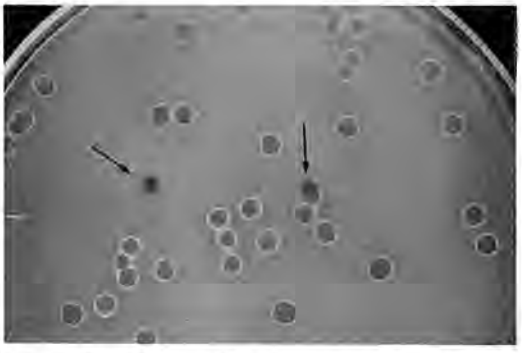

FIGURE 2. *Mutant colonies among colonies of parent strain.*

Colonies of the parent strain have hemolytic zones around them and are lighter in color, because erythrocytes covering them are lyzed. The mutant colonies, shown by arrows, have no hemolytic zone and are darker because they are covered with erythrocytes.

2. *Test for phospholipase A activity with the cell lysate of the mutants*

For testing phospholipase A deficiency in these mutants, their cell lysates were obtained by treatment with colistin plus lysozyme or by sonication without separation into supernatant and debris, and were tested for phospholipase A activity on ^{14}C-labelled phosphatidylethanolamine (^{14}C-PE). Fig. 3 shows the radiochromatogram obtained with bacteria which had been treated with colistin and lysozyme. The lysates of the mutant strains did not hydrolyze ^{14}C-PE. However, the lysate from the parent strain as well as that from a strain (C17), which had showed delayed hemolysis on the replica plate described in the preceding paragraph, degraded ^{14}C-PE to free fatty acids (FFA) and lysophosphatidylethanolamine (LPE). Similar results were obtained with lysates prepared by sonication.

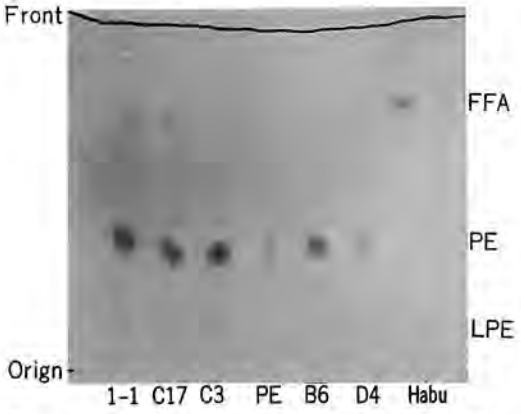

FIGURE 3. *Radiochromatogram for detecting phospholipase A activity of cell lysates from E. coli B/SM, strain 1-1 and its mutants.*

Young cultures of the parent (1-1) and mutant strains were washed and resuspended in Tris-buffered saline at a concentration of 1.0×10^{10} bacteria/ml. 1.0 ml of the suspension was mixed with 0.1 ml of a mixture of 22,000 U/ml colistin plus 1,100 μg/ml lysozyme, and incubated at 37 C for 30 min. A mixture of 1.0 ml of 250 μg/ml purified "Habu" venom pjospholipase A and 0.1 ml of colistin+lysozyme was also set up. Then 0.1 ml of solution containing 0.12 M $CaCl_2$+12 mg/ml streptomycin and 25 μl of ^{14}C-PE solution in methanol were added to each mixture. The mixtures were incubated at 37 C for 120 min. After incubation, each mixture was extracted and chromatographed as described in the Materials and Methods.

1-1, parent strain; C17, a mutant strain which showed delayed appearance of a hemolytic zone; C3, C3PLAL; B6, B6PLAL; D4, D4PLAL; Habu, purified "Habu" venom phospholipase A; PE, 5 μl of ^{14}C-PE as reference. FFA, free fatty acids; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine.

3. *Phospholipid compositions and surface antigens of the mutants*

Phospholipase A activity is known to be important in the metabolism of glycerol-containing lipids in many organisms. Therefore, defect in this enzyme might cause some change in the lipid composition of the organism.

The parent and mutant strains were grown in Tris-glucose medium containing ^{14}C-[U]-glucose at 37 C for 16 hr with vigorous aeration. After thorough washing, each was extracted and subjected to two-dimensional chromatography as described in the Materials and Methods. The autoradiograms showed little or no difference in the lipids compositions of the parent strain and all three mutant strains (data not shown).

