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Acquired Thermotolerance and Stressed-Phase Growth of the Extremely Thermoacidophilic Archaeon *Metallosphaera sedula* in Continuous Culture

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The response of an extremely thermoacidophilic archaeon, *Metallosphaera sedula* (growth temperature range, 50 to 79°C; optimum temperature, 74°C; optimum pH, 2.0), to thermal stress was investigated by using a 10-liter continuous cultivation system. *M. sedula*, growing at 74°C, pH 2.0, and a dilution rate of 0.04 hr^{-1}, was subjected to both abrupt and gradual temperature shifts in continuous culture to determine the responses of cell density levels and protein synthesis patterns. An abrupt temperature shift from 74 to 79°C resulted in little, if any, changes in cell density and a small increase in total protein per 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 of up to 81°C, and the washout temperature could be increased to 82.5°C. Continuous culture operation at 81°C for 100 h (stressed phase) led to an approximately sevenfold lower steady-state cell density than that observed for operation at or below 79°C. However, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (both one and two dimensional) revealed significantly higher levels (sixfold increase) of a 66-kDa stress response protein (MseHSP60), immunologically related to Thermophilic Factor 55 from *Sulfolobus shibatae* (J. D. Trent, J. Osipiuk, and T. Pinkau, J. Bacteriol. 172:1478–1484, 1990). If the acclimated culture was returned to a lower temperature (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 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.

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 to 100°C (20), was found to produce increased levels of a 98-kDa protein when the growth temperature was shifted from 95 to 102°C (8). Furthermore, some degree of thermotolerance could be conferred for survival of ES4 at 105°C by first exposing cells grown at 95°C to a temperature of 102°C for periods of 90 min. Another hyperthermophile, *Pyrodictium occultum*, produced increased levels of an ATPase complex (i.e., thermosome) when culture temperature was shifted from 102 to 108°C (18, 19). This complex, constituted of 56- and 59-kDa polypeptides, was a significant fraction of total cellular protein at 108°C (73%) but was 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 growth temperatures (T_{opt}) and maximal growth temperatures (T_{max}) 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, and it has a subunit molecular mass 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 a molecular mass of 64 to 66 kDa for cells shifted from 70 to 85°C. Trent et al.

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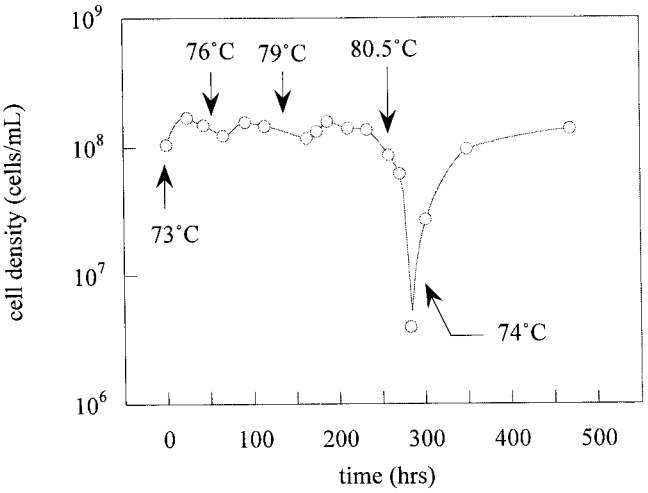

FIG. 1. Heat shock of *M. sedula* in 10-liter continuous culture. The cell density levels prior to (0 to 250 h), during (250 to 300 h), and after (300 to 500 h) heat shock are shown. Arrows indicate the temperature shifts made at the specified time points. Temperature shifts were made from 73 to 79°C (*M. sedula* normal growth range) to 80.5°C (heat shock) and back to 74°C (T_{opt}). The onset of washout occurred at 80.5°C.

