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HIGH ACTIVITY OF ACID PHOSPHATASE OF *PSEUDOMONAS PSEUDOMALLEI* AS A POSSIBLE ATTRIBUTE RELATING TO ITS PATHOGENICITY

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SUMMARY: Phosphatase activities were compared quantitatively among selected species of pseudomonads. *P. pseudomallei* showed the highest activity of a bell-shaped pH pattern with a peak at around pH 5.0. *P. cepacia* had a similar pattern of milder intensity. In contrast, *P. aeruginosa* revealed an alkaline phosphatase activity with a pH optimum higher than 8.0, but the level of activity was much lower than those of the above two species. The enzymatic reactions of other species were slight or negligible at their optimum pH in the same test system. These data were discussed in reference to their growth behavior in different pH environments and also in connection with such recent information that the high activity of microbial acid phosphatase may be a favorable attribute to their intracellular parasitism.

INTRODUCTION

One of the present authors has once studied on mycobacterial phosphatase with special concern to species-specific pH activity patterns (1). The results revealed that each species has its characteristic pH optimum in the acid side, and that the level of activity is particularly high in the pathogenic species.

This kind of study was recently extended by us to pseudomonads as a part of our project on *Pseudomonas pseudomallei*. Our observations gave an impression that bacterial acid phosphatase may have some relation to the mechanism of

pathogenicity at least partly. Such idea is recently emerging also from the enzymatic and metabolic studies on leishmania species (2) as a possible mechanism to make them survive within the phagolysosomal acid environment.

The present paper describes *p*-nitrophenyl phosphatase activities of selected species of pseudomonads, comparing both their qualitative (pH activity pattern) and quantitative (intensity) aspects. Discussions will be made on their biological significance.

MATERIALS AND METHODS

Microorganisms: Twenty-five strains of 11 species of pseudomonads were employed to test for their phosphatase activities. They were of clinical or environmental origin and stored at room temperature in stock medium; 1.0% Bacto casitone (Difco, Detroit, MI), 0.3% yeast extract (Difco), 0.5% NaCl, and 0.4% agar (Difco), with pH adjusted to 7.0-7.2. Most of these strains were sent to our laboratory for confirmation (3) and identified by the standard biochemical and chemical tests (4,5). The reference and type strains were obtained from the culture collections of Department of Microbiology, Gifu University School of Medicine, Japan; Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Thailand; and Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand. These strains are listed in Table I along with the strain numbers.

Before use, they were subcultured on trypton glucose extract (TGE) agar (Difco). A colony developed on this agar was transferred to 3 ml of nutrient broth (Difco). After 18 to 20-hr of incubation at 30 C, the broth culture was inoculated on TGE agar with a cotton swab. The 18-20 hrs growth was then suspended in an appropriate amount of physiologic saline, and its optical density was adjusted to 10 with a Hitachi spectrophotometer model 100-20 set at 420 nm.

Substrate and buffer: As a substrate for the phosphatase reaction, *p*-nitrophenyl phosphate was employed. A fresh 0.2% solution was prepared immediately before each experiment. Acetate buffer solutions ranging from pH 3.2 to 6.22 were prepared in 0.1 M. For pH 6.5 buffer solution, 1.16 g of maleic acid was dissolved in 40 ml of distilled water and 4% NaOH was added to make its pH to 6.5. Then, the total volume was made up to 100 ml with distilled water. Neutral and alkaline buffer solutions ranging from pH 7.0 to 10.2 were prepared as 0.1 M Tris-HCl buffer.

