Antioxidative defense systems of anaerobic sulfate-reducing microorganisms

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Sulfate-reducing bacteria (SRB) are traditionally considered as strict anaerobic microorganisms. But there are a lot of data up to date about survival and metabolic activity of SRB in biotopes periodically exposed to oxygen. The main factor of O2 toxicity is the action of products of oxygen incomplete reduction, so called the reactive oxygen species (superoxide anion radical, hydrogen peroxide and hydroxyl radical). The main targets of them are cell macromolecules. Also molecular oxygen is able to directly inactivate key enzymes of sulfate reduction metabolism. The SRB have thus developed complicated and tightly regulated systems of physiological and enzymatic mechanisms of oxidative stress response which provide their relative aerotolerance. They can form aggregates in the presence of O2, establish symbiotic relationships with aerobic microorganisms and possess aerotaxis. In addition to classical enzymes (superoxide dismutase, catalase, peroxidases) which are usual for aerobes, the cells of many SRB possess also unique alternative enzymes with superoxide reductase (desulfoferrodoxin, neelaredoxin) and peroxidase (rubrerythrin, nigerythrin) activities.

Keywords: sulfate-reducing bacteria; oxidative stress; aerotolerance; reactive oxygen species; superoxide dismutase; catalase; desulfoferrodoxin; neelaredoxin; rubrerythrin; nigerythrin.

1. Introduction

Sulfate-reducing bacteria (SRB) are traditionally considered as strict anaerobic microorganisms. But there are a lot of data up to date about survival and metabolic activity of SRB in such zones of biotopes where oxygen exposure temporarily exists – surface sea waters, cyanobacterial mats, activated sludge, water sediments, intestines of termites, etc. SRB represent a group of prokaryotes, morphologically and metabolically highly diverse, which gain energy by coupling the oxidation of great variety of low-molecular mass organic compounds or molecular hydrogen to reduction of sulfate (SO4\(^{2-}\)) in sulfide (H2S, HS\(^{-}\)) [1]. One of the most abundant biotopes in which sulfate reduction coexists withoxic conditions is present in cyanobacterial mats, where photosynthetic oxygen synthesis, sulfate reduction and microbial sulfide oxidation overlap and create steep opposed gradients of oxygen and sulfide fluctuating in the diurnal cycle [2, 3]. It should be noticed that the sulfate reduction rates in mats under oxic conditions during the daytime often exceed those observed at night under anaerobic conditions [4].

In the case of anaerobic organisms, the toxicity of oxygen is a combination of at least three factors. The main factor is the action of products of oxygen incomplete reduction so called the reactive oxygen species (ROS) – superoxide anion radical (O2•\(^{-}\)), hydrogen peroxide (H2O2) and hydroxyl radical (HO•). The main targets of ROS are cell macromolecules: nucleic acids, lipids and proteins. Secondly, the high redox potential induced by the presence of O2 represents an additional reason restricting the availability of anaerobes in oxygenated environments because of displacement of thermodynamic equilibrium and the following failure of metabolic processes with initiation of adverse reactions. Finally molecular O2 is able to directly inactivate key enzymes of sulfate reduction metabolism.

SRB have developed complicated and tightly regulated systems of behavioral and enzymatic mechanisms of antioxidative defense which are responsible for a relative aerotolerance of SRB and elimination of ROS. Behavioral responses of SRB to oxygen include aggregation, symbiotic relationships with aerobic microorganisms, migration to anoxic zones and aerotaxis [5]. Enzymatic mechanisms involve O2 reduction that uses cytoplasmic, periplasmic and membrane-bound oxygen reduction chains as well as ROS scavenging reactions. In addition to classical enzymes of antioxidative defense (superoxide dismutase, catalase, peroxidases) which are usual for aerobic microorganisms and eukaryotes, the SRB cells, and specially Desulfovibrio species, possess also unique highly effective alternative enzymes with superoxide reductase (desulfoferrodoxin, neelaredoxin) and NADH-dependent peroxidase (rubrerythrin, nigerythrin) activities [6, 7].

2. Ecological aspect of physiological responses of SRB to oxygen exposure

2.1 Effects of oxygen on sulfate-reducing bacteria

Some species of SRB are able to survive after a short-time of exposure to O2 and they exhibit high oxygen tolerance even when grown in pure cultures [8]. The high number of SRB, found in the oxic environments, indicates that these organisms are able to deal with temporary exposures to elevated O2 concentrations up to 1.5 mM [9].
Thus, *Desulfovibrio desulfuricans* NCIB 8301 grown on agar media in the presence of oxygen survived well over the first 24 h but the cell viability decreased rapidly for longer exposure times; thereafter, live cells were still detected in colonies for one month [10]. *D. desulfuricans* is capable to grow slowly at low O₂ partial pressures (10-15 mm Hg); the cell yield fell drastically with an increase of the oxygen partial pressure, which was correlated with a decrease of both lactate utilization and hydrogen sulfide production [10].

The growth of *Desulfovibrio gigas* in a lactate/sulfate containing medium is suppressed in the presence of 5 µM O₂; the cell division resumed as soon as anaerobic conditions were restored, even if air exposure lasted for 24 h [11]. Under the aerobiosis, non dividing cells of *D. gigas* were still capable of oxidizing pyruvate to acetate. When grown in a lactate/sulfate medium, *D. gigas* utilizes O₂ and synthesizes NTPs. Nevertheless, the observed increase in NTP levels may indicate that, under the aerobic conditions, O₂ inhibits processes leading to ATP dissipation [11].

