AFFINITY AND RESPONSE OF *BURKHOLDERIA PSEUDOMALLEI* AND *BURKHOLDERIA CEPACIA* TO INSULIN

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Abstract. The cells of *Burkholderia pseudomallei, B. cepacia* and *Pseudomonas aeruginosa* grown on agar plates were stained with fluorescently-labeled insulin. The former two species were stained positively indicating insulin binding but *P. aeruginosa* was not. Insulin exposure reduced phospholipase C and acid phosphatase activities of *B. pseudomallei* but did not affect those enzymatic activities of *B. cepacia* in the employed experimental conditions. It is suggested that *B. pseudomallei* have insulin receptors which may be associated with a signal transfer system involving phospholipase and protein tyrosine phosphatase.

INTRODUCTION

Recently, Woods *et al* (1993) suggested that serum insulin lavels may play a significant role in modulating the pathogenesis of *B. pseudomallei*, especially in melioidosis patients with diabetes mellitus as the underlying disease. This suggestion was made on the basis of their *in vitro* and *in vivo* experiments to reduce the growth rate of *B. pseudomallei* by insulin. The affinity of insulin to *B. pseudomallei* cells was also demonstrated by 125_I-binding studies. In other words, they explained the mechanism of frequent opportunistic occurrence of melioidosis in diabetes patients by the decreased level of insulin.

This information stimulated greatly our ongoing study though from the viewpoint of a different research interest. We have been concerned with acid phosphatase (tyrosine phosphatase) of *B. pseudomallei* claiming that it is a cell-surface glycoprotein enxyme (Kanai and Kondo, 1991; Kondo *et al*, 1991b, 1994) and that it may have a protein tyrosine phosphatase (PTP) function as a part of a signal transfer system to manage to environmental stimuli (Kanai and Kondo, 1994).

During our study several papers reported that protein tyrosine phosphatases are insulin receptors (Hashimoto *et al*, 1992; Ide *et al*, 1994). This information led us to an assumption that the interaction between *B. pseudomallei* and insulin, if any, may start from the binding of insulin to the cell-surface PTP. The present paper reports the affinity of insulin by *B. pseudomallei* and *B. cepacia* cells as revealed by fluorescently-labeled insulin and the effects on their acid phosphatase and phospholipase C activities.

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MATERIALS AND METHODS

Bacterial strains

Local strains of *B. pseudomallei* (UB16, UB26, UB42, UB46, UB54, UB122, UB165, UB173) and *B. cepacia* (UB77, UB140, UB145, UB146) were employed together with 2 strains (ATCC 27853, UB156) of *Pseudomonas aeruginosa*. The strains of *Burkholderia* had been isolated from melioidosis patients admitted in Sappasitthiprasong Ubon Hospital and identified on the basis of growth on selective medium, peculiar order, characteristic colony morphology, and various biochemical tests including oxidase, fatty acid GLC pattern, and pH activity pattern of acid phosphatase (Konda *et al*, 1991a). Further confirmation was made by immunofluorescence assay using antibodies against the protein fraction and endotoxin of *B. pseudomallei* (Petkanchanapong *et al*, 1992).

Culture

In order to obtain the constant growth conditions, stock culture was transferred to tripton soy

broth (TSB) and the overnight growth at 37°C was then subcultured onto tripton soy agar (TSA) plates. In most cases, one-day culture was employed to prepare bacterial suspensions.

Reagents

Insulin preparations from bovine pancreas, unlabeled (27.5 unit per mg) or labeled with fluorescein isothiocyanate (1 mole FITC per mole insulin) were obtained from Sigma (St Louis, MO, USA), together with other common chemicals such as vanadate, zinc chloride, p-nitrophenyl-phosphate, and p-nitrophenylphosphorylcholine.

Exposure of bacterial suspension to insulin, zinc chloride and sodium vandate

The exposure was carried out in an incubation system consisting of 0.3 ml of bacterial suspension and 0.2 ml of insulin suspension (10 mg per ml). For this system, aqueous bacterial suspensions of *B. pseudomallei* and *B. cepacia* were prepared and standardized to optical density of 0.8 at 420 mμ in the defined procedure. 0.5 ml of water was further added to the mixture tubes. A similar mixture without insulin served as a control.

After termination of incubation at 37°C for a given time, 0.1 ml aliquots of each mixture were sampled for the assay of phospholipase C and acid phosphatase activities as described later.

