APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1990, p. 1255–1262
0099-2240/90/051255-08$02.00/0

Vol. 56, No. 5

Role of Polysulfides in Reduction of Elemental Sulfur by the Hyperthermophilic Archaebacterium *Pyrococcus furiosus*†

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Received 27 November 1989/Accepted 20 February 1990

Polysulfides formed through the breakdown of elemental sulfur or other sulfur compounds were found to be reduced to H_2S by the hyperthermophilic archaebacterium *Pyrococcus furiosus* during growth. Metabolism of polysulfides by the organism was dissimilatory, as no incorporation of ^{35}S-labeled elemental sulfur was detected. However, [^{35}S]cysteine and [^{35}S]methionine were incorporated into cellular protein. Contact between the organism and elemental sulfur is not necessary for metabolism. The sulfide generated from metabolic reduction of polysulfides dissociates to a strong nucleophile, HS^-, which in turn opens up the S_8 elemental sulfur ring. In addition to H_2S, *P. furiosus* cultures produced methyl mercaptan in a growth-associated fashion.

Despite the apparent significance of elemental sulfur in the growth of bacteria at elevated temperatures (especially those capable of growing at or above 100°C, i.e., hyperthermophiles), very little information is available on how particular bacteria interact with sulfur. It is well established that sulfur is the element with the largest number of allotropes which exist in both chain and ring forms (20). The most thermodynamically stable form of elemental sulfur found under normal biocompatible temperatures and pressures is orthorhombic α-sulfur (22). This allotrope consists of eight sulfur atoms in the form of staggered crownshaped rings and has a melting point of 112°C. At temperatures around 95°C, α-sulfur converts into monoclinic β-sulfur, which melts at 119.6°C. This also is a cycloocta-S allotrope which, like α-sulfur, is essentially water insoluble (20). Therefore, to utilize sulfur, both mesophilic and thermophilic bacteria must either have direct contact with the solid sulfur substrate or mediate conversion of the sulfur ring into a soluble compound.

For several mesophilic bacteria, experimental evidence suggests that a sulfur solubilization step is involved in the metabolism of elemental sulfur. For example, the sulfur reduction reaction catalyzed by membrane fractions of the eubacterium *Spirillum* sp. strain 5175 was shown to be facilitated by addition of thiols and sulfides, which cleave S-S bonds by nucleophilic attack (33). Similarly, it has been suggested that polysulfide chains formed by the reaction of sodium sulfide and sulfur are involved in the reduction of sulfur by cytochrome c_3 of *Desulfovibrio desulfuricans* (6). On the other hand, sulfur oxidation by *Thiobacillus thiooxidans* is thought to involve both direct attachment of the bacteria to the solid sulfur and its activation by a membrane-bound thiol group or by reaction with glutathione (15, 27, 32).

In this work, the bacterium-sulfur interactions of the hyperthermophilic marine archaebacterium *Pyrococcus furiosus* were examined. This anaerobic heterotroph was isolated from geothermally heated sulfataric sediments off the coast of Vulcano, Italy (7). This bacterium grows optimally at approximately 100°C and can modify its metabolism depending on whether it is grown with or without elemental sulfur (7, 16). Without sulfur, CO_2 and H_2 are produced, with the latter eventually becoming inhibitory for growth. With sulfur, only trace amounts of H_2 can be detected and H_2S is produced in a growth-associated fashion (19).

Because several heterotrophic bacteria have been isolated from extreme temperature niches which mediate the reduction of elemental sulfur to sulfide, insights gained from the study of *P. furiosus* will likely be applicable to other related organisms. For example, sulfur utilization may be a key factor in the function of an organism at the most extreme temperatures. In a larger sense, the role of sulfur-reducing bacteria in the ecological systems characteristic of extreme temperature environments is still not understood. The purpose of this study was to provide some information that may be useful along these lines.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *P. furiosus* DSM 3638 was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Federal Republic of Germany. Cells were cultured in media based on artificial seawater (ASW) supplemented with 0.1% yeast extract and 0.5% tryptone. The ASW is a modification of the formulation of Kester (17). Equal volume of solution A (47.8 g of NaCl per liter, 8.0 g of Na_2SO_4 per liter, 1.4 g of KCl per liter, 0.4 g of $NaHCO_3$ per liter, 0.2 g of KBr per liter, 0.06 g of H_3BO_3 per liter) and solution B (10.8 g of $MgCl_2 \cdot 6H_2O$ per liter, 1.5 g of $CaCl_2 \cdot 2H_2O$ per liter, 0.025 g of $SrCl_2 \cdot 6H_2O$ per liter) were mixed and supplemented with an additional salts (AS) solution (50× AS solution is 12.5 g of NH_4Cl per liter, 7.0 g of K_2HPO_4 per liter, and 50.0 g of sodium acetate per liter) after autoclaving. Cells were grown in sealed 125-ml culture vials containing 50 or 100 ml of medium. Anaerobic conditions were obtained by heating the medium at 98°C for 15 to 30 min, flushing with helium for 3 min, and adding 20 ml of 25-g/liter $Na_2S \cdot 9H_2S$ per liter of medium. Resazurin (1 mg/liter) was used as a redox indicator. Elemental sulfur (J. T. Baker Chemical Co., Phillipsburg, N.J.) was added in the form of crystalline sulfur flowers (10 g/liter) unless otherwise stated. All cultures were incubated at 98°C in an oil-filled bath (New Brunswick Scientific Co., Inc., Edison, N.J.) under quiescent conditions. Although no bacterial settling has been observed in *P. furiosus* cultures, the vials were gently mixed before sampling. Growth was monitored by cell enumeration by using epifluorescence microscopy