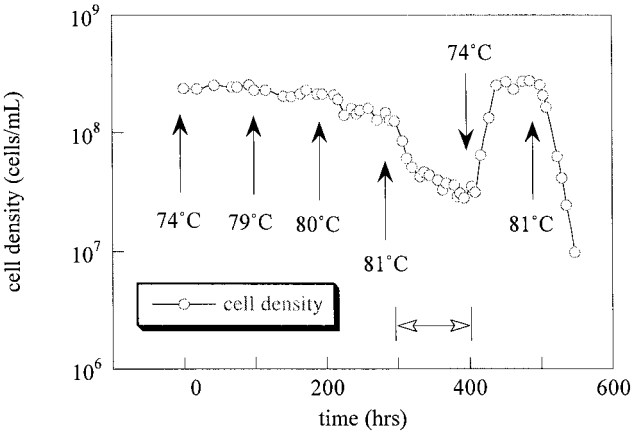

FIG. 3. Gradual and abrupt exposure to thermal stress on *M. sedula* in 10-liter continuous culture. This experiment examined whether thermotolerance acquired by *M. sedula* through gradual exposure to thermal stress created any residual capacity to withstand subsequent abrupt thermal stress. Arrows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

(22) showed that *Sulfolobus shibatae* B12 exhibited a heat shock response when the cells were shifted to 88°C, accompanied by the increased synthesis of a predominant 55-kDa protein (Thermophilic Factor 55 or TF55). *S. shibatae* was found to acquire some thermotolerance at 92°C by a prior incubation of cells at 88°C for 60 min or longer. This major heat shock protein in *S. shibatae* was shown to be unrelated to DnaK or GroEL but similar to a eukaryotic protein family known as t-complex polypeptide-1 (TCP1) (23), whose role as a molecular chaperone has not been established. Peeples and Kelly (17) showed that *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 h after heat shock in batch cultures.

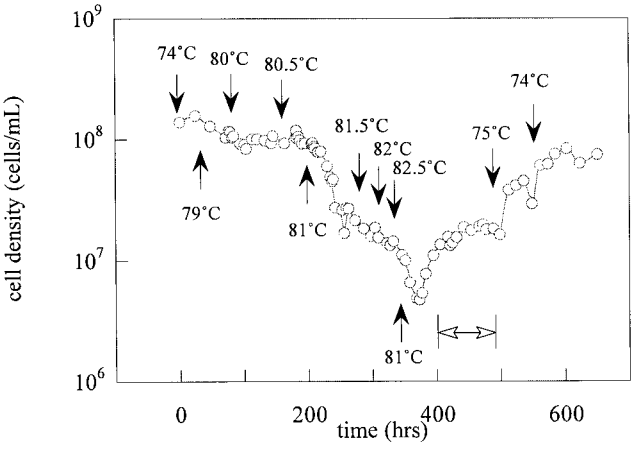

FIG. 2. Thermal acclimation and heat shock of *M. sedula* in 10-liter continuous culture. The culture was subjected to gradual increases in temperature over the course of the experiment. Temperature shifts were made from 74°C→79°C (*M. sedula* normal growth range) to 80°C→80.5°C→81°C→81.5°C→82°C→82.5°C→81°C (thermal stress) to 75°C→74°C (optimal growth range). The cell density levels prior to (0 to 100 h), during (100 to 500 h), and after (500 to 650 h) exposure to thermal stress are shown. Arrows indicate the temperature shifts made at the specific time points. Double-headed arrow indicates the 81°C stressed phase. Note that the cell density is about sevenfold lower at 81°C compared to 74°C. The onset of washout occurred at 82.5°C.

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 they referred to this complex as the archaeal HSP60 (designated here as SshHSP60). The α and β subunits (molecular masses of 59.72 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, Peeples and Kelly (17) showed that *M. sedula* could be maintained in continuous culture for extended periods of time. The study of heat shock response and thermotolerance in continuous culture is advantageous since complications arising from transient growth phases and rates can be eliminated. The work reported here examines the response of *M. sedula* 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.

