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Characteristics of Lytic and Non-Lytic Derived Strains of *Pseudomonas aeruginosa.** (31330)

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One of the common cultural characteristics associated with many strains of *Pseudomonas aeruginosa* is the spontaneous production of turbid plaque-like erosions on themselves when cultivated on tryptone agar media(1,6, 10). Lysis in most strains is accompanied by a metallic iridescence, while other strains form flecks of metallic patches on agar media (4), but do not exhibit visible lysis. The mechanism by which lysis occurs is presently unknown since little or no infectious phage, bacteriocines, or lytic enzymes appear to be detectable in individual plaques or in the harvested growth media(2). Consequently, the term "auto-plaque" has been adopted to describe this self-lytic phenomenon and to differentiate it from the plaques obtained with phage plated on sensitive indicator strains, the phage carrier state(9), and virulent mutant phage(5). Previous studies by Berk and Gronkowski(2) indicated that non-lytic strains (AP^-) could spontaneously revert or give rise to lytic strains (AP^+). However, detection of naturally occurring AP^- cells in lytic cultures has not been successful, nor have mutagenic and curing technics been effective in converting lytic cells to the non-lytic state(3). However, studies to be described herein indicate that under certain environmental conditions, AP^- cells can be isolated from AP^+ cultures. Therefore, the purpose of this report is to compare some of the cultural characteristics of 7 AP^- strains with their respective lytic parent cultures.

Materials and methods. The cultures of *P. aeruginosa* used in this study were obtained from various clinical sources and the departmental stock culture collection. Each culture was streaked for isolated colonies and a pure culture of each phenotype was obtained by picking individual colonies and re-streaking on tryptone agar media. Cultures which produced auto-plaques in areas of confluent growth and in individual colonies within 16 to 48 hours were labelled AP^+ to differentiate them from stable cultures which did not exhibit visible lysis (AP^-) at 37°C.

All strains examined for auto-plaque formation were cultured on a medium composed of 2% tryptone (Difco), 1% glucose, and 0.5% sodium chloride, while all stock cultures were maintained on a rehydrated tryptose medium (Difco) at 4°C after an initial incubation at 37°C for 24 hours. Tryptose agar was also used for determination of pigment production at 37°C and subsequently at 4°C. Chlororaphin and oxychlororaphin crystal formation was observed to occur on tryptone-glucose agar or broth media after 5 to 7 days incubation at 37°C, but was not detectable on tryptose medium(7).

Auto-plaque stimulation was demonstrated

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by use of demethylchlortetracycline Difco antibiotic discs (30 μg) which were placed on the surface of tryptose agar media which had been previously swabbed with 24-hour AP⁻ strains of *P. aeruginosa*. Induction of auto-plaque formation in areas around each sensitivity disc was detectable after 16 to 24 hours incubation at 37°C. Antibiotic sensitivity studies were performed with Difco sensitivity discs and employed the following sulfonamides in concentrations of 50, 150, and 300 μg, respectively: sulfisomidine, sulfadiazine, sulfathiazole, sulfamethoxypyridazine, sulfisoxazole, sulfamerizine, sulfamethylthiadiazole, and triple sulfa (sulfadiazine, sulfamethazine, sulfamerazine). The following antibiotics were also employed: chloramphenicol (5, 10, 30 μg), erythromycin (2, 15, 15 μg), kanamycin (5, 10, 30 μg), neomycin (5, 10, 30 μg), novobiocin (5, 10, 30 μg), penicillin (2, 5, 10 units), streptomycin (2, 5, 10 μg), tetracycline (5, 10, 30 μg), chlortetracycline (5, 30 μg), demethylchlortetracycline hydrochloride (5, 30 μg), dihydrostreptomycin (2, 10 μg), nitrofurantoin (50, 300 μg), oleandomycin (2, 15 μg), polymyxin B (50, 300 units), vancomycin (5, 30 μg) and viomycin (2, 10 μg).

