GROWTH AND SURVIVAL OF *PSEUDOMONAS PSEUDOMALLEI* IN ACIDIC ENVIRONMENTS

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SUMMARY: A study was made on the growth and survival of *Pseudomonas pseudomallei* in culture environments differing in nutrients, initial pH, and aeration, in comparison with *Pseudomonas cepacia* and *Pseudomonas aeruginosa*. The observations led us to a view that *P. pseudomallei* has the highest adaptability to acidic environments among the three species. Unlike the other species, it grew in heart infusion broth of initial pH 4.5 under aeration and survived keeping a high level (10^9 per ml) of viable counts for as long as 30 days. This sort of adaptation was found to be more evident in the media of poor nutrition and under limited aeration.

INTRODUCTION

*P. pseudomallei*, the aetiological agent of melioidosis, is a normal inhabitant of soil and water and indigenous to the natural environments of Southeast Asia between 20°N and 20°S (1). Though moist soils contain a greater number of organisms, they can survive the dry season for many months (2). Such

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persistence in soil has been demonstrated by field survey and in a laboratory model (3-5). In the rainy season, the organisms harbored in soil may come up to the surface of the water-table and then multiply in the stagnant water and muddy sites such as rice paddy (6).

One of the interesting aspects of the survival of *P. pseudomallei* in natural environments is that pH varying with the depth of soil has little or no effect on their growth (7). In agreement with this finding, Yabuuchi et al. (8) have reported that the growth in brain heart infusion broth of pH 4.5 is one of the unique characters of *P. pseudomallei* and *P. cepacia*. In our previous study (9), this was partially confirmed by the time-course observation of in vitro growth (turbidity). The study was then extended to a larger scale of experiments including the viability test. The results showed the tenacious nature of *P. pseudomallei* to survive unusual environmental conditions.

**MATERIALS AND METHODS**

*Microorganisms:* The *P. pseudomallei* strain employed in this study was NCTC 4845 designated as DMS 0634 in our laboratory. This strain was kindly supplied by Dr. Kriangsak Saithanu, Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University. *P. cepacia* was JCM 5510 (DMS 0704) which also came from Dr. Saithanu's laboratory. *P. aeruginosa* was ATCC 27853 (DMS 0551) which was supplied by Dr. Toshio Shimada, Department of Bacteriology, the National Institute of Health, Tokyo.

*Culture medium:* Heart infusion broth (Difco, Detroit, MI) was employed at three different pH values (4.5, 7.0 and 9.5) adjusted with 1N NaOH or 1N HCl. Modified minimum medium (Difco) was as follows in the composition: 0.7 g of K$_2$HPO$_4$, 0.2 g of KH$_2$PO$_4$, 50 mg of sodium citrate, and 0.1 g of (NH$_4$)$_2$SO$_4$ dissolved in 100 ml of water. This was adjusted to pH 7.0 and sterilized at 121°C for 15 min. Then, 1 ml of a sterilized MgSO$_4$ solution of 10 mg per ml was added to the main solution. For preparation of the minimum medium of pH 4.5, KH$_2$PO$_4$ was used in an amount of 0.9 g instead of 0.2 g of the original composition, and K$_2$HPO$_4$ was omitted. Citric acid was replaced with sodium citrate. For preparation of the minimum medium of pH 9.5, 0.9 g of K$_2$HPO$_4$ was used as sole phosphate salt. Any liquid medium was dispensed into screw-capped pyrex tubes of 30-ml capacity (1.5 × 18 cm) in 10-ml amounts. For plate counting, tripticase agar medium was employed.

*Measurement of bacterial growth and viable counts:* A single colony of an overnight culture on tripticase agar was inoculated into 3 ml of heart infusion
broth, which was incubated overnight. A 0.2-ml portion of this culture was then inoculated into 10 ml of heart infusion broth, which was brought to shaking culture or to standing culture at 37 C. When the modified minimum medium was employed, the inoculum was prepared by suspending the pure bacterial culture in sterilized saline solution to a density of McFarland No. 4. A 0.2-ml portion of this bacterial suspension was inoculated into 10 ml of the modified minimum medium.

With time intervals, bacterial growth was followed by measuring the turbidity with a Coleman spectrophotometer at 600 nm for the heart infusion broth culture or at 500 nm for the minimum medium culture. At the same time, the viability was determined by plate counting with a 0.1-ml aliquot withdrawn from each culture and its dilutions.

**Measurement of medium pH:** The pH change of the culture medium during incubation was followed with pH-test paper with time intervals. One or two drops of the liquid taken out aseptically with a pasteur pipette were applied on the paper.

