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Bioenergetic Studies of Coal Sulfur Oxidation by Extremely Thermophilic Bacteria

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Abstract

Although there are many potential benefits in using microorganisms to reduce the sulfur content of coal, the primary disadvantages remain relatively low desulfurization rates. Thermoacidophilic microorganisms have been considered for inorganic sulfur removal from coal because of expected improvements in rates of both biotic and abiotic sulfur oxidation reactions with increasing temperature. Efforts to optimize the performance of such systems have relied mostly on modifications in growth media and manipulation of process parameters, often without careful examination of the consequences to the microorganism's metabolism and bioenergetics. Thus, the physiological basis for any improvements in sulfur removal rates are not always clear and may be difficult to implement on a large scale. Without appropriate insights, attempts to improve or include key metabolic pathways in promising thermoacidophiles through genetic manipulation, at the time this becomes feasible for these organisms, will not have any basis. In this study, the bioenergetic response of the extremely thermoacidophilic archaeon, *Metallosphaera sedula*, to environmental changes have been examined in relation to its capacity to catalyze pyrite oxidation in coal. Given an appropriate bioenergetic challenge, the metabolic response was to utilize additional amounts of energy sources (i.e., pyrite) to survive. Of particular interest were the consequences of exposing the organism to various forms of stress (chemical, nutritional, thermal, pH) in the presence of coal pyrite. Several approaches to take advantage of stress response to accelerate pyrite oxidation by this organism were examined, including attempts to promote acquired thermal tolerance to extend its functional range, exposure to chemical uncouplers and decouplers, and manipulation of heterotrophic and chemolithotrophic tendencies to optimize biomass concentration and biocatalytic activity. Promising strategies were investigated in a continuous culture system. This study identified environmental conditions that promote better coupling of biotic and abiotic oxidation reactions to improve biodesulfurization rates of thermoacidophilic microorganisms.

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Executive Summary

The response of an extremely thermoacidophilic archaeon *Metallosphaera sedula* (growth temperature range 50-79°C, T_{opt} 74°C and pH_{opt} 2.0) to thermal stress was investigated using a 10-liter continuous cultivation system. *M. sedula*, growing at 74°C, pH 2.0, and dilution rate of 0.04 hr^{-1} , was subjected to both abrupt and gradual temperature shifts in continuous culture to determine the response of cell density levels and protein synthesis patterns. An abrupt temperature shift from 74°C to 79°C resulted in little, if any, changes in cell density and a small increase in total protein/cell. When the culture temperature was shifted further to 80.5°C, cell density dropped to below 5×10^6 cells/ml from 10^8 cells/ml, leading to washout of the culture. Operation at this temperature and slightly higher temperatures, however, could be achieved by exposing the culture to thermal stress more gradually (0.5°C increments). As a result, stable operation could be maintained at temperatures up to 81°C, and the washout temperature could be increased to 82.5°C. Continuous culture operation at 81°C for 100 hours ("stressed phase") led to an approximately 7-fold lower steady state cell density than observed for operation at or below 79°C. However, SDS-PAGE analysis (both one- and two-dimensional) revealed significantly higher levels (6-fold increase) of a 66 kDa stress response protein (MseHSP60), immunologically related to Thermophilic Factor 55 from *Sulfolobus shibatae* (Trent, J. D., J. Osipiuk, and T. Pinkau. 1990. *J. Bacteriol.* **172**:1478-1484). If the acclimated culture was returned to lower temperatures (i.e., 74°C), the amount of MseHSP60 returned to levels observed prior to thermal acclimation. Furthermore, when the previously acclimated culture (at 81°C) was shifted back from 74°C to 81°C, without going through gradual acclimation steps, the result was the immediate onset of washout, suggesting no residual thermotolerance. This study shows that gradual thermal acclimation of *M. sedula* could only extend the temperature range of stable growth for this organism by 2°C above its maximal growth temperature, albeit at reduced cell densities. Also, this

investigation illustrates the utility of continuous culture for characterizing heat shock response and assessing maximum growth temperatures for extremely thermophilic microorganisms.

The biooxidation capacity of an extremely thermoacidophilic archaeon *M. sedula* (DSMZ 5348) was examined under bioenergetic challenges imparted by thermal or chemical stress in regard to its potential use in microbial bioleaching processes. Within the normal growth temperature range of *M. sedula* (70-79°C) at pH 2.0, upward temperature shifts resulted in bioleaching rates that followed an Arrhenius-like dependence. When the cells were subjected to supraoptimal temperatures through gradual thermal acclimation at 81°C (Han, C. J., Park, S. H., Kelly, R. M. 1997. Appl. Environ. Microbiol. **63**: 2391-2396), cell densities were reduced but 3 to 5 times faster specific leaching rates (Fe^{3+} released from iron pyrite/cell/hr) could be achieved by the stressed cells compared to cells at 79°C and 73°C, respectively. The respiration capacity of *M. sedula* growing at 74°C was challenged by poisoning the cells with uncouplers to generate chemical stress. When the protonophore 2,4-dinitrophenol (5-10 mM) was added to a growing culture of *M. sedula* on iron pyrite, there was little effect on specific leaching rates compared to a culture with no protonophore at 74°C; 25 μM levels proved to be toxic to *M. sedula*. However, a significant stimulation in specific rate was observed when the cells were subjected to 1 μM nigericin (+135%) and 2 μM (+63%); 5 μM levels of the ionophore completely arrested cell growth. The ionophore effect was further investigated in continuous culture growing on ferrous sulfate at 74°C. When 1 μM nigericin was added as a pulse to a continuous culture, a 30% increase in specific iron oxidation rate was observed for short intervals, indicating a potential positive impact on leaching when periodic chemical stress is applied. This study suggests that biooxidation rates can be increased by strategic exposure of extreme thermoacidophiles to chemical or thermal stress and this approach should be considered for improving process performance.

**I. Bioenergetic response of the extreme thermoacidophile
Metallosphaera sedula to thermal and nutritional stress**

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I. ABSTRACT

The bioenergetic response of the extremely thermoacidophilic archaeum *Metallosphaera sedula* to thermal and nutritional stress was examined. Continuous cultures (pH 2.0, 70°C and dilution rate of 0.05 hr⁻¹), in which levels of casamino acids and ferrous iron in growth media were reduced by a step-change of 25-50%, resulted in higher levels of several proteins on SDS-PAGE including a 62 kDa protein immunologically related to the molecular chaperone designated as Thermophilic Factor 55 (TF55) in *Sulfolobus shibatae* (Trent, J. D., J. Osipiuk and T. Pinkau. 1990. J. Bacteriol. **172**:1478-1484). The 62 kDa protein was also noted at elevated levels in cells shifted from 70°C to either 80°C or 85°C. The proton motive force (Δp), transmembrane pH (ΔpH) and membrane potential ($\Delta \psi$) were determined for samples obtained from continuous culture (pH 2.0, 70°C and dilution rate of 0.05 hr⁻¹) and incubated under nutritionally and/or thermally stressed and unstressed conditions. At 70° under optimal growth conditions, *M. sedula* was typically found to have a Δp of approximately -190 to -200 mV, resulting from an intracellular pH of 5.4 (extracellular pH 2.0) and a DY of +40 to +50 mV (positive inside). When cells were shifted to either 80°C or 85°C, DY decreased to near zero and internal pH approached 4.0 within four hours after the shift; respiratory activity, as evidenced by iron speciation in parallel temperature-shifted cultures on iron pyrite, had ceased at this point. If cultures shifted from 70°C to 80°C were shifted back to 70°C after four hours, cells were able to regain pyrite oxidation capacity and internal pH increased to near normal levels after 13 hours. However, $\Delta \psi$ remained close to zero, possibly the result of enhanced ionic exchange with the media upon thermal damage to the cell membrane. Further, when *M. sedula* was subjected to an intermediate temperature shift from 73°C to 79°C, an increase in pyrite dissolution (ferric iron levels doubled) was noted over the unshifted control at 73°C. 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 for acidophilic chemolithotrophs.

I. INTRODUCTION

The 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 metals 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 metals leaching and sulfur oxidation processes as competitors to, or potential improvements on, mesoacidophile-based leaching technologies (1,10). Whether in the recovery of precious metals or in the 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 their ability to conserve energy through cellular processes that maintain a high transmembrane pH gradient (8,11,23,24,28). The extracytoplasmic oxidation of sulfur and metal sulfides has been linked to the generation of energy through chemiosmotic coupling in chemolithotrophic acidophiles (8). These bioenergetic modes thus take advantage of extremely acidic conditions to provide some or all of the cell's energetic requirements. It would seem 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 exposed, would rapidly inactivate biomolecules in the cytoplasm. However, this margin of error is relaxed somewhat by an intracellular non-diffusible net positive charge or Donnan potential which gives rise to a positive (inside) membrane potential (11,22,23,24).;

additional protection has been proposed to result from the contribution of a proton diffusion potential (23). The positive membrane potential ($\Delta\psi$) makes the acidification of the cytoplasm self-limiting by opposing the movement of protons across the otherwise ion-impermeable membrane (23).

There has been increasing interest in the mechanisms by which organisms respond to thermal stress and the possible technological consequences of this response (25). Acidophilic organisms may be more susceptible to thermal stress than neutrophiles 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 (15,16,36). When the extremely thermophilic archaeum *S. acidocaldarius* was subjected to temperature shift from 70°C to 85°C, two proteins of molecular mass 64-66 kDa, which were major cellular proteins at 70°C, were produced at increased levels at 85°C (15,16). Trent et al. (36) showed that *Sulfolobus shibatae* possessed an acquired thermotolerance when it was acclimated to sub-lethal temperatures for sufficient periods of time, prior to exposure to even higher temperatures; a prominent heat shock protein (Thermophilic Factor 55 or TF55) was identified and 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 for thermoacidophiles to encounter various types of stress that may arise from their use in oxidation of metal sulfides in ores and inorganic sulfur in coal, the stress response of these organisms merits additional study.

To investigate further the response of thermoacidophilic microorganisms to stress, the archaeum *Metallosphaera sedula* was examined. This thermoacidophile, isolated by Huber et al. (14) from a solfataric field in Italy, grows optimally at 70-75°C, and over a pH range of 1-4.5. Clark et al. (7) and Peeples and Kelly (31) have shown that *M. sedula* oxidizes both coal and mineral pyrite at rates 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 their prospects for application in a variety of bioprocesses in addition to gaining insight into the basis for metabolic function in extreme environments.

