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Influence of oxygen on sulfate reduction and growth of sulfate-reducing bacteria

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Abstract. The ambivalent relations of sulfate-reducing bacteria to molecular O_2 have been studied with ten freshwater and marine strains. Generally, O_2 was reduced prior to sulfur compounds and suppressed the reduction of sulfate, sulfite or thiosulfate to sulfide. Three strains slowly formed sulfide at O_2 concentrations of below 15 µM (6% air saturation). In homogeneously aerated cultures, two out of seven strains tested, *Desulfovibrio desulfuricans* and *Desulfobacterium autotrophicum*, revealed weak growth with O_2 as electron acceptor (up to one doubling of protein). However, O_2 was concomitantly toxic. Depending on its concentration cell viability and motility decreased with time. In artificial oxygen-sulfide gradients with sulfide-containing agar medium and also in sulfide-free agar medium under an oxygen-containing gas phase, sulfate reducers grew in bands close to the oxic/anoxic interface. The specific O_2 tolerance and respiration capacity of different strains led to characteristically stratified gradients. The maximum O_2 concentration at the surface of a bacterial band (determined by means of microelectrodes) was 9 µM. The specific rates of O_2 uptake per cell were in the same order of magnitude as the sulfate reduction rates in pure cultures. The bacteria stabilized the gradients, which were rapidly oxidized in the absence of cells or after killing the cells by formaldehyde. The motile strain *Desulfovibrio desulfuricans* CSN slowly migrated in the gradients in response to changing O_2 concentrations in the gas phase.

Key words: Sulfate reduction – Aerobic growth – Oxygen toxicity – Oxygen-sulfide gradients

Several recent studies indicate that dissimilatory sulfate reduction proceeds with high rates in the oxic zones of sediments or microbial mats (Battersby et al. 1985; Cohen 1989; Jørgensen and Bak 1991; Canfield and DesMarais 1991; Fründ and Cohen 1992). High numbers of sulfate-reducing bacteria were counted near the oxic/anoxic interfaces in such environments (Laanbroek and Pfennig 1981; Jørgensen and Bak 1991), and it was demonstrated that sulfate-reducing bacteria remain viable for hours or even days when exposed to O_2 (Hardy und Hamilton 1981; Cypionka et al. 1985; Fukui and Takii 1990). Bacteria carrying out dissimilatory sulfate reduction in the presence of O_2 have not been identified so far. It was found, however, that sulfate-reducing bacteria can respire with O_2 (Abdollahi und Wimpenny 1990; Dilling and Cypionka 1990). They oxidize H_2, various organic compounds or even sulfur compounds coupled to aerobic respiration, with rates comparable to those of aerobic bacteria. Respiration allows the formation of ATP, but aerobic growth has hitherto not been demonstrated (Dilling and Cypionka 1990; Dannenberg et al. 1992).

The capacity for aerobic respiration does not explain sulfate reduction under oxic conditions. Why should bacteria able to reduce O_2 reduce sulfate under oxic conditions? This question involves ecological as well as thermodynamic aspects. Usually facultatively aerobic bacteria reduce O_2 prior to other electron acceptors, although exceptions are known: *Thiosphaera pantotropha* carries out denitrification in the presence of O_2 (Robertson and Kuenen 1983; Bell and Ferguson 1991). *Desulfobulbus propionicus* reduces sulfate prior to nitrate (Widdel and Pfennig 1982), while *Desulfovibrio desulfuricans* prefers nitrate over sulfate as electron acceptor (Seitz and Cypionka 1986).

In the present study with several pure cultures of sulfate-reducing bacteria we describe the influence of O_2 on the reduction of inorganic sulfur compounds to sulfide, and on growth, viability and motility of the cells in homogeneously aerated cultures and in artificial oxygen-sulfide gradients.

