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Biological and Abiological Sulfur Reduction at High Temperatures†

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Reduction of elemental sulfur was studied in the presence and absence of thermophilic sulfur-reducing bacteria, at temperatures ranging from 65 to 110°C, in anoxic artificial seawater media. Above 80°C, significant amounts of sulfide were produced abiologically at linear rates, presumably by the disproportionation of sulfur. These rates increased with increasing temperature and pH and were enhanced by yeast extract. In the same medium, the sulfur respiration of two recent thermophilic isolates, a eubacterium and an archaebacterium, resulted in sulfide production at exponential rates. Although not essential for growth, sulfur increased the cell yield in both strains up to fourfold. It is suggested that sulfur respiration is favored at high temperatures and that this process is not limited to archaebacteria, but is shared by other extreme thermophiles.

Several modes of bacterial anaerobic respiration are known, in which various inorganic compounds, primarily sulfate, nitrate, or carbon dioxide, may serve as terminal electron acceptors. The role of elemental sulfur as an oxidant in anaerobic respiration has been less investigated.

The reduction of elemental sulfur yields less energy with a given reductant than the other electron acceptors mentioned above (22). At the range of temperatures for mesophilic growth and below, elemental sulfur is relatively uncommon as an oxidant. Microbial sulfur reduction is often observed, however, in nonspecific side reactions (19). Several photosynthetic bacteria reduce sulfur to sulfide at very low rates in the course of fermentative, anaerobic dark metabolism (23, 24). In the cyanobacterium *Oscillatoria limnetica* dark sulfur reduction appears to be respiratory but does not support growth (16). *Beggiatoa* sp. was reported to reduce internally stored sulfur to sulfide (15). The metabolic significance of this reaction is unknown.

In mesophilic bacteria "true" sulfur respiration has been described only in a few cases. *Desulfuromonas acetoxidans*, a nonfermentative anaerobe, utilizes acetate, ethanol, or propanol while stoichiometrically reducing sulfur to sulfide (17). A few sulfate-reducing bacteria (2), a *Campylobacter* sp. (13) and a *Spirillum* sp. (26), have also been demonstrated to support growth to various degrees by sulfur respiration.

Recent comprehensive studies on newly described extremely thermophilic archaebacteria have shown the frequent occurrence of sulfur respiration. It has been found in all anaerobic thermoacidophiles (9, 21, 27–30), as well as in the thermophilic methanogens (20). This fact has been interpreted as indicating a close evolutionary relationship between these two branches of the archaebacteria (20).

The correlation between thermophily and the reduction of elemental sulfur, coupled to respiratory metabolism or not, is apparent. We have recently observed the production of sulfide in uninoculated, sulfur-containing anaerobic media of varied composition, serving as controls for growth of thermophilic isolates from marine hot springs (S. Belkin and H. Jannasch, Arch. Microbiol., in press). The present paper describes the experimental conditions under which this abiological process takes place and discusses the occurrence of biological sulfur reduction and its possible role in the metabolism of extremely thermophilic bacteria.

MATERIALS AND METHODS

Isolates. The two thermophilic strains used in this study were isolated from a shallow submarine thermal spring off the coast of Lucrino (Bay of Naples, Italy). Both isolates are heterotrophic and obligate anaerobes. Strain NS-E is a rod-shaped eubacterium, with an optimum growth temperature of 77°C at pH 7.5. Strain NS-C is an irregular coccoid archaebacterium, growing optimally at 88°C and pH 7.2. Detailed descriptions of these strains will be published elsewhere (Belkin and Jannasch, in press; S. Belkin, C. Wirsen, and H. Jannasch, manuscript in preparation).

Media and growth conditions. For all experiments and for routine culture maintenance, 17-ml Hungate-type test tubes (Bellco Glass, Inc.) were used, containing 10 ml of the appropriate medium, added under a nitrogen gas flow. The tubes were then flushed for 15 min with hydrogen or with nitrogen gas passed through heated (350°C) copper shavings.

