Energy Metabolism Response to Low-Temperature and Frozen Conditions in *Psychrobacter cryohalolentis*[▽]

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Studies of cold-active enzymes have provided basic information on the molecular and biochemical properties of psychrophiles; however, the physiological strategies that compensate for low-temperature metabolism remain poorly understood. We investigated the cellular pools of ATP and ADP in *Psychrobacter cryohalolentis* K5 incubated at eight temperatures between 22°C and −80°C. Cellular ATP and ADP concentrations increased with decreasing temperature, and the most significant increases were observed in cells that were incubated as frozen suspensions (<−5°C). Respiratory uncoupling significantly decreased this temperature-dependent response, indicating that the proton motive force was required for energy adaptation to frozen conditions. Since ATP and ADP are key substrates in metabolic and energy conservation reactions, increasing their concentrations may provide a strategy for offsetting the kinetic temperature effect, thereby maintaining reaction rates at low temperature. The adenylate levels increased significantly <1 h after freezing and also when the cells were osmotically shocked to simulate the elevated solute concentrations encountered in the liquid fraction of the ice. Together, these data demonstrate that a substantial change in cellular energy metabolism is required for the cell to adapt to the low temperature and water activity conditions encountered during freezing. This physiological response may represent a critical biochemical compensation mechanism at low temperature, have relevance to cellular survival during freezing, and be important for the persistence of microorganisms in icy environments.

Although more than 80% of the biosphere (by volume) is permanently below 5°C and most of the biomass is microbial (42), very little is known about the physiology of cold-adapted microorganisms. A number of recent studies have reported metabolic activity at subzero temperatures in environmental samples, including permafrost at −10°C (22, 47), snow samples at −17°C (10), sea ice at −20°C (24), and soil at −39°C (36), and under laboratory conditions between −15°C and −196°C (12, 25, 31). Cells are partitioned into the aqueous phase of ice during freezing (1, 24, 29), and metabolism likely occurs in the solute-rich interstitial liquid veins that exist between three-grain crystal boundaries (40) but may not be solely restricted to this microenvironment (e.g., see references 48 and 55).

Growth and metabolism are clearly limited at low temperature because of lower membrane fluidity (19), reduced affinity of enzymes for their substrates (17), decreased thermal energy and reaction rates, and increased aqueous viscosity (14). Cold adaptations to counteract these detrimental effects include increasing the lipid unsaturation level in membranes (50), increasing the concentration of enzymes (60), and expressing isozymes with optimal activities at low temperature (20). A variety of cold shock proteins are induced at temperatures below an organism's optimum that stabilize and interact with nucleic acids, proteins, or ribosomes and effect gene expression (37, 44). Low temperature also enhances the uptake or synthesis of compatible solutes, which serve to neutralize osmotic pressure and maintain cell turgor pressure at high salinity (27,

28). When the temperature is low enough for ice formation, cells are subjected to additional stressors such as mechanical disruption, oxidative damage, and osmotic imbalance (54). Some bacteria increase their survival during freezing by producing proteins that prevent the growth or recrystallization of ice crystals (45, 46). In yeast, freeze resistance has been linked to the expression of aquaporin proteins, which are thought to enhance the efflux of water from the cell and prevent intracellular ice crystal formation (53).

The hydrolysis of ATP into ADP is the main energy source for most endergonic metabolic reactions in the cell. Because of their key role in the transfer of biochemical energy, the concentration and ratio of adenylates provide information on the metabolic state of the cell (e.g., see references 8 and 30). Napolitano and Shain (33, 34) recently reported that psychrophilic microorganisms and oligochaete worms increase their cellular ATP concentration at decreasing temperatures despite having slower growth rates. These authors suggested that this may be a physiological response for offsetting the decreased diffusion rate at low temperature, thus permitting cells to maintain biochemical processes under these conditions. It has also been shown that an *Escherichia coli* mutant that maintained an elevated ATP concentration was more tolerant to storage for several days at 0°C than the wild type (32).

Macromolecular synthesis is an energy-requiring process that has been documented in bacteria at temperatures well below the freezing point (12, 25). In this study, we investigated the temperature-dependent variation of ATP and ADP in *Psychrobacter cryohalolentis* K5, a eurythermal psychrophilic (T_{opt} = 22°C) bacterium isolated from Siberian permafrost that is capable of growth at temperatures as low as −10°C (3). A range of temperatures within its growth range were examined (22°C to −5°C), as well as temperatures at which cell suspensions were completely

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frozen ($-15°C$ to $-80°C$). The key observations in this study were that respiring cells increase their adenylate concentrations in response to low-temperature and freezing conditions and are capable of generating ATP at temperatures as low as $-80°C$. We discuss the potential physiological advantages of this strategy at very low temperatures, as well as the implications of our results for understanding the metabolic status of cells during freezing and long-term frozen storage.

