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Distinctions in adenylate metabolism among organisms inhabiting temperature extremes

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Abstract Microbiota from multiple kingdoms (e.g., Eubacteria, Fungi, Protista) thrive at temperature optima ranging from 0–20°C (psychrophiles) to 40–85°C (thermophiles). In this study, we have monitored changes in adenylate levels and growth rate as a function of temperature in disparate thermally adapted organisms. Our data indicate that growth rate and adenylate levels increase with temperature in mesophilic and thermophilic species, but rapid losses of adenosine 5′-triphosphate (ATP) occur upon cold or heat shock. By contrast, psychrophilic species decrease adenylate levels but increase growth rate as temperatures rise within their viable range. Moreover, psychrophilic ATP levels fell rapidly upon heat shock, but dramatic gains in ATP (~20–50%) were observed upon cold shock, even at sub-zero temperatures. These results suggest that energy metabolism in thermophiles resembles that in mesophiles, but that elevated adenylate nucleotides in psychrophiles may constitute a compensatory strategy for maintaining biochemical processes at low temperature.

Keywords Adenylate · Algae · ATP · Bacteria · Bioenergetics · Fungi · Psychrophile · Thermophile

Introduction

A fundamental requirement for all living organisms is the necessity to balance energy production with consumption. For organisms inhabiting environments at extreme temperatures (e.g., psychrophiles, thermophiles), energy levels must be adjusted accordingly in order to maintain biological processes at viable levels. In general, rates of biochemical reactions double with every ~10°C rise in temperature (i.e., the Q_{10} relationship); consequently, organisms increase the production rate of high energy phosphates [(e.g., adenosine 5′-triphosphate (ATP)] as temperatures rise to keep up with demand, and most also elevate steady-state ATP levels with temperature gain (Fedorow et al. 1998; English and Storey 2000; Napolitano et al. 2004). An exception to this paradigm has been observed in the glacier ice worm, *Mesenchytraeus solifugus*, and its associated microbiota, which paradoxically increase ATP levels as temperatures fall (Napolitano et al. 2004; Napolitano and Shain 2004). It has been proposed that these cold-adapted organisms use elevated ATP levels to compensate for reductions in molecular motion at low physiological temperature. Other compensatory changes associated with temperature adaptation include adjustments in membrane viscosity (Hazel 1995), mitochondrial density (Johnston et al. 1988; Guderley 1998; Johnston et al. 1998), metabolic rate (Peck 2002), specific enzyme activities (Crockett and Sidell 1990), and modulation of the cellular environment (e.g., pH, Pörtner et al. 1998).

Single-cell microbiota (e.g., algae, bacteria, fungi) have invaded and adapted to almost every niche in the biosphere, including thermally challenging environments uninhabitable by most (e.g., glacier ice, thermal hot springs). In this study, we were interested in comparing the energetics of adenylate metabolism among microorganisms that thrive at temperature extremes. Specifically, we examined the relationship between adenylate levels (i.e., energy supply) with growth rate (i.e., energy demand) in disparate extremophiles. Our results demonstrate that the psychrophiles invariably elevate adenylate levels as temperatures fall, despite declining growth rates, while thermophiles increase both adenylates and growth rate as temperatures rise within their viable temperature range. The stoichiometry of these changes target pivotal enzymes in cellular metabolism that influence adenylate ratios and the adenylate pool size.

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Materials and methods

Cultures

Psychrophilic bacterial strain *Psp1* (*Pseudomonas* sp.) was obtained by dissociating glacier ice worms (*Mesenchytraeus solifugus*) between two glass slides and culturing the extract on LB agar at 0°C. Designation of *Psp1* in the genus *Pseudomonas* was based on rRNA sequencing (Accugenix, Newark, Del., USA). *Janthinobacterium lividum* was purchased from ATCC (strain 14487). Psychrophilic algae (*Raphidonema nivale*) and fungi (*Crytococcus sp.*) were isolated by plating meltwater from Byron Glacier, Alaska, on carbon-free (CCMP DY-V) and YEPD medium, respectively, as described (Napolitano and Shain 2004). Antarctic algal strain *Chlamydomonas sp.* was purchased from CCMP (1619 from Lake Bonney, Antarctica). Mesophilic species *Escherichia coli* (strain HB101), *C. moewussi* (Carolina Biological), and *Saccharomyces cerevisiae* (Fisher) were cultured by standard procedures. Thermophiles *Thermus thermophilus* (ATCC 27634), *Cyanidium* sp. (CCME 5634), and *Sporotrichum thermophile* (ATCC 28811) were maintained as suggested by the supplier.