When zinc chloride and sodium vanadate were the test materials, basically the same procedure was employed.

Assy for phospholipase C activity

Phospholipase C activity was assayed by the method of Berka *et al* (1981). The incubation mixture consisting of 0.8 ml of 0.1M tris-HCl buffer (pH 7.74), 0.1 ml of 10 mM p-nitrophenyl phosphorylcholine and 0.1 ml of the whole bacterial cell suspension preincubated mixture with or without insulin was left to stand at 37°C for a given time, usually 1 to 2 hours. At the termination of incubation, 1 ml of 0.5M NaOH was added to each reaction mixture to stop the enzymatic reaction and to develop the yellow color of released p-nitrophenol. Futhermore, one ml of water was added. The optical density of the tubes was measured by Coleman spectrophotometer set at 420 mμ and the released amount of p-nitrophenol was calculated from the standard curve.

Assay for acid phosphatase activity

Acid phosphatase activity was assayed in a reaction mixture consisting of 0.8 ml of buffer, 0.1 ml of the bacterial suspension preincubated with or without insulin, and 0.1 ml of p-nitrophenyl phosphate solution (0.2%). For pH range from 3.19 to 6.22, acetate buffer solutions were prepared at 0.1M. For pH range higher than 6.5, 0.1M Tris-HCl buffer solutions were employed.

The reaction mixture was incubated in a water bath at 40°C for 30 minutes. To stop the reaction and develop the yellow color of released p-nitrophenol, 1.0 ml of 0.5M NaOH was added. After further addition of one ml distilled water the reaction mixture was subjected to colorimetric determination with a Coleman spectrophotometer set at 420 mμ. To the blank tube NaOH solution was added at zero time.

Fluorescence-staining

The colonies on TSA were scraped off and suspended in PBS to the density of around 0.4 at OD 420 mμ. One drop there of was placed on a glass slide and air dried. Then the smear was fixed with acetone for 15 minutes and dried. For fixation, formalin is not recommended. Thus fixed smears were covered with 20 μl of a previously diluted solution of FITC-labeled insulin, and incubated in a moist chamber at 37°C for 45 minutes. The treated smears were washed three times with PBS.

Lipid analysis to detect phospholipase activity

The reaction mixture consisting of 0.8 ml of 0.1M buffer solution, 0.1 ml of substrate (lipid) suspension and 0.1 ml of bacterial suspension (OD 0.8-0.9 at 420 mμ) was incubated at 37°C for a given time. The substrate was egg yolk lecithin or oleoyl lysophosphatidylcholine, each of 10 mg per ml. The incubation time was overnight for the former lipid and 3 hours for the latter. After termination of the incubation, 4 ml of chloroform-metha-

nol (2 : 1) was added to extract lipids with occasional shaking and left to stand for a while. The lower layer of the mixture was separated and evaporated at dryness. The residue here obtained was subjected to thin-layer chromatography with silica gel G-60 activated at 110°C for 30 minutes. The solvent system was hexane-ether-acetic acid (90 : 10 : 1) for neutral lipid analysis and chloroform-methanol-H_2O (65 : 25 : 4) for phospholipid analysis.

RESULTS

Fluorescence-staining

Seven *B. pseudomallei* strains and four out of 4 *B. cepacia* strains were stained positively with FITC-labeled insulin, but 2 strains of *P. aeruginosa* here employed were negative (Table 1). However, the level of fluorescence in the insulin-stained cells was lower (Fig 1-B) than that (Fig 1-A) stained by the indirect immunofluorescence assay as reported previously by the present authors (Nargourt *et al*, 1993). Besides, the stained cells showed a clear cell shape and dimension in the latter case but those in the former were more ill defined and aggregated. The difference between *Burkholderia* and *Pseudomonas* in the staining was distinct.

Table 1

Affinity to FITC-labeled insulin of *B. pseudomallei* and *B. cepacia*.