MATERIALS AND METHODS

Culturing and incubation conditions. *P. cryohalolentis* K5 was cultured aerobically by shaking at 200 rpm at 15°C in M9 mineral medium (Difco, Inc.) supplemented with 24 mM acetate, vitamins (American Type Culture Collection MD-VS; 1%, vol/vol) and a trace mineral solution (American Type Culture Collection MD-TMS; 1%, vol/vol). The optical density of cultures was periodically measured at 620 nm, and 0.1-ml aliquots of cells at the early stage of stationary phase were transferred into 1.5-ml tubes. Prior to incubation, the number of CFU per milliliter was determined in triplicate by standard dilution plating and ranged from $1.50 \times 10^8 \pm 0.16 \times 10^8$ to $1.92 \times 10^9 \pm 0.14 \times 10^9$ CFU ml^{-1} between experiments. Triplicate samples of the cell suspensions were incubated for up to 88 h at 22°C, 15°C, 5°C, 0°C, $-5°C$, $-15°C$, $-20°C$, and $-80°C$ without shaking. The cell suspensions placed at temperatures below $-5°C$ froze within 1 h and were incubated as ice. In some experiments, a temperature-equilibrated solution of 5 M NaCl was added to selected liquid samples to achieve a final concentration of 1 M.

Experiments were also carried out in the presence of the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma), which inhibits oxidative phosphorylation by uncoupling the proton motive force. CCCP was dissolved in 100% dimethyl sulfoxide and was shown to prevent the growth of *P. cryohalolentis* in liquid culture at a concentration of 15 μM. For the adenylate assays, cells were amended with 15 μM CCCP and incubated for 15 min at 22°C to ensure the penetration of CCCP into the cells, and aliquots of the culture were distributed as described above. Identical cell suspensions without CCCP were analyzed in parallel and served as controls.

The rate of cooling was measured for incubation at temperatures below freezing ($-15°C$ to $-80°C$) with loggers equipped with thermal probes (Hobo). The probes were placed into a microcentrifuge tube containing 70% ethanol, and the temperature was logged for about 1 h, with one measurement every 10 s. The average rates of cooling were 0.32°C min^{-1} at $-15°C$, 0.73°C min^{-1} at $-20°C$, and 1.91°C min^{-1} at $-80°C$. It took samples initially at 15°C approximately 90 min to reach $-15°C$, 36 min to reach $-20°C$, and 45 min to reach $-80°C$. The temperature at which freezing initiated in the cell suspensions was about $-10°C$, and this was reached in 19 min at $-15°C$, in 9 min at $-20°C$, and in 6 min at $-80°C$.

Adenylate extraction and sample preparation. The total adenylate pool was extracted by the addition of ice-cold trichloroacetic acid (TCA; Fisher) to a final concentration of 0.7%, followed by vigorous mixing. For frozen samples, TCA was layered over the ice surface and samples were then rapidly melted (within 2 min) during constant vortexing. Procedural blanks consisted of TCA and molecular biology grade water (DNase, RNase, and protease free; Acros Organics) which were processed in parallel. The samples were incubated on ice for about 1 h before measurement. The possibility that freezing enhanced the adenylate extraction efficiency was investigated by measuring the ATP concentration in cell suspensions before and after freezing in the presence or absence of TCA (0.7%) by immersion of the samples in liquid nitrogen.

The concentrations of ATP, ADP plus ATP, and AMP plus ADP plus ATP were measured by using the firefly luciferase/luciferin assay. For each measurement, two samples were run in parallel. One set was prepared with Tris-acetate buffer (TAB; 0.05 M Tris buffer, Tris base [molecular biology grade; Fisher], adjusted to pH 7.75 with acetic acid [glacial acetic acid certified ACS Plus; Fisher]), and the second was prepared identically but contained 10^{-5} M ATP (Sigma) that was dissolved in TAB and served as an internal standard. For ATP measurements, the solution consisted of 10 μl of extracted sample and 10 μl of either TAB or the ATP standard in TAB. For ADP and ATP and AMP, ADP, and ATP, 10 μl of the extracted sample was mixed with 10 μl of either the ADP or AMP "cocktail" (described below) and incubated at 37°C for 15 min, and then 10 μl of TAB or ATP standard in TAB was added to the sample.

ADP was measured after enzymatic phosphorylation to ATP, and the $2\times$ ADP cocktail consisted of 200 mM triethanolamine (Fisher), 2 mM $MgCl_2$ (Fisher), 240 mM KCl (Fisher), 4.5 mM phosphoenolpyruvate (PEP; phosphoenolpyruvic

acid monopotassium salt; Acros Organics), and 4.2 U ml^{-1} pyruvate kinase (PK; MP Biomedicals) (35). PEP and PK solutions were prepared as follows. A 0.1 M PEP solution was made in 0.05 M Tris-Cl (pH 7.2), and the pH was adjusted to 7.2 with 1.7 M KOH after dissolution. One milliliter of this solution was added to a vial containing 1,000 U ml^{-1} PK to obtain a PEP-PK solution at respective concentrations of 44 mM and 420 U ml^{-1} in 21 mM Tris-Cl. The AMP cocktail was identical to the ADP cocktail but also included 3 U ml^{-1} myokinase (catalog no. M5520-1KU; Sigma) (35). Samples were kept on ice during preparation but were equilibrated to room temperature (22°C) prior to measurement.

