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Inhibition of respiratory oxidation of reduced sulfur compounds by intact cells of *Thiobacillus denitrificans* (strain RT) grown on thiosulfate

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Abstract. Thiobacillus denitrificans strain RT, an obligate sulfur-oxidizing chemolithoautotroph, was grown under microaerophilic conditions with thiosulfate as the only energy source. The rates of tetrathionate, thiosulfate, elemental sulfur (S°) and sulfite oxidation were measured respirometrically with an oxygen electrode, using actively growing cells. Cells oxidized thiosulfate, elemental sulfur (S°) and sulfite, but not tetrathionate. The thiosulfate-oxidizing activity and elemental sulfur-oxidizing activity (SOA) were almost totally inhibited by 50 μM myxothiazol (>80%), an inhibitor of the quinone-cytochrome b region, and by 10 μM of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) (>82%). Sulfite-oxidizing activity was also significantly inhibited (>60%) by 50 μM myxothiazol and 10 μM CCCP. 1 mM KCN totally inhibited (>90%) all respiratory activities.
This study confirms that a sulfur-oxidizing activity appears during microaerophilic growth of *Thiobacillus denitrificans* strain RT on thiosulfate. The SOA is linked to the respiratory chain, probably releasing electrons in the quinone-cytochrome b region.
Key words. Thiobacillus denitrificans; chemolithotrophy; thiosulfate; elemental sulfur; respiratory activity inhibitors.

The obligate autotroph *Thiobacillus denitrificans* is a facultative anaerobe deriving energy from the oxidation of reduced sulfur compounds using either nitrate and nitrite or oxygen as the terminal electron acceptor. In *T. denitrificans*, evidence has been presented for oxidative as well as substrate level phosphorylation in energy production[1].
Recently, we reported the presence of an elemental sulfur oxidizing activity (SOA) linked to the respiratory chain in Thiobacilli. In *Thiobacillus tepidarius* as well as in *Thiobacillus versutus* and *Thiobacillus novellus* grown on thiosulfate, the SOA was significantly inhibited by the inhibitors acting in the quinone-cytochrome b region, and by KCN acting at the end of the respiratory chain[2–5]. In contrast, in cells of *Thiobacillus denitrificans* grown aerobically on thiosulfate, Justin and Kelly[6] measured a very weak SOA in the presence of reduced glutathione. The thiosulfate-oxidizing activity was only weakly inhibited (<15%) by inhibitors acting in the quinone-cytochrome b region[7].
Here we report the respiratory oxidation rates of thiosulfate, tetrathionate, S° and sulfite for *Thiobacillus denitrificans* strain RT grown on thiosulfate as the sole energy source, as well as the effect of different respiratory chain inhibitors on these activities.

Materials and methods

Thiobacillus denitrificans, strain RT[8] (DSM 807) was obtained from the German collection of microorganisms (DSM) in Braunschweig. *T. denitrificans* was grown chemolithoautotrophically under microaerophilic conditions (5 kPa oxygen, 10 kPa CO_2, 75 kPa nitrogen) with agitation (150 rpm) at 28 °C, in a basal mineral medium (DSM 113) containing 0.2% (w/v) $NaHCO_3$ and 20 mM potassium thiosulfate[8]. The final pH was adjusted to 7.0. The purity of each culture was routinely checked.
For measurement of respiratory activity, cells were taken from exponentially growing cultures. At the time of harvest, the pH of the cultures was between 6.5 and 6.7. The respiratory activity was measured polarographically with an oxygen electrode at 28 °C in growth medium minus $NaHCO_3$ and thiosulfate.
Contrary to what has been observed with other thiobacilli[2–5], respiratory activities on sulfur compounds measured in cells from concentrated suspensions (13–18 mg DW ml^{-1}) were rapidly lost and were 5–10 times lower than respiratory activities measured directly from agitated cultures as described below. Oxygen consumption was calculated on the basis of 230 nmol O_2 ml^{-1} in air saturated medium at 28 °C, and expressed in nmol O_2 consumed mg $protein^{-1}$ min^{-1}.
To obtain significant and reproducible values it was essential to measure the respiratory activities with cell material prepared as follows. Cells from exponentially growing cultures were washed and concentrated under sterile conditions by centrifugation at 12,000 × g for 5 min at 22 °C. Cells were resuspended at a final biomass concentration of 0.3–0.4 mg DW ml^{-1}, in complete mineral medium, containing $NaHCO_3$ and potassium thiosulfate. Cultures were incubated under normal air pressure at 28 °C with agitation at 150 rpm. At different times 1 ml of culture was centrifuged at 8000 × g for 10 min at 22 °C. The cell pellet was resuspended in 0.5 ml mineral medium lacking $NaHCO_3$ and

potassium thiosulfate, placed in a respiratory cuvette containing 0.5 ml of the same mineral medium (without $NaHCO_3$ and potassium thiosulfate), and precalibrated at 230 nmol O_2 ml^{-1} in air-saturated medium at 28 °C. The endogenous respiration was followed for 3–4 min and then the inorganic sulfur compounds were added as electron donor. Under these conditions the respiratory activities of the culture were reproducible over 3–4 h of incubation. Respiratory activities obtained by this procedure were similar to those determined directly from washed cells grown under microaerophilic conditions.

