NUTRITIONAL STUDIES ON THE "AUTO-PLAQUE" PHENOMENON IN *PSEUDOMONAS AERUGINOSA*

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Received for publication 2 May 1963

ABSTRACT

BERK, RICHARD S. (Wayne State University, Detroit, Mich.). Nutritional studies on the "auto-plaque" phenomenon in *Pseudomonas aeruginosa*. J. Bacteriol. **86**:728–734. 1963.—Examination of 20 cultures of *Pseudomonas aeruginosa* indicated that 18 cultures possessed the common property of spontaneously lysing to form "plaques" or erosions on themselves in the absence of a sensitive indicator strain. Maximal lysis and plaque production was found to occur on a medium with a Tryptone concentration of 2.0 to 2.5%. Reduction of the Tryptone concentration to 0.5% or less supported growth, but was usually inadequate for support of lysis. However, addition of L-asparagine or L-arginine to 0.5% Tryptone induced lysis. Examination of five strains of *Pseudomonas*, which routinely exhibited the autolytic phenomenon, indicated that all were both lysogenic and pyocinogenic when tested against other *Pseudomonas* strains on both 2 and 0.5% Tryptone. Culturing of autolytic strain Pa-1 on a simple medium composed of glucose and inorganic salts appeared to be inadequate for "auto-plaque" formation, although lysis occurred occasionally when a yeast extract concentration of 1% was incorporated into the medium. Suppression of auto-plaque formation was also effected by growing the culture on Technicon dialyzing membrane D3 overlaid on 2% Tryptone, although lysogenic lysis of an indicator strain was demonstrable on the membrane.

There exists in certain organisms a highly unusual and bizarre lytic phenomenon which has never been completely understood. The lytic phenomenon has been temporarily termed the "auto-plaque" phenomenon by this investigator, and basically represents the formation of "plaques" or lytic erosions in cultures of *Pseudomonas aeruginosa* grown on solid media in the absence of a sensitive heterologous indicator strain. The first observation of this visible lysis of *Pseudomonas* cultures was made by Hadley (1924), who noted the spontaneous lysis and appearance of circular pitted or pocked areas on cultures grown on agar media. Hadley recognized that the lysis was precipitated by some unrecognized factor of the environment, but he did not investigate the phenomenon on a nutritional basis. Other workers occasionally reported the same occurrence in *Pseudomonas* cultures (Rabinowitz, 1934; Fastier, 1945; Don and van den Ende, 1950; Warner, 1950). However, no direct evidence is yet available which clearly accounts for the widespread occurrence of the autolytic phenomenon in cultures of *P. aeruginosa*. Recently, Lindegren and Bang (1961) and Lindegren, Bang, and Hirano (1962) reported the occurrence of possibly the same phenomenon in certain yeast cultures.

Recent reinvestigation of this phenomenon has been undertaken in this laboratory in an effort to better understand and characterize the basic lytic process. This has been mediated primarily through the use of a nutritional approach. Consequently, the purpose of this report is to describe the basic phenomenon and to demonstrate its nutritional dependence.

MATERIALS AND METHODS

Organisms. The culture of *P. aeruginosa* used throughout this study was obtained from a patient and will be referred to as Pa-1 to differentiate it from other strains with similar lytic properties. The other strains are designated as P11 P12, P13, and P14.

Cultivation of cells. Routine cultivation of *Pseudomonas* strains was carried out on a 2% Tryptone medium (Difco) containing 1% glucose, 0.5% sodium chloride, and 1.5% agar. Certain studies were also carried out on a simple medium composed of the following (per liter): glucose, 10 g; NH_4Cl, 10 g; $MgSO_4$, 1 g; K_2HPO_4,

1 g; $CaCl_2$, 1 μg; $FeSO_4$, 1 μg; and agar, 15 g. The pH was adjusted to 7.0. Yeast extract was occasionally added in variable amounts when called for.