I. MATERIALS AND METHODS

Cell cultivation. *Metallosphaera sedula* (DSM 5348) was obtained from the Deutsche Sammlung von Microorganismen (DSM; Braunschweig, Germany). Cells were cultivated in a basal medium containing 0.4 g/l K_2HPO_4 , 0.4 g/l NH_4SO_4 and 0.4 g/l $MgSO_4 \cdot 6H_2O$ (Fisher Scientific, Raleigh, NC). 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/ml. Batch and small scale continuous cultivation of cells were performed as described previously (31). A 10 liter dual-limited chemostat (0.5 g/l $FeSO_4 \cdot 7H_2O$; 0.2 g/l Casamino acids (Sigma, St. Louis, MO)) was used to generate biomass for bioenergetic studies. Cells to be used for bioenergetic measurements and leaching studies were harvested and centrifuged at $10,000 \times g$ for 30 minutes. Cell pellets were washed in 10 mM H_2SO_4 (pH 2.0) and resuspended in a 5 - 10 ml of desired buffer at a concentration of 10^9 - 10^{10} cells/ml.

Batch cultures. For small cultures (50 ml) autoclaved basal medium was added to serum vials or shake flasks and supplemented with one of the following: $FeSO_4 \cdot 7H_2O$ (0.5 g), NIST standard reference pyrite (NIST 8455) (National Institute for Standards and Technology, Gaithersburg, MD) (1-2 g). Vials for heterotrophic or mixotrophic growth studies were also supplemented with

200 μ l of 5% acid hydrolyzed casein (casamino acids, Sigma A-2427). 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 inorganic substrate. The flask was then heated to culture temperature and aerated before inoculation. Inocula were obtained from small shake flask cultures.

Continuous culture. Figure 1 is a schematic of the 10 liter continuous culture apparatus modified from 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 μ m 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 μ m filter at a rate of 60 cc/min. The system was inoculated and run in batch mode for 3 to 4 days prior to initiation of continuous operation. Continuous culture was initiated by pumping fresh medium containing salts and organic substrates (pH 2.0) and a slurry of inorganic substrate (pH 2.0) into the round bottom flask containing cells at mid-log phase ($0.5-1.0 \times 10^8$ cells/ml). The dilution rate for most experiments was 0.05 hr^{-1} (corresponding to a 14 hour doubling time). Cells and spent media were collected in sterile polypropylene catch vessels. Cell product (4-8 L) was stored at 4°C (up to 12 hours) 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/ml. Significant build-up of iron oxides on the reactor walls was observed after extended periods of operation such that 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.

Iron Assays. Samples of cell culture media were analyzed for total and ferrous iron using a 1,10-phenanthroline assay (31). Ferric iron was determined by difference. Iron conversion or speciation as the result of biological action was calculated by subtracting iron levels detected in abiotic controls from iron levels 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 hour prior to the addition of radioactive probes. ^{14}C -labeled salicylic acid (1.3 μM ; 50mCi/mmol) and ^{14}C -labeled thiocyanate (0.58 μM , 50mCi/mmol) were used for ΔpH and $\Delta\psi$, respectively. Cell volume was determined using ^{14}C -labeled inulin and $^3\text{H}_2\text{O}$. All radiolabelled probes were purchased from Dupont NEN (Wilmington, DE) except for thiocyanate which was purchased from Amersham (Arlington Heights, IL). Cell suspensions were incubated with probes for 10 min and then centrifuged at 13,000 x 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 level of intracellular and extracellular probes. Disintegrations per minute were counted in 5 ml of Cytoscint ES (ICN, Irvine, CA) on a Beckman LS 21000 Scintillation counter. Transmembrane pH gradient and transmembrane potential were calculated as 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 based on cells de-energized with 10% butanol (Fisher Sci, Raleigh, NC) (uptake ratios were 32 and 6.3 for SCN^- and salicylic acid, respectively.). An iterative procedure was used to calculate the $\Delta\psi$; convergence was generally achieved after 3 to 5 iterations.

Preparation of cell extracts. Cells were centrifuged at 8000 rpm for 25 minutes. Supernatant was discarded and cell pellets were washed twice with 10 mM H₂SO₄ at pH 2. Cells were resuspended in 10 mM Tris buffer at pH 7.4. Cells were disrupted at 69000 kPa in a French Pressure Cell. Lysates were centrifuged for 30 minutes at 4000 rpm. Lysates were retained for gel electrophoresis and immunoblot analyses. Total protein was determined with the coomassie blue G-250 binding assay (Bio-Rad Laboratories, Richmond, CA).

Gel electrophoresis. *M. sedula* cell extracts were run on SDS-PAGE gels (19). Samples were boiled in dissociation buffer containing 1% SDS and 100 mM DTT for 5 minutes prior to loading on 12.5% discontinuous SDS-PAGE gels. Typically, 10-30 µg of protein were loaded in a given lane with equal amounts used for each lane for a particular gel. Pre stained molecular weight markers (GIBCO-BRL, Gaithersburg MD) for protein size references. Gels were silver stained using the method of Merrill et al. (27) with a Bio-Rad Silver Stain kit (161-0443 Bio-Rad Laboratories, Richmond, CA) or transferred to nitrocellulose (2) for Western blot analysis. When protein concentrations were very low, extracts were placed directly on nitrocellulose. Volume of extracts in these dot blots were typically 10 µl (5 ng of protein). Proteins were blotted against antibodies to Themophilic Factor 55 (obtained from J. Trent, Argonne National Laboratories) in 1% gelatin. Secondary antibody was a goat-anti-rabbit alkaline phosphatase conjugate (Sigma, St Louis, MO). Bands were developed using the Fast NBT/BCIP substrate/ buffer system (Sigma, St. Louis, MO).

Temperature shift experiments. For the preliminary characterization of stress response in *M. sedula*, cells were subjected to thermal stress. Cells grown in shake flasks or harvested from continuous culture were subjected to temperature shifts of 6,10 or 15°C as in heat shock experiments established in the literature (12,15,16,36). Cells were incubated at the normal growth conditions until in log phase (for shake flask cells) or for at least one hour (for cells harvested from

continuous culture). Cell extracts from normal and heat shocked cells were Western-blotted with antibodies generated against the chaperone from *Sulfolobus shibatae*, Thermophilic Factor 55 (anti-TF55).

I. RESULTS

Proton motive force (Δp) measurements. 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 harvested from rich media (10 g/l ferrous sulfate and 0.2 g/l casamino acids at pH 2.0 and 70°C) and assayed immediately, presumably an indication of metabolic state. To test the stability of the measured bioenergetic parameters over the time frame necessary to conduct the experiments described here, $\Delta\psi$, ΔpH and Δp were determined for cells harvested from continuous culture, stored for up to 12 hours at 4°C and then brought back to normal growth conditions. These cells were incubated in minimal media (with 0.02 g/l casamino acids and 0.05 g/l $FeSO_4 \cdot 7H_2O$) at 70°C and pH 2.0 for one hour prior to sampling for bioenergetic measurements. These parameters varied only slightly over a four hour period: $\Delta\psi$ remained constant at +25 mV, ΔpH varied from -200 to -215 mV (corresponding to an internal pH (pH_i) of 5.0-5.2) and Δp varied from -175 to -190 mV. Standard deviation of triplicate measurements of ΔpH and $\Delta\psi$ were typically less than $\pm 10\%$.

Effect of media composition on Δp and nutritional stress. *M. sedula* appears to grow mixotrophically on media containing ferrous iron and casamino acids supplemented with yeast extract (31). When both organic and inorganic nutrient levels were reduced to 50% and 25% of base medium concentrations of ferrous sulfate (10 g/l) and casamino acids (0.2 g/l) in continuous culture at pH 2.0, 70°C and a dilution rate of 0.05 hr^{-1} (doubling time of 13.9 hours), cell densities dropped from 2.2×10^8 cells/ml to 6.1×10^7 cells/ml (50%) and 2.1×10^7 cells/ml

(25%). SDS-PAGE of *M. sedula* cell extracts showed higher levels of protein bands at molecular masses of 62, 49, 45, 40 and 35 kDa (see Figure 2a). By Western blot analysis, the protein band at 62 kDa cross-reacted with anti-TF55 (heat shock protein from *S. shibatae* (36)) antibodies (see Figure 2b); this species appears to be a major constituent of cell protein both under strict nutrient limitation and normal cell growth.

Figure 3 shows that $\Delta\psi$, ΔpH and Δp for harvested cells, that were stored at 4°C before being processed and 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 internal pH ranged from 4.1 (-140 mV) for basal salts alone to 4.8 (-190 mV) for low levels of maltose, $\Delta\psi$ varied only slightly from +15 to +25 mV; thus, Δp varied from about -135 mV on basic salts to -170 mV on maltose. These parameters were different for cells incubated directly after harvesting and processing (i. e., with no storage at 4°C) in richer media (harvested from aerated cultures grown on 10 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 2.0 g/l casamino acids) where values of +40 mV, -230 mV (ΔpH of 3.4 units or pH_i of 5.4), and -190 mV for $\Delta\psi$, ΔpH and Δp , respectively, were measured. The differences presumably reflect the bioenergetic state of *M. sedula*.

Temperature Stress, Δp and Iron Oxidation. *M. sedula* were concentrated 30-fold from 10 liters of continuous culture growing at 70°C and pH 2.0 (0.05 hr^{-1}) to provide a uniform sample of cells in the same metabolic state. Dense cell suspensions (concentrated to 2×10^9 cells/ml) were incubated simultaneously with either FeS_2 (1% wt/vol) or $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05% wt/vol) under otherwise identical conditions. The presence of FeS_2 precluded the measurement of Δp due to non-specific binding of probes to pyrite, so that parallel incubations were used to correlate the energetic responses to bioleaching. Iron speciation for both biotic and abiotic cases was done during the course and at the end of pyrite leaching experiments.

For experiments in which temperature was to be shifted, it was of interest to manipulate cultures in ranges where stress response 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 was shifted by transferring bottles to different oil shaker baths at 70°C, 80°C and 85°C. These temperatures were chosen based on results from other reported heat shock experiments with related thermoacidophiles (15,36), given knowledge of the growth range of *M. sedula* (14). It was desired to have a sub-maximal temperature (70°C), a temperature at the high end of the growth range (80°C) and a potentially lethal temperature (85°C). Time intervals between shifts were also chosen based on literature reports. For example, Trent et al. (36) showed that *S. shibatae* was more resistant to thermal stress at 92°C when cultures were pre-incubated for 2-4 hours at 88°C; shorter pre-incubation periods did not enhance survival. The time course to follow response was thus at least four hours. In all cases where temperature shifts were done, Western dot blot analysis of *M. sedula* cell extracts with antibodies directed against the TF55 heat shock protein from *S. shibatae* (see Figure 2 and Trent et al. (36)) showed evidence of a stress response by intensity of cross-reactivity compared to unstressed cells at 70°C (data not shown).

