JOURNAL OF BACTERIOLOGY, Aug. 1972, p. 343-350

Vol. 111, No. 2
Printed in U.S.A.

Ecology of Sulfur-Oxidizing Bacteria in Hot Acid Soils

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Received for publication 27 March 1972

Hot acid soils in Yellowstone National Park are rich in elemental sulfur and harbor extensive populations of sulfur-oxidizing bacteria. *Thiobacillus thiooxidans* is found at temperatures below 55 C, and at temperatures from 55 to 85 C *Sulfolobus acidocaldarius* is present. The distribution of these bacteria as a function of temperature was measured by a most-probable-number dilution method, and their activity in situ was assessed by use of a new technique permitting measurement of $^{14}CO_2$ fixation. From these data it is concluded that sulfur-oxidizing bacteria are responsible for production of sulfuric acid in these acidic thermal habitats. Physical and chemical parameters of this unusual soil habitat were also measured and are described.

The development of an isotopic technique for measuring CO_2 fixation in soils (17) has permitted direct in situ measurement of the activity of sulfur-oxidizing chemoautotrophic bacteria. The present work uses this technique to study the activity and assess the distribution of sulfur-oxidizing bacteria in sulfur-rich hot acid soils of Yellowstone National Park. Such soils are widespread in many vapor-dominated thermal areas of Yellowstone Park (18). These areas, sometimes called solfataras, are generally quite limited in size and usually occupy hillsides, plateaus, small ravines or shallow hollows (2). The discharge of hot water in such areas is meager, and springs, if present, are generally quite small. The most acidic springs, with *p*H values less than 2.0, usually have no outflow. In much of a solfatara area springs are absent, and the area is characterized by acidic soils of various temperatures, the soils being heated by steam rising to the surface. Solfatara soils abound with crystals, veins and lumps of elemental sulfur that form as a result of spontaneous oxidation of hydrogen sulfide present in the steam. The acidity of a solfatara is due to sulfuric acid, which is generally considered to arise by microbial oxidation of elemental sulfur (15).

Sulfur-oxidizing autotrophs resembling the acidophilic organism *Thiobacillus thiooxidans*, but growing at temperatures up to 55 C, have been isolated from acidic thermal habitats by several workers (9, 12, 14, 16; J. A. Brierley, Ph.D. thesis, Montana State Univ., Bozeman, 1965). Recently Brock et al. (4) have isolated members of a new genus of sulfur-oxidizing acidophilic bacteria, *Sulfolobus*, the type species of which is *S. acidocaldarius*. *Sulfolobus* grows and oxidizes elemental sulfur over a temperature range of 55 to 85 C.

In the present work we have studied the distribution of *Thiobacillus* and *Sulfolobus* as a function of soil temperature and *p*H in several thermal areas of Yellowstone Park. By simultaneously measuring bacterial numbers by a most-probable-number method and bacterial activity by isotopic CO_2 fixation, we have been able to obtain information on the distribution and activity of these bacteria in high-temperature systems.

MATERIALS AND METHODS

Culture techniques. The basal salts medium of Allen (1) adjusted to *p*H 3.0 was used. Elemental sulfur separately sterilized by steaming on 3 successive days was added to a final concentration of about 0.1%. Cultures were prepared in either 56-ml screw-capped bottles with 15 ml of medium or in 18 by 150 mm screw-capped culture tubes with 5 ml of medium. After inoculation, 100% CO_2 was passed into the gas space for about 10 sec, and the screw caps were quickly closed. If such a CO_2 enrichment was not done, growth was poor or negligible. All incubations were carried out in constant-temperature water baths. Stock cultures of *T. thiooxidans* were maintained at 30, 45, and 55 C, and those of *S. acidocaldarius* at 55, 65, and 70 C; a variety of other temper-

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atures were used in isolation and most-probable-number counting.

To observe organisms attached to natural sulfur crystals, acridine orange staining was performed and fluorescence microscopy with a vertical illuminator was used. Growth in tubes was determined by visual observation of turbidity and by a lowering of the *p*H of the medium, and was confirmed by microscopy examination with a Carl Zeiss phase-contrast microscope. Since *T. thiooxidans* is rod-shaped and *S. acidocaldarius* is a lobed sphere, the two organisms are easily distinguished microscopically.

