JOURNAL OF BACTERIOLOGY, May 1973, p. 706-710

Vol. 114, No. 2
Printed in U.S.A.

# Oxidation of Elemental Sulfur by *Sulfolobus acidocaldarius*

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Received for publication 8 September 1972

Oxidation of elemental sulfur by *Sulfolobus acidocaldarius*, an autotroph which grows at high temperatures and low pH, was examined by use of $^{35}S$-labeled elemental sulfur. When cultured at pH 3.2 and 70 C, *S. acidocaldarius* oxidized elemental sulfur essentially quantitatively to sulfuric acid. Oxidation rate paralleled growth rate and decrease in pH of the culture medium. Elemental sulfur was not oxidized under these conditions if the culture was poisoned with formaldehyde. During the growth phase, the proportion of cells attached to the sulfur crystals increased progressively, and in the later phases of growth over 10 times more cells were attached to sulfur than were free. Doubling times for eight strains growing on elemental sulfur varied from 37 to 55 h. The organism grows much more rapidly on yeast extract than on sulfur. In a medium containing both sulfur and yeast extract, sulfur oxidation was partially inhibited, although growth was excellent.

Brock et al. (2) have reported the isolation of a new species of bacteria designated *Sulfolobus acidocaldarius* from both thermal acid soils and acid hot springs. The organism has been found in a number of geothermal areas where there is an abundant source of sulfur, low pH (less than 3.0), and high temperature (65 to 90 C). *S. acidocaldarius* grows heterotrophically on yeast extract and autotrophically on elemental sulfur (2).

The purpose of this paper is to characterize the autotrophic growth of this organism on sulfur and to demonstrate the oxidation of $^{35}S$-labeled elemental sulfur to sulfate. The attachment of *S. acidocaldarius* cells to sulfur crystals was also studied.

## MATERIALS AND METHODS

**Organisms.** The eight strains of *S. acidocaldarius* used in this study were isolated by Brock et al. (2) from acid hot springs in Yellowstone National Park, El Salvador, and Italy. Strain 129-2, not cited by Brock et al. (2), was isolated from the same source as strain 129-1. The pH and temperature of the acid hot springs from which these organisms were isolated ranged from pH 1.7 to 2.5 and from 65 to 85 C.

**Culture technique.** The basal salts medium was that described by Allen (1) except that the trace elements were omitted. The medium contained: $(NH_4)_2SO_4$, 1.3 g; $KH_2PO_4$, 0.28 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2 \cdot 2H_2O$, 0.07 g; and distilled water, 1,000 ml; the pH was adjusted to 2.5 to 3.2 with 10 N $H_2SO_4$. To 250-ml prescription bottles which contained 62.5 mg of $^{35}S$-labeled elemental sulfur was added 40 ml of basal salts medium; sterilization was by Tyndallization. The medium was inoculated with 10% (vol/vol) of an actively growing culture and was bubbled for 1 min at room temperature with 5% $CO_2$ in air. The container was then sealed and incubated at 70 C. At intervals during growth, a 3-ml sample was removed and the bottle was regassed. The pH, optical density (Bausch & Lomb Spectronic 20 colorimeter, 550 nm), direct cell count, and the quantity of radioactive sulfate were determined for the sample.

**$^{35}S^0$ preparation.** $^{35}S$-labeled elemental sulfur in benzene (New England Nuclear Corp.) was added to 2 g of sulfur powder NF (Fisher Scientific Co.) dissolved in 10 ml of $CS_2$ and thoroughly mixed. The $CS_2$ and benzene were removed by evaporation. The resulting radioactive sulfur crystals were dried and pulverized to make a fine powder. The final specific activity usually was about 225,000 counts per min per mg of $^{35}S$-labeled elemental sulfur.

**Detection of $^{35}SO_4$.** The substrate, $^{35}S^0$, is insoluble in the culture medium, whereas the oxidized product is soluble. Therefore, the oxidized product was separated from the substrate by filtration. A 1.0-ml sample of the culture supernatant fluid was passed through a membrane filter (0.45-$\mu$m pore size; Millipore Corp.). A volume of 0.1 ml of the filtrate was added to a scintillation vial containing 10 ml of scintillation fluid and counted in a Packard Tri-Carb scintillation counter (Packard Instrument Co., Inc.). The scintillation fluid contained 0.5% (wt/vol) 2,5-diphenyloxazole (Beckman Instrument Co.) and 10% napthalene (J. T. Baker Chemical Co.) in 1,4-dioxane (J. T. Baker Chemical Co.).