Adenylate measurements

Batch suspension cultures were grown at appropriate temperatures without forced aeration; bacterial and fungal cultures (except *S. thermophile*) were rotated at 220 rpm on an orbital shaker. Cultures were collected during log phase by centrifugation at 10,000 *g* for 30 s, weighed to the nearest 0.1 mg (wet weight), and deproteinated in 50 mM HEPES, pH 7.4 (170 µl) containing 50 mAU/ml proteinase K (Novagen) at 50°C for 30 min, as described (Napolitano et al. 2004). Adenylate extraction efficiencies did not differ significantly from perchloric acid deproteination (Lowry and Passonneau 1972) in side-by-side comparisons, but proteinase K extractions did not require the physical disruption of cells (e.g., pestle/mortar), which inevitably led to losses in small sample sizes (1–3 mg used here). Adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), and ATP levels were determined simultaneously in aliquoted tubes, respectively, by converting the former (i.e., AMP, ADP) to ATP with myokinase/pyruvate kinase (Sigma) and making the appropriate mathematical calculations, as described (Adam 1963). All samples were coupled with luciferin-luciferase assays (Cal-Biochem), as described (Lowry and Passonneau 1972), and monitored with a Beta-Scout luminometer (PerkinElmer). The adenylate energy charge (AEC) was calculated by the following equation: AEC = [ATP] + 1/2[ADP]/[ATP] + [ADP] + [AMP] (Atkinson 1968).

Temperature shock

Cultures maintained at a median temperature within their viable range (e.g., normally near the growth optimum of each respective species) were placed ~10–20°C below (cold shock) or above (heat shock) that temperature. In most cases, the "shock" temperatures fell within the viable temperature range of the organism. After a 120-min incubation period at the cold shock or heat shock temperature, respectively, cells were collected and assayed for adenylate nucleotides as described above.

Growth rate

Cultures grown at appropriate temperatures were monitored for changes in optical density at defined time intervals, using a UV/VIS spectrophotometer (Abs_{540} and Abs_{600}, Beckman).

Statistical analysis

Data points are reported as means ± SEMs. At least three independent samples were analyzed for each data point, comprising ~2–3 mg of cells per sample. ANOVA analysis and Student's *t*-tests were conducted where appropriate.

Results

Psychrophilic, mesophilic, and thermophilic microorganisms from three kingdoms (i.e., Eubacteria, Protista, Fungi) were cultured under appropriate conditions at a minimum of three independent temperatures within their viable temperature range, respectively. For example, the mesophilic bacterium, *Escherichia coli*, was maintained at 10, 20, 37, and 42°C and displayed sustained growth at all of these temperatures. For all species examined, growth rate increased with temperature within their viable range, peaked at a temperature optimum, and then declined as temperatures increased further (Fig. 1). In general, mesophilic growth rate optima were higher than psychrophilic and thermophilic representatives within the same kingdom.

In mesophilic and thermophilic species, ATP profiles reflected changes in growth rate as a function of temperature with some exceptions, e.g., *E. coli* ATP levels continued to rise at 42°C, even though growth rate decreased (Fig. 1). In these cases, it appears that growth-related processes were more sensitive to temperature than ATP synthetic pathways within a narrow, elevated temperature window. In all psychrophilic species examined, ATP levels increased upon declining temperatures, even though growth rates increased with temperature (Fig. 1). Thus, ATP level and growth rate were positively correlated in both mesophilic and thermophilic species,

