

Properties of the Glucose Transport System in Some Deep-Sea Bacteria

EDWARD F. DELONG^{†*} AND A. ARISTIDES YAYANOS

Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093

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Many deep-sea bacteria are specifically adapted to flourish under the high hydrostatic pressures which exist in their natural environment. For better understanding of the physiology and biochemistry of these microorganisms, properties of the glucose transport systems in two barophilic isolates (PE-36, CNPT-3) and one psychrophilic marine bacterium (*Vibrio marinus* MPI) were studied. These bacteria use a phosphoenolpyruvate:sugar phosphotransferase system (PTS) for glucose transport, similar to that found in many members of the *Vibrionaceae* and *Enterobacteriaceae*. The system was highly specific for glucose and its nonmetabolizable analog, methyl alpha-glucoside (a-MG), and exhibited little affinity for other sugars tested. The temperature optimum for glucose phosphorylation *in vitro* was approximately 20°C. Membrane-bound PTS components of deep-sea bacteria were capable of enzymatically cross-reacting with the soluble PTS enzymes of *Salmonella typhimurium*, indicating functional similarities between the PTS systems of these organisms. In CNPT-3 and *V. marinus*, increased pressure had an inhibitory effect on a-MG uptake, to the greatest extent in *V. marinus*. Relative to atmospheric pressure, increased pressure stimulated sugar uptake in the barophilic isolate PE-36 considerably. Increased hydrostatic pressure inhibited *in vitro* phosphoenolpyruvate-dependent a-MG phosphorylation catalyzed by crude extracts of *V. marinus* and PE-36 but enhanced this activity in crude extracts of the barophile CNPT-3. Both of the pressure-adapted barophilic bacteria were capable of a-MG uptake at higher pressures than was the nonbarophilic psychrophile, *V. marinus*.

The phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) is widely distributed among the eubacteria. In general, most facultative anaerobes and some strict anaerobes which use the Embden-Meyerhoff pathway for sugar catabolism use the PTS for active sugar transport across the cell membrane (22). The glucose PTS system is composed of two general (non-sugar specific) soluble proteins (Enzyme I and HPr), which transfer a phosphate group from PEP to a third, soluble, sugar-specific protein, Enzyme III. Enzyme III then transfers the phosphoryl moiety to a sugar-specific integral membrane protein, Enzyme II^{Glc}, which concomitantly phosphorylates and transports a given sugar across the membrane (26). Although the system is somewhat complex, it is one of the best understood of the bacterial transport systems. On the basis of physiological, biochemical, and genetic evidence, all the specific PTS proteins are thought to have arisen from a common ancestral gene, possibly one that coded for a PEP-dependent sugar kinase (28).

Many marine bacteria, including those in the genus *Vibrio*, are known to possess a PTS for transport of glucose, fructose, and other sugars across the membrane (1, 3, 9, 12). The biochemical properties of these glucose transport systems in several marine isolates appear to be structurally and functionally analogous to those found in the well-studied enteric bacteria *Escherichia coli* and *Salmonella typhimurium* (9, 12). Recently, a mannitol PTS uptake system in a marine *Vibrio* isolate was described which appeared to undergo an adaptive shift, resulting in a lower K_M for transport after 5 weeks of starvation (3). The fact that the

PTS is present in many marine bacteria, in conjunction with the observation that glucose utilization is common to marine microbial populations (30), suggests that the glucose PTS is both functional in and widely utilized by indigenous marine bacteria in their natural environment.

The existence of barophilic bacteria, first alluded to in 1949 (34), is now unequivocal (33). These heterotrophic eubacteria grow optimally or exclusively at hydrostatic pressures greater than 1.01×10^5 Pa (1 atm). Taxonomic studies have indicated that at least some barophiles are closely related to, but distinct from, members of the genus *Vibrio* (6, 15). Most are psychrophilic, and there is a general tendency for an inverse relationship between growth rate and depth of origin (10, 32). Studies of the biochemistry and physiology of these deep-sea microbes are now providing general insight into the strategies of adaptation of these bacteria to the deep-sea environment (4, 5, 11).