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To confirm that the radioactivity in the filtrate was $^{35}SO_4^{2-}$, a sample of the filtrate was treated with an excess of 1.0 M $BaCl_2$. All of the radioactivity was precipitated. Additionally, none of the radioactivity in the filtrate was extractable with carbon disulfide.

## RESULTS

When *S. acidocaldarius* was grown at 70 C on $^{35}S$-labeled elemental sulfur as a sole source of energy, radioactive sulfate accumulated in the medium and the pH decreased (Fig. 1). When the initial pH of the medium was between 2.0 and 3.8, elemental sulfur was oxidized to sulfate, and the final pH values of the growth medium were 1.1 to 1.5 regardless of the initial pH values. If the initial pH values exceeded 4.0, then neither growth nor sulfur oxidation was detected after 18 days of incubation. Brock et al. (2) found a pH range of 1 to 5.9 for heterotrophic growth on 0.1% yeast extract with an optimum of pH 2 to 3.

The effect of sulfur concentration upon the rate of sulfur oxidation was examined over a concentration range from 0.1 mg of $S^0$ per ml to 10 mg of $S^0$ per ml. The optimal substrate concentration for both growth and sulfur oxidation was a concentration of 1.5 mg of $S^0$ per ml. There was a negligible increase in the total quantity of elemental sulfur oxidized as the concentration of elemental sulfur was increased to 10 mg of $S^0$ per ml.

Since *S. acidocaldarius* grows at a fairly high temperature, it was considered that either the quantity of dissolved oxygen or the quantity of dissolved carbon dioxide might be limiting for growth. At 70 C the solubilities of $O_2$ and $CO_2$ are, respectively, about one-half and one-third the solubility in water at 25 C (5). To examine whether the quantity of $CO_2$ was limiting, we increased the partial pressure of $CO_2$ by bubbling the culture medium with 5% $CO_2$ in air before sealing the bottle. The highest rate of growth and sulfur oxidation was achieved when the culture medium was bubbled with 5% $CO_2$ in air each time the container was opened. The rate of growth and sulfur oxidation decreased slightly when the culture was sealed with no supplemental $CO_2$. When the growth container was left open to the atmosphere, the rate of sulfur oxidation decreased threefold. However, sulfur oxidation ceased when the culture was continuously bubbled with 5% $CO_2$ in air.

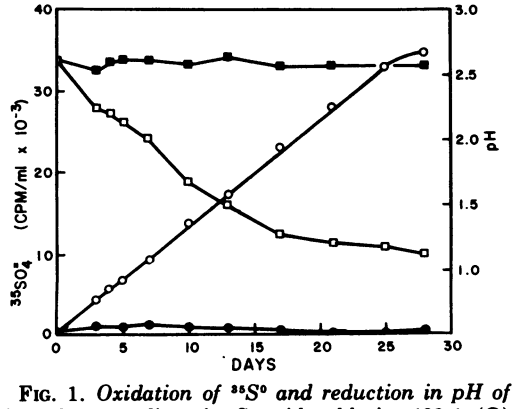


FIG. 1. *Oxidation of $^{35}S^0$ and reduction in pH of the culture medium for S. acidocaldarius 129-1.* (●) *$^{35}SO_4^{2-}$ formation in uninoculated control;* (■) *pH change in uninoculated control;* (○) *$^{35}SO_4^{2-}$ formation in inoculated sample;* (□) *pH change of inoculated sample.*

