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Bacterial incorporation of leucine into protein down to −20 °C with evidence for potential activity in sub-eutectic saline ice formations ☆

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Abstract

Direct evidence for metabolism in a variety of frozen environments has pushed temperature limits for bacterial activity to increasingly lower temperatures, so far to −20 °C. To date, the metabolic activities of marine psychrophilic bacteria, important components of sea-ice communities, have not been studied in laboratory culture, not in ice and not below −12 °C. We measured [^{3}H]-leucine incorporation into macromolecules (further fractionated biochemically) by the marine psychrophilic bacterium *Colwellia psychrerythraea* strain 34H over a range of anticipated activity-permissive temperatures, from +13 to −20 °C, including expected negative controls at −80 and −196 °C. For incubation temperatures below −1 °C, the cell suspensions [all in artificial seawater (ASW)] were first quick-frozen in liquid nitrogen. We also examined the effect of added extracellular polymeric substances (EPS) on [^{3}H]-leucine incorporation. Results showed that live cells of strain 34H incorporated substantial amounts of [^{3}H]-leucine into TCA-precipitable material (primarily protein) down to −20 °C. At temperatures from −1 to −20 °C, rates were enhanced by EPS. No activity was detected in the killed controls for strain 34H (or in *Escherichia coli* controls), which included TCA-killed, heat-killed, and sodium azide- and chloramphenicol-treated samples. Surprisingly, evidence for low but significant rates of intracellular incorporation of [3H]-leucine into protein was observed for both ASW-only and EPS-amended (and live only) samples incubated at −80 and −196 °C. Mechanisms that could explain the latter results require further study, but the process of vitrification promoted by rapid freezing and the presence of salts and organic polymers may be relevant. Overall, distinguishing between intracellular and extracellular aspects of bacterial activity appears important to understanding behavior at sub-freezing temperatures.

Keywords: [^{3}H]-leucine; Psychrophiles; Marine bacteria; *Colwellia psychrerythraea*; Vitrification; Exopolymers; Sub-eutectic; Cryopreservation; Saline ice

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The mechanisms enabling bacteria to remain active at very low temperatures are of considerable importance to the fields of polar microbial ecology, astrobiology, cryopreservation, and medical or industrial applications [31]. Evidence for bacterial activity to −15 °C has been obtained in Siberian permafrost samples [15], to −20 °C in Arctic winter sea ice [21], and indirectly to −40 °C in ancient glacial ice [30]. Only a few laboratory studies have reported bacterial activity in pure culture at temperatures below −10 °C; e.g., by re-freezing isolates obtained from glacial ice cores at −15 °C [9] or by suspending permafrost bacteria in brine to −20 °C [2,19]. To date, the activity of obligately psychrophilic marine bacteria (organisms defined by minimal, optimal, and maximal growth temperatures of $\leqslant 0$, $\leqslant 15$, and $\leqslant 20$ °C and a requirement for sea salts) has only been examined at temperatures below −10 °C in culture media kept liquid with glycerol (at −12 °C [4]). Their potential for activity while frozen in ice remains to be explored.

Marine psychrophiles are important inhabitants of sea ice [12], which is an important component of the Earth's cryosphere and global climate system [12,34]. A fundamental question underlying the behavior of bacteria in sea ice or any frozen environment is; how do bacteria manage to persist and remain active when temperatures drop well below freezing? Based on microscopic and molecular analyses of winter sea ice and its microbial inhabitants, attachment to ice or particle surfaces appears to be an important aspect of bacterial activity, especially at temperatures of $\leqslant 5$ °C [21], as is the presence of extracellular polymeric substances (EPS) thought to function as cryo- and osmo-protectants [25]. EPS are known in milder environments to facilitate the attachment process itself [6], a role that could be particularly important in a habitat like sea ice where high salt concentrations tend to reduce the electrostatic forces that typically account for the initial stage of bacterial attachment.

In this work, we investigated the lower temperature range for activity by the obligately psychrophilic and salt-requiring Arctic marine bacterium, *Colwellia psychrerythraea* strain 34H [18]. Specifically, we studied leucine incorporation from 13 to −196 °C in artificial seawater (ASW) both with and without EPS. *C. psychrerythraea* strain 34H is already known for its remarkable adaptations to low temperature: e.g., swimming speeds at −10 °C comparable to mesophilic bacteria at 37 °C [20] and production of unusually cold-active enzymes [18]. A variety of possible specific cold adaptation strategies was recently revealed in this organism by whole genome sequence analysis; of interest here is the encoding of large numbers of proteins, enzymes, and polysaccharides apparently destined for export from the cytoplasm [28]. Given this feature of the organism and the potential roles of organic exopolymers in facilitating microbial persistence in very cold sea ice, we included the presence or absence of added EPS as an experimental factor. Prior to this study, the observed minimal growth temperature for strain 34H in marine media was −5.9 °C, with a generation time of 4.1 days [17].

Materials and methods

We measured bacterial metabolic activity by tracking [^{3}H]-leucine incorporation into protein (a standard method to assess bacterial protein synthesis [24]), motivated by the recent work of Christner [9] studying the activity of psychrotolerant bacteria isolated from freshwater (glacial) ice (along with *Escherichia coli* controls [9]). The methods described by Christner [9] were followed closely. Our approach differed, however, in several important aspects. Washed cultures of strain 34H, were resuspended in artificial seawater (defined below) rather than distilled water in keeping with the marine origin and salt requirements (growth optimum at 3.5% sea salts) of the strain. [^{3}H]-leucine incorporation into protein was distinguished from the residual fraction of total TCA-insoluble material by adding a protein-hydrolysis step to Christner's biochemical fractionation protocol [9]. We also expanded the number and range of test conditions to include eight temperatures between 13 and −196 °C, amendment with EPS, and additional controls (e.g., EPS in cell-free artificial seawater, heat-killed cells, and sodium azide-treated cells). Finally, for incubations below −1 ° C, when initial sample freezing in a −80 °C freezer failed to yield consistently low time-zero values, we switched to more rapid freezing in liquid nitrogen (LN_2; −196 °C) which solved the problem.

