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Minimal sulfur requirement for growth and sulfur-dependent metabolism of the hyperthermophilic archaeon *Staphylothermus marinus*

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Summary *Staphylothermus marinus* is an anaerobic hyperthermophilic archaeon that uses peptides as carbon and energy sources. Elemental sulfur (S°) is obligately required for its growth and is reduced to H_2S. The metabolic functions and mechanisms of S° reduction were explored by examining S°-dependent growth and activities of key enzymes present in this organism. All three forms of S° tested—sublimed S°, colloidal S° and polysulfide—were used by *S. marinus*, and no other sulfur-containing compounds could replace S°. Elemental sulfur did not serve as physical support but appeared to function as an electron acceptor. The minimal S° concentration required for optimal growth was 0.05% (w/v). At this concentration, there appeared to be a metabolic transition from H_2 production to S° reduction. Some enzymatic activities related to S°-dependent metabolism, including sulfur reductase, hydrogenase, glutamate dehydrogenase and electron transfer activities, were detected in cell-free extracts of *S. marinus.* These results indicate that S° plays an essential role in the heterotrophic metabolism of *S. marinus*. Reducing equivalents generated by the oxidation of amino acids from peptidolysis may be transferred to sulfur reductase and hydrogenase, which then catalyze the production of H_2S and H_2, respectively.

Keywords: electron transfer activity, glutamate dehydrogenase, hydrogen, hydrogenase, hyperthermophile, sulfide, sulfur reductase.

Introduction

Many microorganisms reduce elemental sulfur (S°) to H_2S (Fauque et al. 1991). Microbial S° reduction was first discovered in *Desulfuromonas* and *Desulfovibrio* (Pfennig et al. 1976, Biebl et al. 1977). Currently, more than 90 species of archaea and bacteria, most of which are strictly anaerobic hyperthermophilic microorganisms (Adams 1994, 1999, Kelly and Adams 1994, Stetter 1996), are known to reduce S° to H_2S (Hao 2003). The metabolic mechanisms, bioenergetic benefit and the nature of the enzymes involved in microbial S° reduction are unclear (Adams 1999) because only a few S°-reducing species have been studied in detail. These include the mesophilic bacterium *Wolinella succinogenes* (Schröder et al. 1988, Schauder and Kröger 1993, Jankielewicz et al. 1995, Krafft et al. 1995), moderately thermophilic iron-oxidizing bacteria (Sugio et al. 1998, Ng et al. 2000) and the hyperthermophilic archaea *Pyrodictium brockii* (Pihl et al. 1991, 1992, Maier 1996), *Pyrodictium abyssi* (Dirmeier et al. 1998) and *Pyrococcus furiosus* (Ma et al. 1993, 2000, Ma and Adams 1994, Adams et al. 2001, Schut et al. 2001). Among the latter, only *P. brockii* and *P. abyssi* are obligatory S° reducers. *Wolinella succinogenes* and *Pyrodictium* species appear to conserve energy by S° respiration (Schröder et al. 1988, Pihl et al. 1991, 1992, Schauder and Kröger 1993, Maier 1996, Dirmeier et al. 1998), which occurs in a membrane-bound complex consisting of at least hydrogenase and sulfur reductase. Hydrogenase oxidizes H_2, and the electrons are transferred to sulfur reductase, which reduces S° to H_2S (Hedderich et al. 1999). Elemental sulfur respiration generally benefits organisms by coupling S° reduction to ATP synthesis. However, sulfur reductase has not been identified in the membranes of *P. furiosus*, a heterotrophic S°-reducer that has been studied intensively, and in which H_2S production has been demonstrated as an energy-conserving process (Schicho et al. 1993). On the contrary, its sulfur-reducing activities were found only in the cytoplasm of the cell. *Pyrococcus furiosus* can grow both with and without S°, suggesting that the energy-conserving mechanism in this heterotrophic S°-reducing organism is more complicated.

