JOURNAL OF BACTERIOLOGY, Oct. 1972, p. 372-380

Vol. 112, No. 1
Printed in U.S.A.

Variable Ammonia Production Among Smooth and Rough Strains of *Pseudomonas pseudomallei*: Resemblance to Bacteriocin Production

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Received for publication 2 March 1972

The colonial morphology of some strains of *Pseudomonas pseudomallei* was correlated with certain biochemical and physiological traits. After 3 days of growth on Wahba or heart infusion agars, smooth-colony strains generated toxic amounts of ammonia. Under the same conditions, the rough strains simultaneously produced oxalic acid which decreased the inhibitory concentration of ammonia. The ammonia-ammonium concentrations in smooth cultures exhibited certain bacteriocin-like characteristics. An unusually stable, smooth strain (strain 165) was chosen to compare and emphasize any differences with typical, rough strain 7815. Three-day-old smooth cultures grown on Wahba agar containing 3% (w/v) glycerol demonstrated ammonia toxicity. The substitution of glucose for glycerol completely obviated this toxicity. In highly aerated Wahba broth containing glucose, the amount of ammonia found in strain 165 smooth cultures and the amount of oxalic acid found in strain 7815 rough cultures were greatly reduced. In Difco nitrate broth smooth strain 165 did not form gas, and it reduced nitrate to nitrite only. Strain 7815 produced a gas and reduced both nitrate and nitrite.

Antibiosis among bacterial strains is a common occurrence and is sometimes useful as an aid in laboratory identification. One of the most striking antagonisms in this respect is attributed to a class of compounds called bacteriocins. The bacteriocins are generally defined as protein-like, naturally occurring bacterial antibiotics which act principally on strains of the same species as the producer or on genetically related species. Some bacteriocins act on species of other genera (4, 25).

Our efforts to find bacteriocins among strains of *Pseudomonas pseudomallei* proved fruitless. However, another type of antagonism was found which resembled bacteriocin antibiosis, but was essentially due to ammonia excretion. This paper describes the conditions under which ammonia toxicity was observed and some interesting correlations among colonial morphology, physiological and genetic traits.

MATERIALS AND METHODS

Strains and cultural conditions. Eighteen isolates of *P. pseudomallei* were obtained from water, soil, and animal sources in Vietnam, Malaysia, and Thailand. *P. mallei* (synonym *Actinobacillus mallei*, strains 4 and 3873) and *P. cepacia* (synonym *P. multivorans*, strains 17616 and 17759) were described previously (26). *P. aeruginosa* pyocin indicator strains were obtained from R. J. Zabransky (38). Strains were maintained on Difco brain heart infusion agar containing 3% (w/v) glycerol (glycerol agar).

Survey for inhibiting strains. The survey procedures were essentially those of Darrell and Wahba (6), except that the basal medium (36) was heart infusion broth (Difco) containing 10^{-5} M iodoacetic acid, 0.1% sodium citrate, 0.1% K_2HPO_4, 0.0018% phenol red dye, and 2% agar (Wahba agar). In the survey, a candidate inhibitor strain was streaked on Wahba agar and incubated at 37 C for 3 days. The cultures were then exposed to $CHCl_3$ vapors for 30 min, the growth was scraped off with a glass slide, and the cultures were chloroformed again for 30 min and aired for 1 hr (treated primary cultures). Indicator strains were streaked at right angles across the scraped area and incubated at 37 C overnight (secondary indicator cultures), and the degree of inhibition was recorded. All other modifications are noted in the text.

