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Sulfur cycling in *Catenococcus thiocyclus*

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Abstract

Since its isolation from marine volcanic areas, *Catenococcus thiocyclus* has been known to be able to oxidize thiosulfate to tetrathionate, but the benefits gained from the reaction were unknown. The energy to be gained from such a reaction is so small (1 electron per mol of thiosulfate, compared with 8 electrons if the thiosulfate is oxidized to sulfate) that it seemed unlikely to be a useful metabolic reaction. However, continuous culture experiments have now revealed that *C. thiocyclus* is able to gain metabolically useful energy from this oxidation (biomass yields increased by approximately 20% after the addition of 7.75 mM thiosulfate to medium containing 20 mM acetate) by combining it with the chemical reduction of the tetrathionate by sulfide. The enzymes for thiosulfate oxidation appear to be constitutive. Moreover, with a suitable primary energy source (e.g. glucose), *C. thiocyclus* can reduce sulfur (S°) to sulfide and Fe^{3+} to Fe^{2+}. A chemical reaction then generates FeS. Such reactions may have important implications for the sulfur cycle at oxic:anoxic interfaces in marine and freshwater systems.

Keywords: Sulfur cycle; *Catenococcus thiocyclus*; Thiosulfate; Tetrathionate; Sulfide

1. Introduction

The ability of heterotrophic bacteria to oxidize thiosulfate to tetrathionate has been known for quite some time [1-5], but the benefit gained from the reaction has never been conclusively established. In some cases, notably among marine isolates [4], stimulation of growth rates and slight increases in final culture densities was observed with pH-controlled batch cultures. However, the contribution that thiosulfate oxidation to tetrathionate makes to the metabolic activity of such cultures has never been accurately quantified. Heterotrophic, thiosulfate oxidizing bacteria are frequently isolated from marine samples (e.g. [3,6,7]) and it seems inescapable that some advantage must be gained from such a widespread property. This paper will describe investigations into the oxidation of reduced sulfur compounds by a recently described marine heterotroph, *Catenococcus thiocyclus* [8].

2. Materials and methods

2.1. Organism

Catenococcus thiocyclus LMD 92.12 was isolated from a near-shore sulfidic hydrothermal area [8].

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2.2. Media and culture methods

The mineral medium for batch cultures contained the following (g l^{-1}): NH_4Cl, 0.5; $MgSO_4.7H_2O$, 0.3; $CaCl_2.2H_2O$, 0.1; KH_2PO_4 and K_2HPO_4 between 2 and 10 depending on the required pH and buffering capacity; yeast extract, 0.05; NaCl, 20; trace elements [9], 1 ml. Unless otherwise stated, 10 mM acetate was provided as the carbon source.

The medium for the chemostats contained (g l^{-1}); K_2HPO_4, 0.8; KH_2PO_4, 0.3; NH_4Cl, 0.4; $CaCl_2.2H_2O$, 0.15; $MgSO_4.7H_2O$, 0.5; NaCl, 20; yeast extract, 0.05; and 0.4 ml of trace element solution. Sodium acetate (20 mM) was supplied as the limiting substrate. When appropriate, 32 mM KNO_3 was used.

Batch cultures were made in Erlenmeyer flasks on a rotary shaker. Continuous cultures were made in chemostats fitted with dissolved oxygen and pH control. The temperature was maintained at 28°C, the pH at 7.0 and the stirrer above 400 rpm.

2.3. Analytical techniques

Thiosulfate and tetrathionate were measured by cyanolysis [10]. Sulfur was extracted from biomass pellets with methanol (overnight, 28°C) and then determined cyanolytically as for tetrathionate. Sulfite was measured colorimetrically, by the method described by Trüper and Schlegel [11]. Sulfate was determined with the HPLC using a Nucleosyl anion exchange column with 0.04 M sodium salicylate (pH 4.0) as eluent and a Waters R.I. detector. In some cases, sulfate was also determined turbidometrically [12]. Sulfide was measured colorimetrically after stabilization of samples by precipitation with zinc acetate [11].