Just as thermophilic bacteria have evolved such that their membranes, catabolic enzymes, and transcriptional and translational machinery function optimally in high temperature regimes (2, 7), analogous deep-sea bacterial structures and functions can be expected to have evolved to suit the low temperature and high pressure of the deep sea. Adaptation of deep-sea bacteria to low temperature and high hydrostatic pressure presumably may be detected in many of their physiological and biochemical characteristics. Pressure is known to affect a variety of biological processes at several hierarchical levels of organization (8), and so biological adaptation to this physical parameter is unlikely to be a single-site phenomenon. However, the membrane and membrane-associated functions have been implicated as major sites of pressure perturbation (14, 18) and so possibly exhibit detectable pressure adaptations. In a continuing effort to characterize autochthonous deep-sea bacteria, particularly

* Corresponding author.

† Present address: Department of Biology, Jordan Hall 138, Bloomington, IN 47405.

with respect to their environmental physiology and ecology, this study reports on the presence and some properties of the glucose PTS in several deep-sea isolates.

MATERIALS AND METHODS

Strains and media. Marine broth (type 2216; Difco Laboratories, Detroit, Mich.) buffered with 20 mM MOPS (morpholinepropanesulfonic acid [pH 7]) and 0.2% glucose, or basal medium supplemented with 20 amino acids (1 mg each per ml), 20 mM MOPS (pH 7.0) and 0.2% glucose or succinate (5) were used throughout this study. Organisms were cultured in polyethylene bags at 2°C, and kinetics of growth were monitored as previously described (5). CNPT-3 and PE-36 are gram-negative, heterotrophic, psychrophilic barophiles. *Vibrio marinus* ATCC 15381 is a marine psychrophile (18) and was purchased from the American Type Culture Collection, Rockville, Md. CNPT-3, isolated from a depth of 5,800 m, is spiral shaped and exhibits a maximal growth rate at a pressure of 500×10^5 Pa and a temperature of 10°C. PE-36 is vibrio shaped, originates from a depth of 3,584 m, and has a maximal growth rate at a pressure of 350×10^5 Pa and a temperature of 11°C. For all experiments, PE-36 was grown at 374×10^5 Pa, CNPT-3 was grown at 578×10^5 Pa, and *V. marinus* was grown at 1.01×10^5 Pa. These organisms were grown and maintained at 2°C. *S. typhimurium* LJ144, which carries the F' 198 episome encoding the *pts* operon and which exhibits fivefold-elevated activities of the soluble PTS components, was used as a source of the PTS-soluble proteins (27). This organism was grown in Luria broth (17) at 35°C.

Preparation of crude extracts and membranes. Cells were harvested in mid to late exponential phase and frozen at -20°C until needed. Cells were thawed in 5 to 10 ml of 20 mM Tris hydrochloride buffer (pH 7.5) containing 2 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. Cell suspensions were then sonicated for three 15-s intervals on ice, or until cells were completely lysed as judged by microscopic observation. Lysates were subjected to low-speed centrifugation ($3,000 \times g$, 5 min, 4°C) to remove unlysed cells. For preparation of washed membranes, these crude extracts were ultracentrifuged at $100,000 \times g$ for 2 h at 4°C. Membrane pellets were washed (20 mM Tris hydrochloride [pH 7.5]-2 mM dithiothreitol-0.1 mM phenylmethylsulfonyl fluoride) and recentrifuged twice. Membranes were then resuspended in 2 ml of wash buffer and stored in 0.4-ml portions at -80°C until use. The crude extracts and washed membrane preparations described above were used in all subsequent experiments.