The final concentrations of the respiratory substrates were 20 mM for thiosulfate, hydrophilic S° and sulfite, and 10 mM for tetrathionate. Hydrophilic S°, physiologically free of thiosulfate, was prepared by a thiosulfate acidification procedure previously described[2]. In the presence of sulfite, the respiratory activity must be measured carefully, as in most cases sulfites oxidize spontaneously in solution[4]. Tetrathionate solution was freshly prepared to prevent chemical decomposition at neutral pH.

Stock solutions of myxothiazol (5 mM, Boehringer), an inhibitor of the respiratory chain in the quinone-cytochrome b region[9], and carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 mM) were freshly prepared in ethanol. The final concentration of ethanol present in the assays did not affect the respiratory activities. KCN, an inhibitor of the terminal cytochrome oxidase, was dissolved (0.1 M) in 0.2 M sodium-phosphate buffer at pH 7.5. The respiratory activities were measured from constant respiratory slopes, and the percentage inhibition was determined according to previously published methods[3–4].

Growth was followed as a function of turbidity at 436 nm using 1-cm cuvettes in a Perkin-Elmer (Lambda 6) spectrophotometer. Dry weight was determined from the washed cell mass dried at 70 °C under vacuum for 48 h. Total protein was obtained according to Beffa et al.[4], and the protein concentration was measured by the Bradford assay[10].

Results and discussion

Cells of *Thiobacillus denitrificans* growing aerobically on thiosulfate possess relatively similar thiosulfate-oxidizing activity, elemental sulfur-oxidizing activity (SOA) and sulfite-oxidizing activity, when these activities were measured by the new procedure described here (fig. and table 1). The SOA was two orders of magnitude higher than the activity measured previously with cell-free extracts in the presence of glutathione[6]. Tetrathionate-oxidizing activity was absent in thiosulfate-grown cells (table 1).

When young cells of *T. denitrificans* grown on thiosulfate were washed by the standard procedure and the pellet stored in a highly concentrated form (>5 mg DW cells/ml) prior to the respiratory activity measurements, they showed low respiratory activities in the presence of sulfur compounds and these activities were rapidly lost over 10–30 min (table 2).

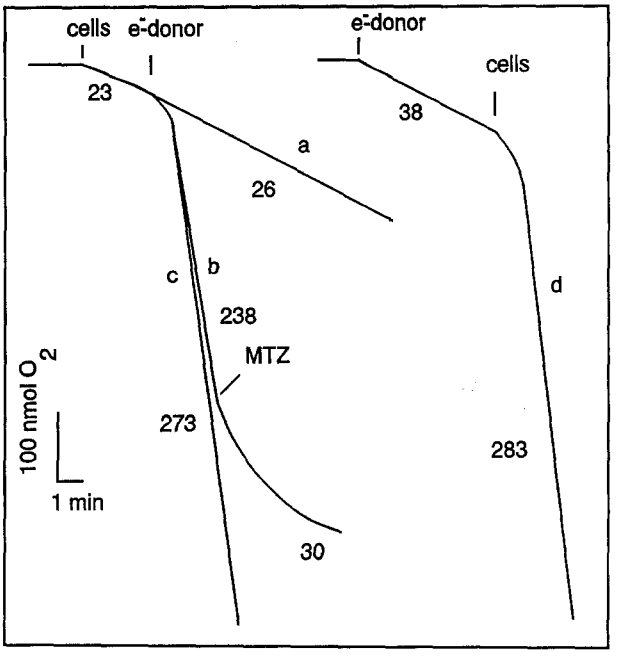

Illustration of respiratory traces of the tetrathionate- (a), elemental sulfur- (b), thiosulfate- (c) and sulfite-oxidizing (d) activities of cells of *Thiobacillus denitrificans* (type strain) grown on 20 mM thiosulfate. Experimental conditions and final concentrations of respiratory substrates and myxothiazol were the same as in table 1. The number beside each respiratory trace indicates the rate of oxidation in nmol O_2 mg protein^{-1} min^{-1}.