Cells of *Desulfovibrio vulgaris* Hildenborough also are able to consume oxygen and they possess aerotaxis in the direction of preferred sub-microaerophilic O₂ concentrations (0.02-0.04%), a level which also supported growth [12]. The air exposure for 2 h has an insignificant effect on survivability of *D. vulgaris* [13] whereas 1 h exposure to 100% O₂ causes five-fold decrease of the cell viability [14].

It has been shown [15] that in homogeneously aerated cultures of several SRB species, the rate of sulfide formation from the reduction of either sulfate, sulfite or thiosulfate decreases as the O₂ concentration increases, and is abolished above 15 µM O₂. Viability, cell motility and ability to cell division decrease with time when cells are exposed to oxygen. In continuous cultures of *Desulfovibrio oxyclinae* sulfate reduction is inhibited in the presence of 1% O₂ [16]. Moreover, oxygen induces morphological changes as it has been observed in the case of *Desulfovibrio* strains, developed atypically elongated cells [17]. This toxicity of O₂ might be related to the sensitivity of several proteins isolated from SRB: hydrogenases (key enzymes of *Desulfovibrio* energetic metabolism), L(+)-lactate dehydrogenase, NAD-dependent alcohol dehydrogenase, aldehyde dehydrogenase [18], some enzymes involved in protein and nucleic acids synthesis, and in cell division [14].

### 2.2 Sulfate-reducing bacteria in cyanobacterial mats

SRB are often found in the photooxygenic zone of cyanobacterial mats, where the gas ratio ranges from total O₂ saturation during the daytime to anaerobic conditions and high level of H₂S in the night-time. Cyanobacterial mats provide an interesting stratified system to study the behavioral patterns of SRB in response to oxygen. It has been shown, that during the day the most probable number (mpn) count of SRB in the upper layer of cyanobacterial mats from the Solar Lake (Sinai, Egypt) is 20-times lower than at dark in the absence of O₂ [19].

Within the oxic surface layer of the hypersaline cyanobacterial mat of the Solar Lake and in the mat layers below, diurnally migrating cells of *Desulfonema* are found in variable densities of 10⁴ to 10⁶ cells ml⁻¹. The number of cultured SRB in the surface aerobic layer and the level of sulfate reduction during the daytime are comparable, and sometimes even higher than identified in the interior of the mat. Facultative aerobic respiration, filamentous morphology, motility, migration to deep anoxic mat layers, and aggregates formation are the main adaptations of SRB from the Solar Lake to the mat matrix and to diurnal oxygen stress [20].

*D. oxyclinae* D22, isolated from the upper layer of cyanobacterial mats from the Solar Lake during the day, does not show any migration reaction while *D. oxyclinae* strains N24 and N13, dominate in the dark, migrate depending on the O₂ concentration. Strain N13 dominates aerotaxis inside cyanobacterial mats, forming bands around the O₂ bubbles on the boundary of aerobic and anaerobic zones. Cells perform circular motion in the bands which is uncharacteristic for anaerobic environment. In a full aeration all isolates are able to respire with oxygen as electron acceptor and hydrogen, lactate or sulfide as electron donors. Ethanol, which can be used for sulfate reduction, is not metabolized with the participation of O₂. Strain N13 supports growth under aerobic conditions up to 2 days, but cell division is inhibited and the length of cells increased to 5 times [19]. Thus, oxygen has the toxic effect on substrate oxidation as well as on anaerobic processes.

A chemostat culture of *D. oxyclinae* isolated from the oxic layer of a hypersaline cyanobacterial mat was grown in anaerobic conditions and then subjected to exposure with 1% O₂ [9]. 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. The O₂ consumption rate 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 observations indicated possible oxygen-dependent enhancement of growth. After 140 h of growth under oxygen exposure, the presence of cell aggregates 0.1 to 3 mm in diameter was observed. Formation of SRB cell clusters occurs within a cyanobacterium *Microcoleus chthonoplastes*-dominated layer of a cyanobacterial mat under daily exposure to O₂ concentrations of up to 900 µM [9]. Aggregates can only consist of bacteria, or include particles of inorganic nature, such as FeS, which provide additional mechanical strength and resistance to O₂.

In natural habitats, *D. oxyclinae* exists in close relationship with aerobic microorganisms, consuming O₂, in particular with heterotroph *Marinobacter* sp. Probably, SRB *in vivo* form small aggregates surrounding layers inhabited by symbiotic aerobes. A chemostat coculture of *D. oxyclinae* and *Marinobacter* sp. strain MB, isolated from the oxic zone of a cyanobacterial mat, was grown for 1 week under anaerobic conditions and then exposed to a flux with 5% O₂ [21].

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Sulfate reduction persisted under these oxic conditions, though the amount of reduced sulfate decreased by 45%. The coculture consumed oxygen effectively, and no residual \( \text{O}_2 \) was detected during the growth. The proportion of \( D. \ oxyclinae \) cells in the coculture decreased from 86% under anaerobic conditions to 70% in the microaerobic sulfate-reducing mode and 34% in the microaerobic sulfate-depleted mode. The patterns of consumption of electron donors and acceptors suggested that when oxygen was supplied in the absence of either sulfate or thiosulfate, \( D. \ oxyclinae \) performed incomplete aerobic oxidation of lactate to acetate. This is the first observation of oxygen-dependent growth of a SRB. Nevertheless, pure cultures of SRB, capable of aerobic growth in the absence of \( \text{SO}_4^{2-} \), have not been obtained, so the oxygen respiration is attributed, primarily, the defensive function in microaerophilic conditions [21].

