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Apparent Phosphate Retrieval System in *Bacillus cereus*

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Bacillus cereus **secretes three different phospholipases C. We studied the effect of P_i levels in the growth medium on the production of these exoenzymes. Production of both phosphatidylcholine-preferring phospholipase C and sphingomyelinase C was repressed by P_i in the growth medium, whereas production of phosphatidylinositol phospholipase C was unaffected. We also found that *B. cereus* secretes a phosphate-repressed alkaline phosphatase activity. Together with a previously reported highly efficient, active uptake system for P_i, these three phosphate-repressed exoenzyme activities seem to be part of a phosphate retrieval mechanism that operates under growth-limiting concentrations of P_i. In natural soil systems, which are the natural habitats of *B. cereus*, the scarcity of P_i is the major growth-limiting factor. A phosphate-repressed metalloprotease activity was also detected in culture supernatants of *B. cereus*. It is unclear whether this exoenzyme activity also participates in the proposed phosphate-scavenging system.**

It has been known for many years that during growth *Bacillus cereus* secretes three different phospholipase C enzymes, viz., a phosphatidylinositol-hydrolyzing enzyme (PI-PLC), a sphingomyelinase C (SMase C) which also possesses activity hemolytic to mammalian erythrocytes (5), and a phospholipase C which attacks phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (PC-PLC) (23). The genes that encode PC-PLC and SMase C have recently been cloned and sequenced (2, 8, 9, 30). However, the physiological function of these three exoenzymes has not been established. *Pseudomonas aeruginosa* also secretes a PC-PLC which, together with a heat-stable hemolysin and alkaline phosphatase (APase), seems to be part of a phosphate retrieval system operating under growth-limiting concentrations of P_i (3, 13). *B. cereus* is a soil bacterium, and P_i is an important limiting factor for biological growth in natural soil systems which is commonly present at levels some 2 to 3 orders of magnitude lower than those of other major nutrient ions (18). We studied the production of certain extracellular enzyme activities of *B. cereus* relative to P_i concentrations in the medium and concluded that *B. cereus* operates a P_i-repressed phosphorus retrieval-scavenging system.

MATERIALS AND METHODS

Organism and culture conditions. *B. cereus* SE-1 was grown in 250-ml acid-washed flasks in PHG1 or PHG2 medium. *B. cereus* SE-1 is derived from *B. cereus* ATCC 10987 by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenesis and overproduces PC-PLC fivefold per culture volume compared with the parent strain when grown to the stationary phase in rich media (9). PHG1 medium is a P_i-free synthetic medium with the following composition: 25 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (HEPES) buffer (pH 7.5), 0.4% glucose, 4% 100×-concentrated Eagle basal medium amino acid solution without glutamine, 0.01% L-alanine, 0.02% L-aspartate, 0.02% L-glutamate, 0.007% glycine, 0.005% L-proline, 0.01% L-serine, 0.03% L-glutamine, 70 mM NaCl, 8 mM $(NH_4)_2SO_4$, 5 mM KCl, 1.7 mM trisodium citrate, 0.4 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.025 mM $ZnSO_4$, 0.09 mM $FeSO_4$, and 0.025 mM $MnSO_4$. PHG2 medium is PHG1 medium supplemented with the medium described by Zwaal et al. (31) at 4% (vol/vol). When required, the P_i concentration of the culture medium was adjusted by addition of 1 M NaH_2PO_4–Na_2HPO_4 (pH 7.4). Cultures were inoculated 1/100 or 1/50 with a fresh overnight culture that originated from a single colony. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}). One OD_{600} unit was found to correspond to a total cell count of 1.1×10^8 cells per ml. All culture samples were appropriately diluted before determining the OD_{600} to ensure perfect linearity between OD_{600} measurements and total cell counts.