Biomass was routinely monitored by measuring its optical density at 430 nm against water. Protein was measured by the Lowry method or with the micro-biuret technique [13]. If sulfur compounds were present in the biomass, the Lowry method was used after extraction with methanol overnight. In all cases, BSA (Sigma) was used as the standard. Total organic carbon was measured using a Beckman model 915B Tocamaster.

Substrate-dependent oxygen uptake for the calculation of K_m and V_{max} was measured polarographically, using a Clark-type oxygen electrode (Yellow Springs Instruments) mounted in a thermostatically controlled cell which was closed, except for a small hole through which additions could be made. K_m and V_{max} were calculated from Direct Linear plots using specifically designed software.

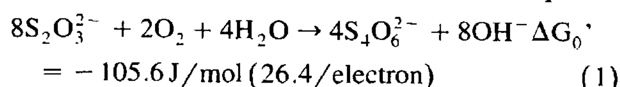
Biomass for experiments with whole cells was washed and suspended in 0.1 M phosphate buffer, pH 7.0, with 2% NaCl. Cell extracts were prepared by sonicating cells suspended in phosphate buffer, pH 7.0, and then centrifuging at $20000 \times g$ for 20 min to remove whole cells and debris.

Ferrous iron was measured colorimetrically using *o*-phenanthroline as described in the Standard Book of ASTU methods [14]. 0.5 ml of cell suspension or 0.2 ml cell extract was added directly to 4.5 ml of the reaction mixture. After colour development, the mixture was centrifuged to remove cells and debris before measurement.

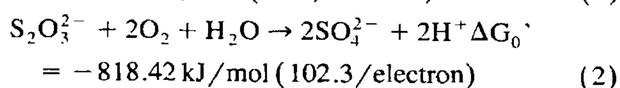
3. Results

3.1. Influence of thiosulfate on yields from acetate-limited chemostat cultures

The addition of increasing amounts of thiosulfate to the culture medium resulted in proportionate increases in the biomass yield of acetate-limited cultures, as shown by protein and total organic carbon determinations (Table 1). Growth at low dissolved oxygen concentrations (20–30% of air saturation) gave lower yields than when higher oxygen levels (75–80% of air saturation) were used. Given the homogeneity of the culture, this drop in cell density must have been due to physiological effects associated with the dissolved oxygen concentration. All thiosulfate was converted to tetrathionate, sulfate production was never detected. The stoichiometry of thiosulfate oxidation was in the range of 1 mol O_2:3.8–4.2 mol $S_2O_3^{2-}$, which corresponds with the amount of oxygen needed to oxidize thiosulfate to tetrathionate (Eq. 1), rather than to sulfate (Eq. 2).



$$8S_2O_3^{2-} + 2O_2 + 4H_2O \rightarrow 4S_4O_6^{2-} + 8OH^- \Delta G_0' = -105.6\,J/mol\,(26.4/electron) \quad (1)$$



$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+ \Delta G_0' = -818.42\,kJ/mol\,(102.3/electron) \quad (2)$$

Table 1
Influence of $S_2O_3^{2-}$ on yields of acetate-limited chemostat cultures of *Catenococcus thiocyclus*

Culture conditions	$S_2O_3^{2-}$ (mM) added	Protein yield (g/mol acetate)	Biomass yield (g/mol acetate)
$D = 0.075\ h^{-1}$	0	7.44	11.02
O_2 = 75–80% air sat.	3.1	7.82	12.48
	7.75	8.88	15.08
$D = 0.05\ h^{-1}$	0	4.89	7.99
O_2 = 20–30% air sat.	3.25	5.16	8.96
	6.1	5.33	9.39
	12.0	5.33	9.78

All $S_2O_3^{2-}$ was converted to $S_4O_6^{2-}$.
The dilution rate (D) was 0.05–0.075 h^{-1}.
Biomass was calculated from total organic carbon (TOC) data using the general formula for biomass ($C_1H_2O_{0.5}N_{0.25}$)

