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Bioenergetic Response of the Extreme Thermoacidophile *Metallosphaera sedula* to Thermal and Nutritional Stresses

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The bioenergetic response of the extremely thermoacidophilic archaeon *Metallosphaera sedula* to thermal and nutritional stresses was examined. Continuous cultures (pH 2.0, 70°C, and dilution rate of 0.05 h^{-1}) in which the levels of Casamino Acids and ferrous iron in growth media were reduced by a step change of 25 to 50% resulted in higher levels of several proteins, including a 62-kDa protein immunologically related to the molecular chaperone designated thermophilic factor 55 in *Sulfolobus shibatae* (J. D. Trent, J. Osipiuk, and T. Pinkau, J. Bacteriol. 172:1478–1484, 1990), on sodium dodecyl sulfate-polyacrylamide gels. The 62-kDa protein was also noted at elevated levels in cells that had been shifted from 70 to either 80 or 85°C. The proton motive force (Δp), transmembrane pH (ΔpH), and membrane potential (Δψ) were determined for samples obtained from continuous cultures (pH 2.0, 70°C, and dilution rate of 0.05 h^{-1}) and incubated under nutritionally and/or thermally stressed and unstressed conditions. At 70°C under optimal growth conditions, *M. sedula* was typically found to have a Δp of approximately −190 to −200 mV, the result of an intracellular pH of 5.4 (extracellular pH, 2.0) and a Δψ of +40 to +50 mV (positive inside). After cells had been shifted to either 80 or 85°C, Δψ decreased to nearly 0 mV and internal pH approached 4.0 within 4 h of the shift; respiratory activity, as evidenced by iron speciation in parallel temperature-shifted cultures on iron pyrite, had ceased by this point. If cultures shifted from 70 to 80°C were shifted back to 70°C after 4 h, cells were able to regain pyrite oxidation capacity and internal pH increased to nearly normal levels after 13 h. However, Δψ remained close to 0 mV, possibly the result of enhanced ionic exchange with media upon thermal damage to cell membranes. Further, when *M. sedula* was subjected to an intermediate temperature shift from 73 to 79°C, an increase in pyrite dissolution (ferric iron levels doubled) over that of the unshifted control at 73°C was noted. The improvement in leaching was attributed to the synergistic effect of chemical and biological factors. As such, periodic exposure to higher temperatures, followed by a suitable recovery period, may provide a basis for improving bioleaching rates of acidophilic chemolithotrophs.

Dissimilatory oxidation of metal sulfides (e.g., iron pyrite), dissolved metal cations (e.g., Fe^{2+}), and elemental sulfur by the mesophilic eubacterium *Thiobacillus ferrooxidans* and other acidophiles has had more than ecological significance, as it has been the basis for industrial processes for metal recovery and coal desulfurization (3, 29). Although these bioprocesses generally require less energy and have fewer adverse environmental consequences than physicochemical routes, they can be slower, more difficult to control, and more easily disrupted by variations in processing conditions. Several thermoacidophilic organisms, such as those in the archaeal genera *Sulfolobus* (4) and *Acidianus* (34), have been evaluated for metal leaching and sulfur oxidation processes as competitors of or potential improvements on mesoacidophile-based leaching technologies (1, 10). In the recovery of precious metals or removal of metal sulfides, such as pyrite from coal, expanded utilization of thermoacidophiles requires an appreciation of the underlying mechanisms of function and survival in hot, acidic environments.

Microorganisms that thrive in acidic environments are uniquely characterized by the ability to conserve energy through cellular processes that maintain a high transmembrane pH (ΔpH) gradient (8, 11, 22, 23, 28). Extracytoplasmic oxidation of sulfur and metal sulfides has been linked to the generation of energy through chemiosmotic coupling in chemolithotrophic acidophiles (8). Thus, these bioenergetic modes take advantage of extremely acidic conditions to provide some or all of the cell's energetic requirements. It seems that the margin for error in these environments is small. Only the cell membrane provides physical protection from an environment in which the pH is so low that if the cell were exposed, the pH would rapidly inactivate biomolecules in the cytoplasm. However, this margin of error is relaxed somewhat by an intracellular nondiffusable net positive charge or Donnan potential which gives rise to positive (inside) membrane potential (Δψ) (11, 22, 23, 25); additional protection has been proposed to result from the contribution of proton diffusion potential (22). Positive membrane potential makes acidification of the cytoplasm self-limiting by opposing the movement of protons across the otherwise ion-impermeable membrane (22).

There has been increasing interest in the mechanisms by which organisms respond to thermal stress and the possible technological consequences of this response (24). Acidophilic organisms may be more susceptible to thermal stress than neutrophiles are because of the critical need to maintain the integrity of the plasma membrane to prevent acidification of the cytoplasm. Certainly, stress response proteins have been identified in mesoacidophiles and thermoacidophiles (6, 15, 16, 36). When the extremely thermophilic archaeon *Sulfolobus acidocaldarius* was subjected to a temperature shift from 70 to 85°C, two 64- to 66-kDa proteins which had been major cellu-

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lar proteins at 70°C were produced at increased levels (15, 16). Trent et al. (36) have shown that *Sulfolobus shibatae* possesses acquired thermotolerance when it becomes acclimated to sublethal temperatures for sufficient periods prior to exposure to even higher temperatures; a prominent heat shock protein (thermophilic factor 55) was identified, shown to be related to a chaperone component in the eukaryotic cytosol, and proposed to be representative of a new class of molecular chaperones (35). Given the potential of thermoacidophiles to encounter various types of stress that arise from their use in oxidation of metal sulfides in ores and inorganic sulfur in coal, the stress responses of these organisms merit additional study.

To investigate further the response of thermoacidophilic microorganisms to stresses, the archaeon *Metallosphaera sedula* was examined. This thermoacidophile, isolated by Huber et al. (14) from a solfataric field in Italy, grows optimally at 70 to 75°C and over a pH range of 1 to 4.5. Clark et al. (7) and Peeples and Kelly (31) have shown that *M. sedula* oxidizes both coal and mineral pyrite at rates that are higher than those observed for several other mesoacidophilic and thermoacidophilic species, warranting its closer examination as a metal- and sulfur-leaching biocatalyst. Here, the objective was to study the ability of *M. sedula* to work against membrane destabilization brought about by thermal stress. Insights into the mechanisms by which thermoacidophiles survive in and exploit extreme conditions for bioenergetic benefit are important in improving the prospects for their application in a variety of bioprocesses, in addition to gaining insight into the basis for metabolic function in extreme environments.