Chemicals. Phenylmethylsulfonyl fluoride, 1,10-phenanthroline, *N*-tosyl-L-phenylalanine chloromethyl ketone, *p*-nitrophenylphosphorylcholine, 2-(*N*-hexadecanoyl)amino-4-nitrophenylphosphorylcholine, azocasein, bovine serum albumin, soybean trypsin inhibitor, and all individual amino acids except L-serine (which was supplied by E. Merck AG, Darmstadt, Federal Republic of Germany) were from Sigma Chemical Co., St. Louis, Mo. 4-Nitrophenylphosphate bis (cyclohexylammonium) salt was from Janssen Chimica, Beerse, Belgium. Phosphatidylinositol from *Saccharomyces cerevisiae* was supplied by Koch-Light Laboratories Ltd., Colnbrook, England. L-3-Phosphatidyl[2-^{3}H]inositol was purchased from Amersham, Buckinghamshire, England. 100×-concentrated Eagle basal medium amino acid solution without glutamine was from GIBCO-Bethesda Research Laboratories, Paisley, Scotland. Calf intestinal alkaline phosphatase was purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany. All other chemicals used were of the analytical reagent grade of purity.

P_i determination. The method of Ames (1) was used for P_i determinations. To determine very low concentrations of P_i, 250-μl portions of sample were mixed with 100 μl of a freshly prepared 1:1 mixture of 7.14% (wt/vol) ascorbic acid in H_2O and 1.8% (wt/vol) ammonium molybdate in 5 N H_2SO_4 and the reaction mixture was incubated for 20 min at 45°C. After cooling to room temperature, the A_{640} was recorded. The peak absorption of the reduced phosphomolybdate complex is at approximately 820 nm (1), but we obtained more stable readings at 640 nm.

Enzyme assays. PC-PLC activity was assayed by the

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colorimetric method of Kurioka and Matsuda (14) by mixing 300 μl of substrate in buffered sorbitol with 50 μl of culture supernatant (undiluted or diluted in buffer containing 0.1 mg of bovine serum albumin per ml). The final composition of the assay mixture was 17 mM *p*-nitrophenylphosphorylcholine, 45 mM HEPES-NaOH (pH 7.5), 50% (wt/wt) D-sorbitol, 1.0 mM $MgCl_2$, 0.1 mM $ZnSO_4$, and 0.1 mg of bovine serum albumin per ml. SMase C also hydrolyzes *p*-nitrophenylphosphorylcholine but with only 5 to 6% of the activity of PC-PLC. Measured PC-PLC activities were not corrected for contributions from SMase C.

SMase C activity was determined as described by Tomita et al. (26), with sodium deoxycholate included at a final concentration of 0.07% (wt/vol).

In certain preliminary experiments, the very sensitive coupled assay system using calf intestinal alkaline phosphatase as originally described by Krug and Kent (12) was also used to determine PC-PLC and SMase C activities.

PI-PLC was assayed essentially as described by Ikezawa and Taguchi (6), in a total volume of 40 μl and with the modification that L-3-phosphatidyl[2-^{3}H]inositol was added to the reaction mixture (12,500 cpm per assay). Following extraction, a sample of the upper aqueous phase was subjected to scintillation counting.

Alkaline phosphatase was assayed by the procedure described by Keesey (11), while protease activity was determined by using azocasein as the substrate by the method of May and Elliott (17). One unit of protease activity was defined as causing an initial increase in OD_{440} of 0.001/min at 37°C in this system.

APase and PC-PLC assays were performed at either 22 or 37°C; the temperatures used are specified in the figure legends. The other activities were always assayed at 37°C. For the activities of enzymes other than protease, 1 U of activity causes hydrolysis of 1 μmol of the substrate per min in the relevant assay system.

Addition of P_i to the assay systems for APase, PC-PLC, PI-PLC, and SMase C at the highest concentrations encountered from P_i transferred from culture supernatants had no effect on the observed enzyme activities; hence, the activity profiles seen cannot be ascribed to direct inhibition effects of P_i on enzyme activity.