In quantitative agglutination tests of these mutant strains against rabbit antiserum to the parent strain all three mutants gave the same titer as the parent. Moreover, as shown in a separate paper (Inoue et al., 1974b), the sensitivities of all these mutants to immune bactericidal activity mediated by the complement and antiserum against the parent strain were similar to that of the parent. Therefore, these mutants seem to show little, if any, modification of surface antigenic structure.

4. *Sensitivities of the mutants to bacteriophages*

The mutants are all sensitive to all the T series bacteriophages, as shown in Table 1. They are insensitive to λ phage, like the parent strain.

TABLE 1. *Plating efficiencies of various bacteriophages on E. coli B/SM and its phospholipase A-deficient mutants*

Host Bacteria	Plating efficiency[a]						
	T1	T2	T3	T4	T5	T6	T7
E. coli B/SM, 1-1	1.0	1.0	1.0	1.0	1.0	1.0	1.0
C3PLAL	1.07	0.78	1.00	0.68	1.15	1.07	0.87
B6PLAL	0.91	0.85	0.91	0.72	1.11	0.88	0.97
D4PLAL	0.97	0.83	0.96	1.04	1.23	0.82	0.88

[a] Efficiencies were compared with those of strain 1-1.

5. *Phospholipid metabolism in the mutants while supporting replication of bacteriophages*

Cronan and Wulff (1969) proposed that infection of *E. coli* B with bacteriophage T4 induces phospholipid hydrolysis resulting in production of free fatty acid, and that this degradation causes lysis of infected bacteria in addition to phage-controlled lysozyme production. This proposal was denied by Josslin (1971). However, this problem has not been settled yet (Cronan and Wulff, 1971). Thus it

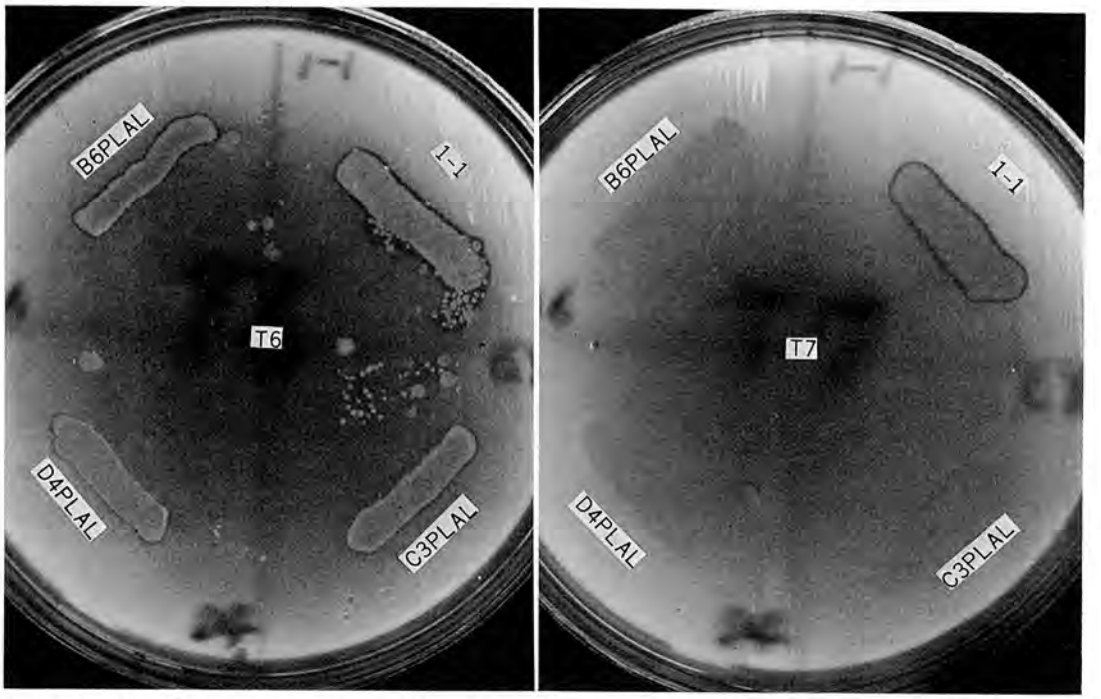

FIGURE 4. *Hemolytic zones around streak inocula in soft agar containing sheep erythrocytes, lecithin, Ca^{++} and bacteriophage.*

Hemolytic zones are seen around the parent strain 1-1, and mutant strains infected with T6 phages. Streaks with hemolytic zones are observed clearly because of lysis of erythrocytes above them, while streaks of mutant strains infected with T7 are obscure because of covering erythrocytes. See text for details.

is very interesting to see if a phospholipase A-deficient mutant of *E. coli* B can produce phospholipase A when it is infected by a bacteriophage.