MATERIALS AND METHODS

Cell cultivation. *M. sedula* DSM 5348 was obtained from the Deutsche Sammlung von Microorganismen (Braunschweig, Germany). Cells were cultivated in a basal medium that contained 0.4 g of K_2HPO_4 per liter, 0.4 g of NH_4SO_4 per liter, and 0.4 g of $MgSO_4 \cdot 6H_2O$ (Fisher Scientific, Raleigh, N.C.) per liter. Cell samples (1 ml) were fixed with glutaraldehyde, diluted appropriately, and stained with acridine orange for enumeration by epifluorescence microscopy (37). Typically, cultures were inoculated to a starting density of 10^5 cells per ml. Batch cultivation and small-scale continuous cultivation of cells were performed as described previously (31). A 10-liter dual-limited chemostat (0.5 g of $FeSO_4 \cdot 7H_2O$ per liter, 0.2 g of Casamino Acids [Sigma, St. Louis, Mo.] per liter) was used to generate biomass for bioenergetic studies. Cells to be used for bioenergetic measurements and leaching studies were harvested and centrifuged at 10,000 × *g* for 30 min. Cell pellets were washed in 10 mM H_2SO_4 (pH 2.0) and resuspended in 5 to 10 ml of desired buffer at a concentration of 10^9 to 10^{10} cells per ml.

(i) Batch cultures. For small cultures (50 ml), autoclaved basal medium was added to serum vials or shake flasks and supplemented with either $FeSO_4 \cdot 7H_2O$ (0.5 g) or standard reference pyrite (NIST 8455; National Institute for Standards and Technology, Gaithersburg, Md.) (1 to 2 g). Vials for heterotrophic and mixotrophic growth studies were also supplemented with 200 μl of 5% acid-hydrolyzed casein (A-2427; Sigma). Vials were incubated at 75°C and shaken at 100 rpm. Larger batch cultures were grown in a 3-liter round-bottom flask (2-liter working volume) with a heating mantle and air sparge. Sterile basal medium, supplemented with appropriate organic substrates, was autoclaved and then added to the flask with an inorganic substrate. Then the flask was heated to culture temperature and aerated before inoculation. Inocula were obtained from small shake flask cultures.

(ii) Continuous culture. Figure 1 is a schematic of our 10-liter continuous culture apparatus, modified from that of Brown and Kelly (5). Basal salts with Casamino Acids (pH 2.0) were autoclaved and pumped into the reactor. The inorganic feed was filter sterilized through a 0.2-μm-pore-size filter to avoid contamination of the culture. The culture was agitated at 200 rpm with an impeller and aerated by sparging with air filtered through a 0.2-μm-pore-size filter at a rate of 60 cm^3/min. The system was inoculated and run in batch mode for 3 to 4 days prior to the initiation of continuous operation. Continuous culture was initiated by pumping fresh medium that contained salts and organic substrates (pH 2.0) and a slurry of inorganic substrate (pH 2.0) into the round-bottom flask that contained cells at mid-log phase (0.5×10^8 to 1.0×10^8 cells per ml). The dilution rate for most experiments was 0.05 h^{-1} (corresponding to a 14-h doubling time). Cells and spent media were collected in sterile polypropylene catch vessels. Cell products (4 to 8 liters) were stored at 4°C (up to 12 h) for later analysis or processed immediately for bioenergetic studies. Samples were taken regularly to check for cell density, which typically varied from 1×10^8 to 2×10^8 cells per ml. Significant buildup of iron oxides on the reactor walls was observed after extended periods of operation, so the system had to be taken down and acid cleaned after 3 to 4 months of operation. A smaller-volume continuous culture (2-liter working volume) was also run in parallel to maintain the culture and serve as an inoculum for the larger culture.

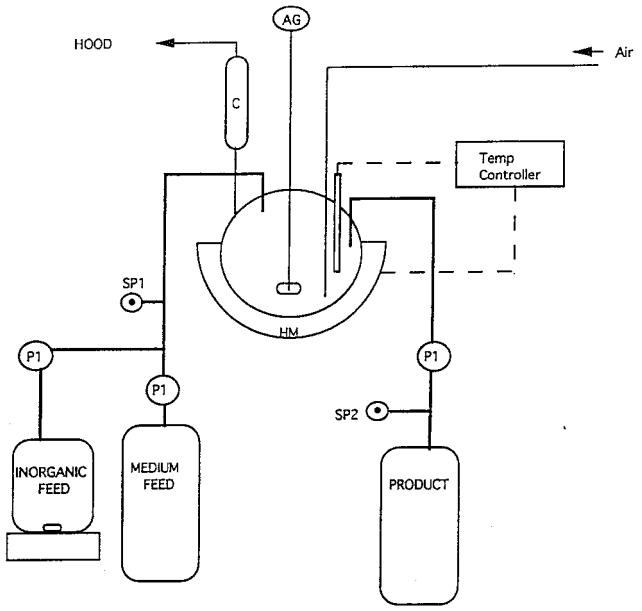


FIG. 1. Schematic of continuous culture system used for the growth of *M. sedula*. This is a modification of the system described by Brown and Kelly (5). Temp, temperature; SP, sample point; P1, main pump; HM, heating mantle; C, condenser; AG, agitator; ——, gas line; ▬, liquid line; – – –, signal line.

Iron assays. Samples of cell culture media were analyzed for total iron and ferrous iron amounts by 1,10-phenanthroline assay (31). The amount of ferric iron was determined by the difference. Iron conversion or speciation as a result of biological action was calculated by subtracting the iron levels detected in abiotic controls from those detected in the presence of *M. sedula.*

Proton motive force measurement. Cells harvested from continuous culture were shaken (100 rpm) at the desired temperature (70, 80, 85, or 25°C) for at least 1 h prior to the addition of radioactive probes. ^{14}C-labeled salicylic acid (1.3 μM; 50 mCi/mmol) and ^{14}C-labeled thiocyanate (0.58 μM; 50 mCi/mmol) were used for ΔpH and Δψ, respectively. Cell volume was determined with ^{14}C-labeled inulin and ^3H_2O. All radiolabeled probes but one were purchased from Dupont, NEN (Wilmington, Del.); thiocyanate was purchased from Amersham (Arlington Heights, Ill.). Cell suspensions were incubated with probes for 10 min and then centrifuged at 13,000 × *g*. Supernatant samples (100 μl) were taken, with the balance of supernatant removed by aspiration. Cell pellets were extracted with 1.0 M formic acid (100 μl). Cell pellets and supernatant samples were added to scintillation vials to measure the levels of intracellular and extracellular probes. Disintegrations per minute in 5 ml of Cytoscint ES (ICN, Irvine, Calif.) were counted on a Beckman LS 21000 scintillation counter. Transmembrane pH gradient and transmembrane potential were calculated as previously described by Rottenberg (32), with a background correction for probe binding according to the methods of Zaritsky et al. (38). In brief, nonspecific probe binding was assumed to occur according to a log mean concentration gradient across the cell membrane. An uptake ratio was calculated on the basis of cells de-energized with 10% butanol (Fisher Scientific) (uptake ratios of 32 and 6.3 for thiocyanate and salicylic acid, respectively.). An iterative procedure was used to calculate Δψ; convergence was generally achieved after 3 to 5 iterations.