Ammonia assay. Portions of bacterial broth cultures were sterilized by shaking with chloroform. The bacterial cells and chloroform were separated from the aqueous phase by centrifugation and discarded. In the assay, the aqueous ammonia and ammonium were converted to gaseous NH_3 by increasing the *p*H of the solution. The total ammonia evolved was trapped in a boric acid solution and titrated with sulfuric acid in the presence of bromcresol green (35). The *p*K equilibrium of ammonia/ammonium was taken to be *p*H 8.9. Above this *p*H, the predominant molecular species was assumed to be ammonia; below this *p*H, the predominant species was considered to be ammonium or ammonium salts. The assay was expressed as grams of NH_4^+ per 100 g of solution. Heat was needed to drive the NH_3 out of whole agar cultures. The elevated temperatures apparently increased the amounts of NH_3 due to deamination of cultural components; therefore, this method was used sparingly for agar cultures and the results were cautiously interpreted.

Oxalic acid assays. Agar cultures were examined microscopically (60-fold magnification) for the presence of oxalate crystals. Broth cultures were sterilized by shaking with $CHCl_3$. The chloroform and cells were discarded after centrifugation. Oxalic acid assays were performed by the method of Lewis and Weinhouse (18).

Tests for nitrate and nitrite. The organisms were grown in 8 ml of Difco nitrate broth at 37 C. A 0.5-ml amount of 20% sodium acetate was added to each culture just prior to testing. The presence or absence of nitrate and nitrite was determined by the methods described in the 9th edition of the *Difco Manual*.

Staining of PBHB granules. Cultures were grown in β-hydroxybutyrate (BHB) broth. Stained preparations were made according to the method described by Hugh (10). Poly-BHB (PBHB) granules retained the Sudan black B stain and appeared dark blue under microscopy examination.

Enumeration of bacteria. Samples (1 ml) of cultures were diluted by 10-fold increments. One-tenth-milliliter amounts of each dilution were spread over the dried surfaces of glycerol agar plates and incubated at 37 C. The colonies were counted on a Bactronic colony counter (New Brunswick Scientific Co., New Brunswick, N.J.).

Enzymes and other tests. Pronase was obtained from Calbiochem, Gaithersburg, Md., and trypsin was obtained from Fisher Scientific Co., Silver Spring, Md. Bovine plasma albumin fraction V was obtained from Armour Pharmaceutical Co., Kankakee, Ill. The *p*H of fluids was crudely determined by the response of the phenol red dye indicator. More exact *p*H determinations were made with a Beckman SS-3 *p*H meter (Beckman Instruments, Inc., Palo Alto, Calif.) on fluids which had previously been sterilized with chloroform. The methods described by Osman (23) were used to search for phage.

RESULTS

Identifying inhibitor-producing strains. Separation of bacteriocin-producing and indicator strains of *P. pseudomallei* was best accomplished by using the single-streak method of Wahba (36). With this method, the inhibition was most pronounced and consistent when the inhibitory strains were incubated for 3 or more days at 37 C. Eight out of 18 strains tested produced an inhibitor in agar cultures which inhibited the subsequent growth of all other strains, including themselves. The other 10 strains did not cause any type of growth inhibition. Phage were not detected in any of the inhibitor strains.

Cultural characteristics. After a few days of incubation at 37 C on cystine Trypticase agar (CTA; BBL, Baltimore, Md.), the inhibitory strains were usually nonviable. The phenol red dye in the medium indicated increasingly alkaline conditions as these cultures became sterile. On MacConkey agar, these strains first appeared as colorless colonies. On continued incubation the colonies acquired a slight pink color. When the inhibitor-alkaline strains were grown on glycerol agar the young colonies had a smooth-appearing texture. After 48 hr of incubation some of the colonies became rough, but the majority remained smooth.

In contrast, the noninhibiting strains were viable for many weeks on CTA medium and elicited neutral to acid conditions in the culture medium. On MacConkey agar these strains formed red to dark-red colonies, often mimicking coliform lactose fermenters. On glycerol agar they always grew out as the rough colony type.

Occasionally, the smooth strains gave rise to rough variants. This was accompanied by a loss of inhibitor production and a change from alkaline to acid conditions in Wahba agar cultures. For this reason, 0.0018% phenol red was incorporated into Wahba agar as an inhibition indicator. If the medium was alkaline by day 3 of culture, the agar was inhibitory.