Materials and methods

Organisms and cultivation

The freshwater strains *Desulfovibrio desulfuricans* CSN (Cypionka 1989) and Essex 6 (DSM 642), *Desulfovibrio vulgaris* (DSM 2119),

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Desulfomicrobium baculatum (formerly *Desulfovibrio baculatus*, DSM 1743), *Desulfobulbus propionicus* (DSM 2043), and the strains 3a and 1a (isolated from a settling tank of the municipal sewage plant in Konstanz) were cultivated at 29 °C in the medium described by Cypionka und Pfennig (1986). The marine strains *Desulfobacterium autotrophicum* (DSM 3382), *Desulfobacter hydrogenophilus* (DSM 3380) and the strain c2 (isolated from the top layer of mediterranean littoral sediment, in Cervo, Riviera dei Fiori, Italy) were supplied with increased concentrations of NaCl (350 mM) and $MgCl_2$ (14 mM). Lactate (20 mM) or H_2 (H_2/CO_2, 80/20, v/v) served as electron donor, and acetate (2 mM) as additional carbon source during growth with H_2. Sodium dithionite (< 30 µM) was added as reducing agent until the redox indicator resazurin (0.2 mg/l) was colourless.

Measurement of aerobic respiration and sulfide formation

Aerobic respiration and sulfide formation were measured simultaneously with electrodes. The reaction chamber (3 ml) of a stirred O_2 electrode chamber (electrode at the bottom; Hansatech, Bachofer, Reutlingen, FRG) was closed by a stopper which contained a small combined pH electrode (Ingold, LoT 406-M4), a sulfide electrode (1 mm ∅, silver wire coated with Ag_2S), a platinum electrode (0.8 mm ∅, coated with platinum black), and a hollow needle used as in- and outlet (Cypionka 1989). The silver anode of the O_2 electrode was separated from the reaction chamber by a salt bridge that prevented contact with sulfide. The signals of the electrodes were amplified and processed by an analogue/digital converter (DAS-8PGA, Keithley) on a personal computer (AT 386SX, Highscreen, Aachen, FRG).

Washed cells were added to N_2-saturated phosphate buffer (25 mM) at 29 °C. For marine strains the medium was supplemented with 400 mM KCl. By flushing the assay with N_2 for 15 min, the O_2 concentration was not lowered below 15 µM. After addition of the cells, however, O_2 was completely removed within 1 min.

Aerobic growth experiments

Aerobic growth in homogeneously aerated cultures was studied in stoppered 500 ml-glass bottles. Prior to the addition of O_2 by a syringe, the headspace (400 ml) was gassed with a mixture of 80 vol% H_2 as electron donor and 20 vol% CO_2. The medium (100 ml) contained acetate (2 mM) as carbon source and thiosulfate (150 µM) as sulfur source. The bottles were vigorously shaken (100 rpm.) at 29 °C. Viable cell counts were determined in duplicate deep agar dilution series (Pfennig at al. 1981; Cavalli-Sforza 1972) with the appropriate growth, lactate (20 mM) as electron donor, and dithionite as reducing agent. The O_2 concentration in the gas phase was measured with a gas chromatograph. Protein was determined according to Schmidt et al. (1963).

Artificial oxygen-sulfide gradients

Oxygen-sulfide gradients (Nelson and Jannasch 1983; Cypionka et al. 1985; Wimpenny and Jones 1988) were generated in glass tubes half filled with sulfate-free mineral medium which additionally contained agar (0.36%, w/v), lactate (20 mM), resazurin (1 mg/l) and Na_2S (250 µM), and were inoculated with about 10^7 cells per ml. The gas phase consisted of 20% CO_2 and varying amounts of N_2 and O_2.

The oxygen-sulfide gradients were characterized by means of microelectrodes. The O_2 concentration was measured by a steel needle electrode (Diamond Electro-Tech, model No. 760) combined with an Ag/AgCl electrode and a Keithley 485 Picoammeter. Sulfide microelectrodes were constructed from silver wire (0.1 mm ∅), which was glued (UHU plus sofortfest) into a glass capillary of 1 mm outer diameter.

Sulfidation was carried out according to Cypionka (1986). The microelectrodes were simultaneously lowered by a micromanipulator into tubes cut off near the agar surface. Control experiments revealed that there were only minor pH changes which did not significantly interfere with the sulfide measurements in the gradients.