Staphylothermus marinus is an anaerobic hyperthermophilic S°-reducer that grows at temperatures up to 98 °C, with an optimal temperature of 85 or 92 °C, depending on the growth substrates (Fiala et al. 1986). It uses peptides as carbon and energy sources, and does not grow in the absence of S°. These unique physiological properties make *S. marinus* a suitable subject for studying S°-dependent metabolism of hyperthermophiles. We report here the determination of the minimal S° requirement for growth and S°-dependent peptide metabolism of *S. marinus*, as well as the activities of sulfur reductase, hydrogenase and glutamate dehydrogenase and electron transfer activities present in *S. marinus*. The results indicate a link

between peptide metabolism and S° reduction in *S. marinus*.

Materials and methods

Growth conditions

Staphylothermus marinus (DSM 3639) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany and grown at 85 °C in modified 0.8× SME medium (Stetter et al. 1983). This medium contained (in g l^{-1}): NaCl, 16.0; $MgSO_4 \cdot 7H_2O$, 2.8; $MgCl_2 \cdot 6H_2O$, 2.2; $CaCl_2 \cdot 2H_2O$, 0.6; KH_2PO_4, 0.5; KCl, 0.26; NaBr, 0.04; H_3BO_3, 0.012; $SrCl_2 \cdot 6H_2O$, 0.006; citric acid, 0.004; $Ni(NH_4)_2(SO_4)_2$, 0.0016; KI, 0.00004; granulated yeast extract, 1.0; Bacto-Peptone, 5.0; and Resazurin (Sigma), 0.00025 as well as 8 ml of trace mineral solution (pH 7.0). The trace mineral solution contained (in g l^{-1}): $MgSO_4 \cdot 7H_2O$, 3.0; nitrilotriacetic acid (Sigma), 1.5; NaCl, 1.0; $MnSO_4 \cdot 2H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.1; $CoSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.01; $KAl(SO_4)_2 \cdot 12H_2O$, 0.01; H_3BO_3, 0.01; and $Na_2MoO_4 \cdot 2H_2O$, 0.01. Sublimed S° (Fisher Scientific, Canada) or colloidal S° (Fluka, Buchs, Switzerland) or polysulfide (0.5 M, prepared from sublimed S° (Ma and Adams 1994)) (1%, w/v) was added unless otherwise specified. The growth pH (measured at room temperature) was adjusted to 6.5.

Staphylothermus marinus was routinely cultivated in 50-ml serum bottles (Wheaton, Millville, NJ) containing 25 ml of medium. Large-scale cultures in 10-, 15- or 20-l glass carboys (Corning, Corning, NY) were harvested by continuous centrifugation at 13,000 *g* (Sharples super centrifuge). Cells were frozen in liquid nitrogen and stored at –80 °C.

Growth was monitored by direct cell counting with a Petroff-Hausser bacteria counting chamber (0.02 mm deep) and a Nikon Eclipse E600 phase-contrast light microscope.

Preparation of cell-free extracts

Frozen *S. marinus* cells were thawed anaerobically in about three volumes of 20 mM Tris-HCl buffer (pH 7.8) containing 1 mM sodium dithionite and 1 mM dithiothreitol, and suspended for 15 min. After adding DNase I (0.0002%, w/v), the suspension was incubated at 37 °C for 15 min with constant stirring. Cell lysis was verified by phase-contrast microscopic examination and protein determination. The suspension was centrifuged (Sorvall RC-5B centrifuge, SS-34 Rotor) at 8000 *g* and 4 °C for 30 min to remove cell debris and S° particles. The resulting supernatant was dispensed into anaerobic 1.5-ml vials under nitrogen.

Protein concentrations were routinely determined by the Bradford method, with bovine serum albumin (Bio-Rad) as a standard (Bradford 1976).

Enzyme assays

Glutamate dehydrogenase (GDH) activity was determined in anaerobic glass cuvettes by monitoring glutamate-dependent reduction of NADP spectrophotometrically at 340 nm and 80 °C (Ma et al. 1994*a*, Robb et al. 2001). One unit of GDH activity was defined as the formation of 1 µmol of NADPH per min.

Electron transfer activity (ETA) was routinely determined by measuring the NAD(P)H-dependent reduction of benzyl viologen spectrophotometrically at 580 nm and 80 °C (Ma and Adams 1999). One unit of ETA was defined as the reduction of 2 µmol of benzyl viologen per min.