Bacterial strain and culture conditions

Colwellia psychrerythraea strain 34H (ATCC No. BAA-681; GenBank Accession No. AF396670), originally isolated from Arctic marine sediments [16] and representing a genus common in sea ice [3], was cultured from frozen stocks stored in glycerol at −80 °C. Cultures were grown at −1 °C without shaking in

half-strength Marine 2216 broth (DIFCO laboratories, Detroit, MI) to $OD_{600\,nm}$ of 0.4 until early stationary growth phase (2 weeks or less, except for biochemical fractionation experiments when the cultures were 3 weeks old). Cells were harvested by centrifugation at 3400*g* for 20 min at 4 °C. After one wash with chilled (−1 °C), 0.2 μm-filtered and autoclaved artificial seawater (ASW; 24 g NaCl, 0.7 g KCl, 5.3 g $MgCl_2$, 7.0 g $MgSO_4 7H_20$, 1.3 g TAPSO [3-[*N*-tris(hydroxymethyl)methylamino]-2-hydroxypropane-sulfonic acid] buffer in 1 liter of distilled water, with pH adjusted to 7.5 using 0.2 N NaOH), cells were resuspended in chilled ASW to $OD_{600\,nm}$ of 0.2. Splits of the cell suspensions were amended with EPS, obtained as described below, to achieve a final concentration of 1.5 μg C ml^{-1}, the median bulk EPS concentration found in winter sea-ice samples [25]. From each prepared cell suspension, subsamples of 100 μl were removed, fixed in 2% formaldehyde, and stored at 4 °C until these time-zero bacterial concentrations were determined by epifluorescence microscopy using the DNA-specific stain 4′,6′-diamidino-2-phenylindole 2HCl (DAPI), as described by Junge et al. [21]. In all cases (with or without EPS), 500-μl aliquots of the live-cell suspensions (and various cell-free and killed-cell controls described below) were placed into 1.5-ml Eppendorf tubes on ice for immediate use in the [3H]-leucine incubation experiments.

EPS preparation

Extracts of extracellular polymeric substances (EPS) were obtained from the spent growth medium of *C. psychrerythraea* strain 34H, essentially as described by Huston et al. [18]. Cultures were grown in half-strength Marine 2216 broth at 2 °C to late-log phase and centrifuged at 1500*g* for 50 min at 2 °C. The supernatant was passed through a GFF filter, amended with 1.5-times volume of chilled 95% ethanol, and incubated overnight at 2 °C. The resulting EPS-rich precipitate was then collected by centrifugation at 11,400*g* for 20 min, repeating the ethanol wash and centrifugation steps twice. To remove low molecular weight compounds (that might serve as readily utilizable substrates), the final pellet was dissolved in distilled water and dialyzed for 48 h at 2 °C using Spectra Por dialysis tubing (2000–3500 Da cutoff). The resulting dialyzate was stored either at −20 °C or at −80 °C until use. Its EPS content was quantified using the colorimetric phenol–sulfuric acid method (6).

[^{3}H]-leucine incorporation assay

To determine rates of [^{3}H]-leucine incorporation in the prepared cell suspensions, time-course experiments were conducted at known growth-permissive temperatures of 13, 4, −1 °C (sampling at 0, 0.5, 1, 2, and 20 h) and at much lower temperatures of −10, −15, −20, including anticipated negative controls at −80 and −196 °C (sampling at 0, 0.5, 1, 2, 3, 4, 5, 10, and 113 days). Triplicate samples for each time point received 100 μl of a chilled working solution of [^{3}H]-leucine (10 μCi ml^{-1}; diluted 1:100 from stock of [^{3}H]-leucine [ICN Biomedicals, Catalog No. 20036E; 40–60 Ci ml^{-1} in sterile 2:98 ethanol–water mixture]). For routine negative (killed) controls, 100 μl of 50% chilled trichloroacetic acid (TCA) were added to each of triplicate samples before tracer addition. Cell-free controls (ASW only and ASW with EPS) were also included.

For experiments at 13, 4 or −1 °C, samples were immediately transferred to an incubator unit (cold room or freezer unit) held at the desired temperature (±1 °C). For experiments at −10 °C or lower (and in one experiment at 4 °C for comparative purposes), samples were first rapidly frozen, within seconds of adding the [^{3}H]-leucine, by placing the 1.5-ml Eppendorf tubes directly into LN_2 (−196 °C) in an insulated flask. A 2-mm thermistor probe (YSI Precision Temperature Group Inc.) used to measure temperature evolution in ASW sample tubes showed that when tubes were immersed in LN_2 prior to incubation the sample temperature dropped to below −80 °C within 10 s. Adding the [^{3}H]-leucine tracer to the sample tubes before immersion in LN_2 amounted to an additional 10–20 s before the sample reached −80 °C. This LN_2 quick-freezing step was necessary to avoid significant exposure of cells to tracer at temperatures above that desired for incubation. After at least 5 min (and at most 30 min) in LN_2, tubes were transferred directly to various freezer units held at the desired incubation temperatures of −10, −15, −20 or −80 °C (±2 °C). For incubations at −196 °C, samples were left in the insulated flask, replenishing the LN_2 as needed.