a-MG and glucose uptake. Cells in mid to late exponential phase were harvested by centrifugation ($7,700 \times g$, 10 min, 4°C) and washed twice in ice-cold basal medium. Cells were then resuspended to a density of 1×10^7 to 4×10^7 cells per ml in basal media containing 0.01% yeast extract, 0.05% peptone, and 20 mM MOPS. Uptake was initiated by adding 1 ml of this cell suspension to 20 μ l of ¹⁴C-labeled glucose or methyl alpha-glucoside (a-MG; 1 mM; 5 μ Ci/ml). All experiments were performed at 0°C unless otherwise indicated. Uptake was terminated by filtration on 0.45- μ m-pore size membrane filters (Millipore Corp., Bedford, Mass.), immediately followed by filtration of 10 ml of ice-cold artificial seawater. The filters were placed in 10 ml of Filter Solve (Beckman Instruments, Inc., Fullerton, Calif.) for scintillation counting. Rate measurements were taken over the linear portion of the initial uptake curve, and for all strains uptake at 0°C was linear for at least the first 20 min. To test the effect

of pressure on uptake, after addition of labeled sugar the cell suspension was drawn into a 1-ml transfer pipette (Sarstedt, Inc., Princeton, N.J.), which was immediately sealed with a hand-held heat sealer (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.), with care to avoid the inclusion of air bubbles. The mixture was subsequently placed in a pre-equilibrated 30-ml pressure vessel and then brought to the appropriate pressure. This entire operation took 2 min; consequently, an atmospheric pressure control ($t = 2$ min) was subtracted from each pressure uptake value. After 20 min of incubation at 0°C, the vessel was decompressed and the cells were immediately filtered as described above.

In vitro sugar phosphorylation. For PEP-dependent sugar phosphorylation, reaction mixtures contained: 12.5 mM MgCl₂, 25 mM KF, 2.5 mM dithiothreitol, 50 mM MOPS (pH 7.2), 10 μ M ¹⁴C sugar, and 5 mM PEP. The reaction volumes were 0.6 ml, and approximately 300 to 500 μ g of crude extract protein was added per tube to initiate the reaction. For sugar/sugar phosphate transphosphorylation assays, reaction mixtures contained: 12.5 mM MgCl₂, 25 mM KF, 2.5 mM dithiothreitol, 25 mM MES ([2-*N*-morpholino]ethanesulfonic acid) (pH 6.0), 10 μ M [¹⁴C]a-MG, and 10 mM glucose-6-phosphate. The reaction was initiated by adding 500 to 1,000 μ g of membrane protein in 0.6 ml total volume. The reactions were terminated by application to an anion exchange column (31), which was immediately washed with 20 ml of nanopure H₂O (Sybron Corp., Boston, Mass.). The sugar phosphate was eluted with two 3-ml volumes of 1 M LiCl. Beta Phase (10 ml; West Chem, San Diego, Calif.) was added to the eluate for subsequent scintillation counting. Pressure experiments were performed as described above for uptake. All experiments were conducted at 0°C, unless otherwise indicated.

Other methods. Cell numbers were determined with a Coulter counter (model Z; Coulter Electronics, Inc., Hialeah, Fla.) as previously described (32). Protein concentration was determined by the method of Lowry et al. (13).

Chemicals and radiochemicals. Peptone, yeast extract, and marine medium 2216 were purchased from Difco. [¹⁴C]a-MG and [¹⁴C]D-glucose were purchased from Amersham Corp., Arlington Heights, Ill. MOPS was purchased from United States Biochemical Corp., Cleveland, Ohio. All other reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Glucose and a-MG uptake. The approximate growth pressure optima at 2°C of the strains used in this study were as follows: PE-36, 200×10^5 Pa; CNPT-3, 300×10^5 Pa; *V. marinus*, 1.01×10^5 Pa. Under growth conditions identical to those used for barophiles, *V. marinus* ATCC 15381 did not exhibit a barophilic growth response at 2°C. At pressures of 1.01×10^5 , 101×10^5 , and 202×10^5 Pa, generation times for *V. marinus* were, respectively, 5.8, 6.4, and 7.3 h. All three of these psychrophilic marine strains exhibited uptake of both glucose and a-MG at 0°C, which was saturable at about 15 μ M. Uptake was linear for at least 20 min at 0°C in all three isolates. The apparent K_{ms} for uptake of a-MG at atmospheric pressure and at 690×10^5 Pa, estimated from Lineweaver-Burk plots, were, respectively: PE-36, 10 and 12 μ M; CNPT-3, 7 and 2 μ M; *V. marinus*, 8 and 3 μ M. These K_{ms} for a-MG uptake are in the same vicinity as that of *Serratia marinorubra*, which is 5 μ M at 20°C (9). The transport system was specific for glucose and a-MG. Other sugars, including mannose, which is structurally quite simi-