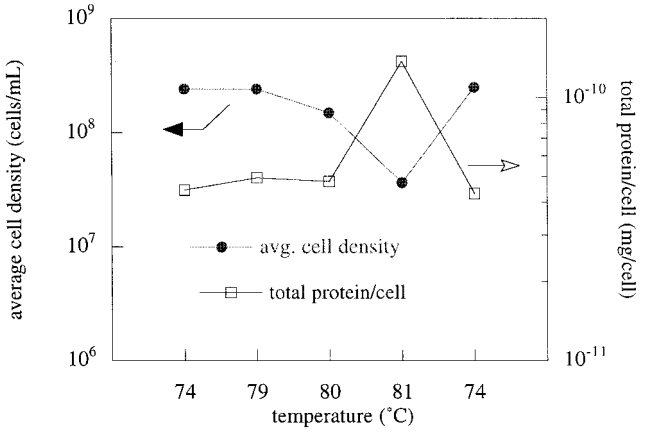

FIG. 4. Levels of total protein per cell and average cell densities. The data shown were obtained from the conditions described in the legend for Fig. 3. The plot shows the increased amount of total protein per cell (about four to fivefold) and the reduction of cell density (about sevenfold) at 81°C (stressed phase) relative to other phases. This increase in total protein per cell at 81°C appears to be mostly the result of overproduction of the heat shock protein MseHSP60 (see Fig. 5 and 6).

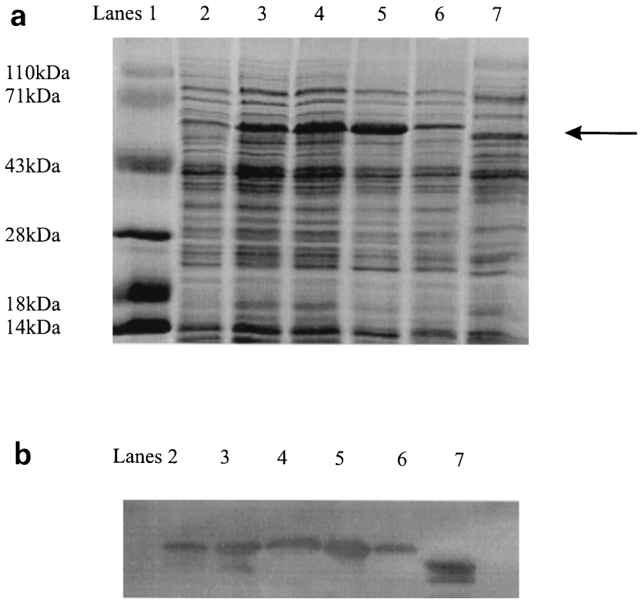

FIG. 5. Effects of temperature and thermal acclimation on protein patterns in *M. sedula* cell extracts. Samples were obtained from the conditions described in the legend for Fig. 3. (a) Samples run on an SDS–12.5% PAGE gel and stained with Coomassie blue. The arrow to the right indicates the locations of heat shock protein MseHSP60 in lanes 2 to 6. Lanes: 1, prestained protein molecular weight standards (as described in Materials and Methods); 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) appear to be slightly lower in molecular mass than MseHSP60. (b) Western blot (against TF55 antibodies) analysis of *M. sedula* cell extracts shown in panel a. Only bands from *M. sedula* and *S. shibatae* in the range of 60 to 70 kDa cross-reacted with TF55 antibodies. Contents of lanes are as described above. Cell extracts from *S. shibatae* show that two bands cross-reacted with the antibodies (lane 7), presumably the α and β subunits of SshHSP60. Note that there are increased levels of cross-reactivity of the MseHSP60 bands as the culture temperature is increased.

MATERIALS AND METHODS

Cell cultivation. *M. sedula* (DSMZ 5348) was obtained from the Deutsch Sammlung von Microorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and cultivated by a slightly modified method from that previously described (17). A 10-liter glass chemostat (Ace Glass, Vineland, N.J.) was used for thermal stress response studies. The continuous culture was agitated at 150 rpm with an impeller and aerated by sterile (0.2-μm-pore-size filter) house air at a rate of 100 cm^3/min. Residence time distribution studies showed that this 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, Ill.), connected to a Teflon-coated probe, was used to monitor and adjust the temperature of the culture. Temperature control was typically ± 0.2°C, as confirmed periodically with a mercury glass thermometer inserted into the culture. A dilution rate of 0.04 h^{-1} (corresponding to a 17-h 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 by epifluorescence microscopy (27).