*Thiobacillus thiooxidans* (7, 11), *T. ferrooxidans* (6), and *Ferrobacillus ferrooxidans* (3) have been reported to attach to solid surfaces, in particular to insoluble substrates such as elemental sulfur. Brock et al. (2) also reported that *S. acidocaldarius* cells are often seen attached to sulfur crystals. The extent to which *S. acidocaldarius* attached to sulfur crystals during growth was determined by staining cultures grown on elemental sulfur with acridine orange and examining them with a fluorescence microscope equipped with a vertical illuminator (Carl Zeiss Universal). A differential cell count was made to determine the fraction of organisms attached to sulfur compared with the number of unattached organisms (Table 1). During the first 3 to 6 days of growth, the number of unattached organisms exceeded the number of attached organisms, and there was a direct correlation between sulfur oxidation and the numbers of unattached organisms. After 6 days of growth, sulfur oxidation continued to increase while the proportion of free-floating organisms decreased. After 17 days of growth, the sulfur crystals were covered with microcolonies, which often contained more than 100 organisms per colony, and over 10 times as many organisms were attached as free. McGoran et al. (6) reported that about 77% of *T.*

TABLE 1. *Attachment of S. acidocaldarius to elemental sulfur*[a]

| Time (days) | Cells/ml unattached | Optical density | Relative cell numbers | | Ratio U:A |
|---|---|---|---|---|---|
| | | | Unattached (U) | Attached (A) | |
| 0 | $2.6 \times 10^7$ | 0.009 | 407 | 0 | — |
| 3 | $9.0 \times 10^7$ | 0.018 | 525 | 6 | 87:1 |
| 6 | $3.5 \times 10^8$ | 0.056 | 1,011 | 83 | 12:1 |
| 10 | $5.6 \times 10^8$ | 0.072 | 129 | 342 | 1:3 |
| 13 | $8.1 \times 10^8$ | 0.091 | 273 | 1,658 | 1:6 |
| 17 | $2.1 \times 10^9$ | 0.112 | 88 | 1,560 | 1:18 |

[a] Strain 129-1, incubation at 70 C.

*ferrooxidans* cells were associated with sulfur crystals and questioned the validity of growth determination and generation times based upon the free cell count of organisms that attach to solid surfaces. However, the data show that the direct cell count of the unattached organisms of *S. acidocaldarius* is a good approximation of growth when limited to the early exponential phase of growth.

Figure 2 shows typical growth curves for eight strains of *S. acidocaldarius* grown on elemental sulfur at 70 C. Doubling times of 36.8 h for six strains and 55.3 h for two strains were determined based upon direct cell counts during the first three generations when the number of unattached organisms greatly exceeded the number of attached organisms. Brock et al. (2) reported doubling times of 6.5 to 8 h for several strains on 0.1% yeast extract.

The rate of sulfur oxidation by eight strains of *S. acidocaldarius* is shown in Fig. 3. The data presented in this figure were obtained in the same experiment as the data in Fig. 2, so that the two figures can be directly compared. Six strains, 106-3, 115-2, 129-1, 132-1, 136-1 and 140-5, oxidized sulfur at a similar rate, whereas strain 129-2 had a faster rate and strain 98-3 had a slower rate. Strain 98-3 was found to have an optimal temperature between 75 and 80 C,

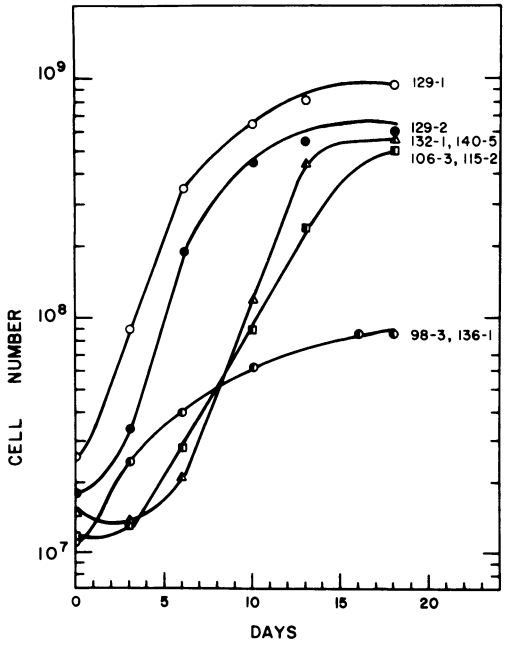


FIG. 2. *Growth rates of eight strains of S. acidocaldarius on elemental sulfur at 70 C. The numbers represent only free-floating cells. Compare with the data of Fig. 3, obtained in the same experiment.*