TABLE 1. Inhibition of glucose uptake by competing sugars

Competing sugar ^a	% Inhibition of glucose uptake ^b		
	<i>V. marinus</i>	PE-36	CNPT-3
Glucose	90.1	96.2	95.2
α -MG	93.4	95.6	97.7
Mannose	0	7.0	33.6
Fructose	18.0	33.3	30.5
Mannitol	0	16.0	23.7

^a All competing sugars added at 1 mM; glucose added at 10 μ M.

^b Relative to control without added competing sugar.

lar to glucose, showed little or no inhibition of uptake of D-glucose (Table 1). The uptake system was inducible in *V. marinus* and CNPT-3; cells grown in glucose minimal medium exhibited uptake rates about three to fivefold greater than those of cells grown in succinate minimal medium (data not shown).

PTS activity in vitro. PEP-dependent phosphorylation of α -MG, indicative of a glucose PTS, was demonstrable in the crude extracts of all three strains studied (Fig. 1). PEP was the most efficient phosphoryl donor tested. As is generally the case (22), the soluble PTS enzymes were rate limiting in crude extracts, which could be demonstrated by including additional PTS soluble proteins or membranes in the reaction mixture. Although ATP was not an efficient phosphoryl donor, glucose-6-phosphate was, reflecting the sugar/sugar phosphate transphosphorylation activity known to be catalyzed by Enzyme II^{Glc} (27). PEP-dependent sugar-phosphorylating activity in crude extracts was temperature dependent, the Q₁₀s being greatest in organisms originating from lesser depths (Fig. 2). The soluble PTS enzymes of *S. typhimurium* were capable of passing the phosphoryl group from PEP to the Enzyme II^{Glc} of *V. marinus*, PE-36, and CNPT-3. This functional homology between the Enzyme II^{Glc} of these marine psychrophilic and barophilic bacteria and *S. typhimurium* is illustrated in Table 2. α -MG phosphorylation was increased above the background about fivefold when the soluble PTS proteins of *S. typhimurium* were

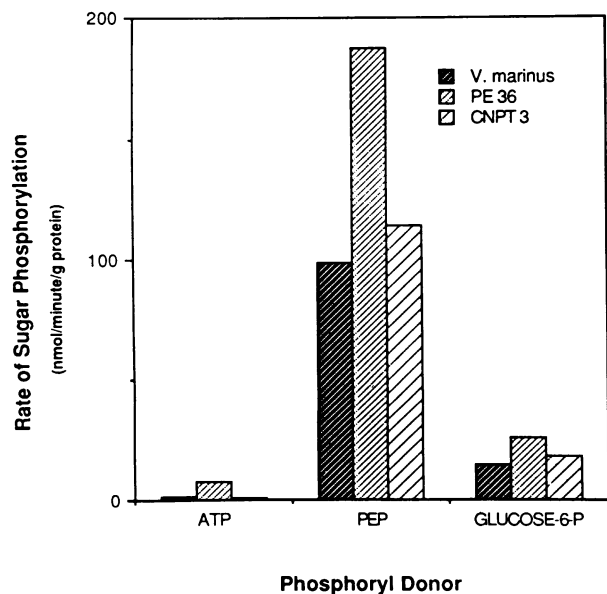


FIG. 1. In vitro α -MG phosphorylation catalyzed by crude extracts in the presence of different phosphoryl donors.