S. shibatae (DSMZ 5389) was obtained from the DSMZ and cultivated by using the culture media compositions provided by the 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 7,500 rpm (9,500 × *g*) in a Sorvall RC5C centrifuge (DuPont, Newtown, Conn.) 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 resuspended in 100 mM sodium phosphate buffer (pH 7.5) and then disrupted with a sonicator (model XL2020; Heat Systems Inc., Farmingdale, N.Y.) and/or a French pressure cell press (SLM Instrument Inc., Urbana, Ill.) at 16,000 lb/in^2. Lysates were centrifuged again at 4,000 rpm for 30 min. Soluble cell extracts were stored at −20°C for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and immunoblotting. Total protein concentration was determined by a dye-staining method (1) with bovine serum albumin as a standard (Bio-Rad Laboratories, Richmond, Calif.).

One-dimensional SDS-PAGE. A modified Laemmli method (13) was used to separate *M. sedula* cell extracts on SDS-PAGE gels. Cell extracts were incubated at 100°C in 5× dissociation buffer (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 gels. For one-dimensional SDS-PAGE, equal amounts of total protein (typically 40 μg) were loaded in each lane. Gels were stained with Coomassie blue. The following prestained 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 with an Eagle Eye II system (Stratagene, La Jolla, Calif.).

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (16) by Kendrick Labs, Inc. (Madison, Wis.) as follows. Isoelectric focusing (IEF) was carried out in glass tubes (inner diameter, 2.0 mm) with 2.0% ampholines (pH 4 to 8) (BDH; Hoefer Scientific Instruments, San Francisco, Calif.) for 9,600 V · h. Forty nanograms of an IEF internal standard, tropomyosin protein, with lower reference point at a molecular weight of 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 using 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 immunoglobulin-horseradish peroxidase conjugate (Amersham, Little Chalfont, England). Detection was performed by utilizing the ECL Western blotting kit (Amersham).

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. The dilution rate of the culture was 0.04 h^{-1}. Cells were harvested from continuous culture after the new steady state was achieved, typically after three to four reactor volume changes, which corresponds to 95 and 98% approaches to steady state, respectively, based on mixing characteristics.

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 in experiments in which some degree of thermal acclimation is sought, sudden temperature shifts in 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 T_{opt} of 74 to 75°C and a T_{max} of 79°C. In 10 liters (working volume) of continuous culture at pH 2.0 and dilution rate 0.04 h^{-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 (Fig. 1), the culture temperature was shifted from 73 to 76 to 79°C over a period of 220 h, with no significant change in cell density. After holding the culture at 79°C for over 100 h (four reactor volume changes), the temperature was shifted to 80.5°C, resulting in a dramatic reduction in culture cell density from 10^8 cells/ml to less than 5×10^6 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 to 74°C, computed from the increase in cell density from 280 to 350 h, corresponded to a doubling time of approximately 17 h; 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 wash out, even at this relatively low dilution rate.

After the sensitivity of the culture to temperature changes around the reported T_{max} was determined, the operation of the culture at and above this temperature was examined, as shown in Fig. 2. Here, the culture was brought first to 79°C from 74°C and then to 80°C and held at 80°C for 125 h. Unlike the first experiment (in which the shift was from 79 to 80.5°C [Fig. 1]), the shift from 79 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 to 81.5 to 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 Fig. 3. If the cells were first acclimated at 79 to 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 that when the culture temperature was shifted back to 74°C, the protein/cell ratio returned to the one observed prior to 81°C exposure. It also shows a slight increase in the protein/cell ratio between 74 and 80°C as temperature increased and a four- to fivefold increase in the protein/cell ratio during thermal stress at 81°C. However, if the culture at 74°C was then shifted abruptly (without first gradual exposure to 79 to 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)

