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Transition from Anaerobic to Aerobic Growth Conditions for the Sulfate-Reducing Bacterium *Desulfovibrio oxyclinae* Results in Flocculation

PAVEL SIGALEVICH, ERAN MESHORER, YAEL HELMAN, AND YEHUDA COHEN*

Division of Microbial and Molecular Ecology, Moshe Shilo Minerva Center for Marine Biogeochemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem 91904, Israel

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A chemostat culture of the sulfate-reducing bacterium *Desulfovibrio oxyclinae* isolated from the oxic layer of a hypersaline cyanobacterial mat was grown anaerobically and then subjected to gassing with 1% oxygen, both at a dilution rate of 0.05 h^{-1}. The sulfate reduction rate under anaerobic conditions was 370 nmol of SO_4^{2-} mg of protein^{-1} min^{-1}. At the onset of aerobic gassing, sulfate reduction decreased by 40%, although viable cell numbers did not decrease. After 42 h, the sulfate reduction rate returned to the level observed in the anaerobic culture. At this stage the growth yield increased by 180% compared to the anaerobic culture to 4.4 g of protein per mol of sulfate reduced. Protein content per cell increased at the same time by 40%. The oxygen consumption rate per milligram of protein measured in washed cell suspensions increased by 80%, and the thiosulfate reduction rate of the same samples increased by 29% with lactate as the electron donor. These findings indicated possible oxygen-dependent enhancement of growth. After 140 h of growth under oxygen flux, formation of cell aggregates 0.1 to 3 mm in diameter was observed. Micrometer-sized aggregates were found to form earlier, during the first hours of exposure to oxygen. The respiration rate of *D. oxyclinae* was sufficient to create anoxia inside clumps larger than 3 μm, while the levels of dissolved oxygen in the growth vessel were 0.7 ± 0.5 μM. Aggregation of sulfate-reducing bacteria was observed within a *Microcoleus chthonoplastes*-dominated layer of a cyanobacterial mat under daily exposure to oxygen concentrations of up to 900 μM. *Desulfonema*-like sulfate-reducing bacteria were also common in this environment along with other nonaggregated sulfate-reducing bacteria. Two-dimensional mapping of sulfate reduction showed heterogeneity of sulfate reduction activity in this oxic zone.

The photic zone of cyanobacterial mats is characterized by extreme diurnal shifts in oxygen and sulfide concentrations from oxygen supersaturation during the light period to anoxic, sulfide-enriched conditions in the dark (25). These changes are a challenge for the organisms living in such a habitat. Daily exposures to high oxygen concentrations may be deleterious to obligate anaerobes such as sulfate-reducing bacteria (SRB). Nonetheless, high numbers of SRB were found in oxic zones of microbial mats (5, 7, 12, 22, 23, 32), indicating that these organisms can deal with temporal exposures to elevated oxygen concentrations of even up to 1.5 mM. Previous research demonstrated the abundance of members of the genera *Desulfovibrio* (18, 26) and *Desulfonema* (22, 23, 32) in the upper layers of hypersaline cyanobacterial mats. Moreover, high rates of sulfate reduction were found in the oxic layers of these microbial mats (8, 11). Attempts were made to isolate oxygen-tolerant SRB from these environments (13a, 16, 17). However, all SRB isolated to date required anoxic conditions for growth. Although the notion that SRB are strictly anaerobic was recently reconsidered (14, 19), the evidence of oxygen-dependent growth is still scarce.

A *Desulfovibrio* strain isolated from a hypersaline microbial mat was shown to be able to produce cell protein via oxygen respiration, though cell division was inhibited (18). *Desulfovibrio vulgaris* Hildenborough was found to be capable of slow linear aerobic growth under very low concentrations of oxygen. Oxygen concentrations of only 0.07% were toxic to this organism (15).

D. oxyclinae (17), isolated from the upper layer of a hypersaline microbial mat, was initially obtained as a coculture with an oxygen-scavenging aerobe. These cocultures were found to reduce sulfate under aeration. This SRB was repeatedly isolated from consortia and obtained in axenic cultures (30).

D. oxyclinae was demonstrated to be capable of aerobic respiration, although only at low oxygen concentrations. It was found to form aggregates when subjected to oxygen (17). Flocculation was expected to be one of the mechanisms protecting *D. oxyclinae* from the toxic effects of oxygen. The central part of such an aggregate could remain anaerobic even under exposure to a moderate oxygen flux as the result of aerobic respiration by the surface cells.

In this report, we present the results of experiments on the continuous culture growth of *D. oxyclinae* under anaerobic conditions and the sequence of events occurring after exposure to oxygen culminating in cell flocculation. An attempt was made to demonstrate the ecological importance of flocculation of SRB in their natural environment. We demonstrate (i) the occurrence of aggregates of SRB within the upper layers of cyanobacterial mats by in situ hybridization and (ii) the activity of sulfate reduction by two-dimensional mapping of sulfate reduction activity across the oxygen-sulfide gradient.

* Corresponding author. Mailing address: Division of Microbial and Molecular Ecology, Moshe Shilo Minerva Center for Marine Biogeochemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem 91904, Israel. Phone: 972 2 6585110. Fax: 972 2 6528008. E-mail: yehucoh@vms.huji.ac.il.