At designated time points during an incubation period, triplicate samples were removed from their incubators for immediate addition of 100 μl of chilled (−1 °C) 50% TCA. Samples still frozen after adding the TCA were thawed as quickly as possible by continuous mixing at room temperature (at most for 10 min; with sample temperatures remaining below 4 °C, as verified by thermistor probe). All

samples were placed on ice for a total cold TCA-precipitation period of 30 min. The TCA-insoluble material was pelleted by centrifugation at 18,000*g* for 15 min, resuspended in 500 μl of chilled 50% TCA, and centrifuged again at 18,000*g* for 10 min. The washed pellet was then resuspended in 500 μl chilled 70% ethanol and centrifuged at 18,000*g* for 10 min. The final pellet was either resuspended in 500 μl of ASW and transferred to a vial containing 4 ml of Biofluor scintillation cocktail (Perkin-Elmer) or else 1000 μl of Biofluor cocktail was added directly to the pellet as described by Christner [9]. Radioactivity (dpm) in the samples was quantified by liquid scintillation counting, using a Tri-Carb 2900TR liquid scintillation analyzer (Packard Bioscience Company).

When the triplicate samples containing live cells incubated at −80 and at −196 °C yielded mean dpm values after 1 day that were significantly higher than those for triplicate time-zero and TCA-killed controls, we conducted additional shorter-term experiments using the same approach described above but with samples removed for analysis at 0, 1, 2, 4, 8, and 24 h. To minimize variability in the data, since low if not near-blank levels of radioactivity were expected in these samples, the final pellets (in triplicate) of ethanol-washed TCA-insoluble material were not resuspended in ASW and transferred to new vials. Instead, 1 ml of Biofluor scintillation cocktail was added directly to each of the Eppendorf tubes, which were then placed in separate scintillation vials and assayed for radioactivity as described above.

Biochemical fractionation

To determine if radioactivity added in the form of [^{3}H]-leucine was incorporated into protein, biochemical fractionation was performed on the total TCA-precipitated material obtained from samples incubated at −10, −15, −20, −80 (for 151–178 days), and −196 °C (for 20 days), essentially as described by Christner ([9]; adopted from [23]). Fractionation experiments were performed at the end of our incubation periods following Christner's longer incubation time schedule and thus to accommodate a comparison with his results. After thawing, each of the triplicate samples was split into two aliquots (300 μl each) and processed to obtain TCA-insoluble material as described above. One set of pellets was assayed for total radioactivity, while the second underwent biochemical fractionation. The latter pellets were resuspended in 30 μl of a 1:1 ethanol–ether mixture, incubated at 37 °C for 30 min, and centrifuged at 18,000*g* for 10 min. The supernatant (ethanol–ether soluble = lipids) from each tube was assayed for radioactivity. The remaining pellets were resuspended in 30 μl 5% TCA, incubated at 95 °C for 30 min, and centrifuged at 18,000*g* for 10 min. The supernatant (hot TCA soluble = nucleic acids) from each of these tubes was also assayed for radioactivity. Protein remaining in the pellets was hydrolyzed in 30 μl of 6 N HCl [23] by incubation at 105 °C for 17 h. After centrifugation at 18,000*g* for 10 min, the supernatant (= proteins) was assayed for radioactivity. The final pellet (= residual) in each tube was resuspended in 1 ml of scintillation liquid and analyzed directly for radioactivity as described above.

Additional controls

To determine if the radioactive tracer might bind abiotically to EPS, control samples (in triplicate for each time point) of cell-free ASW amended with EPS (same concentration used for live-cell incubations) were included. To further examine possible abiotic mechanisms for [^{3}H]-leucine incorporation in killed-cell suspensions, particularly at the extreme temperatures tested, samples of cell suspensions pretreated with heat, sodium azide or chloramphenicol were added to the routine TCA-killed controls. Heat-killed controls where the pH/chemical environment would better resemble that in live samples were prepared to account for the possibility that the use of strong acid (TCA) might have altered the adsorption of tracer. For heat-killed controls, live-cell suspensions were prepared as described above, but first incubated at 46 °C for 1 h prior to tracer addition. For sodium azide and chloramphenicol treatment, 100 μl of 1.2 g l^{-1} sodium azide solution (final concentration of 0.2 g l^{-1}) or 0.15 g l^{-1} chloramphenicol solution (final concentration of 25 μg ml^{-1}) were added to triplicate samples of live-cell suspensions prepared as described above and incubated for 10 min on ice before tracer addition. After tracer addition, quick-freezing (if destined for incubation at −10 °C or lower) and subsequent incubation, all of these control samples were processed for total TCA-insoluble material as described above.

Rate calculations

Incorporation rates of radioactivity (dpm) were calculated from the linear portions of the time-

course curves and scaled to bacterial number in order to cross-compare experiments involving different starting concentrations of bacteria. Linearity was assumed in the cases where saturation was reached before the first time point for the experiment—typically 12 h. In order to cross-compare with literature values of bacterial metabolic rates, rates were converted from nmol [^{3}H]-leucine incorporated per unit time to grams of leucine carbon incorporated per grams of bacterial carbon per hour, assuming 65 fg C bacterium^{-1} (as in [30]).

Results

Bacterial counts and conditions

Even though the $OD_{600\,nm}$ of all cell suspensions had been adjusted to the same value (0.2) at the start of each experiment, direct counts revealed a range of concentrations from 1.2×10^7 to 1.8×10^8 bacteria ml^{-1}. The higher cell concentrations were observed in the live-cell suspensions prepared early in the study for longer-term incubation experiments conducted across the full spectrum of temperatures examined. The lowest concentrations were observed in later cell suspensions used for short-term incubation experiments at −80 and −196 °C, where a tendency for cell clumping was occasionally noted. Cell clumping was pronounced in EPS-amended live-cell samples before they were subjected to chloramphenicol and sodium azide treatments.