Results. Initial studies were designed to determine whether auto-plaque producing cultures (AP+) routinely elicited detectable non-lysing cells (AP⁻). Forty-eight recently acquired AP+ cultures were streaked for isolated colonies on several occasions, but such strains usually exhibited homogeneous populations of AP+ colonies. Spreading of 200 to 300 colonies on the surface of tryptone agar also yielded only self-lysing colonies. Occasionally, one or two AP⁻ colonies could be visually detected, but they immediately reverted to the lytic state upon subculture. However, true breeding AP⁻ colonies were isolated by routine streaking from 7 of the 48 AP+ stock cultures which had been stored for 30 days on refrigerated (4°C) tryptose agar slants. All of the 7 lytic cultures usually exhibited confluent lysis at 37°C within 18 hours, whereas the new variants were completely free of auto-plaques.

Comparative growth studies of the 7 AP+ cultures with their respective AP⁻ variants were performed with aerated broth cultures. The parent strains usually grew at the same or slightly faster rate than their AP⁻ variants. No visible lysis of AP+ cultures was detected turbidimetrically in a Coleman spectrophotometer at 660 mμ over an 18 to 24 hour incubation period. Although visible lysis of AP+ strains has previously been detected only on agar media, the lytic phenomenon was found to be reproducible in liquid media if a loopful of an AP+ culture was carefully placed on the surface of tryptone broth contained in petri plates. As the cultures grew they spread from the center point of inoculation to form a thin pellicle which subsequently exhibited auto-plaques over the entire broth surface. All AP+ stock cultures grown under these static conditions were capable of eliciting the lytic phenomenon on the broth surface.

One of the most striking differences noted between the 7 AP+ strains and their derived AP⁻ cultures was the alteration in pigmentation noted on tryptose media incubated at 37°C for 24 hours. These results are itemized in Table I and indicate that when the blue pigment pyocyanin was present it was subsequently lost in the conversion from the AP+ to AP⁻ state. For example, strain W_2AP+ produced both pyocyanin and fluorescin, while its AP⁻ variant exhibited only green pigmentation. Of the 7 sets of cultures only the RMAP pair exhibited identical pigment formation at 37°C. In addition, the AP⁻ variants of S_{11}AP+ and W_2AP+ were no longer able to elicit the green and yellow crystals of chlororaphin and oxychlororaphin (7) that had been produced by the parent strains on tryptone agar. Of the 7 sets of cultures only one exhibited striking changes in morphology upon loss of the lytic character. Culture C_{17}AP+ produced typical short Gamma-negative rods usually characteristic of *P. aeruginosa*, while each colony of the AP⁻ variant produced short cells as well as long thin "filaments" or chains of cells. The average length of the "filament" forms was about 10 to 30 μ.

Another unusual characteristic observed was the appearance of pink to purple-pink pigmentation of many AP+ stock cultures

TABLE I. Comparative Cultural Characteristics of AP+ Strains with Their Respective AP− Variants.

Culture	Pigmentation at 37°C 24 hr	Pigmentation at 4°C 30 days	Demethylchlortetracycline inducible* 30 μg disc	Crystal formation 5-7 days
$S_{11}AP^+$	dark green	pink-purple		+
$S_{11}AP^-$	none	yellow-green	—	—
C_9AP^+	blue-green	pink		—
C_9AP^-	faint yellow-green	yellow-green	—	—
$C_{16}AP^+$	green	brown-green		—
$C_{16}AP^-$	grey-green	" "	+	—
$RMAP^+$	yellow-green	yellow-green		—
$RMAP^-$	" "	" "	—	—
W_2AP^+	blue-green	pink		+
W_2AP^-	green	brown-green	+	—
W_7AP^+	blue-green	pink		—
W_7AP^-	green	yellow-green	—	—
$C_{17}AP^+$	none	yellow-green		—
$C_{17}AP^-$	dark green	brown-green	—	—

* AP+ strains were not tested since they normally exhibited confluent lysis within 18 hr, thus making interpretation of the results quite difficult.

maintained for 3 to 4 weeks in loosely capped tryptose agar slants. The appearance of the new pigment seemed to be correlated primarily with strains producing blue-green or dark green pigmentation, which was subsequently lost or replaced during refrigeration. The appearance of pink pigmentation also appeared to be dependent upon aerobiosis since tightly stoppered cultures exhibited markedly slower rates of pigment formation. In this case, the new character was not observed until 2 to 3 months later. At the present time, none of 19 non-inducible stock AP− cultures has been observed to form pink pigmentation during refrigeration; however, only one culture was a pyocyanin producer.