RESULTS

Secretion of PC-PLC, SMase C, PI-PLC, and APase at different P_i concentrations in the medium. To evaluate the effect of the P_i concentration in the medium on phospholipase C and APase production, culture supernatant activities of PC-PLC, SMase C, PI-PLC, and phosphomonoesterase were determined after growth of *B. cereus* SE-1 for 8 h in PHG2 medium containing different initial levels of P_i. PI-PLC activities were in essence unaffected by P_i levels in the medium, whereas the other three activities were markedly higher in media with low initial P_i concentrations (Fig. 1). The phosphomonoesterase activity observed was assayed at different pH values and found to be optimal at strongly alkaline pH values (ca. pH 10) and can thus be justifiably termed APase activity (data not shown). The results presented in Fig. 1 suggest that production of PC-PLC, SMase, and APase exoenzymes is repressed by P_i in the medium. Initial P_i levels below 0.7 mM resulted in markedly reduced growth rates, and it was at these growth-limiting P_i levels that APase, PC-PLC, and SMase C activities were maximally produced in the culture supernatant. These experiments were also performed with the parent strain, *B. cereus*

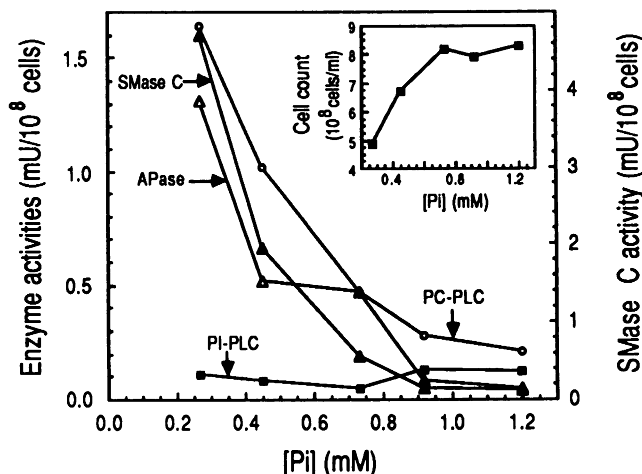

FIG. 1. Secretion of PC-PLC, SMase C, PI-PLC, and APase at different P_i concentrations in medium. Culture supernatants were assayed for the respective enzyme activities (see Materials and Methods) after growth of *B. cereus* SE-1 in PHG2 medium for 8 h. APase and PC-PLC were assayed at 22°C. The left axis represents APase, PI-PLC, and PC-PLC activities. The inset shows cell density after 8 h of growth as a function of the initial P_i concentration in the medium.

ATCC 10987. Exactly the same pattern of enzyme activities was seen, but the growth rate of the parent strain was generally slower in PHG2 medium than was that of strain SE-1 (data not shown).

Phosphate shock experiment. To examine more closely the P_i-mediated repression of these three culture supernatant activities, a phosphate shock experiment was performed. *B. cereus* SE-1 was grown in PHG2 (initial P_i concentration, 0.26 mM) for about 6 h. Then, P_i was added to 2.2 mM. Following addition of P_i, growth recommenced within 10 min while the activities of the three exoenzymes (expressed as activities per 10^8 cells) first declined after about 60 min (Fig. 2). In fact, maximum activities were seen 50 to 60 min after P_i addition. APase showed the most pronounced P_i repression, with a 10-fold decrease in activity per cell, while the rate of PC-PLC and SMase C production was reduced by only about 50% 3 h after the phosphate shock. However, when the phosphate shock was performed in the early log phase, the fall in the production of PC-PLC and SMase C activities was very much more pronounced and repression was evident about 30 min after P_i addition (data not shown).