Preparation of cell extracts. Cells were centrifuged at 8,000 rpm in a DuPont Sorvall RC5C centrifuge for 25 min. The supernatant was discarded, and cell pellets were washed twice with 10 mM H_2SO_4 (pH 2). Cells were resuspended in 10 mM Tris buffer (pH 7.4). Cells were disrupted at 69,000 kPa in a French pressure cell. Lysates were centrifuged for 30 min at 4,000 rpm. Lysates were retained for gel electrophoresis and immunoblot analyses. The amount of total protein was determined by Coomassie blue G-250 binding assay (Bio-Rad Laboratories, Richmond, Calif.).

Gel electrophoresis. *M. sedula* cell extracts were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (19). Samples were boiled in dissociation buffer that contained 1% SDS and 100 mM dithiothreitol for 5 min prior to being loaded on discontinuous SDS–12.5% PAGE gels. Typically, 10 to 30 μg of protein was loaded in a given lane, with an equal amount

used in each lane on a particular gel. Prestained molecular weight markers (GIBCO-BRL, Gaithersburg, Md.) were used for protein size references. Gels were silver stained by the method of Merril et al. (27) with a Bio-Rad silver stain kit (161-0443) or transferred to nitrocellulose (2) for Western blot (immunoblot) analysis. When protein concentrations were very low, extracts were placed directly on nitrocellulose. The typical volume of an extract on these dot blots was 10 μl (5 ng of protein). Proteins were blotted against antibodies to thermophilic factor 55 (obtained from J. Trent, Argonne National Laboratories) in 1% gelatin. The secondary antibody was a goat anti-rabbit alkaline phosphatase conjugate (Sigma). Bands were developed by using the fast Nitro Blue Tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) substrate-buffer system (Sigma).

Temperature shift experiments. For preliminary characterization of the stress response in *M. sedula*, cells were subjected to thermal stress. Cells that had been grown in shake flasks or harvested from continuous culture were subjected to temperature shifts of 6, 10, or 15°C, as established for heat shock response experiments in the literature (12, 15, 16, 36). Cells were incubated under normal growth conditions until they reached log phase (for shake flask cells) or for at least 1 h (for cells harvested from continuous culture). Cell extracts from normal and heat-shocked cells were Western blotted with antibodies generated against the chaperone from *S. shibatae*, thermophilic factor 55.

RESULTS

Proton motive force measurements. The proton motive force values determined for *M. sedula* varied for the range of conditions studied. Greater absolute values of Δp were typically seen for actively growing cultures that had been harvested from rich media (10 g of ferrous sulfate per liter and 0.2 g of Casamino Acids per liter at pH 2.0 and 70°C) and assayed immediately, presumably an indication of metabolic state. To test the stability of the bioenergetic parameters measured over the time frame necessary to conduct the experiments described here, the Δψ, ΔpH, and Δp of cells that had been harvested from continuous culture, stored for up to 12 h at 4°C, and then returned to normal growth conditions were determined. These cells were incubated in minimal media (with 0.02 g of Casamino Acids per liter and 0.05 g of $FeSO_4 \cdot 7H_2O$ per liter) at 70°C and pH 2.0 for 1 h prior to being sampled for bioenergetic measurements. These parameters varied only slightly over 4 h. Δψ remained constant at +25 mV, ΔpH varied from −200 to −215 mV (corresponding to an internal pH [pH_i] of 5.0 to 5.2), and Δp varied from −175 to −190 mV. The typical standard deviations of triplicate ΔpH and Δψ measurements were less than ±10%.

Effects of medium composition on Δp and nutritional stress. *M. sedula* appears to grow mixotrophically on medium that contains ferrous iron and Casamino Acids supplemented with yeast extract (31). When both organic- and inorganic-nutrient levels were reduced to 50 and 25% of the base medium concentrations of ferrous sulfate (10 g/liter) and Casamino Acids (0.2 g/liter) in continuous culture at pH 2.0, 70°C, and a dilution rate of 0.05 h^{-1} (doubling time, 13.9 h), cell densities dropped from 2.2×10^8 cells per ml to 6.1×10^7 and 2.1×10^7 cells per ml, respectively. SDS-PAGE of *M. sedula* cell extracts showed higher levels of protein bands with molecular masses of 62, 49, 45, 40, and 35 kDa (Fig. 2A). By Western blot analysis, the 62-kDa protein band cross-reacted with anti-thermophilic factor 55 (heat shock protein from *S. shibatae* [36]) antibodies (Fig. 2B); this species appears to be a major constituent of cell protein under both strict nutrient limitation and normal cell growth.

Figure 3 shows that the Δψ, ΔpH, and Δp of harvested cells that had been stored at 4°C before being processed, resuspended in buffer (pH 2.0), and heated to 70°C varied slightly in the presence or absence of low levels of ferrous iron, Casamino Acids, or maltose. The pH_i ranged from 4.1 (−140 mV) for basal salts alone to 4.8 (−190 mV) for low levels of maltose. The Δψ varied only slightly (from +15 to +25 mV); thus, the Δp varied from about −135 mV on basic salts to −170 mV on maltose. These parameters were different for cells that had been incubated directly after being harvested and processed (i.e., with no storage at 4°C) in richer media (harvested from aerated cultures grown on 10 g of $FeSO_4 \cdot 7H_2O$ per liter and 2.0 g of Casamino Acids per liter), for which values of +40, −230 (ΔpH of 3.4 units; pH_i, 5.4), and −190 mV were measured for Δψ, ΔpH, and Δp, respectively. These differences presumably reflect the bioenergetic state of *M. sedula.*