Sulfur reductase activity was determined by measuring H_2S production by methylene blue formation (Chen and Mortenson 1977, Ma and Adams 2001). The assay was carried out in sealed 10-ml serum bottles with H_2 in the headspace at 80 °C (Ma and Adams 2001). One unit of sulfur reductase activity was defined as 1 µmol of H_2S produced per min.

Hydrogenase activity was measured by H_2 production with dithionite-reduced methyl viologen as the electron donor (Ma and Adams 2001). Hydrogen production was quantitatively determined by gas chromatography (Model 910, Buck Scientific, East Norwalk, CT). The gas chromatograph was equipped with a thermal conductivity detector (TCD) and a 1.85 m × 3.2 mm, 60/80 molecular sieve 5A, S.S. column (Supelco, Bellefonte, PA). The carrier gas was nitrogen, the pressure was 11 psi (7.6×10^4 Pa) and the flow rate was 16 ml min^{-1}. The injector, TCD and column temperatures were 110, 100 and 60 °C, respectively. One unit of hydrogenase activity was defined as 1 µmol H_2 produced per min. Hydrogen oxidation activity was determined spectrophotometrically by measuring H_2-dependent reduction of benzyl viologen at 580 nm and 80 °C (Ma and Adams 2001).

Results

S°-dependent growth

We confirmed that *S. marinus* requires S° for growth (c.f. Fiala et al. 1986). Next, we explored the possibility that S° functions as a physical support. Because of the low solubility of S° (5 µg l^{-1}, Schauder and Kröger 1993), it was possible that *S. marinus* cells attached to S° particles for growth. However, no attachment of the cells to S° particles was observed by phase-contrast microscopy, and no growth occurred in the presence or absence of 3% (w/v) sterile pure sand (BDH Chemical, U.K.) in S°-free medium (Figure 1). The growth of *S. marinus* in the presence of S° or S° plus sand was similar (data not shown). These results suggest that S° is not a physical supporting material for the attachment of *S. marinus* cells. There was also no growth when S° was omitted or replaced by other sulfur-containing compounds such as Na_2SO_4, $Na_2S_2O_3$, Na_2SO_3, cysteine, cystine or methionine (Figure 1). Likewise, 0.5 mM Na_2S did not enable fermentative growth on peptides, indicating that S° was not required solely as a sulfur nutrient for the cells. These results show that S° is essential for the growth of *S. marinus,* which is in agreement with the previous report of Fiala et al. (1986).

Colloidal S° or polysulfide could be substituted for sublimed S° in the growth medium (Figure 1). Similar growth occurred when sublimed S° was replaced by the same amount of colloidal S° or 3 mM polysulfide. However, when the concentration

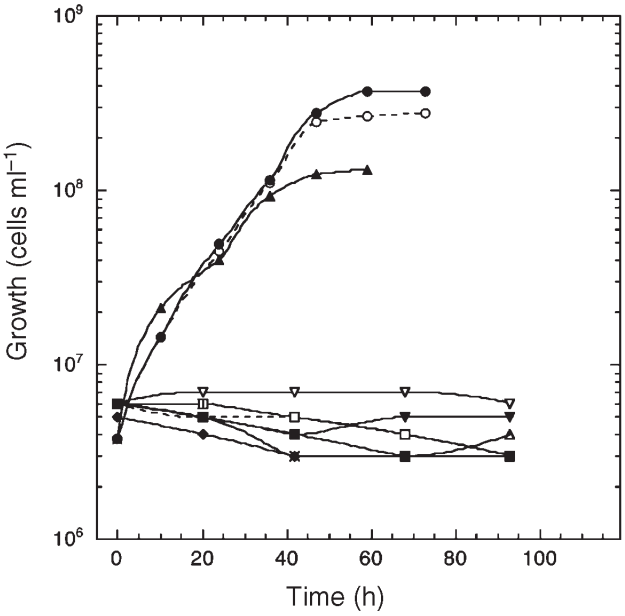

Figure 1. The S°-dependent growth of *S. marinus*. *Staphylothermus marinus* was grown in 25 ml of modified 0.8× SME medium containing (◆) no S°, (●) sublimed S° (0.05%, w/v), (○) colloidal S° (0.05%, w/v), (▲) polysulfide (3 mM), (□) Na_2SO_4 (10 mM), (×) Na_2SO_3 (10 mM), (▼) $Na_2S_2O_3$ (10 mM), (△) cysteine (10 mM), (▽) cystine (10 mM), (■) methionine (10 mM) or (◫) sand (3%, w/v) at 85 °C. Cell growth was monitored by direct cell counting.

