

Exoprotease Activity of Two Marine Bacteria during Starvation

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Exoprotease activity during 120 h of total energy and nutrient starvation was examined in two marine bacteria, *Vibrio* sp. strain S14 and *Pseudomonas* sp. strain S9. The activity was determined by spectrophotometric measurement of the rate of release of soluble color from an insoluble azure dye derivative of hide powder (hide powder azure). Starved cells of both strains (5 h for S14, and 4 or 24 h for S9) showed greater extracellular proteolytic activity than at the onset of starvation. The exoprotease activity of cells starved for longer periods of time then decreased, but was found to be present at significant levels throughout the starvation period studied (120 h). The accumulation of exoprotease activity in the bulk phase during starvation indicated that both strains constitutively excreted extracellular proteases. As deduced from experiments with chloramphenicol, de novo protein synthesis during starvation was required for the production and/or release of the exoproteases into the surrounding environment. The degradation of hide powder azure allowed an immediate increase in respiration rate, also by long-term-starved cells. This suggests that metabolic systems are primed to respond to the availability of substrates, allowing the cells to recover rapidly. The regulation of exoprotease activity was also studied and found to be different in the two strains. Casamino Acids repressed exoprotease activity in *Pseudomonas* sp. strain S9, whereas a mechanism similar to catabolite repression was found for *Vibrio* sp. strain S14 in that glucose repressed activity and cyclic AMP reversed this effect. The exoproteases appeared to be metalloproteinases because the addition of EDTA to cell-free starvation supernatants from both strains significantly inhibited the activity of the proteases.

The marine environment is relatively dilute with respect to energy and nutrient sources (3). Furthermore, only 5 to 10% of the dissolved combined amino acids (comprising protein and peptides) are utilized directly by bacteria in the form of amino acids or small peptides (3). In order to most effectively obtain energy and nutrients in the oligotrophic environment, the marine copiotroph (18) must utilize an efficient substrate-scavenging tactic during both growth and non-growth conditions. The switch from low- to high-affinity uptake of amino acids by starved cells (14) is one example of this, and exoprotease activity is another.

In aggregates or microniches where surfaces provide for accumulation of substrate, intense nutrient cycling takes place. This can be attributed partly to the exoprotease activity of the microorganisms associated with these surfaces (10). It has been concluded that exoproteases are usually cell bound, since little protease activity is found in sterile filtered seawater (11, 21). Although it would not seem beneficial for microorganisms to excrete proteins, especially during nutrient-limited conditions, the formation and release of exoproteases could be advantageous for microniche-associated bacteria because the release of extracellular proteases would allow substrate scavenging over a larger area.

The continued synthesis of proteins by marine bacteria during long-term energy and nutrient starvation has been demonstrated both by measurements of the relative rate of protein synthesis (17) and by the incorporation of radioactivity into specific proteins (16). Therefore, the synthesis of proteins leading to exoprotease activity during long-term starvation appears to be feasible. Previous studies have also demonstrated the synthesis of starvation-related proteins which improve the uptake capacity of cells at low substrate concentrations (8, 9, 14).

The regulation of starvation proteins has only been studied in a few systems, e.g., the regulatory role of cyclic AMP (cAMP) on proteins induced by glucose or succinate starvation in *Escherichia coli* (20) and on proteins induced during nicotinate depletion in *Salmonella typhimurium* (22). Studies on the regulation of exoprotease synthesis in gram-negative bacteria have demonstrated the presence of different mechanisms: catabolite repression (5–7, 23); repression by casamino acids (23); and induction by amino acids (7, 25). The role of such regulatory mechanisms in the marine environment has not yet been studied. A regulatory role for cAMP in the marine environment has been suggested by Ammerman and Azam (2). They speculated that cAMP produced by algae could act as an exogenous signal for bacteria, indicating the metabolic and nutritional status of the microenvironment. In the present study, the formation, release, and regulation of exoprotease activity during starvation of *Vibrio* sp. strain S14 and *Pseudomonas* sp. strain S9 were examined.