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with acridine orange stain (10). Cells were also grown in a 3-liter round-bottom flask containing 1 liter of medium supplemented with 10 mM sodium polysulfides (see below). The pH of the medium was adjusted to 8.7 to prevent conversion of the polysulfides to colloidal sulfur. The culture vessel was sparged continuously with prepurified N_2 (Linde Gases, Baltimore, Md.), and the temperature was maintained at 98°C by using a heating mantle and a temperature controller (Cole-Parmer Instrument Co., Chicago, Ill.).

Gas analysis. Gas analysis was done by using a 3700 gas chromatograph (Varian, Sunnyvale, Calif.). Carbon dioxide, hydrogen, and hydrogen sulfide were detected by using a thermal conductivity detector after separation on a Hayesep-N column (Alltech Associates, Inc., Applied Science Div., State College, Pa.) at 80°C. Methyl mercaptan was detected by using a flame photometric detector after separation on a Chromosil-330 column (Supelco, Bellefonte, Pa.) at 28°C. Peak area integration was performed with a microcomputer, and gas concentrations were determined on the basis of calibration curves prepared with standard gases.

Dialysis bag experiments. Sulfur particles were physically separated from the bacteria by placing the sulfur in a Spectra/Por (Spectrum, Los Angeles, Calif.) dialysis bag with a nominal pore size limited to 6- to 8-kilodalton (kDa) molecules containing 3 ml of growth medium. The bags were sealed to ensure that the bacteria had no direct access to the sulfur and then placed in the culture vials before inoculation. Integrity of the dialysis membranes was checked after each experiment by visual inspection and by determining whether any bacteria were present inside the bags by using epifluorescence microscopy.

Colloidal sulfur preparation. A hydrophilic colloidal sulfur suspension was prepared by the procedure of Janek (12). In short, solution I was prepared by mixing 1.5 ml of 0.57 M Na_2SO_3 with 50 ml of 0.53 M Na_2S and then adding 2.5 M H_2SO_4 dropwise until the solution turned slightly turbid. Solution II was made by adding 3 ml of concentrated H_2SO_4 to 48.5 ml of 0.57 M Na_2SO_3. The colloidal sulfur formed when solution I was slowly added to solution II while stirring. The sulfur was then washed twice by centrifugation at 5,000 rpm (1,000 × *g*) for 15 min with distilled water and finally suspended in 300 ml of water.

Sulfur-containing model compound studies. To determine whether *P. furiosus* could reduce other sulfur-containing molecules, different organic sulfur compounds were tested. Cystine, dimethyl disulfide, methyl trisulfide, ethyl disulfide, trithiane, sodium thiosulfate, cysteine, and tetrahydrothiophene were added to the growth medium at a concentration of 0.64 mg of sulfur per ml. Sulfur reduction was monitored by measuring H_2S formation during growth of the bacteria.

Polysulfide preparation and assay. A 0.5 M polysulfide solution was prepared by reacting 12 g of Na_2S with 1.6 g of elemental sulfur in 100 ml of anoxic water (11). Polysulfide formation was measured by cold cyanolysis (31). In short, 1 ml of 0.1 M Tris (pH 8.7) and 0.1 ml of 0.2 M NaCN were added to a 1-ml sample and incubated at 30°C for 1 h. The reaction mixture was then cooled, 1.7 ml of 2.5% zinc acetate was added, and the precipitate formed was pelleted by centrifugation at 5,000 rpm (1,000 × *g*) for 3 min. Thiocyanate formation was determined by adding 0.05 ml of 0.75 M $Fe(NO_3)_3$ in 20% HNO_3 to 0.95 ml of the reaction supernatant. Absorbance was determined at 460 nm, and concentrations were calculated by using a standard curve prepared with sodium thiocyanate and are reported as concentrations of thiocyanate formed (millimolar). It is important to note that by this method it is not possible to determine the precise nature or the exact concentration of the polysulfides in solution because of the lack of information about the length distribution of the molecules. Because the system is not at equilibrium during growth of the bacteria and the length of the polysulfide molecules is a function of the growth stage and medium composition, no attempt was made to determine the chain length distribution. Under the assay conditions used and in the absence of cupric ions, only the polysulfides reacted to form thiocyanate. No interference was found with sulfide, cystine, cysteine, or elemental or colloidal sulfur.