Table 1. Respiratory activities of cells of *Thiobacillus denitrificans* (type strain) grown on 20 mM thiosulfate.

Respiratory substrates	Respiratory activities (nmol O_2 mg protein^{-1} min^{-1})	% inhibition by MTZ	CCCP	KCN
Tetrathionate	<5	-	-	-
Thiosulfate	250	80	82	91
S°	215	93	95	92
Sulfite	245	65	70	90

The final concentrations of the respiratory substrates were 20 mM for thiosulfate, hydrophilic S° and sulfite, and 10 mM for tetrathionate; those of the inhibitors were 50 µM for myxothiazol (MTZ), 1 mM for KCN, 10 µM for CCCP. The respiratory activities were measured as described in 'Material and methods', from cells freshly harvested during their exponential growth phase. The low endogenous respiratory activities (23 nmol O_2 mg protein^{-1} min^{-1}) have been subtracted, as well as the inhibition of the endogenous respiratory activity by MTZ, KCN, and CCCP.

Table 2. Respiratory activities of cells of *Thiobacillus denitrificans* (type strain) grown on 20 mM thiosulfate.

Respiratory substrates	Respiratory activities (nmol O_2 mg protein^{-1} min^{-1}) A	B	% inhibition by KCN
Tetrathionate	<3	<2	>90
Thiosulfate	85	19	>90
S°	60	8	>90
Sulfite	105	35	>90

The final concentrations of the respiratory substrates and inhibitor were the same as in table 1. The respiratory activities were measured from cells freshly harvested during their exponential growth phase, and stored prior the respiratory activities measurements at high cell density (25 mg DW cells/ml). The low endogenous respiratory activities (3 nmol O_2 mg protein^{-1} min^{-1}) have been subtracted, as well as the inhibition of the endogenous respiratory activity by KCN.
A, respiratory activities measured after 10 min of storage; B, respiratory activities measured after 30 min of storage.

Schedel and Trüper[11] have reported anaerobic oxidation of thiosulfate and S° in *T. denitrificans* strain RT during chemolithotrophic growth on thiosulfate. In this case, thiosulfate was split by rhodanese to sulfite and S°, and S° was rapidly oxidized to sulfate. Our results suggest a similar pathway in cells of *T. denitrificans* grown under aerobic conditions on thiosulfate.

Better growth of *T. dentrificans* using thiosulfate as the unique electron donor was observed under microaerophilic conditions than under normal air pressure. Growth yield was 30–40% greater at the lowest dissolved oxygen concentration (12–54 µM, or 5–23% air saturation) than at higher dissolved oxygen concentrations (104–216 µM, or 44–88% air saturation), indicating oxygen to be a growth-inhibitor[6]. In contrast, *Thiobacillus versutus* showed good growth on thiosulfate under normal air conditions as well as under microaerophilic conditions (2.5 KPa O_2 or 12.5% air saturation), but with S° as substrate only growth under microaerophilic conditions (2.5 KPa O_2) was observed[2,3]. In cells of *T. versutus*, the respiratory oxidation rate of thiosulfate under normal air and under microaerophilic conditions was 3–4 times higher than the activity measured in cells of *T. denitrificans* grown under microaerophilic conditions[2,3]. In *T. versutus* grown on thiosulfate under normal air conditions the thiosulfate-oxidizing activity was about 30% higher than the activity measured in cells grown under microaerophilic conditions, but the ratio of thiosulfate-oxidizing activity to sulfur oxidizing activity remained the same[2,3]. We suggest that in some Thiobacilli the high respiratory oxidation rate of thiosulfate under normal air conditions is probably essential for lowering the endocellular oxygen pressure, and permitting SOA expression.

Elemental sulfur occurs mainly in the form of S_8 ring. Generally it is believed that S° is insoluble in water, but it has been shown that it is soluble in very small quantities[12] (approximatively 5 µg/l). In contrast, all inorganic sulphur anions are easily soluble in water.