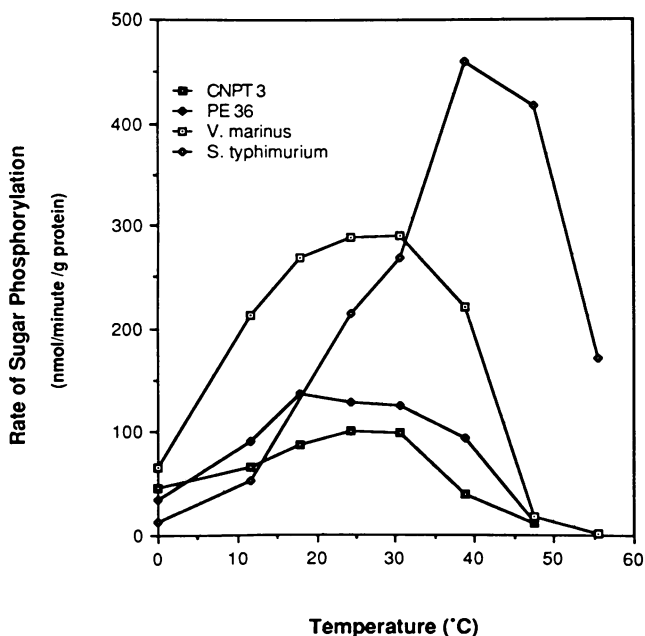


FIG. 2. In vitro α -MG phosphorylation catalyzed by crude extracts as a function of temperature.

mixed with the membrane fractions of *V. marinus*, PE-36, and CNPT-3.

Effects of pressure on α -MG uptake and PTS activities. Of the three strains studied, pressure inhibited the rate of α -MG uptake in *V. marinus* to the greatest extent, with no detectable uptake occurring at pressures greater than 800×10^5 Pa (Fig. 3). Uptake was also inhibited in isolate CNPT-3, to a lesser extent. α -MG uptake rates in isolate PE-36 were stimulated by increased pressures, up to 800×10^5 Pa. Rates of in vitro sugar phosphorylation catalyzed by crude extracts were pressure inhibited in *V. marinus* and PE-36, with about 25% of the activity remaining at 500×10^5 Pa (Fig. 4). In contrast, CNPT-3 crude extracts exhibited a marked pressure activation of PEP-dependent sugar phosphorylation, up to about 500×10^5 Pa. α -MG/glucose-6-phosphate transphosphorylation, catalyzed by membrane preparations of *V. marinus* and PE-36, exhibited small increases in activity as pressure was increased to 200×10^5 Pa, and a steady decrease in activity was observed as pressure was increased beyond this point (Fig. 5). This membrane-bound transphosphorylation activity catalyzed by membranes from CNPT-3, however, was greater at higher pressures than at 1 atm (101.29 kPa).

TABLE 2. Enzymatic cross-reactivity of *Salmonella* soluble PTS proteins with membranes of psychrophilic marine strains

PTS protein	Source of membrane protein ^a		
	<i>V. marinus</i>	PE-36	CNPT-3
<i>S. typhimurium</i> soluble protein ^b	20 ^b	16	17
Membrane protein ^c	16	6.0	7.6
<i>S. typhimurium</i> soluble protein plus membrane protein	230	166	115

^a α -MG phosphorylation (cpm/s) at 15°C.

^b Concentration of soluble proteins was approximately 400 μ g/ml.

^c Concentration of membrane protein was approximately 500 μ g/ml.