MATERIALS AND METHODS

Growth medium. One liter of the synthetic growth medium used in all continuous culture experiments contained the following: NaCl, 50.0 g; KCl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 2.5 g; K_2HPO_4, 0.5 g; NH_4Cl, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.08 g; vitamin solution, vitamin B_{12} solution, and thiamine solution, 1 ml of each; ascorbate-

thioglycolate reducing solution, 10 ml (34); SL7 mineral solution, 1 ml (3); and resazurine, 10 mg. The medium was prepared by mixing the sterile stock solution (10 ml liter^{-1}; KCl, 10%; $MgCl_2 \cdot 6H_2O$, 25%; NH_4Cl, 10%; $CaCl_2 \cdot 2H_2O$, 0.8%) with a 5% solution of NaCl. Subsequently, 1 ml of 50% K_2HPO_4 per liter was added. This procedure minimized precipitation and prevented wall growth in the growth vessel, which occurred in previous experiments (17). Sodium sulfate and carbon sources were added as sterile stock solutions to the final concentrations specified below.

Bacterial strain. *D. oxyclinae* strain P1B (DSM 11498) was isolated previously in our laboratory from the oxic zone of the Solar Lake mats (17).

Continuous culture experiments. Growth experiments were carried out in a 1.2-liter fermentor manufactured at the Biological Center of the University of Groningen (Haren, The Netherlands), equipped with controls for temperature, pH, and dissolved oxygen. The growth medium contained 10 mM Na_2SO_4 and 20 mM DL-sodium lactate. Growth temperature was maintained at 35°C; pH was kept at 7.6 ± 0.2. Dissolved oxygen was measured using a 900 series New Brunswick (Edison, N.J.) oxygen electrode. This electrode was recalibrated after the experiment and found not to deteriorate as a result of exposure to sulfide. As determined chemically, the steady-state oxygen concentration in the sterile growth medium under the aerobic incubation conditions was 8.2 μM. The mixing rate was 1,500 rpm; the dilution rate was set at 0.05 h^{-1} since μ_{max} was determined to be 0.06 h^{-1}. During the anaerobic stage, the growth vessel was gassed with oxygen-free nitrogen. During the aerobic stage, gassing was performed with 4 parts of nitrogen and 1 part of a mixture containing 90% N_2, 5% O_2, and 5% CO_2; the gassing rate was 0.5 1 min^{-1}, corresponding to an oxygen flux of 45 μmol min^{-1}. At every sampling, 1-ml aliquots were withdrawn and incubated aerobically in sulfate-free growth medium with 20 mM lactate to ascertain that the culture was free from aerobic contamination.

Estimation of cell growth. Total cell numbers were determined by microscopic counting of at least 500 cells in Petroff-Hausser chamber. The number of sulfate-reducing cells was determined by the most-probable-number (MPN) method in eight replicate fivefold dilutions on Nunclon 96-well plates in the defined multipurpose medium with ascorbate-thioglycolate reducing solution (35) supplemented with 50 mg of $FeSO_4 \cdot 7H_2O$ per liter. The multiwell plates were incubated in an anaerobic hood.

Analytical procedures. Protein was determined by the Lowry method (20) on washed cell pellets. Elemental sulfur, known to interfere with the analysis, was never found in the samples. Sulfate was determined by the turbidimetric method (13a) as modified by H. Cypionka (personal communication), and sulfide was determined by the methylene blue method (6).

Measurement of respiration rates. Rates of aerobic respiration and thiosulfate reduction were measured in a 2-ml reaction chamber with built-in electrodes for oxygen, sulfide, and pH (9). The rates of aerobic respiration, sulfide production, and consumption were determined under lactate excess. To determine the rate of aerobic respiration, 100 μl of oxygen-saturated water containing 139 nmol of O_2 was injected into the chamber, and the rate of oxygen consumption was determined. The rate of thiosulfate reduction was measured by the rate of sulfide production from initial thiosulfate concentrations of 50 to 100 μM. Prior to measurement, the samples were aseptically washed with sterile medium without carbon and sulfur sources, resuspended in sterile medium without carbon and sulfur sources, and incubated under nitrogen for 30 min. Protein concentrations in the cell suspension in the chamber were determined by the Lowry method after the measurement.

Calculation of oxygen consumption and sulfate reduction rates in the growth vessel. The in situ rate of oxygen consumption in the growth vessel was calculated from the known oxygen input and corresponding oxygen saturation level in the sterile growth vessel, and residual oxygen concentration was determined by the submerged oxygen electrode. The in situ rate of sulfate reduction was calculated from the sulfate concentration in the medium reservoir and measured level of residual sulfate in the growth vessel.

Effect of oxygen on cell aggregation. Anaerobically grown cells of *D. oxyclinae* were washed and resuspended in mineral medium with 10 mM lactate. The cell suspension was studied in a 31.5-ml stirred, thermostatic turbidimetric cuvette at 35°C, using a nephelometric 90° measurement ratioed to sum of transmitted light and forward scatter light measurement in a Hach model 18900 ratio turbidimeter (Loveland, Colo.). The range of turbidity fluctuations was recorded for 1 min with 5-min intervals. To obtain single oxygen pulses, a known amount of oxygen-saturated water was injected into the cuvette. Constant oxygen input was achieved by passing 5% oxygen at a rate of 5 ml min^{-1} through a U-shaped silicone tube submerged in the cuvette.

In situ hybridization. Solar Lake mat samples of 0.5 by 0.5 by 1.0 cm were fixed on site for 10 h at room temperature in 4% paraformaldehyde in phosphate-buffered saline solution prepared in 8% NaCl. The samples were then washed twice with phosphate-buffered saline and dehydrated in increasing ethanol concentration series. The samples were then embedded in paraffin and cut into 5-μm slices by a cryogenic microtome (model CM300; Leica, Nusslauch, Germany). The slides were hybridized with fluorescein-labeled probe SRB385 (2) and treated as described by Manz et al. (21). Fluorescence was examined by a Zeiss LSM510 confocal laser scanning microscope in the laboratory of M. Wagner, Technical University, Munich, Germany.