Phosphate starvation experiments. The effects of shifting a high-P_i culture in which the three enzyme activities were repressed to P_i starvation conditions were studied. *B. cereus* SE-1 was first grown for 10 h in PHG2 with an initial P_i concentration of 3.9 mM. The cells were then quickly pelleted, washed, and suspended in PHG2 containing 0.26 mM P_i. A biphasic growth process was observed in which production of PC-PLC, SMase C, and APase increased when the P_i concentration decreased below 0.20 mM toward the end of the first growth step (Fig. 3). A transient increase in the P_i level of the medium occurred at the onset of the second growth step. Biphasic growth with a transient increase in the P_i concentration was verified in another experiment in which more frequent sampling was performed (Fig. 4). In this experiment, protein-free PHG1 medium was used in the P_i starvation part. Interestingly, while the production of PC-PLC activity was, once initiated, a fairly smooth process and a continuous buildup in this activity was ob-

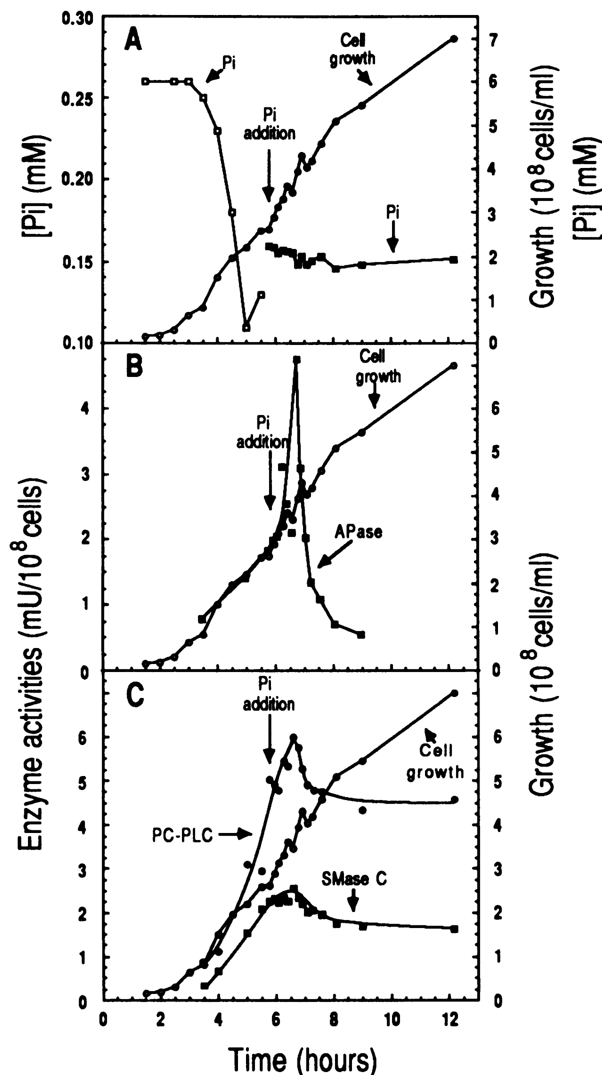

FIG. 2. Phosphate shock experiment. *B. cereus* SE-1 was grown in PHG2 medium (initial P_i concentration, 0.26 mM) for about 6 h. P_i was then added to 2.2 mM. Culture supernatants were assayed for P_i and enzyme activities as described in Materials and Methods. PC-PLC was assayed at 37°C, while APase was assayed at 22°C. In panel A, the P_i concentrations before and after P_i addition are shown on the left and right axes, respectively. On the right axis, the same scale is used for both cell growth and P_i concentration.

served, marked losses of both SMase C and APase activities were observed halfway through the second growth step (data not shown). The decreases in activities per 10^8 cells were far too large to be accounted for simply by bacterial multiplication without enzyme production and represented large absolute decreases in enzyme activity per milliliter of culture.