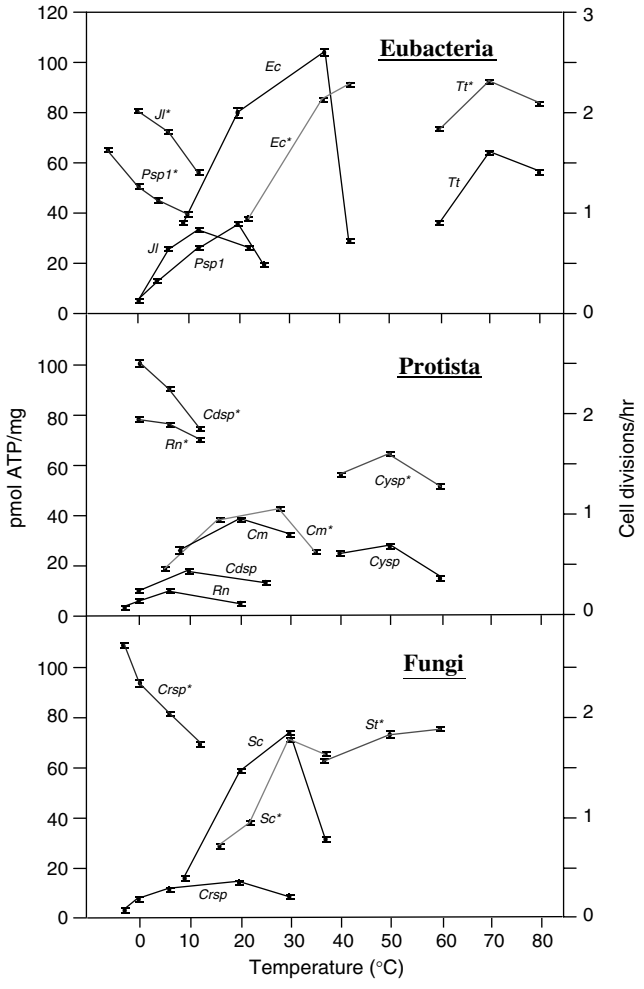


Fig. 1 Adenosine 5′-triphosphate (ATP) vs growth rate profiles as a function of temperature change in diverse microbiota. Mesophilic (*Ec*,*Cm*, *Sc*) and thermophilic (*Tt*, *Cysp*, *St*) species displayed gains in both ATP levels (*asterisk*) and growth rate as temperatures increased to a thermal optimum ($P < 0.05$), upon which both variables usually declined. Psychrophilic (*Jl*, *Psp1*, *Cdsp*, *Rn*, *Crsp*) growth rates displayed a similar profile, but ATP levels invariably increased as temperatures fell ($P < 0.05$). All data points represent equilibrated values, allowing at least 24 h at each respective temperature before taking measurements. Note that ATP levels were not determined throughout the viable growth range of all organisms, but representative temperatures identify the trend in which ATP levels changed in those species. *St Sporotrichum thermophile* (Fungi) growth rates were not determined, since the organism is filamentous, but the diameter of individual hyphal clusters increased with temperature. *Cm Chlamydomonas moewussi*; *Cdsp Chlamydomonas* sp.; *Crsp Crytococcus* sp.; *Cysp Cyanidium* sp.; *Ec Escherichia coli* (strain HB101); *Jl Janthinobacterium lividum*; *Psp1*, *Psp2 Pseudomonas 1, 2* sp.; *Rn Raphidonema nivale*; *Sc Saccharomyces cerevisiae*; *St Sporotrichum thermophile*; *Tt Thermus thermophilus*

but inversely related in psychrophiles. Although absolute ATP levels vary considerably between species, it is noteworthy that psychrophilic algal (*Chlamydomonas* sp.) and fungal species (*R. nivale*, *Crytococcus* sp.) displayed ATP levels equal to, or greater than, their mesophilic and thermophilic counterparts, respectively, even though growth rates of the former were significantly lower.

To monitor changes in ATP levels as a function of rapid temperature change, all species were heat- and cold-shocked for 120 min at temperatures ~10–20°C above and below a median temperature within their viable range, respectively (Fig. 2). For example, *T. thermophilus* equilibrated to 70°C was heat-shocked at 90°C and cold-shocked at 50°C. For all species examined, ATP levels dropped significantly upon heat shock ($P < 0.01$), with psychrophiles displaying the most substantial losses (e.g., ~60–80% loss of ATP in 120 min). Both mesophiles and thermophiles lost ATP upon cold shock, at levels comparable with those observed when heat-shocked (~20–40%). In contrast, psychrophiles from all three kingdoms displayed significant gains in ATP after cold shock (~20–50%), even when placed at sub-zero temperatures (e.g., *Pseudomonas* sp.).