Measurement of ATP concentration by luminescence. Stock solutions of luciferase (8.6×10^6 U ml^{-1} in 1.0 M TAB [pH 7.75]; catalog no. L9506-1MG; Sigma) and D-luciferin (3 mM in 0.05 M TAB [pH 7.75]; catalog no. AC33705-0500; Acros Organics) were prepared in molecular biology grade water and stored at $-80°C$. The luciferase/luciferin reagent was freshly prepared before each series of measurements and consisted of 100 U ml^{-1} luciferase and 0.140 mM D-luciferin. Measurements were made with a 20/20n luminometer (Turner Biosystems). Light emission per second was averaged over a period of 3 s, with no delay after autoinjection of 100 μl of the luciferase/luciferin reagent. The concentrations of ATP; ATP and ADP; and AMP, ADP, and ATP per cell were calculated with the equation [ATP], [ADP + ATP], or [AMP + ADP + ATP] = $Q_{std} \times [I_{smp}/(I_{smp+std} - I_{smp})]/(V_{smp} \times D_f \times C_{cell})$, where I_{smp} and $I_{smp+std}$ are measures of luminescence in relative light units for the sample and for the sample plus the ATP standard, respectively; Q_{std} is the amount of ATP (moles) added to the sample (standard); V_{smp} is the volume of extracted sample (milliliters) analyzed; D_f is the dilution factor of the sample after the addition of TCA for extraction, and C_{cell} is the concentration of cells in the suspension determined by dilution plating (CFU per milliliter). The concentrations of ADP and AMP were obtained by subtracting the concentrations of ATP and ATP plus ADP from ATP plus ADP and AMP plus ADP plus ATP, respectively. Procedural blanks provided the level of background associated with these measurements, which were subtracted from all values. All measurement values reported are the means of replicate samples ($n = 3$), and statistical analysis was performed with the F test (analysis of variance).

Calculations of ATP accumulation by respiration. The rate of respiratory ATP accumulation (in moles per cell per hour) was calculated as follows: $\{[ATP_{(t2)} - ATP_{(t1)}] - [ATP(CCCP)_{(t2)} - ATP(CCCP)_{(t1)}]\}/(t2 - t1)$, where ATP and ATP(CCCP) are the concentrations of ATP (in moles per cell) in untreated cells and in cells treated with CCCP, respectively, at times t1 and t2 (2.5 and 21 h, respectively).

RESULTS

Effect of temperature on the cellular concentration of ATP and ADP. During logarithmic growth at 15°C, the ATP/ADP ratio reached a maximum value of 9.0, decreasing to values of around 1 as cell populations entered the stationary phase. Cells were harvested from the early stationary phase of growth, and the concentrations of ATP and ADP after subsequent incubation at temperatures between 22°C and $-80°C$ for 88 h are shown in Fig. 1. The lowest concentrations of ATP per cell were measured at temperatures between 5°C and 22°C ($7.40 \times 10^{-20} \pm 0.95 \times 10^{-20}$ to $8.31 \times 10^{-20} \pm 1.25 \times 10^{-20}$ mol $cell^{-1}$), and highest were measured between $-20°C$ and $-80°C$ ($7.44 \times 10^{-19} \pm 0.67 \times 10^{-19}$ and $7.60 \times 10^{-19} \pm 1.66 \times 10^{-19}$ mol $cell^{-1}$, respectively). The ADP concentration was lowest at 5°C ($1.62 \times 10^{-19} \pm 0.19 \times 10^{-19}$ mol $cell^{-1}$) and increased significantly with temperature ($P = 0.05$; Fig. 1) to $3.71 \times 10^{-19} \pm 0.18 \times 10^{-19}$ mol $cell^{-1}$ at 22°C. However, ADP concentrations were highest at subzero temperatures between $-15°C$ and $-80°C$ and ranged from $1.13 \times 10^{-18} \pm 0.15 \times 10^{-18}$ to $2.04 \times 10^{-18} \pm 0.28 \times 10^{-18}$ mol $cell^{-1}$. The ADP concentration was ~1.5 times higher than that of ATP between $-5°C$ and $-20°C$, and the concentration increase of both adenylates with decreasing temperature was exponential ($r^2 = 0.96$ for ATP and $r^2 = 0.99$ for ADP; $n = 9$) and statistically significant ($P < 0.01$; Fig. 1). AMP concentrations were more than an order of magnitude less than those of ATP
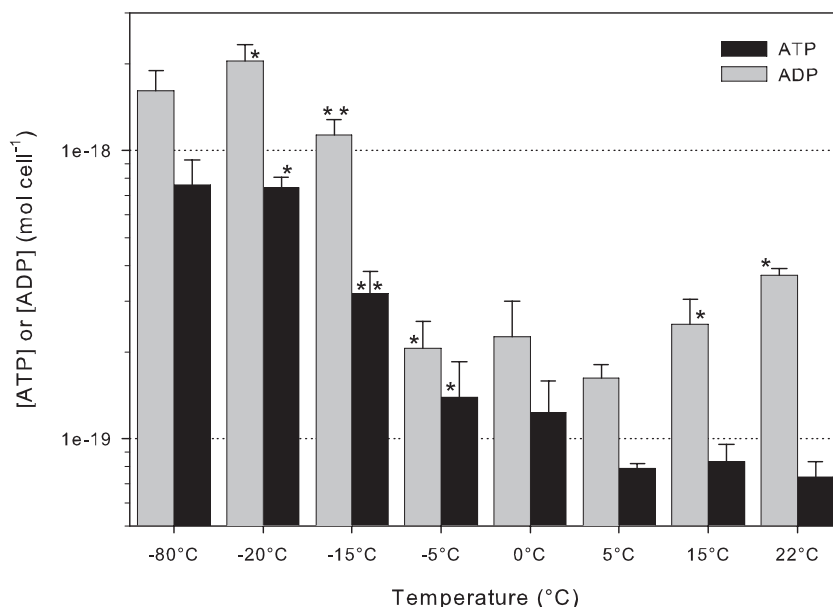
FIG. 1. Cellular concentrations of ATP and ADP in *P. cryohalolentis* K5 at temperatures of −80°C to 22°C after 88 h of incubation. Samples at temperatures of ≥−5°C remained liquid for the duration of the experiment. Errors bars denote the standard deviation from the mean ($n = 3$). An asterisk indicates a significant difference ($P < 0.05$) from the value measured at the next warmer (right side of error bar) or colder (left side of error bar) temperature (for ATP, $P_{-5°C/-15°C} = 0.01$ and $P_{-15°C/-20°C} < 0.01$; for ADP, $P_{22°C/15°C} = 0.05$, $P_{-5°C/-15°C} < 0.01$, and $P_{-15°C/-20°C} < 0.01$).