A most-probable-number technique was used to estimate the number of sulfur-oxidizing chemoautotrophs in the soil samples. Selective enrichments were made on Allen's medium at *p*H 3.0 amended with sterile elemental sulfur. Serial 10-fold dilutions were prepared in five replicates for each soil sample. Tubes were incubated in the dark for 14 days at the in situ temperature of the soil, and all tubes were observed for growth as previously described. The final *p*H of each sample was measured to determine the extent of acid production, and the most probable numbers of the organisms per gram of soil were determined by using the tables of Cochran (6).

Habitat measurements and sampling. Terrestrial and aquatic temperatures were measured using a telethermometer (Yellow Springs Instrument Co., model no. 42SC) and a "banjo" probe (model no. 408). The small surface area (83 mm^2) and quick response time (3 sec) of the probe permitted temperature measurements in a localized area. The probe and telethermometer were periodically checked with a standard mercury thermometer.

Soil samples were collected at 5-cm depth intervals, with a calibrated stainless-steel hand corer. Each sample was homogeneously mixed, and soil moisture was determined gravimetrically by placing soil samples into tared 35-mm screw-capped film cans directly in the field. In the laboratory the samples were weighed and dried to a constant weight at 110 C with the lids loose. The samples were then placed in a desiccator to cool and reweighed, and the amount of water lost was expressed as a percentage of the soil weight. For measuring the *p*H of very acid soils, the dilution technique of Doemel and Brock (7) was used. Soil sulfate determinations were made by the barium chloride turbidimetric method (3), and the data are presented as sulfate anion parts per million (ppm).

The methods used to measure the amount of elemental sulfur in solfatara soils will be reported in detail elsewhere (10). For most of the present work a direct spectrophotometric method was used after extracting the sulfur into carbon disulfide, since elemental sulfur shows a sharp absorption maximum at 382 nm in this solvent. Soils were extracted by shaking 5-g samples for 3 hr with 20 ml of CS_2 in 6-ounce screw-capped bottles. Spectral analyses were carried out in a Beckman DB-G grating spectrophotometer at 382 nm with a 10-mm light path using unamended CS_2 as a blank.

Measurement of $^{14}CO_2$ fixation in soils. The details for this technique have been reported elsewhere (17). Briefly, soil samples were placed in tared 5-ml serum bottles, capped with rubber serum stoppers, and preincubated for 20 min at the desired temperature. Incubation temperatures in the field were obtained along the thermal effluent of a hot spring which provided a range of temperatures from 25 to 90 C. After temperature equilibration, 0.1 or 0.2 ml of air was removed from the vial and an equivalent volume of gaseous $^{14}CO_2$ was injected. The exact amount of $^{14}CO_2$ used in each experiment (generally 200,000 counts/min) was measured by injecting an identical amount into a scintillation vial containing the liquid scintillation counting fluid and CO_2-binding agent. All incubations were carried out in the dark. Although preliminary experiments indicated that $^{14}CO_2$ uptake was linear for at least 6 hr with 0.2 μCi of $^{14}CO_2$ per sample, 4-hr incubations were generally used. Incorporation was stopped by the addition of 2 ml of 1 N perchloric acid, and the samples were returned to the laboratory for processing.

For assaying incorporated radioactivity, the samples were subjected to wet oxidation after removal of inorganic carbonate, as described by Smith et al. (17). The $^{14}CO_2$ released was trapped in phenethylamine in a toluene-based liquid scintillation fluid and counted in a Beckman β-mate II, LS-100 or LS-233 liquid scintillation counting system.

RESULTS

Habitats. Several solfatara regions in Yellowstone National Park were chosen for extensive study. Such areas are recognized by the sparseness or absence of vegetation, the reddish or whitish color of the soil, and the frequent occurrence of patches of sulfur crystals encrusting the rocks and soil particles. In winter, these areas are usually snow-free, and condensing steam reveals the presence of fumaroles. However, not all areas of steaming ground are solfatara areas. If the vapors are low in H_2S, elemental sulfur and sulfuric acid are not formed and the soil is of neutral or alkaline *p*H. Although several areas were sampled, for simplicity, the data for only one area, that at Roaring Mountain, will be presented. The data for the other areas can be found elsewhere (C. B. Fliermans, Ph.D. thesis, Indiana Univ., Bloomington, 1972). Roaring Mountain is a barren, white ridge 169 m (555 ft) high which is located 7.2 km (4.5 miles) north of Norris Junction on the Grand Loop Road. The study area was established on a level plain at the southwestern base of the mountain.