MATERIALS AND METHODS

Organisms, cultivation, and starvation conditions. The marine *Vibrio* sp. strain S14 (CCUG 15956) resembles *Vibrio proteolyticus* ATCC 15338 but is atypical in four characteristics: (i) it accumulates poly- β -hydroxybutyrate (PHB) during growth, (ii) it is negative for amylase, (iii) it does not grow at 40°C, and (iv) it does not swarm on solid complex medium. The marine *Pseudomonas* sp. strain S9 has been described by Wrangstadh et al. (26). Both strains were grown to exponential phase in VNSS containing 1.0 g of peptone, 0.5 g of yeast extract, 0.5 g of glucose, 0.5 g of starch, 0.01 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.01 g of Na_2HPO_4 per liter of nine-salt solution (NSS) (15). NSS was used for washing the cells and in the starvation regimen when cells were starved for glucose, amino acids, nitrogen, and phosphorus (15). Starvation regimens were prepared as described

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by Albertson et al. (1). Cell densities were monitored spectrophotometrically at 610 nm to provide similar conditions (5×10^7 cells per ml at the onset of starvation corresponds to an A_{610} of 0.1) between experiments.

Exoprotease activity of cells and filtered supernatants. At selected times of starvation, cells were centrifuged and resuspended in fresh NSS to the same optical density. Hide powder azure (HPA; Sigma Chemical Co.) was added to 20 ml in a 50-ml flask (final concentration, 1 mg/ml). Samples were incubated on a rotary shaker (150 rpm) at 26°C, and 1.5-ml samples were withdrawn, filtered (0.2- μ m pore size; Nuclepore), and measured spectrophotometrically at 595 nm.

To determine whether protein synthesis during starvation was required for exoprotease activity, 5-h-starved cells were washed and suspended in NSS containing chloramphenicol (100 μ g/ml). After HPA was added, the exoprotease activity was monitored for 3 h. To determine the exoprotease activity in the filtered supernatant, starved-cell suspensions were centrifuged and the supernatant was filtered (0.2- μ m pore size) prior to HPA addition (1 mg/ml). Samples were incubated and assayed as described above. To determine whether the exoprotease activity in the filtered supernatants was due to small cells passing through the 0.2- μ m filter, samples were plated on nutrient agar. To further establish whether nonrecoverable but substrate-active cells could be the source of exoprotease activity in the supernatant, the initial rate of incorporation of L-[4,5- 3 H]leucine in the supernatant and cell suspension from cells starved for 24 h was determined by the method of Nyström et al. (17). As a further control that the exoprotease activity in the supernatant was due to deliberate protein synthesis and release and not to cell lysis, 8-h-starved cells were washed and suspended in NSS containing chloramphenicol (100 μ g/ml) and incubated for 10 h. The supernatants were then isolated and assayed for protease activity as described above.

Respiration measurements. In order to determine whether starved cells could respire the degradation products of HPA, the rates of oxygen consumption by starved-cell suspensions of *Vibrio* sp. strain S14 and *Pseudomonas* sp. strain S9 were measured by using the oxygen monitor of Hansatech (DW1). HPA (1 mg/ml) was added, and 2-ml samples were taken throughout an 8-h incubation period. Respiration rates were compared with those of the controls without added HPA.

Regulation of exoprotease activity. For studies on the regulation of exoprotease activity, glucose (4 g/liter), Casamino Acids (4 g/liter), or cAMP (1.99 mM) was added to suspensions of exponential-phase cells that had been washed twice, suspended in NSS, and incubated for 5 h (*Vibrio* cells) or 2 h (*Pseudomonas* cells) before measuring exoprotease activity. The shorter incubation time used for S9 cells is a result of the significantly greater exoprotease activity of these cells than of S14 cells. These concentrations of glucose and Casamino Acids are routinely used in growth media in our laboratory, although a relatively wide range of concentrations was found to give similar results. The concentration of cAMP corresponds to the lowest level which resulted in a significant response in these experiments. The addition of glucose, Casamino Acids, and cAMP was also tested on filtered supernatants from starved cultures to elucidate at which level, regulation of exoprotease synthesis or steric effects on exoproteases, the additions affected exoprotease activity.

