JOURNAL OF BACTERIOLOGY, Sept. 1973, p. 732-737

Vol. 115, No. 3
Printed in U.S.A.

Dissimilatory Reduction of Inorganic Sulfur by Facultatively Anaerobic Marine Bacteria[1]

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Received for publication 3 May 1973

Three strains, selected from a large number of newly isolated, facultatively anaerobic marine bacteria, reduced inorganic sulfur compounds other than sulfate anaerobically in defined culture media in the following different patterns: (i) sulfite and thiosulfate were reduced to sulfide, and tetrathionate was reduced to thiosulfate; (ii) tetrathionate was reduced to thiosulfate only; or (iii) thiosulfate was reduced to sulfide only when pyruvate was the substrate. Comparison of anaerobic growth in the presence or absence of inorganic sulfur compounds indicated true dissimilatory reductions.

The dissimilatory reduction of inorganic sulfur compounds to sulfide is generally assumed to be carried out only by the obligately anaerobic bacteria of the genera *Desulfovibrio* and *Desulfotomaculum*. Apart from this transformation, the occurrence of sulfide in natural environments is largely related to the desulfurylation of organic compounds. However, several early studies have indicated that thiosulfate, elemental sulfur, and sulfite, but not sulfate, could be reduced to sulfide by certain facultative anaerobic bacteria.

As early as 1912, it was recognized that a variety of nonsulfate reducing bacteria could form sulfide from elemental sulfur and thiosulfate, and in a few cases from sulfite in protein-free media (13). Later, Tanner (18) described thiosulfate reduction in protein-free medium by "fluorescent" bacteria from water. However, no attempts were made in these studies to grow the bacteria under anerobic conditions, and incubation periods as long as 30 days were required to demonstrate sulfide formation. In addition, quantitative methods for the determination of sulfide formed and thiosulfate utilized were not done. Wilson (26) described sulfite reduction by *Salmonella* sp., but his media were based on nutrient agar or MacConkey agar. In a series of papers, Tarr (19, 20, 21) described the production of sulfide from thiosulfate by *Proteus vulgaris* in nongrowth experiments performed under anaerobic conditions. Sulfide formed by washed cell suspensions from cysteine and thiosulfate was found to arise from different pathways. However, the production of sulfide from thiosulfate was much slower than from cysteine, and the bacterial suspensions used in the experiments were grown aerobically in protein-containing medium. The significance of thiosulfate reduction in *Proteus* is still undetermined (1). Sulfide is also formed from thiosulfate by *Bacterium paratyphosum B* (10) and *Citrobacter* (4) after depletion of tetrathionate, but the reduction is slow in both organisms and in *Citrobacter* is considered to be non-enzymatic.

Dissimilatory reduction of tetrathionate to thiosulfate, on the other hand, has been demonstrated in the latter two strains (4, 6). Although tetrathionate reductase is widespread among other genera of the *Enterobacteriaceae*, particularly *Salmonella*, *Proteus*, *Arizona*, *Serratia*, and *Providencia* (9, 9), anaerobic respiration has not been shown in these cases. Tetrathionate reductase has also been found in several strains of *Pasteurella septica* (7), and evidence for its activity in a soil pseudomonad has been described (23).

In the present communication, we report on growth experiments demonstrating dissimilatory reduction of thiosulfate and sulfite by some newly isolated (24; J. H. Tuttle and H. W. Jannasch, Mar. Biol., in press) facultatively anaerobic marine bacteria. Physiologically the isolates resemble the thiobacilli-type bacteria studied by Trautwein (22), Starkey (15, 16), and Trudinger (23). Details of the aerobic metabolism of reduced sulfur compounds by our isolates will be discussed in a later communication.