Two-dimensional submillimetric mapping of sulfate reduction across oxygen-sulfide gradients. Silver foil of 0.125-mm thickness (Sigma) was cut into 10- by 10-mm squares, which were then washed extensively with hexane, water, and acetone to remove surface contamination, exposed to 0.1 N HNO_3 for 1 min to form an outer layer of silver oxide, and washed extensively with double-distilled water. $Na_2{}^{35}SO_4$ solution of high specific activity (Amersham, Little Chalfont, United Kingdom) was diluted in 2% sodium silicate solution to the final concentration of 0.1 mCi ml^{-1}. Silver foils were immersed into the radioactive solution thrice followed by drying by infrared lamp. Homogeneity of the coating on the silver foils was examined by phosphor imaging. Homogeneously coated silver foils were then inserted vertically into a cyanobacterial mat sample from Solar Lake, Sinai (16), and incubated in the light at 800 μE min^{-1} cm^{-2} and 35°C for 40 min. Silver foils were then washed extensively with 0.1 N Tris buffer (pH 8.7) until no radioactivity could be detected in the washing solution. $Ag_2{}^{35}S$ trapped on the silver foil as a result of sulfate-reducing activity was mapped by phosphor imaging. Profiles of oxygen, pH, and sulfide as well as rates of oxygenic photosynthesis were obtained simultaneously using specific microelectrodes (25) in a cyanobacterial mat sample under experimental conditions identical to those used for the sulfate reduction mapping.

RESULTS AND DISCUSSION

Continuous culture experiment. *D. oxyclinae* was initially grown in the growth vessel of the chemostat in an anaerobic batch culture mode under nitrogen gassing until sulfate was completely depleted from the medium. At this point, the cell concentration reached 6.7 × 10^8 ml^{-1} and the protein level was 33.8 mg liter^{-1}. Growth yield of the batch culture was 3.6 g of protein per mol of sulfate consumed. Medium flow was then set at a dilution rate of 0.05 h^{-1}. The anaerobic chemostat was maintained for 436 h under gassing with nitrogen. At this point, 1% oxygen was added to the incoming gas. Activities of *D. oxyclinae* under anaerobic conditions and after exposure to oxygen are presented in Fig. 1; average values for the different stages are given in Table 1. The sequence of activities of this organism during anaerobic growth and in the transition from anoxic to oxic conditions can be divided into four stages according to the calculated apparent efficiency of sulfate reduction and the overall appearance of the culture. These stages differ also in the calculated oxygen consumption and measured rates of oxygen uptake and thiosulfate reduction.

MPN counts demonstrated that *D. oxyclinae* persisted in the growth vessel throughout the entire experiment at the growth rate of 0.05 h^{-1}. The culture was axenic during all the stages of the experiment. Tests for aerobic contamination performed at each sampling point were all negative.

During the initial 122 h of the anaerobic growth stage, sulfate concentration decreased from 10 mM to the residual concentration of 0.2 mM and remained stable during the next 314 h of steady-state chemostat culture (stage I [Fig. 1]). The concentration of dissolved sulfide was low as a result of intense nitrogen gassing at 0.5 1 min^{-1}. Lower gassing rates were found to result in suppression of sulfate reduction, presumably due to inhibition by sulfide (data not shown). During stage I, protein concentration and cell numbers also remained stable. At this stage, 2.4 ± 0.1 g of protein was produced per mol of sulfate consumed in the chemostat, corresponding to the calculated sulfate reduction rate of (2.0 ± 0.2) × 10^{-11} μmol of sulfate cell^{-1} min^{-1}. The rates of thiosulfate reduction and of aerobic respiration measured in washed cell suspensions in the respiration chamber were 1.2 × 10^{-11} and 1.9 × 10^{-11} μmol min^{-1} cell^{-1}, respectively (Fig. 1; Table 1). No cell aggregates were observed.

When at the end of the steady-state anaerobic stage the chemostat was bubbled with 1% O_2 in the incoming gas mixture, *D. oxyclinae* was not washed out. During the first 42-h transition phase, stage II, the calculated sulfate reduction rate decreased by 20%, the residual sulfate concentration increased from 0.2 to 4.6 mM, and the sulfide concentration decreased twofold. Cell numbers nevertheless remained stable (Fig. 1, upper panel), and the culture remained macroscopically ho-