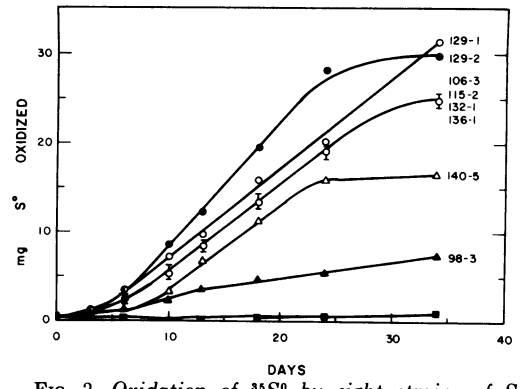


FIG. 3. *Oxidation of $^{35}S^0$ by eight strains of S. acidocaldarius. Compare with the growth data of Fig. 2.*

which probably accounts for the low rate of sulfur oxidation at 70 C.

All strains of *S. acidocaldarius* oxidized elemental sulfur to sulfate at temperatures between 67 and 75 C. However, strain 129-1 oxidized sulfur over a broad temperature range (55 to 84 C), whereas strains 129-2 and 136-1 were restricted to temperatures from 62 to 75 C. At the lower and upper temperature limits for sulfur oxidation, the lag phase was increased to at least 21 days and the rate of sulfur oxidation was reduced. Strains 98-3 and 132-1 had the highest temperature optimum for growth. The upper temperature limit for sulfur oxidation by these two strains has not been determined, but sulfur is readily oxidized at 84 C.

A sulfur balance was determined for strains 129-1 and 136-1: The growth medium containing unattached organisms and radioactive sulfate was removed by a pipette from the $^{35}S^0$. The residual sulfur was washed with water, dried, dissolved in 1 ml of $CS_2$, and diluted with 9 ml of benzene. The quantity of radioactivity in the unoxidized substrate was determined on a 0.1-ml sample of the dissolved and diluted $^{35}S^0$. The $CS_2$ and benzene were then evaporated from the sulfur and the weight of the sulfur was determined gravimetrically. The unattached organisms were collected on a membrane filter (0.22-$\mu$m pore size; Millipore Corp.). The filter and organisms were washed, dried, inserted into a scintillation vial with 10 ml of scintillation fluid, and counted to determine the amount of radioactive sulfur incorporated into the organisms. The quantity of radioactivity in the filtrate, which contained the radioactive sulfate, was determined directly. Table 2 shows the disappearance of elemental sulfur with the concomitant appearance of sulfate. The quantity of sulfur incorporated into the organisms was probably much

TABLE 2. *Sulfur balance, S. acidocaldarius 129-1*

| Distribution of sulfur | Percentage of total sulfur | | | |
|---|---|---|---|---|
| | 10-day culture | 18-day culture | 34-day culture | Control |
| $S^0$ | 79 | 58 | 36 | 94 |
| $SO_4^{2-}$ | 12 | 30 | 51 | 2 |
| Incorporated into organisms | 1 | 3 | 4 | 0 |
| Total | 92 | 91 | 91 | 96 |

greater than shown since many of the organisms were attached to the sulfur crystals.

Brock et al. (2) showed that *S. acidocaldarius* is a facultative autotroph, since the organism grows autotrophically on sulfur and heterotrophically on yeast extract. Therefore, the effect of yeast extract upon sulfur oxidation was examined (Fig. 4). During the early phases of growth, sulfur oxidation was greatly depressed if yeast extract was in the medium, although growth was considerably better (Table 3). In the later phases of growth, oxidation of sulfur occurred even in the presence of yeast extract, although the total quantity of sulfur oxidized was reduced. At a concentration of 0.5% (wt/vol) yeast extract, both growth and sulfur oxidation were totally inhibited. $CO_2$ fixation is also inhibited by yeast extract supplementation (Shivvers and Brock, unpublished data).

## DISCUSSION

The strains of *S. acidocaldarius* available in culture oxidize elemental sulfur to sulfate over a temperature range from 55 to 84 C. The maximal rate of sulfur oxidation occurs at 70 to 75 C. No attempts were made to examine any of the intermediates in the metabolic pathway since most of the intermediate sulfur compounds decompose at the temperature and pH at which the organism grows.

