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Phosphate and Carbon Source Regulation of Alkaline Phosphatase and Phospholipase in *Vibrio vulnificus*

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In this study, the effects of phosphate concentration and carbon source on the patterns of alkaline phosphatase (APase) and phospholipase (PLase) expression in *Vibrio vulnificus* ATCC 29307 were assessed under various conditions. The activities of these enzymes were repressed by excess phosphate (4 mM) in the culture medium, but this repression was reversed upon the onset of phosphate starvation in low phosphate defined medium (LPDM) containing 0.2 mM of phosphate at approximately the end of the exponential growth phase. The expressions of the two enzymes were also influenced by different carbon sources, including glucose, fructose, maltose, glycerol, and sodium acetate at different levels. The APase activity was derepressed most profoundly in LPDM containing fructose as a sole carbon source. However, the repression/derepression of the enzyme by phosphate was not observed in media containing glycerol or sodium acetate. In LPDM-glycerol or sodium acetate, the growth rate was quite low. The highest levels of PLase activity were detected in LPDM-sodium acetate, followed by LPDM-fructose. PLase was not fully repressed by high phosphate concentrations when sodium acetate was utilized as the sole carbon source. These results showed that multiple regulatory systems, including the phosphate regulon, may perform a function in the expression of both or either APase and PLC, in the broader context of the survival of *V. vulnificus*.

Keywords: *Vibrio vulnificus*, alkaline phosphatase, phospholipase, phosphate, carbon source

Vibrio vulnificus is a marine pathogen, and is known to cause septicemia in humans following the consumption of raw seafood or via wound infection. This particular variant of septicemia is fatal in over 50% of cases (Blake *et al.*, 1979). As *V. vulnificus* experiences two totally different environments, i.e. seawater and host animals, the pathogen is likely to be equipped with a variety of systems for responding to environmental changes that might prove a threat to its survival. As a pathogen, *V. vulnificus* is known to harbor a number of virulent factors, including several toxins (Linkous and Oliver, 1999; Gulig *et al.*, 2005). These factors are also likely to be regulated for more effective utilization of the gene pool. Therefore, the systems that respond to environmental changes and those that regulate virulence may be associated closely with the overall prosperity of the bacteria throughout its life cycle. In an effort to understand the networking among the systems, two genes that might be involved in both systems were selected; one of these is the alkaline phosphatase (APase) gene, and the other is the phospholipase (PLase) gene.

APase (orthophosphate monoester phosphohydrolase; EC 3.1.3.1) is ubiquitous among all organisms, thereby indicating its fundamental functions in metabolism (Trowsdale *et al.*, 1990). In prokaryotes, it has been identified as a phosphate scavenger under conditions in which organic phosphate is scarce and limits further growth. The APase gene of *E. coli* has been identified as a member of the *pho* regulon regulated by PhoB-PhoR, a two-component signal transduction system (Wanner, 1993). The regulon is derepressed upon phosphate starvation in the medium. Constitutive APase expression has been demonstrated to be coupled to hyper-invasiveness in a clinical isolate of entero-invasive *E. coli* (Sinai and Bavoil, 1993).

PLases have been previously associated with virulence in many pathogens (Portnoy *et al*, 1981; Mengaud *et al*, 1991; Titball, 1993; Barker *et al.*, 2004). Due to their catalytic capability, they may also be involved in scavenging phosphate in cases in which it constitutes a bacterial growth-limiting factor. PLase C of *Pseudomonas aeruginosa* has been well characterized with regard to its role in phosphate scavenging and pathogenesis (Berka *et al.*, 1981; Gray *et al.*, 1982). *Vibrio* species have also been shown to generate PLases, and some of them have been identified as hemolysins (Testa *et al.*, 1984; McCarter and Silverman, 1987; Pal *et al.*, 1997). Other PLases have been demonstrated to be involved in the induction of apoptosis (Kischnek and Gulbins, 2006) and the regulation of flagella (Schmiel *et al.*, 2000), thereby indicating that they may be under the control of multiple regulatory systems.