$^{35}S^0$ assimilation. ^{35}S-labeled elemental sulfur was used to determine whether *P. furiosus* was able to assimilate this sulfur species into organic cellular compounds. Two different methods were used to test elemental sulfur assimilation. In the first, radioactive sulfur (28.0 mCi/mg; Amersham Corp., Arlington Heights, Ill.) was diluted with cold elemental sulfur to a final activity of 0.5 μCi/mg. A uniform dilution was obtained by dissolving the sulfur in toluene, evaporating the solvent, and reducing the solid to a fine powder with a mortar and pestle. One hundred milligrams of this diluted, radioactive sulfur was added to culture vials containing 100 ml of medium. The bacteria were then grown in the presence of diluted radioactive sulfur. In the second method, the cells were first grown to the stationary phase in medium containing regular sulfur and then pelleted and washed twice with defined medium. The defined medium consisted of ASW supplemented with a mixture of amino acids (0.08 g of Glu and Gly per liter; 0.06 g of Arg per liter; 0.04 g of Thr, His, Ile, Lys, Leu, and Asn per liter; 0.03 g of Phe, Ser, Ala, and Tryp per liter; 0.02 g of Val, Gln, Asp, and Pro per liter). The sulfur-containing amino acids cysteine and methionine were purposely left out of the formulation. The cells were then suspended in defined medium, transferred to growth vials, and incubated at 98°C for 2 h before addition of ^{35}S-labeled elemental sulfur. Radioactive sulfur (28.0 mCi/mg; Amersham) was added in the form of a toluene-medium suspension to a final concentration of 20 μCi/ml. The cultures were then incubated for another 2 h at 98°C. To determine sulfur uptake, cells from 1-ml culture samples were pelleted and washed three times by centrifugation in an Eppendorf microcentrifuge with fresh medium. The pellets were then suspended in 0.1 ml of medium, and the incorporated radioactivity was measured by scintillation counting in a liquid scintillation spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). To determine whether $^{35}S^0$ was incorporated into cell proteins, cell pellets from 100-ml cultures were lysed by sonication and the total cell extracts were fractionated on a 10% polyacrylamide gel containing sodium dodecyl sulfate. The gels were then dried and applied to X-ray film (Kodak XR) and exposed at −70°C for 2 weeks.

[^{35}S]methionine and [^{35}S]cysteine incorporation. [^{35}S]methionine and [^{35}S]cysteine uptake and incorporation studies were done using the defined medium described above. Radiolabeled cysteine (1,083 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) and radiolabeled methionine (1,169 Ci/mmol; Dupont, NEN) were added to final concentrations of 0.15 μg/liter (1.25 μCi/ml) and 0.19 μg/liter (1.25 μCi/ml), respectively. Incorporation of radioactivity was monitored as described in the previous section.

Nucleophile studies. To identify effective sulfur nucleophiles and to determine the sulfur solubilization kinetics, different nucleophilic agents and various pH values were tested. For this, 50 mg of diluted, ^{35}S-labeled elemental sulfur (0.5 μCi/mg; see section on $^{35}S^0$ assimilation) was

placed in dialysis bags as previously described. The bags were then immersed in culture vials containing 100 ml of medium supplemented with the nucleophilic agents (0.5 g of Na_2S per liter [2.1 mM], 1.0 g of cysteine per liter [8.26 mM], 0.4 g of NaOH per liter [20 mM], and methyl mercaptan at 530 ppm [530 μl/liter]). The following buffers were also tested: 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid (EPPS) (pH 8.0), 50 mM sodium phosphate buffer (pHs 7.0 and 9.0), and ASW (pHs 7.0 and 5.50). Sulfur solubilization was determined by measuring radioactivity in the liquid outside the dialysis bag.

RESULTS

Cell-sulfur interactions. To determine whether elemental sulfur reduction by *P. furiosus* requires direct contact of the bacteria with sulfur, elemental sulfur was physically separated from the bacteria by placing it in a closed dialysis bag (pore size limited to 6- to 8-kDa molecules) as described previously. This arrangement ensured that the cells had no direct access to the sulfur but small soluble molecules could diffuse in either direction across the dialysis membrane. Sulfur reduction was determined by measuring H_2S production in the gas phase. Representative results of these experiments are presented in Fig. 1. Hydrogen sulfide production was growth associated in both culturing configurations (elemental sulfur exposed to cells and in dialysis membranes). The extent of growth and the final hydrogen sulfide levels were both considerably lower when the elemental sulfur was shielded from the cells. This difference was even greater if smaller amounts of sulfur were placed in the dialysis bag and indicates that diffusion limitations were likely present in the system. However, formation of significant amounts of sulfide in the culture vials with the sulfur placed in dialysis bags relative to uninoculated controls indicated that sulfur reduction can occur without direct contact of cells with elemental sulfur. These results also suggest that a soluble intermediate was formed from elemental sulfur. This soluble sulfur molecule was able to diffuse out of the dialysis bag and become available to the bacteria for reduction.

In light of other studies that focused on aqueous sulfur chemistry (20, 22), the most likely elemental sulfur solubilization reaction is formation of polysulfides. Analysis of the growth medium outside the dialysis membranes showed that polysulfides were present in both inoculated and uninoculated culture vials. Polysulfides were also present in supernatant samples from culture vials with sulfur in suspension (Fig. 1C). Interestingly, polysulfide concentrations increased faster in inoculated samples than in uninoculated samples, suggesting that the metabolism of the organism produces a sulfur-solubilizing agent. Moreover, the decrease in polysulfide concentrations observed between 5 and 10 h after inoculation showed that the bacteria metabolized the polysulfides and reduced them to H_2S.

Polysulfide formation. To investigate the nature of the sulfur-solubilizing agents present in the culture medium, different sulfur nucleophiles were tested. Nucleophiles were chosen because the redox potential of the growth medium was very low and therefore did not favor electrophilic sulfur attack. The specific nucleophiles selected corresponded to compounds present in the medium: sodium sulfide, cysteine, hydroxide ions, and methyl mercaptan (see the section on methyl mercaptan production).