To gain a perspective on the changes in adenylate metabolism after cold shock and after organisms had equilibrated to different temperatures, respectively, ATP, ADP, and AMP were monitored in psychrophiles and thermophiles exposed to appropriate conditions (Fig. 3). Four general trends were observed. First, ratios between ATP:ADP:AMP remained constant in all species when allowed to equilibrate to temperatures within their viable range. Second, total adenylate nucleotides (TAN) decreased with temperature in thermophiles, but TAN levels increased as temperatures fell in psychrophiles, similar to ATP profiles observed in Fig. 1. Third, cold shock-induced ATP gains in psychrophiles were stoichiometrically related to ADP losses, with little change in TAN levels, suggesting a direct phosphorylation of ADP into ATP under these conditions. Fourth, cold shock-induced ATP loss in thermophiles was correlated with gains in ADP and/or AMP with concomitant losses in TAN, suggesting widespread loss of high-energy phosphates and depletion of the adenylate pool.

Discussion

The rates of biological processes generally increase with rising temperatures in accordance with the Arrhenius principle, until a threshold is reached (Pörtner et al. 1998). This trend was observed for the growth rates of all species examined here, and has been reported for a variety of metabolic parameters (e.g., respiration) in diverse organisms (Goodman 1971; Sommer et al. 1997; Johnston et al. 1998). By this criterion, the observation that adenylate levels increased with growth rate (and temperature) in thermophilic algae, bacteria, and fungi is not unexpected. Specifically, an increase in energy demand (deduced from increased growth rate) appears to coincide with gains in energy supply (i.e., adenylate levels), as observed in all mesophilic species examined to date (Napolitano and Shain 2004). In contrast, the apparent widespread strategy of raising adenylate levels