Table 1. Influence of O_2 on sulfate reduction

Strain	Rate of sulfide formation[a] (nmol min^{-1} mg $protein^{-1}$) Oxygen concentration		
	> 15 µM	< 15 µM	0 µM
Desulfovibrio desulfuricans CSN	0	9.5	28.5
Desulfovibrio vulgaris Marburg	0	12.0	41.0
Desulfobulbus propionicus	0	0.0	61.0
Desulfobacter hydrogenophilus	0	4.0	36.0

[a] The experiments were performed with washed cells at a protein concentration of 0.2 mg/ml

Results

Influence of O_2 on sulfide formation from sulfate, thiosulfate or sulfite

The reduction of sulfate, sulfite or thiosulfate to sulfide by washed cells was sensitive to O_2. There was no sulfide formation at O_2 concentration above 15 µM (6% air saturation). In the presence of 15 µM O_2 the rates of sulfide formation from sulfate was diminished by at least two thirds (Table 1). Only in a few cases weak sulfide formation was observed when the addition of lactate significantly accelerated the aerobic respiration rates (to 150 nmol O_2 per min and mg protein, Fig. 1). After complete O_2 consumption, sulfate was reduced at maximum rates (Table 1, Fig. 1). Similar results were obtained with thiosulfate or sulfite instead of sulfate. The rates of chemical oxidation of sulfide in the presence of O_2 were very low and thus did not lead to errors in the measurement of sulfide formation. Except *Desulfobulbus propionicus*, the strains tested were not able to oxidize sulfide with O_2 (Dannenberg et al. 1992).

Table 2. Aerobic growth of sulfate-reducing bacteria in homogeneously aerated cultures[a]

Strain[b]	O_2 concentration (% of Headspace volume)	O_2-dependent increase of OD (OD_{436})	Final protein concentration (mg ml^{-1})
Desulfovibrio desulfuricans Essex	0.0	0	0.022
	0.4	0.045	0.026
	1.5	0.085	0.039
Desulfovibrio desulfuricans CSN	0.0	0	0.021
	0.4	0.011	0.036
	1.5	0.013	0.037
Desulfobacterium autotrophicum	0.0	0	0.010
	0.4	0.052	0.013
	1.5	0.017	nd

[a] Growth conditions as in Fig. 2; the medium for *Desulfobacterium autotrophicum* contained sulfate instead of nitrate

[b] Strains tested that did not grow aerobically: *Desulfomicrobium baculatum*, and the strains 1a, 3 a, c2

nd = not determined

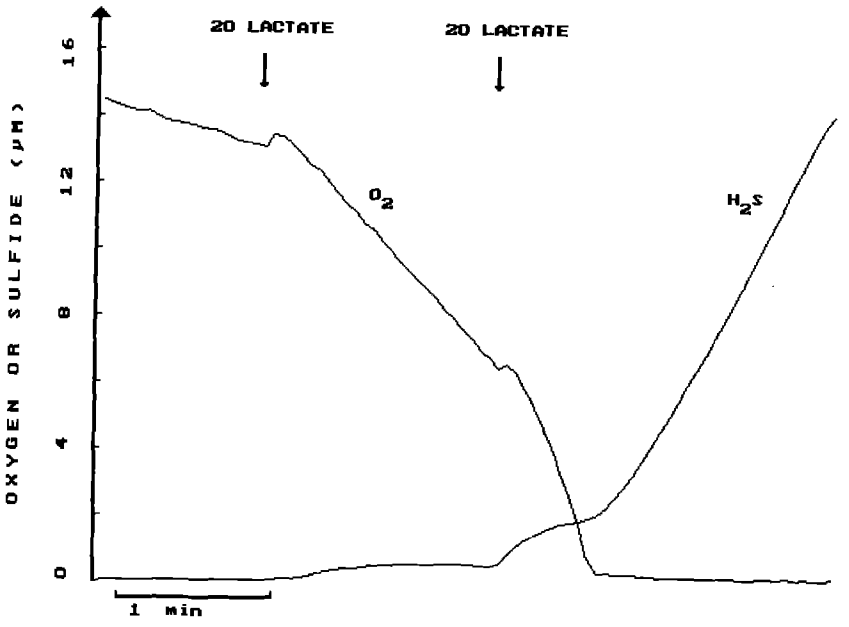

Fig. 1. Influence of O_2 on sulfate reduction by *Desulfovibrio vulgaris*. The protein concentration was 0.17 mg ml^{-1}, and the pH 7.0. The numbers at the arrows indicate additions in nmol