MATERIALS AND METHODS

Bacteria. The isolates were obtained from enrichment cultures prepared with thiosulfate-mineral medium as previously described (24). Strain 12W was a

[1] Contribution no. 3052 from the Woods Hole Oceanographic Institution.

pseudomonad from Eel Pond, Woods Hole; strain 16B, also a pseudomonad, was found in samples collected from the Black Sea at 190 m depth, i.e., below the oxygen-sulfide interface. Strain 38Ch was a nonmotile, gram-negative, rod-shaped bacterium obtained from a deep North Atlantic sediment sample.

Media and culture conditions. The bacteria were cultured in a basal salts medium of the following composition: K_2HPO_4 (0.5 g); KH_2PO_4 (0.5 g); $MgSO_4 \cdot 7H_2O$ (0.25 g); $(NH_4)_2SO_4$ (3.0 g); $NaHCO_3$ (0.5 g); NaCl (20.0 g); and distilled water (1 liter). Substrates (sodium pyruvate, lactate, or acetate) were added to give a final concentration of 6 mM. Inorganic sulfur compounds ($Na_2S_2O_3 \cdot 5H_2O$, Na_2SO_3, or $K_2S_4O_6$) were added in concentrations of 5.0 to 10.0 mM. Nitrate (KNO_3) as an electron acceptor was used at a concentration of 0.2% (wt/vol). The pH was set at 7.5 with NaOH, and the medium was sterilized by filtration through membrane filters (3-μm pore size).

The medium was aseptically transferred into aspirator bottles, purged with O_2-free N_2 gas for 1 h and dispensed into sterile, 65-ml glass-stoppered bottles. Inocula consisted of a loopful of bacteria grown on thiosulfate-seawater medium slants. The bottles were filled to overflowing with medium, stoppered, and incubated at 21 ± 1 C for 6 days unless specified otherwise. After incubation the culture fluids were analyzed for sulfide, thiosulfate, tetrathionate, trithionate, sulfite, cell protein, and pH as described below.

Chemicals and chemical determinations. $K_2S_4O_6$ was prepared from reagent grade $Na_2S_2O_3 \cdot 5H_2O$ by cold iodine oxidation as described by Roy and Trudinger (12). Aqueous solutions of the product gave a single spot on ascending paper chromatograms developed in pyridine:acetic acid:*n*-butanol:water (20:6:30:24). The product was assayed as tetrathionate according to the procedure of Kelly et al. (5).

Tetrathionate, trithionate, and thiosulfate were determined by the cyanolysis method of Kelly et al. (5). Sulfide was removed from the samples before polythionate determination by adding 2.0 ml of 2.6% zinc acetate followed by 0.5 ml of 1.4 M K_2HPO_4 to 2.5 ml of the sample. The precipitate was removed by centrifugation at 6,000 × *g* for 10 min, and the supernatant fluid was assayed for polythionates. The sulfide removal procedure did not interfere with the polythionate determinations.

Sulfide was determined by the method of Gilboa-Garber (3), which is unaffected by the presence of thiosulfate in the sample. Sulfite was measured by the difference in iodometric titrations in the presence and absence of 40% Formalin. Samples were titrated in an equal volume of 10% acetic acid. Before titration, sulfide was removed from the samples by bubbling with O_2-free N_2 following the addition of 1 drop of 4 N HCl.

The high potential of abiogenic interactions between a number of sulfur compounds during the various growth experiments requires the detailed presentation of control data in Tables 1 through 5.

Protein was determined by the method of Lowry et al. (8) after digestion of the cells with 10% trichloroacetic acid. pH was measured with a Metrohm expanded scale pH meter (Brinkman Instruments, Inc., Great Neck, N.Y.).

RESULTS

Reduction of tetrathionate and thiosulfate. Table 1 summarizes the ability of the three isolates to grow anaerobically on three selected substrates in the presence of three electron acceptors. Protein concentrations of less than 0.3 μg/ml are not regarded as a significant indication of growth. Isolate 16B fermented lactate and pyruvate slowly during the 6-day incubation period, causing a net decrease of the pH as compared with the controls. Slight pH increases in the controls are due to chemical reactions (see Table 2). The apparent growth of isolate 38Ch with acetate and pyruvate in the absence of an electron acceptor and with lactate in the presence of thiosulfate or tetrathionate resulted from a precipitate that interfered with the protein determination.