Membranes. Three dialyzing membranes were routinely used and overlaid on the Tryptone agar surface as described by Birch-Hirschfeld (1934). Membranes were purchased from Technicon Co., Chauncey, N.Y. The membranes were Cupriphane, D3, and D300. Occasionally, membranes were coated with antistatic compound #79 (Merix Chemical Co., Chicago, Ill.), diluted 1:1 with distilled water, by dipping in a prepared bath for a few seconds, and then allowed to dry.

Pyocin. Detection of *Pseudomonas* bacteriocins was performed according to the agar stab-agar overlay technique described by Fredéricq (1948).

Lysogeny. Detection and demonstration of lysogeny in cultures of *P. aeruginosa* was performed by streaking test strains against an indicator lawn with cells in the log phase of growth in Tryptone broth or other media. When other media were used to determine their ability to support lysogenic lysis of indicator strains in the absence of auto-plaque formation, both the test strains and indicator strains were cultured in the medium to be tested two to three times before initiation of the experiment.

Amino acid supplements. Saturated solutions of 19 amino acids were singly prepared, neutralized to pH 7.0, and sterilized by passage through a Seitz filter. Approximately 0.1 ml of each acid was placed in the center of 0.5% Tryptone medium containing 0.5% agar. The solution was allowed to diffuse throughout the medium setting up a concentration gradient. After the plates were dried, they were inoculated with the test organism to determine which amino acids played an active role in stimulating auto-plaque formation on a minimal Tryptone medium. The amino acids used were the following: L-lysine, L-leucine, DL-histidine, L-glutamate, L-asparagine, L-arginine, DL-phenylalanine, DL-threonine, DL-α-alanine, L-cysteine, L-proline, DL-valine, L-hydroxyproline, DL-aspartate, L-glutamine, tyrosine, L-cystine, and DL-tryptophan.

Production of auto-plaques. Lysis was usually obtained by swabbing a 24-hr broth culture onto a Tryptone medium or by picking a colony from agar media, dispersing it in saline, and then swabbing 0.1 ml of the suspension onto the agar or agar-membrane surface. Lysis of cells was visually observed after 4 to 36 hr of incubation at 37 C. Occasionally, lysis was so pronounced that discrete lytic clearings were no longer present owing to confluent lysis.

RESULTS

General characteristics. Initial observations on the autolytic phenomenon were first made with the lysogenic strain Pa-1, which previously did not exhibit self-lysis until several months of subculture on Tryptone medium. The initial appearance of the auto-plaques occurred spontaneously and resembled plaques obtained when virulent phage lyses a sensitive indicator strain. Strain Pa-1 almost never exhibited complete lysis of itself down to the agar surface, but rather exhibited what appeared to be a turbidlike plaque. In addition, the circular appearance of the clearings was irregular or ragged rather than perfectly circular. On other occasions, the culture did not exhibit discrete plaquelike clearings of the surface growth but rather a general lytic erosion of the culture. Concomitantly with the spontaneous appearance of auto-plaques on Pa-1 grown on agar media, a shift from the highly mucoid, stringy cell mass to a nonmucoid, rough-appearing culture was apparent in broth cultures. Occasionally, plaquelike areas were seen on the pellicle formed on the surface of broth cultures. Microscopy of this autolytic culture did not disclose granules or any other unusual morphological structures as previously noted by Hadley (1924). An iridescent, metallic sheen almost always accompanied auto-plaque formation when cultures were grown on Tryptone agar media. The metallic-appearing material seemed to be lipoidal in nature, since it floated off the culture when water was added. Although it has not been identified as yet, it is most likely an unlysable remnant of the lysed cells.

One of the unusual characteristics of the lytic phenomenon in strain Pa-1 was its highly variable nature. Size, numbers, and general appearance of plaques appeared to vary somewhat from experiment to experiment. In addition, the time of their appearance during growth was also highly variable. In some cases, lysis was seen to occur concomitantly with the appearance of growth and continued to progressively increase in size and numbers over a several-day period. Consequently, with a heavy inoculum, both growth and lysis was visible 4 to 6 hr after inoculation

of 2% Tryptone, and resembled the rapid appearance of autolytic clearings on certain yeast cultures described by Lindegren et al. (1962). However, for the most part, lysis usually began to appear after 8 to 12 hr or later.