or below the level of detection (data not shown) and were highly variable between samples.

Effect of uncoupling of oxidative phosphorylation on ATP and ADP concentrations and the ATP/ADP ratio. Figure 2 shows temporal changes over 45 h in cellular ATP and ADP concentrations and the ATP/ADP ratio for samples incubated at temperatures between 22°C and −80°C in the presence and absence of CCCP. At time zero, the ATP and ADP concentrations were nearly equivalent, with an ATP/ADP ratio of 0.98. Similar to the results shown in Fig. 1, there was an inverse relationship between the ATP concentration and incubation temperature (Fig. 2A). At temperatures between 0°C and −80°C, the ATP concentration increased during the first 21 h, but only samples incubated under frozen conditions (<−5°C) had higher ATP concentrations after 45 h of incubation than at time zero. The ATP concentration after 45 h in samples at 0°C to −15°C decreased relative to values at 21 h, measurements during this time interval at −20°C were nearly identical, and the ATP concentration continually increased over time for samples at −80°C. In general, the ATP concentration in cells incubated at temperatures above 0°C continually decreased over time. The ADP concentration increased over time during the first 21 h before apparently reaching a steady state by 45 h (Fig. 2C), with the exception being samples at −20°C, which continued to increase between 21 and 45 h. Very similar concentrations of ADP were observed between 22°C and −5°C, but the values increased with decreasing temperatures between −15°C and −80°C. For all time points, the highest concentrations of ATP and ADP were observed at −80°C (Fig. 2A and C). The ATP/ADP ratio continually decreased during the course of the experiment and was highest after 45 h at −5°C (~0.4; Fig. 2E).

When cellular respiration was uncoupled with CCCP, the concentration of ATP at each temperature and time point was significantly lower than that in unamended cell suspensions (Fig. 2A and B), except at 22°C, where values were higher than those in respiring cells after 45 h. The cellular ADP concentration was largely unaffected by the presence of CCCP (Fig. 2C and D), but similar to ATP, the ADP concentration was higher at 22°C after 45 h than in the unamended controls. The presence of the uncoupler greatly accelerated the decrease in the ATP/ADP ratio over time at all temperatures, and after 45 h, the ratio was significantly lower (at 95% confidence) in cells incubated in the presence of CCCP than in respiring cells at all temperatures except −20°C and −15°C ($P = 0.16$ and $P = 0.07$, respectively). There were significant changes in the ATP/ADP ratio at all temperatures in untreated cells during incubation, but in the presence of the uncoupler, temporal variations were minimal after 2.5 h.