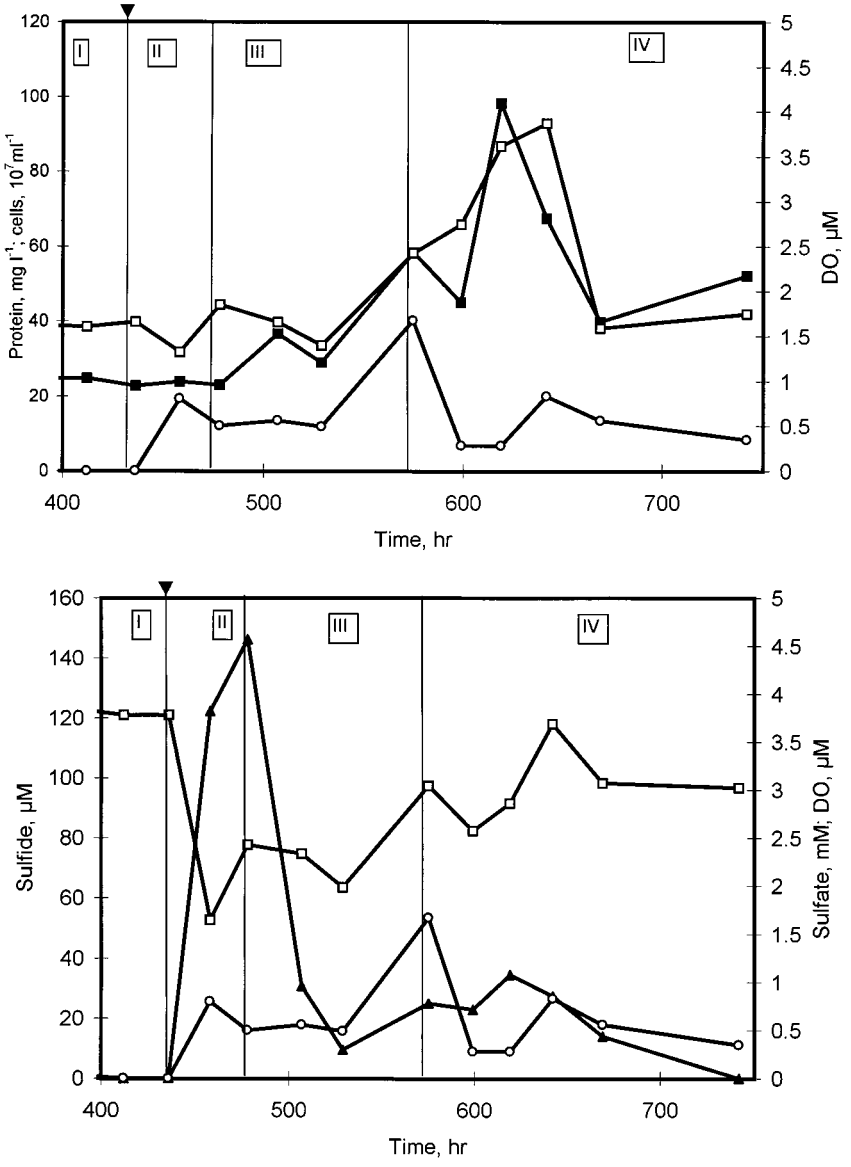

FIG. 1. Growth parameters of *D. oxyclinae* in anaerobic chemostat culture and under 1% oxygen in the incoming gas grown on 20 mM lactate. (Upper) Protein concentration (■), cell numbers (□), dissolved oxygen (DO; ○, solid line); (Lower) concentrations of sulfide (□), sulfate (▲), and dissolved oxygen (○). Arrowheads indicate initiation of oxygen supply.

mogeneous. At this oxygen flux of 45 μmol min^{-1}, most of the oxygen was utilized; only a residual oxygen concentration of 0.7 ± 0.2 μM was detected in the growth vessel. The calculated growth yield increased to 3.4 ± 0.8 g of protein per mol of sulfate consumed. This increase may indicate the use of intermediate sulfur compounds at a considerably lower K_m than that of sulfate (32a).

A marked increase in sulfate reduction indicated the onset of stage III, which continued for the following 97 h. Elevated sulfate reduction may be a result of the utilization of the intermediate sulfur electron acceptors generated during stage II, probably by chemical oxidation of sulfide. Residual sulfate concentration decreased sharply and later stabilized at 0.6 mM (Fig. 1, lower panel). Although this was evidence of sulfate reduction activity restored to a level similar to that found at stage I, residual sulfide concentration did not change. Protein concentration increased steadily from 23.8 to 58.3 mg liter^{-1}, and MPN counts also increased. The yield increased further to 4.4 ± 1.2 g of protein per mol of sulfate consumed. The calculated in situ rate of oxygen consumption in the chemostat increased threefold, to (9.7 ± 1.2) × 10^{-11} μmol of O_2 cell^{-1} min^{-1} compared to stage II, while the rate of sulfate reduction did not change significantly, remaining at (1.7 ± 0.5) × 10^{-11} μmol cell^{-1} min^{-1}. The rates of both oxygen consumption and thiosulfate reduction measured on washed cell suspensions in the respiration chamber increased 1.8-fold compared to stage I, to 3.4 × 10^{-11} and 2.2 × 10^{-11} μmol cell^{-1} min^{-1}, respectively (Table 1). The culture remained macroscopically homogeneous throughout this stage.

The onset of stage IV was indicated by formation of cell aggregates of up to 3 mm, with no marked wall growth. This heterogeneous culture was no longer a true chemostat, and

TABLE 1. Growth parameters of *D. oxyclinae* grown on 20 mM lactate in anoxic chemostat culture and under gassing with 1% oxygen

Stage	Flow (h)	Mean ± SD										
		Dissolved oxygen (μM)	Sulfate consumed (mM)	Residual sulfide (μM)	Protein (mg liter^{-1})	Cells (10^8 ml^{-1})	Protein		Oxygen respiration rate (μmol of O_2 min^{-1} cell^{-1} [10^{-11}])		Reduction rate (μmol min^{-1} cell^{-1} [10^{-11}])		MPN (10^8 ml^{-1})
							Per cell (mg [10^{-11}])	Per mol of SO_4^{2-} consumed (mg mol^{-1})	Calculated	Measured[a]	SO_4^{2-}, calculated (mean ± SD)	$S_2O_3^{2-}$, measured[a]	
I	122–436	0.0	9.8 ± 0.1	129 ± 11	22.2 ± 2.6	4.1 ± 0.3	57.9 ± 5	2.4 ± 0.1		1.9	2.0 ± 0.2	1.2	3.1 (0.8–3.6[b])
II	436–478	0.7 ± 0.2	5.8 ± 0.4	65 ± 13	23.4 ± 0.5	3.8 ± 0.6	61.2 ± 9.9	3.4 ± 0.8	3.3 ± 0.5	ND[c]	1.6 ± 0.4	ND	2.5 (0.8–2.8)
III	478–575	0.5 ± 0.1	9.4 ± 0.3	69 ± 6	32.8 ± 3.9	3.6 ± 0.3	82.7 ± 18.6	4.4 ± 1.2	9.7 ± 1.2	3.4	1.7 ± 0.5	2.2	3.9 (1.3–4.5)
IV	575–742	0.7 ± 0.5	9.3 ± 0.4	97 ± 11	60.1 ± 19.2	6.4 ± 2.1	97.2 ± 20.4	6.5 ± 2.3	6.0 ± 2.3	1.7	1.4 ± 0.5	6.3	13.6 (4.5–15.6)