For abiological sulfide production, an artificial seawater medium (ASW) of the following composition was used: NaCl, 430 mM; $(NH_4)_2SO_4$, 7.6 mM; $MgSO_4 \cdot 7H_2O$, 6.1 mM; KH_2PO_4, 3.1 mM; $CaCl_2 \cdot 2H_2O$, 2 mM; trace elements solution (3), 1 ml liter^{-1}; resazurine, 0.6 mg liter^{-1}. The medium was supplemented with steam-autoclaved sulfur (100 mg 10 ml^{-1}, or as indicated) in the absence or presence of the indicated concentrations of yeast extract. The pH was controlled by the addition of 50 mM bis-Tris propane {1,3-bis[tris(hydroxymethyl)-methylamino]-propane} buffer for the pH range 6.3 to 8.0 or 50 mM MES (2[*N*-morpholino]ethanesulfonic acid) buffer for pH values lower than 6.3.

Both isolates were routinely grown in 2216 marine broth (Difco Laboratories), diluted to half-strength with synthetic seawater (Turks Island Salt; 14). The medium was supplemented with a vitamin mix (thiamine-HCl, biotin, and B_{12}, 40 μg liter^{-1} each), resazurine (0.6 mg liter^{-1}), and 20 mM PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) buffer, at pH 7.2 (for strain NS-C) or 7.5 (for strain NS-E). Sodium thioglycolate (to 2 mg liter^{-1}) was injected into the N_2-flushed tubes, which were then preincubated at the given growth temperature. After preincubation of about 1 h,

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during which the resazurine turned colorless, the tubes were inoculated (to 10^6 cells ml^{-1} from a 18- to 24-h culture) and incubated without shaking.

For measurements of biological sulfur reduction, cells of both strains were grown under conditions allowing optimal growth without sulfur. For strain NS-C the medium was the supplemented marine broth described above. Strain NS-E was grown in ASW, with the addition of yeast extract (2 g $liter^{-1}$) and glucose (1 g $liter^{-1}$). Steam-autoclaved sulfur was added at 100 mg 10 ml of $medium^{-1}$. At different time intervals during the course at 24 h, liquid samples were withdrawn for sulfide determination or cell counts or both.

Incubation at temperatures up to 100°C was carried out in covered thermostated water baths. Temperature variations did not exceed 1°C. For incubation at 110°C, a small autoclave (Wisconsin Aluminum Foundry, Inc.) was used, with temperature variations of 2°C and at a pressure of 0.4 atm (40.5 kPa) above ambient.

Sulfide determination. The determination of sulfide was carried out according to Cline (6), with slight modifications to allow for the small sample volumes and the wide range of sulfide concentrations encountered. A measured sample of reagent (0.08 ml) was introduced into a nitrogen-flushed 1.0-ml syringe and then brought to 1.0-ml volume by withdrawal of liquid sample through the rubber septum of the Hungate tube immediately upon its removal from the water bath. All sulfide determinations were done on duplicate tubes. For further verification of the assay method, duplicate samples were also assayed with an Orion sulfide electrode (model 94-16A, in conjunction with model 90-02 reference electrode, using a Fisher Accumet pH meter model 825 MP). The two procedures agreed within 10%.

To assess the total amounts of sulfide actually produced, immediate sampling from the hot tubes, at various temperatures and pH values was compared with samples withdrawn after being cooled to room temperature. As expected from the decreased solubility of H_2S at high temperatures (5), the amount of H_2S present in the 7-ml gas phase as compared with that present in the 10 ml of hot medium depended on temperature, pH, and sulfide concentration. At 98°C, pH 7.5, and in the presence of 1 mM total sulfide, about 50% of the sulfide was in the gas phase. The data reported in this paper are based on measurement of dissolved H_2S at the temperature stated. The sulfide production rates, therefore, must be considered as minimum values.

Cell counts. After fixation of samples in 0.5% glutaraldehyde in autoclaved and filtered (0.2 μm; Gelman Acrodisc) seawater and staining with acridine orange (0.01%), cells were counted by epifluorescent microscopy (12).

RESULTS

Abiological sulfide production from elemental sulfur. Abiological sulfide production was measured in relation to temperature, pH, the concentration of elemental sulfur, and the concentration of yeast extract, in the presence of N_2 or H_2.

The production of sulfide was limited to temperatures above 80°C (Fig. 1), the rates increasing with increasing temperature. Sulfide production was linear with time for at least 24 h, but was preceded by a lag period (Fig. 1, inset). The length of this period before the resazurine became colorless varied with temperature from approximately 9 h at 88°C to <30 min at 110°C. This lag was probably due to initial reduction of traces of dissolved oxygen before the accumulation of measurable sulfide. It should be noted that even at 75°C small amounts of sulfide could be measured after incubation periods of 48 h.