Unshifted Control Experiments (25°C and 70°C). Since the 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), making iron speciation an important tool in assessing biological oxidation activity. Decline of Fe^{3+} in solution results from the precipitation of Fe^{3+} -hydroxy complexes (i. e., jarosites) which has been reported in iron leaching processes at elevated temperatures (1,7). Thus, Fe^{3+} concentrations reflect the balance of oxidation and precipitation rates within a given experiment. The level of total iron in solution was the same in both the presence and absence of *M. sedula* (Figure 4) at room temperature (25°C)

over a period of 20 hours. However, in the presence of *M. sedula*, 75% of total iron was as Fe^{3+} , while for the abiotic case, Fe^{3+} was 30% of total iron, suggesting some biological activity even at reduced temperatures. ΔpH values remained relatively stable over 20 hours of incubation at 25°C (Figure 4a); ΔpH was approximately constant at -206 mV (pH_i of 5.5) (Note: all internal pH's calculated from ΔpH (mV) are corrected for temperature; see (8)), $\Delta\psi$ remained between approximately +80 mV to +100 mV and Δp between -110 mV to -130 mV. When incubated at 70°C, ΔpH increased slightly from -235 mV (pH_i of 5.5) to -250 mV (pH_i of 5.7) while $\Delta\psi$ began to drop after 3 hours from +108 mV to +50 mV after 20 hours, thereby increasing the proton motive force from -135 mV to -200 mV (Figure 5a). This increase in proton motive force was accompanied by an increase in biological iron oxidation in the parallel experiment on iron pyrite. Net increases in Fe^{3+} in solution indicate that the rate of formation of this species from Fe^{2+} exceeds that lost to precipitation. For these experiments, the total amount of iron in solution at 70°C was over 2 times that at 25°C for both the *M. sedula* and cell-free cases after 20 hours. However, for *M. sedula* at 70°C, total iron levels (171 mg/l) were higher than abiotic levels at 70°C (140 mg/l), with 88% of the iron as Fe^{3+} . The cell-free case had only 3.4% of the iron in the Fe^{3+} state. Cell densities measured in media containing pyrite remained stable over a 20 hour period for both temperatures. Inspection of pyrite under phase contrast and epifluorescence microscopy (37) also indicated significant amount of cell attachment. For the biotic case at 70°C, the rate of appearance of Fe^{3+} in solution (9.0 mg/l-hr) was comparable to the rate of disappearance of Fe^{2+} (9.3 mg/l-hr). In the absence of *M. sedula* at 70°C, Fe^{2+} appeared at a rate of 9.4 mg/l-hr, while Fe^{3+} concentration decreased at a rate of 4.8 mg/l-hr (See Figure 5b). Thus, in the absence of cells, the rate of appearance of Fe^{3+} from abiotic reactions did not offset precipitation.

Temperature shift 1: (70°C --> 85°C). When *M. sedula* was shifted from 70 to 85°C, internal pH decreased as did the positive $\Delta\psi$ (see Figure 6), presumably reflecting thermal damage to the cell membrane. These values appear to continue to decrease through the 13 hour timepoint

after heat shock with the internal pH dropping from 5.5 to 4.3 and $\Delta\psi$ falling from 80 to -5 mV. These concurrent decreases resulted in a slight increase in Δp , albeit probably for respiratory inactive cells. Before the shift, Fe^{3+} and Fe^{2+} concentration profiles resemble the non-shifted cultures at 70°C. After the shift, no significant increase in total iron released was detectable and this value remained constant at 121 mg/l total Fe in solution. The level of Fe^{3+} in solution decreased such that it was 9% of the total iron in solution, suggesting that bioleaching activity has stopped.

Temperature Shift 2: (70°C --> 80°C). Cells shifted from 70°C to 80°C on iron pyrite created only a slight increase in Fe^{3+} above abiotic controls. Proton motive force increased initially due to a drop in $\Delta\psi$, which was similar to the 70°C case. But, after 4 hours at 80°C, the internal cytoplasm had decreased from pH 5.5 to 4.6, in contrast to remaining constant for the unshifted case. Eventually, the $\Delta\psi$ stabilized at +10 mV while the internal pH continued to fall to 3.4 after 17 hours post-shift. This resulted in a decrease in Δp to about -70 mV. Ferric iron generation in solution after the shift occurred at low rates such that Fe^{3+} concentrations did not decline until after 4 hour at 80°C, at which point respiratory processes are presumably severely compromised. Ferrous iron levels continued to increase, indicating abiotic dissolution of FeS_2 with cessation of bioconversion (Figure 7).

Temperature Shift 3: (70 °C--> 80°C --> 70°C). Cells shifted from 70°C to 80°C and then back to 70°C (after 4 hours at 80°C) exhibited the iron oxidation and proton motive force phenomena of both 80°C and 70°C incubations (See Figure 8). Although pH_i is close to one unit lower than at this point in the 70°C unshifted case, Δp is similar because $\Delta\psi$ is significantly lower (close to 0 versus +65 mV in the unshifted case). At the end of the two temperature shifts, Fe^{3+} made up 64% of the iron in solution. However, the total iron concentration (250 mg/l) was higher than for the non-shifted 70°C case (171 mg/l). The Fe^{2+} release rate at 80°C was nearly

double that at 70°C, while ferric ion levels and cell densities dropped. When returned to 70°C, Fe³⁺ generation resumed at rates approximating those at the unshifted 70°C conditions. In the absence of *M. sedula*, Fe²⁺ was also released at high rates upon the shift to 80°C, although ferric ion concentrations remained near zero. Total iron in solution for the abiotic case was 160 mg/l, with 12% as Fe³⁺.

Influence of temperature shift on iron oxidation. In the 70-80-70°C shift experiment, there was a net increase of total iron released over the unshifted 70°C case. This may have been primarily the result of additional abiotic release of Fe²⁺ at the higher temperature which was rapidly oxidized to Fe³⁺ by cells shifted back to 70°C. At 80°C, the cells shifted from 70°C were probably unable to immediately take advantage of the increase of solubilized energy substrate. Thus, an intermediate shift was tried in actively growing *M. sedula* cultures to see if this strategy could be used to increase leaching rates. Shake flask cultures of *M. sedula* on FeS₂ (2% wt/vol) 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 the temporary increase in cell density; however, cell density declined to prior levels shortly afterwards. Shifted cultures exhibited a 2-fold higher ratio of ferric to ferrous iron in solution over those maintained at 73°C (0.39 at 79°C vs. 0.18 at 73°C). Although cell extracts for both shifted and non-shifted cases showed cross reactivity with anti-TF55 antibodies (Figure 9), at 79°C cultures exhibited stronger cross-reactivity than at 73°C, providing evidence of a heat shock response.

I. DISCUSSION

Table 1 is a list of the $\Delta\psi$, ΔpH and Δp values reported for various acidophilic microorganisms in addition to the thermophilic neutrophile *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. Values for $\Delta\psi$,

ΔpH and Δp 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 that measured for many mesoacidophiles but comparable to that reported for *S. acidocaldarius* ($\text{pH}_i=5.6$, $\text{pH}_o=3.5$ at 45°C) (21) (see Table 1). Comparisons of internal pH in various acidophilic mesophiles and thermophiles is interesting in view of the potential temperature effect on *in vivo* and *in vitro* cytoplasmic buffering capacity and the differences in cell membrane characteristics between archaea and eubacteria. Also, note that pH_i for *M. sedula* incubated at 25°C was similar to that at 70°C . In the studies of a similar organism, *S. acidocaldarius*, for which components of the respiratory chain have been examined (17,20, 30,33) Lüben and Schäfer (21) found $\Delta\psi$ to be inside negative in contrast to the positive inside values found for *M. sedula* in this study and reported values for other mesoacidophiles (see Table 1). *T. ferrooxidans* was found also to have a inside negative $\Delta\psi$ when incubated at pH 3.0, in contrast to its optimum pH of about 2.0 (see Table 1); since the *S. acidocaldarius* study was done at pH 3.5, this may reflect the variation of this parameter with decreasing transmembrane pH gradient.

In previous continuous culture studies, *M. sedula* fared best in media containing both ferrous iron and casamino acids (31). Attempts to separate autotrophic and heterotrophic growth of *M. sedula* resulted in limited success. Reducing the level of organic substrate, while increasing the levels of inorganic substrate, resulted in a reduction in biomass yield and higher iron turnover. However, complex organic media components could not be completely eliminated without the death of the cells. Similarly, elimination of inorganic substrates resulted in cell death. Here, cutting back on both Fe^{2+} and casamino acids in continuous culture resulted in increased levels of a 62 kDa protein immunologically related to TF55 (see Figure 2). It remains to be seen if separate changes in levels of either Fe^{2+} or casamino acids will elicit a stress response.

Shifting *M. sedula* from 70°C to either 80°C or 85°C had a significant and negative effect on cellular function. The shift to 85°C (Figure 6) causes respiratory activity to decrease as indicated by iron speciation. Although proton motive force is fairly constant in the period after heat shock, pH_i has dropped from 5.5 to 4.4 and $\Delta\psi$ has fallen to approximately zero after 13 hours. Thermal damage to the cell membrane likely circumvented the regulated routes for proton and ion exchange with the extracellular environment. It would seem that the primary protection to acidification of the cytoplasm at this point is its intracellular non-diffusible net positive charge which might be augmented by the protonation of titrable cellular constituents (24). The shift to 80°C caused 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 temperature shift is reflected in changes in pH_i and $\Delta\psi$: pH_i dropped from 5.5 to 4.6 in the first four hours after shift, at which time $\Delta\psi$ fell to near zero. After 17 hours at 80°C, pH_i had dropped further to 3.3. It is interesting that cells shifted back to 70°C at four hours were able to recover respiratory activity (Figure 8); 13 hours after being returned to 70°C, pH_i was 4.8 although $\Delta\psi$ was at or below zero. The recovery of iron-oxidizing activity seems to be done without the aid of a positive $\Delta\psi$ and may be a reflection of a concerted cellular effort to pump protons out in addition to significant changes in levels of ionic species resulting from 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 could translate to higher turnover rates of inorganic energy sources. In addition, at higher temperatures, the kinetics of chemical and biochemical FeS_2 oxidation to Fe^{2+} are enhanced. This combination of effects, if properly balanced, could be exploited to increase iron and/or sulfur oxidation rates. This scenario may explain the results shown in Figure 9. By choosing an intermediate temperature shift (73°C to 79°C), Fe^{3+} levels were significantly higher than in cultures maintained at 73°C. It follows that the main benefit of short term temperature shift

for active cultures is the boost in available energy substrate (i.e., Fe^{2+}) in culture and may even lead to a short-term boost in growth kinetics. However, this benefit is realized only if the cells are in a metabolically-competent state which supports iron/sulfur-oxidizing activities. *M. sedula* was able to stabilize the pH of its cytoplasm for a short period of time under moderate levels of thermal stress (see Figure 8), although longer periods of incubation at high temperatures results in cell death with lower levels of FeS_2 converted (see Figure 7). Further work to determine the point at which metabolic recovery from thermal stress is possible (both as a function of 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 response of extremophilic microorganisms. From an evolutionary perspective, insights into how biological systems adapted to mesophilic/neutrophilic conditions may be gained through studies of thermoacidophilic adaptation to thermal and nutritional stress. Further, the utility of thermoacidophiles for biotechnological applications will depend on better understanding on their physiology and bioenergetics and insights into how to manipulate their metabolic features to achieve bioprocessing objectives.