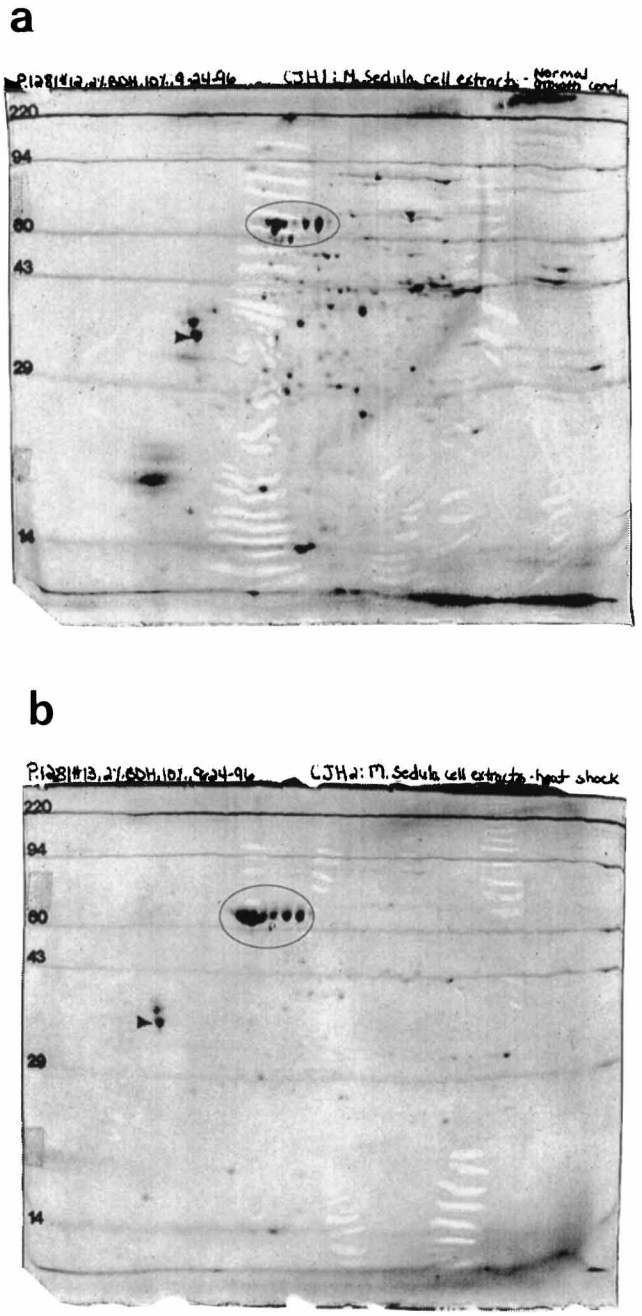

FIG. 6. Two-dimensional BDH(2%)/SDS(10%) polyacrylamide gel analysis of proteins from *M. sedula* at 74°C (a) and 81°C (b) in continuous culture. Two-thirds less protein (16.9 μg) was loaded at 81°C compared to that at 74°C (50 μg) to resolve the isoforms of MseHSP60. For comparison and quantification of spots, the spot volume minus background volume was multiplied by 2.9 to correct for loading. In general, spot intensities were similar in both cases except for those proteins corresponding to MseHSP60 (circled). Quantification showed that the MseHSP60 isoforms increased approximately 6-fold at 81°C compared to 74°C, and in particular, one isoform (MW 66,899 and pI 5.8) increased 73-fold at the higher temperature. A total of eight to nine isoforms could be identified. An IEF internal standard (MW 33,000 and pI 5.2) was added and is indicated by the arrowheads.