Determination of phosphatase activity: Phosphatase activity was determined with the reaction system consisting of 0.8 ml of a buffer, 0.1 ml of a bacterial suspension, and 0.1 ml of a substrate solution. The reaction mixture was incubated in a water bath at 40 C for 30 min and then 1.0 ml of 0.5 M NaOH and 1.0 ml of

Table I. The list of pseudomonads employed in this study

Pseudomonas species	strain No.	Source
<u>P. pseudomallei</u>	NCTC 4645	Dr. Kriangsak Saithanu ¹
	472/30	Blood-Chulalongkorn Hospital
	443/31	Blood-Chulalongkorn Hospital
	446/31	Blood-Choomporn Hospital
<u>P. cepacia</u>	JCM 5510	Dr. Kriangsak Saithanu
	668/31	Blood-Chulalongkorn Hospital
	613/31	Catheter-Chulalongkorn Hospital
	615/31	Catheter-Chulalongkorn Hospital
<u>P. aeruginosa</u>	JCM 5516	Dr. Kriangsak Saithanu
	JCM 5517	Dr. Kriangsak Saithanu
	ATCC 15692	Dr. Somporn Srifuangfung ²
	ATCC 27853	Dr. Toshio Shimada ³
<u>P. putida</u>	JCM 6160	Dr. Kriangsak Saithanu
	JCM 6157	Dr. Kriangsak Saithanu
	601/31	Blood-Chulalongkorn Hospital
<u>P. fluorescens</u>	406/31	CSF-Chulalongkorn Hospital
	240/31	Tap water-Prapokkla Hospital
	242/31	Blood-Barnpoong Hospital
<u>P. diminuta</u>	ATCC 11568	Dr. Eiko Yabuuchi ⁴
	=Gifu 657	
<u>P. mendocina</u>	ATCC 25411	Dr. Eiko Yabuuchi
	=Gifu 1953	
<u>P. pickettii</u>	Gifu 1934	Dr. Eiko Yabuuchi
<u>P. stutzeri</u>	338/31	Cervical swab-Trang Hospital
	380/31	Air-Pisanulook Hospital
	533/31	Blood-Cholpratar Hospital
<u>P. alcaligenes</u>	ATCC 14909	Dr. Eiko Yabuuchi
	=Gifu 2492	
<u>P. pseudoalcaligenes</u>	ATCC 17440	Dr. Eiko Yabuuchi
	=Gifu 363	

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distilled water were added, thus stopping the reaction and developing the yellow color of released *p*-nitrophenol. After centrifugation at 2,000 rpm for 10 min, the clear supernatant of each reaction mixture was subjected to colorimetric determination with a Hitachi spectrophotometer set at 420 nm. To the blank tube NaOH was added at zero time.

RESULTS

Biochemical and Biological Characters of Pseudomonad Strains Employed

Before the start of the present study, biochemical and biological characters of the pseudomonas strains to be employed were reexamined by the conventional methods (4,5).

The results are shown in Table II identifying their species.

Species Difference in the pH Activity Pattern of Phosphatase

Twenty-five strains of 11 different species were subjected to quantitative determinations of their phosphatase activities at a wide pH range from 3.2 to 10.2. Marked differences were noted from one strain to another in both the pH activity pattern and the reaction intensity. An example is shown in Fig. 1, in which the pH activity curve is compared among three species, *P. pseudomallei*, *P. cepacia*, and *P. aeruginosa*. The activity of *P. pseudomallei* was particularly high showing a pattern of acid phosphatase with a pH optimum of 5 to 6. The activity of *P. cepacia* was lower, though the pH activity pattern was rather similar to that of *P. pseudomallei*. In contrast, *P. aeruginosa* showed a pattern of alkaline phosphatase as observed in *E. coli* (6). The level of reaction, however, was much lower than those of the above two species.

Such observations were repeated and extended to the other species and strains. The results are summarized in Fig. 2, in which the level of comparative activity and the pH range for that are shown in combination. Generally speaking, each species has its characteristic phosphatase activity without much strain deviation. *P. putida*, *P. picketti* and *P. alcaligenes* showed typical pH ranges of alkaline phosphatase similar to that of *P. aeruginosa*, though the latter two species showed very low activities in the reaction. The activities shown by *P. stutzeri* and *P. pseudoalcaligenes* are of acid phosphatase, but the reactions were of negligible levels. *P. fluorescens* and *P. mendocina* exhibited definite acid phosphatase activities with rather narrow pH ranges. *P. diminuta* was unique in having a

Table II. Biochemical and biological characters of pseudomonads employed in this study