Bacteria belonged to genera \( \text{Desulfonema} \) and \( \text{Desulfococcus} \) are dominant forms of photoxic and chemocline zones of the mats from the Solar Lake [4, 19, 20]. The representatives of \( \text{Desulfobacter} \) are most prevalent at depths corresponding to the chemocline zone during the day and the anaerobic zone at night. Profile of sulfate reduction in the bacterial community is heterogeneous: local anaerobic microniches populated by metabolically active SRB are formed in aerobic zone [4, 19]. Thus, recognized genera of SRB are not necessarily restricted by high \( \text{O}_2 \) levels in the mat community, and significant sulfur cycling within the chemocline is possible. Glycolate, secreted by cyanobacteria during the photosynthesis, stimulates the respiration of \( \text{Desulfonema} \) in the aerobic surface layer. The distribution of SRB allows associated sulfur-oxidizing chemo- and phototrophic microorganisms to take more sunlit areas with better \( \text{O}_2 \) access [4, 9, 19]. In the cyanobacterial mat from the saline evaporation pond in Baja California, \( \text{Desulfobacter} \) and \( \text{Desulfobacterium} \) are also restricted to greater depths while the representatives of \( \text{Desulfococcus} \) and \( \text{Desulfovibrio} \) are predominant in the upper part of the well-aerated photosynthetic zone [22].

SRB in the fully oxic photic zone of mats have to compete with numerically dominant and metabolically more diverse aerobic heterotrophic bacteria for organic substrates. Aerobic respiration by SRB contributes to organic carbon mineralization in the oxic zone of microbial mats as daytime porewater concentrations of low-molecular-weight carbon compounds are above typical half-saturation constants. Thus, the strain SRB D2, isolated from the photic zone of a hypersaline microbial mat (Lake Chiprana, Spain), respired pyruvate, alanine, and \( \alpha \)-ketoglutarate but not formate, lactate, malate, succinate, and serine at significant rates under fully oxic conditions. Dehydrogenases of only the former substrates are likely oxygen-tolerant enzymes as all substrates supported anaerobic sulfate reduction. Although strain SRB D2 appeared phylogenetically closely related to the oxygen-tolerant \( D. \ oxyclinae \), substrate spectra were essentially different [23].

2.3 Sulfate-reducing bacteria in activated sludge

SRB are widespread microorganisms in activated sludge of wastewater treatment facilities, periodically exposed to \( \text{O}_2 \). The presence of SRB in large bacterial flocs was demonstrated by FISH and PCR-based detection of genes coding for metabolic inactivation. Lactate-dependent anaerobic substrate spectra were more thermodynamically preferable [24].

Washed cell suspensions of \( D. \ desulfuricans \) strain DvO1, isolated from the highest sulfate reduction positive mpn dilution of an activated sludge sample, were aerated at 50% atmospheric \( \text{O}_2 \) saturation in sulfide-free media for 33 h in the presence or the absence of lactate as external electron donor. The cell suspension aerated in the absence of lactate showed insignificant endogenous \( \text{O}_2 \) reduction rates. In contrast, the cell suspension aerated in the presence of lactate sustained high rates of \( \text{O}_2 \) reduction during the entire aeration period. Strain DvO1 remained viable throughout the 33-h aeration irrespective of the presence of lactate; however, the \( \text{O}_2 \) exposure resulted in a dose-dependent reversible metabolic inactivation. Lactate-dependent anaerobic sulfate-reducing activity recovered quickly upon anaerobiosis, and was more aerotolerant than lactate-dependent oxygen-reducing activity [25].

2.4 Sulfate-reducing bacteria in wastewater biofilms

The SRB activity in biofilms is considered as the main reason of corrosion of industrial water pipe-lines and water supply facilities. Taking into consideration the fact that SRB are able to extract smallest \( \text{Zn} \) particles from water with the formation of \( \text{ZnS} \) pellet, the water quality control systems can be created on SRB basis [26].

The vertical distribution of SRB in photosynthetic biofilms from the trickling filter of a sewage treatment plant was investigated with \( 16S \) rRNA-targeted oligonucleotide probes. A negative correlation between the distribution of SRB and the measured \( \text{O}_2 \) profiles was found. It was also shown that SRB were located in the biofilms irregularly, being present in all states from single cells to dense clusters of several thousand cells. The distribution differed in light- and dark-incubated samples; in both cases the SRB were largely restricted to anoxic layers [27]. Microsensor measurements for \( \text{O}_2 \) showed that anoxic zones developed in the aerobic bacterial biofilm within a week and that oxygen level decreased during all stages of biofilm development. Sulfate reduction was first detected after 6 weeks of growth, although favourable conditions for SRB growth were presented from the first week. \textit{In situ} hybridization with a 16S rRNA probe for SRB revealed that they were present in high numbers (~10^6 cells/ml) in all stages of biofilm development, both in the oxic and anoxic zones. Hybridization analysis of the DGGE profiles with taxon-specific oligonucleotide probes showed that \( \text{Desulfobulbus} \), \( \text{Desulfovibrio} \) and \( \text{Desulfovibrio} \) were the most numerically
important SRB in all biofilm samples as well as in the bulk activated sludge. It was found that not all SRB detected by molecular methods in the wastewater biofilm were metabolically active [28].