In this report, we demonstrated that the production of APase and PLase in *V. vulnificus* was regulated in the same manner by the phosphate concentration, but in a different manner by the carbon source used. The effects of other environmental factors on the production of the two enzymes are also discussed herein. The results implied that the production of both enzymes was controlled by a network of complicated regulatory systems for optimal survival and vir-

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ulence under fluctuating environmental conditions.

Materials and Methods

Bacterial culture conditions

V. vulnificus (ATCC 29307) was cultured in Luria-Bertani medium [LB; Bacto-tryptone 1.0% (w/v), yeast extract 0.5% (w/v)] containing 3.0% (w/v) NaCl at 30°C or 37°C with shaking (200 rpm). The effects of various growth conditions on APase and PLase production were monitored using a defined medium [100 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10 mM NH_4Cl, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.4% (w/v) fructose or other carbon source, 0.02% (w/v) casamino acid, 0.1 mM $FeCl_2$, 4 mM or 0.2 mM K_2HPO_4].

APase assay

A time-course assay of the APase activity of *V. vulnificus* was conducted using a defined medium containing high (4 mM; HPDM) or low (0.2 mM; LPDM) concentrations of phosphate and fructose as the carbon source (0.4%, w/v). An aliquot of the culture was obtained every hour and the growth was monitored via measurements of OD_{595}. APase activity in the cell lysates was assayed using *p*-nitrophenyl phosphate (*p*NPP; Sigma Co., USA) as the substrate. The cell lysates (250 μl) were mixed with 1 ml of pre-warmed 1 mM *p*NPP in 0.5 M CHES buffer (pH 10.0) containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$. The reaction mixture was then incubated at 37°C until yellow color developed, after which the reaction was stopped by the addition of 13% (w/v) K_2HPO_4 (500 μl). The optical density of the supernatant was then measured at 420 nm after 5 min of centrifugation of the reaction mixture at 12,000 rpm. One unit of APase activity was defined as the quantity of enzyme required to hydrolyze 1 μmol of *p*NPP per min. Specific activity was calculated as units of activity per ml culture divided by $O.D._{595}$ (Lee *et al.*, 1991). Each value is expressed as the mean of at least duplicate experiments.

Phosphate shock experiment

The direct regulation of APase or PLase expression by phosphate concentration was confirmed via the addition of an excess quantity of phosphate during growth under phosphate-limiting conditions. *V. vulnificus* was cultured in LPDM (0.2 mM K_2HPO_4) until the enzymes were induced at 37°C with shaking at 200 rpm. Upon the onset of the derepression of the enzyme synthesis, 4 mM of K_2HPO_4 was added and the cells were allowed to grow further under the same conditions. Cell growth and APase or PLase activity in the medium was monitored throughout the culture.

PLase assay

The time-course assay of the PLase activity of *V. vulnificus* was conducted using an artificial substrate, *p*-nitrophenyl phosphorylcholine (*p*NPC; Sigma Co., USA; Kurioka and Matsuda, 1976). The cell lysates (100 μl) were mixed with pre-warmed 50 mM *p*NPC (60 μl) dissolved in dH_2O and 2 M Tris-HCl (pH 8.0) containing 1 mM $ZnCl_2$ (140 μl). The reaction mixture was then incubated for 10 min at 37°C and the reaction was stopped by boiling for 3 min and mixing with 800 μl of EtOH and 200 μl of 2 M Tris-HCl (pH 8.0). The optical density of the supernatant was measured at 420 nm after 5 min of centrifugation at 15,000 rpm (Kuroshima and Hayano, 1982). One PLase unit was defined as the quantity of enzyme required to release 1 mmole of *p*-nitrophenol at 37°C for 1 h. Each of the values is expressed as the mean of at least duplicate experiments.