Species	Total strains tested	No. of tests	
		Positive	Negative
B. pseudomallei	8	7	1
B. cepacia	4	4	0
P. aeruginosa	2	0	2

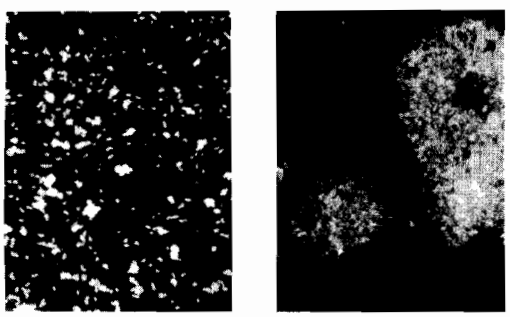

Fig 1–Staining of *Burkholderia pseudomallei* cells with specific antiserum (A) or FITC-labeled insulin (B).

pH activity patterns of phospholipase C

Before coming into the experiments to examine the effects of insulin and other agents on the phospholipase C activities of *B. pseudomallei* and *B. cepacia*, their pH-activity patterns in the enzymatic reactions were studied each employing several strains and arranging a wide pH-range from 3.8 to 8.4. The results are summarized in Fig 2, which indicates that the optimal pH is 7.2 for *B. pseudomallei* and 5.6 for *B. cepacia*.

Insulin-inhibition of phospholipase C

First, a time-course observation was made following the phospholipase C activities of *B. pseudomallei* (UB16) cells exposed to insulin in three

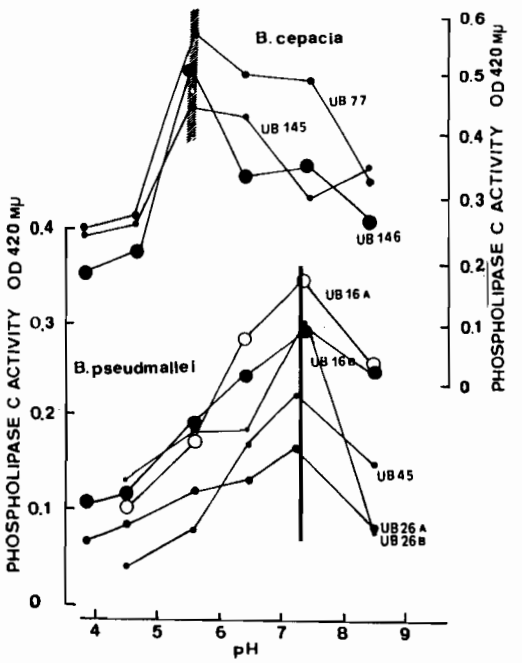

Fig 2–pH-dependent patterns of phospholipase C activity in *B. pseudomallei* and *B. cepacia*.

different concentrations, ranging from 13.8, 22.5 and 90 units per ml. Inhibition of the enzymatic activity was manifested in a dose-response manner as shown in Fig 3. Then, a further study was designed to compare such inhibitory effects between *B. pseudomallei* and *B. cepacia* employing 4 and 3 strains, respectively. The evaluation of the effects was made by per cent change of the specific activity in the insulin-treated cells.

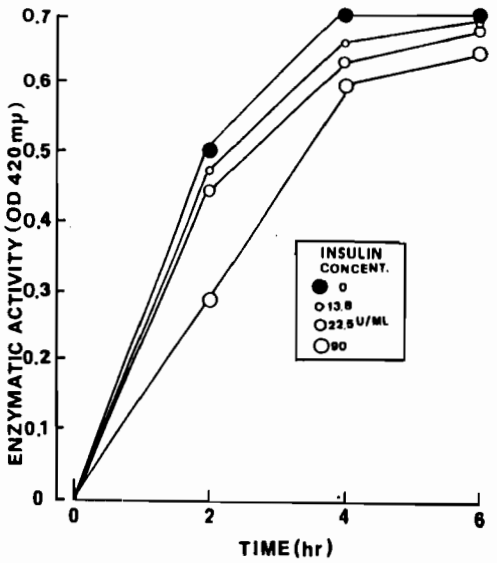

Fig 3–Inhibition by insulin of phospholipase C activity of *B. pseudomallei* (UB16), dose-response in time course observation.

The results are shown in Table 2. The inhibitory effects of insulin on phospholipase C activity were manifested only on *B. pseudomallei* and not at all in *B. cepacia.*

Insulin-inhibition of acid phosphatase activity

A preliminary test for insulin effect on acid phosphatase activity was conducted with *B. pseudomallei* (UB26) and *B. cepacia* (UB77). They were exposed to two concentrations of insulin, 0.1 mg and 1mg per ml, at five different pH environments from 4.6 to 8.5, for one hour. The results are shown in Fig 4. As is clear in this figure, the insulin effect on the enzymatic activity of *B. pseudomallei* is remarkable, but almost negligible on *B. cepacia.*

On the basis of this experience, a confirmation study was carried out with 55 units of insulin per ml at pH 5.57, comparing the effects on specific acid phosphatase activities between the two species of *Burkholderia.*

The results are shown in Table 3. This concentration of insulin inhibited the enzymatic activities in 5 cases out of six with three strains of *B. pseudomallei* (9 to 29% reduction).

