biology letters
Biol. Lett. (2008) 4, 53–56
doi:10.1098/rsbl.2007.0432
Published online 20 November 2007
Evolutionary biology

An AMP nucleosidase gene knockout in *Escherichia coli* elevates intracellular ATP levels and increases cold tolerance

Brittany A. Morrison and Daniel H. Shain*

Biology Department, Rutgers The State University of New Jersey, Camden, NJ 08102, USA
**Author for correspondence* (*dshain@camden.rutgers.edu*).

Disparate psychrophiles (e.g. glacier ice worms, bacteria, algae and fungi) elevate steady-state intracellular ATP levels as temperatures decline, which has been interpreted as a compensatory mechanism to offset reductions in molecular motion and Gibb's free energy of ATP hydrolysis. In this study, we sought to manipulate steady-state ATP levels in the mesophilic bacterium, *Escherichia coli*, to investigate the relationship between cold temperature survivability and elevated intracellular ATP. Based on known energetic pathways and feedback loops, we targeted the AMP nucleotidase (*amn*) gene, which is thought to encode the primary AMP degradative enzyme in prokaryotes. By knocking out *amn* in wild-type *E. coli* DY330 cells using recombineering methodology, we generated a mutant (AMNk) that elevated intracellular ATP levels by more than 30% across its viable temperature range. As temperature was lowered, the relative ATP disparity between AMNk and DY330 cells increased to approximately 66% at 10°C, and was approximately 100% after storage at 0°C for 5–7 days. AMNk cells stored at 0°C for 7 days displayed approximately fivefold higher cell viability than wild-type DY330 cells treated in the same manner.

Keywords: psychrophile; energetics; adenylates; cold tolerance

1. INTRODUCTION

The evolutionary steps leading to cold temperature adaptation (i.e. psychrophilic organisms) remain an intensely investigated topic in modern biology. Changes in membrane fluidity (Russell 1997; D'Amico *et al.* 2006), protein structure and enzyme activity (Marshall 1997; Nakagawa *et al.* 2003), respiration (Stokes & Larkin 1968; Pörtner *et al.* 1998) and, in some cases, depression of the protoplasmic freezing point (Davies *et al.* 2002) appear to be important components of the evolutionary processes that permit life to survive and function at low physiological temperatures (i.e. less than 20°C). Less often considered is the contribution of energy metabolism, specifically the requirement that cells maintain biochemical reactions at sufficient levels to sustain life at cold temperatures. In the context of the Q_{10} relationship, which predicts an approximately twofold decrease in the reaction rate for every drop of 10°C, the ability to maintain biochemical reactions at viable levels becomes increasingly more challenging as temperatures approach 0°C. Nonetheless, disparate organisms ranging from psychrophilic microbes (e.g. algae, bacteria and fungi) to glacier ice worms and Antarctic fish thrive in cold environments (Johnston 1990; Napolitano & Shain 2004).

We have shown previously that intracellular ATP (5′-adenosine triphosphate) levels are relatively high in psychrophilic organisms, and that these levels increase paradoxically as temperatures decline, even though growth rates increase with temperature (Napolitano & Shain 2004, 2005; Napolitano *et al.* 2004). This has been interpreted as a compensatory mechanism by which temperature-dependent reductions in molecular motion (i.e. number of molecular collisions) and Gibb's free energy of ATP hydrolysis (i.e. joules) are offset by gains in [ATP], which drives many unfavourable reactions.

In this study, we aimed to elevate ATP levels in the mesophilic bacterium, *Escherichia coli*, to determine whether this manipulation affected its ability to tolerate cold temperature. To change intracellular ATP levels, we targeted the bacterial AMP nucleosidase (*amn*) gene, the functional counterpart of eukaryotic AMP phosphatase, which are key regulators of the adenylate pool size (i.e. AMP, ADP, ATP). Specifically, AMP phosphatase and AMN remove AMP from the adenylate pool in response to excess ATP, thus countering a constitutive influx of AMP from de novo and salvage pathways (Atkinson 1977; Ataullakhanov & Vitvitsky 2002). We hypothesized that downregulating the bacterial *amn* gene would increase steady-state intracellular ATP levels, and therefore may enhance cold temperature tolerance. Indeed, *amn* gene knockouts in *E. coli* strain DY330 increased ATP levels by more than 30% across its viable temperature range, and *amn* mutants displayed significantly higher viability than wild-type cells after cold temperature storage.