Exoprotease characterization. As a preliminary characterization of the exoproteases, a variety of protease inhibitors having different specificities (4) were added to filtered super-

natants from cells starved for 5 h. Specifically, 2.5 mM disodium EDTA (to inhibit metalloproteinases), 1 μ M leupeptin (an inhibitor of cysteine and serine proteinases), 1 mM phenylmethylsulfonyl fluoride (PMSF, an inactivator of serine proteinases), or 1 μ M pepstatin (an inhibitor of acidic proteinases) was added, and exoprotease activity was measured after 6 h of incubation for *Vibrio* sp. strain S14 and 3 h for *Pseudomonas* sp. strain S9.

Osmotic shock. Periplasmic proteins were obtained by osmotic shock in distilled water as follows. A starvation suspension (120 ml) was centrifuged and suspended in 1 ml of distilled water for 20 min. After centrifugation, the supernatant was filtered (0.2- μ m pore size).

RESULTS

Exoprotease activity and release during starvation. The exoprotease activity of *Vibrio* sp. strain S14 and *Pseudomonas* sp. strain S9 during 120 h of starvation is shown in Fig. 1. The multiphasic kinetics of the curves reflect the sensitivity of the method and not the induction of exoprotease activity by HPA. HPA was found to be completely insoluble in NSS, so a mechanism for induction by HPA seems unlikely. Throughout the starvation period studied, S9 showed greater exoprotease activity per culture density than did S14. It was not determined whether this difference between the strains was due to a difference in exoprotease synthesis and release rates or to a difference in the affinity of the exoproteases for HPA. After 5 h (S14) and 4 h (S9) of starvation, both strains demonstrated an increased rate of extracellular HPA degradation compared with that at the onset of starvation. The exoprotease activity of S9 cells starved for 24 h was as high as that of cells starved for 4 h. Other substrates (casein, albumin) were also degraded by these organisms, but the kinetics of degradation were only determined for HPA. Washed cells of S9 starved for 13 days were also found to degrade HPA (data not shown). To normalize for variations in cell densities between experiments and also to account for changes in biomass during starvation, the A_{595} of the HPA filtrate was divided by the A_{610} of the culture, which was adjusted to 0.1 at the onset of starvation. Strain S9 was particularly sensitive to cell concentrations; even a doubling in the number of cells per milliliter in the starvation regimen resulted in a marked decrease in normalized exoprotease activity, although the same pattern of increased exoprotease activity during starvation was demonstrated.

To indicate whether de novo protein synthesis was required for the exoprotease activity seen in the assay, 5-h-starved and washed cells were suspended in NSS containing 100 μ g of chloramphenicol per ml. This concentration inhibited the initial rate of protein synthesis, measured as the incorporation of leucine in *Vibrio* sp. strain S14 by 95% and in *Pseudomonas* sp. strain S9 by 84%, but had no effect on the viability of cells during the incubation, as determined by CFU. In comparison to the controls, the exoprotease activity in S14 was inhibited by 94% and in S9 by 87% after 3 h of incubation with chloramphenicol. In another experiment, 8-h-starved cells were washed and subdivided into two samples: one received chloramphenicol, and the other served as the control. After an additional 10 h of starvation, the supernatants were collected and the exoprotease activity was compared. No exoprotease activity was found in the chloramphenicol-treated cells, clearly demonstrating that only cells with intact protein synthesis activity are able to synthesize and release exoproteases into the supernatant.