Production of protease activity during P_i starvation. The marked decreases in APase and SMase activities noted above could be explained by secretion of protease activity from the bacteria. In an experiment in which *B. cereus* SE-1 was grown in low-P_i growth medium, it was found (Fig. 5) that protease and APase activities appeared in the culture supernatant when P_i levels in the medium were below 0.05 mM. In an experiment similar to the one shown in Fig. 1, except that PHG1 medium was used, it was found that the amount of protease activity secreted increased dramatically

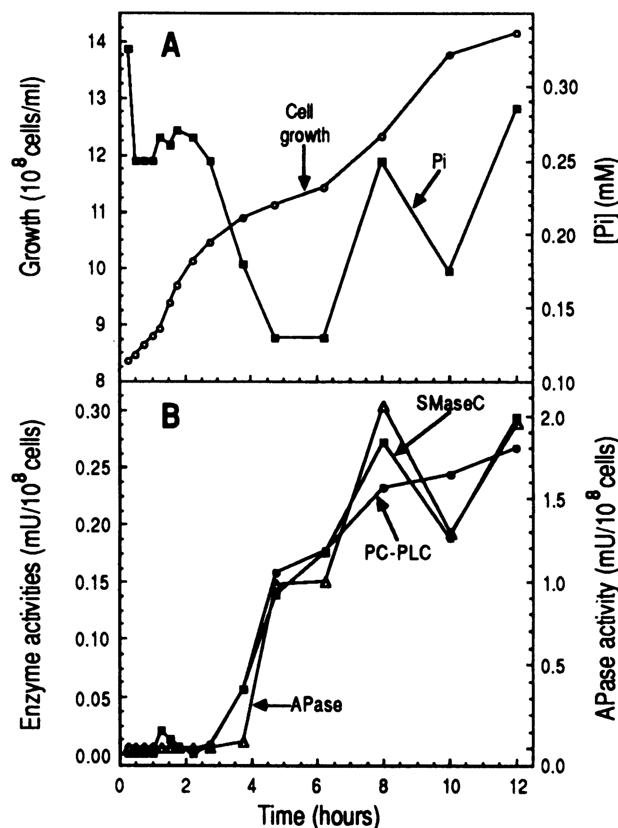

FIG. 3. Phosphate starvation experiment. Before P_i starvation, *B. cereus* SE-1 was grown for 10 h in PHG2 medium (initial P_i concentration, 3.9 mM). The cells were then pelleted, washed, resuspended, and grown in PHG2 containing 0.26 mM P_i. Cell growth and P_i concentration in the medium are shown in panel A. PC-PLC, SMase C (left axis), and APase (right axis) culture supernatant activities (see Materials and Methods) are shown in panel B. Both PC-PLC and APase were assayed at 22°C.

with decreasing P_i concentration in the culture medium (data not shown). This suggests that protease production-secretion by *B. cereus* SE-1 is also P_i repressed. In an attempt to characterize this extracellular protease activity, a sample of protease activity-containing culture supernatant was incubated with a series of standard protease inhibitors. It was found that of the inhibitors used, only EDTA and 1,10-phenanthroline were able to cause total inactivation-inhibition, thereby suggesting the presence of a metal-dependent protease(s) (Table 1).

DISCUSSION

Secretion of PC-PLC, SMase C, and APase activities by *B. cereus* appears to be a P_i-repressed process. In media in which P_i levels are not growth limiting, modest amounts of these enzymes are secreted, mostly in the early and mid-log growth phases. In media in which P_i levels are growth limiting, high levels of these enzymes are secreted during a biphasic growth process in which a transient P_i increase in the medium occurs along with a recommencement of cell growth. This combination of P_i-repressed exoenzyme activities could well be part of an effective phosphorus retrieval mechanism in *B. cereus*. PC-PLC and hemolytic SMase C in combination have the capacity to attack phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and