Figure 1 also shows that sulfide production under H_2 gas phase was not significantly higher than under N_2. Although in other experiments H_2 seemed to have a minor positive effect (see Fig. 2 and 3), it is apparently not used as the primary reductant. This function, furthermore, cannot be attributed to one of the minerals comprising the seawater medium used in the experiments, since in distilled water sulfide production from sulfur was not significantly different (data not shown).

Sulfide production was enhanced to a certain extent by yeast extract (Fig. 1 and 2), which may indicate that the added organic matter supplies additional reducing power. The rate was doubled in the presence of 1 g of this substrate $liter^{-1}$, but higher concentrations of yeast extract had no further effect (Fig. 2). It has also been suggested (21) that the presence of yeast extract results in a better dispersion of elemental sulfur in liquid media.

Sulfide production rates were positively correlated with the concentration of elemental sulfur (Fig. 3). The rates, however, were not directly proportional to the amounts of sulfur, an effect possibly caused by the heterogeneous dispersion of the sulfur in the liquid medium. In most cases the powdered sulfur tended to remain at the bottom of the unmixed test tube, resulting in a limited contact with the medium.

Sulfur reduction at 98°C was highly pH dependent, the rates increasing with increasing pH. Sulfide concentrations measured after 24 h of incubation at various pH values showed a 60-fold increase over 1 pH unit (Fig. 4). According to the pH dependency of H_2S solubility, only a 10-fold increase over a single pH unit should occur. This indication of an increasing sulfide production at increasing pH values was confirmed by H_2S measurements in the gas phase (data not shown). At pH values higher than 7.5, the medium turned yellow-orange during incubation at 98°C, probably due to the formation of polysulfides. These are probably the product of a reaction between elemental sulfur and newly formed sulfide, although the possibility that the polysulfides may be instrumental in H_2S formation cannot be discounted.

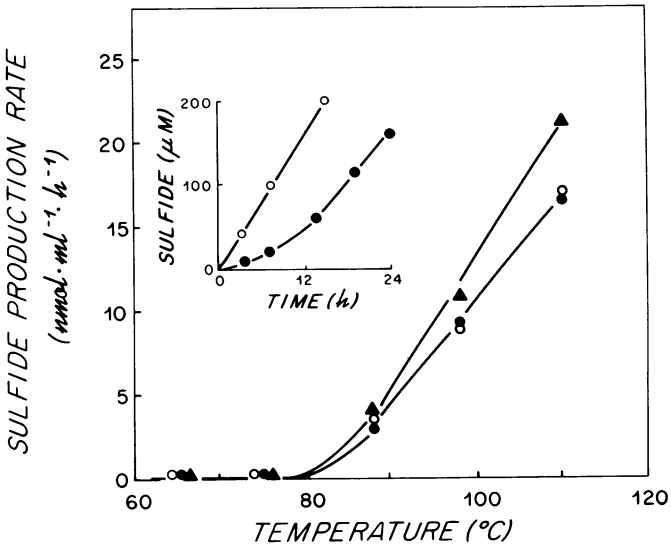

FIG. 1. Temperature dependence of abiological sulfur reduction in ASW at pH 7.5 during a 24-h incubation under N_2 (●), H_2 (○), or in the presence of 2 g of yeast extract $liter^{-1}$ under N_2 (▲). (Inset) Abiological sulfur reduction at 88°C (●) and 110°C (○) under N_2.

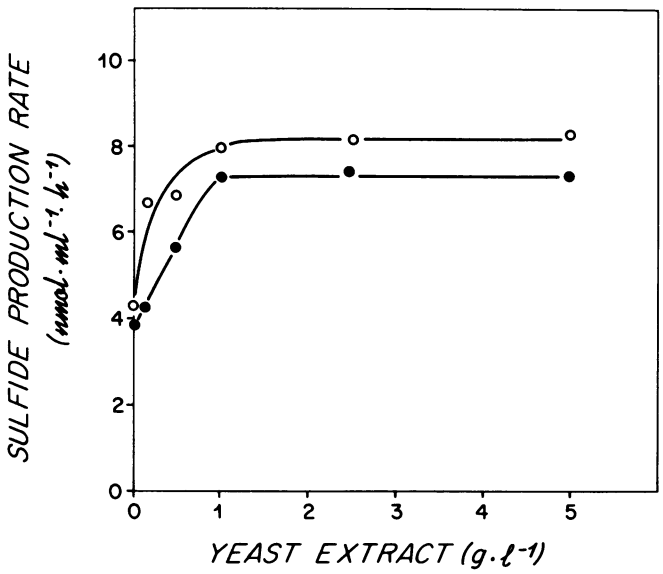

FIG. 2. Effect of yeast extract on abiological sulfur reduction in ASW at pH 7.5 during a 24-h incubation at 98°C under N_2 (●) or H_2 (○).