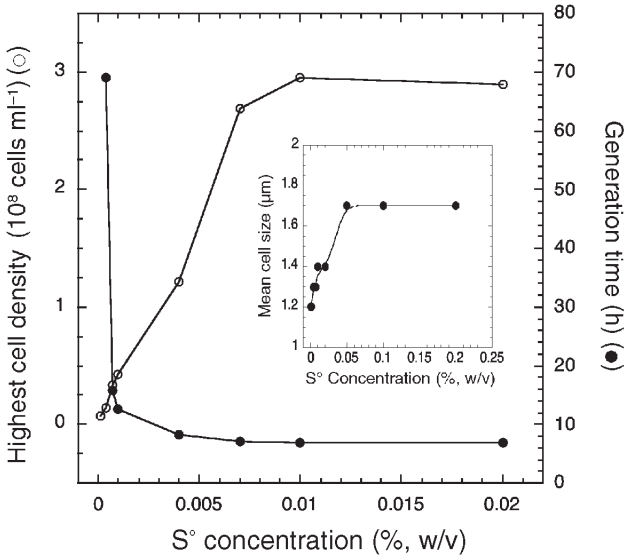

Figure 2. Effects of S° concentration on growth rate, yield and cell size of *S. marinus*. Open circles denote cell density at the end of growth, and closed circles denote generation time. The inset shows the mean cell size of *S. marinus*. Cells were grown in 25 ml of modified 0.8× SME medium at 85 °C. Cell size was determined by phase-contrast microscopy. Mean cell size (means ± SD) was determined from 20 cells at late log-phase.

of polysulfide was above 5 mM, growth of *S. marinus* was greatly inhibited (data not shown). A possible explanation is that the polysulfide reacts with inorganic components in the medium and impedes use by *S. marinus* of essential inorganic ions. When both sublimed and polysulfide S° were present in the medium, growth remained the same as in the presence of sublimed S° only. Thus, the addition of both forms of S° had no synergic effect on growth.

Minimal concentration of S° required for growth

Because S° was indispensable for growth of *S. marinus*, the minimal amount of sublimed S° required for optimal growth was determined. Generation time, highest cell density and mean cell size were used to compare growth in S° concentrations from 0 to 3% (w/v). Generation time decreased and final cell density increased with an increase in S° concentration from 0 to 0.01% (Figure 2), and there was no significant change in either parameter when S° concentrations were ≥ 0.01%. Mean cell size was affected by S° concentrations < 0.05%, and smaller cells were observed at low S° concentrations (Figure 2). Even at 0.0005% S°, some S° particles were visible under the microscope during growth. Therefore, small cell size was not caused by exhaustion of S°. When S° concentrations were ≥ 0.05%, all three parameters were constant. Therefore, 0.05% S° was considered to be the minimal S° concentration required by *S. marinus* for optimal growth.

Production of H_2 and H_2S

To investigate the extent to which *S. marinus* utilizes S°, the end products of S°-dependent metabolism were analyzed, with the emphasis on H_2S and H_2 production during growth (Table 1 and Figure 3). More H_2S was produced when higher concentrations of S° were present in the medium. No H_2S was produced in the absence of S°. Production of H_2S in the presence of 0.05% S°—the minimal S° concentration required for optimal growth of *S. marinus*—was about 6.50-fold more than in the presence of 0.005% S°, confirming that the higher concentration of S° resulted in higher H_2S production.

The amount of H_2 produced decreased as the concentration of S° increased above 0.005% (Table 1 and Figure 4). In the presence of 0.005% S°, 1.9 μmol H_2 ml^{-1} of culture was produced in 72 h, whereas only 0.24 μmol H_2 ml^{-1} was produced during the same period of time in the presence of 1% S°. Although growth was not detected in S°-free medium, H_2 production was detected at a rate of 0.018 μmol ml^{-1} h^{-1} of culture. The production of H_2 indicates that the cells were still metabolically active even though no net increase in cell number was observed.