Nitrate supported anaerobic growth of all the isolates regardless of the substrate. Isolate 12W was the most active nitrate reducer. Strain 16B grew in the presence of tetrathionate with a concomitant decrease of the pH regardless of the substrate. Strain 12W grew with tetrathionate as the electron acceptor with lactate as the substrate only. With acetate as the substrate, thiosulfate did not serve as an electron acceptor for any of the isolates. It supported growth of isolate 16B, however, in the presence of pyruvate or lactate and 38Ch in the presence of pyruvate only.

The various products of the tetrathionate and thiosulfate reduction for the three substrates used are given in Table 2. The fact that considerable concentrations of trithionate are formed in the tetrathionate-containing cultures and controls indicates either the alkaline hydrolysis of tetrathionate to form trithionate and elemental sulfur or chemical disproportionation of tetrathionate to form trithionate and pentathionate (12). The former seems more likely, since the samples from the 10 mM tetrathionate cultures appeared to contain elemental sulfur after the samples had been frozen and thawed, in this particular case, before the polythionate determinations. This reaction appeared to be minimized in later experiments where lower concentrations of tetrathionate were used and the samples were not frozen before the assay (Table 3). Iodometric titration of the samples before freezing confirmed that thiosulfate was formed only in all tetrathionate cultures of isolate 16B and the lactate-tetrathionate culture of isolate 12W. Approximately 2 mol of thiosulfate per mol of tetrathionate were formed

TABLE 1. *Anaerobic growth and final pH of isolates on three substrates in the presence or absence of exogenous electron acceptors*[a]

Isolate	No acceptor		0.2% KNO_3		10 mM $K_2S_4O_6$		10 mM Na_2SO_3	
	Final pH	Cell protein (μg/ml)	Final pH	Cell protein (μg/ml)	Final pH	Cell protein (μg/ml)	Final pH	Cell protein (μg/ml)
Expt 1								
12W	8.02	0.1	8.44	42.8	7.58	0.0	8.13	0.1
16B	8.05	0.1	7.86	10.5	7.01	0.3	8.13	0.0
38CH	8.05	(0.3)	7.97	11.6	7.58	0.1	8.13	0.0
Control	8.06	0.0	8.03	0.0	7.58	0.0	8.15	0.0
Expt 2								
12W	8.00	0.0	7.99	80.0	7.46	0.0	8.06	0.0
16B	7.08	0.6	6.78	15.8	4.90	18.9	6.91	7.1
38Ch	8.00	(0.4)	6.89	22.0	7.50	0.0	7.80	1.1
Control	8.02	0.0	8.06	0.0	7.51	0.0	8.07	0.0
Expt 3								
12W	7.91	0.0	8.33	52.8	4.98	11.4	8.07	0.1
16B	7.88	0.3	6.64	12.2	4.86	12.2	8.12[b]	1.8
38Ch	7.96	0.0	7.81	4.0	7.75	(0.9)	8.07	(1.1)
Control	7.96	0.0	8.03	0.0	7.78	0.0	8.09	0.0

[a] Substrates were 6 mM acetate (expt 1), pyruvate (expt 2), and lactate (expt 3). Initial pH was 7.5.
[b] pH was determined after sulfide was removed by bubbling with O_2-free N_2.