Results and Discussion

Phosphate regulation of APase expression in V. vulnificus

The expression pattern of APase in *V. vulnificus* in accordance with phosphate concentration was monitored via time-course assays using a defined medium (Fig. 1). High phosphate concentration (4 mM) in the defined medium resulted in the repression of APase activity during 12 h of culture. However, APase expression was derepressed in LPDM (0.2

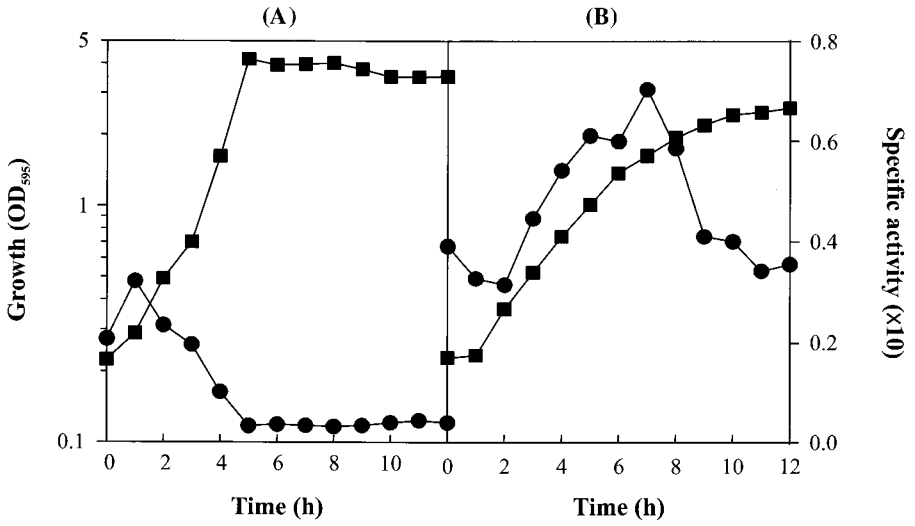

Fig. 1. Effect of phosphate concentration on APase expression in *V. vulnificus*. APase expression (•) and growth (■) profiles of *V. vulnificus* were monitored in either high phosphate (A) or low phosphate (B) defined medium at 37°C.

mM) at the early logarithmic growth phase, and continued to increase until the beginning of the stationary growth phase. The derepression level of APase specific activity was approximately two-fold. For other bacteria, APase has been shown to be derepressed upon phosphate starvation at concentrations below 0.1 mM (Spencer *et al*, 1981; Hulett and Jensen, 1988). In *V. vulnificus*, APase was derepressed when the phosphate concentration in the medium decreased to 0.08 mM (data not shown). The APase activity observed at the beginning of culture in both HPDM and LPDM was likely attributable to the activity carried over from the overnight pre-culturing of *V. vulnificus*.

In an attempt to verify that the repression/derepression of APase activity was indeed regulated by the phosphate concentration, we conducted a phosphate shock experiment via the addition of excess phosphate (4 mM) immediately after the derepression of the enzyme during culturing in LPDM (Fig. 2A). APase expression was repressed instantly upon the addition of phosphate, but the growth rate was improved almost to the control levels in HPDM. This shows that phosphate concentration in growth medium may actually constitute the critical factor in the growth and regulation of APase expression in *V. vulnificus*.

Phosphate regulation of PLase expression in V. vulnificus

PLase expression in *V. vulnificus* was monitored under the same conditions described above (Fig. 3). In HPDM, PLase activity, which was monitored by measurements of capability to hydrolyze an artificial substrate, *p*NPC, was maintained at the basal level. In LPDM, however, it became derepressed