The colorless aerobic sulfur-oxidizing bacteria, in one form or another, are to be found in almost every life-supporting environment where reduced sulfur compounds occur. The range of habitats is very wide. In some cases, the primary reduced sulfur compounds available tend to be sulfides (including minerals e.g. pyrite and metal sulfides) and S°, and were produced by microbial sulfate reduction and degradation of organic sulfur compounds, or by chemical reaction. Sulfide is also formed geologically in hydrothermal vents. Sulfur anions are formed principally by different microbial activities and by chemical reaction of sulfides and polysulfides with oxygen[13–15]. Colorless sulfur-oxidizing bacteria live in marine and fresh water sediments, soils, soil aggregates, waste water treatment systems, acid mine drainage water, and geothermal fields, to name but a few habitats. In some of these ecophysiological niches microaerophilic conditions are common, and in particular sulfide:oxygen gradients occur in stratified water bodies as well as in soil aggregates and sediments[15]. Recently several different mesophilic and thermophilic strains of sulfur-oxidizing bacteria have been isolated from young and mature compost heaps of garden waste (Beffa et al. unpublished). Several of these strains grow chemolithoautotrophically on inorganic sulfur compounds, such as S°, but only under microaerophilic conditions (2.5–5 KPa O_2). However, they grow very easily on organic compounds under normal air pressure. In conclusion we suggest that microaerophilic conditions of niches, in stratified macro- and micro-environments where reduced sulfur compounds are found, could be one of the crucial determinants for the proliferation of sulfur-oxidizing bacteria.

50 µM myxothiazol, an inhibitor of the respiratory chain in the quinone-cytochrome b region[9], inhibited the SOA completely, the thiosulfate-oxidizing activity strongly, and the sulfite-oxidizing activity significantly (table 1). KCN almost totally inhibited all the respiratory activities (table 1). The almost complete inhibition of the SOA by myxothiazol suggests that electrons produced during S° oxidation enter the respiratory chain in the quinone-cytochrome b region. Similarly, we observed the participation of cytochrome b during sulfite oxidation. These results are partly in contrast with those presented by Peeters and Aleem[7], who showed very poor suppression of thiosulfate-oxidizing activity by inhibitors of the quinone-cytochrome b region in cells of *T. denitrificans* grown under aerobic conditions on thiosulfate. Inhibitors acting on the terminal part of the respiratory chain strongly inhibited the thiosulfate- and sulfite-oxidizing activities[7]. Furthermore, in cells grown anaerobically on thiosulfate and nitrate, these activities were strongly inhibited by both quinone-cytochrome b and terminal respiratory chain

inhibitors[7]. Strong inhibition of SOA and significant inhibition of thiosulfate oxidizing activity by inhibitors in the quinone-cytochrome b region have been reported for different thiobacilli[3–5,16,17]. The redox potentials at pH 7.0 ($E_{m7.0}$) for some inorganic sulfur compound couples were −45 mV to −120 mV for S°/SO_3^{2-}, −200 mV to −250 mV for S°/SO_4^{2-}, and for SO_3^{2-}/SO_4^{2-} −510 mV[18]. The redox potential ($E_{m7.0}$) of the NAD^+/NADH couple was −320 mV, and the most common quinones present in the nonphototrophic bacterial redox systems showed $E_{m7.0}$ of about +70 mV for ubiquinone (QH_2/Q) and −70 to −100 mV for menaquinone (MKH_2/MK)[18,19]. The $E_{m7.0}$ values of bacterial cytochromes generally lie between −100 mV and +500 mV[19]. According to these redox potentials, we suggest that the electrons produced by oxidation of S° to sulfite or sulfate are sufficiently electronegative to enter the respiratory chain at the level of ubiquinone-cytochrome b, as shown by the inhibition of SOA by myxothiazol.

The uncoupler CCCP (10 μM final concentration) was a potent inhibitor of the SOA (95%), and inhibited relatively strongly the thiosulfate- and sulfite-oxidizing activities (table 1). Strong inhibition of SOA by an uncoupler has previously been reported in *Thiobacillus ferrooxidans* grown on thiosulfate[20], in *Thiobacillus acidophilus* grown on thiosulfate[21], and in *Thiobacillus tepidarius* grown on tetrathionate[4,22], thiosulfate[4,22] or crystalline elemental sulfur[4]. In contrast, in *T. acidophilus* grown on thiosulfate[21] and *T. tepidarius* grown on thiosulfate[4], tetrathionate[4] or crystalline elemental sulfur[4] the thiosulfate-, tetrathionate- and trithionate-activities were less inhibited by an uncoupler. The total inhibition of SOA by the uncoupler CCCP suggests that the state of the membrane potential plays an essential role during S° oxidation[20]. This might imply that an active transport step is involved in S° oxidation or in the activation of the sulfur molecule, as has been previously suggested for *T. ferrooxidans*[20]. In addition, in intact cells of *T. tepidarius* grown on thiosulfate, the ATP-synthesis inhibitor DCCD has been shown to inhibit ATP production without affecting the proton membrane potential or NAD(P) reduction. In contrast the uncoupler FCCP, affecting both the proton membrane potential and ATP synthesis, strongly inhibited NAD(P) reduction[22]. In conclusion it appears that the proton potential could be the direct energy donor for activation of the S° molecule.