TABLE 2. *Reduced sulfur compounds in spent cultures of isolates grown on three substrates in the presence of tetrathionate or thiosulfate as electron acceptors*[a]

Acceptor (10 mM)	Isolate	Sulfur compound (μmol/ml)			Total sulfur[b] (μg of atoms/ml)
		$S_2O_3^{-2}$	$S_4O_6^{-2}$	$S_3O_6^{-2}$	
Expt 1					
$K_2S_4O_6$	12W	0.00	8.20	4.36	45.88
	16B	1.86	5.98	5.58	44.38
	38Ch	0.00	7.88	5.06	46.70
	Control	0.00	8.12	4.58	46.22
$Na_2S_2O_3$	12W	10.28	0.14	0.03	21.21
	16B	9.87	0.16	0.37	21.49
	38Ch	9.83	0.21	0.02	20.56
	Control	10.06	0.16	0.06	20.94
Expt 2					
$K_2S_4O_6$	12W	0.00	8.06	3.38	42.38
	16B	11.00	1.80	4.72	43.36
	38Ch	0.00	8.08	3.82	43.78
	Control	0.00	7.98	3.82	43.38
$Na_2S_2O_3$	12W	9.98	0.15	0.20	21.16
	16B	8.43	0.42	0.32	19.50[c]
	38Ch	8.83	0.22	0.36	19.62[c]
	Control	9.71	0.19	0.07	20.39
Expt 3					
$K_2S_4O_6$	12W	10.34	4.43	1.64	43.32
	16B	11.03	3.97	1.82	43.40
	38Ch	0.00	9.97	0.20	40.48
	Control	0.03	9.78	1.57	43.89
$Na_2S_2O_3$	12W	10.08	0.12	0.18	21.18
	16B	9.47	0.06	0.11	20.88[d]
	38Ch	10.91	0.00	0.00	21.82
	Control	10.63	0.05	0.00	21.46

[a] Substrates were as in Table 1.
[b] Sum of $S_2O_3^{-2}$, $S_4O_6^{-2}$, and $S_3O_6^{-2}$ sulfur.
[c] Traces of S^{-2} sulfur were detected.
[d] 1.37 μg of atoms per ml of S^{-2} sulfur was detected.

by isolate 16B in pyruvate and lactate medium and by isolate 12W in lactate medium only. The concomitant decrease of pH suggests the reaction $S_4O_6^{-2} + 2H = 2S_2O_3^{-2} + 2H^+$.

Thiosulfate was not reduced by any of the isolates when acetate was given as the substrate. In pyruvate medium, isolates 16B and 38Ch reduced thiosulfate and produced sulfide in detectable quantities. The low values of total sulfur in these cultures were caused by the exclusion of sulfide sulfur from the calculations. In the lactate medium, thiosulfate was reduced only by isolate 16B. In this case, the production of sulfide was determined as 1.37 μg of atoms per ml (in 6 days).

No evidence for the presence of reduced sulfur compounds was found in any of the cultures which contained a large amount of sulfate (24 mM) but did not contain added tetrathionate, thiosulfate, or sulfite.

More detailed data for the formation of sulfide in pyruvate media by isolate 16B are given in Table 3. Since excess tetrathionate in the spent culture media appeared to prevent the formation of sulfide, the concentrations of the two acceptors were lowered to 5 mM. As a result, sulfide was indeed found in the tetrathionate cultures. An initial decrease of the pH followed by an increase in the cultures with added tetrathionate suggests that thiosulfate is formed first and successively utilized to form sulfide. In the thiosulfate cultures, reasonably good agreement was found between the amount of sulfide formed and the amount of thiosulfate lost. This suggests that both sulfur atoms of thiosulfate are reduced to sulfide.

Sulfite analyses resulted in the detection of small amounts in the thiosulfate cultures but not in the controls and led to the assumptions that (i) thiosulfate might be reductively cleaved into sulfide and sulfite, and (ii) the latter might be utilized further as an intermediate electron acceptor.

Reduction of sulfite. Tables 4 and 5 present data of three independent growth experiments, each with pyruvate and lactate as substrates and sulfite as the electron acceptor. Whereas some fermentation occurred only in the pyruvate medium, sulfite supported growth in the presence of both substrates (Table 4). The major products of sulfite reduction (Table 5) were sulfide and thiosulfate. Difficulty with pyruvate interference with the sulfite analyses was encountered in the uninoculated controls. However, the interference was not a problem in the cultures since the pyruvate had been consumed (unpublished data). Isolates 12W and 38Ch did not grow in sulfite media.