Biochemical comparison of the 7 AP+ strains with their corresponding non-lytic variants did not exhibit any qualitative changes in motility, gelatinase, litmus milk reaction, nitrate reduction, or urease activity. In addition, no fermentation of glucose, mannitol, sucrose, or lactose was observed. The results of drug sensitivity studies employing varying levels of 8 sulfa drugs and 16 antibiotics were qualitatively identical, although small quantitative differences in sensitivity were occasionally detected. Previously, it was noted(3) that several antibacterial agents stimulated lysis of AP+ cultures. Consequently, the 7 AP− strains were grown in the presence of 30 μg discs of demethylchlortetracycline in order to determine whether any of them were inducible. Other antibacterial agents were not similarly employed since demethylchlortetracycline consistently exhibited the most dependable lysis-stimulation of inducible strains. Only 2 of the 7 strains were induced to form turbid auto-plaques, whereas the remaining cultures retained their resistance to lysis. These data can be seen in Table I. Sub-culture of cells present in the surviving lawn in close proximity to the antibiotic disc indicated that the stimulation was of a temporary nature since no autoplaque formation subsequently occurred on the antibiotic-free tryptone media. The lytic parent strains were not tested with the antibiotic since they all exhibited confluent lysis within 18 hours, thus making the experiment too difficult to interpret accurately.

Subsequent studies with the 2 inducible strains described in Table I indicated that they spontaneously reverted to the AP+ state by some unknown self-inducing mechanism after many serial transfers, whereas the remaining 5 AP− cultures never exhibited autoplaque formation. In addition, examination of 35 AP− strains which exhibited metallic flecks or silvery patches on colony growth

in the absence of lysis were all found to be demethylchlortetracycline-inducible. On the basis of past observations, all of these cultures are expected to revert spontaneously to the AP+ state at some time in the future, especially after having been serially transferred on tryptone agar media for many generations. The symbol AP_i^- was adopted to denote non-lytic strains which lyse spontaneously or are auto-plaque inducible with certain antibiotics (3).

Discussion. The detection of AP− variants from refrigerated tryptose agar slants suggests that these variants are normally produced under routine cultural conditions, but at a frequency too low to detect them readily. It would appear that refrigeration somehow selects or favors survival of variants already present, otherwise they would not have been detected by routine streaking methods. An alternate hypothesis can also be postulated suggesting that refrigeration may convert some AP+ cells into AP− variants by some unknown mechanism akin to "curing." However, in view of recent studies by Howarth and Dedman(8) the former hypothesis is more probable since these investigators were able to isolate brown mutants of *P. aeruginosa* which arose spontaneously from yellow-green strains cultivated on sulfathiazole-containing media. It was demonstrated that the variants normally occurred with a frequency of 10^{-7}, but had a selected growth advantage in the presence of sulfathiazole.

The mechanism by which pink pigmentation is produced in the cold by many AP+ strains, but not by AP− cultures is not clear. However, it is most probably due to the conversion of the blue pyocyanin pigment to a pink color under acid conditions, which can be duplicated by acidification of chloroform extracts of the blue pigment. The production of pink pigmentation would therefore be dependent on accumulation of acidic products under aerobic conditions during prolonged refrigeration. Since the auto-plaque phenomenon is seldom associated with yellow and yellow-green cultures, the appearance of pink pigmentation would not be expected to occur in AP− cultures even if the final pH of the culture medium were identical to that of AP+ strains. Certainly, from a genetic point of view, the qualitative alterations in phenotypic characters upon conversion of the AP+ to AP− state suggest that pigmentation, lytic character, and possibly even crystal formation may all be interdependent. In addition, the presence of two types of AP− variants suggests that there may be quantitatively different degrees of immunity to lysis. These would range from little or no immunity in AP+ cultures, partial immunity in the AP_i^- state, and "absolute" immunity in non-inducible strains. The appearance of these varying levels of immunity is of particular interest, since it partially supports previous studies (2) wherein a mucoid, non-lytic culture (M^+AP^-) gave rise to non-mucoid lytic variants (M^-AP^+). In addition, these data help explain previously encountered observations of AP− cultures suddenly undergoing auto-plaque formation by some type of self-inducing mechanism. With regard to this latter phenomenon it is conceivable that demethylchlortetracycline-inducible AP− cultures are really lytic, but are of a very low order as opposed to the rapid confluent lysis noted in many AP+ strains. Conceivably, a few pin-point plaques could easily be masked by slime production which would accumulate in highly mucoid cultures.

