Plaquelike Clearings Induced by Antimicrobial Agents on Lawns of *Pseudomonas aeruginosa*

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Plaques similar in appearance to those induced by phage were observed adjacent to chloramphenicol and tetracycline discs on *Pseudomonas aeruginosa* lawns used for the determination of antibiotic susceptibility. Thirteen strains were selected for study, 10 of which exhibited the plaquing phenomenon. The ability to form plaques induced by tetracycline was not related to any of the biochemical properties of the strains studied, their overall antimicrobial susceptibility pattern, or their pathological source. Some pseudomonad strains were capable of pyocin production; however, the relationship between plaque formation and pyocin production was not apparent. Supernatant fluids of resuspended plaque contents of eight strains originally demonstrating clearings could induce plaques on sensitive indicator lawns only when collected from tetracycline-induced plaque areas; supernatant fluids of the same strains could not produce clearings without previous exposure to the drug. Of the eight supernatant fluids capable of plaque induction, three were active on their homologous indicator lawns. In a subsequent survey of 95 *P. aeruginosa* strains, it was found that 28 isolates exhibited plaques. Of these, 17 were associated with tetracycline, 7 were associated with chloramphenicol, 3 were associated with triple sulfa; and 1 was associated with nalidixic acid.

In the course of examining the antimicrobial susceptibility patterns of *Pseudomonas aeruginosa*, it was observed that distinct, discrete areas of clearing appeared around the periphery of certain high concentration antibiotic-impregnated paper discs, i.e., tetracycline and chloramphenicol. These areas seemed to conform with Warner's (5) criteria for plaques, as opposed to iridescent clearings, since they were (i) uniform and did not tend to increase in size, (ii) appeared in less than 16 hr, (iii) were well defined and circular, (iv) were not covered with iridescence, and (v) were not associated with subsurface crystals. The phenomenon reported here appeared on otherwise smooth, even, confluent lawns of growth and is not concerned with autolytic strains. Our first impression regarding the orgin of these clearings was that they were caused by a virulent infection of the pseudomonads by bacteriophage, but, since the clearings did not appear throughout the lawn of growth, it was assumed that the antibiotics were in some manner responsible for the lytic phenomenon (Fig. 1).

Another possibility as regards the etiological nature of the plaquing agent (PA) is that it could be a bacteriocin, specifically a pyocin. Since it is known that some strains of *P. aeruginosa* harbor bacteriophages and pyocin concomitantly, either might be under constant repression except in the presence of the antibiotic inducer. Therefore, this investigation was undertaken to uncover the following: the relationship, if any, between the appearance of plaques and the organism's pathological origin, biochemical properties, and antimicrobial susceptibility pattern; the association between plaque production and pyocin formation; the feasibility of isolating PA; and the detection of any lytic reactions that might occur when indicator strains included within the study are exposed to isolated PA.

MATERIALS AND METHODS

Antibiotic sensitivity (AS) agar (Albimi) was used exclusively for all plating procedures, and tryptic soy (TS) broth (Albimi) served as the soluble medium in tests requiring a broth culture. Where methods were employed to detect activity of phage, the TS broth was fortified with 0.1 M $CaCl_2$. Soft-agar methods requiring 0.7% agar were satisfied by adding the appropriate amount of agar agar (Albimi) to TS broth.

Pseudomonas strains. A total of 13 strains were collected from routine clinical specimens; 3 did not demonstrate the phenomenon and were included as controls. Strains were identified as *P. aeruginosa* on the basis of the following tests: colonial morphology on MacConkey agar, indole production, motility (semisolid), citrate utilization, lactose and glucose fermen-

tation (Russell double sugar), and cytochrome oxidase. In addition, strains were tested for fluorescence (Sellers medium), alkaline reaction in litmus milk, reduction of nitrate, motility, and hemolysin production (sheep blood). Fermentation of glucose, lactose, sucrose, mannitol, dulcitol, arabinose, maltose, and inulin was tested at a 1% concentration of the carbohydrates in a peptone broth base containing bromocreosol purple indicator and was observed daily for 72 hr.