In the case of *B. cepacia*, however, the specific activity was increased in 3 out of 4 cases.

Table 2

Insulin (55 U/ml) effects on phospholipase C activity of *B. pseudomallei* and *B. cepacia.*

Species	Strains	Specific activity* of phospholipase C		
		Control	Insulin exposed	%Reduction
B. pseudomallei	UB16	0.160	0.077	52
,,	UB26	0.056	0.040	29
,,	UB54	0.124	0.098	21
,,	UB54	0.042	0.030	29
B.cepacia	UB145	0.260	0.260	0
,,	UB145	0.305	0.305	0
,,	UB146	0.256	0.256	0

* PNPC mM/2 hours/0.1 ml bacterial suspension of the standardized turbidity (OD: 0.24 at 420 mμ). The enzymatic activity was assayed at the optimal pH of each species, pH 7.74 for *B. pseudomallei* and pH 5.6 for *B. cepacia.*

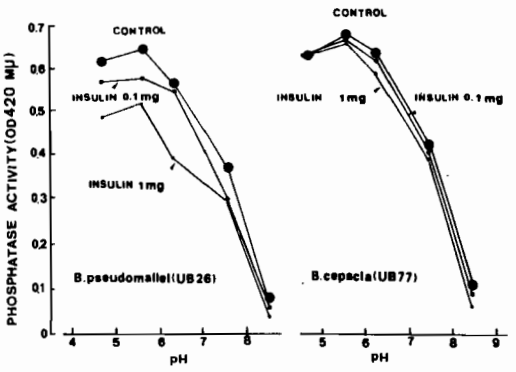

Fig 4–Effects of insulin on acid phosphatase activities of *B. pseudomallei* and *B. cepacia*.

Vanadate- and zinc chloride-inhibition of phospholipase C and acid phosphatase activities of *B. pseudomallei*

Vanadate and zinc chloride are general inhibitors of acid phosphatase. For reference to insulin effects, a study was made to see the effects of 0.1% vanadate on phospholipase C and acid phosphatase activities of *B. pseudomallei* (strain UB16) in the same experimental system as before. The results are shown in Fig 5. Here is shown that the *Pseudomallei* cells exposed to 0.1% are markedly inhibited in both enzymatic activities.

The exposure effect of 0.1% zinc chloride on

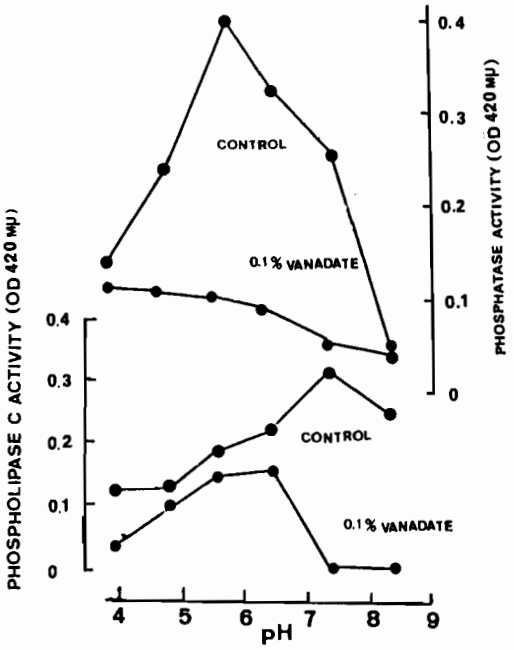

Fig 5–Inhibition by vanadate of phospholipase C and phosphatase activities in *B. pseudomallei* (UB16).

Table 3

Insulin (55 U/ml) effects on p-nitrophenyl phosphatase activity of *B. pseudomallei* and *B. cepacia*.