The investigation of successive development of SRB community structure and in situ sulfide production activity within a biofilm, growing under microaerophilic conditions, showed that the most active sulfate reduction zone moved upward to the oxic-anoxic interface and intensified with time. This result corresponded with an increase in SRB populations in the surface layer of the biofilm. The number of cells, hybridized with SRB385 and SRB660 probes, significantly increased in the biofilm surface layer during eight-week cultivation, while those populations were relatively unchanged in the deeper part of the biofilm, probably due to substrate transport limitation [29]. The phylogenetic identification and substrate uptake patterns of SRB inhabiting a sewer biofilm with oxygen, nitrate, or sulfate as an electron acceptor was performed with the use of MAR-FISH analysis. It was demonstrated that Desulfobulbus, hybridized with SRB660 probe, was a dominant SRB subgroup in the biofilm, accounting for 23% of the total SRB. Approximately 9 and 27% of detected Desulfobulbus cells could take up labelled propionate with O₂ and NO₃⁻, respectively, as an electron acceptor, which might explain the high abundance of this species in various oxic environments [30].

3. Molecular mechanisms of SRB responses to oxygen

3.1 Toxic effects of molecular oxygen

Products of incomplete reduction of oxygen – superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (HO) – are highly toxic to cells. For instance, these reactive oxygen species (ROS) induce peptide bond breaks and the formation of cross-linked protein aggregates, depolymerization of nucleic acids, impair of sulfhydryl groups and [4Fe–4S] clusters (with concomitant iron release into the cytosol) in enzymes, oxidation of membrane lipids, polysaccharide molecules, and polyunsaturated fatty acids [31-33]. Inhibition of cell growth and DNA synthesis by oxygen was demonstrated in D. oxyclinae and D. vulgaris cells [11, 19]. In addition to the ROS effects, oxidative stress is also a consequence of the direct inactivation of key enzymes of sulfate reduction (hydrogenase or lactate dehydrogenase) by O₂ and of the modification of environmental parameters like redox potential and pH.

Hydrogen peroxide is the most stable intermediate in the process of O₂ reduction. H₂O₂ is capable of reacting with ferrous iron and other transition metals, forming more power oxidants, such as hydroxyl radicals (in the course of Fenton reaction: H₂O₂ + Fe²⁺ → Fe³⁺ + HO + HO⁻), as well as alkoxyl radicals and iron-oxygen complexes. In addition to H₂O₂, organic peroxides may be formed in the cells, initiating a chain reaction of lipid oxidation and leading to membrane and DNA molecule damages [34]. Superoxide radical which is the most widespread free radical damages whole cells, in addition to subcellular structures and macromolecules. Moreover, O₂⁻ is characterized by considerable diffusion capacity and exhibits greater selectivity in its reactions with intracellular proteins than other ROS. In addition, O₂⁻ and H₂O₂ may be formed in the course of auto-oxidation of reduced cytochromes or flavoproteins, and as a result of UV irradiation [33]. Besides, the auto-oxidation of H₂S, normally produced by SRB, could also result in the formation of reactive species, which therefore increases the cell sensitivity to O₂ [8].

Usually, oxidative stress in microorganisms is induced by O₂⁻ and H₂O₂ at relatively low intracellular concentrations (nmolar and µmolar, respectively) [35]. Superoxide dismutase (SOD), catalase, peroxidases and oxidases represent the first line of efficient enzymatic antioxidative defense, which prevent the accumulation of ROS and eliminate of those which production cannot be avoided. The expression of certain genes encoding the components of such systems is controlled by general regulatory mechanisms [31].

3.2 Oxidative stress responses of SRB on transcriptional level

Little data has been accumulated on the regulation of oxidative stress mechanisms in strict anaerobes; nevertheless some of them possess enzymes, which are induced under unfavorable oxic conditions [5]. Multiple antioxidative defense pathways were identified in SRB and proposed to be specifically expressed under different concentrations of oxidizing agents, contributing to ability of SRB to survive periodic aerobiosis in their econiches.

The global expression levels of oxidative stress response genes in D. vulgaris Hildenborough differ depending on ROS concentration. Concerted up-regulation of the genes belonged to predicted peroxide stress response regulon (PerR) was observed in response to low O₂ levels (0.1%) [36]. In contrast, long air exposure was highly detrimental to the cell viability and caused dramatic changes at the transcriptome level (393 genes were up-regulated and 454 genes were down-regulated) [36]. The expression levels of many genes, encoding enzymes of central metabolic pathways and antioxidative defense systems, decreased significantly during 4-h air exposure, but abundance of proteases and chaperons increased [36]. Transcripts analyses revealed that the PerR regulon of D. vulgaris also exhibited opposite regulation in the presence of 0.1 mM and 0.3 mM H₂O₂ and revealed that genes encoding a SOD, a superoxide reductase, a nigerinhyrin and a thiol peroxidase, in addition to the PerR regulon, belong to the H₂O₂ stimulon [37]. A global transcriptomic analysis pointed out that H₂O₂ as well as redox potential shift increased the expressions of the genes of D. vulgaris involved in ROS detoxification, thioredoxin-dependent reduction system, protein and DNA repair,
and decreased those involved in sulfate reduction, lactate oxidation and protein synthesis [38]. In addition, deletion mutants analysis suggested that PerR and Fur (ferric uptake regulator) regulons are functionally overlapped in response to H$_2$O$_2$ stresses [38].