After initial isolation and identification, the 13 pseudomonad strains selected for study were subjected to additional biochemical tests (as noted in *Bergey's Manual*) to determine whether plaquing strains were altered to such an extent as to change the organism's metabolic apparatus. Results indicated that all strains used in this study conformed to the properties of the "type" strain on the basis of the preliminary identification tests and their reactions in the following additional tests: positive for motility, gelatin liquefaction, fluorescence, nitrate reduction, hemolysis; negative for indole, glucose, lactose, sucrose, mannitol, dulcitol, arabinose, maltose, and inulin; and alkaline in litmus milk.

Antibiotic susceptibility testing. Susceptibility of pseudomonads to antimicrobial agents was determined by two different methods. In the standard disc method, antibiotic-impregnated discs (BBL) were used. Agar gradient plates containing tetracycline (TC) and chloramphenicol (CP) were also employed to determine strain susceptiblity and for the large scale production of plaques. Alternatively, plaques were formed utilizing multiple placement of either TC or CP antibiotic discs on *Pseudomonas* lawns.

Pyocin formation and susceptibility. To determine the presence of pyocin and its effect upon other strains, all strains selected for study were typed against each other (Fig. 2) by the method of Darrell and Wahba (2).

PA isolation. Suspect PA was isolated by one of the two antibiotic plaque induction techniques noted above. Plaque areas were removed with a loop, inoculated into TS broth containing 0.1 M $CaCl_2$, and agitated for 2 to 3 minutes; the supernatant fluid was collected in a sterile tube. Chloroform was not added to supernatant fluids (precipitation of medium was observed when augmented with chloroform), but supernatant fluids were stored at 4 C and checked for bacterial contamination before use. The agent prepared in this manner represented the "concentrated" source of the potential PA.

Plaque demonstration. Plaques could be demonstrated by preparation of a soft-agar overlay consisting of 1 ml of TS broth containing 0.1 M $CaCl_2$ combined with 2 drops of a 4-hr culture of a potential indicator strain and 2.0 ml of 0.7% TS agar (4). The mixture was poured over an AS plate and allowed to solidify. Supernanant fluids of PA prepared from the various strains were dropped (spotted) with a capillary pipette over this lawn; plates were incubated overnight and observed the following day for clearings (Fig. 3).

RESULTS

The typical distinct, discrete areas of clearing which appeared around the periphery of 30-μg TC discs can be observed in Fig. 1. Plaques were circular, uniform in size, and well defined.

Table 1 records the antimicrobial susceptibility patterns of the study strains. In the three instances in which plaques were produced adjacent to CP discs, the organism was resistant to the antimicrobial action of drug. For TC, 9 of the 10 strains which produced plaques were sensitive to the antibiotic, whereas 1 was resistant. In strain 571, both CP and TC induced plaques. When the pseudomonad was sensitive to TC, plaques could be observed on the bacterial lawn at a distance from the disc where the drug concentration gradient was minimal. Thus, sensitivity or resistance to TC and CP could not be correlated with plaque formation.

When the TC inhibitory concentration of plaquing strains was determined by the agar gradient technique, it was found that organisms which originally exhibited plaques around a 30-μg TC disc retained the ability to form plaques when exposed to graded concentrations of the antibiotic. However, the number of plaques which appeared did not seem to be proportional to the concentration of antibiotic, and macroscopic observation of the plates revealed the same number of plaques in an area of high TC concentration as were found in areas of lesser drug concentration, demonstrating an "all or none" response.

Of the 13 strains selected for study, 8 were isolated from urine, 3 were isolated from wounds, and 1 was isolated from sputum. Six of the eight urine strains were plaque formers. In this small sample, there did not appear to be any obvious

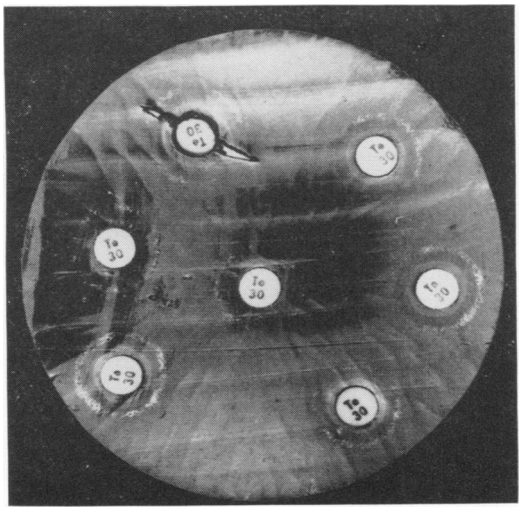

FIG. 1. *Plaques observed on a lawn of Pseudomonas aeruginosa as concentric rings around TC discs.*

relationship between plaque production and pathological origin.