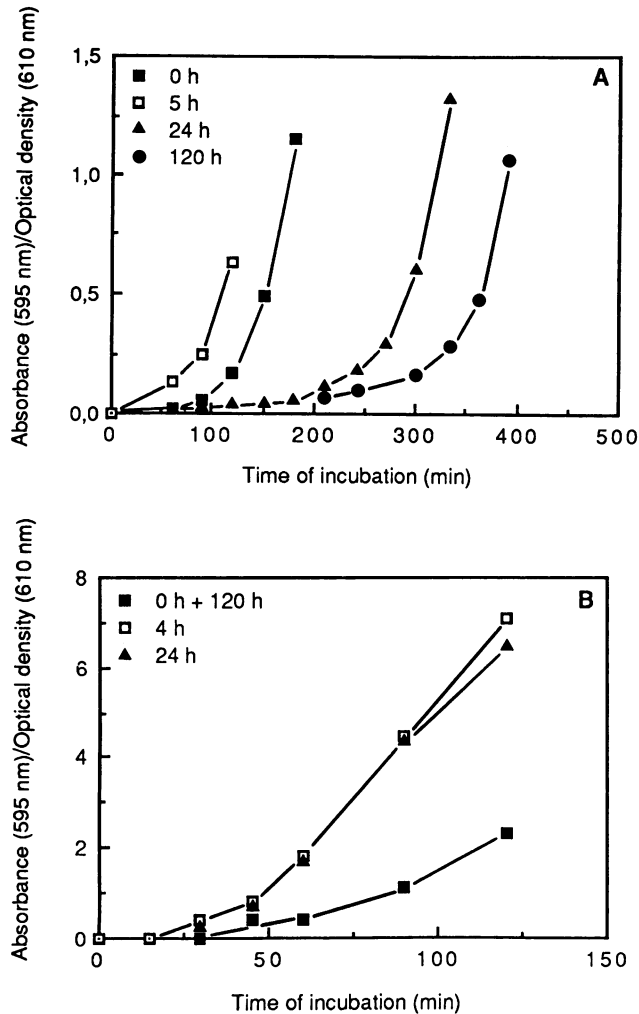


FIG. 1. Synthesis of exoproteases by starved cells of *Vibrio* sp. strain S14 (A) and *Pseudomonas* sp. strain S9 (B) as determined by the increase in A_{595} due to the release of soluble color from the insoluble substrate HPA divided by the A_{610} of the cell suspension. Cells were starved in NSS, washed at the appropriate times to remove accumulated extracellular proteases, and resuspended to the same cell density in NSS containing HPA. The experiments were repeated at least twice, with insignificant deviations between experiments. Representative results are presented.

The accumulation of exoprotease activity in the supernatant during starvation is shown in Fig. 2. The exoprotease activity in *Pseudomonas* sp. strain S9 appeared to be cell bound in cells starved for 4 h and subsequently released, although no further accumulation between 24 and 96 h of starvation was found (Fig. 2B). In *Vibrio* sp. strain S14, however, there was continuous accumulation of activity during 120 h of starvation (Fig. 2A). To ensure that the activity found was not a result of small cells passing through the 0.2- μ m filters, the supernatants were plated on nutrient agar, but no colonies were formed. The possibility that small, substrate-active, but nonrecoverable cells were responsible for the exoprotease activity was determined by comparing the incorporation rate of L-[4,5- 3 H]leucine in the filtered supernatant and cell suspension. The rate of incorporation in the filtered supernatant collected from 24-h-starved cells was 0.09% of the cell suspension of S14 and