Biological sulfur reduction. The biological reduction of elemental sulfur at high temperatures was tested with two of our thermophilic sulfur-reducing bacterial isolates. Since thermophilic sulfur reduction has so far only been reported from members of the archaebacteria (9, 20, 21, 27–30), one of our isolates (NS-E) is unusual. Its probable eubacterial affiliation has been determined by its sensitivity to chloramphenicol, streptomycin, and vancomycin (100 μg ml^{-1}) and by the presence of murein in its cell walls (Belkin et al., in preparation).

During growth of strain NS-E at its optimal growth temperature (77°C) and pH (7.5), sulfur was actively reduced to sulfide (Fig. 5A). In accordance with the data presented in Fig. 1, there was very little sulfide production at this temperature in the absence of cells. Furthermore, the same minute rate of sulfur reduction was observed when growth of the organism was inhibited by chloramphenicol or when the yeast extract and glucose were replaced by acetate, which cannot be utilized by strain NS-E. In accordance with these observations, biological sulfide production ceased upon the onset of the stationary growth phase.

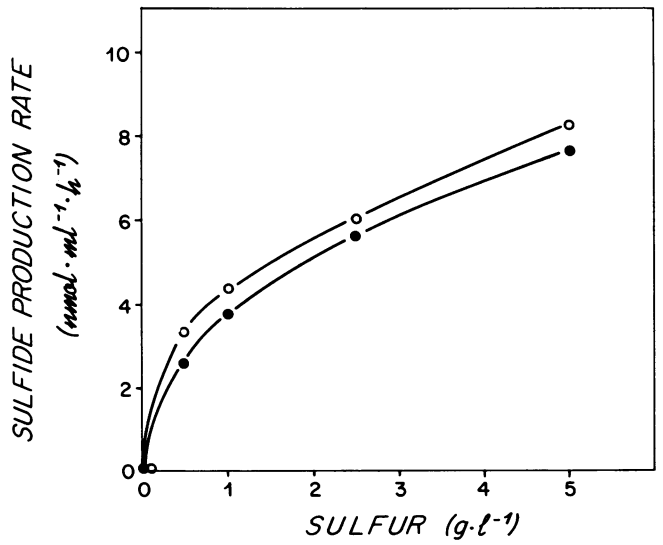

FIG. 3. Effect of elemental sulfur on abiological sulfur reduction in ASW at pH 7.5 during a 24-h incubation at 98°C under N_2 (●) or H_2 (○).

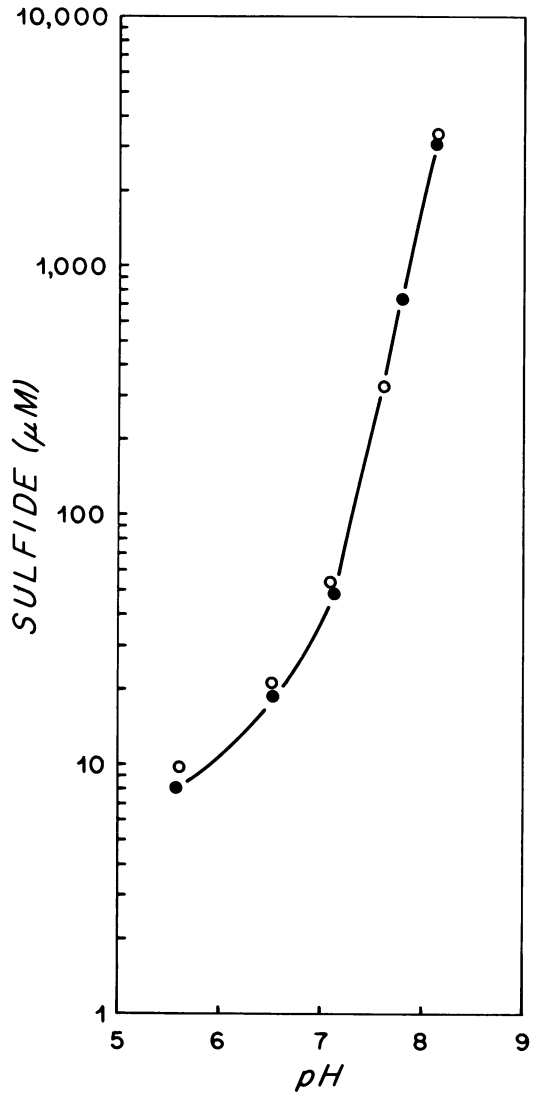

FIG. 4. Effect of pH on abiological sulfur reduction in ASW at 98°C, under N_2 (●) or H_2 (○). Final (24 h) pH and sulfide concentrations are presented. The pH at that point is up to 0.1 unit lower than the initial value.