DISCUSSION

The facultatively anaerobic bacteria described in this report have been found to metabolize reductively various inorganic sulfur compounds other than sulfate. The three different isolates studied reduced either tetrathionate to thiosulfate (strain 12W), thiosulfate to sulfide (strain 38Ch), or tetrathionate to thiosulfate, thiosulfate to sulfide, and sulfite to thiosulfate and sulfide (strain 16B). Evidence that these reductions involve dissimilation of inorganic sulfur are (i) the failure of the bacteria to grow anaerobically in the absence of a reduced sulfur compound, or (ii) the significant increase of anaerobic growth in the presence of a reduced sulfur compound. The observed increase may be an expression of increased growth rate, increased cell yield, or both. The reduction of tetrathionate by the isolates 12W and 16B appears to be consistent with that described for *Bacterium paratyphosum-B* (6) and *Citrobacter* (4). Although none of the isolates were able to use sulfate as an electron acceptor, it is clear that assimilatory reduction of sulfate occurs when sulfate is the sole sulfur source in growing cultures.

It is not readily apparent from our growth experiments whether thiosulfate is a true intermediate in the reduction of sulfite by isolate

TABLE 3. *Reduced sulfur compounds in spent cultures of isolate 16B grown on pyruvate (6 mM) in the presence of electron acceptors in two independent experiments*[a]

Acceptor (5 mM)	Expt no.	Sulfur compound (μmol/ml)					Total sulfur[b] (μg of atoms/ml)
		$S_2O_3^{-2}$	$S_4O_6^{-2}$	$S_3O_6^{-2}$	SO_3^{-2}	S^{-2}	
$K_2S_4O_6$	1	9.70 (0.00)	0.00 (4.56)	0.49 (0.76)	0.07 (0.06)	0.07 (0.00)	21.01 (20.58)
	2	7.63 (0.00)	0.24 (4.21)	0.22 (0.00)	—	0.37 (0.00)	17.25 (16.84)
$Na_2S_2O_3$	1	4.26 (4.96)	0.00 (0.04)	0.12 (0.07)	0.02 (0.00)	1.39 (0.00)	10.29 (10.29)
	2	4.12 (5.08)	0.00 (0.04)	0.19 (0.06)	0.07 (0.00)	1.38 (0.00)	10.26 (10.50)

[a] Values for uninoculated controls are in parentheses.
[b] Sum of S^{-2}, $S_2O_3^{-2}$, $S_4O_6^{-2}$, $S_3O_6^{-2}$, and SO_3^{-2} sulfur.

TABLE 4. *Anaerobic growth and final pH of isolate 16B in medium with and without sulfite*[a]

Expt no.	Donor (6 mM)	No acceptor		Na_2SO_3 (5 mM)	
		Final pH	Cell protein (μg/ml)	Final pH	Cell protein (μg/ml)
1	Pyruvate	7.01 (7.93)	5.9 (0.0)	7.23 (7.85)	15.4 (0.1)
2	Pyruvate	6.90	5.2	7.21	13.3
3	Pyruvate	6.97	5.7	7.22	15.5
1	Lactate	7.85 (8.00)	0.2 (0.0)	7.68 (7.95)	10.3 (0.0)
2	Lactate	7.84	0.2	7.68	8.7
3	Lactate	7.85	0.2	7.70	8.6

[a] Cultures were incubated for 12 days; initial pH was 7.5. Values for uninoculated controls are in parentheses.