The inhibition of sulfite-oxidizing activity by CCCP remains to be understood. In *T. denitrificans* (strain RT) a siroheme-containing sulfite reductase has been isolated and purified[23]. With reduced viologen dyes the enzyme reduced sulfite to sulfide, thiosulfate and trithionate. In many properties *T. denitrificans* sulfite reductase closely resembled desulfoviridin, the dissimilatory sulfite reductase of *Desulfovibrio* species[23]. The successive oxidation of these products by intermediary S° production could explain the effect of CCCP on sulfite-oxidizing activity. Further investigations will be necessary to elucidate this aspect. It is also very important to stress that the effect of the respiratory chain inhibitors myothiazol and HQNO and uncoupler CCCP on the oxidation of sulfites should be interpreted with caution. Sulfite solutions are readily autooxidized in air, a process catalyzed by a number of metal ions. This abiotic oxygen consumption may result in an overestimation of the biotic sulfite-oxidizing activity, even in its complete absence. The fresh sulfite solution can be partially stabilized with 5 mM ethylenediaminetetraacetate (EDTA). Generally, in order to take into account the residual spontaneous oxidation, sulfite was always added to the oxygen electrode unit prior to the cell suspension. However, a possible stimulation of sulfite autooxidation by intracellular metal ions cannot be excluded. In addition, we have observed that the abiotic sulfite autooxidation (control) is partially inhibited by myxothiazol and HQNO at 10–50 μM (final concentration), and by the uncoupler CCCP at 5–10 μM (final concentration). Abiotic inhibition of sulfite-oxidizing activity by these inhibitors may result in an overestimation of the percentage inhibition, or even in an erroneous assumption of its presence. In the conditions used in this study, the autooxidation of S°-, thiosulfate-solutions was not observed, and tetrathionate-solutions remained stable for some hours at pH 7.2.

Chemostat studies on various thiobacilli have revealed important differences in the growth rates and growth yields on thiosulfate[24–29]. These may be explained by the existence of fundamental differences in the electron transport mechanism and modes of energy conservation among these organisms. On the basis of the recently published results on SOA and thiosulfate-oxidizing activity in actively growing cells of *T. versutus*, *T. tepidarius*, *T. novellus*, *T. denitrificans*[2–5] and literature on growth rates and growth yields on thiosulfate, a speculative correlation between S° metabolism and growth rates or yields on thiosulfate can be established. Apparently, a high growth rate on thiosulfate can be correlated with a high SOA rate, and higher yield implies a lower ratio of thiosulfate-oxidizing activity/SOA (table 3).

It is certain that several specific oxidation pathways are expressed in chemolithoautotrophic sulfur-oxidizing bacteria during the oxidation of different inorganic sulfur compounds. However, it seems that the intermediary production of S° and the SOA are one common system in these bacteria. Further studies on SOA activity during the oxidation of reduced sulfur compounds, including chemical analyses and measurments of growth rate and growth yields, are indispensible to elucidate the possible central role of S° and SOA in the metabolism of chemolithoautotrophic sulfur-oxidizing bacteria.

Table 3. Summary of the respiratory activities, yield (Y_{max}) and growth rate (μ_{max}) of different Thiobacilli grown chemolithoautotrophically on thiosulfate as the sole energy source. The respiratory activities are data from references 2–5

Strains	Respiratory activities (nmol O_2 mg protein^{-1} min^{-1})					Y_{max}[a]	μ_{max}[b]
	TTOA	SUOA	TOA	SOA	TOA/SOA		
T. versutus	0	85	720	135	5.3	6.4–8.1	0.10
T. tepidarius	260	0	1400	330	4.2	11	0.41
T. denitrificans	0	245	250	215	1.2	14.7	0.13–0.14
T. novellus	0	715	275	70	3.9	(c)	0.05

TTOA, tetrathionate-oxidizing activity; SUOA, sulfite-oxidizing activity; TOA, thiosulfate-oxidizing activity; SOA, elemental sulfur-oxidizing activity.
[a]g dry weight mole thiosulfate consumed^{-1}; data from refs 24–26.
[b]h^{-1}; data from refs 26–29.
[c]a Y_{max} value with thiosulfate for *T. novellus* is not available[24].

Abbreviations. CCCP, carbonyl cyanide m-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; DW, dry weight; FCCP, carbonyl cyanide p-fluoromethoxyphenylhydrazone; HQNO, 2-N-hepthyl-4-hydroxyquinoline; MTZ, myxothiazol.

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