A genome-wide transcriptomic analysis of D. vulgaris showed that 130 genes were responsive to 1-h air exposure [39]. For instance, amino acid biosynthetic pathways of D. vulgaris were induced by oxidative stress. The genes, encoding ruberythrin (rbr) and thioredoxin reductase (trxB) were also up-regulated, suggesting an important antioxidantive role of these proteins. In contrast, the expression of rubredoxin oxidoreductase (rbo), superoxide dismutase (sodB) and catalase (katA) genes were not changed in response to oxidative stress. Comparison of cellular reactions to oxidative stress and heat shock identified 66 genes that showed a similar drastic response to both of them, implying that these genes might be part of the general stress defensive system in D. vulgaris [39].

When D. vulgaris cells were exposed to the highest concentration of oxygen (100% O$_2$, 1 h) that SRB are likely to encounter in natural habitats, 307 genes were responsive, with cellular roles in energy metabolism, protein and nucleic acid synthesis, cell division, transport and regulatory functions, including multiple genes encoding heat shock proteins and peptidases [40]. The oxidative defense response concentrated on damage repair by metal-free enzymes. These data, together with the down-regulation of the Fur operon, which restricts the availability of iron in order to decrease the possibility of ROS generation, and the lack of response of the H$_2$O$_2$-sensing regulator operon, suggest that a major effect of high oxygen stress is the inactivation of multiple metalloproteins as a consequence of oxidative damage to their metal clusters [14, 40]. Thus, transcriptional responses of SRB to oxidative stresses are flexible and correlate with cell needs under specific conditions.

### 3.3 Molecular mechanisms of oxygen reduction by SRB

SRB not only survive oxygen exposure for at least days, but many of them even reduce O$_2$ to H$_2$O. It was shown that 14 tested freshwater or marine strains were able to reduce oxygen with at least one substrate as electron donor, including lactate, formate, pyruvate, ethanol, acetate, propionate, butyrate, hydrogen as well as sulfite or thiosulfite. The capability of O$_2$ reduction may be related to an adaptation to sulfate limitation in corresponding biotopes [41].

In D. vulgaris and D. desulfuricans, oxygen reduction was coupled to proton translocation and ATP conservation. In these species, the periplasmic fraction, which contains hydrogenases and low redox multiheme c-type cytochromes, was found to catalyze reduction of O$_2$ with high rates [42]. In D. gigas, a cytoplasmic rubredoxin oxidase was identified as an oxygen-reducing terminal oxidase (Table 1).

Desulfovibrio strains generally exhibit O$_2$ reduction rates comparable to those of aerobes, but these rates often decrease by increasing the O$_2$ concentration and slow down after repeated oxygen additions [19, 41]. The highest O$_2$ reduction rate detected so far have been observed in Desulfovibrio termitidis, isolated from termite guts, with more than 1570 nmol O$_2$ min$^{-1}$ mg$^{-1}$ [43]. Nevertheless, despite a high respiration rate and energy coupling, aerobic growth of pure cultures is very poor or absent because O$_2$ blocks sulfate reduction. The oxygen respiratory activity of SRB seems to be a protective mechanism against toxic effects of O$_2$, also providing considerable ecological advantage over aerobic microorganisms through the recovery of anoxic conditions if the habitat turns transiently oxic.

#### Table 1 Proteins involved in cytoplasmic and membrane-bound pathways of oxygen reduction in SRB.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amount of subunits</th>
<th>Molecular mass of the subunits, kDa</th>
<th>Prosthetic groups</th>
<th>Cell localization</th>
<th>Microorganism</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH:rubredoxin oxidoreductase</td>
<td>2</td>
<td>26; 32</td>
<td>FMN; FAD</td>
<td>Cytoplasm, soluble</td>
<td>D. gigas, D. vulgaris, D. desulfuricans</td>
</tr>
<tr>
<td>Rubredoxin</td>
<td>1</td>
<td>6</td>
<td>Fe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rubredoxin:oxygen oxidoreductase</td>
<td>2</td>
<td>43; 43</td>
<td>Fe-Fe; FMN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome bd oxidoreductase</td>
<td>2</td>
<td>29; 40</td>
<td>Heme; Cu</td>
<td>Membrane-bound</td>
<td>D. gigas, D. vulgaris, Desulfotalea psychrophila</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>4</td>
<td>11; 23; 47; 60</td>
<td>Heme; Cu</td>
<td>Membrane-bound</td>
<td>D. vulgaris Hildenborough, D. vulgaris Miyazaki, D. desulfuricans G20</td>
</tr>
</tbody>
</table>