Species	Strains	Specific activity* of phosphatase at pH 5.57		
		Control	Insulin exposed	%Reduction (-) or increase (+)
B. pseudomallei	UB16	0.197	0.140	-29
,,	UB16	0.188	0.160	-15
,,	UB26	0.245	0.173	-29
,,	UB26	0.255	0.255	0
,,	UB54	0.124	0.099	-20
,,	UB54	0.124	0.133	-9
B.cepacia	UB77	0.255	0.246	+4
,,	UB145	0.098	0.100	+2
,,	UB145	0.139	0.178	+28
,,	UB146	0.255	0.255	0

* PNP mM/30 minutes/0.1 ml bacterial suspension of the standardized turbidity (OD: 0.24 at 420 mμ). The enzymatic activity was assayed at the optimal pH (5.6) of species.

Table 4

Effect of zinc chloride (0.1%) on phospholipase C activity and phosphatase activity of *B. pseudomallei* and *B. cepacia*.

Species	Strains	Specific activity* of phospholipase C			Specific activity** of phosphatase		
		Control	$ZnCl_2$ exposed	%Reduction	Control	$ZnCl_2$ exposed	%Reduction
B. pseudomallei	UB16	0.081	0.025	69	0.114	0.052	54
,,	UB26	0.040	0.015	62	0.090	0.065	28
B. cepacia	UB77	0.194	0.070	64	0.160	0.016	90
,,	UB145	0.127	0.067	47	0.124	0.013	90
,,	UB146	0.163	0.052	68	0.164	0.068	59

* PNPC mM/2 hours/0.1 ml bacterial suspension of the standardized turbidity (OD: 0.24 at 420 mμ). The activity was assayed at pH 7.74 for *B. pseudomallei* and pH 5.6 for *B. cepacia*.
* PNP mM/30 minutes/0.1 ml bacterial suspension of standard turbidity as above. The activity was assayed at pH 5.6.

the two enzymatic activities was compared between *B. pseudomallei* and *B. cepacia* in the same experiment system. As shown in Table 4, both enzymatic activities of the two species were inhibited > 50% in most cases.

Lipid analysis to comfirm phospholipase C activities

A synthetic substrate, p-nitrophenyl phosphorylcholine, was employed for detection of phospholipase C activity in *B. pseudomallei*. To confirm the presence of this enzymatic activity, the degradation of the natural substrate, eggyolk lecithin or synthetic substrate, oleoyl-lysolecithin were examined by lipid analysis of the incubation mixture with the bacterial suspension. The degradation of lecithin was very slow. Even when the incubation with whole bacterial cells was made overnight at optimum pH (7.4), only a small portion of lecithin was degraded. Meanwhile, lysolecithin was hydrolysed to a considerable extent in 3 hours incubation as shown in Fig 6, the size of the spotted substrates (LPC) decreased (spots No. 1, 3 on plate B,) and the spots of fatty acids as the final degradation products appeared on TLC plates A (FA). Lipase may have acted together with lysophospholipase. This activity was inhibited almost completely by heating of *B. pseudomallei* cells at 70°C for 30 minutes (Spot No. 2). The lysolecithinase activity was also observed in *B. cepacia* and *P. aeurginosa*.

DISCUSSION

Insulin was shown in this study to have an affinity for *B. pseudomallei* and *B. cepacia* but not for *P. aeruginosa*. This observation suggests a possibility that the factors facilitating insulin binding are rather specific to *Burkholderia* in agreement with the recent taxonomical proposal separating it from *Pseudomonas* (Yabuuchi *et al*, 1992). At the same time, this finding reminded us the information that protein tyrosine phosphatase (PTP) can be an insulin receptor (Hashimoto *et al*, 1992; Ide *et al*, 1994). In fact, we have claimed in our serial studies that acid phosphatase of *B. pseudomallei* and *B. cepacia* is a glycoprotein enzyme with tyrosine phosphatase activity (Kanai and Kondo, 1991; Kondo *et al*, 1994), though the enzyme of *B. pseudomallei* is more glycosylated and has a higher pH optimum than that of *B. cepacia* (Wongwanich *et al*, 1996). In contrast, the phosphatase activity of *P. aeruginosa* has a pH-optimum on the alkaline side and is much lower in the reaction intensity (Dejsirilert *et al*, 1989).