[^{3}H]-leucine incorporation into TCA-insoluble material

At all temperatures examined, live-cell suspensions of *C. psychrerythraea* strain 34H showed significant incorporation of [^{3}H]-leucine into TCA-insoluble material over time. For experiments at growth-permissive temperatures of 13, 4, and −1 °C, radioactive counts in TCA-killed controls were always several orders of magnitude lower than those in live-cell suspensions (e.g., Fig. 1A for −1 °C). For experiments with frozen samples (incubation temperatures $\leqslant -10$ °C, e.g., Fig. 1B for −20 °C), radioactive counts in the TCA-killed controls were also significantly lower than corresponding counts in live samples. In particular, at time points for calculating incorporation at saturation, the median of the averages for triplicate live samples was 11,800 dpm (range: 2960–82,200 dpm; $n = 14$), compared to a median of 300 dpm (range: 164–2040 dpm; $n = 14$) for TCA-killed controls. The cell-specific incorporation rates (dpm bacterium^{-1} day^{-1}) were highest at temperatures of 13, 4, and −1 °C, whether or not EPS had been added (Fig. 2). At non-optimal growth temperatures of 13 °C (higher than optimal), −1 and −20 °C (sub-optimal), the addition of EPS resulted in significantly higher rates than those measured in ASW alone (Figs. 1 and 2). At temperatures of −80 and −196 °C, no consistent relationship between EPS and leucine incorporation was found, since mean rates in EPS-amended samples were higher (−80 °C) or indistinguishable (−196 °C) in one experiment (Fig. 2) and lower in another (compare solid diamonds [EPS added] with crosses [no EPS] in Fig. 4). Closer study (in short-term incubations) of EPS-amended live-cell suspensions at −80 and −196 °C revealed classic enzyme-mediated uptake curves, with steadily increasing incorporation of radioactivity into TCA-insoluble material after 1 h of incubation and saturation after 8 h (Fig. 3).

Cell-specific [^{3}H]-leucine incorporation rates are depicted in Fig. 4. Overall, the rates in live-cell suspensions were several orders of magnitude higher at growth-permissive temperatures than in ice at the much lower temperatures tested, with a maximum of 5.3×10^{-5} g leucine C [g bacterial C]$^{-1}$ [h]$^{-1}$ at 4 °C and minimum of 2.03×10^{-8} g leucine C [g bacterial C]$^{-1}$ [h]$^{-1}$ at −196 °C (for EPS-amended samples, Fig. 4). Temperature dependence appeared to be non-linear (Figs. 2 and 4); however, cross-comparing rates strictly as a function of temperature should be avoided, since samples at the warmer temperatures were incubated in liquid media and not first quick-frozen like the others. [^{3}H]-leucine incorporation experiments done at 4 °C both with and without quick-freezing showed that when live-cell suspensions destined for incubation at 4 °C were first quick-frozen in LN_2, the resulting [^{3}H]-leucine incorporation rate was >100 times slower than the rate obtained when samples were incubated at 4 °C without the quick-freezing step (compare the open square to solid square in Fig. 4). Either the formation of ice or the quick-freezing process itself (inherent to sample incubations at $\leqslant -10$ °C) influenced the ability of the cells to be active thereafter. If quick-freezing was the problem, then the rates determined at $\leqslant -10$ °C may have been underestimates. Furthermore, starting cell cultures differed in concentration and other features (e.g., cell size, age, and clumpiness) in spite of efforts to standardize them. The cellular production of EPS is known to change over cultivation time for strain 34H [17] leaving

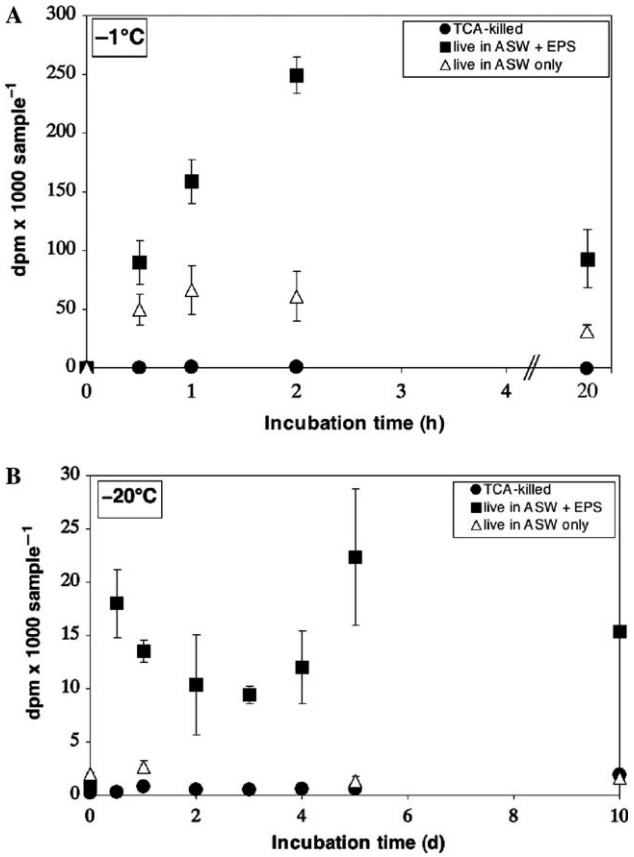

Fig. 1. Incorporation of radioactive [^{3}H]-leucine (measured as dpm per sample) into TCA-insoluble material at −1 (A) and −20 °C (B) in live-cell suspensions of strain 34H in artificial seawater (ASW), with EPS amendment (squares) and without it (triangles), and in TCA-killed controls (circles). Error bars indicate SD of triplicate samples. Note the break in scale on the *x*-axis in (A).