At Roaring Mountain a transect was established which extended westward along the barren plain from the base of the hill into a lodgepole pine forest. At each station of this transect, soil cores were taken within a radius of about 0.1 m from the station marker. Data on the physical and chemical characteristics of

TABLE 1. *Characteristics of the solfatara habitat at Roaring Mountain*

Station	Distance from station 87 (m)	Depth[a] (cm)	Temp 4 Aug. 1971	Moisture (%)	Soil *p*H	SO_4^{2-} (ppm)	S° (ppm)	Station	Distance from station 87 (m)	Depth[a] (cm)	Temp 4 Aug. 1971	Moisture (%)	Soil *p*H	SO_4^{2-} (ppm)	S° (ppm)
87	0	0	28.0	14.3	2.7	260		95	32	0	30.0	13.2	4.0	10	
		5	32.0	14.4	0.7	4,100				5	40.0	19.2	4.5	20	1,000
		10	44.9	15.4	1.6	3,000				10	45.0	16.4	3.7	28	
		15	51.1	15.1	1.3	2,590	1,000			15	46.0	18.8	3.3	20	
		20	57.0							20	52.6				
88	5	0	35.0	12.2	1.2	730	5,000	96	37	0	30.0	17.1	4.7	20	
		5	42.0	13.2	1.0	950	156,000			5	34.0	15.8	4.2	20	
		10	67.0	16.7	1.3	470	110,000			10	37.0	14.7	4.6	25	
		15	81.0	12.4	1.1	245	24,000			15	40.0	25.7	4.1	20	2,000
		20	89.9							20	48.0				
89	10	0	36.0	13.9	2.6	50		97	42	0	29.0	18.6	3.8	20	1,000
		5	47.0	15.2	1.5	480	2,200			5	35.0	19.1	3.6	20	1,000
		10	59.0	22.3	1.5	400	152,000			10	38.5	18.8	3.0	18	<200
		15	79.0	25.9	1.2	690	34,000			15	42.0	19.7	4.9	20	5,000
		20	83.9							20	45.0				
90	15	0	33.0	17.3	3.1	50	4,000	98	47	0	30.0	19.8	4.1	10	2,000
		5	44.0	23.0	3.1	35				5	34.0	15.2	4.4	10	2,000
		10	56.0	22.0	3.1	23	1,000			10	35.5	18.5	4.1	10	<200
		15	71.0	16.6	2.9	50	2,000			15	35.0	18.2	4.3	15	1,000
		20	75.0							20	41.0				
91	16	0	26.0	17.6	4.2	<10		99	52	0	30.0	14.3	4.3	10	15,000
		5	36.0	27.0	3.7					5	29.0	19.4	3.5	30	<200
		10	44.0	21.7						10	30.5	21.2	3.9	20	6,000
		15	51.0	23.4						15	31.0	18.8	4.7	25	<200
		20	60.0							20	32.0				
92	21	0	30.0	20.4	3.8	20		100	57	0	30.0	36.5	4.8	20	35,000
		5	34.0	15.2	4.5	30				5	29.0	18.9	4.8	38	15,000
		10	38.0	20.3	4.0	40	2,000			10	30.0	14.1	4.4	15	6000
		15	42.0	23.8	4.8	20	2,000			15	30.0	16.3	4.4	20	
		20	49.0							20	30.0				
93	26	0	34.0	14.3	3.5	<10	5,000	101	62	0	28.0	55.9	4.3	15	
		5	42.0	14.1	2.6	20				5	28.3	20.5	4.6	10	
		10	50.0	20.0	3.7	40				10	29.0	17.7	5.1	<10	
		15	60.0	20.9	3.7	30	1,000			15	30.0	16.2	4.1	10	
		20	65.0							20	28.1				
94	27	0	34.0	20.9	4.7	<10									
		5	35.2	18.9	4.0										
		10	46.0	14.5	4.9										
		15	50.0	25.6	5.0										
		20	58.0												

[a] Moisture, *p*H, SO_4^{2-}, and S° determined on samples collected from 5-cm segments of soil cores. Thus, 0 represents soil collected from the interval 0 to 5 cm, 15 represents soil collected from the interval 15 to 20 cm, etc. Station 87 was established near the base of the mountain and the transect extended in a westerly direction. For further details, see C. B. Fliermans, Ph.D. thesis, Indiana Univ., 1972.

the soil at different depths at each station are given in Table 1. Although these data were obtained during a single sampling time in mid-summer 1971, temperatures were measured a number of other times over a 2-year period, and the values presented in Table 1 are reasonably representative. Temperatures were highest near the base of the hill and were pro-

gressively lower near the pine forest. At any station, temperature always increased with depth, as would be expected. Small fumaroles present near stations 88 and 89 accounted for the higher temperatures at these stations.