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

Organism	Probes ¹	T (°C)	pH _o	pH _i	ΔpH^2 (mV)	$\Delta\psi^3$ (mV)	Δp^4 (mV)	Reference
<i>Thermus thermophilus</i>	WA TPMP ⁺	70	7.5	7.5	0	-197	-197	26
<i>Sulfolobus acidocaldarius</i>	WA SCN ⁻	45	3.5	5.6	-134	-19	-153	21
<i>Thiobacillus ferrooxidans</i>	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
<i>Metallosphaera sedula</i>	SA SCN ⁻	25	2.0	5.5	-220	+85	-125	This work
	SA SCN ⁻	70	2.0	5.4	-230	+40	-190	This work
<i>Thermoplasma acidophilum</i>	WA SCN ⁻	59	2.0	6.1	-266	+10	-256	28
	TEA ⁺ SCN ⁻	56	2.0	6.5	-296	+120	-176	13
<i>Bacillus acidocaldarius</i>	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

¹Probes

SA	salicylic acid
SCN ⁻	thiocyanate
WA	weak organic acid
TPMP ⁺	triphenylmethylphosphonium
TPB ⁻	tetraphenylboron
TEA ⁺	tetraethylammonium

$$^2\Delta\text{pH} = \text{pH}_\text{O} - \text{pH}_\text{i}$$

³ $\Delta\psi$ is positive when cytoplasm is positive with respect to the surrounding medium

$$^4\Delta\text{p} = DY + 2.303 RT/F * \Delta\text{pH}$$

FIGURE 1. Schematic of continuous culture system used for the growth of *Metallosphaera sedula*. This is a modification of the system described by Brown and Kelly (5).

FIGURE 2. Effect of nutrient limitation on growth of *Metallosphaera sedula*. (a) SDS-PAGE (silver stained) and (b) Western blot (against TF55) of *M. sedula* cell extracts from continuous culture ($D = 0.05 \text{ hr}^{-1}$): L-low molecular mass markers F- fed cells (10 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2g/l casamino acids) S1- nutrient-limited cells (nutrient feed (F) cut by 50%) S2- nutrient-limited cells (nutrient feed (F) cut by 75%) H-high molecular mass markers. Note cross reactivity at 62 kDa against anti-TF55 antibodies in addition to enhanced levels of bands corresponding to lower molecular mass species in nutrient-limited cells at approximately 49, 45, 40 and 35 kDa.

FIGURE 3. Influence of medium composition on bioenergetic parameters for *Metallosphaera sedula*. Measurements are for stock samples that had been grown in continuous culture, harvested, stored at 4°C and then incubated in media at 70°C and pH 2.0. Fresh Ms refers to cells that were harvested and tested immediately.

FIGURE 4. (A) Variation of *Metallosphaera sedula* proton motive force (Δp), membrane potential ($\Delta \psi$) and transmembrane proton gradient (ΔpH) with time at 25°C and external pH 2.0. (B) Changes in iron speciation in the presence of *Metallosphaera sedula* and iron pyrite under identical conditions as in (A).

FIGURE 5. (A) Variation of *Metallosphaera sedula* proton motive force (Δp), membrane potential ($\Delta \Psi$) and transmembrane proton gradient (ΔpH) with time at 70°C and external pH 2.0. (B) Changes in iron speciation in the presence of *Metallosphaera sedula* and iron pyrite under otherwise identical conditions as in (A).

FIGURE 6. (A) Variation of *Metallosphaera sedula* proton motive force (Δp), membrane potential ($\Delta \psi$) and transmembrane proton gradient (ΔpH) with time before and after shift from 70°C $\rightarrow$ 85°C and external pH 2.0. (B) Changes in iron speciation in the presence of *Metallosphaera sedula* and iron pyrite under otherwise identical conditions as in (A).

FIGURE 7. (A) Variation of *Metallosphaera sedula* proton motive force (Δp), membrane potential ($\Delta \psi$) and transmembrane proton gradient (ΔpH) with time before and after shift from 70°C $\rightarrow$ 80°C and external pH 2.0. (B) Changes in iron speciation in the presence of *Metallosphaera sedula* and iron pyrite under otherwise identical conditions as in (A).

FIGURE 8. (a) Variation of *Metallosphaera sedula* proton motive force (Δp), membrane potential ($\Delta \Psi$) and transmembrane proton gradient (ΔpH) with time before and after shift from 70°C -> 80°C -> 70°C and external pH 2.0. (b) Changes in iron speciation in the presence of *Metallosphaera sedula* and iron pyrite under otherwise identical conditions as in (a).

FIGURE 9. Heat Shock of *M. sedula*. (a) Growth of *M. sedula* on FeS₂ at 73°C and temperature shifted from 73°C → 79°C; (b) SDS-PAGE (silver stained); and (c) Western blot (against TF55 antibodies) of *M. sedula* cell extracts. Lanes: (1) *M. sedula* shifted from 73°C → 79°C; (2) *M. sedula* held at 73°C; (3) Low molecular mass markers. After 65 hours, Fe³⁺/Fe²⁺ ratios were 0.39 with the heat shocked cells shifted to 79°C and 0.18 for cells left at 73°C.

II. Acquired thermotolerance and “stressed phase” growth of the extremely thermoacidophilic archaeon *Metallosphaera sedula* in continuous culture

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II. ABSTRACT

The response of an extremely thermoacidophilic archaeon *Metallosphaera sedula* (growth temperature range 50-79°C, T_{opt} 74°C and pH_{opt} 2.0) to thermal stress was investigated using a 10-liter continuous cultivation system. *M. sedula*, growing at 74°C, pH 2.0, and dilution rate of 0.04 hr^{-1} , was subjected to both abrupt and gradual temperature shifts in continuous culture to determine the response of cell density levels and protein synthesis patterns. An abrupt temperature shift from 74°C to 79°C resulted in little, if any, changes in cell density and a small increase in total protein/cell. When the culture temperature was shifted further to 80.5°C, cell density dropped to below 5×10^6 cells/ml from 2×10^8 cells/ml, leading to washout of the culture. Operation at this temperature and slightly higher temperatures, however, could be achieved by exposing the culture to thermal stress more gradually (0.5°C increments). As a result, stable operation could be maintained at temperatures up to 81°C, and the washout temperature could be increased to 82.5°C. Continuous culture operation at 81°C for 100 hours ("stressed phase") led to an approximately 7-fold lower steady state cell density than observed for operation at or below 79°C. However, SDS-PAGE analysis (both one- and two-dimensional) revealed significantly higher levels (6-fold increase) of a 66 kDa stress response protein (MseHSP60), immunologically related to Thermophilic Factor 55 from *Sulfolobus shibatae* (Trent, J. D., J. Osipiuk, and T. Pinkau. 1990. *J. Bacteriol.* **172**:1478-1484). If the acclimated culture was returned to lower temperatures (i.e., 74°C), the amount of MseHSP60 returned to levels observed prior to thermal acclimation. Furthermore, when the previously acclimated culture (at 81°C) was shifted back from 74°C to 81°C, without going through gradual acclimation steps, the result was the immediate onset of washout, suggesting no residual thermotolerance. This study shows that gradual thermal acclimation of *M. sedula* could only extend the temperature range of stable growth for this organism by 2°C above its maximal growth temperature, albeit at reduced cell densities. Also, this investigation illustrates the utility of continuous culture for characterizing heat shock response and assessing maximum growth temperatures for extremely thermophilic microorganisms.

II. INTRODUCTION

As the thermal environment for a particular microorganism moves to higher temperatures, cellular function is eventually compromised to such an extent that growth ceases, despite the stabilizing influences that intrinsic and extrinsic factors might provide for individual biomolecules. The thermal history of each microorganism can affect its upper temperature limit for growth, which may be elevated by thermal acclimation to supraoptimal temperatures (3). Cellular response to supraoptimal temperatures seems to have certain universal features independent of optimal growth temperature; namely, increased synthesis of one or more heat shock proteins thought to be involved in protein turnover and prevention of protein aggregation and denaturation *in vivo* (4,24,25).

The nature of heat shock response by extremely thermophilic microorganisms is intriguing, considering the fact that they thrive at temperatures far above the limits for most cells (5,25). In some organisms, stress induces elevated levels of certain intracellular compatible solutes (2,7,15,28), some of which have been shown to provide thermal stabilization to proteins *in vitro* (21). Evidence has also been provided that thermal stress induces synthesis of specific proteins. The hyperthermophilic archaeon ES4, growing optimally at 98-100°C (20), was found to produce increased levels of a 98 kDa protein when the growth temperature was shifted from 95°C to 102°C (8). Furthermore, some degree of thermotolerance could be conferred for survival of ES4 at 105°C by first exposing 95°C grown cells to 102°C for periods of 90 minutes. Another hyperthermophile, *Pyrodictium occultum*, produced increased levels of an ATPase complex (i.e., thermosome) when culture temperatures were shifted from 102°C to 108°C (18,19). This complex, comprised of 56 and 59 kDa polypeptides, was a significant fraction of total cellular protein at 108°C (73%) but a much smaller fraction at 100°C (11%). A similar ATPase complex has been identified in *Thermoplasma acidophilum* (26).

Most work to date on heat shock and thermal (acclimation) in thermophilic microorganisms has focused on members of the order Sulfolobales in the kingdom crenarchaeota. Many of these chemolithotrophic organisms grow aerobically and typically have optimal (T_{opt}) and maximal (T_{max}) growth temperatures below 100°C. It appears that, unlike many other mesophilic organisms and cells, there is only one prominent heat shock protein noted in the Sulfolobales examined to date, which has a subunit molecular weight of approximately 60 kDa (25). Several Sulfolobales have been studied. Evidence of a heat shock response in *Sulfolobus acidocaldarius* was first reported by Jerez (10), who observed significantly increased levels of a protein with molecular weight of 64-66 kDa for cells shifted from 70°C to 85°C. Trent et al. (22) showed that *Sulfolobus shibatae* strain B12 exhibited a heat shock response when the cells were shifted to 88°C, accompanied by the increased synthesis of predominant 55 kDa protein (Thermophilic Factor 55 or TF55). *S. shibatae* was found to acquire some thermotolerance at 92°C by first incubating cells at 88°C for 60 minutes or longer. This major heat shock protein in *S. shibatae* was shown to be unrelated to DnaK or GroEL, but similar to a family of eukaryotic protein, known as t-complex polypeptide-1(TCP1) (23), whose role as a molecular chaperone has not been established. Peebles and Kelly (17) showed the *Metallosphaera sedula*, which grows optimally at 74°C and pH 2.0 (9), produced increased amounts of a protein immunologically related to TF55 during temperature shift from 73 to 79°C. Shifts from 70 to 80°C were accompanied by significant reductions in cell density (from 10^7 to 10^6 cells/ml) and intracellular pH (from 5.5 to 3.3). Full recovery of the culture could be accomplished if the cells were shifted back to 70 from 80°C within 13 hours after heat shock in batch cultures.