The concentration of AMP was measured after 21 h of incubation (data not shown) and was highly variable, ranging from below the detection limit (i.e., AMP + ADP + ATP ≤ ADP + ATP) at temperatures of −5°C to 15°C to its highest values at −80°C ($1.30 \times 10^{-19} \pm 2.34 \times 10^{-19}$ mol cell^{-1}). In cells treated with CCCP, AMP was below the detection limit at temperatures of −80°C to 5°C and reached a maximum of $2.31 \times 10^{-20} \pm 13.1 \times 10^{-20}$ mol cell^{-1} at 22°C. AMP represented <7% of the total adenylate pool, and in contrast to ATP and ADP, no variation in the AMP concentration with temperature was observed.

Changes in the ATP and ADP concentrations induced by freezing and osmotic shock. Figure 3 shows changes in the ATP and ADP concentrations after cells cultured at 15°C were transferred to −20°C (representing $t = 0$). In this experiment,
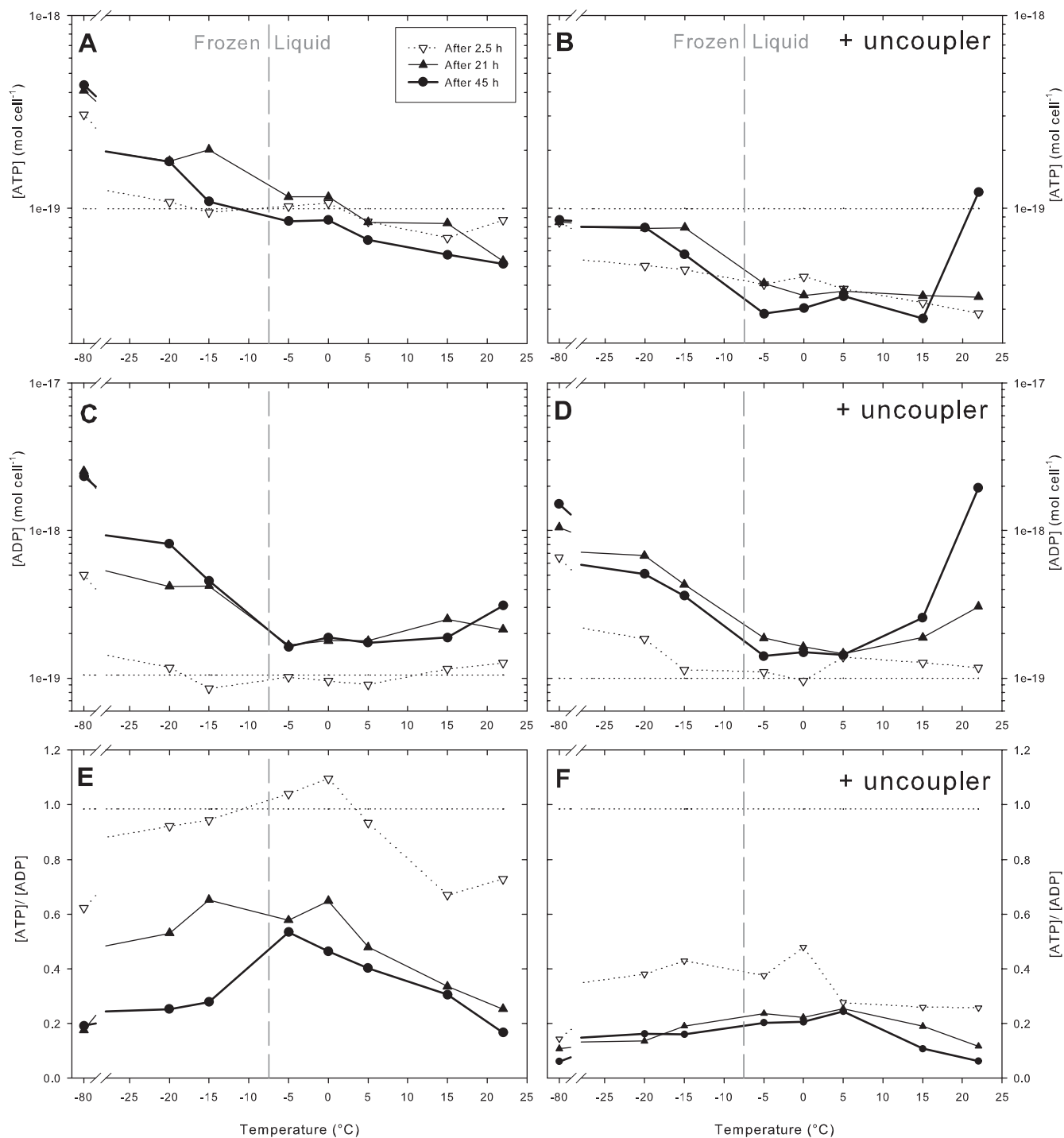
FIG. 2. Time-dependent changes in ATP and ADP concentrations and the ATP/ADP ratio at temperatures between −80°C and 22°C. The cell suspensions in panels A, C, and E were unamended, and those in panels B, D, and F were incubated in the presence of an uncoupler (CCCP). The dashed horizontal line indicates adenylate concentrations and ratios at $t = 0$, open triangles are measurements after 2.5 h, closed triangles are measurements after 21 h, and circles are measurements after 45 h. As indicated by the vertical dashed line, samples remained liquid at temperatures of −5°C and above. The values plotted are averages of triplicate experiments. Errors are not shown for visual clarity, and the coefficients of variation averaged 18 and 26% for ATP and ADP, respectively.