	P. pseudo-Mallei	P. cepacia	P. aeruginosa	P. diminuta	P. fluorescens	P. mendocina	P. putida	P. pickettii	P. stutzeri	P. alcaligenes	P. pseudoalcaligenes
Acid, (OFBM)											
Glucose	+	+	+	-	+	+	+	+	+	-	v
Fructose	+	+	v	-	+	+	+	+	+	-	+
Galactose	+	+	v	-	+	+	+	+	+	-	-
Mannose	+	+	v	-	+	+	+	+	v	-	-
Rhamnose	v	-	v	-	v	-	v	-	v	-	-
Xylose	v	+	v	-	+	+	+	+	+	-	v
Lactose	+	+	-	-	v	-	v	v	-	-	-
Sucrose	v	v	-	-	v	-	-	-	-	-	-
Maltose	+	+	v	-	v	-	v	v	+	-	v
Mannitol	+	+	v	-	+	-	v	v	v	-	-
Pyocyanin	-	-	+	-	-	-	-	-	-	-	-
Nitrate reduction	v	v	v	-	v	+	-	+	+	v	+
Gas from nitrate	+	-	v	-	-	+	-	+	+	v	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydro-lase	+	-	+	-	+	+	+	-	-	v	v
Lysine decarboxy-lase	-	+	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	v	-	-	-	-	-	-	-	-	-
Hydrolysis :											
Urea	v	v	v	-	v	v	v	+	v	v	-
Esculin	v	v	-	-	-	-	-	-	-	-	-
DNA	-	-	v	v	-	-	-	-	-	-	-
Lecithin	v	v	-	-	+	-	-	-	-	-	-
Gelatin	+	v	v	v	+	-	-	v	-	-	-
Acetamide	-	v	v	-	-	-	-	-	-	-	-
Growth on : ss	-	-	v	-	+	v	+	-	v	v	v
Mac Conkey	+	+	+	+	+	+	+	+	+	+	+
Cetrimide	-	v	+	-	+	+	+	-	-	-	v
Acetate	+	+	+	-	+	+	+	+	+	+	+
Growth at 42°C	+	v	+	v	-	+	-	v	v	v	v
Motile	+	+	+	+	+	+	+	+	+	+	+
Polartrichous	>1	>1	1	1	>1	1	>1	1	1	1	1

Sign : + = 90% or more positive after 24-48 hr. of incubation at 30°C
 - = 90% or more negative after 24-48 hr. of incubation at 30°C
 v = more than 10% and less than 90% positive

Fig. 1. pH activity patterns of phosphatase of *P. pseudomallei*, *P. cepacia*, and *P. aeruginosa*.

—●—: *P. pseudomallei* (472/30), —■—: *P. cepacia* (JCM 5510),
—▲—: *P. aeruginosa* (JCM 5516)

fairly high level of activity with a wide pH range from the acid side to the alkaline side, being comparable to *P. cepacia*.

Growth of Pseudomonads in Different pH Environments

With the above observations in mind, an attempt was made to compare the growths of *P. pseudomallei*, *P. cepacia*, and *P. aeruginosa* in brain heart infusion broth whose pH was adjusted to 4.0, 4.5, 5.0, 7.0 and 9.0. Broth with each pH value was dispensed into test tubes in an amount of 3 ml, to which 0.1 ml of exponential growth culture of each species was inoculated. Thereafter, the growth was followed with time intervals by measuring the turbidity at 600 nm.

As shown in Fig. 3, *P. pseudomallei* and *P. cepacia* did grow even in the medium of pH 4.5, but *P. aeruginosa* did not. On the other hand, *P. aeruginosa* grew in the environment of pH 9.5, but the growths of the other two species were negligible. In the pH 5.0 and 7.0 environments, the three strains exhibited good adaptability.

Fig. 2. Grouping of pseudomonads by their phosphatase activity (the level of activity and the pH range for that).