Growth and survival in homogeneously aerated culture

Most of the strains tested – even those isolated from (at least periodically) oxic environments – did not grow in homogeneously aerated cultures at 0.5 to 2% (6 to 25 μM) O_2. Weak aerobic growth was obtained with only three strains (Table 2, Fig. 2). In these cases optical density and protein increase (up to one doubling) were correlated with the O_2 concentration of the gas phase. The growth stopped after a few days. *Desulfobacterium autotrophicum* revealed weak growth only at O_2 headspace concentrations below 1.5%.

Upon prolonged incubation with O_2 the cells lost their motility and the ability to grow in anoxic medium (Table 3).

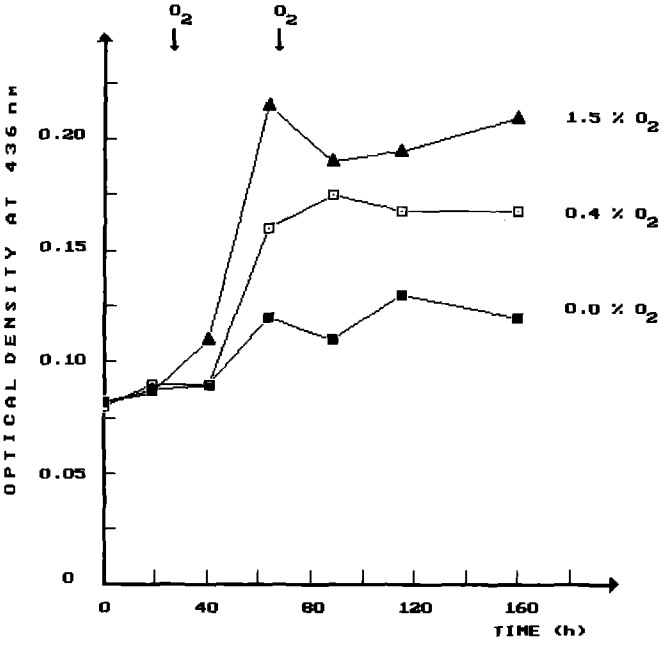

Fig. 2. Aerobic growth of *Desulfovibrio desulfuricans* Essex. The cultures (100 ml medium, 400 ml gas phase with 80% H_2 and 20% CO_2) were vigorously shaken. The buffered mineral medium contained 2 mM acetate as carbon source, 150 μM thiosulfate as sulfur source and 2 mM nitrate as additional electron acceptor. The arrow marks addition of O_2 at the concentration indicated

Activities of sulfate-reducing bacteria in artificial oxygen-sulfide gradients

In counter-rotating oxygen-sulfide gradients in agar medium all sulfate-reducing bacteria tested grew in small colonies, which formed white bands close to the oxic/anoxic interface. Growth depended on the presence of lactate as electron donor and of O_2 as electron acceptor. Free sulfide, however, was not essential since small bands were also obtained in sulfide-free gradients. The localization of the bands varied with the strain (Fig. 3). As shown by microelectrode measurements, overlapping gradients of O_2 and sulfide were built up a few hours after preparation of a gradient. The bacteria formed a visible band within two days (Fig. 4). Within two weeks, this layer moved upwards by 6 mm.