Kagawa et al. (11) found that TF55 was actually composed of two subunits forming two homo-oligomeric rings joined in a structure called a rosettasome, and referred to this complex as the archaeal HSP60 (designated here as SshHSP60). The a- and b- subunits (MW of 59.72 kDa and 59.68 kDa, respectively) were found to be approximately 55% identical at the amino acid level. Western blot analysis using antibodies against these subunits revealed that the SshHSP60 heat

shock protein produced by *S. shibatae* was similar to those found in other members of the crenarchaeota, although not evident in the euryarchaeota. A similar version of SshHSP60 was found in *Sulfolobus solfataricus* (12) and was shown to function as a molecular chaperonin *in vitro* (6).

Given that members of the Sulfolobales appear to contain a single, prominent heat shock protein, they represent an interesting model with which the regulation of thermal stress response can be studied. These organisms are mostly thermoacidophilic, typically growing at high temperatures and low pH. Because contamination is alleviated to a great extent under these extreme growth conditions, Peebles and Kelly (17) showed that a particular Sulfolobale, *Metallosphaera sedula*, can be maintained in continuous culture for extended periods of time. The study of heat shock response and thermotolerance in continuous culture could be advantageous since complications arising from transients in growth phase and rates can be eliminated. The work reported here examines *M. sedula* response to thermal stress and addresses the growth physiology of this organism at supraoptimal temperatures with and without prior thermal acclimation. At the same time it illustrates the utility of continuous culture in studying the stress response of thermophilic organisms.

II. MATERIALS AND METHODS

Cell cultivation. *Metallosphaera sedula* (DSMZ 5348) was obtained from the Deutsch Sammlung von Microorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and cultivated using a slightly modified method from that previously described (17). A 10-liter glass chemostat (Ace Glass, Vineland, NJ) was used for thermal stress response studies. The continuous culture was agitated at 150 rpm with an impeller and aerated by sterile (0.2 μ m filter) house air at a rate of 100 cc/min. Residence time distribution studies showed that reactor

approximated a continuous stirred tank reactor which behaved as a first order system from a mixing standpoint. A Digi-Sense temperature controller (Cole-Parmer, Chicago, IL), connected to a Teflon-coated probe, was used to monitor and adjust temperature of the culture. Temperature control was typically $\pm 0.2^{\circ}\text{C}$, as confirmed periodically with a mercury glass thermometer inserted into the culture. A dilution rate of 0.04 hr^{-1} (corresponding to a 17 hour doubling time) was used to minimize chances of premature washout when thermal stress was applied to the culture. Samples were taken regularly and fixed with glutaraldehyde to monitor cell density using epifluorescence microscopy (27).

Sulfolobus shibatae (DSMZ 5389) was obtained from the DSMZ (Braunschweig, Germany) and cultivated using the culture media compositions provided from DSMZ. *S. shibatae* was grown in batch cultures at 75°C with gentle agitation to late log phase to generate biomass for gel analysis and immunoblotting.

Preparation of cell extracts. Cells were centrifuged at 7500 rpm (9500 g) in a Sorvall RC5C centrifuge (DuPont, Newtown, CT) for 40 min. Supernatant was discarded and pellets were washed at least twice with 10 mM H_2SO_4 at pH 2. To lyse the cells, the pellets were re-suspended in 100 mM sodium phosphate buffer (pH 7.5) and then disrupted using a sonicator (Model XL2020, Heat systems Inc., Farmingdale, NY) and/or a French Pressure Cell Press (SLM Instrument Inc., Urbana, IL) at 16,000 psig. Lysates were centrifuged again at 4000 rpm for 30 min. Soluble cell extracts were stored at -20°C for gel electrophoresis analysis (SDS-PAGE) and immunoblotting. Total protein concentration was determined with a dye-staining method (1) with BSA as a standard (Bio-Rad Laboratories, Richmond, CA).

One-dimensional SDS-PAGE. A modified Laemmli method (13) was used to separate *M. sedula* cell extracts on SDS-PAGE. Cell extracts were incubated at 100°C in 5X dissociation buffer, containing 400 mM Tris pH 6.8, 10% SDS and 400 mM dithiothreitol, for 10 min prior to

loading on 10% or 12.5% discontinuous SDS-PAGE. For one-dimensional SDS-PAGE, equal amounts of total protein (typically 40 μ g) were loaded in each lane. Gels were stained with Coomassie blue. The following pre-stained molecular weight markers (Gibco-BRL, Gaithersburg, MD) were used for protein size references: lysozyme (14,300), β -lactoglobulin (18,200), carbonic anhydrase (27,830), ovalbumin (43,760), bovine serum albumin (71,460), phosphorylase B (110,760), and myosin (216,760). Densitometry was performed using an Eagle Eye II system (Stratagene, La Jolla, CA).

Two-dimensional gel electrophoresis. Two-dimensional gel was performed according to the method of O'Farrell (16) by Kendrick Labs, Inc. (Madison, WI) as follows: Isoelectric focusing was carried out in glass tubes of inner diameter 2.0 mm using 2.0% pH 4-8 ampholines (BDH from Hoefer Scientific Instruments, San Francisco, CA) for 9,600 volt-hrs. 40 ng of an IEF internal standard, tropomyosin protein, with lower reference point at MW 33,000 and pI 5.2, were added to the samples. The following proteins (Sigma Chemical Co., St. Louis, MO) were added as molecular weight standards: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000) and lysozyme (14,000). Gel quantification was performed by Kendrick's Phoretix 2D software.

Immunoblotting. Gels were transferred to nitrocellulose for Western blot analysis. Proteins were probed with antibodies directed against SshHSP60 (provided by J. D. Trent, Argonne National Laboratories) in 1% gelatin. The secondary antibody was a rabbit Ig, horseradish peroxidase conjugate (Amersham, Little Chalfont, Buckinghamshire, England). Detection was performed by utilizing the ECL Western blotting kit (Amersham, Little Chalfont, Buckinghamshire, England).

Thermal acclimation experiments. To study thermotolerance and heat shock response in *M. sedula*, the continuous culture was subjected to various degrees of long-term thermal stress. The continuous culture was shifted abruptly (2°C increment) or gradually (0.5°C increments), after the culture stabilized at a particular temperature. (This is not true, as you have said to reviewer #1. How can we change this?) Dilution rate of culture was 0.04 hr⁻¹. Cells were harvested from continuous culture after the new steady state was achieved, typically after 3 to 4 reactor volume changes which corresponds to 95% and 98% approaches to steady state, respectively, based on mixing characteristics.

II. RESULTS

Response of *M. sedula* to thermal stress in continuous culture. Most studies focusing on the response of cells and organisms to thermal stress are done in batch culture in which abrupt temperature shifts are applied. Even experiments in which some degree of thermal acclimation is sought, sudden temperature shifts to batch cultures are typically used. Here, continuous culture was used to eliminate problems in interpreting results that may arise from transient growth conditions and to permit flexibility in adjusting the time course of temperature shifts.

M. sedula has a reported growth temperature optimum (T_{opt}) of 74°C-75°C, with a maximum temperature for growth (T_{max}) of 79°C. In 10 liter (working volume) continuous culture at pH 2.0 and dilution rate 0.04 hr⁻¹, *M. sedula* was subjected to series of temperature shifts within the normal growth range as well as above it. In the first series of experiments (see Figure 1), the culture temperature was shifted from 73°C -> 76°C -> 79°C over a period of 220 hours, with no significant change in cell density. After holding the culture at 79°C for over 100 hours (4 reactor volume changes), the temperature was shifted to 80.5°C, resulting in a dramatic reduction in culture cell density from 10⁸ cells/ml to less than 5 x 10⁶ cells/ml, indicating the onset of washout.

At this point, the temperature was shifted back to 74°C, resulting in the complete recovery of the culture cell density to over 10^8 cells/ml. The recovery rate after the shift of the cells at 80.5°C to 74°C, computed from the increase in cell density from 280 to 350 hours, corresponded to a doubling time of approximately 17 hours; this is equivalent to the dilution rate. It was apparent from this experiment, that an abrupt temperature shift upwards of only 1.5°C at 79°C was sufficient to cause the culture to washout, even at this relatively low dilution rate.

Having determined the sensitivity of the culture to temperature changes around the reported T_{max} , operation of the culture at and above this temperature was examined, as shown in Figure 2. Here, the culture was brought to 79°C from 74°C and then to 80°C and then held at 80°C for 125 hours. Unlike the first experiment (in which the shift was from 79°C to 80.5°C, as shown in Figure 1), the shift from 79°C to 80°C resulted in little, if any, change in culture cell density. The culture was then shifted in increments of 0.5°C. Upon shifting to 80.5°C from 80°C, no immediate drop in cell density was noted, although a further shift to 81°C caused the cell density to drop to approximately 2×10^7 cells/ml. Subsequent 0.5°C shifts upward in temperature (81°C $\rightarrow$ 81.5°C $\rightarrow$ 82°C) resulted in further decreases in cell density until evidence for the onset of culture washout at 82.5°C was noted. At this point, the culture could be stabilized by decreasing the temperature to 81°C, where a steady state operation could be maintained at a cell density of 2×10^7 cells/ml. By dropping the temperature back to near T_{opt} (74°C), the culture cell density gradually recovered to the same levels ($\sim 10^8$ cells/ml) observed prior to imposition of thermal stress.

The significance of thermal acclimation for *M. sedula* can be seen from the results of the experiment shown in Figure 3. If the cells were first acclimated at 79-80°C, the continuous culture could be operated at 81°C in steady state, albeit at a reduced cell density. If the culture operating at 81°C was shifted back to 74°C, the cell density returned to the levels observed previously at 74°C prior to thermal acclimation. A similar trend was also observed in amounts of total protein per cell. Figure 4 shows when the culture temperature was shifted back to 74°C, the protein/cell level returned to the one observed prior to 81°C exposure. It also shows a slight increase in the

protein/cell level between 74 and 80°C as temperature increases and 5 to 6-fold increase in protein/cell level during thermal stress at 81°C. However, if the culture at 74°C was then shifted abruptly (without first gradual exposure to 79-80°C), cell density plummeted immediately to less than 8×10^6 cells/ml and the onset of culture washout was observed. These results suggest that the culture that had been acclimated to a supraoptimal growth temperature passed along no lasting capacity to withstand an abrupt temperature shift, when it returned to normal growth temperatures.

Characterization of thermal stress response of *M. sedula* in continuous culture.

Studies of other cells and microorganisms exposed to thermal stress have revealed changes in metabolic patterns in addition to increases and decreases in the synthesis or expression of specific proteins (4, 24,25). It was shown previously that *M. sedula* produced increased amounts of an approximately 60 kDa heat shock protein (designated MseHSP60), which cross reacted with antibodies generated against a molecular chaperone/heat shock protein (SshHSP60) identified in *S. shibatae* (17). Changes in the level of MseHSP60 in *M. sedula* was indicative of the organism's response to thermal stress.