In a subsequent survey of 95 *P. aeruginosa* strains isolated in the diagnostic laboratory, it was found that 28 or approximately 30% exhibited plaques. Of these, 17 occurred with TC, 7 occurred with CP, 3 occurred with triple sulfa, and 1 occurred with nalidixic acid. The latter two inhibitors were not observed to be inducers in the first series. Plaques appeared in strains without significant regard to clinical source of specimens.

As the unknown plaquing agent might possibly be a pyocin of lysogenic nature, strains were pyocin-typed and the results are tabulated in Table 2. If one compares the ability to form plaques as noted in the original observations with the production of pyocin, it can be seen that pyocin activity is independent of plaquing ability among the test strains (Table 3).

In experiments in which plaquing agent was successfully isolated from formed plaques induced by TC and the undiluted preparation of soft agar was spotted on indicator lawns, it was observed that only three strains (349, 519, 571) served as suitable indicator lawns as evidenced by innumerable plaques within the spotted zone (Fig. 3). Furthermore, it is noteworthy that each of the above noted strains was susceptible to PA isolated from its respective homologous strain, a reaction which failed to occur with any of the other strains tested (Table 4). When the above procedure was repeated without benefit of the

TABLE 1. *Antimicrobial susceptibility spectrum of test strains (disc method)*[a]

Test strain	Ampicillin (10)μg	Keflin (30 μg)	Chloramphenicol (30 μg)	Colymycin (10 μg)	Furadantin (100 μg)	Mandelamine (3 mg)	Neomycin (30 μg)	Naladixic acid (30 μg)	Polymyxin B (300 units)	Streptomycin (10 μg)	Tetracycline (30 μg)	Triple sulfa (1 mg)
349	R	R	R	S	R	S	S	R	S	R	S[b]	R
379	R	R	MS	S	R	S	S	R	S	S	S	MS
417	R	R	R	S	R	S	R	R	S	R	R	R
442	R	R	R	S	R	R	S	R	S	R	S[b]	MS
519	R	R	R	S	R	S	S	R	S	R	S[b]	R
526	R	R	R	S	R	S	S	R	S	S	S[b]	R
544	R	R	R	S	R	S	S	R	S	R	S[b]	R
562	R	MS	R[b]	S	R	S	S	R	S	S	S	MS
571	R	R	R[b]	S	R	S	S	R	S	R	S[b]	S
605	R	R	S	S	R	S	S	R	S	R	S[b]	S
626	R	R	MS	S	R	S	S	R	S	R	S[b]	MS
629	R	R	R[b]	S	R	S	S	R	S	S	R[b]	R
653	R	R	R	S	R	R	S	R	S	S	S[b]	MS

[a] Abbreviations used are: R, resistant; S, sensitive; MS, moderately sensitive.
[b] Plaques present.

TABLE 2. *Pyocin activity of test strains*

Test strain	Indicator strains												
	349	379	417	442	519	526	544	562	571	605	626	629	653
349													
379						+[a]							
417								+	+	+	+		+
442	+	+			+	+	+	+	+		+	+	+
519			+										
526			+					+				+	
544													
562						+							
571													
605			+			+							+
626					+	+		+					
629													
653	+	+	+		+		+	+		+		+	

[a] Presence of pyocin as indicated by growth inhibition of the indicator strain.

TABLE 3. *Comparison of plaquing phenomenon with pyocin activity*

Test strain	Plaque	Pyocin activity
349	+	0
379	0	+
417	0	+
442	+	+
519	+	+
526	+	+
544	+	0
562	0	+
571	+	0
605	+	+
626	+	+
629	+	0
653	+	+