There is a good correlation between soil *p*H and SO_4^{2-} content, as would be expected in a habitat where acidity is due to sulfuric acid. *p*H values varied from a low of 0.7 to a high of 5.1, the most acidic values being those closest to the base of the hill, where elemental sulfur concentrations were the highest. Elemental sulfur content also varied considerably, the highest concentrations being found at stations 88 and 89, where fumaroles were present.

In addition to the transect at Roaring Mountain, other transects were established at Amphitheater Springs, near Norris Geyser Basin, on the Solfatara Plateau, near Rush Lake, and near Great Fountain Geyser. These were typical solfatara areas except that near Great Fountain Geyser, which was low in sulfur and had soils of higher *p*H, around 4 to 5. In all, over 200 locations at these transects were sampled, and the phsyical and chemical parameters were measured. The data demonstrate that considerable spatial heterogeneity exists. Although replicate samples at a given station gave similar results, differences between adjacent stations could often be quite large. For this reason, temperature was measured each time a sample was taken, and care was taken to insure that incubations were carried out at temperatures close to those measured.

The following is the range of values obtained for the physical and chemical parameters at the various stations: temperature, 8.5 to 92.8 C; *p*H, 0.5 to 6.3; SO_4^{2-}, <10 to 7,100 ppm; S°, <200 to 920,000 ppm; moisture, 4 to 56%.

Relation of habitat temperature to bacterial number and type. Soil samples were collected from selected stations at each transect over a wide range of temperatures, and the numbers of sulfur-oxidizing bacteria were estimated by the most-probable-number method. Incubation temperatures were the same as the temperature of the soil habitat. After incubation for 2 weeks the tubes were examined for presence or absence of growth, and the tubes showing growth at limiting dilution were examined microscopically to determine whether rods (*Thiobacillus*) or lobed spheres (*Sulfolobus*) were present.

The data for two transects are given in Fig. 1 and 2. The data show that *Thiobacillus* was found only at temperatures of 55 C and lower, and *Sulfolobus* at temperature of 50 to 85 C.

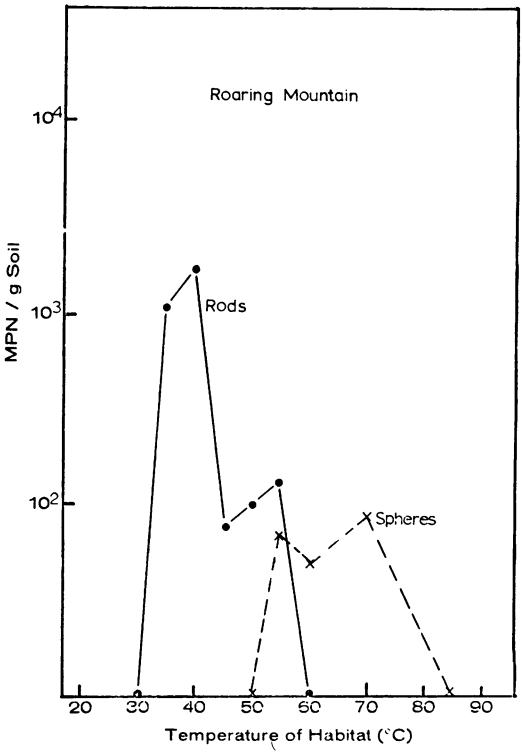

FIG. 1. *Numbers of sulfur-oxidizing bacteria in soils collected at different temperatures along the Roaring Mountain transect, incubations being carried out at the temperature of the habitat. MPN, most-probable-number.*

These data are consistent with the temperature ranges over which these two organisms grow in culture (5, 12, 16; J. A. Brierley, Ph.D. thesis, Montana State Univ., Bozeman, 1965). The data for the four other transects were similar to those presented in Fig. 1 and 2. In all cases, in the temperature ranges of their best growth, numbers of *Thiobacillus* were higher than numbers of *Sulfolobus*. This might reflect a real difference in numbers of these two organisms or it may reflect a lower counting efficiency of *Sulfolobus* than *Thiobacillus* from soil. The data on $^{14}CO_2$ fixation presented below suggest that the difference is real.