Membranes from D. gigas grown in a fumarate/sulfate medium have a fully competent canonical respiratory chain, which allows the reduction of O$_2$ to H$_2$O using NADH and succinate [44]. A membrane-bound terminal oxidoreductase of the cytochrome bd family possesses a significant quinol oxidase activity. The ETC between the fumarate reductase and the cytochrome bd oxidase appears to be able to couple the succinate oxidation with the O$_2$ reduction to H$_2$O. It was suspected that, in addition to cytochrome bd oxidase, D. gigas contained at least another one membrane-bound oxygen...
D. desulfuricans prevents the formation of ROS under microaerophilic conditions [53]. A role for rubredoxin in diverting electron flow proteins (Fig. 1A): a NADH)rubredoxin oxidoreductase (NRO), a rubredoxin (Rub) and a rubredoxin)oxygen partner, ROO. Each ROO monomer is composed of one lactamase)like domain (with Fe)Fe center, where the reduction both FMN and FAD, and is able to reduce rubredoxin from NADH [51]. Rub in turn gives electron to its other redox 2 2 2 oxidative stress has been proposed [53]. A cluster of genes encoding desulfoferrodoxin, Rub and ROO is present in the environment of D. desulfuricans NADH oxidase activity [52]. Thus, such type of cytoplasmic oxygen reduction chain appears to be a conserved and efficient mechanism of O2 elimination among Desulfovibrio species [18]. It was suggested that O2 reduction by ROO prevents the formation of ROS under microaerophilic conditions [53]. A role for rubredoxin in diverting electron flow from the electron transport chain of D. vulgaris Hildenborough to ROO, rubrerythrin and superoxide reductase under oxidative stress has been proposed [53].

![Fig. 1](image.png)

**Fig. 1** (A) Cytoplasmic pathway of O2 reduction of D. gigas [49]; (B) periplasmic pathway of O2 reduction of D. vulgaris [54].

Another mechanism of oxygen reduction has been reported for D. vulgaris Marburg which reduces O2 to H2O with H2 as electron donor and 90% of this O2 reduction activity was found in the periplasmic fraction [54]. The maximum hydrogen oxidation rate was 253 nmol O2 min−1 mg−1 at low oxygen concentrations (up to 30 µM). Hydrogenases and cytochrome c were shown to be involved in this reaction (Fig. 1B). Since this system works with higher rates than sulfate reduction, its antioxidative defense function in D. vulgaris and D. termitidis could be crucial [54]. An increase in the [Fe] hydrogenase and the cytochrome c content in the periplasm of D. vulgaris Hildenborough was observed following either O2 exposure or chromate addition that increased the redox potential of the medium [55]. Viability of the strain lacking the genes, encoding [Fe] hydrogenase (hydAB), after 1-h exposure to oxygen was lower than that of the wild-type [55]. A similar behavior has been also described in D. desulfuricans NCIB 8301 ; when the cells were exposed to oxygen, the same increase in cytochrome c content was detected [10]. Hydrogenases and cytochromes could be either directly involved in the elimination of O2 by its reduction to H2O or involved in a mechanism decreasing the environmental redox potential in order to get the optimal conditions for growth [56].

3.4 Aerotaxis regulation of SRB

In an oxygen gradient, D. vulgaris Hildenborough forms a focused band at an estimated O2 concentration of 0.02–0.04% and D. desulfuricans – ring-shaped bands around air bubbles [12]. The fact that bands are formed at some distance from the air bubble shows that the cells are able of negative aerotaxis. Such cell accumulation also suggests the existence of positive aerotaxis [57].
Cells of *D. vulgaris* Hildenborough possess a chemoreceptor (DcrA) that serves as a sensor of the O₂ concentration or redox potential of the environment [58, 59]. A DcrA homolog was also found in the genome of *D. desulfuricans* G20 [18]. DcrA has the structural design of methyl-accepting chemotaxis proteins from enteric bacteria with an N-terminal periplasmic sensing domain which contains a c-type heme, and a C-terminal cytoplasmic signaling domain [59, 60]. The level of methylation of the signaling domain is influenced by the redox state of the heme group and decreased upon addition of O₂ [59]. The full chemotactic response of *D. vulgaris* is complicated and controlled by at least 15 genes from the *dcr* family [61]. In response to 1-h exposure to 100% O₂, the positive regulation of three genes that encode chemotaxis proteins was observed, among which one belongs to the *dcr* family and one corresponds to the protein of the basal body of flagellum [40].

Mutants lacking *dcrA* show greater resistance to the toxic effects of O₂ than the wild-type cells. The reason for this unusual phenotype is probably a parallel increase in the transcriptional level of the *rbo-rub* operon that encodes for the superoxide reductase and rubredoxin, located immediately downstream from *dcrA*. Thus, DcrA may act under anaerobic conditions as the repressor of the transcription of the *rbo-rub* operon [62].

### 4. Classical enzymes of ROS detoxification

#### 4.1 Superoxide dismutase (SOD)

Superoxide dismutase eliminates O₂⁻ by dismutation to H₂O₂ and O₂. The presence of a SOD activity in anaerobic bacteria has been demonstrated for the first time in *D. desulfuricans* and *Desulfovomaculum nigrificans* [63]. At the same time, SOD was isolated from *D. desulfuricans* Norway 4. The enzyme exhibited similar physico-chemical properties as compared to the Fe-SODs found in aerobic microorganisms [64]. More recently, a Fe-SOD has been purified and characterized from *D. gigas*. This SOD, isolated as a homodimer of 22-kDa noncovalently bound subunits (Table 2), has an EPR spectrum characteristic of high-spin ferric iron in a rhombically distorted ligand field. Like other Fe-SODs, the enzyme of *D. gigas* is sensitive to H₂O₂ and azide but not to cyanide [65].