The increases in yield were unexpectedly high (Table 2). They were compared with those previously obtained with the chemolithoheterotrophic *Thiobacillus* Q. *Thiobacillus* Q oxidizes thiosulfate to sulfate, using the energy thus gained to permit increased acetate assimilation, and giving a higher yield [15]. With *Thiobacillus* Q, 2 mol of thiosulfate produced the same yield increase as 1 mol of acetate. Less energy is generated when thiosulfate is oxidized to tetrathionate (see Eqs. 1 and 2). In this case, 1 mol of acetate might be considered to be equivalent to 16 mol of thiosulfate. However, as can be seen in Table 2, the yield increases (shown as Δ acetate assimilated) found with *C. thiocyclus* were considerably higher than predicted from these assumptions.

3.2. Kinetics of inorganic sulfur compound oxidation

The thiosulfate and sulfur-oxidizing enzymes of *C. thiocyclus* appear to be constitutive. Washed cells grown on acetate as the sole substrate, in both batch and continuous cultures, were able to oxidize both compounds immediately. The K_m values for thiosulfate and sulfide remained in the same order of magnitude, regardless of whether or not these compounds were present in the growth medium (Table 3). As the dissolved oxygen concentration in the continuous cultures fell, the V_{max} for thiosulfate oxidation also fell, even when thiosulfate was present in the growth medium. Sulfide and sulfur were also oxidized, but at much lower rates (Table 3). Sulfide oxidation was not observed with cultures grown at dissolved oxygen concentrations of 30% air saturation, or below.

Table 4 shows the oxidation rates observed with washed *C. thiocyclus* cells that had been grown in the chemostat in the presence of acetate and sulfide. Tetrathionate was not oxidized alone, but stimulated sulfide-dependent oxygen uptake. Moreover, increased tetrathionate concentrations gave increased oxygen uptake rates, and the addition of sulfide to cell suspensions that had completed thiosulfate oxidation stimulated oxygen uptake by an amount proportional to the original thiosulfate concentration.

Table 2
Comparison of the response of similar acetate-limited continuous cultures ($D = 0.05–0.075\ h^{-1}$) of *C. thiocyclus* and *Thiobacillus* Q to different concentrations of thiosulfate

Organism	$S_2O_3^{2-}$ used mmol/g cells	Acetate used mmol/g cells	Acetate dissimilated mmol/g cells	Δ Acetate assimilated	
				observed mmol	predicted mmol
C. thiocyclus	0	90.7	65.3	0	0
	11.9	80.1	54.7	10.6	0.75
	24.7	66.3	40.9	24.4	1.54
Thiobacillus Q	0	62.9	37.5	0	0
	16.5	55.0	29.6	7.9	8
	25.4	50.8	25.4	12.1	12.7
	33	47.2	21.8	15.7	16.5

Thiobacillus Q data from [15].
Δ acetate is the cell yield increase due to thiosulfate, expressed in terms of observed and predicted assimilated acetate (mmol). For a further explanation of the calculations, see the text.