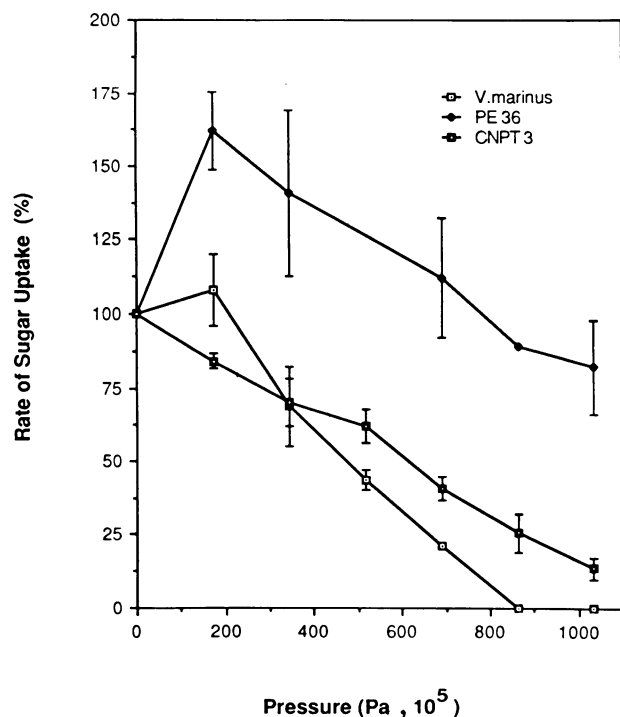


FIG. 3. a-MG uptake in whole cells as a function of pressure. The rate of sugar uptake is shown as the percent uptake at a given pressure, relative to the control rate at atmospheric pressure (1.01×10^5 Pa). Data points represent the mean \pm the standard error of three experiments.

DISCUSSION

Many physiological and biochemical characteristics of marine bacteria may be viewed as adaptations to the physical, chemical, and biological conditions found in the oceanic environment. For example, the nearly constant 480 mM Na^+ present in sea water has given rise to an obligate growth requirement for sodium ions in most marine bacteria (16, 24). Bioluminescence, a characteristic of many symbiotic marine eubacteria, is a trait beneficial to and well exploited by metazoan hosts and hence can be indirectly beneficial to (and presumably selective for) the luminescent bacterial symbiont (19). The ability to survive long periods of starvation is another important trait of some marine bacteria (20), as low nutrient conditions predominate in the most of the open ocean. In addition, the low temperatures (less than 4°C) and pressures greater than 100×10^5 Pa that characterize nearly 75% of the marine environment have resulted in the evolution of barophilic and psychrophilic bacterial species which are readily recovered from cold and deep ocean waters (31a). Specific physiological and biochemical attributes which allow these bacteria to flourish in the deep-sea environment are only now beginning to be understood (4, 5; A. A. Yayanos and E. F. Delong, in H. W. Jannasch, R. E. Marquis, and A. M. Zimmerman, ed., *Current Perspectives on High Pressure Biology*, in press).

The general properties of the PTS in the psychrophilic deep-sea isolates were comparable to the well-studied systems of *E. coli* and *S. typhimurium*. This is demonstrated by the enzymatic cross-reactivity between the *S. typhimurium* soluble PTS enzymes and psychrophile membrane-bound proteins at 15°C (Table 2), sugar specificity (Table 1), and

PEP dependence (Fig. 1). Relatively high activities of uptake and a-MG phosphorylation at 2°C were observed, reflecting the psychrophilic nature of the isolates. This low-temperature activity is negligible in well-studied enteric bacteria; one reason for this is that Enzyme I, which is active in vivo and in vitro as a homodimer, is inactivated by dissociation to the monomer at low temperatures (26). This is reflected in Fig. 3, which demonstrates that rates of sugar phosphorylation in *S. typhimurium* crude extracts are the most sensitive to decreasing temperature, of the strains studied here.

Several studies have focused on the effect of pressure on membrane-mediated transport processes. Shen and Berger (29) found that pressure decreased the rate of a-MG uptake in *E. coli*. Paul and Morita (21) observed that pressures of 400 atm inhibited uptake of several amino acids in a marine bacterium by about 80%, relative to the uptake at 1 atm. Respiration of these same substrates was relatively unaffected by increased hydrostatic pressure. Other studies have demonstrated that transport of other amino acids, sugars, and nucleotides is generally inhibited by increasing hydrostatic pressure. The results reported here, which to our knowledge are the first studies of a specific transport system in pressure adapted bacteria, tend to show a similar trend. However, a-MG transport in *V. marinus*, which has the shallowest depth of origin and the lowest growth pressure optima, was most strongly inhibited by increased pressure. Under the growth conditions we used, *V. marinus* was not barophilic, unlike PE-36 and CNPT-3. Beyond 800×10^5 Pa, there was no measurable uptake in *V. marinus*, whereas the two barophiles retained at least 25% of the atmospheric uptake activity at this pressure. Notably, a-MG transport in