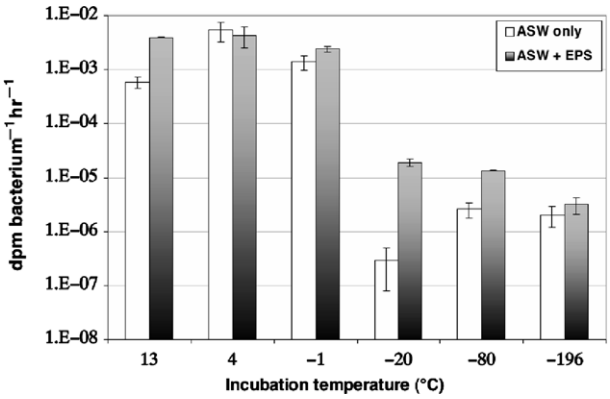

Fig. 2. Incorporation rates of radioactive [^{3}H]-leucine (calculated as dpm incorporated per bacterium per hour) into TCA-insoluble material across the range of test temperatures (from 13 to −196 °C) in live-cell suspensions of strain 34H prepared in artificial seawater only (light bars) or with EPS amendment (dark bars). Rates were scaled to bacterium based on direct counts of starting cell suspensions. For −10 and −15 °C, only rates for EPS-amended samples are available; all samples incubated at ⩽−10 °C were first quick-frozen in LN_2. Error bars indicate SD of triplicate samples.

some (younger) sample preparations containing more EPS (as cell coats) than others. How differences in cellular EPS production and composition may influence the vitrification process (or ice recrystallization process during warming), cell clumpiness or bacterial activity remains to be explored.

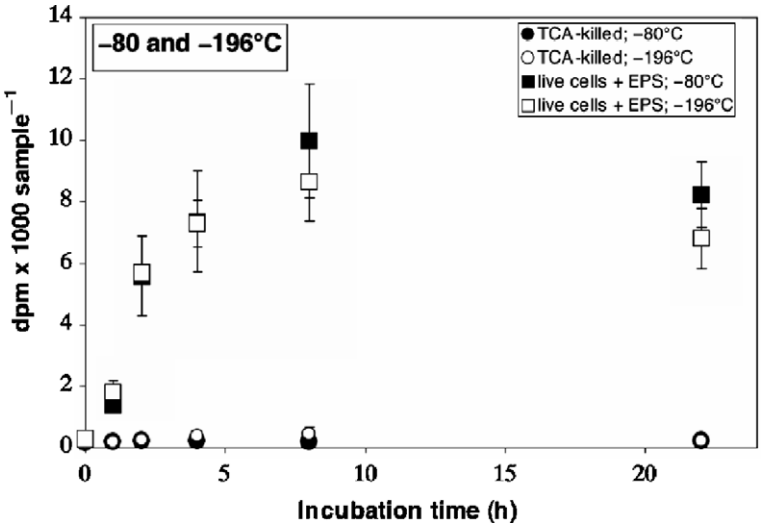

Fig. 3. Incorporation of radioactive [^{3}H]-leucine (measured as dpm per sample) into TCA-insoluble material at −80 °C (solid symbols) and −196 °C (open symbols) in live samples of strain 34H suspended in ASW amended with EPS (squares) and in TCA-killed controls (circles). Error bars indicate SD of triplicate samples.

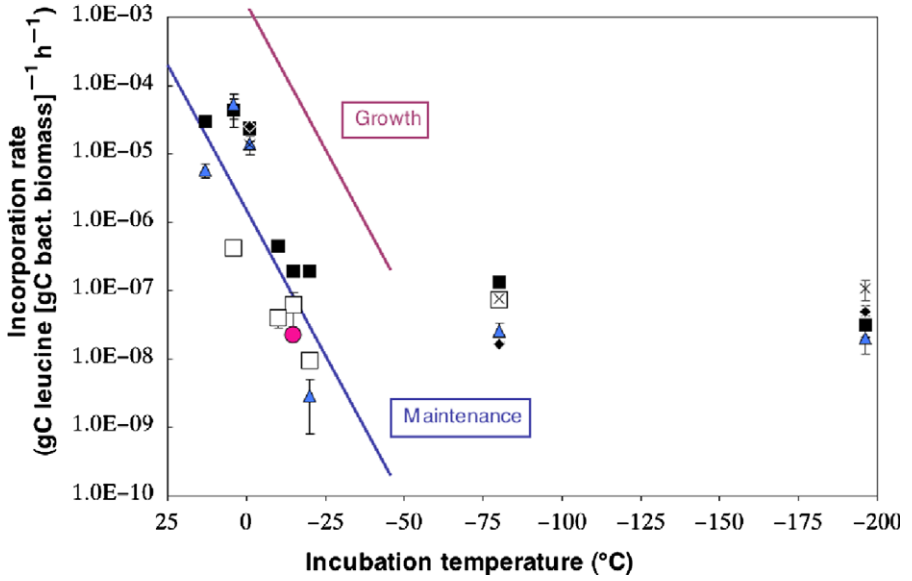

Fig. 4. Incorporation rates of radioactive [^{3}H]-leucine (calculated as gram leucine C per gram bacterial C per hour) into TCA-insoluble material across the range of test temperatures (from 13 to −196 °C) in live-cell suspensions of strain 34H prepared in ASW only (triangles and crosses indicate two different experiments) and with EPS amendment (solid squares, empty squares, and diamonds indicate three different experiments). The circle indicates the rate for psychrotolerant bacterium *Psychrobacter* sp. in distilled water, reported by Christner [9]. The descending lines are theoretical extrapolations of bacterial activity indicative of growth or maintenance metabolism, as reproduced from data points obtained by visual inspection of Fig. 1 in Price and Sowers [30]. Error bars indicate SD of triplicate samples.