When grown on glycerol, Wahba, or some other agar media, many rough or smooth strains will have a pitted or plaqued lawn. This pitted appearance is similar to the autoplaquing found in *P. aeruginosa* (3, 39).

Genetic spectrum of P. pseudomallei inhibition. *P. pseudomallei* has been shown to have a high degree of genetic homology with *P. mallei*, a much lesser degree with *P. cepacia*, and very little, if any, with *P. aeruginosa* (26). When data from a variety of experiments had accumulated, it appeared that the inhibition patterns were somewhat reflective of genetic relatedness to *P. pseudomallei*. These data are summarized in Table 1. Strains of *P. aeruginosa* pyocin indicators, *P. pseudomallei*, *P.*

TABLE 1. *Inhibitory patterns produced by P. pseudomallei within the genus Pseudomonas*

Secondary cultures	Degrees of inhibition caused by primary cultures of *P. pseudomallei* strains[b]								
	165	7919R[a]	7919S	7816R[a]	7816S	7815	7820	321S	292
P. pseudomallei strain									
165	3	—	4	—	4	—	—	4	—
7919	3	—	4	—	4	—	—	4	—
7816	3	—	4	—	4	—	—	4	—
7815	3	—	4	—	4	—	—	4	—
7820	4	—	4	—	4	—	—	4	—
P. mallei strain									
4	4	4	4	—	4	—	—	—[c]	
3873	4	4	4	—	4	—	—	—[c]	
P. cepacia strain									
17616	4	3	4	2	3	2	2	3[c]	
17759	2	—	3	—	3	—	—	—[c]	
P. aeruginosa									
12 strains	—				—			—	—

[a] Rough strains derived from corresponding smooth strains.

[b] Gradation is from 4, total inhibition; 3, only the ends of the streak grew; 2, no growth in the immediate vicinity of the primary culture; 1, less growth over primary culture; to —, no inhibition.

[c] The stocked strains of *P. pseudomallei* 321 were all rough when this series of experiments was done.

cepacia, and *P. mallei* had been streaked across treated primary cultures of *P. pseudomallei*. The smooth strains of *P. pseudomallei* were inhibitory to all of the *P. pseudomallei*, *P. mallei*, and *P. cepacia* secondary cultures. The *P. mallei* strains were later found to be inhibited by alkaline conditions alone. The rough *P. pseudomallei* strains caused variable inhibition of *P. mallei* and *P. cepacia* strains. None of the *P. pseudomallei* strains inhibited any of the 12 *P. aeruginosa* pyocin indicators.

Attempts to extract and characterize the inhibitory substance. Three-day-old inhibitor strain cultures in Wahba broth were centrifuged and sterilized by filtration (0.45-μm membrane filters). These fluids did not demonstrate any antibiotic effect when inoculated onto agar cultures. Broth cultures of the smooth or rough strains were usually near neutrality after 3 days of incubation.

Three-day-old Wahba agar (0.6% agar) cultures of smooth and rough strains were frozen overnight at −20 C. Collapsed agar was removed by centrifugation. The expressed fluids were passed through 0.45-μm membrane filters and stored over chloroform in a refrigerator. The expressed fluids from inhibitor cultures were approximately *p*H 8.6; the noninhibitor cultures were approximately *p*H 6.8. None of the expressed fluids demonstrated any antibiotic activity on agar cultures.

Attempts to isolate and concentrate an inhibitor by vacuum evaporation, ultrafiltration, or ammonium sulfate precipitation techniques were not successful. Wahba agar, buffered to *p*H 8.6 by 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (Tris-hydrochloride) buffer, only slightly retarded *P. pseudomallei* colony size within 24 hr. Colony size was normal at 48 hr.

Sterile dialysis membranes were placed over the agar surfaces of treated primary cultures. Indicator strains were then streaked on each membrane surface. Indicator strains did not grow in the vicinity of primary streaks on alkaline plates. No inhibition was observed on membranes which covered rough (noninhibitory-acid) primary cultures. These membranes were described by the manufacturer as permeable to most molecules of less than 12,000 molecular weight.