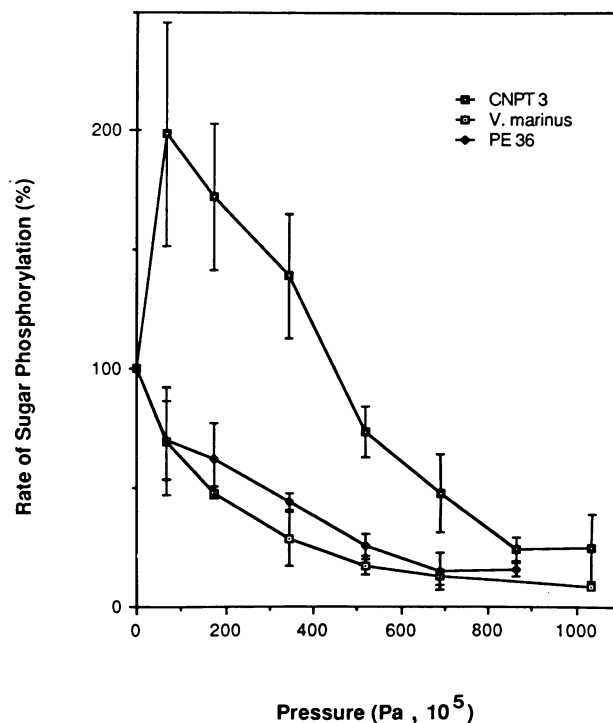


FIG. 4. In vitro a-MG phosphorylation catalyzed by crude extracts as a function of pressure. Values shown are the percent rate at a given pressure relative to the control rate at atmospheric pressure (1.01×10^5 Pa). Data points represent the mean \pm the standard error of three experiments.

PE-36 was stimulated relative to that at atmospheric pressure, up to pressures of 800×10^5 Pa.

Before glucose or a-MG can be transported into the cell, a phosphoryl group must be transferred from PEP through Enzyme I, HPr, and Enzyme III, and finally passed to the integral membrane protein, Enzyme II^{Glc}. As is usually the case for crude extracts (23), the soluble enzymes were rate limiting, so that in vitro assays of pressure effects on a-MG phosphorylation were effectively measuring pressure perturbation of the soluble PTS enzymes. PE-36 and *V. marinus* showed remarkably similar pressure inhibition of in vitro a-MG phosphorylation (Fig. 4). However, as previously noted, a-MG uptake was strongly inhibited in *V. marinus*, and significantly pressure enhanced in PE-36 (Fig. 3). Since pressure affects the in vitro activity of these two strains identically, differences in uptake between the two are probably not attributable to the activity of the soluble PTS components. More likely, the differences in pressure effects on uptake are due to differences in the pressure response of the membrane-bound activity. A relevant observation here is that the fatty acid compositions in the membrane lipids of *V. marinus* and PE-36 are nearly identical (5), so that differences in membrane lipid composition are probably not responsible for the differential uptake response to pressure. The possibility exists that the pressure enhanced uptake observed in PE-36, as compared to *V. marinus*, is due to a difference in the integral membrane protein, the Enzyme II^{Glc}. Adaptation to the deep-sea environment, which in isolate PE-36 is reflected in its barophilic growth response (31a) has presumably also resulted in alterations of the properties of the integral membrane protein, Enzyme II^{Glc}.

In general, pressure inhibits any process which involves a positive volume change, and enhances processes involving negative volume changes (8). One explanation of our results is that volume changes associated with conformational transitions of Enzyme II^{Glu}, which occur during glucose transport and phosphorylation, are more negative in PE-36 as compared with *V. marinus*. Therefore, glucose transport is pressure enhanced in isolate PE-36. An alternative explanation takes into account the fact that the EII is thought to exist as a dimer in its active conformation. Possibly the monomer-dimer equilibria of the Enzyme II^{Glu} in PE-36 is much less pressure sensitive than that of *V. marinus*. Hence, pressure inhibits glucose transport in PE-36 much less.