2. MATERIAL AND METHODS

(a) *Bacterial specimens*

Escherichia coli strain DY330 was generously provided by Dr Donald Court (National Cancer Institute, Frederick, MD), and strain HB101 was purchased commercially (Promega). The psychrophilic bacterium, *Variovorax* sp. (*V*sp1; GenBank accession no. EF681130), was isolated and cultured from the snowpack retrieved from Broken Top Mountain, Oregon, in August 2005, following the procedures as described (Eiler & Bertilsson 2004; Napolitano & Shain 2004).

(b) *Gene knockout strategy*

DY330 AMP nucleosidase (*amn*) knockouts were constructed using recombineering methodology as described by Thompson *et al.* (2005), employing *amn*/chloramphenicol gene-specific oligonucleotides (5′-amn-cat and 3′-amn-cat; table 1). To verify chloramphenicol (*cat*) gene recombination, colony PCR was performed using *cat* primers: 5′-cat and 3′-cat (table 1). To ensure proper recombination, primers were employed (5′-amn and 3′-amn; table 1), which flanked the expected recombination site.

(c) *ATP levels*

Bacterial cells were grown to log phase in LB medium at specified temperatures, upon which intracellular ATP levels were quantitated using a luciferin–luciferase ATP assay (Calbiochem), according to the proteinase K (600 U ml^{-1}; MO BIO Laboratories, Inc.) extraction method described by Napolitano & Shain (2005), with the exception that 40 µl of releasing reagent were added to each

Received 17 August 2007
Accepted 23 October 2007

Table 1. Primer sequences for *amn*<>*cat* recombination.

primers	sequence
5′-amn-cat[a]	5′-TGTGACATACTATCGGATGTGCGGTAATTGTATGGAACAGGAGACACAC**TGTGACGGAAG ATCACTTCGC**
3′-amn-cat[a]	5′-TACTTACTCCTTCACATCTATTTCTGATAAGAAGGTTCAGAACTTATTA**ACCAGCAATAGA CATAAGCG**
5′-cat[b]	5′-TGTGACGGAAGATCACTTCG
3′-cat[b]	5′-ACCAGCAATAGACATAAGCG
5′-amn[c]	5′-ACAATTCCCTGTTTTGCCTGGC
3′-amn[c]	5′-CCCACTGGAATGATCTCCTCG

[a] Retargeting primers contained homologous sequence to *E. coli K12* genes upstream and downstream of *amn*. Bold sequences are homologous to *cat*.
[b] Primers were created within the chloramphenicol gene to verify *cat* recombination.
[c] Verification primers were created upstream and downstream of retargeting primers to ensure proper placement of the *cat* recombination event within the DY330 genome.