Samples taken from the continuous culture experiment shown in Figure 3 were examined by one-dimensional and two-dimensional gel electrophoresis in addition to Western blot analysis. Figure 5 shows one-dimensional SDS-PAGE gel at 74°C (lane 2), 79°C (lane 3), 80°C (lane 4), 81°C (lane 5), and 74°C (lane 6). It is clear that the band corresponding to an approximately 66 kDa protein (MseHSP60) intensifies as the temperature increases from 74°C to 81°C. As the culture is returned to 74°C, the intensity of this band returns to the levels comparable to that observed initially at 74°C. Using densitometry on the SDS-PAGE in Figure 5a, a 3-fold increase in the integrated density value of these 66 kDa bands were measured for the 81°C compared to the 74°C, both before and after thermal stress (data not shown) . Figure 5b confirms this trend in heat shock protein levels by Western blot analysis of the same samples using SshHSP60 antibodies.

To quantify changes in MseHSP60 more accurately under different thermal conditions, two-dimensional electrophoresis was used to compare samples taken from 74°C (before heat shock) and 81°C (stressed phase) continuous cultures (Figure 6). Note that smaller amounts of the thermally stressed sample (81°C) was loaded (approximately one-third the amount of the 74°C sample) to resolve highly expressed 66 kDa MseHSP60. Table 1 shows the intensities of spots associated with prominent 66 kDa polypeptides in the stressed (81°C) and unstressed (74°C) cases. An approximately 6-fold overall increase in MseHSP60 isoforms could be observed in samples from 81°C compared to 74°C. In particular, the volume intensity of one isoform (pI 5.8, MW 668,989) was 73-fold higher in 81°C compared to 74°C. It was also noted that no other prominent polypeptide spot was observed in the thermally stressed sample relative to the unstressed case.

II. DISCUSSION

The work described here illustrates the sensitivity of the extremely thermoacidophilic archaeon *M. sedula* to the shifts in growth temperature at or near the upper growth temperature limit. Thermotolerance is usually defined as a transient resistance to lethal thermal treatment which can be influenced by prior exposure to sub-lethal heat treatment (14). The temperature of the sub-lethal level and length of exposure to this temperature are apparently important in imparting thermotolerance (23). Continuous culture provides a mechanism to adjust these two parameters in a systematic way, as demonstrated here. The use of continuous culture also provides some clear advantages over batch culture approaches for studying thermotolerance and thermal stress as eliminates the complications arising transients in growth phase. It also can be used to select for a culture that manages to grow, and not just to survive, under stressed conditions. By comparing the characteristics of continuous cultures under thermal stress prior to and during washout, insights into the physiological basis for growth in relation to survival may be gained.

Gradual thermal acclimation of the continuous culture of *M. sedula* led to the establishment of a "stressed phase" at 81°C, which could be maintained in steady state for extended periods of time. The culture is particularly vulnerable at this temperature and dilution rate, as even an increase of 0.5°C led to the onset of washout. This stressed phase was characterized by a significant reduction in cell density (~7-fold), in addition to the increased synthesis of a heat shock protein (MseHSP60). This single 66 kDa molecular species appears to be the only prominent heat shock protein in *M. sedula*. This is consistent with other reports focusing on heat shock response in the thermophilic archaea which show that only single major heat shock protein is typically produced (5, 25). Table 1 shows the ratio of MseHSP60 isoforms appearing on two-dimensional electrophoresis at 74°C and 81°C, confirming significant increase in the MseHSP60 level during the thermal stress. It also appears here that the increased level of MseHSP60 is positively related to acquired thermotolerance.

The reasons for the reduced cell density in the "stressed phase" at 81°C is not clear. Peebles and Kelly (17) showed that *M. sedula*, when shifted to temperatures above 80°C for any period of time, appears to lose its capability to maintain internal pH at the levels measured during growth at optimal temperatures. (Presumably, this is the result of damaging membrane integrity and/or functional problems for particular proteins at non-optimal temperatures and pH. In this sense, thermoacidophiles may be more limited in developing thermotolerance because of the potential problem of proton leakage from an external environment at pH 2.0. Efforts are underway to determine if this is the case. There also appears to be no hysteresis involving the development of thermotolerance, at least for the conditions examined in this study. Figure 3 shows that cells returned to 74°C after having grown at 81°C for extended periods are equally sensitive to an abrupt temperature shift to 81°C as cultures that had not been previously acclimated. From this perspective, it appears that thermotolerance is a transient characteristic which is inducible but not permanent

Despite slow, gradual exposure to elevated temperatures, the maximum temperature for stable growth of *M. sedula* could only be extended by approximately 2°C, from 79°C to 81°C. Reported attempts to impart thermotolerance to *S. shibatae* (22) and to *Pyrococcus* strain ES4 (8) were evaluated by the length of survival time at temperatures far in excess of the normal growth temperature. In essence, these experiments focus on the transition from stationary to death phase. In neither case did the cultures survive at supramaximal temperatures for significant periods of time and no net increase or even stabilization in levels of cell density or cell protein was observed. It remains to be seen how approaches such as the one used here will influence the level of thermotolerance of other high temperature organisms and whether long-term acclimation makes any difference compared to short-term exposure to excessive temperatures in this regard. It also raises the question whether the maximal temperature for growth of other extremely thermophilic organisms can be extended by more than a just a few degrees by thermal acclimation.

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Table 1. Polypeptide numbering and isoform ratios for the 66 kDa protein of interest in sample under thermally stressed (left) versus sample under normal growth conditions (right). Protein loading for thermally stressed cell was 34% of that for normal cells to facilitate quantification of intensity spots.

sample at 81°C (left above)	sample at 74°C (right above)
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Spot	pI	MW	Corrected Volume	Spot	pI	MW	Volume	Ratio
8	-	-	2364	-	-	-	-	-
9	5.8	66899	10694	7	5.8	66899	396	73
4	5.9	65494	22337	9	5.9	65494	3286	18
7	5.9	64440	29330	6	5.9	64440	21833	4
2	6.0	65494	19695	8	5.9	65494	4582	11
5	6.0	68849	9708	5	6.0	68849	1688	15
3	6.1	67477	19963	4	6.1	67477	8529	6
6	6.2	67477	19226	3	6.2	67477	15588	3
10	6.3	68506	1308	10	6.3	68506	766	4

Figure 1. Heat shock of *M. sedula* in 10-liter continuous culture. The cell density levels (O) prior to (0 - 250 hours), during (250 - 300 hours), and after (300 - 500 hours) heat shock are shown. Arrows indicate the temperature shifts made at the specified time points. Temperature shifts were made from 73°CÆ79°C (*M. sedula* normal growth range) to 80.5°C (heat shock) back to 74°C (optimal temperature). The onset of washout occurred at 80.5°C.

Figure 2. Thermal acclimation and heat shock of *M. sedula* in 10-liter continuous culture. The culture was subjected to gradual increases in temperature over the course of the experiment. Temperature shifts were made from 74°CÆ79°C (*M. sedula* normal growth range) to 80°CÆ80.5°CÆ81°CÆ81.5°CÆ82°CÆ82.5°CÆ81°C (thermal stress) to 75°CÆ74°C (optimal growth range). The cell density levels (O) prior to (0 - 100 hours), during (100 - 500 hours), and after (500 - 650 hours) exposure to thermal stress are shown. Arrows indicate the temperature shifts made at the specific time points. Double-headed arrow (<f>) indicate the 81°C "stressed phase." Note that the cell density is about 7-fold lower at 81°C compared to 74°C. The onset of washout occurred at 82.5°C.

Figure 3. Gradual and abrupt exposure to thermal stress on *M. sedula* in 10-liter continuous culture. This experiment examined whether thermotolerance acquired by *M. sedula* through gradual exposure to thermal stress created any residual capacity to withstand subsequent abrupt thermal stress. Arrows indicate the temperature shifts made at the specific time points. Double-headed arrow ($\longleftrightarrow$) indicates the "81°C stressed phase" during 300 and 400 hours. Temperature shifts were done in five phases: 0 to 100 hrs, normal to maximal growth range; 100 to 300 hrs, gradual thermal stress; 300 to 400 hrs, stressed phase; 400 to 500 hrs, recovery period; 500 to 600 hrs, abrupt thermal stress and subsequent washout.

Figure 4. The levels of total protein per cell and average cell densities for conditions shown in Figure 3. The plot shows the increased amount of total protein per cell (about 3-fold) and the reduction of cell density (about 7-fold) at 81°C (“stressed phase”) relative to other phases. This increase in total protein per cell at 81°C appears to be mostly the result of over-production of heat shock protein (MseHSP60) (see Figures 5 and 6).

Figure 5. Effect of temperature and thermal acclimation on protein patterns in *M. sedula* cell extracts. Samples were obtained from the conditions run in Figure 3.

(a) Samples run on 12.5% SDS-PAGE and stained with Coomassie blue. The arrow to the right indicates the locations of heat shock protein (MseHSP60) in lanes 2 to 6. Lanes: 1, prestained protein molecular weight standards (as described in Materials and Methods section); 2, 74°C; 3, 79°C; 4, 80°C; 5, 81°C; 6, 74°C; 7, cell extract from *S. shibatae* grown at 75°C. Note that the *S. shibatae* heat shock protein subunits (SshHSP60) appears to be slightly lower in molecular mass than MseHSP60.

(b) Western blot (against Thermophilic Factor 55 antibodies) analysis of *M. sedula* cell extracts from (a). Only bands from *M. sedula* and *S. shibatae* in the range of 60 to 70 kDa cross reacted with TF55 antibodies. Lanes: (see above). Cell extracts from *S. shibatae* show that two bands cross-reacted with the antibodies (lane 7), presumably a- and b- subunits of SshHSP60. Note that there are increased levels of cross-reactivity of MseHSP60 bands as the culture temperatures is increased.

a

b

Figure 6. Two-dimensional BDH(2%)/SDS(10%) polyacrylamide gel analysis of proteins from *M. sedula* at (a) 74°C and (b) 81°C in continuous culture. One-third less protein (16.9 mg) was loaded at 81°C compared to 74°C (50 mg) to resolve the isoforms of MseHSP60. For comparison and quantification of spots, the spot volume minus background volume was multiplied by 2.9 to correct for loading. In general, the spot intensities were similar in both cases except those proteins corresponding to MseHSP60 (circled). Quantification showed that the MseHSP60 isoforms increased approximately 6-fold at 81°C as compared to 74°C, and in particular, one isoform (MW 66,899 Da and pI 5.8) increased 73-fold at the higher temperature. A

total of 8 to 9 isoforms could be identified. An IEF internal standard (MW 33,000 Da and pI 5.2) was added as indicated by the arrow.