Biochemical fractionation

At all temperatures where samples underwent biochemical fractionation (from −10 to −196 °C), the majority of the recovered radioactive fraction was incorporated into the hot HCl extract containing hydrolyzed proteins (e.g., 87% at −10 °C, 60% at −196 °C, Fig. 5A; Table 1). In contrast, the results of fractionating TCA-insoluble material obtained from TCA-killed controls showed that most of the recoverable radioactive material, which was overall an order of magnitude less than for live-cell suspensions

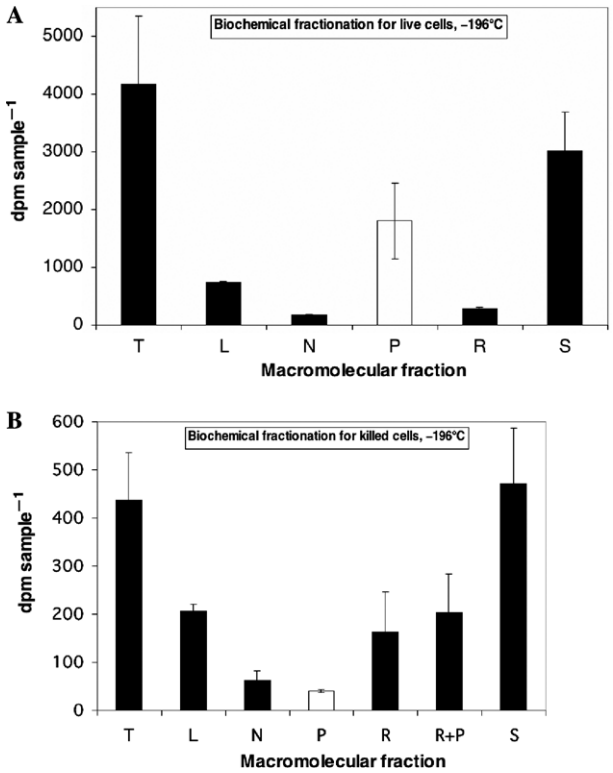

Fig. 5. Biochemical fractionation of TCA-insoluble material from EPS-amended live (A) and TCA-killed (B) samples that had been incubated at −196 °C in the presence of [^{3}H]-leucine for 20 days. Radioactivity (dpm per sample) was recovered from parallel samples of unfractionated TCA-insoluble material (T), from fractions containing lipids (L), nucleic acids (N), proteins (P), residual unknowns (R), and from the sum of the recoverable radioactivity in all fractions (S). Error bars indicate SD of triplicate samples.

at −196 °C, resided in the ether–ethanol extract (lipids) and in the residual fraction (presumably a mixture of macromolecules not extracted in previous steps; Fig. 5B).

Controls

In time-course experiments at −80 and −196 °C, radioactive counts in the live time-zero samples were always significantly lower than radioactive counts in the live samples at saturation, with a median of 916 dpm (range: 293–2010 dpm; $n = 7$) compared to the median of live samples at saturation of 8770 dpm (range: 6530–18,000 dpm; $n = 7$). The median for TCA-killed controls was lower than for the live time-zero samples (226 dpm, range: 164–462 dpm; $n = 7$). Based on results obtained from live-cell suspensions incubated at −1 °C, a maximum of 71 ± 18 dpm ($n = 3$) could have been incorporated per second before samples reached −80 °C.

In additional control samples at −80 °C, including EPS alone (no bacteria) and EPS added to heat-killed cells, no significant incorporation (above dpm values in TCA-killed controls) was observed (Table 2). At −196 °C (and −1 °C), cells of strain 34H treated with the respiratory-oxidase inhibitor sodium azide did not show significant incorporation of [^{3}H]-leucine compared to live-cell suspensions. Treating with the protein-elongation inhibiting chloramphenicol resulted in a marked reduction of [^{3}H]-leucine incorporation to ~20% of that observed for untreated cells (Fig. 6; the same behavior was observed at −1 °C, data not shown). Cells of *E. coli*, whether suspended in distilled water [9] or in ASW with EPS, did not show significant incorporation at −80 or −196 °C.

Discussion

We determined first that [^{3}H]-leucine is an effective tracer of metabolic activity for *C. psychrerythraea* strain 34H in a sea-salt solution, predictably observing highest incorporation rates at the growth-permissive temperatures of 13, 4, and −1 °C (the organism's optimal growth temperature is 8 °C [16]). That the organism then also incorporated this

Table 1
Fractionation of TCA-insoluble material from samples incubated at subzero temperatures in the presence of [^{3}H]-leucine for 5–6 months[a]

Incubation temperature (°C)	Incubation time (days)	Macromolecular fraction	[^{3}H]-leucine incorporation (dpm)
−10	151	Total TCA-insoluble material	39,200 ± 9440
		Ethanol–ether soluble (lipid)	2320 ± 396
		Hot 5% TCA soluble (nucleic acid)	1340 ± 460
		Hot 6 N HCl soluble (protein)	25,000 ± 2770
		Residual	No data
−15	174	Total TCA-insoluble material	29,200 ± 2440
		Ethanol–ether soluble (lipid)	738 ± 148
		Hot 5% TCA soluble (nucleic acid)	303 ± 108
		Hot 6 N HCl soluble (protein)	15,400 ± 5840
		Residual	4200 ± 1020
−20	177	Total TCA-insoluble material	54,600 ± 4,830
		Ethanol–ether soluble (lipid)	1,030 ± 140
		Hot 5% TCA soluble (nucleic acid)	806 ± 646
		Hot 6 N HCl soluble (protein)	31,600 ± 18,900
		Residual	6820 ± 2860
−80	178	Total TCA-insoluble material	43,400 ± 3,690
		Ethanol–ether soluble (lipid)	1020 ± 117
		Hot 5% TCA soluble (nucleic acid)	1160 ± 388
		Hot 6 N HCl soluble (protein)	20,400 ± 6350
		Residual	7670 ± 4780

[a] Values are means ± standard deviation ($n = 3$).