On the basis of the amounts and rates of ^{35}S released from the dialysis bag (Fig. 2A), the tested nucleophiles were divided into two groups. The first group corresponded to the compounds that were effective sulfur-solubilizing agents. They included cysteine and sodium sulfide. Interestingly, the rates and amounts of ^{35}S released by these nucleophiles were identical to the release obtained when the bacteria were grown in the medium (Fig. 2A). These results are in agreement with the hypothesis that the sulfide produced by bacterial sulfur reduction serves as a sulfur nucleophile that further solubilizes sulfur. The second group corresponded to compounds that did not significantly promote sulfur solubilization. Both methyl mercaptan and hydroxyl ions showed release curves that were indistinguishable from the release kinetics of the medium alone. It is important to note that

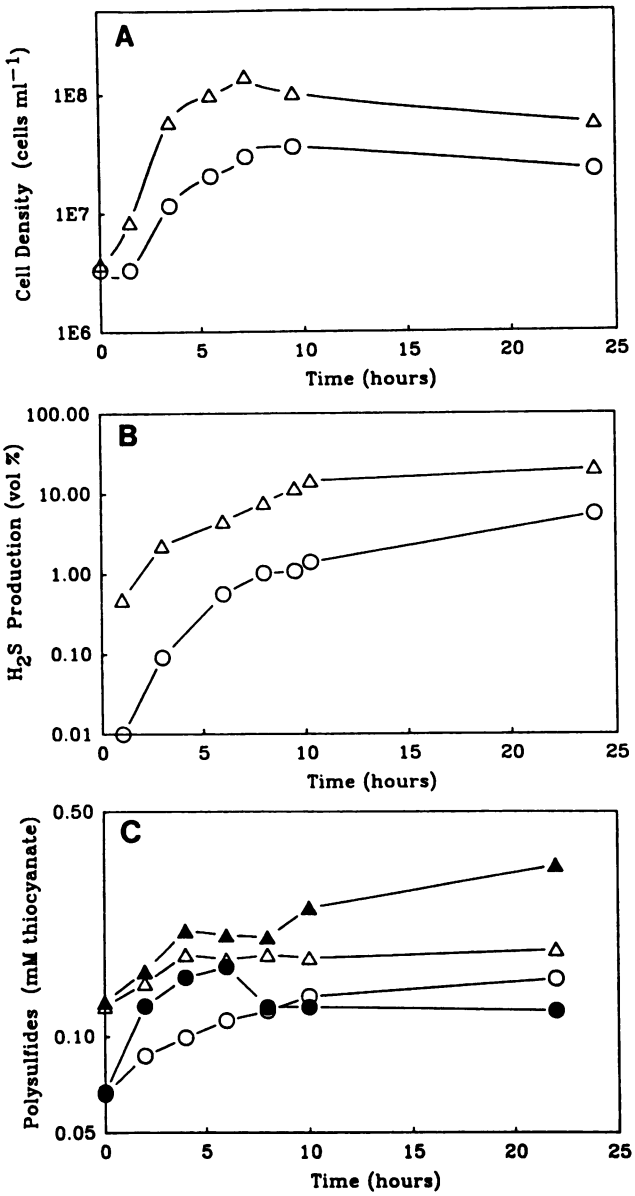

FIG. 1. Cell-sulfur interactions. (A) Growth of *P. furiosus* on sulfur placed inside a closed dialysis membrane (pore size limited to 6- to 8-kDa molecules) (○) and on sulfur dispersed in growth medium (△). (B) Hydrogen sulfide production under the culture conditions used for panel A. (C) Polysulfide concentration in medium samples from cultures used for panel A (solid symbols). Open symbols correspond to uninoculated controls. The datum points represent averages of duplicate experiments.