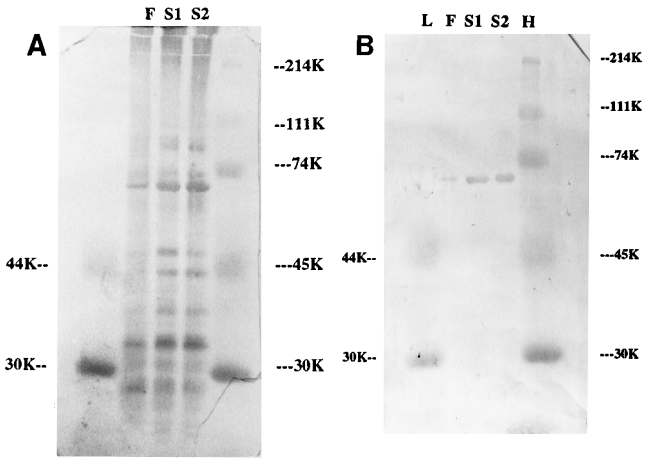


FIG. 2. Effects of nutrient limitation on the growth of *M. sedula*. SDS-PAGE (silver-stained) (A) and Western blot (against thermophilic factor 55) (B) analyses of *M. sedula* cell extracts from continuous culture (dilution rate of 0.05 h^{-1}). Lanes: L, low-molecular-mass markers; F, fed cells (10 g of $FeSO_4 \cdot 7H_2O$ per liter, 0.2 g of Casamino Acids per liter); S1, nutrient-limited cells (nutrient feed [lane F] cut by 50%); S2, nutrient-limited cells (nutrient feed [lane F] cut by 75%); H, high-molecular-mass markers. Note cross-reactivity at 62 kDa against anti-thermophilic factor 55 antibodies, in addition to enhanced levels of bands corresponding to lower-molecular-mass species of approximately 49, 45, 40, and 35 kDa, in nutrient-limited cells.

Temperature stress, Δp, and iron oxidation. *M. sedula* cells were concentrated 30-fold from 10 liters of continuous culture grown at 70°C and pH 2.0 (dilution rate, 0.05 h^{-1}) to provide a uniform sample of cells in the same metabolic state. Dense cell suspensions (concentrated to 2×10^9 cells per ml) were incubated simultaneously with either FeS_2 (1% [wt/vol]) or $FeSO_4 \cdot 7H_2O$ (0.05% [wt/vol]) under otherwise identical conditions. The presence of FeS_2 precluded the measurement of Δp because of the nonspecific binding of probes to pyrite, so parallel incubations were used to correlate energetic responses to bioleaching. Iron speciation for both biotic and abiotic cases was done during the course of and at the end of pyrite leaching experiments.

For experiments in which a temperature shift was to occur, it was of interest to manipulate cultures into ranges in which stress responses had been demonstrated for related organisms. It was also of interest to draw relationships between observed changes in cellular energetics and established cellular stress responses. Culture temperature shifts were carried out by transferring bottles to different oil shaker baths at 70, 80, and 85°C. These temperatures were chosen on the basis of results from other reported heat shock experiments with related thermoacidophiles (15, 36), as well as knowledge of the growth range of *M. sedula* (14). A submaximal temperature (70°C), a temperature at the high end of the growth range (80°C), and a potentially lethal temperature (85°C) were desired. The time intervals between shifts were also chosen on the basis of the literature. For example, Trent et al. (36) showed that *S. shibatae* was more resistant to thermal stress at 92°C after cultures

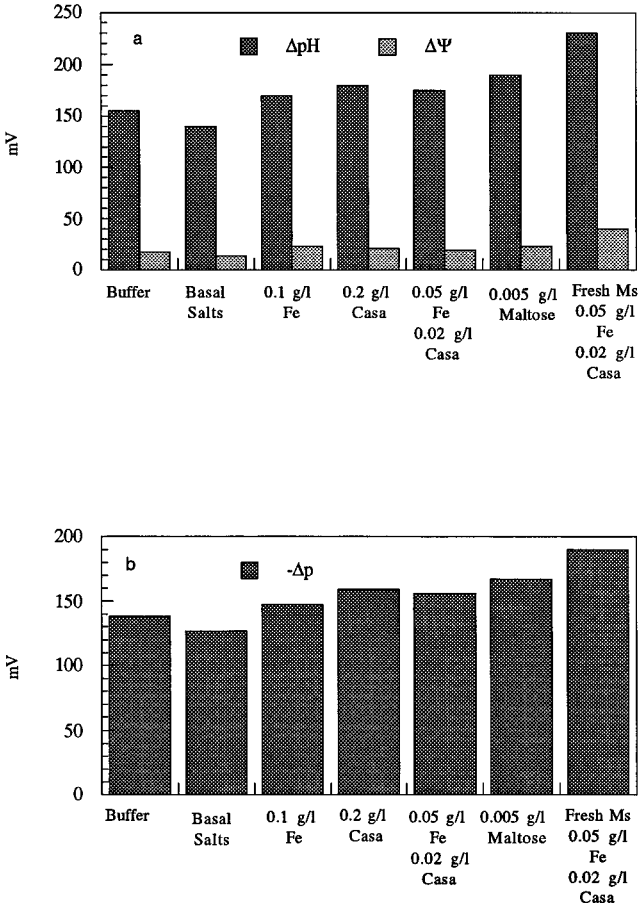


FIG. 3. Influence of medium composition on bioenergetic parameters (ΔpH and Δψ [a] and Δp [b]) for *M. sedula*. Measurements are for stock samples that had been grown in continuous culture, harvested, stored at 4°C, and then incubated in the indicated medium at 70°C and pH 2.0. Fresh Ms, cells that were harvested and tested immediately. Casa, Casamino Acids.

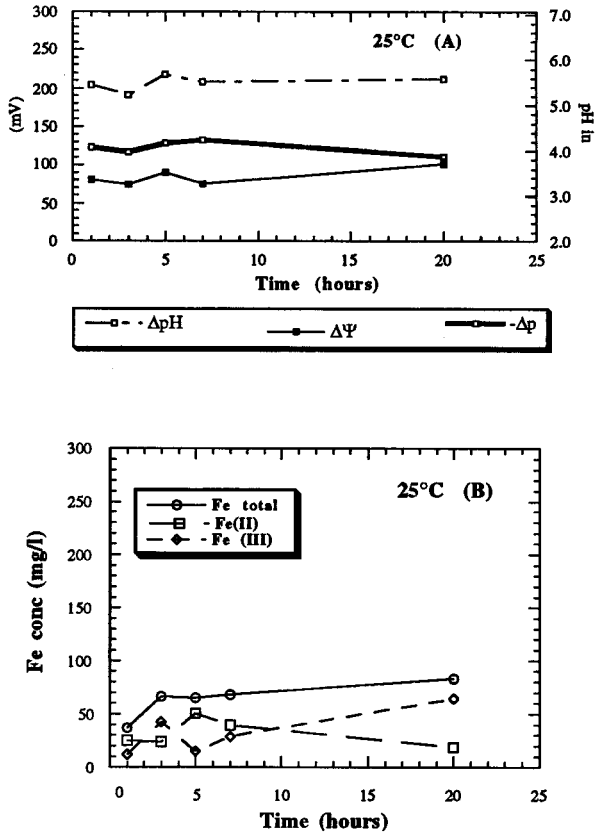


FIG. 4. (A) Variations in *M. sedula* proton motive force (Δp), membrane potential (Δψ), and transmembrane proton gradient (ΔpH) over time at 25°C and pH 2.0. (B) Changes in iron speciation in the presence of *M. sedula* and iron pyrite under otherwise identical conditions to those used in panel A.