CNPT-3, the most barophilic of the strains studied, exhibits considerable pressure sensitivity in a-MG transport mediated by whole cells. This may indicate that the glucose transport system is not a major adaptive site in determining the barophilic phenotype, though adaptation to pressure in this system may occur secondarily, as appears to be the case for PE-36. It is clear, however, that the glucose uptake system of CNPT-3 remains functional at pressures greater than that of the shallower water isolate, *V. marinus*. In addition, the soluble PTS proteins of CNPT-3 appear to be much less pressure sensitive than those of PE-36 or *V. marinus* (Fig. 4).

The K_m s of uptake in the three strains are comparable to those measured for the PTS sugar transport systems of other marine bacteria (3, 9). Since glucose concentrations in sea water rarely exceed $1 \mu\text{M}$ (30), the PTS in marine bacteria should function most optimally when these bacteria are in zones of high nutrient concentration, as epiphytes or gut symbionts, or associated with detrital material or fecal pellets. For deep-sea bacteria this may be a predominant mode of existence, as in situ measurements of heterotrophic activity in the abyssal water column and sediments have

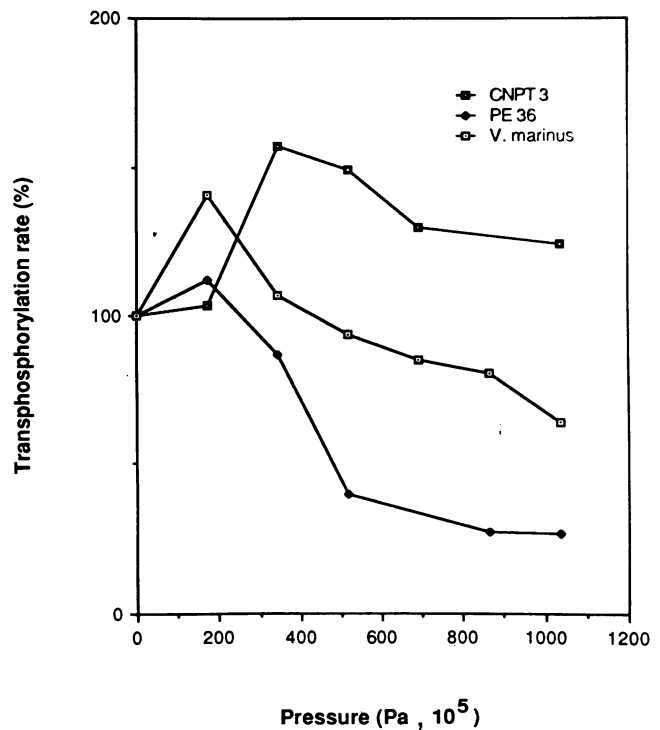


FIG. 5. In vitro a-MG/glucose-6-phosphate transphosphorylation catalyzed by membrane preparations, as a function of pressure. Values shown are the percent rate at a given pressure, relative to the control rate at atmospheric pressure (1.01×10^5 Pa).

often been very low (11). However, the association of barophiles with deep-sea animals and sediments (25), as well as their relatively rapid growth under deep-sea conditions (31a, 32), indicates their potentially significant contribution to deep-sea heterotrophic processes. The PTS of these psychrophilic and barophilic bacteria, with a K_m of transport around $5 \mu\text{M}$, is probably most used in transient zones of high nutrient concentration, such as on or near sedimenting particulates and "food falls," or in the guts of metazoans, where a large proportion of deep-sea heterotrophic activity probably takes place. The bacterial PTS, a widely distributed, energy-efficient transport system, appears well suited to the needs of deep-sea bacteria, as is evidenced by its functionality in these organisms under in situ conditions of temperature and pressure.

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