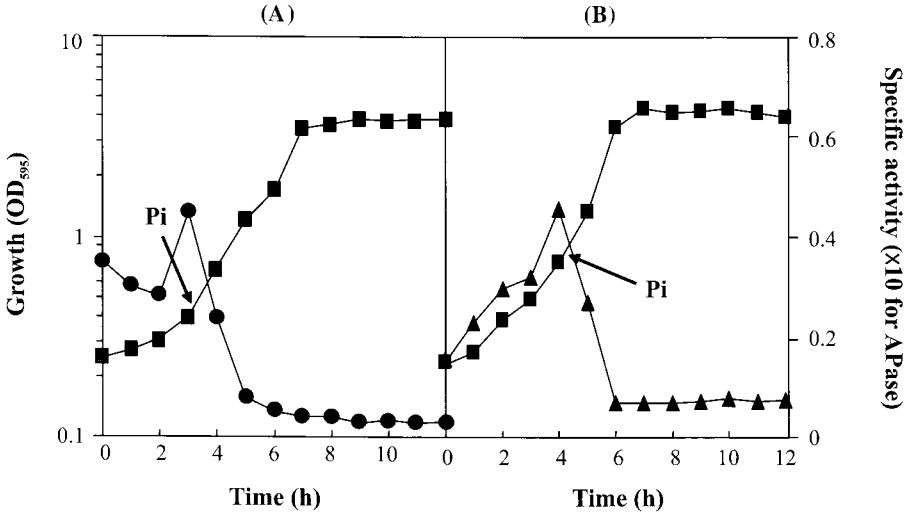

Fig. 2. Effect of phosphate shock on the expression of APase (A) and PLase (B) in *V. vulnificus*. The effects of phosphate on the expression of the enzymes was determined by adding excess phosphate (4 mM) immediately after the derepression of the enzymes was observed during culture in LPDM at 37°C. The graphs marked with squares represent the growth; circles, APase activity; triangles, PLase activity.

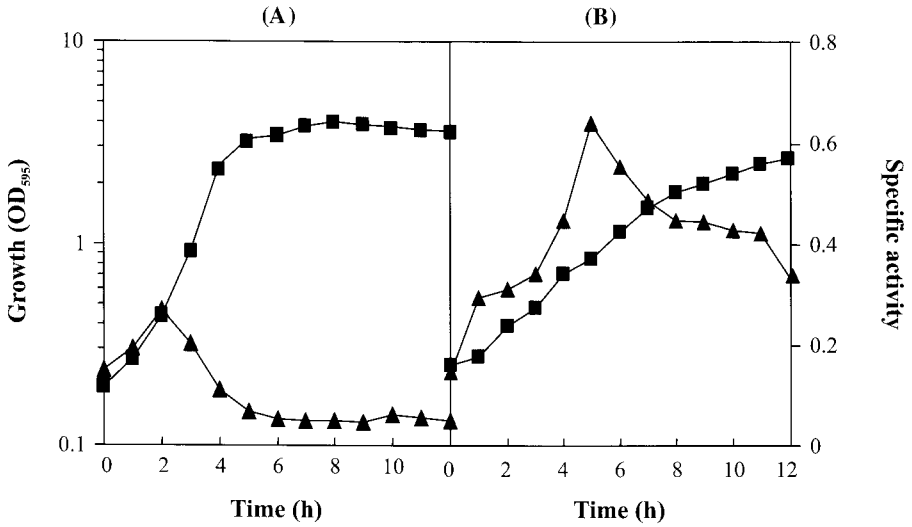

Fig. 3. Effects of phosphate concentration on PLase expression in *V. vulnificus*. PLase expression (▲) and growth (■) profiles of *V. vulnificus* were monitored in either high phosphate (A) or low phosphate (B) defined medium at 37°C.

upon the depletion of phosphate during the early logarithmic phase, and began to decline as the culture reached stationary phase. The PLase activity was derepressed by more than 3-fold. Under both culture conditions, PLase activity was observed at the very early growth phase (hour 0-2), which may indicate the presence of phosphate-irrepressible PLase in *V. vulnificus*.