had been preincubated for 2 to 4 h at 88°C; shorter preincubation periods did not enhance survival. Thus, the time course to follow response was at least 4 h. In all cases in which temperature shifts occurred, Western dot blot analysis of *M. sedula* cell extracts with antibodies directed against the thermophilic factor 55 heat shock protein from *S. shibatae* (Fig. 2) (36) showed evidence of a stress response by the intensity of cross-reactivity, compared with that of unstressed cells at 70°C (data not shown).

(i) Unshifted control experiments (25 and 70°C). Since extracytoplasmic oxidation of Fe^{2+} is mediated by an electron transport chain in chemolithotrophs, inorganic substrate turnover is related to proton motive force parameters. In addition, *M. sedula* oxidizes FeS_2 to Fe^{2+} and Fe^{3+}, maintaining high ratios of Fe^{3+} to Fe^{2+} in solution (7) and making iron speciation an important tool in assessing biological oxidation activity. A decline in the amount of Fe^{3+} in solution is a result of the precipitation of Fe^{3+}-hydroxy complexes (i.e., jarosites), which has been reported for iron leaching processes at elevated temperatures (1, 7). Thus, Fe^{3+} concentrations reflect the balance between oxidation and precipitation rates within a given experiment. The total iron levels in solution were the same in both the presence and absence of *M. sedula* (Fig. 4) at room temperature (25°C) over 20 h. However, in the presence of *M. sedula*, 75% of total iron was Fe^{3+}; for the abiotic case, Fe^{3+} was 30% of total iron, suggesting some biological activity even at reduced temperatures. Δp values remained relatively stable over 20 h of incubation at 25°C (Fig. 4A). ΔpH was approximately constant at −206 mV (pH_i, 5.5) (note that all pH_is calculated from ΔpH [in millivolts] were corrected for temperature [8]). Δψ remained between approximately +80 and +100 mV, and Δp remained between −110 and −130 mV. When cells were incubated at 70°C, ΔpH increased slightly from −235 mV (pH_i, 5.5) to −250 mV (pH_i, 5.7) and Δψ began to drop from +108 mV after 3 h to +50 mV after 20 h, thereby increasing the proton motive force from −135 to −200 mV (Fig. 5A). This increase in proton motive force was accompanied by an increase in biological iron oxidation in the parallel experiment with iron pyrite. Net increases in Fe^{3+} concentrations in solution indicate that the rate of formation of this species from Fe^{2+} exceeds the rate of loss to precipitation. For these experiments, the total amount of iron in solution at 70°C was more than two times greater than those at 25°C for both the *M. sedula* and cell-free cases after 20 h. However, the total iron level (171 mg/liter) for *M. sedula* at 70°C was higher than that for the abiotic case at 70°C (140 mg/liter), with 88% of total iron as Fe^{3+}. The cell-free case had only 3.4% of its total iron in the Fe^{3+} state. The cell densities measured in media that contained pyrite remained stable over 20 h at both temperatures. Inspection of pyrite under phase-contrast microscopy and epifluorescence microscopy (37) also indicated a significant amount of cell attachment. For the biotic case at 70°C, the rate of appearance of Fe^{3+} in solution (9.0 mg/liter/h) was

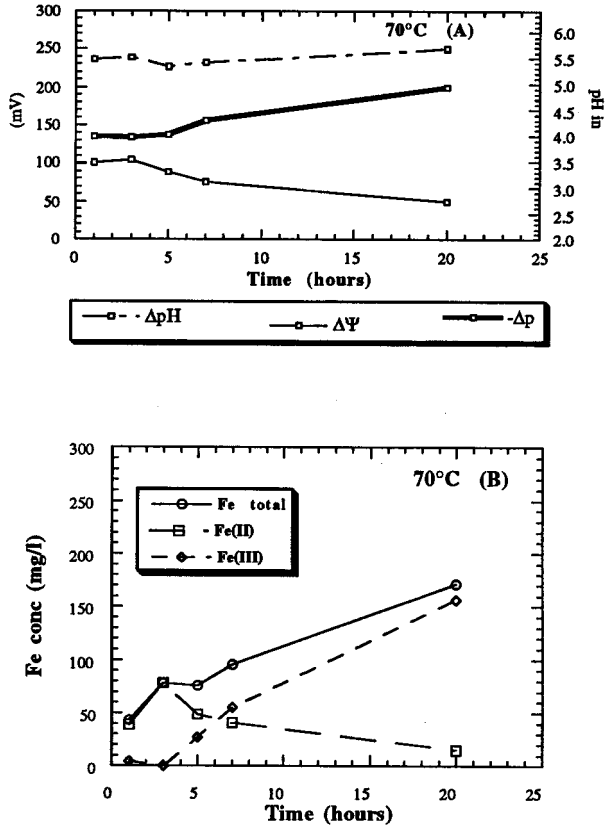


FIG. 5. (A) Variations in *M. sedula* proton motive force (Δp), membrane potential (Δψ), and transmembrane proton gradient (ΔpH) over time at 70°C and pH 2.0. (B) Changes in iron speciation in the presence of *M. sedula* and iron pyrite under otherwise identical conditions to those used in panel A.