Distribution of Thiobacillus and Sulfolobus with relation to pH. The *p*H optimum for *T. thiooxidans* in culture is about 2.5, with a range from 0.9 to 4.5 (13), whereas the *p*H optimum for *S. acidocaldarius* in culture is between 2.5 and 3.0, with a range of 1.0 to 5.9 (4). Both of these organisms are found over the complete *p*H range of the habitats studied, with *Thiobacillus* being found only at temperatures of 55 C or below, and *Sulfolobus* at temperatures of 55 C and above. It seems

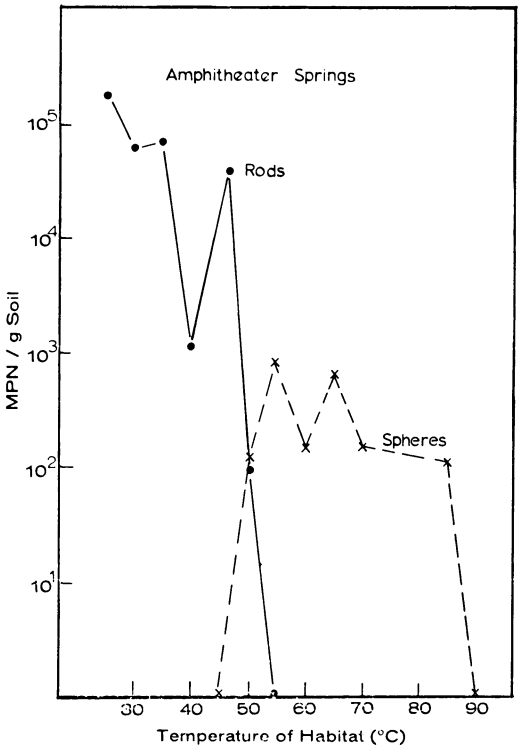

FIG. 2. *Numbers of sulfur-oxidizing bacteria in soils collected at different temperatures along the Amphitheater Springs transect, incubations being carried out at the temperature of the habitat. MPN, most-probable-number.*

clear from these data that it is temperature rather than *p*H which is the key factor controlling the distribution of these two organisms in solfatara soils.

Measurement of chemoautotrophic activity in situ by using $^{14}CO_2$. The isotope technique developed for this work (17) permitted measurement of the activity of chemoautotrophic bacteria under in situ conditions. Preliminary studies showed that the rate of $^{14}CO_2$ incorporation was greatest when incubations were carried out at the temperature of the habitat; thus, all incubations were at such temperatures. The presence of springs near each study area permitted selection of appropriate incubation temperatures. Viable counts were made on replicate samples from each soil where $^{14}CO_2$ incorporation was studied, and a relationship between numbers of sulfur-oxidizing chemoautotrophs and extent of $^{14}CO_2$ fixation could be derived. The composite data are shown in Fig. 3. In soils having less than 300 to 350 sulfur-oxidizing bacteria per g, uptake of $^{14}CO_2$ is less than 200 counts per min per g, and no correlation exists between viable count and $^{14}CO_2$ fixation. It is likely that $^{14}CO_2$ fixation at these low levels is either nonbiological or is due to heterotrophic organisms. Above 300 to 350 sulfur-oxidizing bacteria per g of soil, the uptake of $^{14}CO_2$ is directly related to the number of sulfur-oxidizing bacteria. In analysis of the data on $^{14}CO_2$ uptake, we have thus selected a value of 200 to 250 counts/min, above which incorporation is considered to reflect activity of sulfur-oxidizing bacteria. In