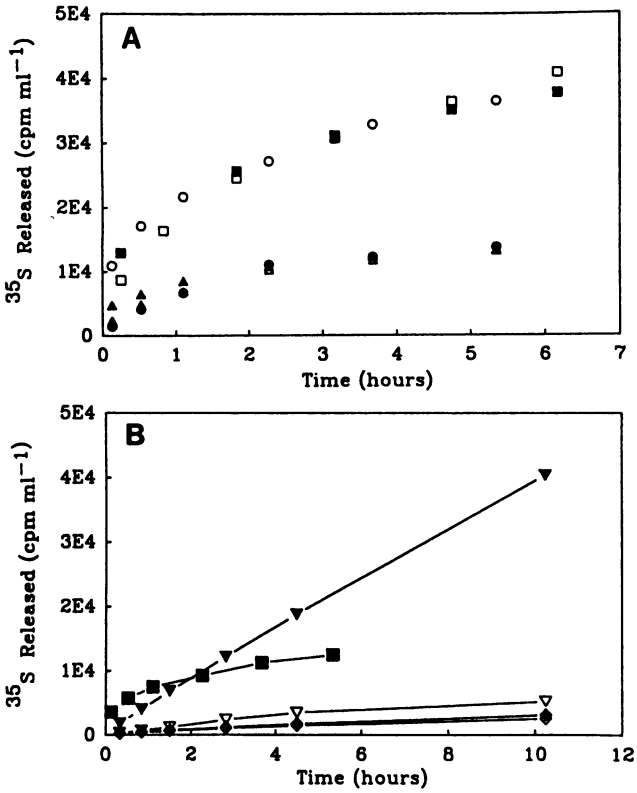

FIG. 2. Sulfur solubilization by different nucleophiles. (A) Release kinetics of ^{35}S-labeled elemental sulfur from dialysis membrane (pore size limited to 6- to 8-kDa molecules) in 0.5 g of Na_2S per liter (○), 1 g of cysteine per liter (□), a growing *P. furiosus* culture (■), 0.4 g of NaOH per liter (●), and methyl mercaptan at 530 ppm (530 μl/liter) (△). The control was growth medium without added nucleophiles (▲). (B) Effect of pH on sulfur solubilization. Release kinetics of ^{35}S-labeled elemental sulfur from dialysis membrane (pore size limited to 6- to 8-kDa molecules) placed in growth medium at pH 5.5 (◆) or 7.0 (◇), 50 mM sodium phosphate buffer at pH 7.0 (▽) or 9.0 (▼), or EPPS buffer at pH 8.0 (■). The datum points represent averages of triplicate experiments.

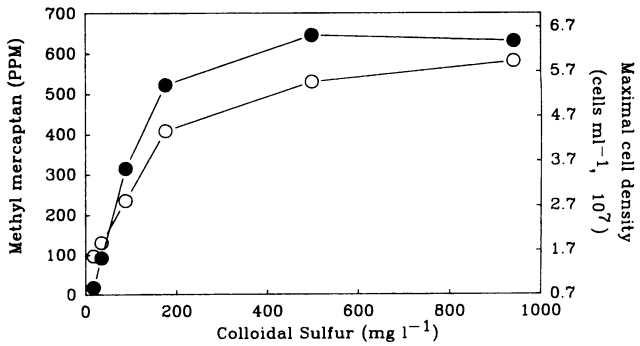

FIG. 3. Total methyl mercaptan production and maximal cell densities in *P. furiosus* cultures grown on different colloidal sulfur concentrations. Symbols: ●, gas phase methyl mercaptan concentration; ○, maximal cell density. The datum points represent averages of four experiments.

some sulfur solubilization did occur in growth medium without added nucleophiles. This was likely due to the presence of trace amounts of sulfur nucleophiles in the yeast extract and the tryptone used in the medium formulation.

Since polysulfides are not stable below pH 8.7 in aqueous solutions (29), it was important to determine the pH dependence of the sulfur solubilization kinetics. Indications are that pH had a very strong effect on sulfur solubilization (Fig. 2B). Essentially no sulfur was solubilized at pH 7.0 and 5.5, independently of the nature of the solution (phosphate buffer or growth medium). At pH 8.0, moderate amounts of sulfur were released, and the release curve reached a plateau after 2 to 3 h. However, at pH 9.0, sulfur solubilization kinetics showed that the rate of release was constant, even after 10 h, and that significant amounts of sulfur were solubilized. These results suggest that under normal growth conditions at pH 7.5, polysulfide availability is limited by the stability of these compounds in solution.

Methyl mercaptan production. The only reported sulfur metabolite produced by *P. furiosus* is H_2S. In this work, by gas chromatography analysis, it was possible to detect formation of methyl mercaptan and carbon disulfide in the gas phase of bacterial cultures grown in sulfur. Carbon disulfide was found to be the result of abiotic reactions, since identical concentrations of this gas were present in uninoculated and inoculated culture vials. Methyl mercaptan production was associated with bacterial growth. Moreover, final mercaptan concentrations were directly proportional to the final cell yields obtained in cultures grown on different concentrations of colloidal sulfur (Fig. 3). It is important to note that the highest mercaptan concentrations produced in the culture vials were 200 times lower than the average H_2S concentrations. Because no methyl mercaptan was produced in cultures grown without sulfur, it is likely that methyl mercaptan is a side product of sulfur metabolism. On the basis of preliminary results, methyl mercaptan was thought to be involved in the sulfur solubilization reactions, but on the basis of the results discussed in the previous section, this seems unlikely. More information is needed to establish the reactions that lead to the production of this mercaptan and how these relate to the overall sulfur metabolism of *P. furiosus*.

Sulfur-containing model compound studies. Different sulfur-containing organic compounds were tested for the ability to be reduced and support growth of *P. furiosus*. The compounds tested included cystine, dimethyl disulfide, dimethyl trisulfide, ethyl disulfide, trithiane, sodium thiosulfate, cysteine, and tetrahydrothiophene. Two of these, cystine and dimethyl trisulfide, could be used as alternative sulfur sources for reduction (Fig. 4A and B). Growth of *P. furiosus* on these model compounds was as good as or better than growth on elemental sulfur, with comparable growth rates and final cell yields (Fig. 4A). Hydrogen sulfide analysis showed that in all cases, gas production was growth associated and that the final sulfide concentration in the gas phase was approximately 10% (Fig. 4B). No H_2S was detected in cultures grown on cysteine, dimethyl disulfide, ethyl disulfide, trithiane, sodium thiosulfate, or tetrahydrothiophene. Also, no sulfide was produced in cultures grown without sulfur.