the initial cell populations were in the late exponential phase of growth and had an ATP/ADP ratio of 1.68 ± 0.52. During the first 75 min, all samples remained liquid and freezing was induced after 78 min in half of the supercooled samples by physical agi-

tation of the tubes. Simultaneously, the effect of osmotic shock on ATP and ADP concentrations was examined by adding NaCl (final concentration of 1 M) to the other half of the samples, which remained liquid for the duration of the experiment.
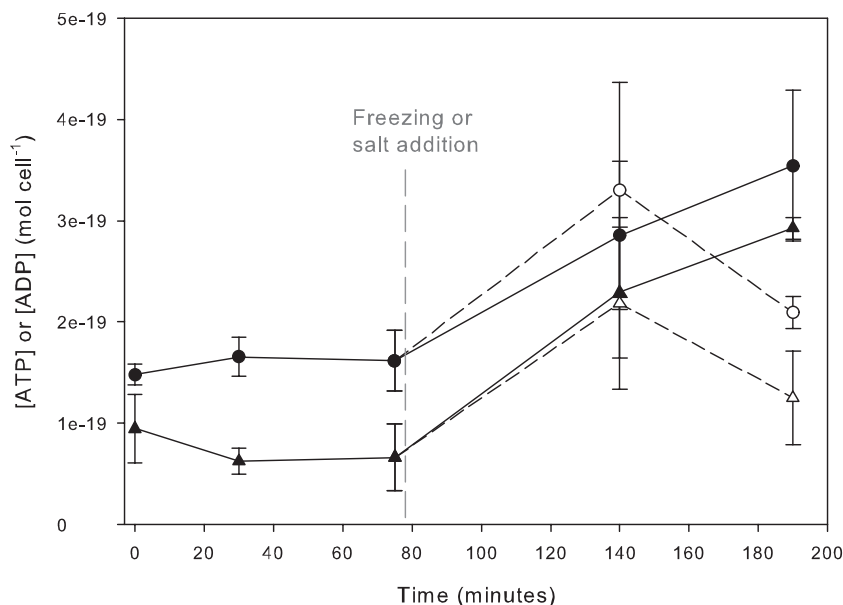
FIG. 3. Concentrations of ATP (circles) and ADP (triangles) in cells cultured at 15°C and subsequently transferred to −20°C (representing $t = 0$). After 78 min of incubation, a final concentration of 1 M NaCl was added to half of the cell suspensions (open symbols) and freezing was induced in the remaining samples (closed symbols after 78 min). Errors bars denote standard deviations from the means ($n = 3$).

Within 1 h of freezing, the concentrations of ATP and ADP continually increased, doubling every 75 min until the end of the experiment ($t = 190$ min, 115 min after freezing). When the cells were osmotically shocked, a similar response ($t = 140$ min) was initially observed and then both the ATP and ADP concentrations decreased between $t = 140$ min and $t = 190$ min to values 1.3 and 1.9 times higher than before the addition of salt, respectively. In a replicate experiment, elevation of the ATP and ADP concentrations in osmotically shocked cells was not restricted to temperatures of −20°C, and the addition of salt elicited a similar response in samples incubated between 15°C and −5°C (data not shown).

DISCUSSION

Experimental comments. Freeze-thaw cycling is commonly used as a method of cell extraction, e.g., for molecules such as proteins (5, 6). The cooling rates at which cells were frozen in our experiments ($<5°C$ min^{-1}; see Materials and Methods) were probably not conducive to the extensive formation of intracellular ice crystals (54), and we observed that freezing at −80°C had little or no effect on the survival of *P. cryohalolentis* K5 cells harvested from the late exponential phase of growth (data not shown). Similar freezing rates have been shown to have a minimal effect on membrane integrity and viability in *E. coli* (9). To explore the possibility that freezing affected the adenylate extraction efficiency, we compared the results obtained with identical cell suspensions that were either liquid or frozen in liquid nitrogen. We confirmed that no such bias was introduced into our analysis but found that the ATP extraction efficiency could be improved by 130% if samples were amended with TCA (0.7%) prior to freezing. Freezing would concentrate the TCA into the aqueous fraction of the ice, where most of the cells were probably located (e.g., see references 1, 24, and 29). The use of a higher bulk TCA concentration would also likely improve the extraction of ATP from the cells, but such an approach is chemically incompatible with the luciferin/luciferase assay. Hence, the addition of a freezing step to the TCA extraction protocol provides a simple method for improving the adenylate yield and could be useful for a number of other cell extraction applications.