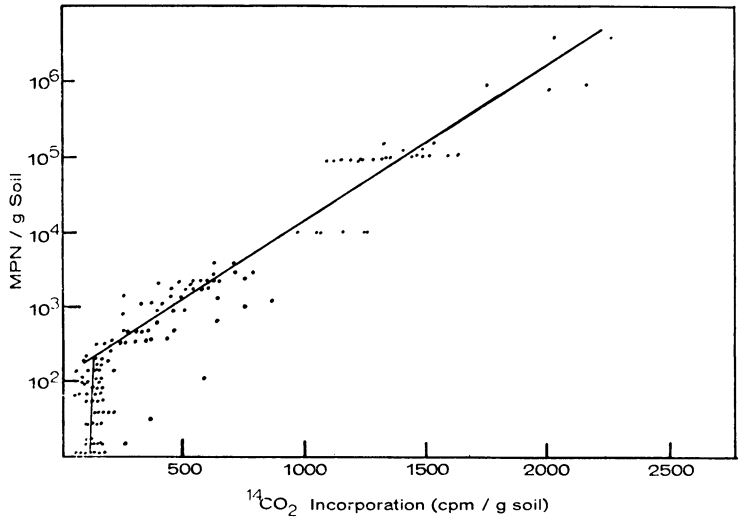

FIG. 3. *Relationship of $^{14}CO_2$ uptake rate and viable count of sulfur-oxidizing bacteria. MPN, most-probable-number.*

one experiment, the effect on $^{14}CO_2$ uptake upon the addition of elemental sulfur to a soil with a fairly low sulfur content (175 μg/g) was studied. When the sulfur content of this soil was increased to 375 μg/g, the rate of $^{14}CO_2$ fixation doubled. These data are consistent with the interpretation that $^{14}CO_2$ fixation is by sulfur-oxidizing autotrophs.

Figure 4 is a composite in which the mean levels of incorporation for samples from the same temperature were averaged and the incorporation is plotted as a function of temperature. It can be seen that the major activity was found at low temperature, 20 to 40 C, but that activity was also found around 70 C. The CO_2 fixation at low temperature clearly reflects the activity of *Thiobacillus*, whereas that at 70 C is *Sulfolobus*. However, since a direct correlation between extent of CO_2 fixation and sulfur oxidation may not exist, we cannot at this time conclude that sulfur oxidation occurs predominately at low temperatures in these solfatara soils.

DISCUSSION

In this work we have studied not only the distribution of numbers and kinds of sulfur-oxidizing chemoautotrophic bacteria, but have also studied their activity in situ by using $^{14}CO_2$. In the solfatara habitat it seems likely that sulfur-oxidizing autotrophs are solely responsible for significant fixation of $^{14}CO_2$ in the dark. The photosynthetic alga *Cyanidium caldarium*, which is sometimes present in solfatara soils at temperatures below 55 C (8), does not fix $^{14}CO_2$ significantly in the dark. Enrichment cultures for nitrifying bacteria using ammonium as sole energy source have always been unsuccessful, whereas sulfur-oxidizing autotrophs can be isolated routinely from these soils, frequently in high numbers. Thus we feel that measurement of $^{14}CO_2$ fixation provides a direct measurement of the activity of sulfur-oxidizing bacteria.

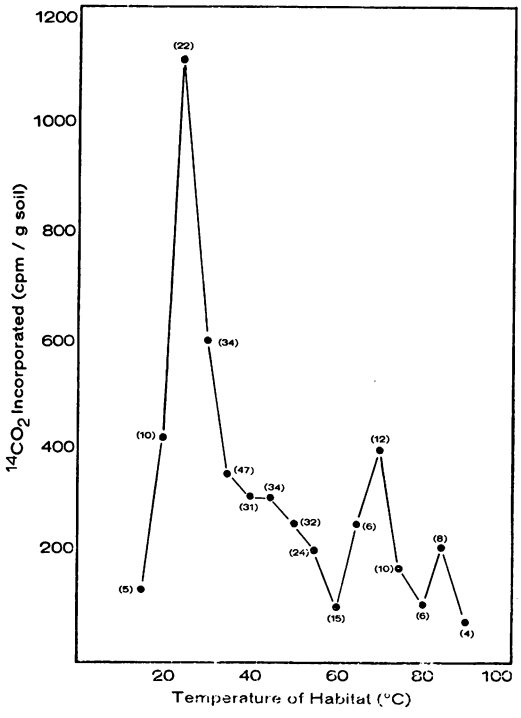

FIG. 4. *Rate of uptake of $^{14}CO_2$ in soils of different temperatures. The number of determinations at each point is indicated in parentheses on the graph and each point is the average of the values obtained.*

The present work has shown the considerable spatial and temporal variability of the solfatara soil environment. At a single location at various depths, wide variation in temperature, *p*H, sulfate, and elemental sulfur concentration occurs. Marked differences were also found from one location to another a few meters away. The most important factor causing such variability is the unpredictable distribution of small fumaroles. Since a fumarole is a source of both heat and hydrogen sulfide which is ultimately oxidized to sulfuric acid, it is understandable that in the vicinity of fumaroles the most acidic conditions are found. The acidophilic sulfur-oxidizing bacteria are probably responsible for the production of most of the sulfuric acid in solfataras.