On the basis of the previous finding that polysulfides are likely to be the actual substrates for bacterial reduction, the medium containing the different model compounds was tested for polysulfide formation. For this, the compounds were incubated at 98°C for 12 h in inoculated and uninoculated culture vials with growth medium. Polysulfide formation was also tested in water containing the different model compounds. In all cases, the model sulfur compound concentration was 0.64 mg/ml of sulfur. Results of these exper-

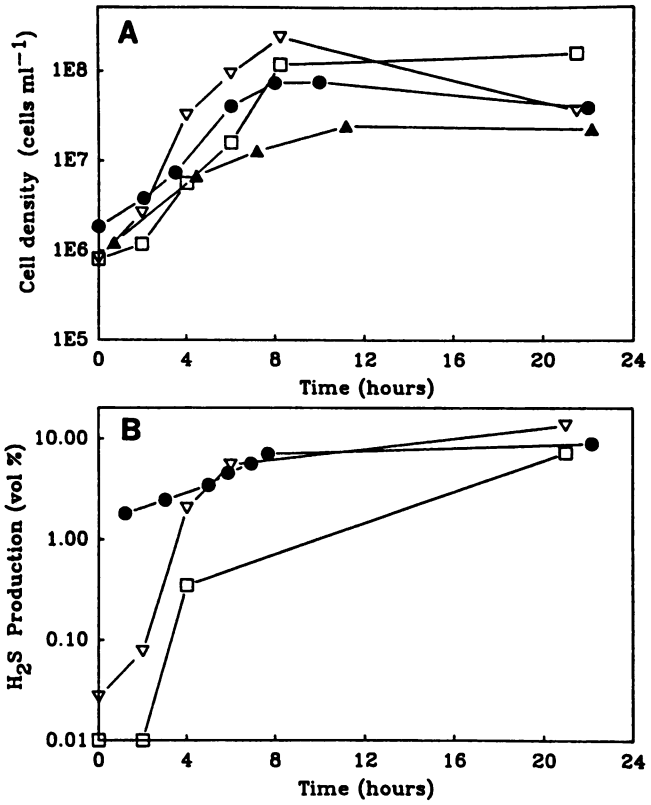

FIG. 4. Growth of *P. furiosus* on different sulfur-containing compounds.(A) Bacterial growth on cystine (▽), methyl trisulfide (●), and sulfur (□); growth on no sulfur is shown for comparison (▲). (B) Hydrogen sulfide production in cultures shown in panel A. The datum points represent averages of duplicate experiments.

iments indicated that there was a direct correlation between H_2S production and formation of polysulfides. Only dimethyl trisulfide and cystine samples tested positive for polysulfides. Dimethyl trisulfide is by itself a polysulfide and produces thiocyanate in the cyanolysis reaction. Cystine, on the other hand, is a disulfide that does not react to form thiocyanate under the reaction conditions of the polysulfide assay. However, when incubated at 98°C in either water or medium, cystine produces reaction products that test positive for polysulfides. The integrity of cystine after incubation at 98°C was also studied by using proton nuclear magnetic resonance spectroscopy, and the results indicate that the molecule is degraded at that temperature. It appears that the disulfide bonds of cystine are broken to form longer polysulfides in a reaction that likely involves nucleophilic attack of a sulfide or hydrosulfide ion (13). The fact that the other disulfide-containing compounds (ethyl disulfide and dimethyl disulfide) did not form polysulfides may be attributed to the higher stability of the S-S bonds in these compounds (18).

Polysulfide utilization. *P. furiosus* was grown in batch fermentations with polysulfides as substrates to test its ability to utilize these sulfur polymers. A 0.5 M hydrogen polysulfide solution was added to the growth medium to a final concentration of 10 mM. Figure 5 shows typical results obtained with these batch fermentations. Bacterial growth on the polysulfides was comparable to growth in elemental sulfur; minimum cell doubling times were between 50 and 55 min, and final cell yields were close to 10^8/ml. Cell growth was accompanied by a decrease in polysulfide concentration

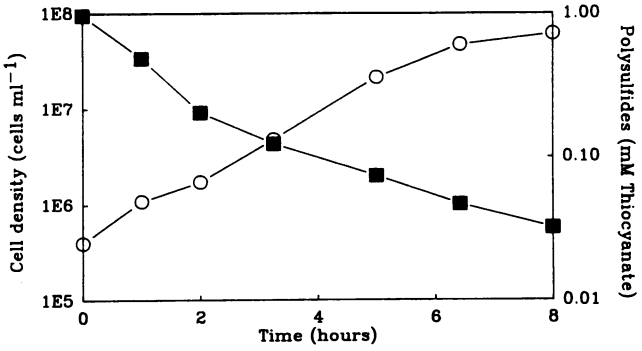

FIG. 5. *P. furiosus* grown in a batch culture containing polysulfides. Symbols: ○, cell density; ■, polysulfide concentration.

(Fig. 5), and the onset of the stationary phase corresponded to exhaustion of the polysulfides, indicating that the cultures were sulfur limited. It was also found that the pH of the culture decreased in parallel with consumption of the polysulfides, from an initial value of 9.2 to approximately 8.3. Higher initial polysulfide concentrations (20 and 30 mM) resulted in high pH values (10.0 to 10.5) in the medium that were inhibitory for bacterial growth. This represents an obstacle to the use of polysulfides as substrates for high biomass yields.