Fig. 3. Comparison of growth by *P. pseudomallei*, *P. cepacia*, and *P. aeruginosa* in brain heart infusion broth of pH 4.0, 4.5, 5.0, 7.0, or 9.0.
—●—: *P. pseudomallei* (472/30), —■—: *P. cepacia* (JCM 5510),
—▲—: *P. aeruginosa* (ATCC 27853)

DISCUSSION

In this study, we evaluated phosphatase activities of members of pseudomonads. Unlike conventional routine methods used in bacterial taxonomy (7,8), evaluation was made quantitatively with a standardized amount of dispersed suspensions. Besides, the enzymatic reaction was determined in a wide range of pH and read in a relatively short period (30 min) of incubation so that species-specific characters may be emphasized. In this test system, it was revealed that each pseudomonas species has its own pH-activity curve of phosphatase. This aspect of the enzymatic reaction may serve as an identification character in pseudomonad taxonomy. Meanwhile, we were interested also in the bacterial phosphatase activity in relation to their pathogenesis and virulence.

In the case of mycobacteria, every tested species showed acid phosphatase activity (1), virulent tubercle bacilli including *M. bovis* being highest in the level of activity. Likewise in pseudomonads, *P. pseudomallei*, the most pathogenic species, had the highest acid phosphatase activity.

Microbial acid phosphatase has been reported as an enzyme associated with the surface membrane (1,9). In leishmania species (e.g., *L. donovani*), the acid phosphatase is also a surface membrane enzyme (10) and reported to be 2.4-fold higher in virulent organisms than in avirulent organisms (11).

P. pseudomallei (12), tubercle bacilli, and *L. donovani* are all intracellular parasites whose residence is the acidic environment of phagolysosomes (13,14). In this context, we are inclined to suspect that strong acid phosphatase might be a favorable attribute of intracellular parasites (2) for their survival, and that the enzymological characteristics of the surface membrane acid phosphatase are apparently adapted to the living environment of the parasite (15).

Yabuuchi et al. (16) have reported that *P. pseudomallei* and *P. cepacia* can grow in brain heart infusion broth of pH 4.5, but *P. aeruginosa* can not. This observation was confirmed by us here together with an additional finding that *P. aeruginosa* can grow vigorously at pH 9.5, but the former two can not. Thomas et al. (17) recommended the use of nutrient agar plates of pH 6.0 for efficient isolation of *P. pseudomallei* organisms from soil samples. All these observations provide evidence showing that *P. pseudomallei* has a good adaptability an acidic environment to survive and multiply.

The physiologic role of microbial acid phosphatase is not clear, and the mechanism for its probable association with pathogenesis is still a matter of discussion. One explanation for the former may be that the enzyme allows the organisms to obtain necessary orthophosphate from organic phosphate compounds

for their nutritional demands. For the latter, a hypothetical explanation is that the microbial phosphatase acting on NADPH oxidase of host phagocytic cells interferes with the production of toxic oxidative radicals, thereby enhancing the survival of the parasite (2). One of the approaches to this problem is to expand the range of test microbial species to see whether or not acid phosphatase is a universal property specific to intracellular parasites. At the same time, explanation must be obtained also about the biological significance of alkaline phosphatase in *P. aeruginosa*. Is it related with the alkaline environment on the mucous membrane or other places?

Evaluation of phosphatase activity for differentiation of pathogenic strains from nonpathogenic strains of the same or similar species has been reported as a rapid method in mycobacteria (18,19), candida (20,21), and staphylococci (22,23). It is interesting that all these microorganisms have a strong interaction with phagocytes and tend to become intracellular parasites. These studies will be worthy of reexamination from a new angle as stated in this paper.

As for the relation between pseudomonas phosphatase and virulence, Liu (24) reported the heat stability of alkaline phosphatase in such pathogenic species as *P. aeruginosa* and *P. pseudomallei*. Our own experiences on this aspect will be reported elsewhere.

Lastly, we must add that such a gene was recently demonstrated in *Salmonella typhimurium*, an intracellular pathogen, that is required as a regulatory element for expression of the virulence function in conjunction with nonspecific acid phosphatase (25).

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