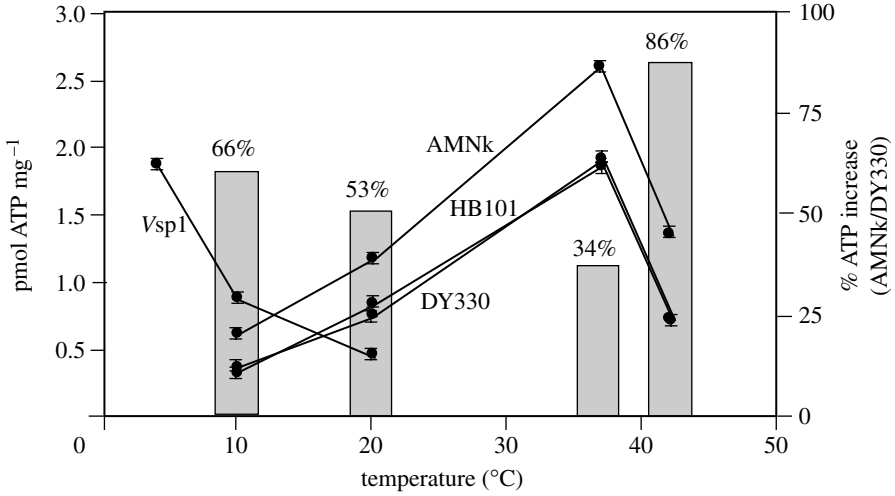

Figure 1. AMNk knockout cells elevated steady-state ATP levels compared with wild-type *E. coli* cells. ATP levels decreased in all mesophilic cells as temperature deviated from 37°C (line graph), but the relative difference in ATP levels between AMNk and DY330 increased (bars). The psychrophilic bacterium, *V*sp1, increased [ATP] as temperature decreased.

sample (60 µl), followed by the addition of 1 µl of luciferin–luciferase mix. Each sample was run in triplicate, and data points indicate the mean±s.e.m. Statistical significance was determined by paired Student's *t*-tests.

(**d**) ***Cold tolerance***
Bacterial cultures (2 ml) were grown to log phase at 37°C. A 50 µl aliquot was pelleted at 16 000*g* for 30 s and resuspended twice in 100 µl cold phosphate-buffered saline (PBS; 8.7 g l^{-1} NaCl, 1.235 g l^{-1} Na_2HPO_4, 0.2038 g l^{-1} NaH_2PO_4), before resuspending in 100 µl cold 1× PBS. Cells were stored at 0°C (ice–water mix) for the whole of the experiment. To establish cell viability at specified time points (e.g. 0, 5 and 7 days), 5 µl of stock cell suspension were diluted 1 : 40 000 in cold 1× PBS, and 10 µl of diluted suspension mix were plated on LB plates (done in triplicate). Following overnight incubation at 37°C, colony forming units were counted (approx. 100–200 per plate at zero time) and the mean±s.e.m. calculated. Data points after zero time were scored as a fraction of viable cells compared with cell counts at the beginning of the experiment (i.e. zero time).

3. RESULTS

The AMP nucleosidase gene (*amn*) was knocked out in *E. coli* DY330 cells by recombineering methodology (Thompson *et al.* 2005). Gene knockouts (AMNk) were verified by colony PCR; two independent colonies were randomly selected from a pool of eight successful recombinants and both were processed in parallel in subsequent analyses, along with wild-type *E. coli* strains DY330 (from which AMNk mutants were derived) and HB101, and the psychrophilic bacterium *V*sp1.

Steady-state ATP levels were monitored in all bacterial strains across their viable temperature ranges (figure 1). In parallel with growth rates (not shown), ATP levels increased with temperature in *E. coli* cells before declining at 42°C. AMNk cells, however, displayed significantly higher ATP levels at all temperatures, ranging from approximately 34% (37°C) to approximately 86% (42°C) more ATP than wild-type DY330 cells. Interestingly, the relative disparity (in terms of % ATP increase) between AMNk and DY330 cells increased as temperature decreased from 37 to 10°C (34–66%; figure 1). *V*sp1 ATP levels increased with declining temperature, consistent with patterns observed in other cold-adapted taxa (Napolitano & Shain 2004, 2005; Napolitano *et al.* 2004).

AMNk cells displayed a significant increase in cell survival compared with DY330 after storage at 0°C; specifically, AMNk cells were approximately 2.5- and approximately 5-fold more likely to survive after 5 and 7 days, respectively (figure 2). Note that steady-state ATP levels were approximately 100% higher in AMNk cells at 5 and 7 days.