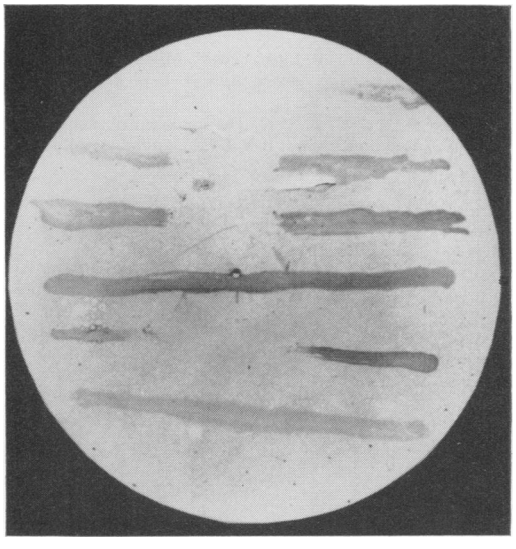

FIG. 2. *Pyocin activity of a Pseudomonas strain previously streaked (vertically), as evidenced by clear areas at the intersections of six horizontally streaked indicator strains.*

test strain's exposure to TC, plaques were absent, demonstrating the necessity for TC treatment.

DISCUSSION

Experiments directed at elucidating the nature of the plaquing agent indicated that some strains capable of forming plaques can also produce pyocin. However, the two phenomena appeared to be independent of one another. According to Holloway (4), pyocin activity can be distinguished from phage on the basis of the clearing surrounding the spotted zone. A large diffuse area appearing at the periphery of the spot would be indicative of pyocin, whereas regular crater-like small depressions occurring within the spot are indicative of virus. We only observe the latter type.

The data from Table 4 and from the comparative series in which supernatant fluids were collected from strains which were never exposed to TC demonstrates that the antibiotic exposure is in some way required for strains to induce the production of plaques on suitable indicator lawns and for some strains to lyse their own lawns. Yet, the capability of the species to form plaques in the presence of TC is independent of the strain's susceptibility to the antibiotic and does not appear to involve any pleiotropic alterations of the several biochemical parameters which were tested.

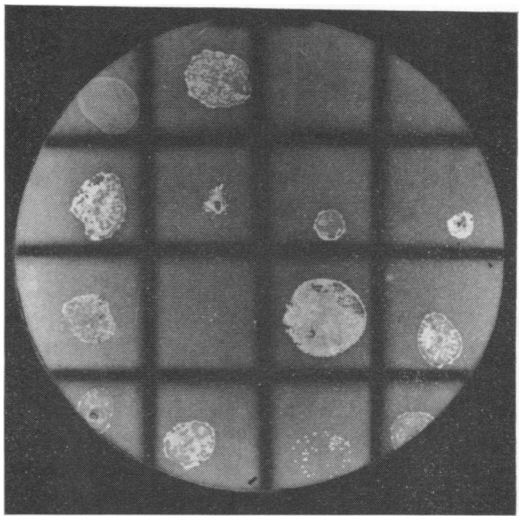

FIG. 3. *Supernatant fluids of broth cultures spotted on a soft-agar lawn of Pseudomonas demonstrating phage-like plaques.*

TABLE 4. *Activity of PA after tetracycline exposure*

Indicator lawn	Test supernatant fluids							
	349	519	526	544	571	605	626	653
349	+[a]		+		+		+	
417								
442								
519	+	+	+	+	+	+	+	+
526								
544								
562								
571	+				+	+	+	
605								
626								
629								
653								

[a] Plaques present.

No encompassing explanation can be proposed for the ability of certain antimicrobial agents to induce plaques in the clinical isolates of *Pseudomonas*, whereas others do not. If the inhibitors known to produce the clearings interfered with membrane synthesis, then the observations would agree with the concept that bacteriocins are specific components of the cell membrane and that their release is mediated by the drugs. However, this is not the case. TC and CP are inhibitors of protein synthesis, the sulfas compounded in the triple-sulfa complex act by interfering with intermediary metabolism, and nalidixic acid inhibits deoxyribonucleic acid synthesis.

Recently, Fedorko et al. (3) observed a phenomenon on *P. aeruginosa* lawns similar to that reported here, but they concluded that the clearings were only an indication of the enhancement of the normal process of clear-spot formation. Our data appear to be in contention with these conclusions. Other workers (1) have reported that certain toxins of *P. aeruginosa* may produce virus-like plaques on monolayers of HeLa cells. The relationship, if any, between the agents responsible for the clearings on the bacterial lawns and mammalian cell lawns is unclear but underscores the potential effects and interactions of bacteria with other diverse host cells.

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