Summary. Permanent auto-plaque negative (AP−) colonies are rarely detectable in AP+ strains cultured on tryptone agar. However, two types of AP− strains can be isolated from AP+ strains that are maintained for one to four weeks on refrigerated tryptose agar slants. One type appears to be permanently non-lytic and non-inducible with antibiotics, while the second type is inducible (AP_i^-) with demethylchlortetracycline and may revert to the lytic state spontaneously. In addition, all AP− strains exhibiting iridescent patches in the absence of visible lysis were found to be inducible auto-plaque producers. Comparative optical density measurements of AP+ cultures with their corresponding AP− strains indicated no significant differences in their growth rate in aerated broth, nor was lysis detectable by this method. However, all AP+ strains exhibited auto-plaque formation in broth-containing petri plates when

allowed to grow as a spreading thin pellicle under static conditions. Biochemical and antibiotic sensitivity comparisons of AP− strains with their corresponding AP+ parent indicated no qualitative differences other than changes in pigmentation at 37°C. In addition, refrigeration of pigmented AP+ strains grown on tryptose agar lead to the appearance of pink pigmentation in many strains, whereas none of the stock AP− cultures ever exhibited this property.

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Some Effects of Ethanol on the Disposition of Palmitate by Intact And Adrenalectomized Rats. (31331)

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A single large dose of ethanol produces a triglyceride fatty liver in normal rats(1,2), but not in adrenalectomized (ADX) rats(3). Adrenalectomy also prevents the development of fatty livers after administration of other agents, such as carbon tetrachloride(4) and ethionine(5). Adipose tissue has been shown to be the main source of the fatty acids deposited in liver as triglycerides (TG) after ingestion of a single dose of ethanol in the intact rat(6). The free fatty acids (FFA) are liberated from adipose tissue and then are transported by the plasma to the liver where they are incorporated into more complex lipids. Since the ADX rat cannot mobilize FFA from adipose tissue as well as the intact rat(7,8), there may be an inadequate supply of FFA in ADX animals for synthesis of liver lipids. In addition, the liver of the ADX rat might extract less FFA from blood plasma or incorporate less FFA into liver lipids. We show in this paper that these last two possibilities are not probable explanations for the inability of ADX animals to develop TG fatty livers.

We have compared in intact and ADX rats the incorporation of labelled palmitic acid into liver lipids, its excretion as $C^{14}O_2$ and the effects produced by ethanol on these processes. The results obtained indicate that the effects of ethanol were qualitatively similar in the intact and the ADX rats.

Methods. ADX female Sprague-Dawley rats (Hormone Assay, Chicago, Ill.), weighing 160 to 180 g, were used. The ADX rats were given a 5% glucose-1% NaCl solution as drinking water until about 18 hours before use which was 4 to 7 days after surgery. All rats were fasted overnight before use, with access to drinking water (1% NaCl solution for ADX rats).

Ethanol, 4 or 6 g/kg as specified in *Results*, was given orally in a 50% (v/v) aqueous solution. Palmitate-1-C^{14} was injected 2 hours or 4 to 5 hours after administration of ethanol. Blood ethanol levels were determined using alcohol dehydrogenase and DPN (Boehringer blood ethanol assay).

Each rat was injected in the tail vein with palmitate-1-C^{14} (Nuclear-Chicago, 29.7 mC/mmole), 0.06 μC/g in a volume of about 0.25 ml. The palmitate was dissolved in a solu-