Table 2
Incorporation of [^{3}H]-leucine into TCA-insoluble material for samples incubated at −80 °C[a]

Incubation temperature (°C)	Incubation time (days)	Experimental parameter	[^{3}H]-leucine incorporation (dpm)
−80	150	Untreated cells	43,400 ± 3690
		5% TCA-killed cells	387 ± 146
		Heat-killed cells	877 ± 238
−80	50	Untreated cells	14,400 ± 3910
		5% TCA-killed cells	538 ± 257
		No cells	824 ± 18
		5% TCA, no cells	977 ± 35

[a] Values are means ± standard deviation ($n = 3$).

amino acid into macromolecules at colder temperatures of −10, −15, and −20 °C adds to the evidence of its remarkable cold adaptation and pushes the current record of −15 °C for bacterial activity in pure culture in artificial ice [9] five degrees lower. These results also support our earlier findings of bacterial activity at −2 to −20 °C in natural winter sea-ice samples [21] and, tangentially, other studies of bacterial activity in non-saline ice formations (summarized by Price and Sowers in [30]).

Our results indicating EPS-enhanced activity of *C. psychrerythraea* strain 34H at 'moderate' temperatures non-optimal for growth (at 13, −1, and −20 °C) suggest that EPS production as a potential stress response may be worth pursuing. At the nanoscale, EPS could trap water molecules in the liquid phase at the cell surface [26], facilitating transport processes for higher activity. At −1 °C, EPS are known to extend the lifetime and enhance activity levels of an aminopeptidase produced by strain 34H [18]; they may also stabilize other enzymes (e.g., permeases) key to substrate transport into the cell. EPS are also thought to act as freezing-point depressants, working in concert with salts to maintain liquid pockets around cells to −20 °C [25]. Especially noteworthy is that our lowest (mean) incorporation rate, in the temperature range of 13 to −20 °C, was for samples incubated at −20 °C without added EPS (Fig. 1B). The significantly higher rate with EPS at this temperature indicates the importance of extracellular polymers under conditions similar to winter sea-ice brines, known to contain high levels of EPS

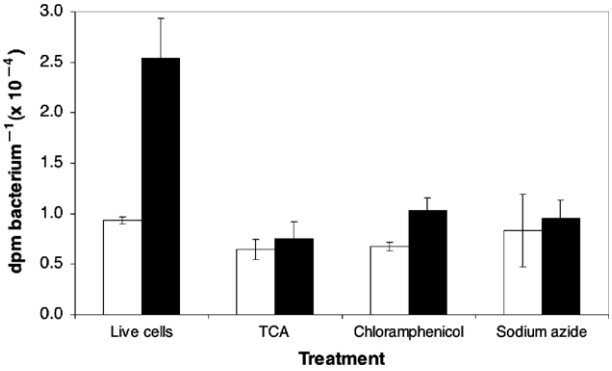

Fig. 6. Incorporation of radioactive [^{3}H]-leucine (calculated as dpm per bacterium) into TCA-insoluble material at time zero (light bars) and after 24 h (dark bars) at −196 °C in live-cell suspensions of strain 34H prepared in ASW amended with EPS and in TCA-killed and chloramphenicol- and sodium azide-treated controls. Dpm were scaled to bacterium based on direct counts of starting cell suspensions. Error bars indicate SD of triplicate samples.

[25]. Furthermore, cells quick-frozen but unprotected by added polymers might have experienced damage during the re-crystallization process that would have occurred during sample warming from −196 °C. How this process and related cell damage may be affected by EPS and salts remains to be explored.

When our measured leucine-incorporation rates by strain 34H are considered in the same terms as those calculated from the literature for other bacteria (in culture or in natural samples) by Price and Sowers [30] at temperatures $\geqslant -20$ °C, the rates agree well with those attributed to maintenance metabolism (Fig. 4). Below −20 °C, Price and Sowers [30] present arguments for bacterial activity becoming exceedingly slow (non-detectable empirically) and restricted to survival functions (DNA repair). Note that on our summary graph (Fig. 4), their linear extrapolation of maintenance activity drops off-axis at about −50 °C. Below the eutectic no pure liquid water should remain in the system beyond quasi-liquid molecular layers of sub-nanometer thickness [37] and possibly small fractions of metastable liquid at supercoolings of less than 25 °C [8]. At our sub-eutectic test temperatures of −80 and −196 °C, however, we measured leucine-incorporation rates in the realm of maintenance activity.

To explain activity detected in the effective absence of liquid water, we first considered whether our findings might be due to procedural artifacts. For example, could the activity have occurred during sample handling, freezing and post-incubation thawing rather than during the sub-eutectic incubation period? Live cells did experience brief exposure to the tracer at 0 °C during the seconds required to add tracer, seal the tube and place it into LN_2; once in LN_2, sample temperature dropped rapidly ($\leqslant$10 s) to −196 °C. Also, when a sample was flooded with TCA to end incubation, some live cells in the ice may not have come into contact with the killing agent instantaneously. That the median radioactivity count for live time-zero samples was higher than for TCA-killed controls does indicate that a small amount of tracer was incorporated in the live samples during these brief exposures to higher temperatures. A 10-s handling period prior to quick-freezing can account for the observed difference, based on the results from live-cell incubations at −1 °C. To account for the higher dpm values from the live samples at saturation point, however, would require holding samples at −1 °C for >90 s (for the minimum live-saturation value of 6530 dpm) or for >4 min (for the maximum of 18,000 dpm) prior to freezing, which never happened. Regardless of how they compared to killed controls, the live time-zero samples accounted for any tracer incorporation that occurred during the handling process. Radioactivity counts in these samples were lower than counts for replicate live samples incubated over time at −80 or −196 °C and always significantly lower than counts at saturation. Instead of random fluctuations around live time-zero values, as might be expected for handling artifacts, the data clearly indicate marked increases in tracer incorporation followed by saturation, mimicking enzyme-mediated uptake kinetics.