^{35}S uptake studies. Growth of *P. furiosus* in diluted radioactive sulfur resulted in cell densities of about 10^8/ml and generation of significant amounts of hydrogen sulfide. On the other hand, growth during incubation of the bacteria with $^{35}S^0$ in defined medium was not quantitated. However, cultures were assumed to be metabolically active during this period because they produced considerable amounts of hydrogen sulfide. Results of $^{35}S^0$ uptake studies showed no detectable radioactivity in bacterial pellets. All of the radioactivity could be easily removed from the bacteria by washing the cell pellets with fresh medium. Despite this, cell pellets from 100-ml cultures were lysed and electrophoresed onto a sodium dodecyl sulfate-polyacrylamide gel to determine whether any ^{35}S was incorporated into cellular protein. Results of this analysis indicated that no radioactivity could be detected in the cell extracts, even after 2 weeks of exposure of the X-ray films to the dried polyacrylamide gels. In contrast, ^{35}S-labeled cysteine or methionine was readily incorporated into cellular proteins. Competition studies with bacteria grown on labeled cysteine with cold methionine and labeled methionine with cold cysteine showed that neither amino acid was preferentially utilized. These results suggest that elemental sulfur metabolism in *P. furiosus* is dissimilatory and that amino acids or sulfur-containing peptides from protein proteolysis can be used as a source of sulfur for the cells.

DISCUSSION

The nature of bacterium-sulfur interactions has been controversial for some time. Two main mechanisms can be proposed for the metabolism of sulfur. (i) Bacteria have to be in direct contact with solid sulfur to metabolize it, or (ii) they use a soluble sulfur compound formed by a chemical or enzymatic reaction. The actual mechanism depends on the bacterial system under consideration. In aerobic sulfur oxidation by *T. thiooxidans*, most evidence indicates that direct contact between the bacteria and solid sulfur is important (2, 28). Moreover, it has been demonstrated that these bacteria

excrete phospholipids that serve as surfactants to promote bacterial attachment (14). Direct bacterial contact with sulfur has also been described in the hyperthermophilic bacteria *Pyrodictium brockii* and *Pyrodictium occultum* (25) and in the moderate thermophile *Sulfolobus mirabilis* (8). However, in these cases, it is not clear that attachment is necessary for sulfur metabolism. On the other hand, it has been suggested that a soluble sulfur intermediate, which has also been called activated sulfur, might be formed during elemental sulfur metabolism (3, 33).

The data presented here show that the hyperthermophilic bacterium *P. furiosus* does not need to be in direct contact with elemental sulfur to metabolize it and that soluble polysulfides serve as substrates for sulfur reduction. Production of H_2S in culture vials in which solid sulfur was separated from the bacteria by dialysis membranes indicates that sulfur reduction can occur without bacterial contact with the substrate. This result also suggests that elemental sulfur reacts to form a soluble sulfur compound which serves as a substrate for the bacteria. When the eight-member sulfur ring is cleaved, a polysulfide is formed. Both ionic and free-radical ring-opening mechanisms have been described for this reaction (9, 20, 21). However, in ionic solutions, elemental sulfur is most susceptible to nucleophilic attack, resulting in formation of polysulfide chains with two negative charges (24). Analysis of culture supernatants of *P. furiosus* grown with sulfur placed in the dialysis membranes and with sulfur dispersed in the medium showed that polysulfides were present. Moreover, polysulfide concentrations increased as growth proceeded, indicating that bacterial metabolism produced a sulfur nucleophile that promoted solubilization. A large number of ions are known to be reactive to sulfur-sulfur bonds (13), and some of these compounds are readily found under the growth conditions of hyperthermophilic bacteria. These include sulfide (S^{2-}), hydrosulfide (HS^-), thiols or mercaptans (e.g., cysteine and methyl mercaptan), and hydroxyl ions. By using ^{35}S-labeled elemental sulfur, it was possible to determine the effectiveness of these nucleophiles as sulfur-solubilizing compounds. The results showed that the solubilization rate was faster and the amounts of sulfur released were higher with cysteine and sodium sulfide than with methyl mercaptan or hydroxyl ions. These results correspond well to the reported S nucleophilicity of the compounds tested (13, 22). In addition, sulfur release kinetics in growing *P. furiosus* cultures were very similar to the kinetics obtained with cysteine and sodium sulfide. As H_2S is the metabolic product of sulfur reduction, these results indicate that sulfide ions (S^{2-} and HS^-) are probably the most important sulfur nucleophiles present in these bacterial systems.

The chemistry of elemental sulfur in aqueous sodium polysulfide solutions is well documented (9, 29, 30). The initial ring-opening reaction

$$S_8 + HS^- \rightleftharpoons S_8S^{2-} + H^+ \quad (1)$$

is followed by rapid chain degradation and establishment of an equilibrium among polysulfide ions of different sizes, sulfide, and hydroxyl ions according to the following reaction (30):

$$S_{m+n}S^{2-} + HS^- + OH^- \rightleftharpoons\!\!\rightleftharpoons S_mS^{2-} + S_nS^{2-} + H_2O \quad (2)$$

However, in the *P. furiosus* system, equilibrium may never be reached, since H_2S concentrations increase as growth proceeds, resulting in a constantly changing mixture of different-length polysulfides. During the initial growth stages, the sulfide levels are relatively low and consequently, the average length of the polysulfide molecules is probably longer than during later growth stages, when sulfide concentrations are high. On the other hand, the growth temperature of this bacterium (98°C) may have a stabilizing effect on the polysulfides in solution, since it has been shown that the mean size of these molecules increases with increasing temperatures (30). Because of the complexity of these reactions, no attempt was made to determine the length distribution of the sulfanes in growing cultures. Further work should establish whether there is a correlation between the length of a polysulfide molecule and its ability to serve as a substrate for reduction. The effect of pH on the formation and stability of the polysulfides is determined by equation 2. Results presented in this paper and previously published data (9) support the idea that the presence of hydroxyl ions affects the equilibria of the polysulfide formation reactions but has a negligible effect on sulfur dissolution.