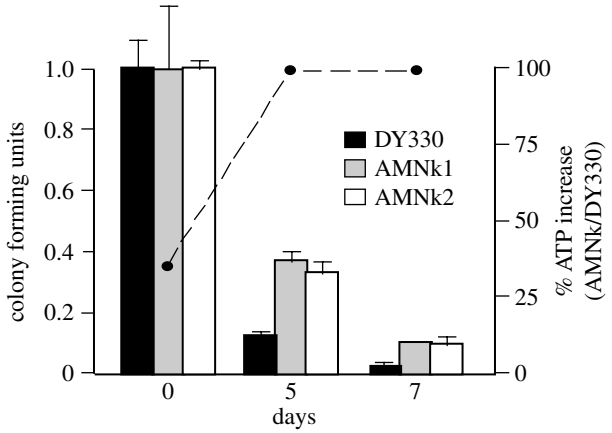

Figure 2. Survivability of AMNk versus DY330 cells after cold storage at 0°C. AMNk cells displayed an approximately 2.5- and approximately 5-fold increase ($p<0.05$) in cold survivability compared with DY330 after 5 and 7 days cold storage, respectively. Data are normalized and presented as a fraction of cells at zero time. ATP levels fell steadily in both AMNk and DY330 cells as a function of time, but were approximately 100% higher in AMNk after 5 days (0.99 ± 0.02 versus 0.48 ± 0.02 pmol ATP mg^{-1}) and 7 days (0.85 ± 0.01 versus 0.41 ± 0.02 pmol ATP mg^{-1}; dashed line, right axis; cf. figure 1).

4. DISCUSSION

The putative role of elevated ATP in enhancing cold temperature survival remains uncertain, yet a striking number of disparate psychrophilic organisms elevate intracellular ATP levels as a function of declining temperature; specifically, North American glacier ice worms (Napolitano *et al.* 2004), psychrophilic bacteria, algae and fungi collected in Alaska (Napolitano & Shain 2004), and an Antarctic psychrophilic alga (Napolitano & Shain 2004). In the current study, we added to this list a snow-inhabiting bacterium, *V*sp1, collected from Oregon, which led us to propose that the ability to elevate intracellular ATP levels at low physiological temperatures probably represents a signature of cold-adapted taxa. And, while the importance of this response may be argued (e.g. a compensatory energetic mechanism or simply a consequence of some other metabolic adaptation), the opposite response (i.e. depleting ATP) clearly has a critical lower limit for all cells, namely the point at which sufficient free energy is no longer available to maintain vital biochemical reactions (Luyet & Gehenio 1940).

Although we were unable to mimic the aforementioned psychrophilic response by an *amn* knockout in *E. coli* DY330 cells, our mutant did maintain higher intracellular [ATP] across its viable temperature range (approx. 10–42°C), and this disparity increased as temperatures declined (i.e. approx. 34% higher ATP levels at 37°C versus approx. 66% at 10°C). The mutant strain also displayed higher cold tolerance than wild-type DY330 cells (i.e. approx. fivefold after storage at 0°C for 7 days). But this change (i.e. *amn* knockout) cannot be the only component (and is not necessarily a component at all) of the characteristic energetic response observed in psychrophiles, since our mutant strain did not directly elevate ATP levels as temperatures declined.

Nonetheless, our data support a myriad of evidence that correlates cold temperature tolerance and adaptation with gains in intracellular adenylate levels (e.g. Southard *et al.* 1985; Churchill *et al.* 1994; Fedorow *et al.* 1998; English & Storey 2000). Interestingly, hibernating prairie dogs, *Cynomys leucurus*, which display elevated intracellular ATP levels, deactivate AMP deaminase (a eukaryotic enzyme functionally related to AMN) but maintain robust adenylate kinase activity ($2\ ADP \leftrightarrow AMP + ATP$; English & Storey 2000). In principle, this combination of events could independently increase intracellular ATP levels, since AMP accumulates in the cell in the absence of AMP deaminase activity (due to constitutive AMP influx), and adenylate kinase functions to maintain relatively constant AMP : ADP : ATP ratios (Atkinson 1977). By analogy, and based on the data presented here, we propose that AMN (and eukaryotic functional counterparts: AMP phosphatase and AMP deaminase) activity levels may be reduced or absent in cold-adapted taxa, and that AMN inactivation coupled with enhanced ATP synthetic reactions at low physiological temperatures may result in the observed cold taxa energetic response (i.e. gain in ATP with declining temperature).

We thank Nina Cosentino (National Cancer Institute, Frederick, MD) for the technical assistance in constructing mutant bacterial strains. This work was supported by NASA grant EXB04-0039-0136 to D.H.S.

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