Although the environment is spatially heterogeneous, the conditions at a single location are sufficiently stable so that bacteria with temperature optima similar to the habitat temperature can develop. Detailed data on variation of temperature with time over a 2-year period for the various transects were given by C. B. Fliermans (Ph.D. thesis, Indiana Univ., Bloomington). Variations of as much as 30 C were found at some depths at certain stations, whereas at other locations, variations of less than 5 C were found. At the Roaring Mountain transect, temperatures were also measured once during the winter (27 January 1971) and were no cooler than summer ones, and in some cases were even hotter. However, at station 88, one of the hotter stations at Roaring Mountain, temperature variation as great as 20 C was found over a single weekly period. Also, diurnal variation in temperature as great as 10 C was found in a single core, although over the same 24-hr period air temperature varied about 25 C. Since the specific

heat of soil is low, it responds fairly quickly to temperature changes.

Soil moisture was relatively constant in the solfatara soils, and was not lower in the hotter than in the cooler areas of the transect (see Table 1). Since steam, the source of heat, is also a source of moisture, this relative constancy in moisture content is perhaps not surprising. However, these solfatara soils are low in water-binding materials such as humus or clay, so the water-holding capacity is probably low. The dominant mineral is quartz, virtually the only mineral which has long stability under hot acid conditions.

It is clear from our study that sulfur-oxidizing chemoautotrophic bacteria are widespread in solfatara soils, and that they are active under in situ conditions. Two kinds of sulfur-oxidizers were found, *Thiobacillus*, which is active over a temperature range of 20 to 55 C, and *Sulfolobus*, which is active over the range of 55 to 85 C. These two organisms are easily distinguished morphologically and hence could be counted separately by the most-probable-number method. Only at 55 C would both organisms be present, although at this temperature neither is optimally active. In addition to viable counting, we also observed these organisms by direct microscopy of soil, either with phase-contrast or fluorescence microscopy. However, we experienced considerable difficulty in carrying out direct microscopic counts, due primarily to the fact that cell numbers were often considerably below the level at which direct counts are feasible, and the distinction between heterotrophic and chemoautotrophic bacteria is not possible. It was for this reason that we performed counts by the most-probable-number procedure.

Data on $^{14}CO_2$ fixation agree reasonably well with the viable counts. The $^{14}CO_2$ fixation data show that not only are the sulfur-oxidizing bacteria present, but that they are active under natural conditions. The data in Fig. 4 show that the greatest activity was found at temperatures around 25 C, but activity was found over a temperature range from 20 to 80 C. A second minor peak of activity at 70 C is due to *Sulfolobus*, whereas the major peak at 25 C is due to *Thiobacillus*. As shown in Fig. 1 and 2, numbers of *Thiobacillus* were significantly higher than numbers of *Sulfolobus*, in agreement with the CO_2 fixation data. These data thus suggest that the main agent in formation of sulfuric acid in solfatara soils is *Thiobacillus*, and that acid formation is most rapid at low temperature. This is consistent with the findings that the greatest concentration of sulfate occurs in the upper soil horizons. Although it is technically more difficult, we hope to confirm this conclusion using ^{35}S-labeled elemental sulfur.

The reduced activity of *Sulfolobus* compared to *Thiobacillus* may be because *Sulfolobus* is less well adapted for growth in soil. *Sulfolobus* is widespread in sulfur-rich hot acid springs in Yellowstone Park, occurring often at very high numbers attached to sulfur crystals. We have found it in large numbers in such springs in the temperature range of 70 to 80 C, and it may be responsible for formation of sulfuric acid in these aquatic systems. Thus *Sulfolobus* may be primarily an aquatic organism, able to establish itself to some extent in soil.

From the data on isotope fraction of ^{32}S and ^{34}S in sulfuric acid in Yellowstone springs (15), it seems likely that oxidation of elemental sulfur to sulfuric acid is a biological process. The present work provides strong support for this conclusion and extends the work of Schoen and Ehrlich (14) to higher temperatures.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Science Foundation (GB-30075), a contract from the Atomic Energy Commission (COO-2161-4), and a Public Health Service training grant (#5T1-GM-503) from the National Institute of General Medical Sciences.

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