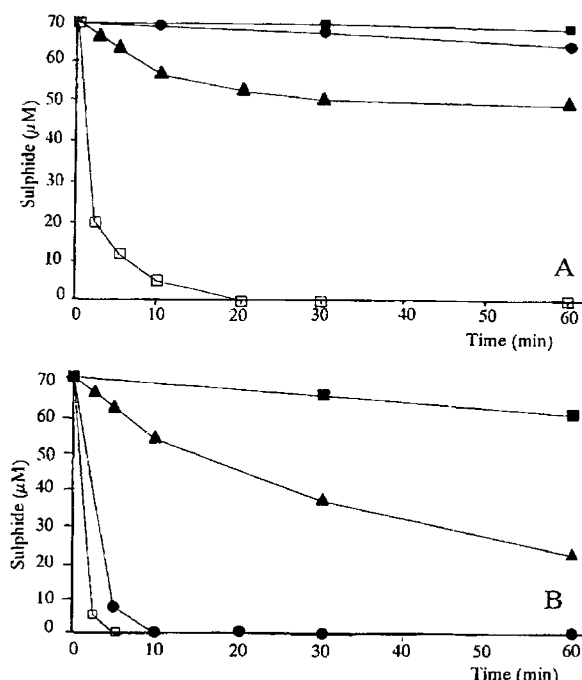


Fig. 1. Sulphide oxidation in the presence of dead (**A**) and living (**B**) *Catenococcus thiocyclus* cells in the presence of thiosulphate or tetrathionate. ■, S^{2-}; ●, S^{2-} with 10 μM $S_2O_3^{2-}$; ▲, S^{2-} with 10 μM $S_4O_6^{2-}$; □, S^{2-} with 100 μM $S_4O_6^{2-}$.

Table 4
Sulfide and tetrathionate-dependent oxygen uptake by *C. thiocyclus*

Substrate	Concentration μM	Oxygen uptake rate nmol min^{-1} mg $protein^{-1}$
S^{2-}	50	6–13
$S_4O_6^{2-}$	10–100	0
$S_2O_3^{2-}$	40	52
$S_2O_3^{2-}$	200	210
50 μM S^{2-} with:		
$S_4O_6^{2-}$	10	35
$S_4O_6^{2-}$	30	74
$S_4O_6^{2-}$	100	144
50 μM S^{2-} added after full oxidation of:		
$S_2O_3^{2-}$	40	48
$S_2O_3^{2-}$	200	101

The cells were harvested from continuous cultures growing on acetate (20 mM) and sulfide (2.5 mM), and washed as described in Materials and methods.

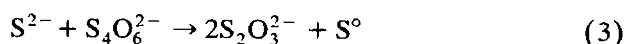
Fig. 1 compares the rates of sulfide oxidation in the presence of different sulfur compounds with dead (A) and living (B) cells. The rapid chemical oxida-

Table 3
The K_m and V_{max} values for oxygen uptake with different reduced sulfur compounds by acetate-grown *Catenococcus thiocyclus*, depending on the growth conditions and presence or absence of reduced sulfur compounds in the growth media

Growth conditions			Kinetic parameters of S-compound oxidation				
culture type	oxygen as % air sat.	S-substrate (mM)	thiosulfate		sulfide		S°
			V_{max}	K_m	V_{max}	K_m	V_{max}
Batch	unknown	–	100–250	100–150	10–20	10	25–45
		$S_2O_3^{2-}$ (10)	300–700	150–200	3–5	nd	15–30
Chemostat	75–80	–	150–300	75–125	2–3	nd	nd
		$S_2O_3^{2-}$ (2.5–10)	520–1100	150–200	nd	nd	nd
		S^{2-} (2.5)	150–380	150	10–15	8	35–45
	20–30	–	10–100	nd	0	nd	47–72
		$S_2O_3^{2-}$ (2.5–12)	190–300	100–160	nd	nd	10–20
	0.5	$S_2O_3^{2-}$ (12)	90	250	nd	nd	nd

nd = not determined.
K_m = μM.
V_{max} = nmol O_2.min^{-1}.mg $protein^{-1}$.

tion of sulfide by tetrathionate (but not by thiosulfate) can clearly be seen in Fig. 1A (Eq. 3). However, in the presence of living cells, sulfide oxidation was apparently strongly stimulated by the addition of thiosulfate as well as tetrathionate (Fig. 1B), presumably because of the 'recycling' of the thiosulfate as biologically produced tetrathionate (Eq. 1).