[a] Measured in cell suspensions in the respiration chamber.
[b] 95% confidence interval.
[c] ND, not determined.

therefore repetitive sampling became variable. As a result, the protein concentration ranged from 29 to 98.2 mg liter^{-1}. This nonhomogeneous culture was maintained for 167 h (Fig. 1). Compared to the previous stage, the sulfate reduction rate did not change significantly and remained at (1.4 ± 0.5) × 10^{-11} μmol cell^{-1} min^{-1}. The average protein concentration was about twice as high as in the anaerobic growth mode, and both cell counts and MPN increased substantially. The calculated apparent growth yield of 6.5 ± 2.3 mg of protein per mol of sulfate consumed was the highest ever recorded for *D. oxyclinae*. The calculated in situ rate of oxygen consumption in the chemostat decreased to (6.0 ± 2.3) × 10^{-11} μmol of O_2 cell^{-1} min^{-1} compared to stage III, while the rate of sulfate reduction did not change significantly, remaining at (1.4 ± 0.5) × 10^{-11} μmol cell^{-1} min^{-1} (Table 1). The rate of oxygen consumption measured on washed cell suspensions in the respiration chamber decreased by 50%, to 1.7 × 10^{-11} μmol O_2 cell^{-1} min^{-1}, while the rate of thiosulfate reduction increased drastically (2.9-fold) compared to stage III, to 6.3 × 10^{-11} μmol cell^{-1} min^{-1} (Table 1). The concentration of residual dissolved oxygen throughout this phase of the experiment fluctuated in the range of 0.1 to 1.7 μM.

The high rate of gas flow applied in this experiment made quantitative estimations of sulfide production impossible. However, the ratio of residual dissolved sulfide per millimole of sulfate reduced may be used to estimate the portion of sulfide not removed by gassing. In the anaerobic coculture of *D. oxyclinae* with *Marinobacter* sp. strain MB this ratio was 43.5 μmol of sulfide per mmol of sulfate reduced (29). As the result of increased gassing rate in axenic culture of *D. oxyclinae* under anaerobic continuous (stage I) this ratio was much lower, 13.6 ± 2.1 μmol of sulfide per mmol of sulfate reduced. During stage II the ratio decreased by 14%, probably due mostly to chemical oxidation of sulfide by oxygen. During stage III it decreased further, to 7.4 ± 1.2 μmol of sulfide per mmol of sulfate reduced. Since the rate of oxygen supply did not change, this decrease can be attributed to biological sulfide oxidation. The subsequent increase of this ratio to 10.6 ± 1.5 μmol of sulfide per mmol of sulfate reduced during stage IV is probably the result of intensified sulfate reduction inside bacterial aggregates.

In this range of oxygen concentrations, the rate of aerobic respiration previously reported for *D. oxyclinae* was 260 nmol of O_2 min^{-1} mg of protein^{-1} (17). In the present experiment, the potential respiration rates measured in the respiration chamber using washed cell suspensions during the anoxic stage were somewhat higher (1.9 × 10^{-11} μmol of O_2 min^{-1} cell^{-1}, which corresponds to 360 nmol of O_2 min^{-1} mg of protein^{-1}), and those obtained during stage III were considerably higher, 3.4 × 10^{-11} μmol of O_2 min^{-1} cell^{-1}, corresponding to 620 to 700 nmol of O_2 min^{-1} mg of protein^{-1} (Table 1). The rate of oxygen consumption by aerobic respiration at stage III was therefore calculated to be (9.7 ± 1.2) × 10^{-11} μmol of O_2 min^{-1} cell^{-1}. During stage IV the measured respiration rate was lower, 1.7 × 10^{-11} μmol of O_2 min^{-1} cell^{-1}, and the calculated rate of oxygen consumption was (6.0 ± 2.3) × 10^{-11} μmol of O_2 min^{-1} cell^{-1}. In the transition between stages III and IV, the measured respiration rate decreased 2-fold whereas the calculated respiration rate decreased only 1.6-fold. This discrepancy may be caused by flocculation. During stage IV, only a fraction of the cells in the growth vessel were in contact with oxygen, resulting in lower calculated respiration rates per cell (Table 1). Both values were well below the oxygen flux of 45 μmol of O_2 min^{-1} applied in this experiment.

The measured values of respiration rates were always less than the calculated ones. This may be partly the result of the

shock received by the cells during washing and resuspension. On the other hand, some portion of oxygen in the growth vessel was bound chemically due to sulfide oxidation.

The yield of 2.4 g of protein per mol of sulfate obtained in the anaerobic continuous culture of *D. oxyclinae* was relatively low. Higher yields of 3.4 g of protein per mol of sulfate consumed were previously reported for this organism in an anaerobic continuous culture (17). The same yield was observed in this study during the batch culture stage. Much higher yields of 9.9 ± 1.6 g (dry weight) per mol of lactate (28) and 5.3 g of protein per mol of lactate (33) were reported for *D. desulfuricans* grown in batch culture on lactate and sulfate. These values correspond to approximately 10 g of protein per mol of sulfate. The yields reported for other *Desulfovibrio* species grown on lactate and sulfate vary from 3.7 to 7.8 g of protein per mol of sulfate (calculated using data from reference 14).