III. Biooxidation capacity of the extremely thermoacidophilic archaeon *Metallosphaera sedula* under bioenergetic challenge

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III. Abstract

The biooxidation capacity of an extremely thermoacidophilic archaeon *Metallosphaera sedula* (DSMZ 5348) was examined under bioenergetic challenges imparted by thermal or chemical stress in regard to its potential use in microbial bioleaching processes. Within the normal growth temperature range of *M. sedula* (70-79°C) at pH 2.0, upward temperature shifts resulted in bioleaching rates that followed an Arrhenius-like dependence. When the cells were subjected to supraoptimal temperatures through gradual thermal acclimation at 81°C (Han, C. J., Park, S. H., Kelly, R. M. 1997. Appl. Environ. Microbiol. **63**: 2391-2396), cell densities were reduced but 3 to 5 times faster specific leaching rates (Fe^{3+} released from iron pyrite/cell/hr) could be achieved by the stressed cells compared to cells at 79°C and 73°C, respectively. The respiration capacity of *M. sedula* growing at 74°C was challenged by poisoning the cells with uncouplers to generate chemical stress. When the protonophore 2,4-dinitrophenol (5-10 mM) was added to a growing culture of *M. sedula* on iron pyrite, there was little effect on specific leaching rates compared to a culture with no protonophore at 74°C; 25 μM levels proved to be toxic to *M. sedula*. However, a significant stimulation in specific rate was observed when the cells were subjected to 1 μM nigericin (+135%) and 2 μM (+63%); 5 μM levels of the ionophore completely arrested cell growth. The ionophore effect was further investigated in continuous culture growing on ferrous sulfate at 74°C. When 1 μM nigericin was added as a pulse to a continuous culture, a 30% increase in specific iron oxidation rate was observed for short intervals, indicating a potential positive impact on leaching when periodic chemical stress is applied. This study suggests that biooxidation rates can be increased by strategic exposure of extreme thermoacidophiles to chemical or thermal stress and this approach should be considered for improving process performance.

III. Introduction

Extreme thermoacidophiles, mostly members of the archaeal order Sulfolobales, represent a unique type of extremophile in that they must deal with high temperature and low pH simultaneously. For example, *Metallosphaera sedula*, an extremely thermoacidophilic archaeon, grows optimally at 74°C and at pH 2.0 (Huber et al., 1989). This organism and other members of the Sulfolobales grow chemolithotrophically by oxidizing reduced iron and sulfur species through a membrane-bound transport system which coordinates electron and proton fluxes to produce ATP (Cobley and Cox, 1983; Lübben and Schäfer, 1989; Schäfer et al., 1990) (see Figure 1). Since these organisms are effective in leaching of metals and sulfur from ores and coal (Clark et al., 1993; Peeples and Kelly, 1993), methods to induce higher rates of metal oxidation or desulfurization are desirable. Unfortunately, the metabolic complexity of iron/sulfur oxidation and the early stage of development for genetic systems in extreme thermoacidophiles make directed approaches to this end very difficult. Therefore, manipulation of process parameters currently represents the most available route for improvement.

Most studies done so far to improve bioleaching rates by extreme thermoacidophiles have dealt with manipulating process parameters only within normal growth ranges of temperature and pH (Boogerd et al., 1990; Kargi and Robinson, 1984; Lindström et al., 1993). Although these microorganisms already contend with growth conditions that are normally debilitating to other living cells and organisms, they can also be subjected to adverse conditions, such as heat shock, starvation, or chemical stress. Such exposures provoke an atypical or stress-induced metabolic response (De Macario and Macario, 1994; Han et al., 1997; Jerez, 1988; Lowe et al., 1993; Peeples and Kelly, 1995; Trent et al., 1994; Trent, 1996). If specific exposure to stress can be utilized and controlled, as has often been tried in medical (Burdon, 1988; Hall and Foster, 1996) and food (Archer, 1996; Jørgensen et al., 1996; Lou and Yousef, 1997; McDonald et al., 1990) applications, a new strategy for improving cellular bioleaching capacity may arise.

Studies of thermal stress in extreme thermoacidophiles have shown that either a small set of proteins or a single protein induced by heat shock has been associated with their prolonged survival at supraoptimal temperatures (Guagliardi et al., 1994; Han et al., 1997; Kagawa et al., 1995; Knapp et al., 1994; Peeples and Kelly, 1995; Trent et al., 1990; Waldmann et al., 1995). The exploitation of stress response in these organisms under bioenergetic challenges to accelerate leaching rates, however, has received little or no attention. Normally the intracellular pH of extreme thermoacidophiles is close to neutral. A significant transmembrane pH gradient must be maintained across the cell membrane which, in fact, is required for chemiosmosis and subsequent electron translocation via iron turn-over (Cobley and Cox, 1983) (see Figure 1). If this energy network were to be disrupted partially or entirely by chemical or thermal stress, one possible result is excess accumulation of protons intracellularly and a drop in internal pH (Peeples and Kelly, 1995). To survive, thermoacidophiles would need to re-establish the proton gradient, possibly through higher iron or sulfur oxidation rates, to generate ATP to compensate for the additional bioenergetic burden. The objective of this study was to investigate whether adaptation or metabolic response of extreme thermoacidophiles to certain bioenergetic challenges (i.e., heat shock or respiratory uncouplers) could be exploited to increase biooxidation rates. If successful, strategic exposure to stress would provide a mechanism to improve bioleaching process performance.

III. Materials and Methods

Cell cultivation. *Metallosphaera sedula* (DSMZ 5348) was obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). Cells were cultivated in a sterilized basal medium consisting of the following components: 0.4 g/L K_2HPO_4 , 0.4 g/L NH_4SO_4 , 0.4 g/L $MgSO_4 \cdot 7H_2O$ (Fisher Scientific, Raleigh, NC). Casamino acids (A-2427; Sigma, St. Louis) (0.2 g/L) were added as an organic carbon source. H_2SO_4 (10 N) was added to the medium to adjust the pH to 2.0. Cultures were inoculated to a starting density of 10^6

cell/mL. Cell samples (500 μ L to 1 mL) were fixed with glutaraldehyde and stained with acridine orange for enumeration by epifluorescence microscopy (Yeh et al., 1987).

Pyrite. 100 grams of -165/+250 mesh (58 to 91 μ m) Reference Material 8455 pyrite was obtained from National Institute of Standards and Technology (NIST, Gaithersburg, MD). Characterization of this reference material has been reported previously (Olson, 1991).

Batch leaching experiments. Small cultures (50 mL) were prepared by adding 1 to 2 grams of standard reference pyrite (NIST 8455) to sterile basal media with Casamino acids in shake flasks. The inoculum was subcultured from a continuous culture and all media were autoclaved prior to the beginning of a particular experiment. Typically, laboratory batch culture leaching experiments were performed at 74°C and pH 2.0 with agitation at 100 rpm in an oil bath (New Brunswick Scientific, Piscataway, NJ). Uninoculated controls were run to account for any abiotic leaching at a particular temperature and/or growth conditions. Typically, the abiotic release of ferric iron from pyrite in the controls was only about 1% of the biotic case.

Continuous culture. A 10-liter glass chemostat (Ace Glass, Vineland, NJ) was used to generate biomass for thermal and chemical stress response studies. The continuous culture was agitated at 150 rpm with an impeller and aerated by sterile (0.2 μ m filter) house air at a rate of 100 cc/min. All chemostat oxidation experiments were performed by feeding filtered (0.2 μ m pore size) and sterilized inorganic substrate (5 grams per liter of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) into the reactor vessel containing sterile basal medium with organic substrate. A dilution rate of 0.04 hr^{-1} (corresponding to a 17 hour cell doubling time) was used to minimize chances of premature washout when thermal or chemical stress was applied to the culture; washout would normally be expected to occur at more than twice this dilution rate (Han and Kelly, data not shown).

Temperature effect on bioleaching of *M. sedula*. Iron pyrite oxidation rates for *M. sedula* in batch culture were determined at 73°C (optimal growth temperature) and 79°C (maximal growth temperature). Equal number of starting cells (i.e. 1.0×10^8 cells/mL) were directly transferred from continuous culture to the pre-heated shake flasks with basal medium at pH 2.0 without iron pyrite. After several hours of incubation at either 73 or 79°C, cell densities reached nearly 10^7 cells/mL at which time iron pyrite (2.0% w/v) was added to the culture. Periodically, samples for cell density enumeration were withdrawn and counted under epifluorescence microscopy with acridine orange stain (Yeh et al., 1987). Iron assay was performed to estimate the amount of total iron, ferrous iron, and ferric iron released in the solution.

Temperature shifts and thermal stress. For the characterization of stress response from temperature elevation or heat shock and corresponding changes in iron leaching capacity of *M. sedula* in batch culture, cells were transferred from the continuous culture and then subjected to varying extents of temperature shifts. Thermal stress to the continuous culture was performed as described previously (Han et al., 1997). Briefly, the continuous culture temperature was shifted gradually (0.5°C increments), after the culture stabilized at a particular temperature. A steady-state thermally-stressed phase (Han et al., 1997) in the continuous culture could be achieved at supraoptimal temperature (81°C) at dilution rate of 0.04 hr^{-1} .

Chemical stress. For the characterization of leaching capacity of chemically-challenged cells in batch culture, various concentrations of 2,4-dinitrophenol (Sigma Chemical, St. Louis, MO) and nigericin (Sigma Chemical, St. Louis, MO) were added after the culture had reached mid-log phase ($\sim 10^7$ cells/mL). To assess the changes in biooxidation rates for chemically-stressed cells in continuous culture, nigericin (1 μM) was either held constant or injected as a pulse to the continuous culture operating at steady-state.

Iron assays. Culture samples were analyzed for total and ferrous iron concentrations by 1,10-phenanthroline-based assay (Peeples and Kelly, 1993). The amount of ferric iron released in the solution was determined by difference.

III. Results

Temperature effect on bioleaching rates within normal growth temperature ranges.

Iron pyrite leaching rates by *M. sedula* were followed at 73°C (near optimal growth temperature) and 79°C (normal maximal growth temperature) over a 4 to 5 day period. Solution cell densities typically climbed from below 10^7 to in excess of 10^8 cell/mL during this period. Pyrite leaching was initiated when the cell density reached mid to high 10^7 cells/mL. Although some cell attachment to iron pyrite was noted when samples were viewed under epifluorescence microscopy, this did not appear to be significant. Based on total protein assay only about 5% of the total protein in a given sample was attributed to attached cells compared to cells in solution (data not shown). Ferric iron levels rose rapidly after about 40 hours of incubation. Bioleaching rates were higher at 79°C (6.7×10^{-7} mg-Fe³⁺/cell/hr) compared to 73°C (4.3×10^{-7} mg-Fe³⁺/cell/hr), corresponding to an Arrhenius-like 60% increase for a 6°C temperature change. Uninoculated controls, however, showed no significant increase in ferric iron levels when temperature shift was made from 73 to 79°C.