$$S^{2-} + S_4O_6^{2-} \rightarrow 2S_2O_3^{2-} + S^\circ \quad (3)$$

When sulfide was supplied to an acetate-limited continuous culture of *C. thiocyclus* grown at a dilution rate of 0.075 h^{-1} (dissolved oxygen 75% of air saturation), the biomass yield fell and sulfur appeared in the culture. After the dilution rate was decreased to 0.055 h^{-1} (Table 5), the biomass density in the culture rose again, and the sulfur gradually disappeared. If tetrathionate was supplied in addition to the sulfide, the yield increased, even though all of the $S_4O_6^{2-}$ was apparently still in the culture medium. Thiosulfate and sulfide were undetectable. Reducing the dissolved oxygen to 45% of air saturation gave a substantial increase in biomass yield and sulfur accumulation (Table 5). A further decrease (to between 20 and 30% air saturation) resulted in a slight fall in the biomass yield and the return of the sulfur concentration to its original level. It was not possible to make a complete sulfur balance as some of the sulfide was stripped by the air flow through the culture.

3.3. Fate of S°

During the chemical reaction between sulfide and tetrathionate, S° precipitated (results not shown). When living *C. thiocyclus* cells were present, less sulfur precipitate was observed. Oxygen uptake experiments had shown that *C. thiocyclus* was able to oxidize elemental sulfur (Table 3). However, cells oxidizing elemental sulfur did not appear to be producing SO_4^{2-}, $S_2O_3^{2-}$ or $S_4O_6^{2-}$. S^{2-} was transiently detected, especially when the cells were incubated at low oxygen concentrations. Sulfide concentrations in cell suspensions under micro-aerobic and anaerobic conditions with different additives were therefore monitored, and the results are shown in Fig. 2. Sulfide rapidly accumulated in the micro-aerobic suspensions, but reached a peak after which it was re-oxidized. In anaerobic suspensions of batch-grown cells, sulfide accumulated, and was not re-oxidized. Cells harvested from batch cultures (and thus grown without substrate limitation) both produced and oxidized the sulfide more rapidly (Fig. 2A) than cells grown under acetate limitation in continuous culture (Fig. 2B). Moreover, the chemostat-grown cells were only active if supplied with additional substrate (acetate or glucose), in contrast to the batch-grown cells. Chemostat-grown cells only produced sulfide anoxically if they were provided with a fermentable (glucose) substrate. Tetrathionate was the end product of the sulfide oxidation. Anaerobic experiments

Table 5
The effect of the presence of reduced sulfur compounds on the growth of acetate-limited *Catenococcus thiocyclus* in the chemostat at different dissolved oxygen concentrations and at different dilution rates

Dissolved O_2 (% air sat.)	D (h^{-1})	Sulfur compound supplied (mM)	Protein (mg l^{-1})	Total organic carbon (mg l^{-1})	S°(μM)
75	0.075	none	168	133	0
	0.075	S^{2-}(2.9)	112	86	43
	0.055	S^{2-}(2.9)	144	114	10
	0.055	$S^{2-} + S_4O_6^{2-}$ (2.9, 0.85)	163	145	10 *
45	0.055	$S^{2-} + S_4O_6^{2-}$ (2.9, 0.85)	230	172	258
20–30	0.055	$S^{2-} + S_4O_6^{2-}$ (2.9, 0.85)	213	161	40

Sulfate was constant at 14.5 mM throughout. Thiosulfate was not detected.
* = tetrathionate present in the culture (0.65–0.73 mM).