Physiological advantage of elevated adenylate concentrations at low temperature. The activity of an enzymatic reaction is a function of the catalytic capacity of the enzyme (k_{cat}), its affinity for a substrate(s) (K_m), and the enzyme concentration. The diminution of k_{cat} and binding capacity (i.e., increased K_m) of an enzyme at low temperature can be counteracted by increasing the enzyme concentration (51, 60) and/or the concentration of the substrate(s) (56, 59). Since ATP is a substrate for many enzymatic reactions, increasing its concentration in the cytoplasm would provide a way to increase the reaction rates of a large number of energy-requiring cellular processes. Moreover, elevating the adenylate concentration will have a positive kinetic effect by increasing the rate of diffusion between the substrate and the enzyme (33).

The concentration of ATP and ADP in the cell at a given time represents a mass balance that is determined by the net rate of phosphorylation and dephosphorylation through substrate level or oxidative phosphorylation and via adenylate kinase, respectively. *P. cryohalolentis* was cultured on a nonfermentable carbon source, and therefore, the observed differences in ATP concentration between the control and CCCP-amended cells provide evidence that ATP was being produced by oxidative phosphorylation at all of the experimental temperatures analyzed.

The growth and metabolic rate of *P. cryohalolentis* follow a predictable Arrhenius relationship between 15°C and −20°C (unpublished data; growth has only been demonstrated down
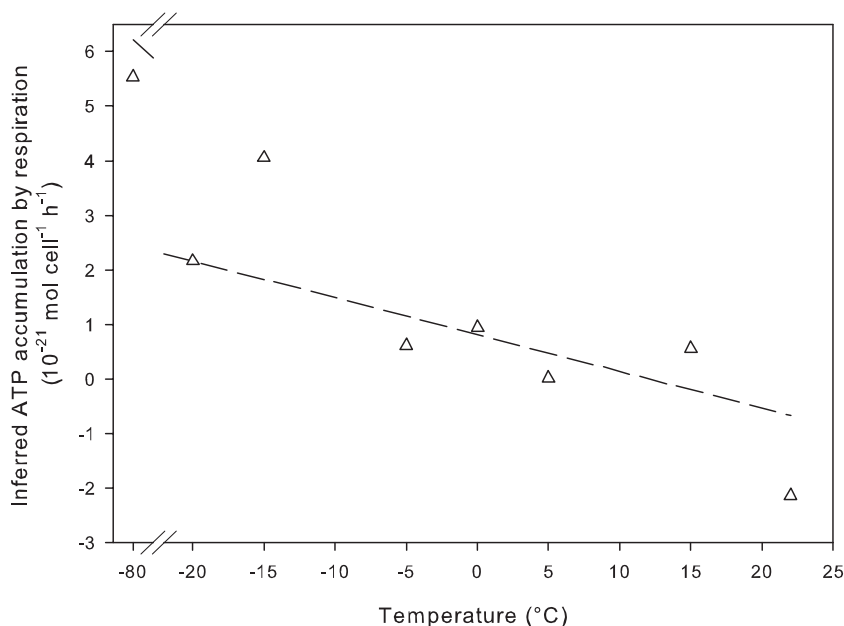
FIG. 4. Linear relationship ($r^2 = 0.79$) between temperature and the inferred rate of cellular ATP accumulation by respiration. Calculations are based on the differences between ATP concentrations in respiring cells and those in cells treated with the uncoupler CCCP between 2.5 and 21 h of incubation.

to $-10°C$ [3]), and the elevated ATP concentrations seen with decreasing temperature could simply be due to the decreased utilization of ATP. However, this does not adequately explain the data, as the ADP concentration also increased with decreasing temperature. Elevation of the ADP concentration would occur principally through the dephosphorylation of ATP or phosphorylation of AMP via adenylate kinase. Since cells inhibit ATP-utilizing and stimulate ATP-regenerating pathways at low energy charge (16), the former mechanism seems an unlikely possibility for the ADP concentrations observed. Furthermore, given that both ADP-generating reactions are dependent on the presence of ATP (15, 32) and the concentration of ADP was 1.8 to 6.0 times higher than that of ATP after 45 h, neither mechanism can stoichiometrically account for the ADP concentrations observed. The total adenylate concentration (ATP plus ADP plus AMP) in respiring cells compared with those in which respiration was uncoupled was not significantly different at a given temperature, but values under both conditions increased at temperatures below freezing. Together, these results imply that there is a noncanonical pathway responsible for the production of ADP and ATP.