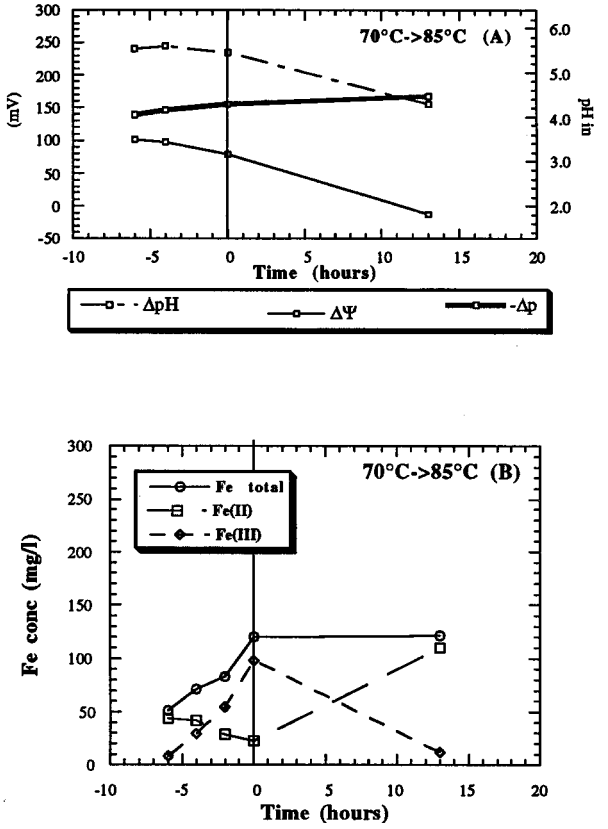


FIG. 6. (A) Variations in *M. sedula* proton motive force (Δp), membrane potential (Δψ), and transmembrane proton gradient (ΔpH) over time before and after shift (indicated by vertical line) from 70 to 85°C at pH 2.0. (B) Changes in iron speciation in the presence of *M. sedula* and iron pyrite under otherwise identical conditions to those used in panel A.

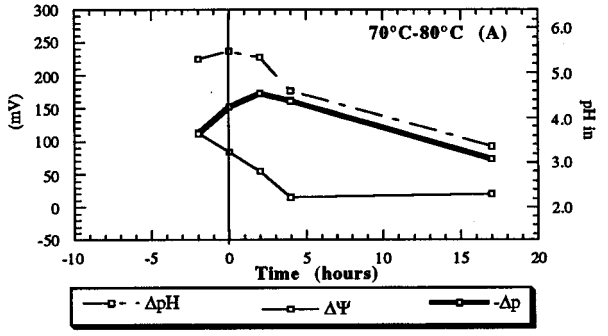
comparable to the rate of disappearance of Fe^{2+} (9.3 mg/liter/h). In the absence of *M. sedula* at 70°C, Fe^{2+} appeared at a rate of 9.4 mg/liter/h and the Fe^{3+} concentration decreased at a rate of 4.8 mg/liter/h (Fig. 5B). Thus, in the absence of cells, the rate of appearance of Fe^{3+} from abiotic reactions did not offset precipitation.

(ii) Temperature shift 1 (70 to 85°C). After *M. sedula* cells had been shifted from 70 to 85°C, both the pH_i and positive Δψ decreased (Fig. 6), presumably reflecting thermal damage to cell membranes. These values appear to continue to decrease through the 13-h timepoint after heat shock, with the pH_i dropping from 5.5 to 4.3 and the Δψ falling from 80 to −5 mV. These concurrent decreases resulted in a slight increase in Δp, albeit probably for respiratory-inactive cells. Fe^{3+} and Fe^{2+} concentration profiles before the shift resemble those of unshifted cultures at 70°C. After the shift, no significant increase in total iron released was detectable; this value remained constant at 121 mg/liter in solution. The level of Fe^{3+} in solution decreased to 9% of the total iron in solution, suggesting that bioleaching activity had stopped.

(iii) Temperature shift 2 (70 to 80°C). Cells that had been shifted from 70 to 80°C on iron pyrite created only slight increases in Fe^{3+} levels above those of abiotic controls. The proton motive force increased initially because of a drop in Δψ, as was the case at 70°C. After 4 h at 80°C, the pH_i of cytoplasm had decreased from 5.5 to 4.6, in contrast to remaining constant in the case of unshifted cells. Eventually, the Δψ stabilized at +10 mV; the pH_i continued to fall to 3.4 at 17 h postshift. This resulted in a decrease in Δp (to about −70 mV). After the shift, ferric iron generation in solution occurred at such low rates that Fe^{3+} concentrations did not decline for 4 h at 80°C, at which point respiratory processes had presumably been severely compromised. Ferrous iron levels continued to increase, indicating abiotic dissolution of FeS_2 with cessation of bioconversion (Fig. 7).

(iv) Temperature shift 3 (70 to 80 to 70°C). Cells that had been shifted from 70 to 80°C (for 4 h) and then back to 70°C exhibited the iron oxidation and proton motive force phenomena of both 80 and 70°C incubations (Fig. 8). Although this pH_i is close to 1 U lower than that at this point in the 70°C unshifted case, the Δp is similar because the Δψ is significantly lower (close to 0 mV versus +65 mV in the unshifted case). After both temperature shifts, Fe^{3+} made up 64% of the total iron in solution. However, the total iron concentration (250 mg/liter) was higher than that for the unshifted 70°C case (171 mg/liter). The Fe^{2+} release rate at 80°C was nearly double that at 70°C, while ferric ion levels and cell densities dropped. Upon returning to 70°C, Fe^{3+} generation resumed at rates approximating those under unshifted 70°C conditions. In the absence of *M. sedula*, Fe^{2+} was also released at high rates upon the shift to 80°C, although ferric ion concentrations remained near 0. The total iron concentration in solution for the abiotic case was 160 mg/liter, with 12% of total iron as Fe^{3+}.

Influence of temperature shift on iron oxidation. In the double temperature shift experiment, there was a net increase in total iron released, compared with that of the unshifted 70°C



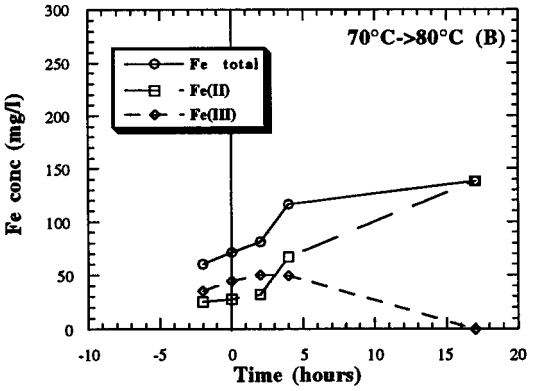
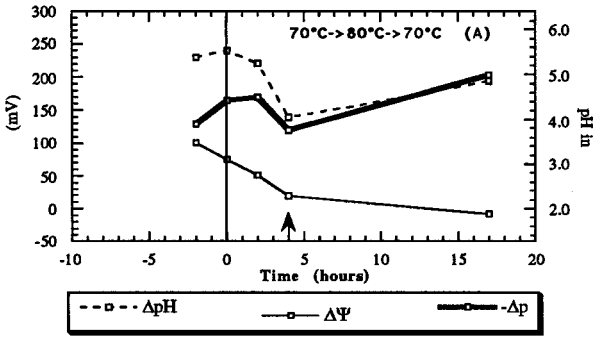


FIG. 7. (A) Variations in *M. sedula* proton motive force (Δp), membrane potential (Δψ), and transmembrane proton gradient (ΔpH) over time before and after shift (indicated by vertical line) from 70 to 80°C at pH 2.0. (B) Changes in iron speciation in the presence of *M. sedula* and iron pyrite under otherwise identical conditions to those used in panel A. Fe(II), Fe^{2+}; Fe(III), Fe^{3+}.