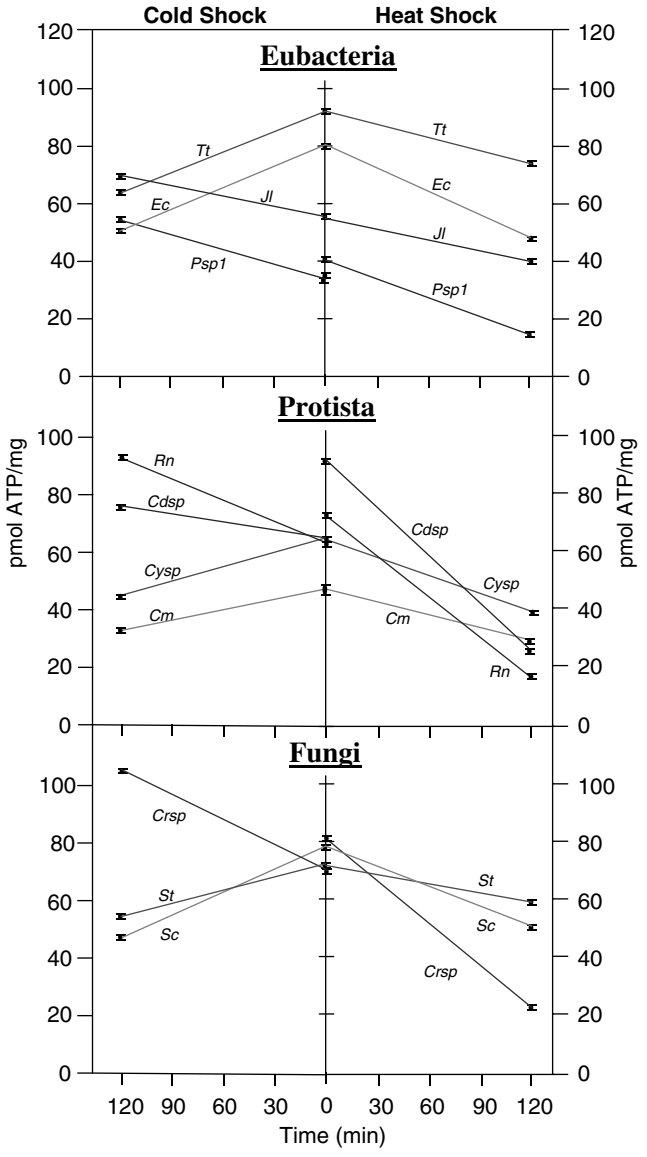


Fig. 2 Changes in ATP levels after 120-min cold- and heat-shocks, respectively. Mesophilic (*Ec*, *Cm*,*Sc*) and thermophilic (*Tt*, *Cysp*, *St*) ATP levels dropped upon cold or heat shock ($P < 0.05$). Psychrophilic (*Jl*, *Psp1*, *Cdsp*, *Rn*, *Crsp*) ATP levels dropped upon heat shock, but increased after cold shock ($P < 0.01$). *Lines* are not intended to indicate a linear relationship between ATP levels and time, but rather identify the absolute change in ATP after 120 min. *Cm* equilibrated at 22°C was cold-shocked at 0°C and heat-shocked at 42°C, displaying ATP losses of 21 and 29%, respectively (22 → 0°C/−21%, 22 → 42°C/−29%); *Cdsp* (10 → 0°C/+17%, 6 → 22°C/−71%); *Crsp* (12 → 0°C/+49%, 6 → 22°C/−73%); *Cysp* (50 → 35°C/−30%, 50 → 65°C/−40%); *Ec* (37 → 0°C/−38%, 37 → 50°C/−43%); *Psp1* (10 → −6°C/+51%, 6 → 22°C/−63%); *Rn* (10 → 0°C/+43%, 6 → 22°C/−77%); *Sc* (30 → 0°C/−42%, 30 → 50°C/−36%); *St* (50 → 30°C/−25%, 50 → 70°C/−17%); *Tt* (70 → 50°C/−30%, 70 → 90°C/−21%). Refer to Fig. 1 for species names

by diverse psychrophiles as temperatures fall remains perplexing.

To maintain physiological processes at cold temperatures, psychrophilic organisms must compensate for reductions in molecular movement by either increasing the intracellular number of molecules (e.g., enzymes), the efficiency of biochemical reactions/processes, and/or by modifying the cellular environment (e.g., pH, membrane viscosity, Hochachka and Somero 1973). Of particular relevance here is that metabolic rate, as measured by oxygen consumption (or respiration), appears to be elevated in some cold-adapted organisms, suggesting at least some level of metabolic compensation at low temperature (Wells 1987; Sommer and Pörtner 1999). While elevated respiration rates may be partially explained by well-documented increases in mitochondrial density at low temperature (Guderley 1998; Johnston et al. 1998), there is also evidence for compensation of enzymatic activity in some energy-related pathways (Wodtke 1981; Crockett and Sidell 1990). Clearly, these changes are likely to enhance the survivability of cold-adapted organisms, but it is unlikely that they play a direct role in the temperature-dependent changes of adenylates observed in this study. Rather, adaptation of key enzymes that control adenylate metabolism are likely to play a role in the dramatic changes in adenylate levels observed at different temperatures, as discussed below.

An overview of adenylate metabolism is shown in Fig. 4. Of the three major pathways leading to ATP production (i.e., chemiosmotic, glycolysis, creatine kinase), the muscle-specific enzyme creatine kinase is not expressed in the microorganisms examined here, and the relatively small ATP yields from the cytosolic, diffusion-dependent reactions composing the glycolytic pathway are particularly susceptible to reductions in temperature. For instance, the onset of anaerobic metabolism has been linked to the lower temperature limit of many cold-adapted organisms (Zielinski and Pörtner 1996; DeWachter and Pörtner 1997; Sommer et al. 1997). The remaining ATP synthetic pathway, i.e., ultimately catalyzed by the F_1F_0 ATP synthase complex in bacteria or mitochondria (Nicholls and Ferguson 2002), is driven by an electrochemical H^+ gradient that is arguably less dependent upon diffusion. In principle, relatively high activities of the catalytic F_1 component of the F_1F_0ATP synthase could account for the rapid gains in ATP observed upon cold shock in psychrophiles, assuming there are no other temperature-dependent bottlenecks in the pathway (e.g., electron transport, proton pumps). Even so, psychrophilic F_1ATP synthase activity almost certainly decreases with temperature based on the Q_{10} relationship and kinetics of other energy-related processes (Guderley 1998; Pörtner et al. 1998). Consequently, rapid ATP gains are likely to result from disproportionately larger declines in energy consumption versus production as temperatures fall. Thermophiles, on the other hand, lost ATP when shocked on either side of their preferred temperature, suggesting that disproportionately larger declines in energy production occurred upon rapid temperature change. The possibility that a homeoviscous response (i.e., reduction in membrane order at lower temperatures, Cossins et al. 1981) contributes to rapid ATP gains in psychrophiles