During stages II and III, the apparent yield of protein per sulfate consumed was greater than in the anaerobic mode of growth. Higher protein concentrations in the presence of oxygen implied lower calculated sulfate reduction rates per milligram of protein. Indeed, the calculated rates of sulfate reduction decreased from $(2.0 \pm 0.2) \times 10^{-11}$ μmol cell^{-1} min^{-1} under anaerobic conditions (stage I) to $(1.7 \pm 0.5) \times 10^{-11}$ μmol cell^{-1} min^{-1} at stage III. In contrast, the rate of thiosulfate reduction as measured in washed cell suspensions in the four-electrode chamber increased during the same period from 1.2×10^{-11} μmol cell^{-1} min^{-1} at stage I to 2.2×10^{-11} μmol cell^{-1} min^{-1} at stage III.

D. oxyclinae has a low affinity for sulfate. Sulfate reduction occurred only when sulfate concentrations were over 30 μM, and the rate of sulfate reduction at this low sulfate concentration was 20-fold less than at 30 mM (17). This does not hamper growth of *D. oxyclinae* in its natural, sulfate-rich environment where sulfate levels of 90 to 120 mM prevail (16). Thiosulfate, on the other hand, was reduced at high rates even at 3 μM. Moreover, unlike sulfate reduction, thiosulfate reduction was found to proceed even in the presence of oxygen, provided the oxygen concentration was below 2 μM. It was previously suggested that thiosulfate was an important natural substrate for *D. oxyclinae* in environments where both sulfate reduction and sulfide oxidation occur (17). It is postulated that in the presence of oxygen, the initial steps of sulfate reduction, namely, formation of adenosine sulfate (APS) and reduction to bisulfite, are impaired to a greater degree than the consequent steps (30).

Although *D. oxyclinae* was demonstrated to oxidize sulfide to sulfate (17), we may speculate that under the experimental conditions when sulfide was in excess, some of sulfide was oxidized incompletely only to thiosulfate or to other intermediate sulfur compounds. This process was expected to result in the formation of free thiosulfate and possibly other sulfur intermediates in the beginning of the transition period upon the initial introduction of oxygen to the anaerobic steady state (stage II). Thiosulfate was indeed found in the growth vessel during stage II at concentrations of about 0.1 mM and was not detected in the growth vessel at later stages. During stages III and IV, thiosulfate was below the detection limit, probably as a result of its rapid recycling.

While the growth rate remained constant, protein content per cell increased. Protein content per cell at stage III was 1.4-fold higher than during the anoxic stage. The enhanced yield can be due to the ability of *D. oxyclinae* to oxidize sulfide aerobically. This process is most efficient when oxygen concentrations are below 30 μM (17). The combination of incomplete oxidation of sulfide to thiosulfate and subsequent thiosulfate reduction may well contribute to the observed increase in yields.

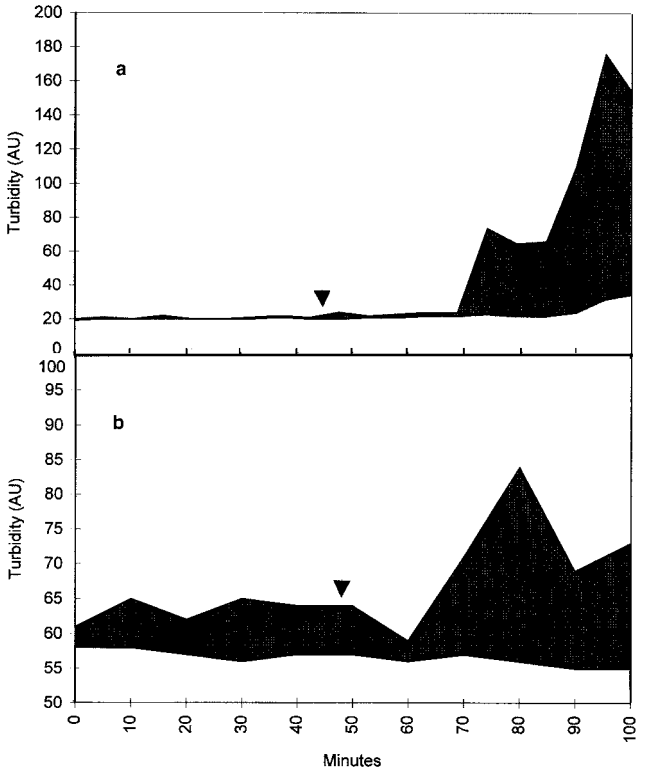

FIG. 2. Initial cell aggregation in a response to exposure to oxygen, based on changes in turbidity in cell suspension of *D. oxyclinae* after addition of oxygen to an anaerobically grown culture. Introduction of oxygen is marked by arrowheads. (a) Response to a single pulse of 87.3 μM oxygen; (b) response to continuous input of 5% oxygen. AU, arbitrary units.

Cell aggregation. Formation of cell aggregates by *D. oxyclinae* under oxygen supply, as well as profuse wall growth was reported previously (17). In the course of this experiment, wall growth was less pronounced and macroscopic cell aggregates of *D. oxyclinae* were observed only after 140 h of exposure to oxygen. Smaller microscopic aggregates were formed earlier, although these micrometer-sized aggregates were easily disrupted during sampling and dilution, as evidenced by the good correlation between cell counts and MPN values.

To determine the initial stage of flocculation, the effect of oxygen on turbidity of the cell suspension was studied. Under anaerobic conditions the culture was homogeneously suspended as single cells, as evidenced by the stable turbidimetric readings. When a single pulse of 87.3 μM oxygen was introduced, initial flocculation was observed after 45 min, as evidenced by increased turbidity fluctuations (Fig. 2a). Continuous input of 5% oxygen resulted in more rapid aggregation of *D. oxyclinae*, but at a smaller range of turbidity fluctuations (Fig. 2b).