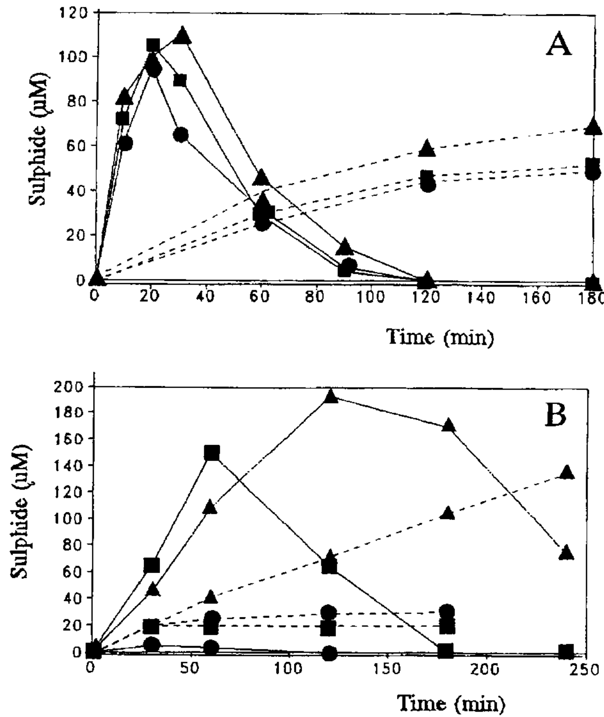


Fig. 2. Reduction of hydrophobic sulphur by suspensions of *Catenococcus thiocyclus* cells from (A) batch and (B) continuous cultures. Solid lines, microaerobic cultures; broken lines, anaerobic cultures; ●, no additions; ■, 20 mM acetate; ▲, 10 mM glucose.

with cell extracts revealed that this sulfide accumulation was NADH dependent, little or no activity was observed with NADPH or organic substrates (Fig. 3).

C. thiocyclus grew aerobically and micro-aerobically in batch cultures supplied with acetate and sulfur, but not anaerobically. Sulfide transiently accumulated in the micro-aerobic culture. Sulfide also

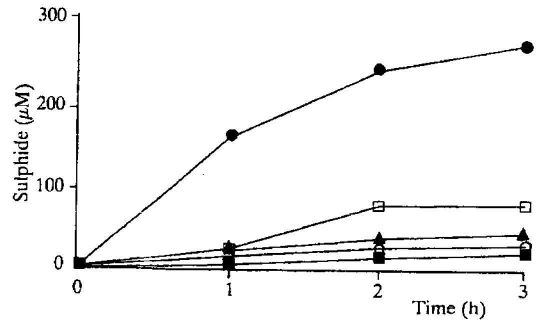


Fig. 3. Reduction of hydrophobic sulphur by extracts of *Catenococcus thiocyclus* from oxygen limited cultures. ■, no additions; O, 20 mM acetate; ▲, 10 mM glucose; □, 1 mM NADPH; ●, 1 mM NADH.

accumulated during anaerobic (fermentative) growth on glucose in the presence of sulfur (Table 6). This was inhibited by oxygen.

3.4. Fe^{3+} reduction

After incubation at low oxygen concentrations, *C. thiocyclus* biomass sometimes had the greenish colour indicative of Fe^{2+}. Subsequent tests with batch cultures showed that this organism was able to grow micro-aerobically on acetate, and anaerobically on glucose with concomitant reduction of Fe^{3+} or elemental sulfur. Sulfide and Fe^{2+} both appeared in these cultures. (Table 6). As indicated above, there was no anaerobic growth on acetate. Similar results were obtained with cell suspensions harvested from chemostat cultures. In subsequent experiments with cell extracts, Fe^{3+} reduction was observed anaerobically with NADH or NADPH as the electron donor (Table 7). It thus appears that the reduction of sulfur

Table 6
Reduction of S° (hydrophobic) and Fe^{3+} (supplied as 300 μM ferric citrate) in batch cultures of *Catenococcus thiocyclus*

Culture conditions		Growth	Product accumulation (μM)	
Oxygen	Substrate (mM)		S^{2-}	Fe^{2+}
aerobic	acetate (20)	+	0	0
micro-aerobic	acetate (20)	+	78 *	32 *
anaerobic	acetate (20)	–	0	0
	glucose (10)	+	100 **	40 **

* = transient accumulation; ** = stable accumulation.

Table 7
Fe^{3+} reduction by cell suspensions and cell extracts of *Catenococcus thiocyclus*

Energy source (mM)		Fe^{2+} accumulation (nmol mg.protein^{-1}.h^{-1})	
		whole cells	cell extracts
aerobic	NADH (1)	nd	0
micro-aerobic	–	15	nd
	acetate (20)	25	nd
	glucose (10)	40	nd
anaerobic	–	22	2
	acetate (20)	25	2
	glucose (10)	60	5
	NADH (1)	nd	60
	NADPH(1)	nd	55

The cells were pre-grown in acetate- and oxygen-limited continuous cultures.
300 μM Fe^{3+} was added as the citrate.
nd = not determined.

and Fe^{3+} serve *C. thiocyclus* as ways of dumping excess reducing power.