Growth inhibition was markedly reversed when treated primary inhibitory cultures were flooded with 1-ml solutions of either 0.2% trypsin in 0.05 M Tris-hydrochloride, *p*H 8.6, or 0.2% Pronase in Tris-hydrochloride buffer, *p*H 8.6, containing 0.1 M $CaCl_2$. The Pronase solution was the most effective. In contrast, a 0.2% bovine plasma albumin solution in 0.05 M Tris-hydrochloride, *p*H 8.6, had no effect on growth inhibition at all, nor did the buffer solutions alone have any effect on the inhibitory process. A 0.2 M acetate buffer of *p*H 3.8 alleviated inhibition to a slight degree, but not dramatically. The assembled results led us to assume that we probably were dealing with an inhibitor which was alkaline, protein-like (activity neutralized by Pronase or trypsin), of

low molecular weight (passed through a dialysing membrane and not precipitated by ammonium sulfate or denatured by chloroform), and somewhat restricted in its activity to certain genetically related organisms. At this point, the results seemed strikingly similar to the D-amino acid toxicity and resistance found in smooth and rough strains of *Brucella abortus* (8).

Chromatographic search for a nitrogenous inhibitor. A highly stable smooth strain (165) and a typical rough strain (7815) were employed for further chemical and physiological studies. Some characteristics of these strains are listed in Table 2. Three-day-old Wahba agar cultures were sterilized by chloroform vapors and frozen. The expressed fluids were then examined by thin-layer chromatography (2, 8) on Dowex AG 50 W-X8 columns (Bio-Rad Laboratories, Richmond, Calif.) and with an automated, amino acid analyzer (21, 28). The tests were designed to elucidate any differences in production of polypeptides or amino acids by smooth or rough strains. It was immediately seen that the fluids of both cultures contained very large amounts of ammonia. Paradoxically, the noninhibitor strain 7815 apparently produced at least three times more ammonia than inhibitor strain 165. The only way we could account for such a large amount of NH_3 and a neutral to slightly acid *p*H in rough cultures was to assume that the ammonia had been neutralized by an acid. This explanation was consistent with Nicholls' obscure finding that copious amounts of oxalic acid are produced by rough-, but not smooth-colony strains of *P. pseudomallei* (22).

A logical explanation for the observed antibiosis was that the smooth cultures excreted ammonia into the agar culture media. The residual toxicity was due to the high concentration of ammonia. However, the ammonia excreted by rough cultures was probably neutralized by the concurrent production of oxalic acid and consequently made nontoxic. An aqueous 0.1 M solution of ammonium oxalate has a *p*H of 6.4 and is easily dissociated during the testing procedures for ammonia (*Merck Index*, 8th ed.).

It also seemed likely to us that there might be a close relationship between colony morphology, ammonia production, and the pathway used for respiration in *P. pseudomallei*, when one considers that the maintenance of smooth *B. abortus* cultures is more dependent on the availability of oxygen than on the components of culture media (1, 29). Even more germane to this study, Stanier et al. (32) found that the smooth-colony forms of *P. stutzeri* were incapable of denitrification, but after one or two passages through nitrate media these strains regained the rough-colony characteristics of fresh-soil isolates and were once again capable of vigorous denitrification. Consequently, we examined the two strains of

TABLE 2. *Cultural characteristics of P. pseudomallei strains after 3 days of incubation*