### RESULTS

In the first experiment, a comparison was made among *P. pseudomallei*, *P. cepacia*, and *P. aeruginosa* concerning their growth behavior in heart infusion broth of three different initial pH values (4.5, 7.0 and 9.5) under shaking culture. The results were most contrasting in these three species of pseudomonads (Fig. 1). In the media of pH 4.5, the *P. pseudomallei* strain made a steep logarithmic growth after a two-day lag period, and reached the maximum turbidity in 5 days. This turbidity was kept at the same level together with persistent viable counts of $10^8$ to $10^9$ for as long as 30 days of the observation period. *P. cepacia* showed a growth tendency similar to that of *P. pseudomallei*, though the maximum level was lower and viable counts decreased down to the undetectable level within 15 days. On the other hand, the *P. aeruginosa* strain did not show any sign of growth at this pH, and the viability began to decrease immediately after inoculation.

In the media of pH 7.0, each of the three strains of pseudomonads grew well under shaking culture. The maximum level of growth was higher in the order of *P. pseudomallei*, *P. cepacia*, and *P. aeruginosa*. The once attained turbidity continued at the same optical density in the former two species, but the optical density of *P. aeruginosa* decreased after the peak growth in 2 days indicating cell lysis. As for viability, *P. pseudomallei* persisted again in a high level ($10^7$ to $10^8$
Fig. 1. Time-course observation of the growth and viable counts of *P. pseudomallei*, *P. cepacia*, and *P. aeruginosa* in shaking culture with heart infusion broth, whose initial pH was adjusted to 4.5, 7.0, or 9.5 per 0.1 ml, but the other two species became negative in plate counting within several days.

In the media of pH 9.5, only *P. aeruginosa* grew under shaking culture. Viable counts of the other two species began to decrease immediately after inoculation and became undetectable within a few days.

The above results aroused our interest in the possible pH change of the medium pH during the bacterial growth. An experiment was undertaken under the same conditions as before to follow the pH change of the medium in 4, 6, 11, 42 and 70 days of culture. The results are shown in Fig. 2. When the medium was not inoculated, any initial pH did not change at all at least during shaking condition at 37 C. In the media of the initial pH 4.5, the tubes inoculated with *P. pseudomallei* turned to pH 7.0 in a few days and kept it constant in the later period of incubation. The media inoculated with *P. aeruginosa*, though the bacterial growth was not evident there, changed the pH up to 9 to 10 in 10 days.
Fig. 2. pH change of heart infusion broth during the growth of P. pseudomallei, P. cepacia, and P. aeruginosa therein. The starting pH of the medium was 4.5, 7.0 or 9.5.

and decreased down to 8 in 70 days. The media inoculated with P. cepacia took the same pattern of pH change as those with P. aeruginosa, though being on a lower level.

In the case of the initial pH 7.0, the media inoculated with P. pseudomallei did not change their pH at all for 70 days. The other two species, however, elevated their medium pH up to around 9.0 in 10 days and kept it constant in the later period of incubation.

In the case of the initial pH 9.5, the media inoculated with P. cepacia did not change the pH at all, probably correlating with no growth and no survival there. In the media inoculated with P. pseudomallei, however, pH decreased down
gradually to neutral in 40 days. On the other hand, *P. aeruginosa* changed the initial medium pH slightly down, and then elevated up to 10 suggesting their metabolic activity.

In standing culture in heart infusion broth (Fig. 3), the general results indicated that the adaptation to the unusual pH environments by any species
could be attained more easily than in shaking culture. Both *P. pseudomallei* and *P. aeruginosa* grew and survived in the media of the initial pH 4.5 for 20 days at least, though the viability of *P. cepacia* decreased down to the undetectable level in 20 days.

In the case of the initial pH 9.5, *P. aeruginosa* grew and survived, and *P. pseudomallei* appeared to take a sign of increase in viable count after the initial decrease by one log level.

The viability of *P. cepacia* went down rapidly from the beginning. Such fragility of *P. cepacia* was evident also in the usual medium of the initial pH 7.0 where the viable cells became undetectable in 13 days.
The last experiment was undertaken to see the mode of growth and viability of the three species when the medium was more inorganic and poor in nutrients. As described before, this minimum medium contained only citrate as carbon source and only ammonium sulfate as nitrogen source. After inoculation with the same strains as employed in the first (Fig. 1) experiment, the media were subjected to shaking culture. With time intervals, viability of the cultures was checked by plate counting as usual. The results are shown in Fig. 4. A survey of this figure in comparison with Fig. 1 indicates that any pseudomonad species can survive in the minimum medium. Even *P. cepacia*, which was most fragile in heart infusion broth, kept the constantly high level of viability in the minimum medium of pH 4.5 or 9.5 for as long as 70 days at least. *P. pseudomallei* persisted in the minimum medium of any pH showing the highest level of viable counts, $10^7$ to $10^8$ per 0.1 ml at pH 4.5 and 7.0, and $10^6$ to $10^7$ per 0.1 ml at pH 9.5. *P. aeruginosa* was the poorest in keeping the viability in acidic environment as we experienced in heart infusion broth. During the course of this experiment, the initial pH of the medium did not change so much, for example from 4.5 to 4.9, from 7.0 to 6.7 and from 9.5 to 8.0 in 70 days, respectively.