Using Fick's first law, and applying the diffusion coefficient of O_2 in water (Ferell and Himmelblau 1967)

Table 3. Toxicity of O_2 to *Desulfovibrio desulfuricans* Essex

Property	Incubation time which resulted in loss of property[a] (days) Oxygen concentration in the gas phase (%)			
	1	2	5	10
Motility	10	4	2	1
Capability of anaerobic growth	12	8	3	2

[a] A property was considered lost when less than 0.01% of the cells still possessed it

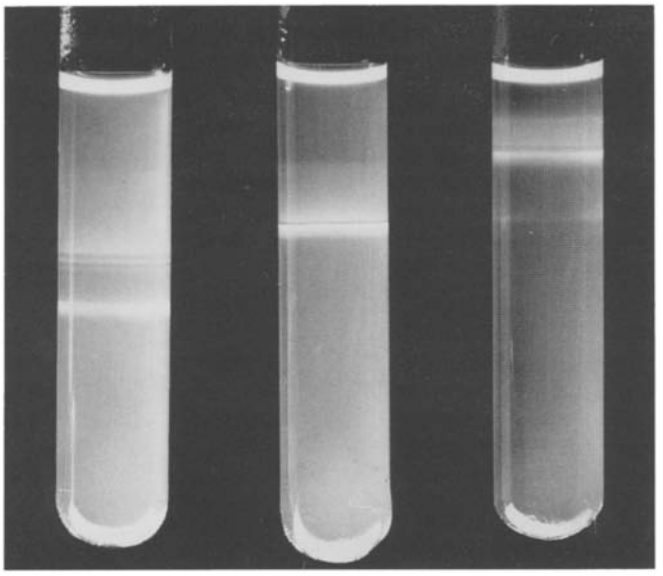

Fig. 3. Growth of the strains 3a, 1a, and *Desulfovibrio desulfuricans* CSN (from left to right) in gradients. The medium contained agar (0.36%, w/v), DL-lactate (20 mM) and sulfide (250 μM). The gas phase contained 21% O_2 and 20% CO_2 and 59% N_2. The tubes were incubated for two weeks at 29 °C

for the gradients, the O_2 flux into the bacterial layer was calculated. With the O_2 gradients getting steeper an increase of O_2 uptake by the bacterial layer from 290 to 1130 nmol per cm^2 and day was obtained. The cells in the layers were counted microscopically in homogenized and diluted samples. An average cell density of 10^9 cells per cm^3 gave a respiration rate of 4×10^{-15} mol $O_2\ d^{-1}\ cell^{-1}$, or about 10 nmol $O_2\ min^{-1}$ and (mg protein)$^{-1}$. Usually there was no or very little (less than 9 μM) free O_2 within the growth zone. Thus, most of the cells appeared to be growing under O_2 limitation. Directly above the bacterial bands the medium turned pink due to the oxidized state of the redox indicator resazurin. The growth zones were not influenced by the presence or absence of resazurin. Within four weeks the bands grew to a thickness of 2 to 10 mm. Gradients without bacteria or after their killing by a drop of formaldehyde were completely oxidized after one day. An increase of the O_2 concentration above the gradients often resulted in intensified growth of the bacteria. In case of doubling the O_2 concentration from 20 to 40%, *Desulfovibrio desulfuricans* CSN formed a new band deeper in the agar within a few days (Fig. 5), while nonmotile strains were killed.