PLase expression in LPDM was shut down to the basal level upon the addition of excess phosphate (4 mM), as was also observed with APase (Fig. 2B). Therefore, both APase and PLase were likely to be controlled by the phosphate concentration in the medium, which showed that they may be under the control of a common regulatory system, most probably by the *phoBR* operon. The coordinated regulation of the two enzymes by phosphate concentration has been reported for pathogens including *P. aeruginosa* (Ostroff and Vasil, 1987) and *Bacillus cereus* (Guddal *et al.*, 1989).

Carbon regulation of APase expression in V. vulnificus

In an attempt to determine factors affecting APase expression other than phosphate concentration in *V. vulnificus*, the effects of five carbon sources on the enzyme were assessed via a time course assay (Fig. 4). As the carbon source, glucose, fructose, maltose, or glycerol was added at a final concentration of 0.4% (w/v); sodium acetate 1.0% (w/v) to either HPDM or LPDM.

The phosphate regulation of APase expression was observed in medium containing glucose, fructose, or maltose with the

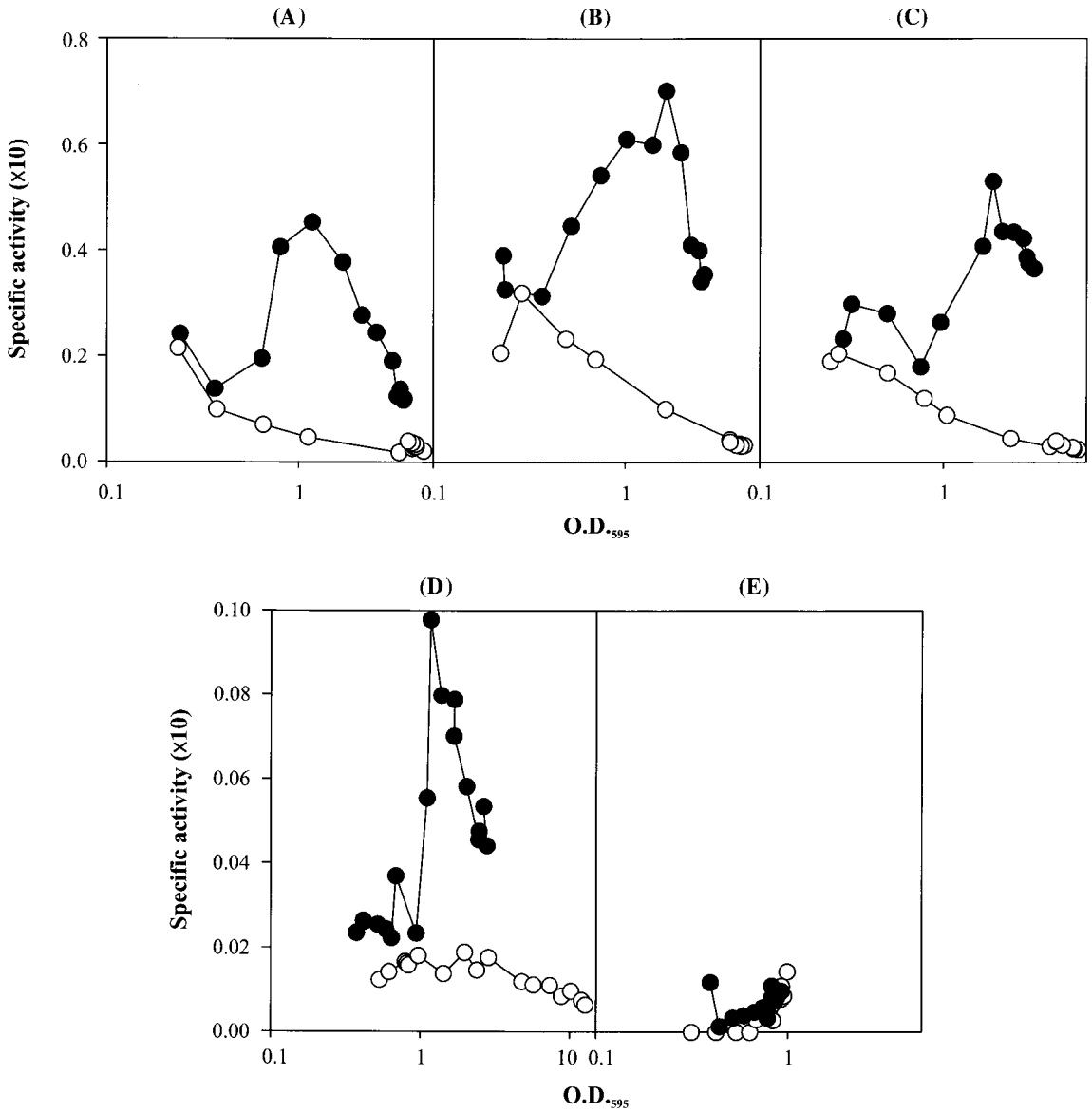

Fig. 4. Effects of carbon source on APase expression in *V. vulnificus*. APase expression was monitored by time-course assay in high or low phosphate defined medium containing various carbon sources. Panel (A) represents the specific APase activity according to growth (OD_{595}) in HPDM (open circles) and LPDM (closed circles) containing glucose as the sole carbon source, (B) fructose, (C) maltose, (D) glycerol, (E), sodium acetate. Note the difference in the scale of the Y-axis in panels D and E.