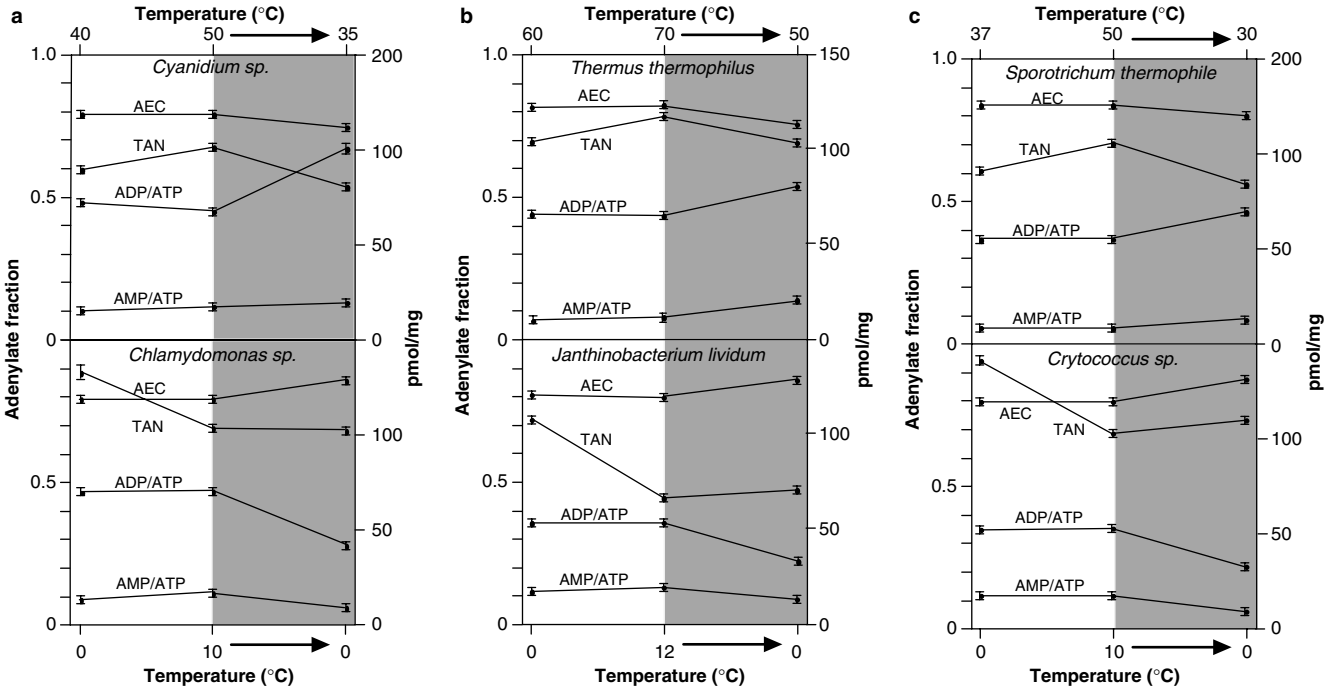
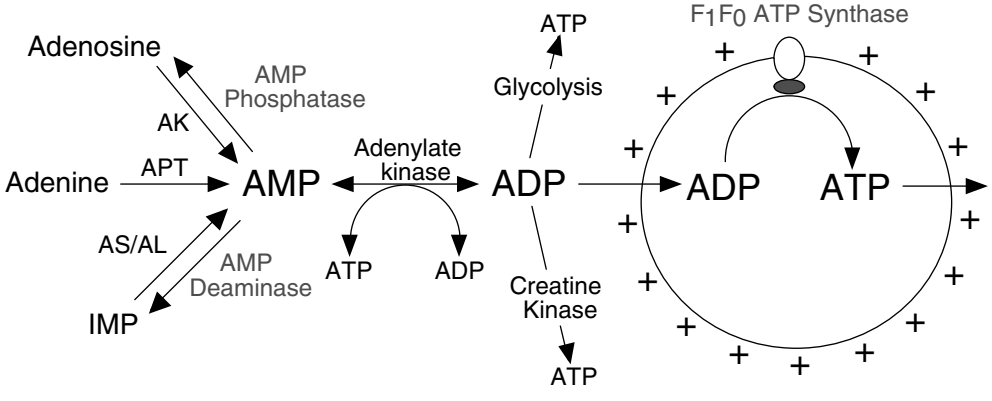