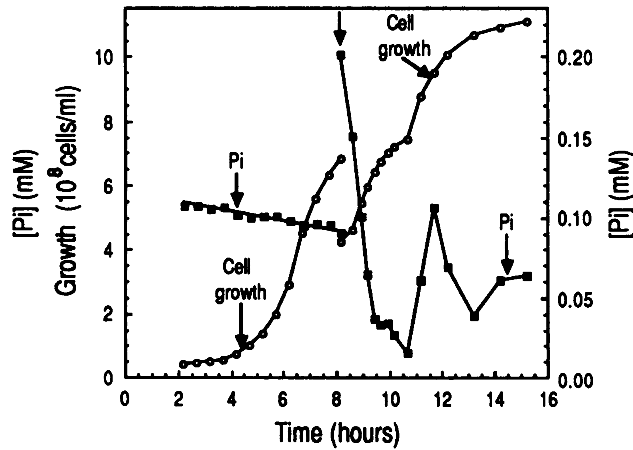

FIG. 4. Phosphate starvation experiment. An experiment parallel to the one whose results are depicted in Fig. 3 was done. Cell growth and P_i concentration in the medium are shown both before and after resuspension in low-P_i medium. The unlabeled vertical arrow near the top indicates the time of resuspension and dilution into low-P_i medium. The P_i levels before and after resuspension are shown on the left and right axes, respectively. Cell growth is shown on the left axis.

sphingomyelin. APase could then liberate P_i from the phosphorylcholine, phosphorylethanolamine, and phosphorylserine reaction products. It is tempting to suggest that precisely this process is responsible for the biphasic growth seen when P_i levels in the medium are growth limiting. Presumably, in this case, the membranes of old and dead cells are degraded by the phospholipases, with concomitant release of P_i due to APase activity. An analogous phosphate retrieval mechanism including P_i-repressed APase, PC-PLC, a hemolytic glycolipid, an outer membrane pore protein, and a periplasmic P_i-binding protein has been postulated for *P. aeruginosa* (3, 4, 13, 19). In *B. cereus*, the membrane-destabilizing function of the hemolytic glycolipid is replaced by PC-PLC and SMase C, which together constitute a functional cytolytic determinant (2). A highly efficient, active P_i uptake system that doubles its rate upon P_i starvation has been documented in *B. cereus* (22). Interestingly, *B. cereus* is also able to utilize aminoalkylphosphonates as P_i sources, and a P_i-repressed uptake system for such natural C-P-bound compounds has been described (15, 21). The most extensively studied bacterial phosphate retrieval system is the phosphate starvation-inducible regulon in *Escherichia coli* (28). Under P_i starvation conditions, a multicomponent gene system (regulon) is induced that includes APase to hydrolyze exogenous organic phosphates to P_i and transport proteins to facilitate uptake. About 20 different P_i-responsive operons are involved (29), and the synthesis of approximately 85 cellular proteins is affected by P_i limitation (7). It is conceivable that both *B. cereus* and *P. aeruginosa* contain such a regulon.

TABLE 1. Effects of various inhibitors on P_i-repressed protease activity[a]

Inhibitor (final concn)	Remaining protease activity (%)
None	100
Soybean trypsin inhibitor (0.5 mg/ml)	103
Phenylmethylsulfonyl fluoride (0.33 mg/ml)	103
N-Tosyl-L-phenylalanine chloromethyl ketone (0.33 mg/ml)	96
Iodoacetate (1 mM)	96
EDTA (1.25 mM)	2
1,10-Phenanthroline (1.25 mM)	0

[a] A culture supernatant containing maximum protease activity was preincubated for 15 min at room temperature with the appropriate inhibitor before protease activity was assayed as described in Materials and Methods.