Culture characteristics	Smooth strain 165	Rough strain 7815
Wahba agar		
*p*H	8.6	6.8
NH_4 (%)	0.025	0.085
Oxalate crystals	None	Many
Growth	Sterile	Viable
Addition of 3% glucose	Acid, noninhibitory	Acid, noninhibitory
Addition of 3% glycerol	Alkaline, inhibitory	Acid, noninhibitory
Wahba broth		
Pellicle	–	+
*p*H	Neutral	Slightly acid or neutral
Gas	–	–
NH_4 (%)	0.006	0.013
Wahba broth plus 0.4% KNO_3		
Pellicle	–	–
Gas	–	+
Nitrate broth		
Gas	–	+
Nitrate reduced	+	+
Nitrite reduced	–	+
Nitrate broth with 3% glycerol		
Gas	–	+
Nitrate reduced	+	+
Nitrite reduced	–	+
Nitrate broth with 3% glucose		
Gas	+[a]	+
Nitrate reduced	+	+
Nitrite reduced	–	+
β-Hydroxybutyrate broth[b]		
Cells retaining Sudan black B stain	Few	Many
Pellicle	–	+
Heart infusion agar, pits (plaques)	Many	Many
Heart infusion agar with 3% glucose, pits (plaques)	Few	Few

[a] A very small amount of gas appeared on the third day.
[b] Observations differed with time and variants of the two strains. Smoothest and roughest isolates were used in this test.

P. pseudomallei in Difco nitrate broth containing Durham tubes. Smooth strain 165 reduced nitrate to nitrite only and did not form any gas, whereas rough strain 7815 reduced nitrate past nitrite and formed a gas in the Durham tube (Table 2).

Approximation of cultural conditions. Experiments were designed to approximate the inhibitory amounts of ammonia in a 3-day-old smooth-strain Wahba agar culture (Table 2). A final concentration of 0.085% ammonium hydroxide in Wahba agar resulted in a *p*H of 8.4. Various amounts of oxalic acid were added to different lots of ammoniated agar so that the media ranged from *p*H 8.4 to 6.7. When cultures were streaked across these media, it was found that *P. pseudomallei* strains were inhibited on agar of *p*H 8.4 (0.0% oxalic acid) and *p*H 8.1 (0.04% oxalic acid). No inhibition occurred at *p*H 7.8 (0.077% oxalic acid). As previously stated, Wahba agar buffered to *p*H 8.6 by Tris-hydrochloride did not dramatically retard the growth of this organism.

Physiologically, the inhibitory action of smooth strain 165 on Wahba agar did not occur when grown on 3% glucose (w/v) in Wahba agar. When 3% glycerol (w/v) was substituted for glucose, strain 165 was as inhibitory as when grown on plain Wahba agar. To facilitate quantitative measurements, an attempt was made to reproduce the oxygen tension of agar cultures in broth cultures. Therefore, 175 ml of Wahba broth was placed into each of three wide-bottomed Fernbach flasks of 2,800-ml capacity. This provided a very shallow medium (ca. 0.5 cm depth) with a large surface area exposed to air. Two other flasks were filled the same way with Wahba broth containing 3% glucose. Except for the controls, each flask was inoculated with 0.1 ml of an overnight brain heart infusion broth culture of either strain 7815 or 165. The cultures were placed in a shake incubator at 37 C and allowed to remain stationary for three hr, and then were shaken overnight. Growth was so heavy by the next morning that shaking was discontinued for the remainder of the experiment. Table 3 summarizes the data obtained from this experiment. In general, these results seemed indicative of those conditions that occurred in agar. As expected, the growth of both strains increased in all media during the first 24 hr. In our interpretation we assumed that an increase in *p*H increased the amount of NH_3 in comparison with ammonium ions or ammonium salts. When the *p*H decreased, we assumed the ammonium and its salt form were favored. Therefore, at the end of 3 days, smooth strain 165 Wahba broth cultures had risen in *p*H from 7.3 to 8.5, the amount of ammonia had increased, the viable cell count had decreased, and there was little, if any, oxalic acid. In contrast, rough strain 7815 had a *p*H decrease from *p*H 7.3 to 6.8, the viable cell count was still elevated, and the broth culture was found to contain approximately two molecules of NH_4 for each oxalic acid molecule, probably in the form of ammonium oxalate. Consequently, we reaffirmed our belief that the inhibitory substance produced by smooth strains on Wahba agar was ammonia and that rough strains neutralized this toxicity by the concurrent production of oxalic acid.