derepression of the enzyme observed at the early logarithmic growth phase (2-3 h into the culture). Among these, the derepression level of the enzyme was highest when fructose was utilized as the sole carbon source (Fig. 4B). The highest levels of enzyme activity were observed at the end of the logarithmic growth phase in medium containing fructose ($O.D._{595}=1.63$) or maltose ($O.D._{595}=1.86$) as the carbon source (Fig. 4B and C), which was not observed in the glucose medium. In the presence of glucose as the carbon source, the enzyme was derepressed during the early logarithmic growth phase, and achieved maximal levels at the mid-logarithmic phase ($O.D._{595}=1.17$), and then began to decline (Fig. 4A). These observations may be suggestive of the existence of an additional regulatory system which performs a function in APase expression separate from the phosphate system. The growth rate in each LPDM may influence the pattern of APase expression, as energy production is closely associated with the availability of phosphate in the cells. Additionally, the APase activity observed at the end of logarithmic phase, which persisted for many hours into stationary phase in the presence of either fructose or maltose might imply the existence of multiple APases under different regulation in the bacteria. Multiple APases forming a gene family in *Bacillus* spp. (Hulett *et al.*, 1986; Hulett *et al.*, 1991) and higher animals (Terao and Mintz, 1987; Weiss *et al.*, 1988) have been previously reported. Actually, two putative APase

