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The anaerobe *Desulfovibrio desulfuricans* ATCC 27774 grows at nearly atmospheric oxygen levels

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Abstract **Sulfate reducing bacteria of the *Desulfovibrio* genus are considered anaerobes, in spite of the fact that they are frequently isolated close to oxic habitats. However, until now, growth in the presence of high concentrations of oxygen was not reported for members of this genus. This work shows for the first time that the sulfate reducing bacterium *Desulfovibrio desulfuricans* ATCC 27774 is able to grow in the presence of nearly atmospheric oxygen levels. In addition, the activity and expression profile of several key enzymes was analyzed under different oxygen concentrations.**
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1. Introduction

The bacterium *Desulfovibrio desulfuricans* ATCC 27774 belongs to the group of the sulfate reducing bacteria that derive their energy from anaerobic respiration. Although named after a single electron acceptor, *Desulfovibrio* species are able to use a wide variety of inorganic compounds, as electron acceptors [1]. In particular, *D. desulfuricans* ATCC 27774 can also use nitrate as the terminal electron acceptor for anaerobic growth, performing a dissimilatory nitrate reduction that yields ammonia [2]. It is well documented that sulfate reducing bacteria are usually found close to oxic habitats and several studies demonstrated that *Desulfovibrio* species survive prolonged exposure to oxygen, by the action of enzymes that enable them to cope with oxygen [3–5]. Analysis of the *D. vulgaris* Hildenborough and *D. desulfuricans* G20 genome sequences shows that they contain genes for membrane bound oxygen reductases of the haem–copper and cytochrome *bd* types. Two oxygen-reducing enzymes have already been isolated from *D. gigas*: the membrane-bound terminal oxygen reductase of the cytochrome *bd* family [6] and a soluble oxygen reductase (ROO) that catalyses the transfer of electrons from rubredoxin to oxygen [7]. Hence, *Desulfovibrio* species seem to have the enzymes required to grow under oxygen respiring conditions. Therefore, we undertook the study of the growth behaviour of a sulfate reducing bacterium in the presence of oxygen, with a *Desulfovibrio* species capable of growth in nitrate, *D. desulfuricans* ATCC 27774, thereby eliminating the possible chemical reactions of reduced sulphur compounds with oxygen which could, at least partially, mask the observations.

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2. Materials and methods

2.1. Cell growth and cell fraction preparation

D. desulfuricans ATCC 27774 was grown anaerobically in a 3 L fermentor (Applikon, Biocontroler 4DI 1030), equipped with pH and pO_2 controllers, in lactate/nitrate medium [8] at 37 °C and under nitrogen (150 ml min^{-1}). Cell exposure to different percentages of oxygen (v/v) was achieved by replacing the nitrogen flux with the required amount of air by means of an Applikon gas mixer. The calibration of the O_2 electrode was performed at the same conditions used for growth. Cells were harvested from stationary phase by centrifugation ($10000 \times g$, 20 min), resuspended in 10 mM MOPS buffer, pH 7.6, disrupted in a French Press and centrifuged for 30 min at $12000 \times g$ to remove cell debris. The supernatant was centrifuged for 2.5 h at $160000 \times g$, allowing the separation of the membrane and soluble fractions. The protein content of the fractions was determined using the BCA Protein Assay Kit (Pierce).

2.2. Haem analysis and enzymatic activities

UV–Visible absorption spectra of the membrane and soluble fractions prepared from cells of *D. desulfuricans* ATCC 27774 grown either anaerobically or in the presence of 18% of oxygen were recorded in a Shimadzu UV-1603 spectrophotometer, at room temperature. Haem extraction was performed as described in [9] and analyzed in a Beckman HPLC system Module 167 with a Waters Nova-Pack C18 column. Type aa_3 cytochrome *c*: oxygen reductase from *Paracoccus denitrificans* and bovine myoglobin (Sigma) were used as standards for haems A and B, respectively.