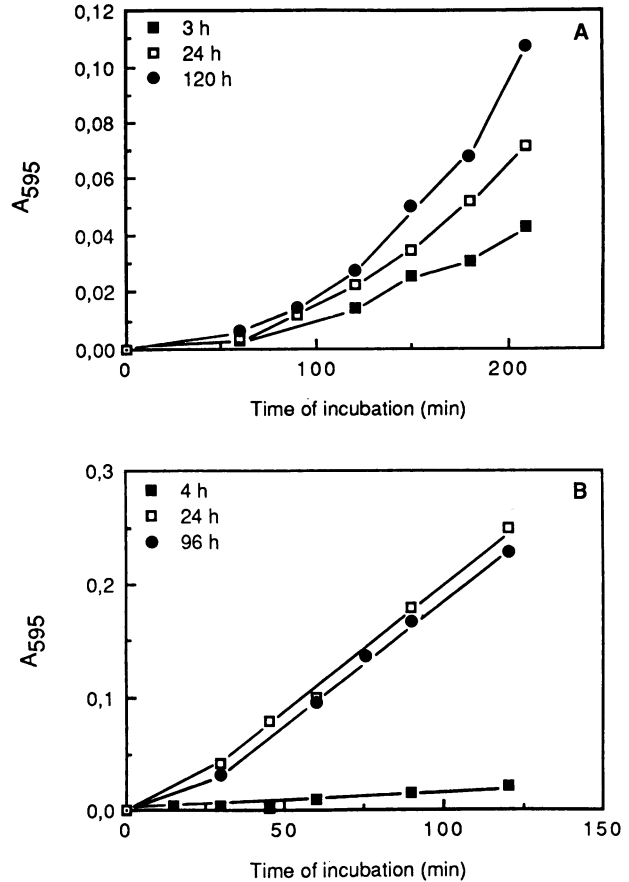


FIG. 2. Accumulation of exoprotease activity in the filtered starvation supernatants of *Vibrio* sp. strain S14 (A) and *Pseudomonas* sp. strain S9 (B). Cells were starved in NSS and centrifuged, and the supernatants were filtered (0.2 μ m pore size) at the appropriate times. HPA was added, and the increase in soluble color was monitored at 595 nm. The experiments were repeated at least twice, with insignificant deviations between experiments. Representative results are presented.

0.1% of that of S9. To determine whether exoprotease activity could be due to leakage of cell material, periplasmic fractions of 24-h-starved S14 were also prepared and assayed for protease activity with HPA (not shown). No activity was detected, indicating that the exoproteases became active when they appeared at or were beyond the outer membrane. The absence of protease activity in the supernatants from chloramphenicol-treated cells (see above) also indicates that the activity is a result of deliberate release and not of passive cell lysis.

The ability of starved cells to utilize the degradation products of HPA is depicted in Fig. 3, in which the increase in respiration rate after the addition of HPA is plotted. Presented are results for 120-h-starved S14 cells and 48-h-starved S9 cells. These results are representative of cells throughout the starvation period examined.

Regulation of exoprotease activity. The effect of Casamino Acids, glucose, and cAMP on exoprotease activity in *Vibrio* sp. strain S14 and *Pseudomonas* sp. strain S9 after 5 and 2 h, respectively, is shown in Fig. 4. These metabolites were added to cell suspensions at the onset of starvation. The addition of Casamino Acids had no significant effect on exoprotease activity in S14, but glucose (4 g/liter) was found

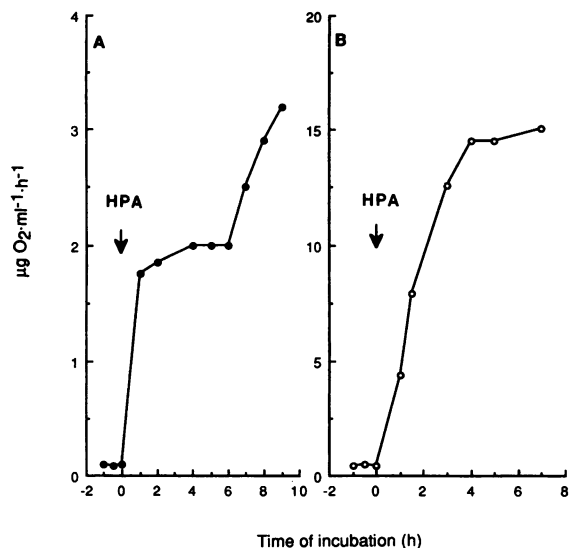


FIG. 3. Changes in the rate of respiration after the addition of HPA to *Vibrio* sp. strain S14 starved for 120 h (A) and *Pseudomonas* sp. strain S9 starved for 48 h (B). HPA was added at time zero (arrow), and the increase in respiration rate was monitored. The experiments were repeated at least twice, with insignificant deviations between experiments. Representative results are presented.

to repress synthesis in S14 by 50%. The addition of cAMP dramatically increased the exoprotease activity in S14, and the repression by glucose was negated by the addition of cAMP (Fig. 4A). The addition of Casamino Acids repressed