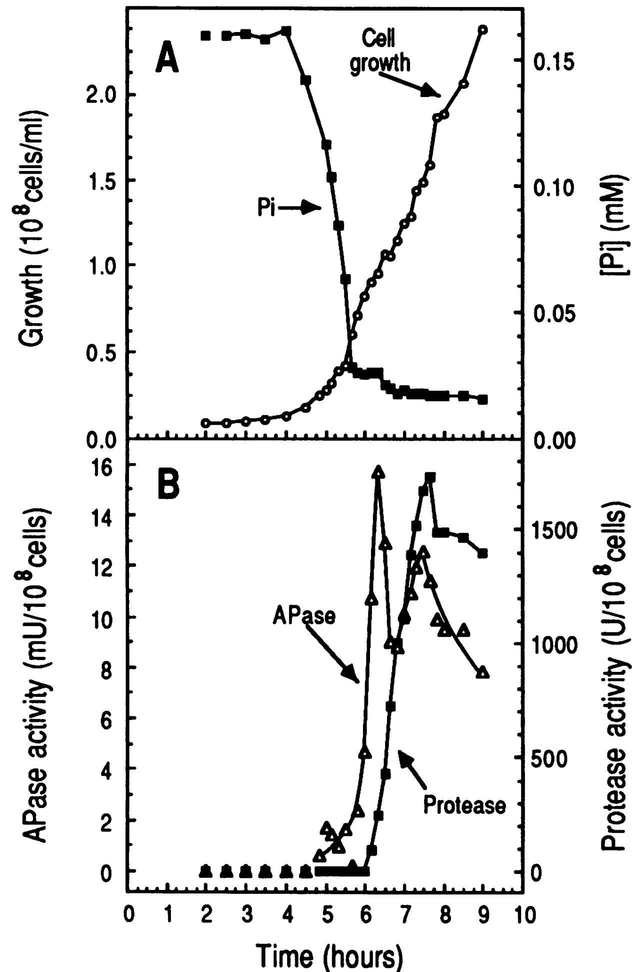

FIG. 5. Production of extracellular protease activity during P_i starvation. *B. cereus* SE-1 was grown in PHG1 containing an initial P_i concentration of 0.15 mM. Panel A shows cell growth and P_i concentration in the medium. Panel B shows secreted APase (left axis) and protease (right axis) activities. APase was assayed at 37°C. The assays were performed as described in Materials and Methods.

The metalloprotease activity of *B. cereus* described in this work is also P_i repressed. However, it is unclear how far this protease activity contributes to the proposed phosphate retrieval mechanism, especially since the protease activity almost certainly attacked SMase C and APase. PC-PLC is an extremely protease-resistant enzyme (C.L., unpublished data), and levels of this enzyme in protein-free growth medium seemed fairly stable.

Differences were noted in the extents of P_i repression of the exoenzymes studied. This was also found for APase, PC-PLC, and heat-stable hemolysin from *P. aeruginosa* (3, 10). In our P_i shock experiments P_i repression of the three enzyme activities was evident about one-half of a generation after P_i addition. A similar slow response to P_i shock was also observed for *P. aeruginosa* PC-PLC (25). This is

different from the extremely rapid repression of APase production seen in *E. coli* upon P_i shock (27). Thus, different regulatory mechanisms must be involved. It has been shown that P_i regulation of *P. aeruginosa* PC-PLC is at the transcriptional level (20). Since the kinetics of repression are similar for the *B. cereus* exoenzymes studied here, their production in response to P_i levels in the medium may also be regulated at the transcriptional level. In this respect, it should be noted that the genes that encode PC-PLC and SMase C are directly linked in the *B. cereus* genome (2, 8, 9, 30).

This is the first report describing secreted APase activity in *B. cereus*. P_i-repressed APase activities have been reported for both *B. subtilis* (16) and *B. licheniformis* (24). The P_i-repressed APase of *B. licheniformis* is secreted, while the activity of *B. subtilis* is not.

Production of PI-PLC exoenzyme activity by *B. cereus* was not influenced by P_i levels in the medium. However, phosphatidylinositol is a very minor phospholipid in biological membranes and has little potential as a source of phosphorus. Presumably, the physiological function of *B. cereus* PI-PLC differs from that of the two other phospholipases C.

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