Nitrite and nitrate reductase activities were performed anaerobically according to [8] and [10], respectively, using 1 mM benzyl viologen as electron donor. Peroxidase activity was measured spectrophotometrically in 10 mM Tris/HCl buffer, pH 7.4, using 31 μg of the soluble fraction, 1.2 mM 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 33.5 mM H_2O_2, with an $\varepsilon_{DAB,460} = 1.68 \times 10^3\ M^{-1}\ cm^{-1}$, which was experimentally determined in this work. Spectrophometric assay of SOD activity was done according to [11]. Catalase activity was determined polarographically in a Clark-type oxygen electrode, YSI Model 5300, Yellow Springs, in 50 mM potassium phosphate buffer, pH 7, using 31 μg of soluble fraction and 335 mM H_2O_2 [12]. The oxidation of 5 mM of ascorbate mediated by 1 mM of tetra-methyl-*p*-phenylenediamine (TMPD) and its inhibition by 20 μM of KCN, was measured in 10 mM MOPS buffer, pH 7.6 at 25 °C in the oxygen electrode. NADH:ferricyanide oxidoreductase and succinate:DCPIP oxidoreductase activities were followed spectrophotometrically, in 10 mM MOPS buffer, pH 7.6 at 30 °C. The reduction of 1 mM of $K_3[Fe(CN)_6]$ by 1 mM of NADH was monitored at 420 nm. For succinate:2,6-dichlorophenolindophenol (DCPIP) oxidoreductase activity, PMS (phenazine methosulfate)-coupled reaction of DCPIP was followed at 578 nm, by mixing 0.1 mM PMS, 0.150 mM DCPIP and 20 mM of freshly prepared succinate. Hydrogenase [13] and superoxide dismutase (SOD)

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[14] activities were assessed on native polyacrylamide gel electrophoresis (PAGE), using 100 μg and 200 μg of soluble fraction for hydrogenase and SOD detection, respectively.

3. Results

3.1. Growth of D. desulfuricans ATCC 27774 in the presence of oxygen

In order to observe the ability of *D. desulfuricans* ATCC 27774 to grow in the presence of oxygen, the cells were cultured in lactate/nitrate medium under nitrogen, in a fermentor equipped with an oxygen electrode. It must be stressed that the medium did not include sulfate or any other sulphur containing compounds, thus eliminating chemical reactions of these compounds with oxygen. At $OD_{600} \sim 0.7$, cells were submitted for 7 h to different percentages of oxygen, namely 5%, 15%, 18% and 21%, by replacing part of the nitrogen flux with the required amount of air. It was observed that cells of *D. desulfuricans* ATCC 27774 could grow at percentages of oxygen as high as 18%, showing the growth curves behaviours similar to the control curve obtained under strictly anaerobic conditions (Fig. 1A). Upon supply of 21% O_2, cells stopped growing but did not exhibit cell death. Cells growing under oxygen atmosphere were effectively consuming oxygen, with a rate that decreased once the stationary phase was reached (Fig. 1B). For all oxygen conditions tested, cells harvested from the stationary phase grew when re-inoculated in fresh media and cultured under strict anoxic conditions. This result shows that the bacterium did not lose the capacity for anaerobic growth, contrary to what was observed for other *Desulfovibrio* strains [15].

Microscope analyses revealed no contaminants in unstained and safranine or methylene blue stained preparations of *D. desulfuricans* ATCC 27774 cells exposed to air. In addition, all *D. desulfuricans* ATCC 27774 cells exposed to air revealed motility and cell division behaviour similar to cells cultured under anoxia. The cells presented the typical morphology of *Desulfovibrio* characterized by quite small cells with straight form, which is quite distinct from most aerobes. Furthermore, the oxygen exposed *D. desulfuricans* ATCC 27774 cells did not develop an atypically elongated form neither tended to form aggregates, as observed for other *Desulfovibrio* strains when submitted to oxygen [15].