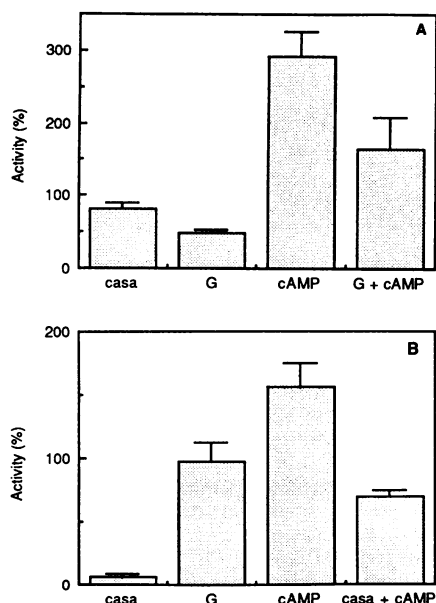


FIG. 4. Effect of Casamino Acids (casa), glucose (G), and cAMP on the synthesis of exoproteases. The additions were added to suspensions of exponential-phase cells that had been washed, resuspended in NSS, and incubated for 5 h (*Vibrio* sp. strain S14) (A) and 2 h (*Pseudomonas* sp. strain S9) (B) before measuring exoprotease activity. The results are given as a percentage of the activity in the NSS control without additions, which was assigned a value of 100%. The bars indicate the standard deviation calculated from three separate experiments.

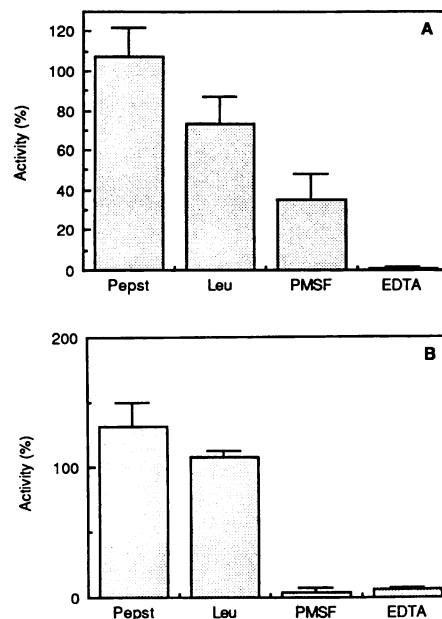


FIG. 5. Effect of pepstatin (Pepst), leupeptin (Leu), phenylmethylsulfonyl fluoride (PMSF), and disodium EDTA on filtered starvation supernatants (5 h) from *Vibrio* sp. strain S14 (A) and *Pseudomonas* sp. strain S9 (B). Cell-free supernatants were incubated for 6 h (S14) or 3 h (S9) with HPA. The results are given as a percentage of the activity in the NSS control without additions, which was assigned a value of 100%. The bars indicate the standard deviation calculated from three separate experiments.

activity in S9 by 92%, and glucose repressed activity by 10%. The addition of cAMP increased activity, although the activity after addition of cAMP in combination with Casamino Acids did not equal the activity in the NSS control (Fig. 4B). To ensure that these results were due to effects on exoprotease synthesis rather than steric effects on the exoproteases, the additions were also made to filtered starvation culture supernatants. No effect was found on the activity of cell-free supernatants.

Inhibition of exoprotease activity. The effect of protease inhibitors on filtered starvation culture supernatants after 5 h of incubation is shown in Fig. 5. The proteases in *Vibrio* sp. strain S14 and *Pseudomonas* sp. strain S9 appeared to be metallo- and serine proteinases, because 2.5 mM EDTA and 1 mM PMSF were found to inhibit activity in both strains, while addition of other inhibitors had little or no effect.

DISCUSSION

Previous studies on the synthesis of exoproteases have mostly been confined to actively growing cultures, in which exoprotease synthesis occurs in the later stages of growth (13). In this study, exoprotease activity during 120 h of total energy and nutrient starvation is demonstrated. Since marine bacteria experience intermittent periods of greatly reduced or totally arrested growth due to nutrient limitation, the degradation of organic polymers via exoprotease activity is an example of how nongrowing bacteria possessing high-affinity amino acid uptake systems (9, 14) may participate in the cycling of nutrients in the marine environment.