4. Discussion

If their biomass yields (11.0 and 16.1 g biomass per mol acetate, respectively (Table 1, or calculated from data in Table 2)) are compared, *C. thiocyclus* appears to be relatively inefficient in its use of acetate, compared to *Thiobacillus* Q. The increase in biomass yield observed when thiosulfate was added to *C. thiocyclus* cultures was considerably higher than might be expected from the free energy of thiosulfate oxidation to tetrathionate (Eqs. 1 and 2; Tables 1 and 2). Thiosulfate oxidation must therefore be causing alterations in the carbon and energy metabolism of *C. thiocyclus*, presumably because the energy thus generated can be used as a substitute for acetate dissimilation, making more acetate available for assimilation, and increasing the yield. This type of metabolic switching between assimilatory and dissimilatory pathways of carbon metabolism in the presence of a secondary energy source (and its possibilities and limitations) has been extensively discussed [16]. As cells grown without reduced sulfur compounds were also able to oxidize thiosulfate (Table 3), it appears that the thiosulfate oxidizing system is constitutive, although the maximum capacity for thiosulfate did increase by at least 2–3 fold on exposure to thiosulfate.

In the presence of (catalytic quantities) of thiosulfate or tetrathionate, supplementing the medium with sulfide also gave a substantial increase in the cell yield. The results shown in Fig. 1 suggest that a combination of chemical and biochemical reactions is occurring. The presence of a small amount of sulfide in the system, permitting the chemical reduction of tetrathionate to thiosulfate thus serves the same purpose as the continuous supply of thiosulfate provided during continuous culture. As both thiosulfate and tetrathionate can be found at detectable levels near the sulfide:oxygen interface in water bodies and sediments [17–19] the reactions occurring in these chemostat cultures may well be more analogous to a natural system. It is therefore suggested that sulfide is probably important to *C. thiocyclus* in its natural environment, because the chemical reaction of sulfide with tetrathionate (Eq. 3; Fig. 1, Tables 4 and 5) makes the thiosulfate available to the bacteria again. This 'recycling' of available thiosulfate in the presence of a constant low input of sulfide would permit significant amounts of energy to be gained from thiosulfate oxidation, even where thiosulfate was only present in amounts too low to appear to be significant from chemical analyses. Tetrathionate-producing bacteria are frequently found in low-sulfide environments [4,5,7,20,21]. It is clear from the results reported here that, even though they cannot oxidize sulfide to any significant degree, these bacteria may contribute significantly to accelerating the rate of chemical sulfide oxidation at oxic:anoxic interfaces by producing tetrathionate. Fe^{2+} could not be used instead of S^{2-} to regenerate thiosulfate because the Fe^{2+} was immediately oxidized by O_2 to Fe^{3+}. The ability of the organism to reduce S° and Fe^{3+} anoxically serves to emphasize the flexibility required of bacteria whose ecological niche is the oxic:anoxic interface. Both S° and Fe^{3+} are abundantly available in the marine volcanic area from which *C. thiocyclus* was isolated.

The metabolism of reduced sulfur compounds by *C. thiocyclus* appears to be controlled by two main factors, the oxygen concentration and the supply of electron donor. Three different patterns of behaviour