Interestingly, when strain 165 was grown in 3% glucose-Wahba broth very little ammonium was detected and little, if any, oxalic acid was found, but the *p*H decreased from 6.5 to 3.8. It appeared that glucose metabolism reduced the amount of ammonium excreted and caused the accumulation of another acidic metabolite. Although the acidity seemed to decrease the viable cell count, it should be remembered that in Wahba agar cultures a 3% concentration of glucose neutralized the inhibitory action of strain 165, even though 3% glycerol did not (Table 2).

The reversal of inhibition by proteolytic enzymes was finally clarified by comparing the action of 0.2% Pronase, trypsin, and albumin solutions (0.05 M Tris-hydrochloride, *p*H 8.6) on treated primary agar cultures of inhibitory strain 165 and uninoculated Wahba agar brought to *p*H 8.7 by NH_4OH. The inhibitory effects on secondary inocula were neutralized completely by Pronase, partially by trypsin, and not at all by albumin.

DISCUSSION

Our original aim was to devise a system for differentiating strains of *P. pseudomallei* by means of bacteriocins. It was to be a system similar to the one used so successfully for the epidemiology of *P. aeruginosa* infections (6, 23). Because none of the strains in this study showed any characteristic of lethal phage production, the inhibition was thought to be bacteriocin-mediated on the basis of Bradley's criteria (4).

In his 1967 review, Bradley described low-molecular-weight bacteriocins as unresolvable in the electron microscope, nonsedimentable, thermostable, and trypsin sensitive. The assumption derived from these characteristics is that the antibiotic is a very small protein. The ability of the inhibitor to pass through dialysis membranes, the neutralization of inhibition by

TABLE 3. *Comparison of P. pseudomallei strains 165 and 7815 grown in shallow broth cultures*

Strain	Medium and time (hr)	Viable cells/ml	Colony type	pH	$NH_4 \times 10^{-3}$ (%)[a]	Oxalic acid (mg/100 ml)
165	Wahba					
	0	2.1×10^5	Smooth			
	24	3.3×10^9	Smooth	8.2	15.51	2.47
	48	3.0×10^9	Smooth	8.1	26.24	0.66
	72	1.0×10^8	Smooth	8.5	26.62	4.02
7815	Wahba					
	0	7.0×10^5	Rough			
	24	9.2×10^9	Rough	7.5	20.00	66.81
	48	1.1×10^{10}	Rough	6.9	55.80	172.57
	72	1.4×10^{10}	Rough (96%)[b]	6.8	74.31	231.52
165	Wahba-3% glucose					
	0	2.1×10^5	Smooth			
	24	3.3×10^9	Smooth	5.9	1.23	9.72
	48	8.9×10^8	Smooth	4.0	1.48	0.91
	72	7.1×10^5	Smooth	3.8	1.14	5.17
Control	Wahba			7.3	1.62	2.29
	Wahba-3% glucose			6.5	1.89	3.13

[a] The NH_3 evolved was expressed as grams of NH_4 per 100 g of sample.
[b] The variant 4% were divided among mucoid and very small, rough colonies.

proteolytic enzymes, and its spectrum of activity led us to believe that *P. pseudomallei* inhibition was due to a low-molecular-weight bacteriocin. Later, we found that aqueous ammonia has similar characteristics, and it was the actual cause of inhibition. Experimentally, the toxic effects of ammonia were neutralized by Pronase and trypsin solutions. This effect was probably due to binding of NH_4^+ cations by enzyme protein (which consequently changed the ratio of NH_3:NH_4^+ and effectively reduced the toxic concentration of ammonia) instead of specific proteolytic enzyme activity (37).