Effect of thermal stress on bioleaching. To determine the effect of thermal stress on bioleaching capacity of *M. sedula*, samples from "stressed phase" continuous culture growing on ferrous sulfate at 81°C (Han et al., 1997) were transferred to batch culture at the same temperature without iron pyrite. Parallel cultures were grown at 73°C and 79°C. After two hours of incubation at the respective temperatures, iron pyrite (2.0% w/v) was added and cell density and iron

concentration (total, ferrous and ferric) were followed as a function of time (Figure 2). After approximately 80 hours, total Fe^{3+} released was highest for the 81°C case (see Figure 2b), despite the fact that this culture had a lower solution cell density at this point (see Figure 2a). As a result, the specific rate was 5-fold and 3-fold higher at 81°C than at 73°C and 79°C, respectively (see Figure 2c). Bioleaching rates accounted for abiotic leaching contributions determined from uninoculated controls. Abiotic leaching contributions, typically less than 1% of biotic case, were similar at all three temperatures.

Effect of respiratory uncouplers on bioleaching. The respiration capacity of *M. sedula* was challenged in batch culture by disrupting the proton and ion pumping capacity with various amounts of a protonophore (2,4-dinitrophenol at 0 to 25 μM) and ionophore (nigericin at 0 to 5 μM). Tables I and II show the specific leaching rate observed in chemically stressed and unstressed (control) cells. The differences between the controls for the two sets of uncoupler experiments arise from the different inocula used in the experiments. *M. sedula*'s bioleaching capacity was completely compromised at 2,4-DNP levels of 25 μM or nigericin levels of 5 μM . At 5 and 10 μM 2,4-DNP, the effect of specific bioleaching rate was slight. However, nigericin at either 1 or 2 μM was found to stimulate bioleaching rates considerably, resulting in 2.4-fold improvement in specific rates at 1 μM concentrations. Figure 3 shows that overall Fe^{3+} release rates also improved in the presence of 1 μM nigericin.

Continuous and pulsed addition of nigericin to continuous culture of *M. sedula*.

Continuous culture (10 L working volume) was employed to determine the response of ferrous sulfate oxidation by *M. sedula* under constant and pulsed addition of nigericin to create respiratory stress. The culture was brought to steady state at 74°C and a cell density of approximately 1.5×10^8 cell/mL (Phase I) before nigericin was added and held constant at 1 μM (Phase II) (see Figure 4a). After nigericin was added, a slight decrease in cell density with time was noted and total Fe^{3+}

concentrations rose initially then declined with time to levels or below those observed in Phase I (Figure 4b). Specific iron oxidation rates increased slightly after the addition of nigericin (Figure 4c) and then returned to levels noted before the ionophore was added.

When nigericin was added as a pulse to bring the system initially to 1 μM , a drop in cell density and total Fe^{3+} release was noted soon after ionophore injection (see Figures 5a and b). Cell densities returned to pre-pulse levels within 25 hours of nigericin injection, although total Fe^{3+} concentrations increased throughout the rest of the experiment. Although not as dramatic as in the batch experiment, specific iron oxidation rates were higher after addition of nigericin (see Figure 5c).

III. Discussion

The work described here suggests a potential strategy to accelerate iron biooxidation rates by extreme thermoacidophiles. By compromising cellular bioenergetics in various ways, iron or sulfur oxidation may be enhanced as a means to compensate for a nonideal situation. In fact, energy coupling by thermoacidophiles at high temperature and low pH may be relatively inefficient even under normal growth conditions in view of the high maintenance coefficients and the high membrane permeability to protons found for these organisms when compared to mesoacidophiles (De Vrij et al, 1988; Farrand et al., 1983; Konings et al, 1992; Kuhn et al., 1980; McKay et al., 1982). On the other hand, Elferink et al. (1994) showed *Sulfolobus acidocaldarius* has a relatively low proton permeability at 80°C compared to *Escherichia coli* or *Bacillus stearothermophilus*. In any case, Extreme thermoacidophiles presumably need to invest additional metabolic energy for maintenance of a suitable intracellular pH under stress.

Within the normal growth temperature range for *M. sedula*, specific iron oxidation rates increase with temperature; at 73 and 79°C, cell densities were comparable although iron pyrite

oxidation rates were 1.6-fold higher at the higher temperature. Through long-term thermal acclimation in continuous culture, *M. sedula* can be stabilized in a heat-shocked state ("stressed phase") at supraoptimal temperatures (i.e., 81°C), although cell densities here are reduced by 5-fold or more (Han et al., 1997). In this stressed state, maintenance requirements should be more significant to overcome additional burdens placed on the cell, such as leakage of protons into the cytoplasm. To compensate, *M. sedula* would need to enhance its proton pumping capacity, possibly fueled by ATP generated through additional dissimilatory iron and sulfur oxidation. This would explain the higher specific bioleaching rates at 81°C.

Collapse of proton pumping circuits under thermal stress can arise from increased membrane permeability to proton leakage. Thermal damage to membrane structural components or partial or total inactivation of proteins involved in maintaining the transmembrane pH potential would lead to an acidification in internal pH for thermoacidophiles. Uncouplers (e.g., protonophores and ionophores) to some extent mimic this effect by directly or indirectly compromising the pH gradient across the cell membrane. Sub-lethal levels of these compounds have been found to induce a compensatory bioenergetic response in mesophilic microorganisms, which has been referred to as "low energy shock" (Lewis et al., 1994). De Melo and co-workers (1996) demonstrated protonophores, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and nisin, caused a decline in proton motive force in *Staphylococcus aureus* and *E. coli*. This could be reestablished by the increased rate of proton extrusion through the respiratory chain and subsequent increase in O₂ consumption. Increased respiration rates were also observed for *Paracoccus denitrificans* under the influence of 2,4-DNP (Stouthamer and Bettenhausen, 1981). Gage and Neidhardt (1993) showed that wild-type *E. coli* could adapt to sub-lethal levels of 2,4-DNP, after an acclimation period, to grow at rates achieved before uncoupler addition. Concomitantly, increased synthesis of 53 proteins was observed in the chemically stressed cells, including a number of heat-shock proteins. Recovery from 2,4-DNP-induced chemical stress was accelerated if pyrroloquinoline quinone (PQQ), a cofactor for glucose dehydrogenase (Matsushita

et al., 1987) was added, leading to the oxidation of glucose to gluconate. They suggested that the electrons generated from glucose oxidation passed through the electron transport chain, thus an alternative energetic pathway was utilized to maintain or reestablish proton motive force.

In this study, sub-lethal levels of 2,4-DNP had little, if any, effect on specific biooxidation rates by *M. sedula*. Other bioenergetic alternatives that did not impact bioleaching rates could have been operable as a means to reestablish proton motive force under "low energy shock" (Lewis et al., 1994). Sub-lethal levels of nigericin, an ionophore, however, were found to stimulate biooxidation rates by *M. sedula*. Why the ionophore effect on iron turnover was more significant than the protonophore is not clear. Perhaps, the different mechanisms by which these uncouplers work to challenge respiration explains the variations in iron oxidation rates. Nigericin catalyzes the carrier-mediated antiport of K^+ for H^+ (Harold, 1986), extruding protons from outside to inside the cell membrane as a means of dissipating the proton gradient. *M. sedula* may have compensated for this by pumping these protons back out through the electron transport chain. The respiration rate would then increase, coupled to the increased rate of ferrous to ferric iron oxidation (see Figure 6). Additional work focusing on other uncouplers and their effect on proton motive force in this system needs to be done to investigate the above hypothesis.

There is still much to be learned about the relationship between chemical and thermal stresses and their impact on biooxidation capacity of extremely thermoacidophilic microorganisms. However, these results indicate this strategy might be exploited to improve biooxidation rates. It remains to be seen if such a strategy can be implemented in an optimal and economic way in a process setting. Alternatively, as techniques for genetic manipulation of these organisms develop, it may be possible to modify key bioenergetic pathways to render them less efficient and thereby improve overall biooxidation performance.

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Table 1. Protonophore (2,4-DNP) effects on specific leaching rates of *M. sedula*.

	pyrite leaching rate (mg-Fe ³⁺ /cell/hr) x 10 ¹⁰	% increase
control	7.0	0
5 µM DNP	5.9	-16
10 µM DNP	7.8	+11
25 µM DNP	-	-

Table 2. Ionophore (nigericin) effects on specific leaching rates of *M. sedula*.

	pyrite leaching rate (mg-Fe ³⁺ /cell/hr) x 10 ¹⁰	% increase
control	1.9	0
1 µM nigericin	4.5	+135
2 µM nigericin	3.1	+63
5 µM nigericin	-	-

Figure 1

Figure 1. Generalized chemiosmotic network for extreme thermoacidophiles under normal growth conditions. This model (modified from Cobley and Cox, 1983) couples the generation of proton motive force to the phosphorylation of ADP and Fe^{2+} oxidation. Outside protons are translocated to inside the cell via an ATPase channel and pumped out through the electron transport chain (ETC). The electrons released from Fe^{2+} oxidation are consumed by oxygen which acts as a terminal electron acceptor.

Figure 2a

Figure 2b

Figure 2c

Figure 2. Temperature effect on bioleaching kinetics by *M. sedula*.

(a) Cell densities at 73°C (optimal growth temperature), 79°C (maximal growth temperature), and 81°C (heat shock) are shown, (b) Ferric iron released by *M. sedula* at 73°C, 79°C, and 81°C corrected for abiotic contributions at these temperatures, (c) Ferric iron released per cell per hour at 73°C, 79°C, and 81°C. Specific leaching rates ($\text{mg-Fe}^{3+}/\text{cell/hr} \times 10^{-10}$) were 4.1 (for 73°C), 6.6 (for 79°C), and 20.0 (for 81°C).

Figure 3

Figure 3. Nigericin effect on overall ferric iron released by *M. sedula* in batch cultures. The amount of ferric iron released in the solution (mg/L) for unstressed cells (control) and stressed (1 μ M, 2 μ M, and 5 μ M nigericin) cells is shown. Arrow indicates the time at which nigericin was added.

Figure 4a

Figure 4b

Figure 4c

Figure 4. *M. sedula* biooxidation kinetics in continuous culture in the presence of 1 μ M nigericin. (a) The cell density level (cells/mL) is shown before (phase I) and after (phase II) 1 μ M nigericin addition, (b) Total, ferrous, and ferric iron released before (phase I) and after (phase II) nigericin addition, (c) Specific leaching rates for before (phase I) and after (phase II) nigericin addition.

Figure 5a

Figure 5b

Figure 5c

Figure 5. Pulse injection of nigericin and its impact on *M. sedula* biooxidation kinetics in continuous culture. (a) Cell density before and after 1 μM pulse of nigericin. Arrow indicates the time point when the pulse of nigericin was added, (b) Overall ferric iron released before and after 1 μM nigericin pulse, (c) Specific leaching rates before and after 1 μM nigericin pulse.

Figure 6

Figure 6. Proposed mechanism for *M. sedula*'s chemiosmotic network under nigericin stress. Nigericin catalyzes the antiport of K^+ for H^+ , leading to a dissipation of the proton gradient. To re-establish the gradient, proton and electron translocations would need to increase, fueled by increased Fe^{2+} oxidation rates.