3.2. Analysis of oxygen detoxifying enzymes of D. desulfuricans ATCC 27774

Since *D. desulfuricans* ATCC 27774 performs nitrate respiration, the influence of oxygen on the activity of the nitrate and nitrite respiring enzymes was analyzed. The results showed that the presence of oxygen did not alter significantly the nitrate and nitrite reductase activities of *D. desulfuricans* ATCC 27774 (Table 1).

To determine whether the haem content changed with the degree of oxygen exposure, UV–Visible absorption spectra of the oxidized and dithionite reduced membrane and soluble fractions prepared from *D. desulfuricans* ATCC 27774 cells grown with zero and 18% oxygen were recorded. The spectra of the membranes displayed an absorption in the Soret region (422 nm) and peaks at 522 and 553 nm, indicating the presence of haem C. Furthermore, only a slight change in the haem composition, reflected in the shift of the Soret band, was observed upon increase of oxygen concentration in both membrane and soluble fractions (data not shown). The HPLC haem analysis of the membrane fractions of the anaerobic and 18% oxygen grown cells revealed the presence of non-covalently bound haem B but not haem A, thus suggesting that the amount of haem A is below detection or, as previously proposed for *D. gigas* [6], these organisms may contain a different type of haem A.

Three hydrogenase-activity stained bands, corresponding to the [Fe]-, [NiFe]- and [NiFeSe]-hydrogenases [16], were detected in the soluble fractions of *D. desulfuricans* ATCC 27774 cells grown either under anaerobic conditions or in presence of 18% O_2 and their intensity did not vary upon exposure to oxygen (Fig. 2A). These results are in agreement with data obtained in *D. vulgaris*, which also showed that the expression of [NiFe]-hydrogenase and [NiFeSe]-hydrogenase is not upregulated by oxygen [17]. However, the increase in expression of the [Fe]-hydrogenase that occurs in *D. vulgaris* upon oxidative stress [17] could not be observed in *D. desulfuricans* ATCC 27774 cells upon exposure to oxygen.

The presence of a wide range of enzymes involved in cell protection against oxidative stress in *Desulfovibrio* species is well documented [6,7,14,15]. In this study, catalase and SOD activities were enhanced by oxygen exposure while the peroxidase activity was not affected (Table 1). The SOD gel activity revealed the presence of three distinct bands and their intensities with or without oxygen were similar (Fig. 2B). At present, the origin of the top band is unknown. The middle band is assigned to the putative Cu,Zn-SOD of *D. desulfuricans* ATCC 27774, which was inhibited upon addition of cyanide, and the lower band corresponds to desulfoferrodoxin (Dfx), a superoxide reductase, based on gel migration of the purified enzyme [14]. These results are in accordance with those obtained for *D. gigas* and *D. vulgaris* that also show no significant change in SOD activity when the strains are exposed to oxygen [18]. The expression of ROO, an enzyme proposed to be involved in O_2 detoxification [7,19], was assayed by immunoblotting detection, and did not change upon exposure to 18% oxygen (data not shown). Similarly, no major variations were observed in the protein level of *D. desulfuricans* ATCC 27774 bacterioferritin and its putative partner, rubredoxin II (data not shown), in spite of the fact that iron-storage enzymes of the ferritin family have been proposed to play a role in oxidative stress response.

The activities of membrane bound respiratory enzymes such as NADH:ferricyanide oxidoreductase and succinate:DCPIP oxidoreductase were also determined. The exposure of cells to oxygen did not increase the activity of these enzymes and oxygen consumption by membrane extracts, assessed through the reduction of oxygen with ascorbate-reduced TMPD, did not vary significantly under oxygenated conditions (Table 1). The available genomes of several *Desulfovibrio* species reveal the presence of genes coding for haem–copper or *bd* type oxygen reductases. Although their presence in *D. desulfuricans* ATCC 27774 has not been yet demonstrated, it is probable that the genome encodes similar enzymes, thus explaining the existence of the TMPD:oxygen oxidoreductase activity in the membrane extracts. The fact that only ~50% of this activity was inhibited with KCN, a specific inhibitor of the haem–copper containing oxidases, suggests the coexistence in *D. desulfuricans* ATCC 27774 of haem–copper enzymes with other oxygen reductases, namely the *bd*-type enzymes, which are KCN insensitive.