TABLE 5. *Reduced sulfur compounds in spent cultures of isolate 16B grown on pyruvate or lactate and with 5 mM sulfite as the electron acceptor*[a]

Expt no.	Donor (6 mM)	Sulfur compound (μmol/ml)					Total sulfur[b] (μg of atoms/ml)
		$S_2O_3^{-2}$	$S_4O_6^{-2}$	$S_3O_6^{-2}$	SO_3^{-2}	S^{-2}	
1	Pyruvate	1.97 (0.06)	0.09 (0.04)	0.04 (0.06)	0.09 (1.92)	0.15 (0.00)	4.66 (2.38)
2	Pyruvate	2.16	0.05	0.00	0.06	0.16	4.74
3	Pyruvate	2.13	0.04	0.06	0.00	0.13	4.73
1	Lactate	0.83 (0.00)	0.09 (0.07)	0.22 (0.00)	0.06 (3.73)	1.66 (0.00)	4.40 (4.01)
2	Lactate	1.17	0.05	0.16	0.09	1.36	4.47
3	Lactate	1.07	0.11	0.13	0.10	1.46	4.53

[a] Cultures were incubated for 12 days. Values for uninoculated controls are in parentheses.
[b] Sum of S^{-2}, $S_2O_3^{-2}$, $S_4O_6^{-2}$, $S_3O_6^{-2}$, and SO_3^{-2} sulfur.

16B. Thiosulfate may be formed by non-enzymatic oxidation of sulfide to sulfur, followed by a condensation of sulfur with remaining sulfite. This formation of thiosulfate has been shown to cause erroneous results in experiments on biological sulfur oxidation (17). On the other hand, enzymatic sulfite reduction to thiosulfate has been shown (Suh et al., Bacteriol. Proc., p. 133, 1968) in extracts of *Desulfovibrio* and cannot be ruled out to participate in the formation of thiosulfate as demonstrated in the present study.

In view of the earlier literature discussed in the introduction, our isolates may represent physiological intermediates between the obligately anaerobic sulfate-reducing bacteria and those which carry out an "incidental" reduction of sulfur compounds (other than sulfate) in analogy to Verhoeven's "incidental nitrate reduction" (25). This question can only be answered by enzymatic studies in which the presence or absence of a low-potential cytochrome, similar to cytochrome *C*3 of *Desulfovibrio* sp. (11), will be decisive. From the inability of our isolates to carry out the dissimilation of sulfate, adenosylphosphosulfate reductase appears to be lacking.

It is of ecological interest that the relatively high concentration of sulfate in seawater and the transformations of sulfur compounds after bacterial sulfate reduction exert a considerable effect on the turnover of carbon compounds (H. W. Jannasch et al., Amer. Ass. Petrol. Geol. Bull., in press). The occurrence of a physiological group of microorganisms capable of utilizing reduced sulfur compounds as electron acceptors may imply a competition with obligately anaerobic sulfate-reducing bacteria for partially reduced inorganic sulfur. The relative abundance of inorganic sulfur intermediates and the redox potential of certain estuarine and offshore seawater can be expected to reflect this competition. Alternatively, the two populations might complement each other. Since sulfate is normally present in excess of oxidizable substrate in seawater, there may be an excretion of partially reduced sulfur compounds by the sulfate-reducing bacteria, whereas the complete reduction to sulfide would be carried out by the organisms unable to utilize sulfate. This situation would be loosely analogous to the relationship between the nitrifying bacteria *Nitrosomonas* and *Nitrobacter* (W. Vishniac, personal communication). In the largest and

most studied anoxic marine basin, the Black Sea, thiosulfate has been found to form in a 1:1 ratio with sulfate as the major product of sulfide oxidation (14). Evidence for varying concentrations of thiosulfate near the interface of oxygen- and sulfide-containing layers of water have been measured in the Cariaco Trench (Tuttle and Jannasch, in press). Studies of the dynamics of chemical sulfide oxidation in seawater have shown thiosulfate to be the major product (2) and even tetrathionate occurring under certain conditions (Chen and Morris, unpublished data). The more detailed role of the physiological type of microorganisms described in the complex processes of sulfur transformations in marine environments remains to be explored.

ACKNOWLEDGMENTS

We thank J. Flanagan for technical assistance and W. V. Vishniac for helpful criticism of this paper.

This research was supported by grants GA 29665 and GA 33405 from the National Science Foundation.

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