The amounts of H_2 and H_2S produced during growth were compared (Table 1). When S° concentrations were below 0.005%, much more H_2 than H_2S was produced. When the S° concentration was 0.01%, the amount of H_2S produced was equal to 85% of the H_2 produced. However, when S° concentrations reached 0.05% and above, H_2S production was more than fivefold greater than H_2 production. Although the final cell densities in the presence of 0.01 and 0.05% S° were similar, H_2S production varied significantly, indicating a metabolic shift from H^+ to S° reduction. Moreover, the amount of total reduced products (H_2S plus H_2) was directly affected by the S° concentration in the medium. As the S° concentration in-

Table 1. Production of H_2S and H_2 by *S. marinus* grown in the presence of different concentrations of S°. Production of H_2S and production of H_2 are expressed as means ± SD of two duplicate experiments. *Staphylothermus marinus* was grown in 25 ml of modified 0.8× SME medium for 72 h at 85 °C. For each condition, a control without inoculum was used.

S° (%, w/v)	H_2S produced (μmol ml^{-1})	H_2 produced (μmol ml^{-1})	$H_2S + H_2$ (μmol ml^{-1})	H_2S/H_2	Final cell density (cells ml^{-1})	Generation time (h)
0.0	0.0	1.31 ± 0.15	1.31 ± 0.15	0.0	0.06×10^8	No growth
0.0001	0.057 ± 0.016	1.63 ± 0.090	1.69 ± 0.11	0.035	0.42×10^8	21.2
0.0005	0.082 ± 0.028	1.65 ± 0.21	1.73 ± 0.23	0.050	0.58×10^8	18.4
0.001	0.087 ± 0.010	1.78 ± 0.11	1.87 ± 0.12	0.049	0.96×10^8	14.7
0.005	0.70 ± 0.16	1.89 ± 0.059	2.58 ± 0.22	0.37	1.68×10^8	7.4
0.01	1.35 ± 0.008	1.56 ± 0.035	2.91 ± 0.04	0.87	2.46×10^8	6.8
0.05	4.55 ± 0.10	0.92 ± 0.11	5.48 ± 0.21	4.95	2.44×10^8	6.3
0.1	4.80 ± 0.085	0.80 ± 0.036	5.60 ± 0.12	6.00	2.41×10^8	6.4
0.5	5.36 ± 0.37	0.39 ± 0.025	5.74 ± 0.40	13.74	2.37×10^8	6.5
1.0	5.96 ± 0.22	0.24 ± 0.015	6.20 ± 0.23	24.83	2.34×10^8	6.5

creased from 0.0001 to 1%, *S. marinus* growth increased such that the total amount of H_2S and H_2 produced per ml of culture increased from 1.69 to 6.20 μmol, and the ratio of H_2S to H_2 rose dramatically from 0.035 to about 25 (Table 1).

The capacity for *S. marinus* H_2 and H_2S production was affected dramatically by the presence of S°, which caused a metabolic transition from proton reduction to H_2S production (Figure 4). Proton reduction was a dominant metabolic process when S° concentration was below 0.05%, and S° reduction became a dominant process when S° concentration was 0.05% or higher. Note that the number of cells in Figure 4 refers to the final cell density, whereas H_2 and H_2S production were monitored throughout the growth period. Thus, the final cell density served only as a reference for comparison of H_2 and H_2S production under the same growth conditions.

Enzyme activities

Some enzyme activities related to S°-dependent metabolism were detected in the cell-free extracts of *S. marinus,* which were prepared separately from six large-scale batches of cells grown in the presence of S° (0.2%). Sulfur reductase (SR) is the enzyme that catalyzes the reduction of S° to H_2S. The SR activities detected in these cell-free extracts varied between 0.018 and 0.092 U mg^{-1}. This high variability may have been caused by oxygen contamination during cell harvesting and enzymatic assays. Sulfur reductase is sensitive to oxygen, and trace amounts of oxygen cause irreversible loss of activity (data not shown). Therefore, the activity of SR might be underestimated.