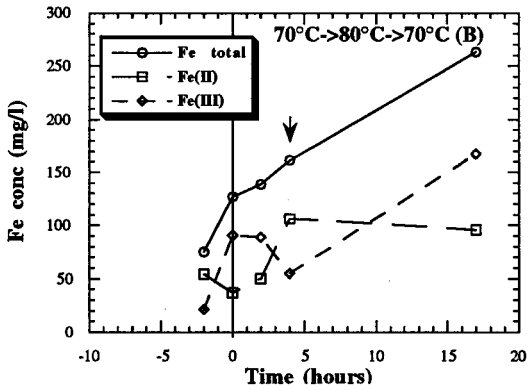


FIG. 8. (A) Variations in *M. sedula* proton motive force (Δp), membrane potential (Δψ), and transmembrane proton gradient (ΔpH) over time before and after shift from 70 to 80°C (indicated by vertical line) and back to 70°C (indicated by arrow) at pH 2.0. (B) Changes in iron speciation in the presence of *M. sedula* and iron pyrite under otherwise identical conditions to those used in panel A. Fe(II), Fe^{2+}; Fe(III), Fe^{3+}.

case. This may have been primarily the result of additional abiotic release of Fe^{2+} at the higher temperature, which was rapidly oxidized to Fe^{3+} by cells when they were shifted back to 70°C. At 80°C, cells that had been shifted from 70°C were probably unable to immediately take advantage of the increase in solubilized energy substrate. Thus, an intermediate shift was tried with actively growing *M. sedula* cultures to see whether this strategy could be used to increase leaching rates. Shake flask *M. sedula* cultures on FeS_2 (2% [wt/vol]) that had been incubated at 73°C were subjected to a 6°C temperature shift during log phase. Cells remained viable and continued to grow after the shift, as indicated by a temporary increase in cell density; however, cell density declined to prior levels shortly thereafter (Fig. 9a). Shifted cultures exhibited a twofold-higher ratio of ferric iron to ferrous iron in solution than that of cultures maintained at 73°C (0.39 and 0.18, respectively). Although cell extracts in both shifted and unshifted cases showed cross-reactivity with anti-thermophilic factor 55 antibodies (Fig. 9b and c), cultures exhibited stronger cross-reactivity at 79°C than they did at 73°C, providing evidence of a heat shock response.

DISCUSSION

Table 1 is a list of the Δψ, ΔpH, and Δp values reported for various acidophilic microorganisms, in addition to those for thermophilic neutrophilic *Thermus thermophilus*. Note that for the neutrophile, the proton motive force is entirely derived from a negative membrane potential, in contrast to the acidophiles, which make use of the transmembrane proton gradient. The Δψ, ΔpH, and Δp values determined for *M. sedula* under the experimental conditions used in this study are comparable to those reported for other respiring thermophilic and mesophilic acidophiles. The pH_i for *M. sedula* determined in this study was lower than those measured for many mesoacidophiles but was comparable to that reported for *S. acidocaldarius* (pH_i, 5.6; external pH, 3.5 [at 45°C]) (21) (Table 1). Comparisons of the pH_is of various acidophilic mesophiles and thermophiles are interesting because of the potential temperature effects on in vivo and in vitro cytoplasmic buffering capacities and the differences in cell membrane characteristics between archaea and eubacteria. In addition, note that the pH_i for *M. sedula* incubated at 25°C was similar to that for *M. sedula* incubated at 70°C. In studies of a similar organism, *S. acidocaldarius*, whose respiratory chain components have previously been examined (9, 17, 20, 30, 33), Lüben and Schäfer (21) found Δψ values to be inside negative, in contrast to the positive inside values found for *M. sedula* in this study and those reported for other mesoacidophiles (Table 1). *Thiobacillus ferrooxidans* also was found to have an inside negative Δψ when it was incubated at pH 3.0, in contrast to its optimum pH of about 2.0 (Table 1). Since the *S. acidocaldarius* study was done at pH 3.5, its results may reflect variations in this parameter with decreasing transmembrane pH gradients.

In previous continuous culture studies, *M. sedula* fared best in media that contained both ferrous iron and Casamino Acids

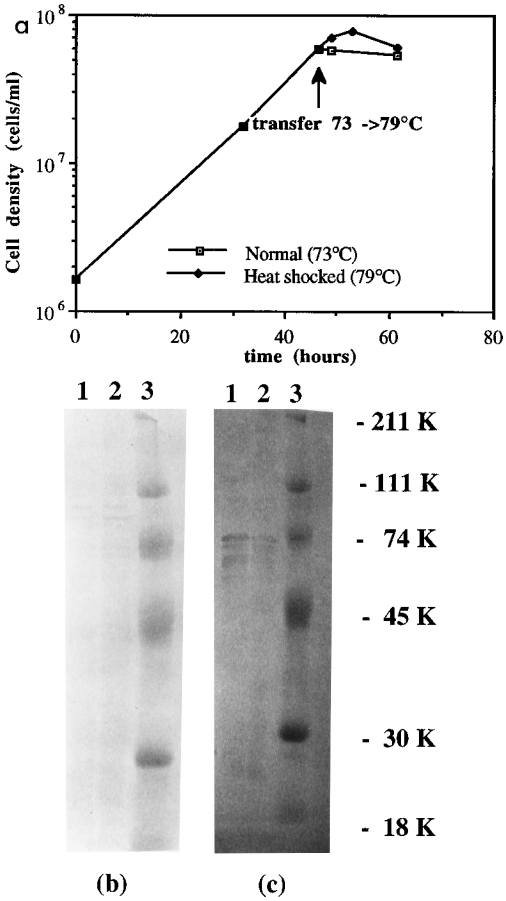


FIG. 9. Heat shock of *M. sedula*. (a) Growth of *M. sedula* on FeS_2 at 73°C and with a temperature shift from 73 to 79°C. (b and c) SDS-PAGE (silver-stained) (b) and Western blot (against thermophilic factor 55 antibodies) (c) analyses of *M. sedula* cell extracts. Lanes: 1, *M. sedula* cells shifted from 73 to 79°C; 2, *M. sedula* cells held at 73°C; 3, low-molecular-mass markers (in kilodaltons [K]). After 65 h, the Fe^{3+}/Fe^{2+} ratios for heat-shocked cells and cells left at 73°C were 0.39 and 0.18, respectively.