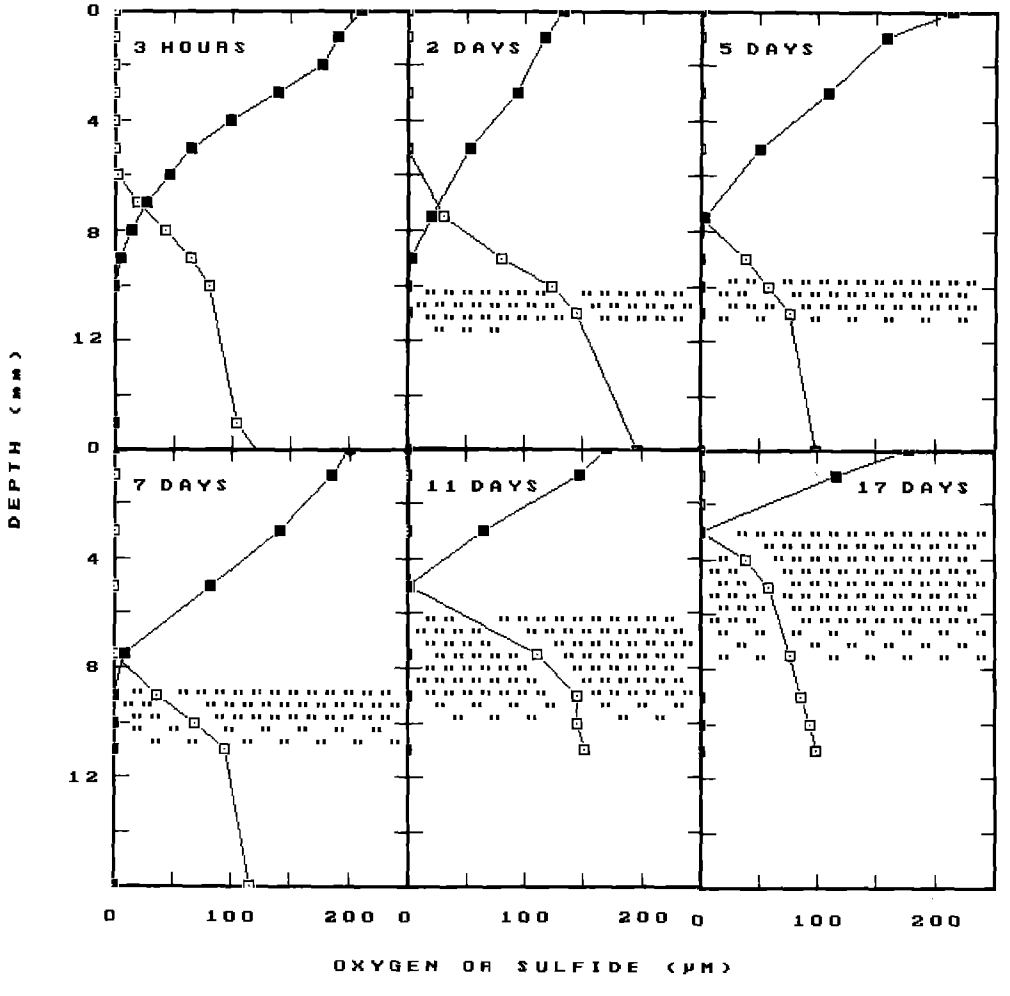

Fig. 4. Development of counter-rotating oxygen-sulfide gradients inoculated with *Desulfovibrio desulfuricans* CSN. Medium as described in Fig. 3. The bacterial bands are indicated by stippling

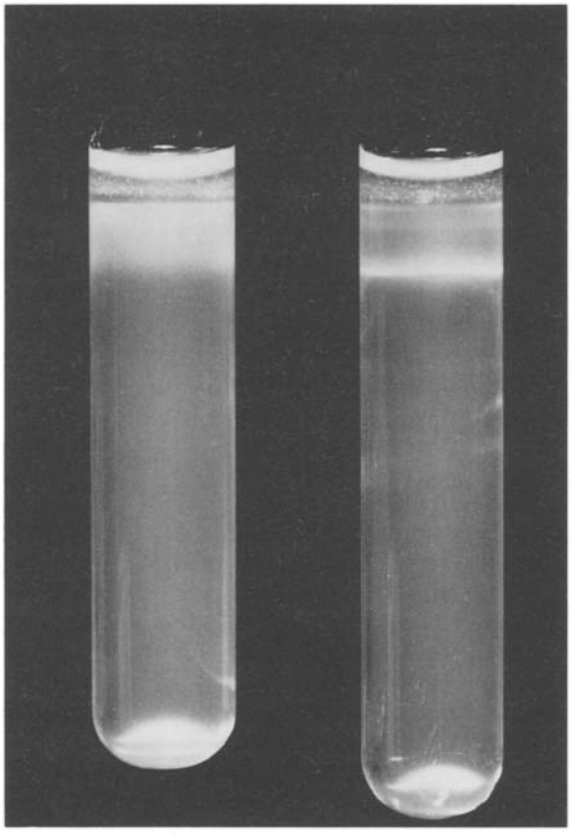

Fig. 5. Response of *Desulfovibrio desulfuricans* CSN upon changing O_2 concentrations. Both gradients were identically produced with 21% O_2 and 20% CO_2 in the gas phase (medium as in Fig. 2). After five days the O_2 concentration in the right tube was elevated to 41%. The pictures were taken after seventeen days