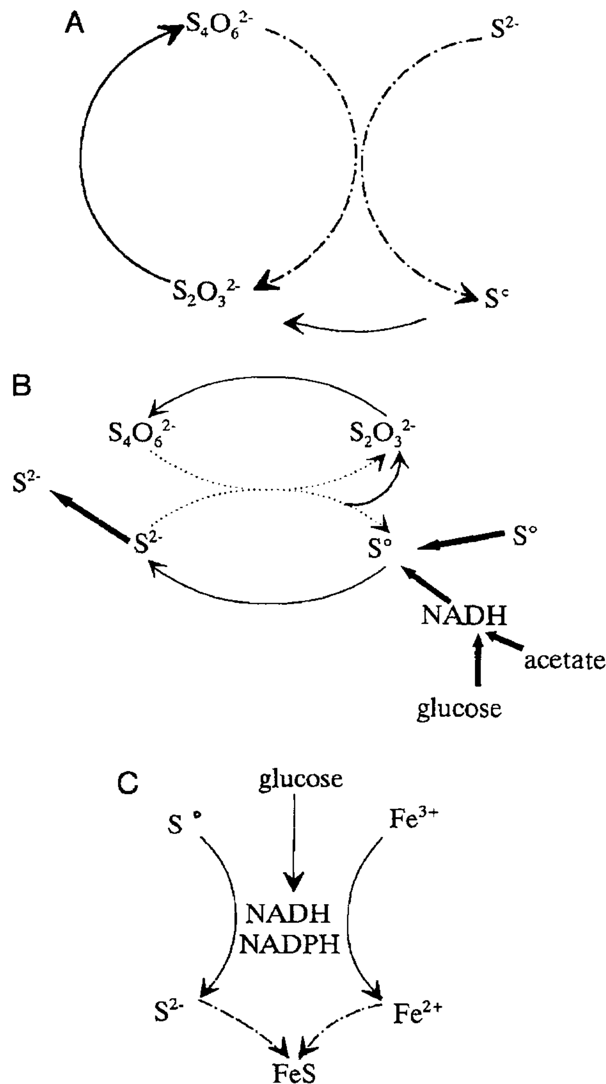


Fig. 4. The metabolism of reduced sulphur compounds under different environmental regimes. **A**: Aerobic, energy limited; **B**: micro-aerobic, energy not limiting; **C**: anaerobic, energy not limiting. Solid lines, biological reactions; broken lines, chemical reactions.

can be distinguished and are summarized in a working model shown in Fig. 4. Under energy limitation, but with oxygen present in excess, the dominant reaction will be tetrathionate production from thiosulfate, as outlined in Fig. 4A. Any available sulfide will be oxidized by the tetrathionate, regenerating thiosulfate for biological use, and producing sulfur, which will accumulate. When oxygen, rather than energy, is the limiting factor, *C. thiocyclus* appears to employ all options at its disposal (Fig. 4B). Thiosulfate will be oxidized to tetrathionate. At the same time, excess NADH would be used to reduce sulfur, generating sulfide. Some of this sulfide would then be used to reduce the tetrathionate, the remaining sulfide being either oxidized to thiosulfate (and hence tetrathionate), or escaping from the culture (see Fig. 2). Anaerobically, *C. thiocyclus* can ferment suitable substrates. When the supply of electron donor is not the limiting factor, excess reducing power can be used to reduce S° and Fe^{3+} to S^{2-} and Fe^{2+}. These last would react chemically to form FeS (Fig. 4C). A similar system, coupling sulfide oxidation and manganese reduction, and resulting in MnS precipitation, was proposed for coastal marine sediments by Burdige and Nealson [22].

From the three schemes outlined in Fig. 4, it is clear that at least some tetrathionate-producing heterotrophs not only gain metabolically useful energy from the reaction, but also substantially influence geochemical reactions in their environs (e.g. accelerating S^{2-} oxidation or promoting FeS formation). It is not yet clear whether *C. thiocyclus* is typical of the group of tetrathionate-producing bacteria, or is specially adapted because of its natural habitat in the waters around marine volcanic vents. However, washed suspensions of heterotrophs isolated from the oxic:anoxic interface in the Black Sea significantly increased their ATP levels when given a pulse of thiosulfate, despite the fact that tetrathionate was the oxidation product (J.G. Kuenen, unpublished results). It seems obvious that a closer study of this hitherto neglected group can shed a great deal of light on the less obvious areas of the sulfur cycle.

Acknowledgements

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