To determine whether this autolytic phenomenon was present in cultures other than Pa-1, 19 other strains of *P. aeruginosa* were examined for appearance of lysis during growth. Of the 19 stock culture strains, 7 exhibited lysis after two subcultures on Tryptone medium, and the others remained nonlytic for many generations. Eventually, only 2 of the 19 did not exhibit the autolytic phenomenon during subculture over a 6-month period. It should be noted that the appearance of the plaques varied considerably from culture to culture, and it was rare to find two lytic cultures which appeared exactly the same. Although a few cultures exhibited clear plaques, the majority exhibited turbid lytic areas. Their size varied from circular, pin-point clearings to large, irregular lysed areas, with or without confluent lysis. Some cultures exhibited a type of generalized surface erosion rather than discrete plaques, and others exhibited both plaques and irregular erosions. One particular culture exhibited a halo about the plaque, similar in appearance to that described by Eklund and Wyss (1962) in *Azotobacter* which they showed to be viral-induced cell-wall depolymerase. Finally, some cultures simultaneously exhibited both large and small plaques which resembled those found in multilysogenic *Pseudomonas* strains (Holloway, Egan, and Monk, 1960). This type of occurrence can be seen in Fig. 1.

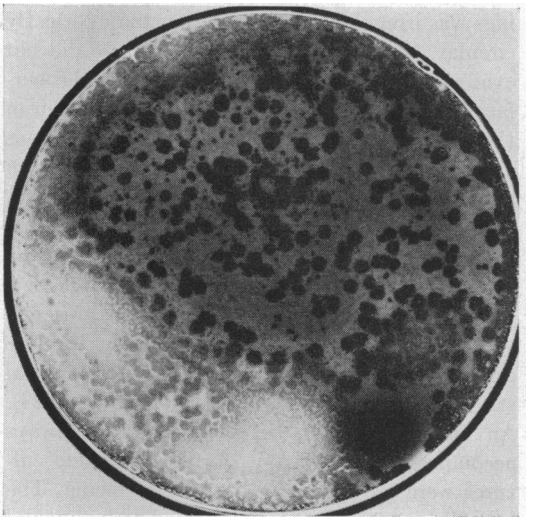

FIG. 1. *Appearance of Pseudomonas aeruginosa Pa-1 grown on Tryptone agar.*

TABLE 1. *Comparison of "plaque" numbers obtained by growth of Pa-1 on coated* and uncoated cellophane membranes overlaid on Tryptone agar*

Membrane	No. of plaques	
	Uncoated	Coated
Cupriphane	>1000	156
D300	110	0
D3	0	0

* Membranes dipped in antistatic solution of compound #79 (Merix Chemical Co., Chicago, Ill.).

Membranes. The nutritional dependence of auto-plaque formation was first investigated during hemolysin studies (Berk, 1962) with the growth of *Pseudomonas* on dialyzing membranes overlaid on a 2% Tryptone agar medium. Three different membranes were examined for their ability to support both growth and hemolysin production by strain Pa-1. However, during this study it was noted that graded autolytic responses were obtained as the membrane type was varied. Cupriphane, which is a very thin membrane, supported a great number of plaques too numerous to count (Table 1). A similar response, but lesser in number and degree of lysis, was obtained with membrane D300, and no lysis of Pa-1 was ever obtained on D3 which is a very thick, opaque, nonionic membrane. However, the ability of Cupriphane and D300 membranes to support lysis was drastically altered by coating them with an antistatic agent to alter or reduce the ionic charge on the membranes. The results (Table 1) indicate that lysis on Cupriphane was considerably reduced, and that lysis was suppressed on coated membrane D300. Despite the absence of lysis on uncoated D3 or coated D300, excellent growth was obtained.

Cultures of Pa-1 grown on the D3 membrane continued to remain stable or nonlytic during prolonged passage on the membrane. However, passage of D3-grown cells to Cupriphane, D300, or 2% Tryptone free from membrane produced plaques which were again suppressed by subsequent growth on Tryptone layered with D3. Despite the absence of plaques, the response of

other *Pseudomonas* strains on the various membranes was not always identical with the response of Pa-1. For example, strain P11 was able to form a limited number of auto-plaques on D3, but in general the lytic reaction was very poor in comparison with membrane-free Tryptone agar.