Discussion

Ambivalent role of O_2 in homogeneously aerated systems

In our study aerobic growth of sulfate-reducing bacteria is described for the first time. O_2 can undoubtedly be a true electron acceptor for sulfate-reducing bacteria. But at the same time it exerts toxic effects even at low concentrations. In homogeneously aerated cultures, we did not observe more than one doubling, and the cells rapidly lost motility and viability. Sulfate reducers isolated from periodically oxic environments (activated sludge, top layer of marine sediment) were no better adapted to oxic conditions than strains from our laboratory collection. Enzymes supposed to be responsible for O_2 tolerance, superoxide dismutase and catalase, have also been detected in some sulfate-reducing bacteria (Hewitt and Morris 1975; Morris 1976; Hatchikian et al. 1977; Hardy and Hamilton 1981; Abdollahi and Wimpenny 1990). We have observed superoxide dismutase activity in *Desulfovibrio desulfuricans* CSN, while catalase was absent (Dilling and Cypionka 1990).

Oxygen prevents the reduction of sulfur compounds, probably by successful competition for electrons. Although the aerobic respiratory chain and the terminal oxidase have not yet been clarified, it appears likely that the same system is used for electron transport to sulfur compounds and to O_2. Accordingly, after increased electron donor supply at very low O_2 concentrations a little sulfide formation could be observed concomitant with aerobic respiration.

Effects of O_2 in stratified systems

The oxygen-sulfide gradients studied are (although still with severe restrictions) better suited to simulate natural environments of sulfate-reducing bacteria than are homogeneously aerated systems. Accordingly, the influence of O_2 was quite different in these gradients. All strains tested were able to grow, at a depth specific for the strain and the O_2 concentration applied. The cells remained viable over weeks and stabilized the gradients. Previous growth experiments in similar oxygen-sulfide gradients (Cypionka et al. 1985) must be regarded in a new context. Originally we had assumed that in those gradients the bacteria reduce thiosulfate or elemental sulfur as major products of chemical sulfide oxidation. Now we know that many sulfate-reducing bacteria are able to oxidize sulfur compounds completely or incompletely with O_2 or nitrate as electron acceptor (Dannenberg et al. 1992). In this regard, the conversions of sulfur compounds in the gradients are comparable to those observed with *Beggiatoa* (Nelson and Jannasch 1983). On the other hand, we have found that sulfide or oxidized sulfur compounds are not essential for the development of the growth bands, since O_2 can be used directly as electron acceptor. The relative contributions of aerobic respiration and chemical oxidation of sulfur compounds cannot be exactly determined. However, at low concentrations the chemical reaction of O_2 and sulfide was slow compared to the biological process (Dannenberg et al. 1992).

The average rates of O_2 uptake by the bacterial bands were in the same order of magnitude as observed in homogeneously aerated cultures (Dilling and Cypionka 1990). Taking into account that only part of the cells in the layer was involved, the rates of aerobic respiration become comparable to those of sulfate reduction in pure cultures (Jørgensen 1978). Therefore, aerobic respiration may play a considerable role in the energy metabolism of sulfate-reducing bacteria at the oxic/anoxic interface. This activity might result in the formation of anoxic microniches (Jørgensen 1977).

Motile cells were able to respond to changing O_2 concentrations by vertical migration. However, these movements were slow, and appear not to be suited to reach the right position in a cyanobacterial mat with rapidly changing O_2 concentrations. Although our study again demonstrates the great versatility of sulfate-reducing bacteria, it is not easily compatible with reports on dissimilatory sulfate reduction in oxic sediments or microbial mats (Canfield and DesMarais 1991; Jørgensen and Bak 1991; Fründ and Cohen 1992). The isolation of oxygen-tolerant sulfate-reducing bacteria that can reduce sulfate instead of O_2 in the presence of both of these electron acceptors remains a challenge for future research.

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