The three mutants and the parent strain were all streaked on 8 TSB agar plates and incubated at 37 C for several hours. Then each plate was covered with 7.5 ml of soft TSB agar containing 2×10^8 washed sheep erythrocytes/ml, 50 μg/ml of egg yolk lecithin, 0.01 M $CaCl_2$, 200 μg/ml of streptomycin and either 5 to 10×10^6 phages/ml of one of the T-series bacteriophages or 100 μg/ml of egg white lysozyme plus 2,000 U/ml of colistin. The plates were further incubated at 37 C. A hemolytic zone appeared around the parent strain 1–1 on all the plates. None of the mutant strains caused hemolysis on plates with T odd phages (T1, T3, T5 or T7) or with colistin plus lysozyme, but caused definite hemolytic zones on plates with T even phages (T2, T4 or T6). Fig. 4 shows the results with T6 and T7. It is uncertain whether this hemolysis observed around the mutant strains is the result of phage-induced phospholipase A. However, it is very interesting that the hemolysis occurs only with T even phages, because it is well known that the metabolism of bacteria infected with T even phages is different from that of bacteria with T odd phages.

One of these mutant strains, D4PLAL, and the parent strain were uniformly labelled with ^{14}C by culturing them in 0.03% glucose Tris medium containing 0.5 μCi/ml of ^{14}C-[U]-glucose at 37 C for 16 hr with vigorous aeration. After washing with Tris buffered saline, they were infected with T1 and T4 bacteriophages at m.o.i.=5.0 and at a concentration of 2×10^9 bacteria/ml. Uninfected bacteria served as controls. After shaking at 37 C for 5 min, the bacteria were separated from the medium by centrifugation at 13,400 *g* for 5 min in the cold. The bacteria was then resuspended at a concentration of 4×10^8 bacteria/ml in TSB containing 200 μg/ml of streptomycin at 37 C and put in a flask equipped with a branched cuvette as a sidearm. The flasks were incubated at 37 C with constant shaking. The optical density of the bacteria was measured in a Coleman Jr. II spectrophotometer, Model 6/20 at about 20 min intervals from the time of their transfer to TSB (Fig. 5). After 60 min, the bacteria were centrifuged, and lipids were extracted from the supernatants and sedimented cells by the method of Bligh and Dyer (1959). These lipid fractions were chromatographed as described in the Materials and Methods.

As shown in Fig. 6, FFA was found in the debris of the parent strain after infection with either T1 or T4. A trace of FFA was also found in the supernatant fractions. No FFA was found in the supernatant or debris of the mutant after infection with T1 and only a

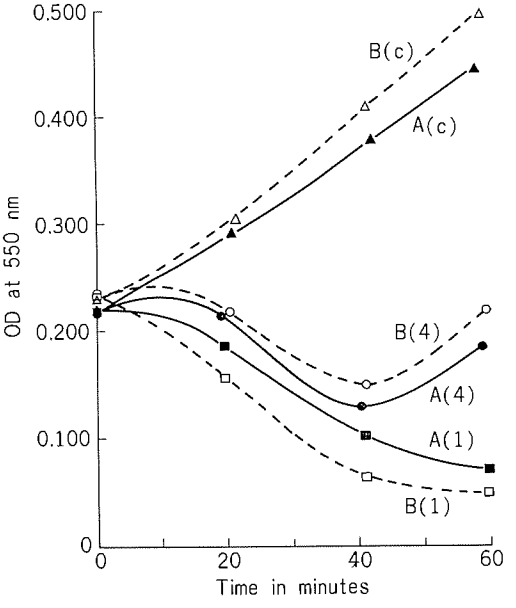

FIGURE 5. *Lysis of the parent and one of the mutant strains after infection with T1 or T4 bacteriophage.*

Time is measured from the time of transfer of bacteria to TSB after centrifugation to separate unabsorbed phages. A(1), mutant strain D4PLAL infected with T1; A(4), strain D4PLAL infected with T4; A(c), strain D4PLAL without phage; B(1), parent strain 1–1 infected with T1; B(4), strain 1–1 infected with T4; B(c), strain 1–1 without phage. See text for details.

trace in the debris after infection with T4. Further investigations are necessary on the relationship between this result and the hemolytic zones observed around the mutant strains infected with T even bacteriophages described above.