Media. Initial nutritional studies were designed to determine what constituent in the 2% Tryptone medium was responsible for support of the autolytic phenomenon. Removal of the glucose or sodium chloride from the medium did not alter the lytic response. However, when Tryptone was removed or diluted, alterations in the response were noted. Media containing concentrations of Tryptone ranging from 0.1 to 4.1% were prepared at 0.2% increments and inoculated with *P. aeruginosa* Pa-1. Examination of the plates after 24 hr of incubation indicated that maximal plaque numbers were obtained at 2 to 2.5% Tryptone with plaques present in all concentrations over 0.8 to 1.0% Tryptone. Excellent growth was obtained in all concentrations, with the exception of 0.1% Tryptone where the growth response was somewhat less than that of other plates. In the range of 0.1 to 0.8% Tryptone, auto-plaques were not usually observed, although this figure varied slightly from experiment to experiment and from batch to batch of Tryptone. In these particular studies, 1% glucose and 0.5% sodium chloride were incorporated into the medium, regardless of the Tryptone concentration. With a 2% Tryptone medium, growth over a wide pH range (from 5.5 to 8.0) was examined but no distinct optimum for maximal auto-plaque production was noted within this range.

Growth of the organism on a simple defined medium was examined to determine what media other than a minimal Tryptone medium could be used to maintain cultures in a nonlytic state. A glucose-inorganic salts medium was found to support growth without the appearance of lysis over a pH range of 5.5 to 8.0. Supplements of yeast extract from 0.1 to 1.0% enhanced growth, although lysis at the latter concentration was observed to occur on rare occasions. Continual subculture of strain Pa-1 on the simple medium did not affect the lytic response when the culture was transferred to 2% Tryptone. In addition, lysis was not observable when Cupriphane or D300 membranes were used as an overlay on the glucose-salts medium.

It should be pointed out that the spontaneous appearance of lysis in cultures of *P. aeruginosa* carried in the laboratory for many months can also spontaneously disappear. In these latter cases, relysis was somehow stimulated or enhanced by use of a Cupriphane membrane overlay on 2% Tryptone medium. Similar results were obtained when a lytic culture of Pa-1 was grown in a candle jar. In this case, auto-plaque formation on 2% Tryptone was either suppressed or retarded and did not appear until after 2 to 3 days of incubation. However, use of the Cupriphane apparently overcame this latency and lysis was obtained within 16 to 24 hr (Fig. 2).

Animal passage. Since strain Pa-1 was originally stable or nonlytic for several months after its isolation from a patient, reversion of the organism to the nonlytic state by animal passage was attempted. An individual colony from Tryptone agar was suspended in 3 ml of saline, and 1 ml was inoculated intraperitoneally into each of three mice. As the mice neared death, they were sacrificed, and the organisms were recovered from the peritoneum, streaked on Tryptone agar, and repassaged through mice. After 10 to 15 passages, including several mouse-to-mouse passages, no loss in plaque-forming ability was observed.

Amino acid supplements. Single additions of 19 amino acids supplemented to various media were examined to determine whether any of these play a role in the control of the autolytic phenomenon. A saturated solution of each, neutralized to pH 7.0, was placed in the center of plates of glucose-salts medium containing 0.7% agar to allow a gradient concentration to develop by diffusion. However, none of the 19 amino acids exhibited stimulatory activity. Similar studies with 2% Tryptone were run in an effort to induce lysis beyond that point normally obtained on this medium, but without effect. However, use of a minimal Tryptone medium of 0.5%, supplemented with the amino acids, proved to be successful. Of the 19 compounds tested, single supplements of either L-asparagine or L-arginine caused the cells to produce plaques on themselves (Fig. 3). Similar results were also obtained with DL-alanine, but these were not consistent. It is interesting to note that in Fig. 3 the plaque size was greatest at the center of the plate where the amino acid concentration was greatest and diminished in size away from the plate center.