In retrospect, it was apparent that a number of bacterial end products might easily be mistaken for bacteriocins. In the propagation and maintenance of *Proteus* and *Chlamydomonas* species, T-mycoplasmas, *Aerobacter aerogenes* and *Escherichia coli*, ammonia toxicity is a recognized threat, and media can usually be designed to minimize ammonia production (7, 19, 30, 31). However, in some bacteriocin systems, like streptococci (13, 15), the toxicities of ammonia, hydrogen peroxide, D-amino acids, and other low-molecular-weight inhibitors are not always taken into consideration as part of the inhibitory phenomenon (8, 9, 27). Even the products of incomplete carbohydrate oxidation, such as formic, acetic, 2-ketoglutaric, fumaric, citric, glycollic, and oxalic acids, are known to be excreted in toxic concentrations by bacteria (9, 31). The interpretations of inhibition patterns are further complicated when production of inhibitors varies or more than one inhibitor is produced. The inhibitory pattern can also be modified by the indicator strain, as when strains which produce peroxidase or catalase neutralize the toxicity of hydrogen peroxide, acid end products neutralize bases, proteins bind fatty acids, and, sometimes, the *p*H of the indicator culture ionizes a molecule into a toxic or nontoxic form. Judging from our experiences and the current bacteriocin literature it would be wise to consider these and other aspects of bacterial interference before assigning a specific epithet to the inhibitory process.

After associating inhibition with colony appearance and ammonia production, we reexamined a relevant article with the misleading title, "Melioidosis, with special reference to the dissociation of *Bacillus whitmori*" (22). In this paper, Nicholls did much more than just attempt to correlate phase dissociation with virulence. In fact, he recorded a number of striking but unexplained colonial, biochemical, and physiological correlations. He noted that smooth strains were generally "suicidal" and caused alkaline conditions in his laboratory

media. The rough colonies were derived from smooth strains. The reverse derivation was not observed. In addition, he found that rough strains produced oxalates and that the more stable smooth strains did not do so until their cultural population started turning rough. No oxalates were found in rough cultures when he incorporated 2 percent glucose into his media.

On the other hand, Tomov (34) did not notice any suicidal phenomenon or antagonism among individual strains of *P. pseudomallei* when he investigated antagonism in the genus *Malleomyces*. This may have reflected his selection of strains or choice of laboratory media. Other workers (11; M. Capponi and P. Sureau, Rapport sur le Fonctionnement Technique de l'Institut Pasteur de Dalat, Viet Nam, 1955, p. 39) have found that *P. pseudomallei* has an antibiotic effect on species of other genera. For the most part, our observations verify and extend Nicholls' findings.

We now know that the alkaline condition in smooth cultures was due to the production of ammonia. As the ammonia accumulated, the concentration became autotoxic and impeded the growth of secondary inocula. This was most notable in Wahba and glycerol agar cultures. When glucose was substituted for glycerol, the ammonia toxicity was not manifested by primary cultures.

Ammonium oxalate is easily dissociated by the testing method used, and it appeared that rough strain 7815 produced even more ammonia and ammonium than smooth strain 165. However, oxalic acid assays of strain 7815 cultures indicated the ammonia was detoxified by simultaneous oxalic acid production. Thus, when rough strains are isolated on MacConkey agar, the slightly acidic ammonium oxalate causes the *p*H indicator to turn the colonies red, thereby giving the false impression that the rough colonies are coliform lactose fermenters. Similar false positives have occurred in the oxidation-fermentation media of Hugh and Liefson (M. Rogul et al., Bacteriol Proc., p. 20, 1969).

In agreement with Nicholls (22), no oxalates were found in cultures when rough strains were grown in high glucose concentrations. The shallow, aerated glucose broth cultures of rough strain 7815 contained hardly any detectable ammonia or ammonium. It was assumed that ammonium was either bound or that its production was suppressed by some aspect of glucose metabolism.