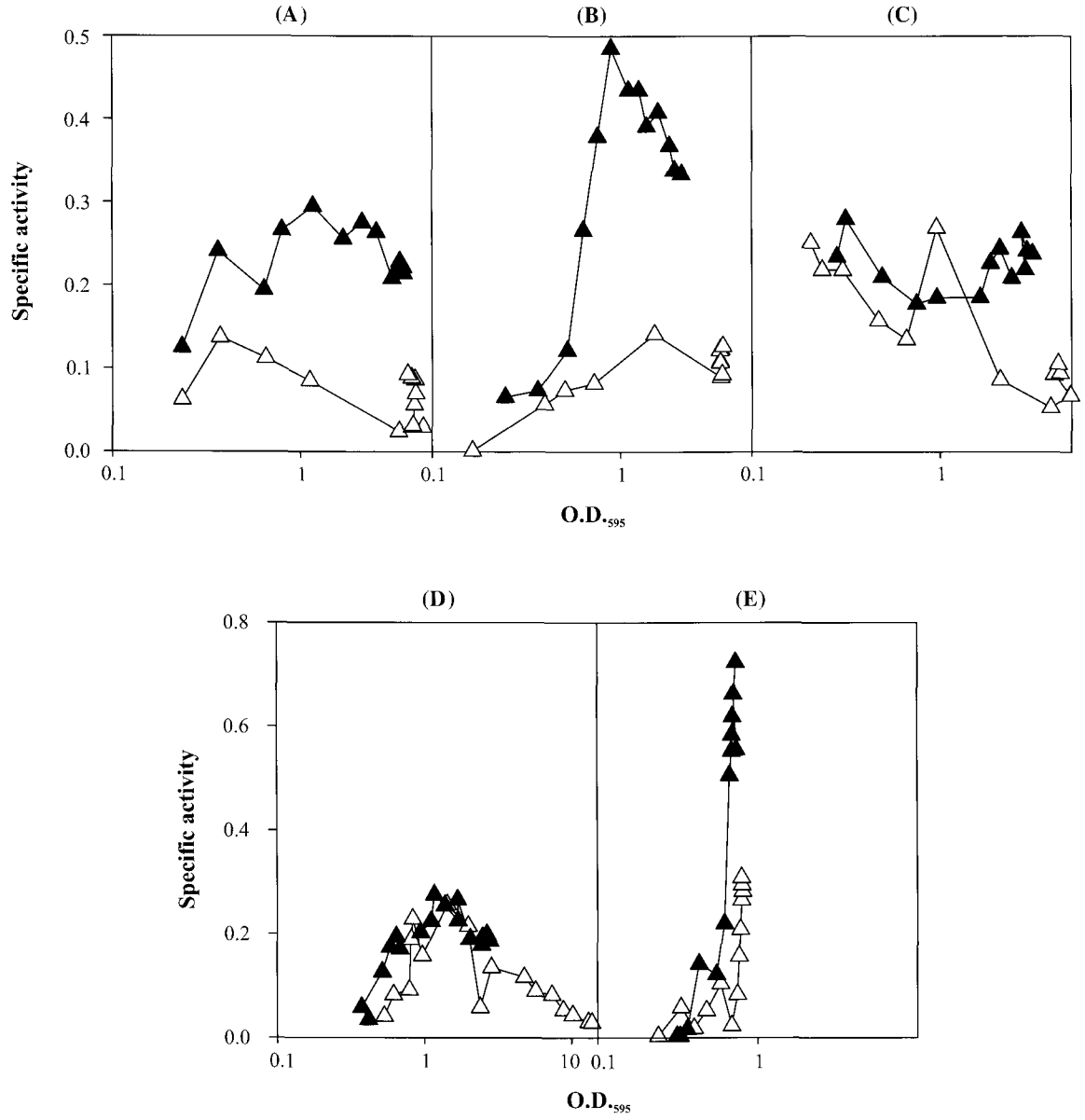

Fig. 5. Effects of carbon source on PLase expression in *V. vulnificus*. PLase expression was monitored via time-course assay in high or low phosphate defined medium containing various carbon sources. Panel (A) represents the specific PLase activity according to growth ($O.D._{595}$) in HPDM (open triangles) and LPDM (closed triangles) containing glucose as the sole carbon source, (B) fructose, (C) maltose, (D) glycerol, (E) sodium acetate.

structural genes have been cloned from *V. vulnificus* in the laboratory (unpublished data).

The bacteria grew at a slower rate in both HPDM- and LPDM-glycerol than in other media, and generated significantly less APase activity in LPDM-glycerol, even though it was allowed to grow to an $O.D._{595}$ as high as that in LPDM containing either fructose or maltose, for an extended time period (Fig. 4D). The onset of depression was also delayed and observed at 6 h into the culture in LPDM-glycerol, even though the culture began at a higher O.D. than did the other LPDMs. The fairly low growth rate of *V. vulnificus* in LPDM-glycerol may somehow influence the APase expression pattern under phosphate-limited conditions. Sodium acetate was not efficiently utilized as a carbon source by the bacteria, and only poor growth and almost no APase expression were observed under both phosphate concentration conditions (Fig. 4E).