Since these two amino acids were most active, they were incorporated singly and together in the

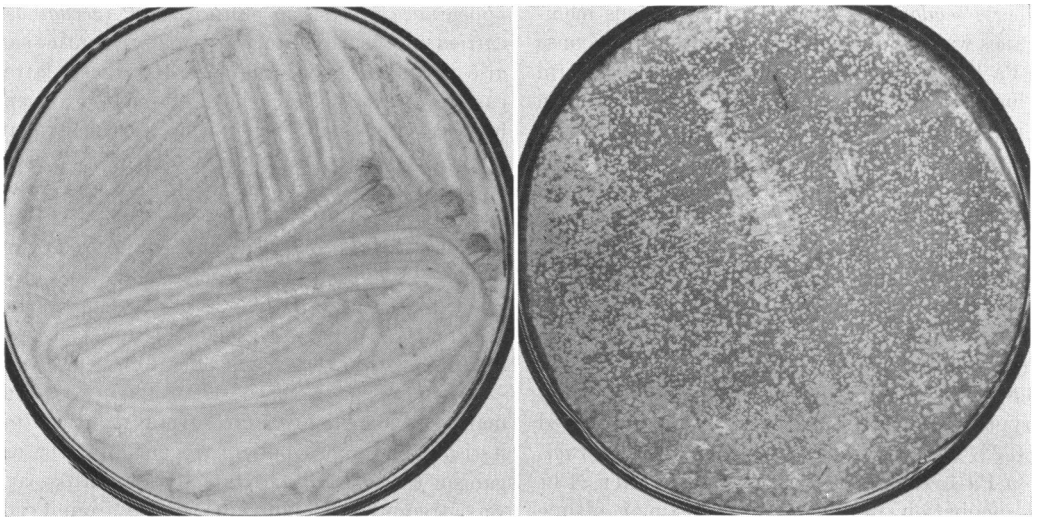

FIG. 2. *Growth of Pseudomonas aeruginosa Pa-1 in a candle jar with and without a Cupriphane overlay Plate on the right shows lysis; no lysis occurred on the left plate in the absence of a membrane.*

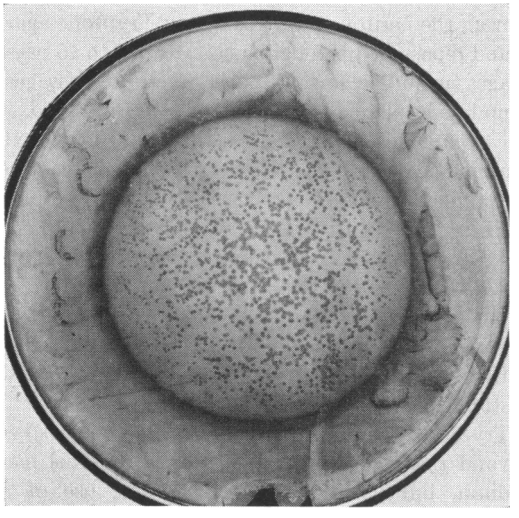

FIG. 3. *Stimulation of auto-plaque formation by* L*-arginine allowed to diffuse from the center of a 0.5% Tryptone-0.5% agar plate.*

glucose-salts medium by use of the gradient technique. However, single additions of the 17 other amino acids to these basal media did not stimulate lysis to occur in strain Pa-1. When additions of more than two amino acids were performed, the compounds were evenly spotted around the edge of the plate and allowed to diffuse toward the center of the plate. The eight amino acids described by Fowler and Cohen (1948) required for the intracellular multiplication of phage in *Escherichia coli* were incorporated into the glucose-mineral medium, but no lysis was noted.