The autoplaquing (autolyzing or autopitting) of pseudomonad cultures is an inconsistent phenomenon which is generally attributed to phage, bacteriocins, or vague metabolic factors (3, 39). Surprisingly, ammonia lysis has been reported in other bacterial systems, but it has not been associated with pseudomonad autoplaquing (7, 16). An attractive hypothesis derived from the presented data was that the pitting in lawns of *P. pseudomallei* cultures reflected the emergence of colonial variants which did not have a mechanism to neutralize toxic amounts of ammonia and were consequently lysed when a critical concentration of ammonia was reached. Pitting does occur in lawns of *P. pseudomallei* strains 165 and 7815 on Difco heart infusion agar. However, the pitting was greatly diminished when glucose was incorporated into the medium (Table 2). The difference was most striking in strain 165 where the pitting was almost totally obviated. There may be other mechanisms operative in the pitting of strain 7815. In another study, we have shown that there is autoplaquing in *P. aeruginosa*, which was probably caused by ammonia lysis and was definitely abolished when high concentrations of glucose were incorporated into the basal media (*unpublished data*).

Other pertinent features of *P. pseudomallei* strains were that in Difco nitrate broth smooth strain 165 reduced nitrate to nitrite only and did not form a gas during the process. In marked contrast, rough strain 7815 produced a gas and reduced both nitrate and nitrite. In general, the rough strains of *P. pseudomallei* did not form their usual pellicle in nitrate broth. It was also our strong impression that rough strain 7815 retained much more Sudan black B stain than did smooth strain 165 after growth in BHB broth, thus indicating the accumulation of PBHB granules in strain 7815. These hydrophobic granules may well be the underlying reason for pellicle formation in rough strains. Because no pellicle formation occurs in nitrate broth, it appears that nitrate reduction or assimilation may be linked in some way with PBHB metabolism. This will have to be studied quantitatively, and in greater depth, because Levine and Wolochow (17) claimed to have found PBHB in a smooth strain but did not indicate the stability of their strain or compare it with their rough strains.

Many of the preceding observations are compatible with the operation of a Krebs (tricarboxylic acid) or glyoxalate cycle. A number of pseudomonads are known to have both the tricarboxylic acid and inducible glyoxalate shunt enzymes. It is widely accepted that PBHB is the storage product derived from the glyoxalate cycle and that oxalic acid is usually

derived from glycine or glyoxalate. The glyoxalate cycle is most active when cells are grown on acetate, other C_2 compounds, or fatty acids whereas it is greatly reduced when cells have to use glucose, certain amino acids, or some tricarboxylic acid cycle metabolites (5, 14, 24).

Occasionally, imbalanced metabolism, due to an excess of nutrient in the medium, will result in the excretion of accumulated intermediate metabolites such as citric, 2-oxoglutaric, or oxalic acid (20, 31). At other times, when the organisms are forced to deaminate and use the carbon skeletons of ribosomal proteins, nucleotides, and amino acids, ammonia will accumulate (12, 20, 33).

Because the rough strain 7815 was found to contain abundant intracellular PBHB granules in BHB broth and excreted large amounts of oxalic acid in media except when glucose was present, we concluded that the glyoxalate cycle was probably operative. The smooth strain did not show any evidence of a glyoxalate cycle.

Most workers agree that in the laboratory smooth strains consistently yield to the rough colonies. We do not know whether this usually occurs as an adaptation or mutation. The stability of smooth strain 165 signifies that, in this case, the colony appearance is basically a genetic trait. In addition, the results of strain 7815 in nitrate and Wahba broths suggested that a nitrite reductase complex and glyoxalate cycle have emerged from smooth strains as a genetically derepressed or an adaptive shift in metabolic pathways.

We are presently devising methods for defining cytochrome content, nitrate and nitrite reducing systems, and the enzymes of the glyoxalate and tricarboxylic acid cycles in *P. pseudomallei* strains.

ACKNOWLEDGMENTS

We thank A. D. Alexander, R. Repaske, and E. S. Windham for their advice and interest during the course of this study. We are also grateful to Nesbitt D. Brown and Charles E. Cain for technical advice and assistance and to K. C. Carr for his enthusiasm and excellent help.

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