Fig. 3 Adenylate profiles of thermophilic versus psychrophilic species. **a** Algae. **b** Fungi. **c** Eubacteria. Acclimated temperature points are shown in the *center* and *left side* of each panel; *shaded regions* represent a 120-min cold shock at indicated temperatures, respectively. Psychrophiles (*bottom* of each panel) elevated total adenylate nucleotides (*TAN*) levels as temperatures fell (*left side, white*) and elevated adenylate energy charge (*AEC*) upon cold shock (*right side, shaded*; $P < 0.05$); the opposite trend was observed in counterpart, psychrophilic species, respectively (*top* of each panel; $P < 0.05$). *AMP* Adenosine 5′-monophosphate, *ADP* adenosine 5′-diphosphate. Data from *Crsp* included for comparison (Napolitano and Shain 2004)

Fig. 4 Overview of adenylate metabolism. The collective data from this study suggest that the F_1F_O ATP synthase (depicted within an inner bacterial/mitochondrion membrane surrounded by H^+ ions) plays a central role in the stoichiometric interconversion of ADP $\leftarrow \rightarrow$ ATP in temperature-shock experiments. Reductions in AMP phosphatase and AMP deaminase activities (which regulate the adenylate pool size by removing AMP in response to fluctuating ATP levels), would lead to an increase in TAN. *AK* adenosine kinase, *AL* adenylosuccinate lyase, *APT* adenine phosphoribosyl transferase, *AS* adenylosuccinate synthetase

seems unlikely due to the short time frame and temperatures involved (e.g., 120 min at 0° or below).

Cold shock-induced gains in ATP observed among psychrophiles remained elevated in the long term and were followed by corresponding gains in ADP and ATP after ~24 h, leading to continued increases in the adenylate pool size (TAN) as temperatures fell (due to technical limitations, it remains unclear how far temperatures could be lowered before TAN levels peaked). Similar gains in TAN were observed in thermophiles with increasing temperature until a threshold was reached, upon which TAN levels dropped in parallel with declining ATP levels and growth rate. The clear difference in the direction of TAN as function of temperature change identifies a major distinction between psychrophilic and thermophilic energetics, and suggests that pivotal enzymes in adenylate metabolism have been selectively modified. In temperate organisms that have been examined, the adenylate pool is supplied constitutively by forward *de novo* and salvage pathways that generate a continuous supply of AMP (Fig. 4). The size



of the adenylate pool is controlled by reverse reactions catalyzed by AMP phosphatase and AMP deaminase, two allosterically regulated enzymes that remove AMP from the adenylate pool based on relative ATP levels (Atkinson 1977; Ataullakhanov and Vitvitsky 2002). When a drop in ATP is detected (e.g., increased demand) these enzymes are inhibited, allowing a net flux of AMP into the pool to replenish adenylate levels. When ATP is abundant the enzymes are activated leading to AMP removal. Thermophilic energetics are consistent with adenylate metabolism in temperate organisms, namely, adenylate levels rise upon an increase in demand (e.g., growth rate), which correlates with temperature gain. That psychrophiles decrease TAN as temperatures rise despite higher growth rates suggests that AMP phosphatase/deaminase counterparts have been modified in these organisms. Specifically, it seems that the activity of these enzyme(s) may be reduced to allow more AMP flux into the adenylate pool at lower temperatures, an arguably easier molecular adaptation compared with the more typical challenges of maintaining enzymatic activity at low temperature.

Collectively, our data target a few key enzymes in adenylate metabolism (i.e., F_1 ATP synthase, AMP phosphatase/deaminase) that may underlie the energetic differences identified between psychrophiles and thermophiles in this study. Clearly, it will be instructive to compare the sequences and activities of these enzymes in thermally challenged organisms to determine whether their properties are consistent with the differences in energy metabolism observed here. Of particular significance will be understanding the mechanism by which psychrophiles increase adenylate levels at reduced temperatures, which appears to constitute an important compensatory strategy for maintaining biochemical processes at low temperature.

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