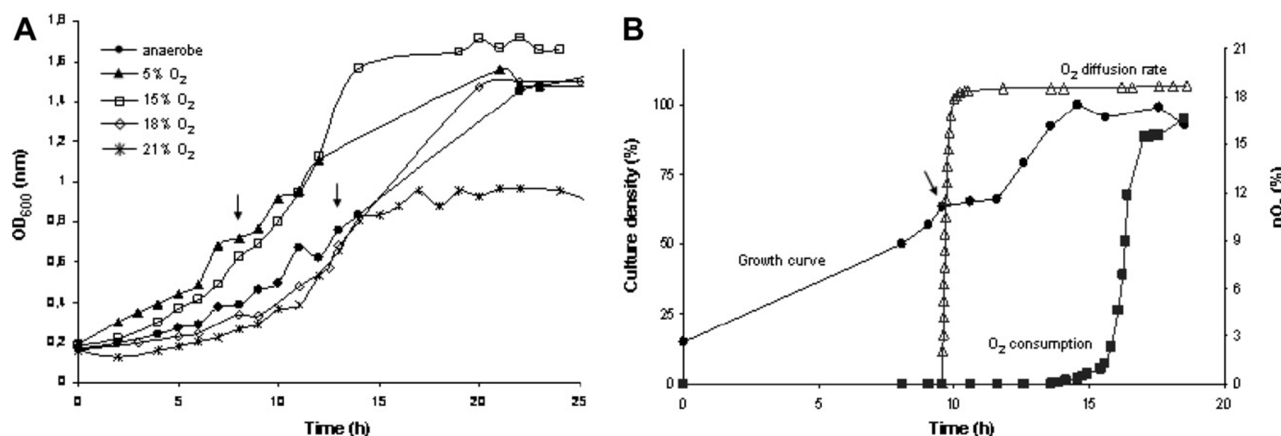

Fig. 1. *Desulfovibrio desulfuricans* ATCC 27774 grows in aerated cultures exhibiting oxygen consumption. *Panel A*: growth curves of *D. desulfuricans* ATCC 27774 obtained under anaerobiosis and with different concentrations of oxygen. At $OD_{600} \sim 0.7$ (black arrow), cells were exposed to an atmosphere containing 5%, 15%, 18% and 21% O_2, for approximately 7 h. *Panel B*: oxygen consumption of *D. desulfuricans* ATCC 27774 cells grown in the presence of 18% oxygen. Culture density values were normalized considering the maximum absorbance measured at the stationary phase.

Table 1
Comparison of several enzymatic activities measured in *D. desulfuricans* cells grown anaerobically (0%) or in the presence of O_2 (18%)

Activity	O_2 (%)	
	0	18
Nitrate reductase[a] (nmol min^{-1} mg^{-1})	8	5
Nitrite reductase[b] (μmol min^{-1} mg^{-1})	46	49
SOD[a,c] (U mg^{-1})	49	73
Catalase[a] (μmol min^{-1} mg^{-1})	78	142
Peroxidase[a](μmol min^{-1} mg^{-1})	3	3
NADH:Ferricyanide oxidoreductase[b] (μmol min^{-1} mg^{-1})	8	7
Succinate:DCPIP oxidoreductase[b] (nmol min^{-1} mg^{-1})	59	61
TMPD: oxygen oxidoreductase[b] (nmol min^{-1} mg^{-1})	7	9
Inhibition of O_2 consumption (%)	40	55

Values are the average of three experiments.
[a]Soluble fractions.
[b]Membrane fractions.
[c]For SOD, one unit of activity (U) is defined as the amount of enzyme required for inhibition of 50% of cytochrome *c* reduction.