Our data suggest that carbon is growth-limiting for *S. acidocaldarius*. This hypothesis is supported by the fact that both growth and sulfur oxidation are stimulated when the partial pressure of $CO_2$ is increased. When carbon is made available by yeast extract supplementation, growth is greatly stimulated and the total quantity of oxidized sulfur is reduced by one-third.

The effect of yeast extract supplementation on autotrophic metabolism is more complex than the simple explanation that growth is greatly stimulated by the greater availability of carbon for assimilation. Yeast extract supplementation affects both energy generation and carbon assimilation. The rate of sulfur oxidation per cell is inhibited up to 30-fold by the presence of yeast extract. Yet, because of the increased growth, the total quantity of sulfur oxidized is only reduced one-third. It can be concluded that *S. acidocaldarius* must be getting energy from the oxidation of both sulfur and organic matter. It is also reasonable to assume that the organic supplement to the growth medium represses the enzymes in the sulfur oxidation pathway. Although the effect of organic supplements on the activity of carboxydismutase has not been examined directly, the preliminary evidence indicates that $CO_2$ assimilation is depressed in cells grown on yeast extract (Shivvers and Brock, unpublished data).

The natural habitat of *S. acidocaldarius* and the evidence that it oxidizes sulfur to sulfate at high temperature and low pH strongly suggest the biological origin of sulfuric acid in acid hot springs and soils. Schoen and Rye (10) examined the sulfur isotope distribution in Yel-

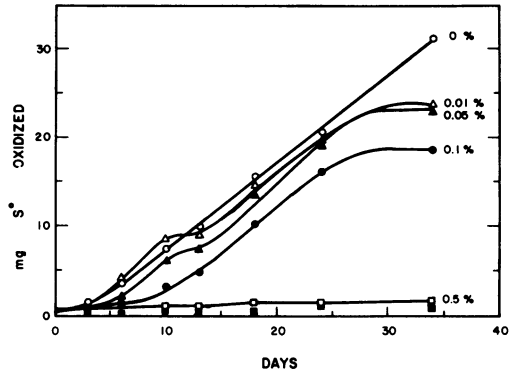


FIG. 4. *Effect of yeast extract in the culture medium on oxidation of $^{35}S^0$. (■) Uninoculated control. No growth occurred at 0.5% yeast extract. At 0.01 to 0.1% yeast extract, growth was better than in sulfur alone (see Table 3).*

TABLE 3. *Effect of yeast extract addition to the culture medium on growth and rate of sulfur oxidation*[a]

| Yeast extract concn (%) | Cell no./ml | Sulfur oxidized (μg/ml) | Sulfur oxidized (μg per ml per $10^7$ organisms) |
|---|---|---|---|
| 0 | $3.48 \times 10^8$ | 95.13 | 2.74 |
| 0.01 | $1.42 \times 10^9$ | 104.05 | 0.73 |
| 0.05 | $4.9 \times 10^9$ | 47.84 | 0.098 |
| 0.1 | $5.5 \times 10^9$ | 39.19 | 0.071 |

[a] Initial inoculum, $2.6 \times 10^7$. Incubation time, 6 days. See also Fig. 4 for complete time course of sulfur oxidation.

lowstone National Park and concluded that hydrogen sulfide autooxidizes to elemental sulfur and then the sulfur is biologically oxidized to sulfuric acid. Schoen and Ehrlich (9) reported that *T. thiooxidans* occurs only at temperatures less than 50 C and suggested that sulfuric acid is formed by *T. thiooxidans* in low temperature soils with the subsequent down slope movement of the acid into high temperature regions (8, 9). Fliermans and Brock (4) studied the ecology of *S. acidocaldarius* in solfatara soils using $^{14}CO_2$ to indicate its presence and were able to show that the organism occurred at temperatures up to 80 C. Recent work (6a) with $^{35}S^0$ has shown that *S. acidocaldarius* oxidizes elemental sulfur to sulfuric acid in its natural environment at temperatures up to 90 C and pH values as low as 1.0. Thus, this organism is an important geochemical agent in hot acid soils. In addition, it is the most thermophilic autotroph available in pure culture.

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