Hydrogenase activities of *S. marinus* were measured as H_2 oxidation or production. However, during the H_2 oxidation assays, an unknown compound in the cell extract (at a concentration of about 7 mM) served as the electron donor for the reduction of benzyl viologen and methyl viologen and interfered with the detection of H_2 oxidation. Hence, H_2 production with sodium-dithionite-reduced methyl viologen as the electron donor seemed to be a more reliable assay of hydrogenase activity, which was relatively low (0.067 ± 0.021 U mg^{-1}). This activity is consistent with the relatively low amount of H_2 produced by cells grown in the presence of S°.

Glutamate dehydrogenase and electron transfer activities were reproducible among various batches of cell-free extracts. Based on the glutamate-dependent reduction of NADP assay, the apparent K_m values for glutamate (in the presence of 0.4 mM NADP) and NADP (in the presence of 2.0 mM glutamate) were 0.12 and 0.04 mM, respectively. The apparent V_{max} was 2.0 ± 0.2 U mg^{-1}. No activity was detected when NADP was replaced by the same concentration of NAD, indicating that GDH in *S. marinus* is NADP-specific. The GDH activity was pH-dependent, with an optimal pH of 8.7. The activity of GDH increased as temperature increased. The optimal temperature was above 95 °C. Assays could not be conducted at temperatures above 95 °C because of the temperature limit of the spectrophotometer and the thermal lability of nicotinamide cofactors. Glutamate dehydrogenase was not oxygen sensitive, and the activity remained stable after 4.5 h of exposure to air at room temperature.

The catalytic properties of ETA were assayed by NAD(P)H-dependent reduction of benzyl viologen. The apparent K_m and V_{max} for benzyl viologen (in the presence of 0.3 mM NADH) were 0.19 mM and 3.6 U mg^{-1}, respectively. The apparent K_m and V_{max} for NADH (in the presence of 1 mM benzyl viologen) were 0.06 mM and 3.8 U mg^{-1}, respectively. In addition to NADH, ETA of *S. marinus* could be assayed with NADPH as substrate. The apparent K_m and V_{max} for NADPH (in the presence of 1 mM benzyl viologen) were 0.018 mM and 0.64 U mg^{-1}, respectively. The optimal pH and temperature for ETA were 9.7 and > 95 °C, respectively. Like GDH, ETA was insensitive to oxygen, and there was no loss of activity after 5 h of exposure to air at room temperature.

Discussion

The growth of *S. marinus* was previously reported to be strictly dependent on S° (Fiala et al. 1986). We have shown that S° did not serve as a physical support for the attachment of cells or as a required sulfur nutrient for anabolism. For instance, growth