Carbon regulation of PLase expression in V. vulnificus

The effects of various carbon sources on the expression of PLase activity in *V. vulnificus* were evaluated under culture conditions identical to those utilized for APase expression (Fig. 5). The repression and derepression of PLase at different phosphate concentrations in the culture medium were observed in all cases, although the repression was not as tight as it was for APase expression. The basal level of PLase activity was higher than that of APase in the presence of excess phosphate, which implies the presence of more than one PLase produced by the bacteria or dual PLase regulation modes in the cell. The repression of PLase in HPDM was tightest when glucose was used as the carbon source (Fig. 5A), which suggests that phosphate-irrepressible PLase may be affected by catabolite repression, which itself is likely exerted by the cAMP-CRP complex. PLase activity was derepressed to a similar level in LPDM containing glucose, maltose, and glycerol (Figs. 5A, 5C, and 5D), whereas fructose and sodium acetate induced its derepression to a significantly higher degree in the medium (Figs. 5B and 5E). The growth rates in the latter two media were lower than those observed in LPDM-glucose or -maltose, thereby suggesting that PLase may also be expressed in a growth rate-dependent manner. In LPDM-glucose and -glycerol, the enzyme was derepressed when the culture achieved the same $O.D._{595}$ of 1.1 (early exponential phase), and this level of activity was sustained at a similar level for 3-4 h (Figs. 5A and 5D). The derepression of the enzyme was observed in later stages of exponential phase ($O.D._{595}$=1.7) in LPDM-maltose than in LPDM-glucose, which may be ascribable to differences in the transportation efficiency of the sugars under phosphate-limited conditions. The derepression pattern of PLase activity in LPDM was similar to that of APase except in LPDM-sodium acetate. The derepressed PLase activity in LPDM-sodium acetate kept increasing up to 15-fold during the rest of the culture period (Fig. 5E). PLase activity in HPDM-sodium acetate also increased over the same period, even though the quantity was significantly lower than in the corresponding LPDM. The results again indicated that multiple PLases were generated by the bacteria, or that a PLase gene was under multiple regulation modes in the cell. The reason that PLase is overexpressed in LPDM-sodium acetate remains to be determined. Another peak of PLase activity was observed in HPDM containing maltose or glycerol (Figs. 5C and 5D), and was detected at 5-6 h of culture ($O.D._{595}$=0.96 and 1.43, respectively) and was not regulated by phosphate concentration.

These results indicate that both APase and PLase are regulated by phosphate concentration, thereby constituting a component of the phosphate starvation response system. They were also expressed in a similar, if not identical, mode in the presence of certain carbon sources, including glucose, fructose, and maltose. However, these enzymes appeared to be under the control of additional regulatory systems which may be unique to the expression of each enzyme when either glycerol or sodium acetate was utilized as the carbon source. The effects of glycerol and sodium acetate on APase and PLase, respectively, were shown to override the phosphate-mediated regulation. Such unique modes of expression for each enzyme were also observed according to temperature, heat-shock, pH, and osmotic pressure (data will be published elsewhere). The involvement of catabolite repression (Wanner *et al.*, 1988; Sage and Vasil, 1997; Puri-Taneja *et al.*, 2006), osmoregulation (Villarejo *et al.*, 1983; Sage and Vasil, 1997), the respiratory regulon along with the sporulation regulon (Sun *et al.*, 1996), and the global regulatory proteins in the regulation (Broich *et al.*, 2006) of APase and/or PLase have been well-documented. On the basis of the results obtained in this study, the regulatory network associated with the stress response and the expression of virulence in *V. vulnificus* warrant further investigation in future studies.

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