Both strains included in this study exhibited an increase in exoprotease activity after 4 h (*Pseudomonas* sp. strain S9) or 5 h (*Vibrio* sp. strain S14) starvation compared with cells at

the onset of starvation. The increase in the degradation of HPA after short-term starvation could be due either to de novo synthesis of proteins involved in the synthesis and/or active release of exoproteases during the initial reorganizational phase of starvation (16) or to an accumulation of exoproteases at the cell surface during starvation. The former hypothesis appears to be correct, since inhibition of exoprotease activity was observed when chloramphenicol was added to cells starved for 5 h. This also indicates that the exoprotease activity found in the filtered supernatant is not due to cell lysis or otherwise passive release of cell material.

In *Pseudomonas* sp. strain S9, the accumulation of exoproteases in the supernatant reached a plateau after 24 h of starvation (Fig. 2B). These cells may be able to sense and regulate exoprotease concentrations, since 24-h-starved cells centrifuged and suspended in fresh NSS demonstrated exoprotease activity (Fig. 1B). The release of exoproteases from S9 into the supernatant first after 4 h of starvation may coincide with the release of an exopolysaccharide which is produced by this organism in response to starvation (26, 27). The importance of this exopolysaccharide, in substrate scavenging either by capturing substrate molecules or by allowing polymer-degrading enzymes to remain associated with the cell, is currently being investigated.

Evidence that the exoprotease activity measured in the filtered supernatant is due to deliberate release rather than the leakage of cell material is summarized by the following results: (i) starved cells of both *Vibrio* sp. strain S14 and *Pseudomonas* sp. strain S9 treated with chloramphenicol did not release exoprotease activity, (ii) no degradation of HPA was caused by the periplasmic proteins, and (iii) a Mu-directed *lac* operon fusion mutant of S9 that did not excrete extracellular proteases was isolated (data not shown). This mutant synthesized cell-bound exoproteases similar to the wild type, but no activity could be detected in the filtered supernatant even after long-term starvation. Therefore, proteins synthesized during starvation are concluded to mediate production and/or active release of exoproteases.

The repression of exoprotease synthesis by amino acids in *Pseudomonas* sp. strain S9 is unusual for gram-negative bacteria, although it is common in *Bacillus* species (19), and a predictable role for amino acids would be to function as end product inhibitors of exoprotease synthesis. In most gram-negative strains, amino acids have been found to induce exoprotease synthesis (19). A model explaining this regulation has been described by Daatselaar and Harder (7). Proteins in the environment liberate amino acids, which act as a signal to bacteria that proteins are present. As a result, exoprotease synthesis is increased. The observed repression by glucose in *Vibrio* sp. strain S14 has also been found in many other strains and can be attributed to a catabolite repression-like mechanism. The increase in exoprotease synthesis due to the addition of cAMP is in agreement with the notion that starving cells would benefit from the induction by cAMP of enzymes enabling the cells to utilize a broader range of substrates, since many cAMP-controlled proteins mediate transport or catabolism of carbon substrates (24). While proteins conferring starvation resistance have been found to be cAMP independent (20), a characterization of cAMP-dependent starvation specific proteins has previously not been made. In the marine environment, S14 may respond to cAMP produced by algae as a signal of the metabolic and nutritional status of the microenvironment (2). Azam and Cho (3) further hypothesized a feedback relationship between algae (exudate) and bacteria (products of mineralization).

The response of marine bacteria to starvation conditions has previously been described as a two-phase process (12); first a series of morphological and physiological reorganizations occur, followed by subsequent long-term survival characterized by low endogenous metabolism. It has been postulated that the changes during the initial phase prepare the cell for long-term survival and rapid recovery when substrate becomes available (P. Mårdén, Ph.D. thesis, University of Göteborg, Göteborg, Sweden, 1987). An example of this is the increase in respiration rate that occurred immediately after the formation of degradation products of HPA, also by long-term-starved cells (Fig. 3). The production of exoproteases may be an important factor in the recovery ability of starved bacteria.

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