We also considered possible abiotic mechanisms for the observed increase of radioactive label at −80 and −196 °C, including physical accumulation of the tracer

between ice crystals and/or freeze-condensation of the label [35]. No significant levels of radioactivity were detected in samples devoid of bacteria (with or without added EPS), indicating that non-specific binding of the tracer to itself or to EPS cannot account for the observed results (Table 2). The possibility that our TCA-killed controls might have been artificially low due to harsh chemistry also fails to explain the live-cell results, since heat-killed controls yielded consistently low counts relative to live samples (Table 2). Treatment with sodium azide, known to inhibit oxidative enzymes involved in the energy (ATP)-generating mechanisms of bacteria, also yielded insignificant leucine incorporation, suggesting that electron transfers are required for the observed process (Fig. 6). At least partially functional machinery for protein synthesis was implied by results of fractionation experiments, showing leucine incorporation mostly into proteins (60% at −196 °C, corresponding to 3.3 leucine molecules bacterium^{-1} h^{-1}, compared to 23,000 molecules bacterium^{-1} h^{-1} at 4 °C; Fig. 5) and by significantly lower rates in samples treated with chloramphenicol (Fig. 6), the bacteriostatic agent known to inhibit protein elongation on the 50S ribosomal subunit at the peptidyl transferase step.

The observed kinetics of leucine incorporation into protein during incubation at sub-eutectic temperatures (Fig. 3) thus cannot be attributed to spurious activity during sample handling or abiotic mechanisms recognizable in comparative experiments. An intracellular mechanism that can proceed at sub-eutectic temperatures is required. The process of vitrification (transition of liquid water to a glassy rather than crystalline state [29]), which occurs during rapid freezing of aqueous solutions to sub-eutectic temperatures, helps to explain our observations. Vitrification is fostered by high concentrations of organic polymers and ions [1] and enhances cryopreservation of uni- and multicellular organisms [13], including detailed preservation of cell structure and chemistry at the nanoscale [26]. For a liquid suspension of EPS-coated bacteria that also contains added EPS and salts, as in this study, the vitrification process can be expected to preserve original spatial gradients of various components inside and outside the cell ([26,14]; *see* Vajda [35] for other possibilities).

We hypothesize that the leucine tracer entered the bacterial cells not at the sub-eutectic incubation temperature but during the first seconds of the freezing process, when liquid water was still present to facilitate cross-membrane transport and the leucine concentration gradient would have favored entry. (Some leucine incorporation also occurred in these seconds, but time-zero controls fully accounted for it, as discussed above.) EPS produced by the test strain (whether added or already present as a natural cell coating) would have facilitated enzyme-mediated leucine transport and enhanced vitrification [27]. As temperature dropped below the eutectic, diffusion-based processes would no longer be influential. Although leucine diffusivity at −80 °C would be orders of magnitude higher than at −196 °C, the drop in diffusivity is sharp below −120 °C [33]; the similarity in measured incorporation rates at −80 and −196 °C argues against a diffusion-limited incorporation process. Instead, only the leucine molecules that had reached enzyme–ribosome–energy complexes in the first seconds of freezing would have been incorporated into protein, according to peptidyl-transfer activity within the ribosome reaction center that proceeds via subtle conformational changes, reactions and electron transfers [5,32] still possible in the glassy state [37]. In fact, only a small number of leucine molecules were incorporated at sub-eutectic temperatures (e.g., ∼2 molecules bacterium^{-1} h^{-1} at −196 °C for a total of ∼400 molecules incorporated after 24 h). Given the typical ribosome content of cultured marine bacteria (many thousands per cell [22]) and average incorporation frequency for leucine (one in six amino acids incorporated [24]), only a small fraction of the ribosomes needed to be active to account for the observed incorporation rates. This hypothetical scenario for sub-eutectic activity defines new territory for further study.

Others have reported that a freezer temperature of −70 or −80 °C is sufficient to achieve negative controls for [^{3}H]-leucine incorporation experiments: neither bacterial isolates suspended in distilled water prior to freezing [9] nor bacterial communities in Antarctic snow [7] incorporated significant amounts of tracer at these temperatures. In the absence of salt or EPS, however, and with the slower freezing rate inherent to placing a sample in a freezer (as opposed to quick-freezing in LN_2), vitrification may not have occurred, leaving more cells vulnerable to ice-crystal damage. In the case of Christner's pure-culture experiments [9], activity during long-term incubation at −70 °C might have been masked by the higher background levels of dpm he reported. Alternatively, *C. psychrerythraea* strain 34H and the type of salt-requiring, EPS-producing cold-adapted organisms it represents may express features that allow for low levels of intracellular activity at such extreme

temperatures. The fact that live cells of *E. coli* did not incorporate tracer at sub-eutectic temperature points to a potentially unique ability of the marine psychrophile tested here.

While freezing to −80 or −196 °C is commonly used to preserve cells [13], the prospects of activity below the eutectic have rarely been considered or documented [36]. Our observations are consistent, however, with indications of (cell-free) enzyme activity below their transition temperatures of −23 to −123 °C [10,11]. They also emphasize that bacterial activity in deeply frozen ice may depend not only on the specific activity and organism considered but also on the distribution of ice, liquid, and glassy phases, as well as organic compounds, in the cell and the surrounding matrix.

The evidence we have presented for incorporation of leucine into proteins at sub-eutectic temperatures involved a multi-phase system in time and space, where liquid was initially available for substrate transport (cell entry) prior to reaching the deeply frozen state. The results may be relevant to organisms in icy habitats that experience periodic phase changes from liquid to solid states of water. Shifts from growth-permissive conditions to a deeply frozen state, and vice versa, occur on Earth in permafrost, surface ice, and the atmosphere, where temperatures fall below −50 °C, and elsewhere in the solar system where known sub-eutectic temperatures at a planetary surface [12] may be coupled to a deeper source of heat.

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