Lysogeny and pyocin production. Several strains of *P. aeruginosa* were surveyed for their ability to synthesize lethal factors such as bacteriophage and pyocin (bacteriocin) under a variety of nutritional conditions. This was done for two reasons: (i) to determine whether the relationship between these various phenomena and auto-plaque formation could be resolved into separate entities on a nutritional basis, and (ii) to develop an assay of the auto-plaque phenomenon which would solely represent the autolytic factor in filtrates possibly containing the other lethal factors. In addition to strain Pa-1, four other strains (P11, P12, P13, and P14) were examined for phage and pyocin production, since all were consistent auto-plaque producers. All five strains were found to be both lysogenic and pyocinogenic when grown on 2% Tryptone. However, in no case was either phenomenon detectable unless an indicator strain other than the test strain was used. Figure 4 shows the five strains streaked against a lawn of Pa-1 for detection of lysogeny. Although several of the strains lysed the indicator lawn, strain Pa-1 did not exhibit this property on itself.

Similar studies were carried out on minimal media in an effort to determine whether the auto-plaque phenomenon in Pa-1 could be nutritionally delineated from lysogeny and pyocin production.

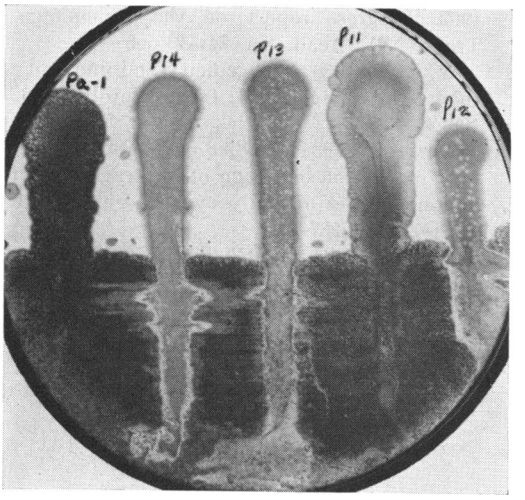

FIG. 4. *Growth of Pseudomonas aeruginosa Pa-1 and four other strains which produced auto-plaques against a lawn of strain Pa-1 for detection of lysogeny. Note that Pa-1 does not exhibit lysis on its own lawn.*

All three phenomena were examined on a simple medium composed of glucose, minerals, and 1% yeast extract. In most studies with this medium, no autolysis was observed, although pyocin production and lysogenic lysis of indicator strains was observed. However, these latter phenomena were very markedly reduced and were sometimes undetectable. Repetition of these studies on 0.1 to 0.5% Tryptone gave stronger viral and pyocin responses, but no auto-plaques were noted. Finally, since strain Pa-1 did not exhibit autolysis on Technicon membrane D3, it was cross-streaked against a known indicator, strain P11, and was found to lyse the culture, although both grew on the membrane.

DISCUSSION

Although auto-plaque formation has been described previously by McCloy (1958) in a strain of *Bacillus cereus* (virulent mutant phage) and by Lindegren et al. (1962) in a few yeasts, the appearance of this lytic phenomenon in the majority of the cultures examined here suggests that it may be a property common to most strains of *P. aeruginosa*. Although the mechanism by which various dialyzing membranes enhance or suppress lysis is not fully understood, most likely it encompasses a form of selective dialysis. The Cupriphane and Technicon D300 membrane apparently allow certain nutritional factors to penetrate the membrane, while concomitantly holding back other factors which may behave in an inhibitory or neutralizing capacity. The apparent suppression of autolysis by membrane D3, although permitting growth to occur, clearly shows the dependency of the autolytic phenomenon on certain nutrients necessary for lysis, but not necessarily required for cell growth. Although the factors in Tryptone responsible for lysis have not been identified, the demonstration that L-asparagine and L-arginine singly added to 0.5% Tryptone stimulate lysis suggests that they are two of several possible inducing factors which may have been diluted out. However, inability to induce autolysis on the glucose-mineral medium with these compounds indicates that the induction process is dependent on one or more unidentifiable factors in Tryptone. Although these two amino acids appear to be two of the eight amino acids required for the intracellular multiplication of *E. coli* phage as previously shown by Fowler and Cohen (1948), it is difficult to say what function they perform in these lytic studies.

ACKNOWLEDGMENTS

The author is indebted to Fred L. Rights, Charles Jeffries, Frank Forshew, and Nuria Kazanas for their suggestions and assistance.

This work was supported by the National Fund for Medical Education.

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