When the same experiment was carried out with the archaebacterial isolate (NS-C, grown at its optimal temperature of 88°C and pH 7.2), similar results were obtained (Fig. 5B). These similarities can be summarized as follows: (i) sulfide production accompanies growth; (ii) as a consequence, and unlike the abiological reaction, sulfide production is exponential rather than linear; (iii) under optimal growth conditions, elemental sulfur does not affect growth rates, but reproducibly enhances final cell yield by a factor of up to 4.

In several points, however, the two isolates differ with regard to sulfur utilization. Growth rates of strain NS-C at

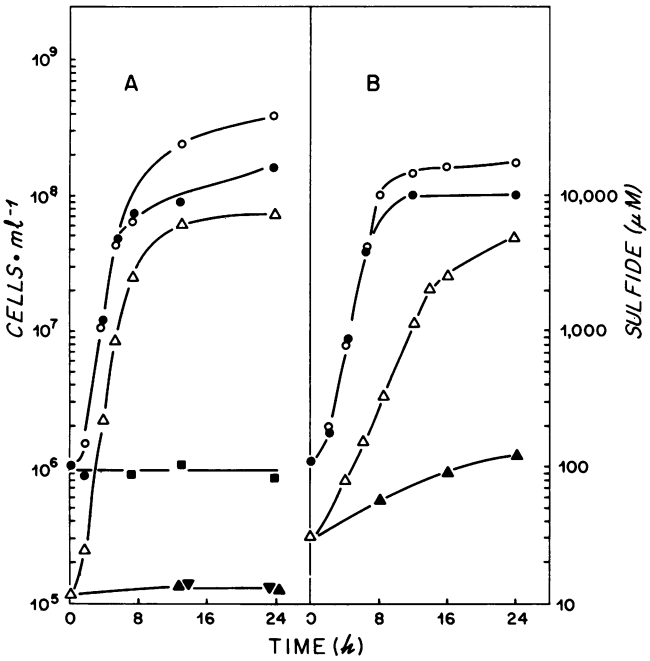

FIG. 5. Growth and sulfur reduction in cultures of two extremely thermophilic bacterial strains. (A) Strain NS-E, grown in ASW with yeast extract (2 g liter^{-1}, glucose (1 g liter^{-1}), and 20 mM PIPES buffer, at pH 7.5 and 77°C. (B) Strain NS-C, grown in marine broth at pH 7.2 and 88°C. Symbols: growth (●) in the absence and (○) in the presence of elemental sulfur; (△) sulfide production in the presence of elemental sulfur; (■) growth in the presence of acetate (3 g liter^{-1}) as sole carbon source; (▼) sulfide production in the presence of acetate or chloramphenicol (100 μg ml^{-1}); (▲) sulfide production in unioculated sulfur-containing medium.

suboptimal yeast extract concentrations are greatly enhanced (up to 3.5-fold) in the presence of sulfur, whereas the effect on the growth rates of strain NS-E under similarly growth-limiting conditions is smaller and variable. On the other hand, whereas in strain NS-E growth parallels sulfide production with doubling times of 40 to 45 min for both parameters, in strain NS-C the doubling time for growth (50 min) is much shorter than for sulfide production (130 min). These dissimilarities may reflect different modes of utilization of the carbon sources as coupled to sulfur reduction. Furthermore, sulfide production of strain NS-C does not stop after the end of the exponential growth phase but continues at a decreased rate, whereas in strain NS-E sulfide generation completely ceases at that point. This can be explained by different sulfide sensitivities in the two organisms. Isolate NS-E is a very active sulfide producer, but its metabolism is completely inhibited when a sulfide concentration of about 10 mM is attained. In contrast, during growth of strain NS-C no inhibitory effect of sulfide is apparent. Here the continued sulfide production during the stationary phase may be the result of maintenance level respiration.