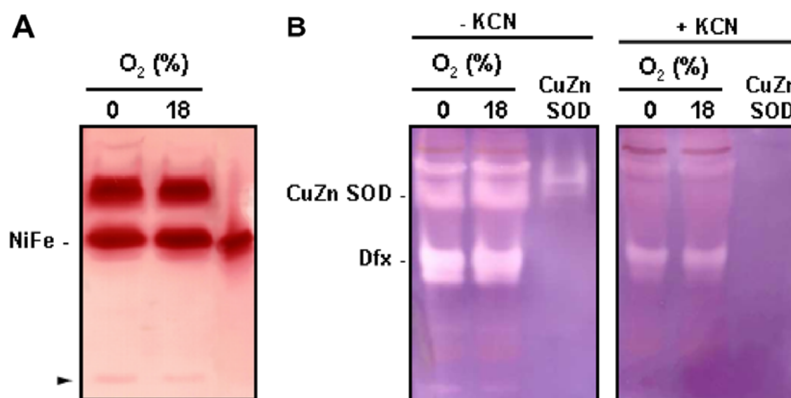

Fig. 2. Native PAGE of the soluble fractions of *Desulfovibrio desulfuricans* ATCC 27774 grown anaerobically and under 18% O_2, showing hydrogenase (A) and SOD (B) staining activities. The *D. desulfuricans* ATCC 27774 NiFe-hydrogenase and bovine CuZn-SOD (Sigma) were used as positive controls.

4. Discussion

In this work, the oxygen reducing systems that enable *D. desulfuricans* ATCC 27774 to cope with oxygen, were characterized. Concerning SOD, the change in the activity of the enzyme followed a pattern similar to that observed for *Desulfovibrio* strains also exposed to oxygen [18]. In contrast with other *D. desulfuricans* strains, namely *D. desulfuricans* (strain

Essex 6) and *D. desulfuricans* B-1388 [15] which are catalase negative, *D. desulfuricans* ATCC 27774 exhibits a positive catalase activity that increases upon exposure to oxygen. We also verified that in *D. desulfuricans* ATCC 27774 the expression of [Fe]-hydrogenase, desulfoferrodoxin, ROO, and bacterioferritin was not elevated by the increase of the oxygen concentration. Furthermore, the increase in the content of the periplasmic cytochromes *c* upon oxygen exposure detected in *D. vulgaris* [17] was not observed in *D. desulfuricans* ATCC 27774. Since the levels of oxygen utilized in this work were much higher than those used in other studies, it is possible that once a certain oxygen concentration is reached, the role of the protection systems changes accounting for the differences observed. Concerning the lack of increase in the activity of oxygen reductases of *D. desulfuricans* ATCC 27774 upon exposure to oxygen, it may result from a differential expression of these enzymes in response to oxygen levels (*e.g.*, *bd* or cbb_3 enzymes are preferentially expressed under quasi anaerobic conditions, while the other types of haem–copper oxygen reductases have a higher expression under higher oxygen concentrations [20]). Studies on *D. gigas* also show that the gene expression level of cytochrome *bd* is not significantly altered upon exposure to oxygen [21].

It has been shown that several sulfate reducing bacteria are able to survive exposure to oxygen, although within limited oxygen concentrations [4]. For example, *D. desulfuricans* NCIB8301 can only grow at low oxygen pressures, with a drastically decrease of the cell yield at oxygen partial pressures greater than 0.4% O_2 [22]. For oxygen concentrations between 0.5% and 2% O_2, weak growth is only observed for two strains of *D. desulfuricans* (Essex and CSN) and for *Desulfobacterium autotrophicum* DSM, which nevertheless display a strong decrease in cell viability and motility for oxygen concentrations higher than 2% [23]. A coculture of *D. oxyclinae* with the aerobe *Marinobacter sp*. consumed oxygen efficiently up to a supply of 5% O_2. However, the portion of *D. oxyclinae* in the coculture decreased significantly upon aeration [24]. Hence, although oxygen tolerance of various sulfate reducing bacteria is well established, the present work shows that *D. desulfuricans* ATCC 27774 exhibits the highest tolerance of all species so far examined, and constitutes the first example of a sulfate reducing bacterium with the ability to grow in a pure culture under nearly atmospheric oxygen levels.

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