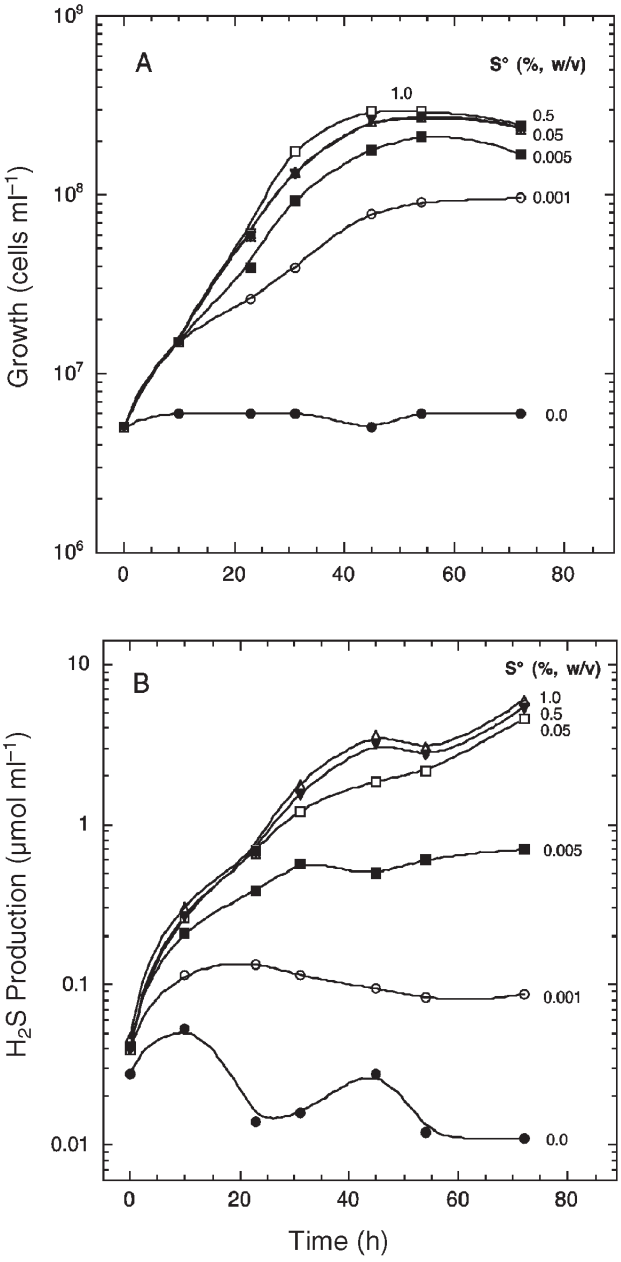

Figure 3. Growth and H_2S production of *S. marinus*. (A) Growth of *S. marinus* in the presence of different concentrations of sublimed S°. Cell density was obtained by direct cell counting. (B) Production of H_2S in the presence of different concentrations of sublimed S°. Cells were grown in 25 ml of modified 0.8× SME medium at 85 °C.

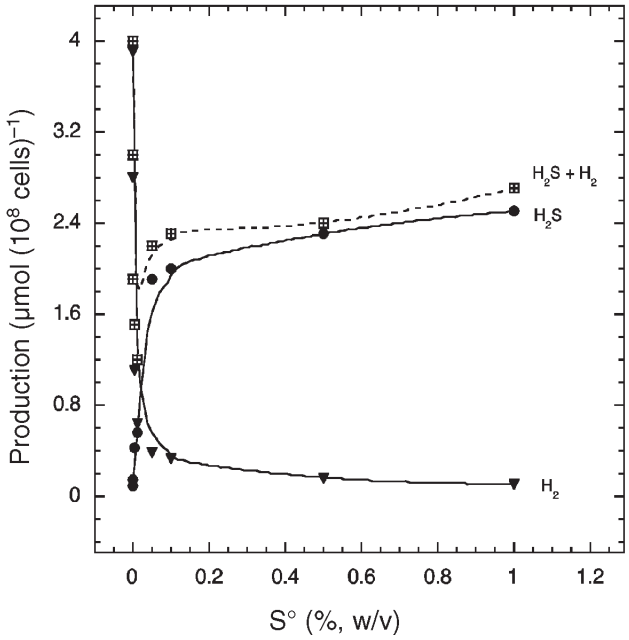

Figure 4. Effects of S° on the production of H_2 and H_2S by *S. marinus*. Production of H_2 and production of H_2S were calculated from the amounts of H_2 and H_2S produced during growth and the cell density at the end of growth. Thus, the units of µmol (10^8 cells)$^{-1}$ are not comparable with specific activities. Cells were grown in 25 ml of modified 0.8× SME medium for 72 h at 85 °C.

was poor at low S° concentrations (0.0001–0.0005%) that would be sufficient to fulfill the sulfur requirement for growth. This characteristic distinguishes *S. marinus* from most hyperthermophilic and heterotrophic S°-reducers for which S° is either not absolutely required for growth or can be replaced by thiosulfate, sulfite, sulfate, ferric iron or cystine as alternative electron acceptors (Stetter 1996, Adams 1999).

The H_2S resulting from the reduction of S° may be used by *S. marinus* for biosynthesis; however, S° was not only a sulfur source. Cell yield was significantly affected by concentrations of S° below 0.01% (w/v), and cell number was proportional to the amount of H_2S produced, suggesting that S° reduction may be an energy-conserving process or may facilitate metabolic processes. We also showed that mean cell size of *S. marinus* is dependent on S° concentration. An increase in cell size (up to 15 µm) was observed when the concentration of yeast extract in the growth medium was increased (Fiala et al. 1986). One can speculate, therefore, that a small increase in nutrient uptake rate, which presumably leads to the increase in cell size of *S. marinus,* may also be achieved by S° reduction.