In an attempt to estimate the size of the initial flakes, shaken batch cultures of *D. oxyclinae* were incubated in sealed bottles for 24 h with 2% O_2 in the gas phase and compared to an anaerobic control. Protein content of the culture and of the fraction filtered through 8-μm-pore-size Nuclepore filters was determined. During the first 10 h of incubation, the fractions of cells in aggregates larger than 8 μm in the presence of oxygen and in the anoxic control were the same. However, after 24 h

of incubation with 2% oxygen, there were 19% more aggregates than in the anoxic control (data not shown).

It was previously demonstrated that oxygen was a preferred electron acceptor for anaerobically grown cells of *D. oxyclinae*. In the respiration chamber, sulfate reduction stopped immediately after the introduction of oxygen and did not resume until all oxygen was reduced (17). In the chemostat culture, however, *D. oxyclinae* continued to reduce sulfate in the presence of dissolved oxygen. One possible explanation to this apparent discrepancy is the need of new enzymes for sulfate reduction in the presence of oxygen. Only when these enzymes are induced in the chemostat can "aerobic" sulfate reduction be demonstrated. In the case of flocculated culture, however, only the outer layers of cell aggregates are exposed to oxygen. Depth of oxygen diffusion through the interface when the rate of oxygen consumption is constant is described by the equation $X = (2C_0D/A)^{1/2}$, where A is the rate of oxygen consumption per unit volume per unit time, C_0 is the concentration of oxygen at the biofilm surface and D is the diffusion coefficient of oxygen (4). Considering the high stirring rate, we can substitute for C_0 the ambient oxygen concentration in solution. Assuming $D = 3 \times 10^{-5}$ cm^2 s^{-1} (A. Wieland, D. van Dusschoten, L. R. Damgaard, D. de Beer, M. Kühl, and H. Van As, unpublished data), $C_0 = 1$ μM, a respiration rate of 260 nmol mg^{-1} min^{-1} (17), and cell density in the aggregates of 1 μm^{-3}, it is possible to calculate that oxygen penetrated only to the depth of 1.5 μm into the aggregates. Hence, in aggregates of 3 μm and larger, the internal cells could perform sulfate reduction in an anaerobic microenvironment.

Aggregation of SRB in a cyanobacterial mat. Flocculation may be one of the mechanisms enabling some SRB to survive under periodic exposure to oxic conditions in their natural habitats. In situ hybridization revealed numerous clusters of SRB in the oxic zone of the Solar Lake cyanobacterial mats. A representative sample taken from 500 μm below the surface, where oxygen concentrations of up to 1.5 mM were detected by oxygen microelectrode at noontime, showed both clustered and individual SRB cells among the filaments of the cyanobacterium *Microcoleus chthonoplastes* (Fig. 3). Similar results for aggregates of SRB were obtained when fluorescein-labeled probe 687, targeted to desulfovibrios, was used (21). Diurnal measurements of sulfate reduction activity in a Solar Lake cyanobacterial mat showed the highest activity at the O_2-H_2S chemocline at noontime (11). These measurements underestimated sulfate reduction in the presence of oxygen because of rapid sulfide reoxidation. Two-dimensional mapping of sulfate reduction by the $Na_2{}^{35}SO_4$-coated silver foil technique allows the detection of sulfate reduction even in the presence of high oxygen concentrations because of the very low solubility of $Ag_2{}^{35}S$ (Y. Helman, unpublished data). Figure 4 shows an example of phosphor imaging of the two-dimensional pattern of sulfate reduction obtained by the silver foil technique correlated to profiles of oxygen, oxygenic photosynthesis rate, sulfide, and pH obtained by specific microelectrodes. Because of changes in specific activity of the radiolabeled sulfate during the experiment, these results cannot be translated to quantitative rates of sulfate reduction; nevertheless, they clearly show the heterogeneity of sulfate reduction activity under oxic conditions. This qualitative mapping shows that active sulfate reduction activity is detected well above the chemocline. Most of this activity is associated with several heterogeneously distributed distinct patches. The combined observations of SRB clumps by in situ hybridization and the heterogeneity of sulfate reduction mapping in the oxic zone of a cyanobacterial mat indicate the existence of anoxic microniches with active SRB in this environment.

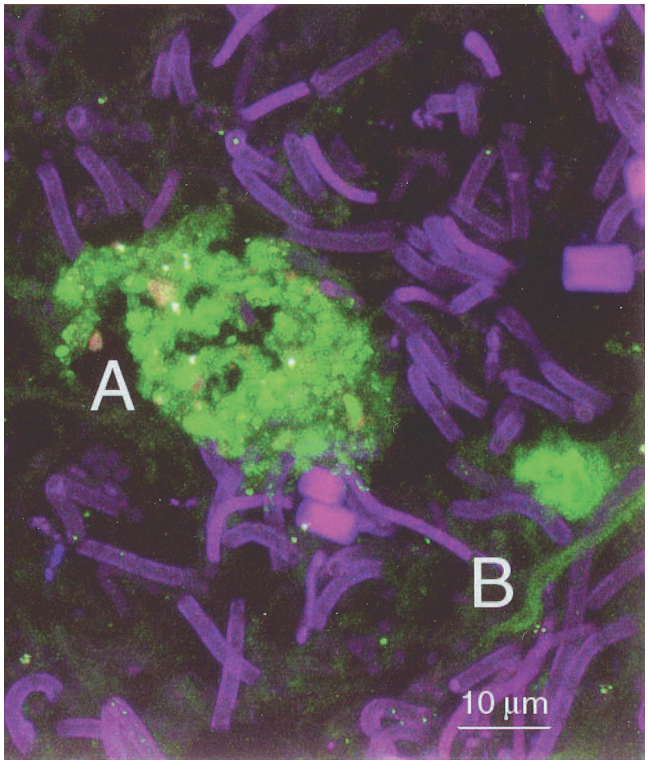

FIG. 3. Confocal laser scanning in situ hybridization with fluorescein-labeled probe SRB385 (green) and autofluorescence of cyanobacteria (purple) in a horizontal transect of a Solar Lake cyanobacterial mat at a depth of 500 μm. (A) Clump of unicellular SRB; (B) filamentous *Desulfonema*-like SRB. Note additional scattered unicellular SRB.