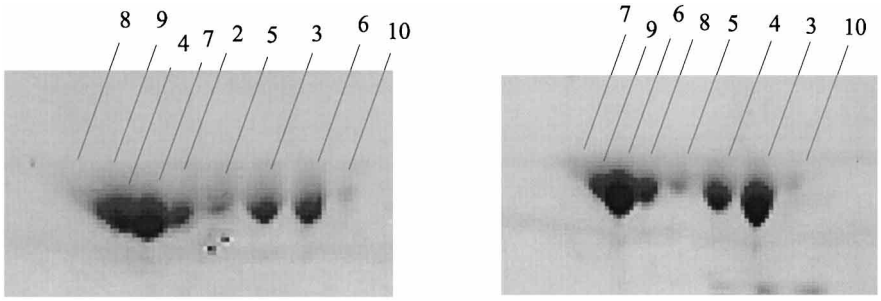

FIG. 7. Polypeptide numbering for the 66-kDa proteins of interest in sample under thermally stressed (left) and sample under normal growth conditions (right). Protein loading for thermally stressed cell was 34% of that for normal cells to facilitate quantification of intensity spots.

TABLE 1. Comparison of MseHSP60 isoforms expressed at 81 and 74°C

Characteristics of MseHSP60 isoforms expressed at:								Ratio
81°C				74°C				
Spot	pI	Mol wt	Corrected vol	Spot	pI	Mol wt	Vol	
8			2,364					
9	5.8	66,899	10,694	7	5.8	66,899	396	73
4	5.9	65,494	22,337	9	5.9	65,494	3,286	18
7	5.9	64,440	29,330	6	5.9	64,440	21,833	4
2	6.0	65,494	19,695	8	5.9	65,494	4,582	11
5	6.0	68,849	9,708	5	6.0	68,849	1,688	15
3	6.1	67,477	19,963	4	6.1	67,477	8,529	6
6	6.2	67,477	19,226	3	6.2	67,477	15,588	3
10	6.3	68,506	1,308	10	6.3	68,506	766	4

identified in *S. shibatae* (17). Changes in the level of MseHSP60 in *M. sedula* were indicative of the organism's response to thermal stress.

Samples taken from the continuous culture experiment shown in Fig. 3 were examined by one-dimensional and two-dimensional gel electrophoresis in addition to Western blot analysis. Figure 5 shows a 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 to 81°C. As the culture is returned to 74°C, the intensity of this band returns to levels comparable to that observed initially at 74°C. By using densitometry on the SDS-PAGE gel shown in Fig. 5a, a threefold increase in the integrated density value of these 66-kDa bands was measured for the cultures at 81°C compared to that of the culture at 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 (Fig. 6). Note that smaller amounts of the thermally stressed sample (81°C) were loaded (approximately one-third the amount of the 74°C sample) to resolve highly expressed 66-kDa MseHSP60. Figure 7 and Table 1 show the intensities of spots associated with prominent 66-kDa polypeptides in the stressed (81°C) and unstressed (74°C) cases. An approximately sixfold 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 66,899) was 73-fold higher at 81°C compared to 74°C (Table 1). It was also noted that no other prominent polypeptide spot was observed in the thermally stressed sample relative to the unstressed case.

DISCUSSION

The work described here illustrates the sensitivity of the extremely thermoacidophilic archaeon *M. sedula* to 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 sublethal heat treatment (14). The temperature of the sublethal 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 it eliminates the complications arising from transient conditions 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 (sevenfold), 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 a single major heat shock protein is typically produced (5, 25). Table 1 shows the ratio of MseHSP60 isoforms appearing on two-dimensional electrophoresis gels at 74 and 81°C, confirming a significant increase in the MseHSP60 level during 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 are not clear. Peeples 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 tem-

peratures. Presumably, this is the result of damaged membrane integrity and/or functional problems for particular proteins at nonoptimal temperatures and pHs. 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 under way 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 as 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 to 81°C. Reported attempts to impart thermotolerance to *S. shibatae* (22) and to *Pyrococcus* sp. 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 were focused 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 in 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 of whether the maximal temperature for growth of other extremely thermophilic organisms can be extended by more than just a few degrees by thermal acclimation.

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