The data presented here also indicate that there is a direct correlation between the formation of polysulfides from different sulfur-containing organic compounds and the production of H_2S by *P. furiosus*. Sulfur reduction was detected only in bacterial cultures grown on cystine and dimethyl trisulfide. Cystine decomposed and formed polysulfides in solution at 98°C. Dimethyl trisulfide is a polysulfide which, under incubation conditions and in the presence of sulfide ions, can react to form longer polysulfide chains (18). On the other hand, no sulfide was produced in the presence of dimethyl disulfide, ethyl disulfide, cysteine, trithiane, sodium thiosulfate, or tetrahydrothiophene, none of which produced polysulfides during incubation at 98°C. These results support the finding that polysulfides are the true substrates for sulfur reduction by *P. furiosus*. They also indicate that care must be taken during identification of alternate sulfur sources for thermophilic bacteria, since the original substrates are likely to degrade at high growth temperatures and give origin to new and completely different sulfur-containing compounds.

Polysulfide utilization by *P. furiosus* was further demonstrated by growth of this bacterium on ASW supplemented with 10 mM polysulfides. Growth rates in these cultures were comparable to those obtained with bacterial cultures grown on elemental sulfur. Final cell densities on 10 mM polysulfides were typically 8.0×10^7/ml $\pm$ 0.5×10^7/ml, which were somewhat lower than the 3.0×10^8/ml $\pm$ 1.0×10^8/ml obtained on elemental sulfur. These lower cell densities are the result of sulfur limitations in the system; the bacteria grow until all of the polysulfides are exhausted. Higher polysulfide concentrations result in high pH values (>10) that are inhibitory for bacterial growth. One way to alleviate this problem might be to use a fed-batch cultivation mode in which the polysulfides are slowly fed to the growing culture. Preliminary results obtained by using this approach indicate that higher cell densities can be achieved (data not shown).

Considering the sulfur-rich environments in which *P. furiosus* thrives (7), it was of interest to determine whether elemental sulfur could be assimilated into cell components. The results obtained indicate that elemental sulfur was not incorporated into the bacterial proteins, even when the cells were grown in defined medium lacking cysteine and methionine. Moreover, elemental sulfur was not transported inside the bacteria, since all of the ^{35}S was easily removed by washing the cells with fresh medium or buffer. On the other hand, [^{35}S]cysteine and [^{35}S]methionine were readily incorporated into cell protein. Taken together, these results suggest that elemental sulfur metabolism in *P. furiosus* is

dissimilatory and that most likely, under natural conditions, these bacteria obtain their cell sulfur from turnover of organic material. Membrane-bound sulfur reductase activity has been described in *Spirillum* sp. strain 5175 (33) and *Wollinella succinogenes* (23). The above-described results suggest that sulfur reduction in *P. furiosus* occurs extracellularly and that it is a membrane-associated event.

The mechanism of sulfur reduction and its significance in the metabolism of *P. furiosus* remain to be determined. Three distinct possibilities are being investigated: (i) sulfur reduction by a mechanism that does not yield energy, (ii) sulfur reduction by an energy-yielding mechanism (i.e., sulfur respiration), and (iii) abiotic reduction of S^0 by hydrogen generated in bacterial metabolism. Possibilities i and ii imply the presence of a protein or set of proteins that reduce sulfur to sulfide. In contrast, mechanism iii does not require direct intervention of enzymes or electron transfer agents. Under the reducing growth environment and at 98°C, sulfur in the form of polysulfides readily reacts with gaseous hydrogen to form H_2S (unpublished data). This suggests that a sulfur-reducing protein is not necessary and supports the idea that sulfur serves as a detoxifying agent for hydrogen (7). Also, the isolation and characterization of the hydrogenase and ferredoxin involved in H_2 generation by *P. furiosus* suggest that this bacterium grows predominantly by fermentation (1, 5). However, more information is needed to preclude the existence of sulfur respiration and to establish the presence or absence of a sulfur-reducing protein.

Identification of water-soluble polysulfides as substrates for sulfur reduction in *P. furiosus* gives a new perspective to the understanding of the ecology and metabolism of hyperthermophilic bacteria. Polysulfides have been shown to be formed under the physico-chemical conditions of seawater (4) and are likely to be present at the isolation sites of these bacteria (deep-sea hydrothermal vents, continental volcanic areas, and shallow submarine geothermal sites). In deep-sea vents, polysulfides can be formed by oxidation of H_2S present in hot vent fluids as they contact cooler marine water (26). In terrestrial and shallow marine volcanic areas, these sulfur polymers can result from reaction of H_2S with the existing sulfur deposits. Therefore, it is very likely that polysulfides are natural substrates for sulfur metabolism in hyperthermophilic bacteria. This ability to metabolize a soluble sulfur substrate would also explain why most of these bacteria are found in suspension and not attached to solid surfaces.

ACKNOWLEDGMENTS

This work was supported in part by the National Science Foundation (grant CBT-8813608) and the Electric Power Research Institute. We thank R. Maier and M. Adams for helpful comments.

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