Both H_2 and H_2S were produced simultaneously during the S°-dependent growth of *S. marinus*, indicating that both S° reduction and H_2 production are important physiological processes. However, the amounts of H_2 and H_2S produced varied depending on the concentration of S°. When the S° concentration was insufficient for optimal growth (< 0.05%), H_2 production was greater than H_2S production. However, H_2S production was nearly four times greater than H_2 production in the presence of ≥ 0.05% S°. This suggests that the metabolism of *S. marinus* shifts from H_2 production to S° reduction under optimal growth conditions and that H_2 production is suppressed by S°, a phenomenon observed in other heterotrophic hyperthermophilic S°-reducers (Ma et al. 1993, 1994*b*). An increase in S° concentration enhanced the growth of *S. marinus* as indicated by an increase in final cell density and a reduction in generation time. Therefore, both the total amount of H_2S and H_2 produced and the ratio of H_2S to H_2 increased with increas-

ing S° concentration, but H_2 production was not completely repressed. Presumably, S° regulates this metabolic shift in *S. marinus*; the mechanism, however, remains unknown.

Although it was not possible to grow *S. marinus* at low S° concentrations in sufficient amounts for enzyme analysis, the enzyme activities determined in cells grown at higher S° concentrations provide valuable information about the S°-dependent metabolic processes of *S. marinus*. Glutamate dehydrogenase is a key enzyme of peptide metabolism and functions as a link between catabolic and biosynthetic pathways (Robb et al. 2001). It was expected to be a key enzyme in *S. marinus*, which grows only on peptide substrates. Glutamate dehydrogenase activities have been detected in several heterotrophic hyperthermophiles (Consalvi et al. 1991*a*, 1991*b*, Robb et al. 1992, Ohshima et al. 1993, Ma et al. 1994*a*, Kobayshi et al. 1995, Aalén et al. 1997, Kujo and Ohshima 1998, Bhuiya et al. 2000). This enzyme can be classified as one of three types based on its coenzyme specificity: NADP-specific, NAD-specific and dual coenzyme specific. Glutamate dehydrogenase of *S. marinus* is of the first type. Compared with enzymes from other hyperthermophiles, it has the lowest K_m for glutamate. This remarkable feature reinforces the importance of GDH in *S. marinus* catabolism.

Electron transfer activity of *S. marinus* showed a distinctively high specific activity of 2.8 U mg^{-1} (mean of seven cultures), which is threefold higher than that of *P. furiosus* (0.7 U mg^{-1}) (Ma and Adams 1999). This strongly indicates that the oxidation–reduction reactions are active in the metabolism of *S. marinus*. Both NADH and NADPH can be utilized as the electron donor, which is also the case in *P. furiosus* and may be common in hyperthermophilic and heterotrophic S°-reducers.

Sulfur reductase activities in *S. marinus* were comparable with those in other S°-reducing prokaryotes. Sulfur reductases of *W. succinogenes* (1.2 U mg^{-1}; Jankielewicz et al. 1995), *P. abyssi* isolate TAG11 (0.56 U mg^{-1}; Dirmeier et al. 1998) and *Acidians ambivalens* (6.3 U mg^{-1}; Laska and Kletzin 2000) have much higher specific activities than that of *P. furiosus* (0.2 U mg^{-1}; Ma et al. 1993), *Thermotoga neapolitana* (0.031 U mg^{-1}; Childers and Noll 1994) and *S. marinus* (0.044 U mg^{-1}). It seems that autotrophic S°-reducers obtaining energy through S° respiration have higher SR activity than the heterotrophic S°-reducers.

Hydrogenases are widely distributed in autotrophic and heterotrophic S°-reducers. Although hydrogenases from other heterotrophic S°-reducers also have SR activity (Ma et al. 1993), their roles in S° reduction were inconsistent with the dramatic decrease in hydrogenase activities during growth with high concentrations of S° (Adams et al. 2001). It is also uncertain if *S. marinus* hydrogenase has SR activity, and we are investigating this possibility further.

On the basis of determined enzymatic activities and the S°-dependent growth of *S. marinus*, the following mode of S°-dependent metabolism in *S. marinus* is proposed: reducing equivalents generated by the oxidation of amino acids from peptidolysis are transferred by ETA to SR and hydrogenase, which then reduce S° and protons to H_2S and H_2, respectively. However, elucidation of the function and regulation of SR and hydrogenase, key enzymes in the S°-dependent transient metabolism of *S. marinus*, requires further study.

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