We used data from the CCCP-uncoupling experiments to estimate the rate of respiration-dependent synthesis of ATP as a function of temperature (Fig. 4). In these calculations, we assumed that the rates of ADP phosphorylation and dephosphorylation of ATP were linear between 2.5 and 21 h (i.e., after samples had adequately equilibrated to the experimental temperature) and that CCCP was 100% efficient in uncoupling respiration. Although we are unable to determine the rate of catabolism or anabolism from the available data, our results suggest that temperature has less of an effect on the production of ATP than on its utilization, resulting in ATP accumulation. The possibility that catabolic reactions were enhanced and those of anabolism were suppressed as an indirect result of

temperature cannot be discounted (i.e., physiological regulation due to a lower energy charge) (2, 16). For example, increased respiration rates at low temperatures have been reported in yeasts (49), fish (23), and plants (52). The total energy present in a cell increased with decreasing temperatures of incubation. Under liquid conditions, the hydrolysis of ATP was more favorable at the lowest temperature due to a higher ATP/ADP ratio (primarily linked to a decreased ADP concentration), whereas both ATP and ADP concentrations increased at subfreezing temperatures (Fig. 1 and 2). The ability to modify ATP and ADP concentrations and the ATP/ADP ratio at low and subzero temperatures could provide a distinct advantage for cellular survival under frozen conditions. Such an energy reserve may be utilized for repair and maintenance pathways during frozen entrapment (e.g., see reference 41) and/or could facilitate the initiation of metabolism when cells are reintroduced to conditions compatible with growth.

Adaptation of cellular metabolism to low temperature and high salinity. Mesophilic and thermophilic microorganisms increase their adenylate pools with increasing rates of growth, but the total adenylate concentration is higher in mesophiles than in thermophiles (58). Napolitano and Shain (33, 34) demonstrated that psychrophiles have a higher adenylate concentration at low temperature than mesophiles, and psychrophilic organisms continued to increase their adenylate content with decreasing temperature. As suggested by Morrison and Shain (32), this implies that increasing the adenylate concentration may play a role in cold tolerance. Bakermans et al. (4) studied the effect of low temperatures (16, 4, and $-4°C$) on protein expression in *P. cryohalolentis* K5 and found that at $-4°C$, the relative abundance of five proteins involved in energy production, protein synthesis, gene expression, and transport was significantly increased. One of the cold-induced proteins identified was a subunit of ATP synthase, suggesting that there is an

"increased need for ATP synthase" and ATP production at subzero temperatures.

Cold-adapted bacteria have been shown to maintain a basal level of metabolic activity in ice (12, 25, 31), and this study constitutes the first analysis of cellular energy metabolism below the freezing point. During freezing, cells are partitioned into the interstitial veins that exist at the grain boundaries of ice crystals (29, 40). The solute-rich liquid in these veins contains ions that were excluded from the bulk solution during freezing, the salinity of which is a function of temperature (11). Hence, cells that survive freezing and metabolize within ice must be able to function at the low temperature and osmotic pressure associated with the high solute concentration in the liquid fraction of the ice. *P. cryohalolentis* is a halotolerant bacterium capable of growth in 1.7 M NaCl and has been shown to metabolize at salinities as high as 2.8 M NaCl (3, 38). Although the specific mechanisms by which *P. cryohalolentis* K5 maintains turgor under osmotic stress have not been identified, its genome includes genes for the synthesis of glycine betaine and transporters (Comprehensive Microbial Resource, http://cmr.jcvi.org/cgi-bin/CMR/shared/MakeFrontPages .cgi?page=geneattribute). The synthesis of compatible solutes is important for adaptation to high salinity and is a process that requires energy in the form of ATP and/or a chemiosmotic gradient. When *E. coli* is osmotically shocked with NaCl, the cell initially balances the sudden increase in osmolarity through the uptake of K^+, and after compatible solutes have been imported or synthesized, the K^+ is actively transported out of the cell via a specific efflux system (27). The genetic response to osmotic shock has been studied extensively and involves the expression of a number of proteins involved in the transport, regulation, and synthesis of compatible solutes (39, 57, 61).

We have extended the energy metabolism measurements of Napolitano and Shain (33, 34) to very low temperatures. Previous work by these authors showed that elevating the total adenylate pool is a physiological strategy for low-temperature adaptation that is widely distributed in the tree of life (i.e., in bacteria, fungi, protists, and animals). Considering the low metabolic rate under frozen conditions, the energy-requiring reactions involved in the synthesis of purine nucleotides seems an unlikely explanation for our results. Instead, we hypothesize that salvage pathways scavenge adenylates from the existing purine nucleotide pool and potentially through the degradation of RNA (e.g., see references 18 and 21). Although ice-entrapped cells are capable of ATP generation via oxidative phosphorylation (Fig. 2A and B), compounds such as phosphoenolpyruvate or GTP may represent other possible sources of high-energy phosphate bonds within the cell.

The ability of microorganisms to remain metabolically active under frozen conditions implies that permanently frozen environments in the biosphere, which harbor a previously unrecognized but globally significant pool of microorganisms and cell carbon (42, 43), may represent active biomes. Our results add to these discussions and provide information that contributes to our understanding of the physiological transition which occurs in cells at low temperature and following the phase change into the frozen state. We have provided unequivocal evidence for the ability of bacteria to conduct oxidative phosphorylation at temperatures as low as −80°C. Although it is not possible to extrapolate the time frame of our experiments over the geological time scales from which viable bacteria have

been documented in natural ice samples (e.g., see references 7 and 13), our results suggest that the longevity of cold-adapted microorganisms under frozen conditions may only be limited by the water activity and the availability of nutrients and redox couples.

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