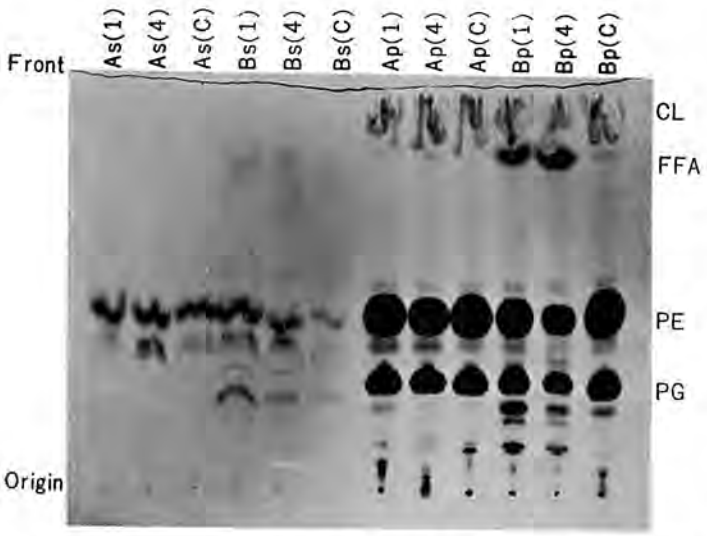

FIGURE 6. *Alteration of lipids of the parent and a mutant, D4PLAL, infected with T1 or T4 bacteriophages.*

Left half, lipid fractions of the supernatants; right half, lipids of the precipitates. Symbols are as described in the legends to Figs. 3 and 5. See text for details.

DISCUSSION

Studies on alteration of phospholipid in *E. coli* B, during the immune bactericidal reaction prompted us to attempt the isolation of a phospholipase A-deficient mutant of this organism.

The isolation procedure outline in Fig. 1 was adopted to avoid using a parent strain defective in cell envelope structure, because the phospholipase A of *E. coli* is known to be membrane-bound and cannot be detected unless the cell envelope has disintegrated. It was not easy to isolate the mutant. First, mutants which showed delayed hemolysis were isolated and we obtained 3 mutant strains from these by repeated treatment with mutagen. The requirement for multiple treatment might be due to the existence of more than one phospholipase A in this organism (Gatt and Barenholz, 1973).

It is still uncertain whether the mutants obtained completely lack phospholipase A. No enzyme activity could be detected in these mutants by usual method, as shown in Fig. 3. However, as shown in a separate paper (Inoue et al., 1974b), slight but definite formation of FFA and LPE was observed in one of these mutants when it was subjected to the immune bactericidal reaction. But this degradation of phospholipid might be due to complement or to a serum phospholipase A other than complement components. The results cannot be interpreted definitely until a set of purified components of complement is available.

Although these mutants were treated with mutagen several times they do not seem to be defective in other important biochemical steps. They all grow normally in a synthetic medium with glucose as the sole carbon source. Two-dimensional chromatographic analysis showed little or no change in their phospholipid composition. The quantitative agglutination test and immune bactericidal test (Inoue et al., 1974b) against rabbit antiserum to the parent strain also showed little or no difference between the antigenic surface structure of these mutants and the parent. Moreover, they all possess receptors for all members of T-series bacteriophages.

The possible participation of bacteriophage-induced phospholipase A in the mechanism of lysis of infected bacteria has been discussed (Cronan and Wulff, 1969 and 1971; Josslin, 1971). The hemolytic zones observed around

the mutant strains infected with T even phages but not T odd phages, as shown in Fig. 4, suggest that T even phages, but not T odd phages, might induce new phospholipase A in the infected bacteria. However, only a trace of FFA was detected in the debris of one mutant infected with T4, while a dense spot of FFA was observed in the lipid fraction of the debris of the T4-infected parent strain, although the turbidities of the two strains decreased similarly. Further investigation is required on this.

Phospholipids are degraded by bacterial phospholipase A during both the immune bactericidal reaction and lysis of phage-infected bacteria. However, the degradation products remain in the debris of phage-infected bacteria, while they are released into the surrounding medium during the immune bactericidal reaction. The degradation seems to be mainly due to the activity of the same bacterial enzyme in both cases, because the mutant deficient in this enzyme produces little or no degradation products. The reason for the difference in distribution of the degradation products is unknown. Further investigations on this problem might throw some light on the mechanism of formation of complement-lesions and of lysis of phage-infected bacteria.

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