DISCUSSION

Our data show that abiological sulfur reduction to sulfide occurs above 80°C, at rates which increase with increasing temperature and pH, and that it depends to a certain extent upon the presence of an organic substrate.

At around 95°C, the crystalline structure of elemental sulfur shifts from the orthorhombic form to less stable monoclinal sulfur (10). Although most published reports on this subject refer to sulfur in organic solutions, they indicate a susceptibility of the S–S bond in the sulfur crystal to cleavage by various agents at high temperatures (10, 19). It is of specific interest that in basic solutions, at temperatures close to 100°C, sulfur disproportionates to sulfide and thiosulfate (18). A similar mechanism may have been active in our experiments, where the reaction was enhanced at high pH values. If that is indeed the case, elemental sulfur serves as both an electron acceptor and an electron donor, with no dependency on an externally supplied reductant. This agrees well with the observation that the reaction was almost as efficient under H_2 or N_2, in double-distilled water as well as in the ASW medium. In qualitative experiments significant thiosulfate was found to be produced at 95°C. To verify quantitatively this disproportionation of elemental sulfur to sulfide and thiosulfate, problems such as product stability, chemical interference in analyses, and temperature effect on sulfide solubility must be dealt with and will be the subject of a future study.

The data of this study imply the importance of abiological controls when measuring sulfur reduction by extremely thermophilic bacteria. The abiological activity will be less significant in cultures of thermoacidophilic strains (9, 21, 27–30) because of the unfavorable pH. It must be taken into account, however, in stoichiometric studies of sulfur respiration in non-acidophilic thermophiles at a well-buffered pH.

The data also imply that part of the sulfide commonly detected in thermal environments may be the result of abiological sulfur reduction. The requirements for this to occur, temperatures above 80°C, pH above neutral, and the presence of sulfur, are often encountered in thermal waters (4).

For mesophilic bacteria at moderate temperatures, on the other hand, elemental sulfur appears to be an unfavorable oxidant. Its reduction yields relatively little energy (22). Furthermore, it is not abundant in aquatic habitats, and its solubility in water approaches zero (5). Nevertheless, a number of microorganisms, such as *Desulfuromonas* (17), *Beggiatoa* (15), or *Spirillum* (26) spp., are able to reduce elemental sulfur with various degrees of efficiency.

In contrast, among the recently described thermophilic archaebacteria, sulfur respiration appears to be widespread and sometimes obligatory (21, 27–30). In addition, strain NS-E, a eubacterial thermophile, is also an active sulfur reducer. This apparent connection between thermophily and sulfur reduction may be related to the fact that all of these organisms have been isolated from sulfur-rich sources. It is tempting to hypothesize, however, that the occurrence of biological sulfur reduction is, similar to the abiological process, temperature related. In other words, elemental sulfur may act as a preferable electron acceptor for thermophilic microorganisms. Its function may be facilitated by chemical changes of sulfur at increasing temperature, such as a possible increase in solubility or the formation of polysulfides (7, 10). It should be noted that sulfate, as the widely distributed and highly soluble electron acceptor for anaerobic respiration, does not commonly occur in thermal waters (8).

Neither of the strains described herein depend on sulfur reduction for growth. However, when sulfur was available it was readily reduced with a considerable enhancement of cell yields but with no effect on the growth rate. Thus, sulfur respiration may increase the efficiency in the utilization of carbon sources and possibly extend the range of utilizable carbon compounds. Since we did not demonstrate the stoi-

chiometric use of sulfur as an electron acceptor in the isolates discussed in this paper, the term "sulfur respiration" should be used with some qualification. This non-obligatory role of sulfur is in contrast to the importance ascribed to its function in several archaebacterial species (9, 21, 27–30) as well as in *D. acetoxidans* (17). Like the latter organism, strains NS-E and NS-C cannot replace elemental sulfur with thiosulfate as an electron acceptor.

During the past decade, the isolation of novel, extremely thermophilic bacteria has demonstrated that life can be found and sustained at 100°C and above (11, 21, 27–30). Under such conditions, reactions considered to be irrelevant to biological processes may prove to carry out important functions. The sulfur reduction at high temperatures appears to be an example of such a case. It remains to be established how widely sulfur respiration occurs among the extremely thermophilic bacteria. That their typical growth conditions favor sulfur reduction suggests that sulfur respiration may be a common phenomenon in marine and terrestrial hot springs.

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