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Molecular mass, kDa</th>
<th>Number of subunits</th>
<th>Specific activity, U/mg</th>
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<tbody>
<tr>
<td></td>
<td>SOD</td>
<td>catalase</td>
<td>SOD</td>
</tr>
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<td>43.0</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td><em>D. desulfuricans</em> B-1388</td>
<td>43.0</td>
<td>-</td>
<td>2</td>
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<td><em>D. gigas</em> ATCC 19364</td>
<td>43.0</td>
<td>186.0</td>
<td>2</td>
</tr>
<tr>
<td><em>D. vulgaris</em> Miyazaki F</td>
<td>43.5</td>
<td>122.0</td>
<td>2</td>
</tr>
</tbody>
</table>

The genes, encoding SOD, have been cloned from *D. vulgaris* strains Hildenborough [66] and Miyazaki F [67]. Genomes analysis of *Desulfovibrio desulfuricans* and *Desulfotalea psychrophila* reveals the presence of a single *sod* gene per genome [18]. In *Desulfovibrio* species, the N-terminal sequence of the SODs contains a twin-arginine motif typical for signal peptides of periplasmic redox proteins, allowing the SOD holoenzyme to be translocated from the cytoplasm into the periplasm using the TAT system [66, 67].

No significant differences in SOD activity have been observed between cells of *D. gigas* exposed to 120 μM O₂ for different times and anaerobic cultures [11]. Likewise, the amount of SOD does not change when *D. vulgaris* is exposed to air for 1 h [13, 14]. On the other hand, O₂ in low concentration (10 mm Hg) induced SOD activity (10-fold increase) in *D. desulfuricans* that improved cell viability under temporary microaerophilic conditions [10].

The deletion of the *sod* gene in *D. vulgaris* Hildenborough induced an increase in the strain sensitivity to externally produced O₂⁻ while exposure to air does not demonstrate any difference [68]. The periplasmic space of *Desulfovibrio* species contains a lot of low redox potential enzymes [69] that might generate O₂⁻ in the presence of O₂. It has been shown that, when cells are exposed to 100% O₂ for a short time, the [Fe] hydrogenase activity is higher in the wild-type strain of *D. vulgaris* than in the ∆*sod* mutant [55]. The periplasmic SOD could be therefore mainly involved in the protection of sensitive Fe-S enzymes against superoxide-induced damages.

#### 4.2 Catalase

Catalase activity, disproportioning of H₂O₂ to O₂ and H₂O, was detected for the first time in *Desulfovibrio* [64]. The enzyme from *D. gigas* is composed of three subunits of 61 kDa (Table 2) and contains only one heme group in an active center per molecule, which may explain for its relatively low specific activity [65]. While *D. vulgaris*, *D. gigas* and *Desulfomicrobium norvegicum* are catalase positive, *D. salexigens* and many strains of *D. desulfuricans* are catalase negative [65]. The activities of three enzymes – catalase, NADPH peroxidase and NADH peroxidase, participating in
H$_2$O$_2$ decomposition, were detected in cell-free extracts of *D. desulfuricans* ATCC 27774 [48]. *Desulfotomaculum* species possess high catalase activity comparable to such in aerobic bacteria [70].

The *kat* gene, encoding a cytoplasmic catalase in *D. vulgaris* Miyazaki F, has been cloned and expressed in *E. coli* [71]. Genomes analysis reveals the presence of the *kat* gene in *Desulfotalea psychrophila* and *Archaeoglobus fulgidus* [18]. In the case of *D. vulgaris*, it is located on a *nif* gene-containing plasmid that is lost during cell cultivation on a medium with ammonium chloride [68]. In *D. gigas*, catalase activity increased as cells are exposed to increasing oxygen concentration (up to 120 µM) and long exposure times led to higher catalase activity [11].

### 5. Alternative enzymes of antioxidative defense

#### 5.1 Desulfoferrodoxin and neelaredoxin

In 2000, Lombard et al. reported [72] that desulfoferrodoxin (Dfx) from *Desulfoarculus baarsii*, which had been already shown to functionally complement an *E. coli* mutant deficient in cytoplasmic SODs [73-74], exhibited a superoxide reductase (SOR) activity, catalyzing the reduction of O$_2^{-}$ to H$_2$O$_2$. This protein has been also purified and characterized from *D. desulfuricans* and *D. vulgaris* Hildenborough [75]. Dfx is a monomer of 14-16 kDa that contains two separate mononuclear non-heme iron centers. Center I is a ferric site with distorted tetrahedral sulfur coordination [Fe(SCys)$_4$] and center II is a ferrous site with a unique square pyramidal structure [Fe(NHis)$_4$(SCys)]. Kinetics and spectroscopic experiments identified center II as the active site for the SOR activity [75-79].

Another enzyme with high SOR activity, named neelaredoxin (Nlr), which contains only one active site, identical to center II of Dfx, has been characterized from *D. gigas* [80]. Both two non-heme iron proteins have been also found in a hyperthermophilic sulfate-reducing archaeon *A. fulgidus* [81]. SOR activity of Nlr may allow cells to eliminate O$_2^{-}$ quickly in a NAD(P)H-dependent pathway. Both enzymes (Nlr, especially) can also act as SOD in conditions of high cellular redox potential in a lack of available electron donor [82, 83].