**DISCUSSION**

The present study revealed two marked events in the growth and survival in the unusual pH environments by three species of pathogenic pseudomonads; *P. pseudomallei*, *P. cepacia*, and *P. aeruginosa*. First, such bacterial adaptation was possible more efficiently in the media of poor nutrition and limited aeration. This may suggest that the low rate of endogenous metabolism is a favorable condition to switch their living pattern adaptable to a new environment, especially improving the chances of survival or prolonged viability.

Secondly, *P. pseudomallei* and *P. cepacia* showed a higher adaptability to acidic environment than *P. aeruginosa*, and the latter, in contrast, is more endurable to the unusual, alkaline condition than the other two species.

The most remarkable example was the case of *P. pseudomallei*. The strain started a vigorous growth after a lag period of 2 days under shaking culture at pH 4.5 and reached the level of $10^8$ to $10^9$ viable count in several days. This viability, together with culture turbidity, persisted at least for 30 days of our observation. *P. cepacia* took the same pattern of growth as did *P. pseudomallei*, though on a
lower level. A big difference, however, was that *P. cepacia* failed to sustain the viability long enough. After 10 days of incubation, viable counts decreased down to the undetectable level. The decline of the viable counts keeping constant turbidity may suggest that death of *P. cepacia* does not accompany autolysis. *P. aeruginosa* did not show any appreciable growth at pH 4.5 and the viable counts declined rapidly soon after inoculation and became undetectable in 5 days. However, the change of medium pH up to higher than 9.0 may indicate the metabolic activity of the inoculum during this period.

On the other hand, the definite growth was observed only in the case of *P. aeruginosa* when the medium was of pH 9.5 and aerated by shaking, though the viability was kept only for the first several days. *P. pseudomallei* and *P. cepacia* failed to grow at all in this alkaline and aerated environment.

Most interesting is the observation that the media of any initial pH turned to neutral sooner or later after inoculation with *P. pseudomallei* and kept it constant. It is not known whether this is a phenomenon correlated only with its persistent viability or there is some causal relationship between the two events. The latter possibility may be greater in view of the results of other experiments.

In the standing culture without forced aeration, both *P. pseudomallei* and *P. aeruginosa* grew and survived at pH 4.5 and 9.5 for 20 days, if not vigorously. *P. cepacia* persisted at pH 4.5 and 7.0 for 10 to 14 days, but died rapidly at pH 9.5 showing again the weakness of the strain in alkaline environment.

In the minimum medium which contains only citrate as carbon source and only ammonium sulfate as nitrogen source, the three species persisted for as long as 70 days of incubation under shaking, regardless of the initial pH level. Only *P. aeruginosa* failed to keep the viable counts long enough at pH 4.5.

In our understanding, “survive” or “persistence” is a nongrowing but living condition. We recognize this situation experimentally when viable counts are constant without the change of total growth (turbidity). This state of bacterial cells is often called “dormant”. However, they must still be active in the metabolic activity to maintain and organize the cell process for adaptation to the environmental changes, even better than the metabolically active cells (10).

In the environment where nutrients are limited, the active aeration did not exert much influence on the persistence of the three species in any pH environment. This may be because that the cells in such starved condition did not have the well-developed oxygen-sensitive metabolic pathways. In other words, they may generate their energy for survival from the microaerophilic or anaerobic pathway. Though pseudomonads are normally described as strict
aerobes (11), the recent studies (12-15) show that this idea is not always correct for some species of pseudomonads, and that the anaerobic respiratory chain is equipped together with the oxygen-dependent channel (11,12). As far as P. pseudomallei is concerned, the adaptability to unusual pH environment is most remarkable in both aerated and unaerated conditions. Though the in vitro situation set up experimentally may differ from those of natural environment, the present observation is compatible with the well-known fact that P. pseudomallei can survive in soil and water for weeks, months, and even years (7,16,17). Not only in such natural environments, P. pseudomallei can persist also in human individuals causing chronic, latent or subclinical melioidosis with occasional relapse or conversion to acute septicemic cases (18-22).

The long persistence of P. pseudomallei cells in the tissue can produce granulomas which are not differentiated from those of tuberculosis on histopathologic ground alone (23,24). Such persistence of P. pseudomallei in the host may be, at least partly, due to their capability to survive in acidic tissue environment such as the phagolysosome of macrophages (25) and in the closed lesions of lower oxygen tension. In addition to metabolic factors, we think that the structural components, especially of the cell surface, are also the important determinants for the survival of P. pseudomallei in the tissue. An attempt is now underway to make clear the nature of pathogenicity and virulence of P. pseudomallei nature along this line.

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REFERENCES