Previously, we were successful in fluorescent staining of *B. pseudomallei* with antisera against an acid phosphatase-active fraction obtained from the culture filtrate of this species. The staining was specific to *B. pseudomallei* and did not produce positive staining for *B. cepacia* (Naigowit *et al*, 1993). *B. pseudomallei* cells were stained in the

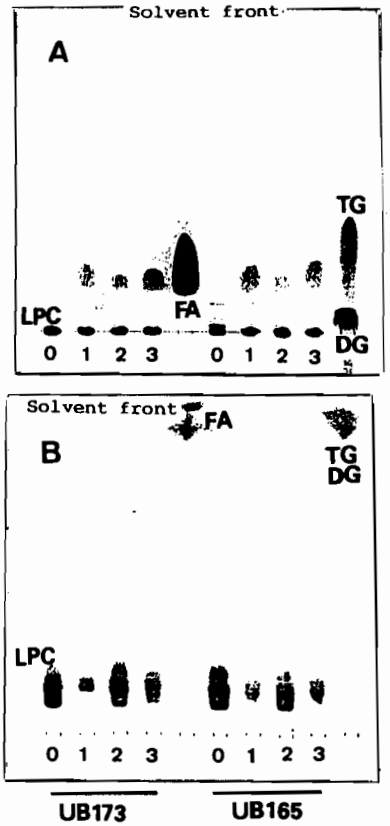

Fig 6–Degradation of lysolecithin by *B. pseudomallei* cells (UB173, UB165)
Solvent system: plate A: hexane-ether-acetic acid (90 : 10 : 1)
plate B: $CHCl_3$-CH_3OH-H_2O (65: 25 : 4)
No. 0 was no incubation control, No. 1 and 2 were incubated at pH 7.44, and No. 3 was incubated at pH 5.6 for 3 hours.
LPC: oleoyl-lysolecithin, FA: oleic acid, TG: triglyceride, DG: diglyceride

bacterial shape and dimension (Fig 1-A). Our subsequent studies (to be published) suggest that the antisera staining is specific to the sugar moiety of the enzyme glycoproteins. In contrast to staining with anti-enzyme sera, the staining with fluorescent insulin revealed rather ill-defined cells or their aggregated mass (Fig 1-B). A hypothesis to explain such staining difference is that the antienzyme sera are species-specific to the sugar moiety of TP and insulin is genus-specific, directed to the enzyme proteins. Further studies are required to prove this hypothesis.

In order to obtain a further evidence for the binding between PTP and insulin, functional analysis was conducted to see the effect of insulin on acid phosphatase activity (representing TP) and phospholipase C activity of *B. pseudomallei* and *B. cepacia*. Insulin inhibited those enzymatic activities of the former species but not those of the latter under the same experimental conditions. Vanadate, a potent PTP inhibitor (Pot *et al*, 1991; Hechit and Zick, 1992), also inhibited the enzymatic activity of *B. pseudomallei*. It is interesting to note here that vanadate has been shown to be a powerful antidiabetic agent in streptozoticin-treated diabetic rats (Shisheva *et al*, 1994). All this information is well explained by the idea that PTP is an insulin receptor and regulator (Hashimoto *et al*, 1992; Ide *et al*, 1994; Goldstain, 1992; Agius and Peak, 1992).

We would like to introduce here the concept that insulin did not arise evolutionarily in the intestinal or neural tissues of primitive vertebrates or complex invertebrates but rather has its molecular origin at least as far back as the simplest unicellular eukaryotes (LeRoith *et al*, 1981). If so, the discussion as above make sense.

The idea that PTP affinity for insulin contributes to the pathogenicity of *B. pseudomallei*, would mean that this species can respond to changing environmental conditions through adjusting its metabolic machinery per the operation of a signal transfer system involving phospholipase C activity (Rhee, 1991; Kircaid, 1991; Stock *et al*, 1989). Guan and Dixon (1991) demonstrated that PTP activity is an essential virulent determinant in Yersinia. This is not incompatible with the concept of "environmental virulence" proposed by Kroll (1990). We think that the same situation is present in *B. pseudomallei*. In this regard, it would be reasonable to think that the high pathogenicity of *B. pseudomallei* is, at least in part, due to its ability to adapt itself to the changing conditions in various environments (Defsirilert *et al*, 1991) so that it can colonize host tissues after chance invasion from the natural environment to produce a wide spectrum of clinical pictures.

The precise mechanism of insulin action on phosphatase and phospholipase C activities of *B. pseudomallei* requires future study (Kanai and Kondo, 1994). The difference in the insulin-sensitivity of the enzymatic activities between the two species is also waiting for future analysis.

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