In the case of Nlr from *D. gigas* [84] and *A. fulgidus* [81, 85], as well as Dfx from *D. vulgaris* [78, 86], superoxide reduction is a fast bimolecular reaction resulting in the formation of a single Fe(III)-hydroperoxide intermediate. In the reaction involving Dfx from *D. baarsii*, a Fe(III)-peroxide species is formed at the first stage, which then undergoes protonation by the solvent and transition into the Fe(III)-hydroperoxide intermediate [87, 88]. During the repeated protonation (by water or a stronger exogenous acid, HX), H$_2$O$_2$ is released, and the vacant sixth iron coordination site is occupied by an anion (OH$^-$ or X$^-$), which may be replaced subsequently by Glu47, a ligand with a higher affinity [86]. While Glu47 might serve to help H$_2$O$_2$ release, Lys48 plays an important role during guiding and binding of O$_2^{-}$ to the iron center II of Dfx (Fig. 2) [77, 79]. Recovery of the SOR leads to a conformation change and breaking the bond with Glu, which promotes more effective interaction with O$_2^{-}$ [81, 82].

Rubredoxins have been shown to be efficient electron donors for SOR in *D. vulgaris* [89] and *A. fulgidus* [90]. In *D. vulgaris* Hildenborough and *D. desulfuricans* G20, the genes, encoding desulfoferrodoxin (SOR), rubredoxin and rubredoxin-oxygen oxidoreductase, are in the same genes cluster. Such gene organization proves that SOR and ROO may collaborate for the detoxification and reduction of O$_2$, entering the cytoplasm, through the use of Rub as a common intermediary electron donor [18, 53].

Comparison of the sensitivity of ∆*sor* and ∆*sod* mutants of *D. vulgaris* to various oxidative stresses indicates that under fully aerated conditions, cytoplasmic SOR is the key oxygen defense enzyme. Whereas SOD is involved in the removal of O$_2^{-}$ in the periplasm under microaerophilic conditions to protect oxygen-sensitive enzymes [66, 68], SOR and SOD are thus complementary components of an efficient superoxide-scavenging cellular system.

**Fig. 2** Superoxide reduction mechanism of desulfoferrodoxin from *Desulfoarculus baarsii* [77, 88].
5.2 Rubrerythrin and nigerythrin

Rubrerythrin and nigerythrin, homodimeric cytoplasmic proteins which exhibit NADH peroxidase activity in vitro, have been isolated from *D. vulgaris* Hildenborough [91, 92]. Rubrerythrin (Rbr) contains a non-sulfur, hemerythrin-like carboxylate-bridged diiron site and a rubredoxin-like [Fe(SCys)₄] site in each of two 23-kDa subunits [91, 93, 94]. The mechanism of hydrogen peroxide reduction by rubrerythrin is shown on Fig. 3. Nigerythrin (Ngr) is a larger protein (54-kDa) that contains the same types of iron sites as rubrerythrin, but has different domain conformation providing higher peroxidase activity [91, 95]. In *D. vulgaris*, while the *rbr* gene has been shown to be co-transcribed with two other genes (encoding a Fur-like protein and a rubredoxin-like protein), the *ngr* gene is monocistronic [96]. A catalytic electron transfer in vitro between rubredoxin and rubrerythrin has been described [89].

The deletion of the *rbr* gene in *D. vulgaris* does not alter the sensitivity of the strain to any oxidative stress [68]. However, this can be explained by the presence of genes encoding a nigerythrin and a second homolog to rubrerythrin that may compensate for the deletion [68]. The expression of *rbr* and, especially, *ngr* from *D. vulgaris* in ΔkatG or ΔkatE strains of *E. coli* significantly increased their resistance to H₂O₂ [66]. It should be noticed that under drastic oxidative stress conditions, *D. vulgaris* reduces the amount of SOR and rubrerythrins in order to limit free Fe²⁺, which is involved in the ROS generation. But two cytoplasmic proteins with a thiol-peroxidase activity are up-regulated in these stress conditions, compensating for this decrease [14, 68].

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Fig. 3 Supposed highly-specific peroxidase catalytic cycle in a Fe-Fe center of rubrerythrin [97].

6. Conclusion

While cellular mechanisms of defense against toxic ROS in aerobic organisms are well described, relatively few data are available about anaerobes. SRB belong to strict anaerobic bacteria that often encounter oxic conditions in their different natural biotopes, and some SRB possess protective aerobic respiration even. They thereby represent organisms of choice to study the variety of antioxidative defense systems in anaerobes which provide a competitive advantage and survival in changing environments. Ecological, biochemical and genetics studies have brought information on the way by which SRB are able to survive temporary oxygen exposure. Efficient response to the oxidative stresses in the SRB cells includes specific behavior (aerotaxis, aggregation) and complicated molecular mechanisms involving classical as well as unique key antioxidative enzymes like superoxide reductase or rubrerythrin. Some of these highly effective enzymes, characterized for the first time in SRB, are in fact largely distributed in the anaerobic forms of life. The main advantage of these systems is the lack of production of oxygen during the catalytic cycle. The differential proteomics and transcriptomics approaches developed on *D. vulgaris* Hildenborough [14, 36] identified several proteins of unknown functions, differentially expressed under drastic oxidative conditions, that might constitute new systems of ROS detoxification to be further studied.
References