Concluding remarks. Oxygen flux of 45 μmol min^{-1} and oxygen uptake of 9.4 ± 0.5 μmol min^{-1} resulted in low concentrations of dissolved oxygen up to 10 μM, as was the case in the chemostat experiment (Table 1). Under these conditions, anoxia can develop in the center of a 10-μm aggregate of *D. oxyclinae*. In aerated environments, anoxia was reported to develop inside the millimeter-sized marine fecal pellets (1), in activated sludge (27), and in nitrifying bacterial aggregates 100 μm below the surface (10), i.e., in relatively large aggregates containing bacteria with high respiration rates at high concentrations of oxygen.

The two SRB aggregates shown in Fig. 3 are small, 10 and 30 to 40 μm. SRB aggregates of similar size were found in the upper oxic layers of wastewater biofilms under aeration (24). Even assuming high respiration rates, these aggregates are too small to become anoxic under 1.5 mM dissolved oxygen as is the case in cyanobacterial mats. Moreover, *D. oxyclinae* has a high respiration rate of 260 nmol mg^{-1} min^{-1} only when oxygen concentrations are not higher than 30 μM (17). Anoxic microniches therefore could not be formed by *D. oxyclinae* alone.

In its natural environment, *D. oxyclinae* occurs in close association with oxygen-scavenging aerobes. Such consortia were repeatedly isolated from Solar Lake mats (29, 31). One of these organisms, *Marinobacter* sp. strain MB, is an aerobe growing under high levels of aeration. A chemostat coculture of *D. oxyclinae* and *Marinobacter* sp. strain MB was growing and reducing sulfate under various levels of oxygen input up to gassing with air. Unlike the axenic culture of *D. oxyclinae*, macroscopic cell aggregates were not observed in this coculture (29). It can therefore be suggested that SRB in oxic en-

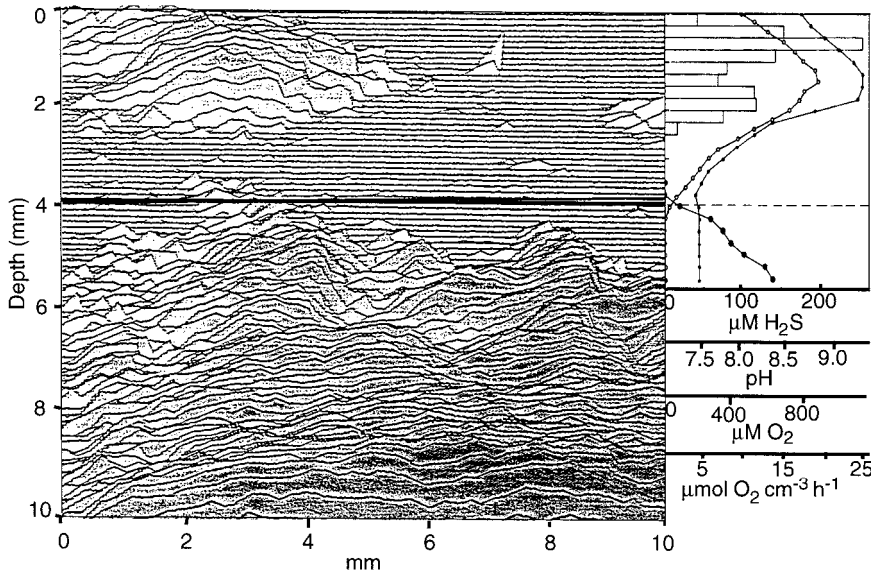

FIG. 4. Phosphor imaging of a two-dimensional pattern of sulfate reduction obtained in a Solar Lake cyanobacterial mat by the silver foil technique, correlated to profiles of oxygen (○), sulfide (●), pH (•), and oxygenic photosynthesis rate (bars) as obtained by specific microelectrodes. The horizontal line indicates area of O_2 and H_2S overlap.

vironments occur mostly in the form of relatively small aggregates surrounded with a layer of oxygen-scavenging aerobes. The actual interaction between *D. oxyclinae* and *Marinobacter* sp. strain MB in mat environments is presently being investigated.

Cell aggregates of tens of micrometers cannot be detected by oxygen microelectrodes as anoxic microniches. Likewise, such environments were not found in a study of two-dimensional O_2 distribution using planar optodes with spatial resolution of 130 μm, although spatial heterogeneity of oxygen concentrations was reported, yet patches of high oxygen net consumption were found to be surrounded by zones of oxygen net production (13). These can be interpreted as clusters of oxygen-scavenging bacteria surrounded by cyanobacterial matrix similar to those shown in Fig. 3. The size of anoxic zones in the upper layer of a cyanobacterial mat must therefore be less than 130 μm.

This is the first report of growth of an SRB under exposure to oxygen. Both sulfate reduction and oxygen respiration occurred in the homogeneous axenic culture, followed by later clumping of the cells. Clumping may be a result of the lack of mechanisms to deal with oxygen radicals in *D. oxyclinae*. Active sulfate reduction by clumps of SRB even in the presence of very high concentrations of dissolved oxygen was demonstrated within the oxic zone of a cyanobacterial mat community.

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