(31). Attempts to separate autotrophic growth and heterotrophic growth of *M. sedula* resulted in limited success. Reducing the level of organic substrate and increasing the level of inorganic substrate resulted in reduced biomass yield and higher iron turnover. However, complex organic medium components could not be completely eliminated without killing cells. Similarly, the elimination of inorganic substrates resulted in cell death. Cutting back on both Fe^{2+} and Casamino Acids in continuous culture resulted in increased levels of a 62-kDa protein immunologically related to thermophilic factor 55 (Fig. 2). It remains to be seen whether separate changes in the level of either Fe^{2+} or Casamino Acids elicit a stress response.

Shifting *M. sedula* from 70 to either 80 or 85°C has significant negative effects on cellular functions. The shift to 85°C (Fig. 6) causes respiratory activity to decrease, as indicated by iron speciation. Although the proton motive force is fairly constant in the period after heat shock, pH_i drops from 5.5 to 4.4 and $\Delta\psi$ falls to approximately 0 mV after 13 h. Thermal damage to the cell membrane likely circumvents the regulated routes for proton and ion exchange with the extracellular environment. It seems that the primary protection for acidification of the cytoplasm at this point is its intracellular nondiffusible net positive charge, which may be augmented by the protonation of titrable cellular constituents (23). The shift to 80°C causes a similar loss in iron-oxidizing activity. It appears, however, that cells continued to be metabolically active in the initial period after this temperature shift, as Fe^{3+} levels rose slightly. The response to this temperature shift is reflected in changes in pH_i and $\Delta\psi$. pH_i dropped from 5.5 to 4.6 in the first 4 h after the shift, at which time $\Delta\psi$ fell to nearly 0 mV. After 17 h at 80°C, pH_i had dropped further to 3.3. It is interesting that cells that had been shifted back to 70°C after 4 h at 80°C were able to recover respiratory activity (Fig. 8); at 13 h after cells had been returned to 70°C, pH_i was 4.8 and $\Delta\psi$ was at or below 0 mV. The recovery of iron-oxidizing activity seems to have occurred without the aid of a positive $\Delta\psi$. This may be a reflection of a concerted cellular effort to pump protons out in addition to significant changes in the levels of ionic species as a result of short-circuiting regulated transport mechanisms after thermal trauma to the cell membrane.

Recovery from thermal stress may require *M. sedula* to devote more of its energy budget to proton pumping, which may translate into higher turnover rates of inorganic energy sources. In addition, at higher temperatures, the kinetics of

TABLE 1. Reported ΔpH, $\Delta\psi$, and Δp values for selected microorganisms

Organism	Probes[a]	Temp (°C)	pH_o[b]	pH_i	ΔpH[c] (mV)	$\Delta\psi$[d] (mV)	Δp[e] (mV)	Reference
Thermus thermophilus	WA, $TPMP^+$	70	7.5	7.5	0	−197	−197	26
S. acidocaldarius	WA, SCN^-	45	3.5	5.6	−134	−19	−153	21
Thiobacillus ferrooxidans	WA, SCN^-	22	1.0	6.8	−340	+70	−270	9
	WA, SCN^-	22	2.0	6.6	−266	+10	−256	9
	DDA^+, TPB^-	22	3.0	6.3	−192	−40	−232	9
M. sedula	SA, SCN^-	25	2.0	5.5	−220	+85	−125	This work
	SA, SCN^-	70	2.0	5.4	−230	+40	−190	This work
Thermoplasma acidophilum	WA, SCN^-	59	2.0	6.1	−266	+10	−256	28
	TEA^+, SCN^-	56	2.0	6.5	−296	+120	−176	13
Bacillus acidocaldarius	WA, SCN^-	50	3.0	5.9	−171	+34	−137	18
	WA, SCN^-	40	3.5	6.2	−230	+10	−220	28
	WA, SCN^-	50	3.5	5.9	−153	+36	−117	18

[a] SA, salicyclic acid; SCN^-, thiocyanate; WA, weak organic acid; $TPMP^+$, triphenylmethylphosphonium; TPB^-, tetraphenylboron; TEA^+, tetraethylammonium.
[b] pH_o, external pH.
[c] $\Delta pH = pH_o - pH_i$.
[d] Positive when cytoplasm is positive with respect to the surrounding medium.
[e] $\Delta p = \Delta\psi + 2.303\ RT/F \times \Delta pH$, where R is the universal gas constant, T is time, and F is the Faraday constant.

chemical and biochemical FeS_2 oxidation to Fe^{2+} are enhanced. This combination of effects, if properly balanced, may be exploited to increase iron and/or sulfur oxidation rates. This scenario may explain the results shown in Fig. 9. By choosing an intermediate temperature shift (73 to 79°C), the Fe^{3+} levels in shifted cultures were significantly higher than those in cultures maintained at 73°C. The main benefit of a short-term temperature shift for active cultures is the boost in available energy substrate (i.e., Fe^{2+}) in culture, possibly even leading to a short-term boost in growth kinetics. However, this benefit is realized only if the cells involved are in a metabolically competent state to support iron- and/or sulfur-oxidizing activities. *M. sedula* was able to stabilize the pH of its cytoplasm for a short period under moderate levels of thermal stress (Fig. 8), although longer periods of incubation at high temperatures resulted in cell death, with lower levels of FeS_2 converted (Fig. 7). Further work to determine the point at which metabolic recovery from thermal stress is possible (as a function of both time and temperature) needs to be done, although this strategy to improve bioleaching rates merits closer examination.

There is still much to be learned about the relationship between stress and the physiological and bioenergetic responses of extremophilic microorganisms. From an evolutionary perspective, insights into how biological systems adapted to mesophilic and neutrophilic conditions may be gained through studies of thermoacidophilic adaptation to thermal and nutritional stresses. In addition, the utility of thermoacidophiles for biotechnological applications depends on a better understanding of their physiology and bioenergetics and insights into how to manipulate their metabolic features to achieve bioprocessing objectives.

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