Oxygen reduction in the strict anaerobe *Desulfovibrio vulgaris* Hildenborough: characterization of two membrane-bound oxygen reductases

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Although *Desulfovibrio vulgaris* Hildenborough (DvH) is a strictly anaerobic bacterium, it is able to consume oxygen in different cellular compartments, including extensive periplasmic O₂ reduction with hydrogen as electron donor. The genome of DvH revealed the presence of *cydAB* and *cox* genes, encoding a quinol oxidase *bd* and a cytochrome *c* oxidase, respectively. In the membranes of DvH, we detected both quinol oxygen reductase (inhibited by heptyl-hydroxyquinoline-N-oxide (HQNO)) and cytochrome *c* oxidase activities. Spectral and HPLC data for the membrane fraction revealed the presence of *α-, b- and d*-type haems, in addition to a majority of *c*-type haems, but no *a*-type haem, in agreement with carbon monoxide-binding analysis. The cytochrome *c* oxidase is of the *cc(o/b)₃* type, a type not previously described. The monohaem cytochrome *c₅₅₃* is an electron donor to the cytochrome *c* oxidase; its encoding gene is located upstream of the *cox* operon and is 50-fold more transcribed than *coxI* encoding the cytochrome *c* oxidase subunit I. Even when DvH is grown under anaerobic conditions in lactate/sulfate medium, the two terminal oxidase-encoding genes are expressed. Furthermore, the quinol oxidase *bd*-encoding genes are more highly expressed than the *cox* genes. The *cox* operon exhibits an atypical genomic organization, with the gene *coxII* located downstream of *coxIV*. The occurrence of these membrane-bound oxygen reductases in other strictly anaerobic Deltaproteobacteria is discussed.

INTRODUCTION

Oxygen is a powerful oxidative molecule used by aerobic forms of life through complex respiratory chains that include at least one membrane-bound terminal oxygen reductase. Surprisingly, the increasing number of sequenced genomes has revealed the presence of genes encoding terminal oxygen reductases not only in aerobic but also in some anaerobic micro-organisms. Although the function of membrane-bound terminal oxygen reductases is readily understood in aerobes, the occurrence of such enzymes in strict anaerobes is more intriguing. However, increasing data suggest that many anaerobic micro-organisms, although unable to use oxygen as terminal electron acceptor for growth, have developed defence strategies to protect themselves against oxidative damage and thus exhibit aerotolerance (Cypionka, 2000; Dolla et al., 2006; Kawasaki et al., 2005, 2009; Le Fourn et al., 2008). Among these micro-organisms, sulfate-reducing bacteria (SRB) are some of the most studied organisms with regard to aerotolerance mechanisms. SRB, which have been regarded for many years as strict anaerobes, are universally distributed in marine and freshwater sediments where sulfate reduction is the dominant biomineralization pathway. SRB gain energy for biosynthesis and growth by coupling oxidation of organic compounds or molecular hydrogen to reduction of sulfate to sulfide (Thauer et al., 2007). Despite the anaerobic nature of this metabolic process, SRB activity is not confined to permanently anoxic habitats. The abundance and metabolic activity of SRB in oxic zones of numerous
biotopes, including marine and freshwater sediments, have frequently been found to be higher than those in neighbouring anoxic zones (Mussmann et al., 2005; Ravensschlag et al., 2000; Sass et al., 1998b). SRB have been detected as active bacteria in high numbers in the uppermost phototrophic layers of microbial mats (Teske et al., 1998), as well as in environments subject to regular periods of oxygen exposure (Ito et al., 2002; Kjeldsen et al., 2004). Even in pure culture, several SRB exhibit relatively high degrees of oxygen tolerance, remaining viable after up to 24 h of exposure to air (Abdollahi & Wimpenny, 1990; Cypionka et al., 1985). However, decreases in viability and cell motility (Marschall et al., 2002; Sigalevich & Cohen, 2000). However, oxygen reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-

SRB might have developed adaptation strategies to protect them against oxygen, including oxygen removal. The ability to reduce oxygen is widespread among SRB (Dannenberg et al., 1992; Fareleira et al., 2003; Fournier et al., 2006; Fröhlich et al., 1999; Kjeldsen et al., 2005; Kuhnigk et al., 1996; Mukhopadhyay et al., 2007; Sass et al., 2002; Sigalevich & Cohen, 2000). However, oxygen reduction by SRB seems to be a protective mechanism against the harmful effects of oxygen rather than an energy-coupled conservation mechanism to allow growth. At the molecular level, membrane-bound oxygen reductases are one of the three systems that could account for oxygen reduction by SRB, the two others being a cytoplasmic reduction chain involving a rubredoxin oxygen oxido-reductase (Frazão et al., 2000; Santos et al., 1993; Wildschut et al., 2006) and a periplasmic reduction involving hydrogenases and cytochromes (cyts) (Baumgarten et al., 2004). The presence of genes encoding a classical oxygen reductase (Hildenborough (DvH), it has been shown that the cyt c oxidase, named ccab, contains two c-type haems in its subunit II (Lobo et al., 2008). The presence of genes encoding both membrane-bound oxygen reductases in several Desulfovibrio isolates from saltmarsh sediments has been shown by hybridization and PCR experiments (Santana, 2008). However, analysis of the genome of Desulfovibrio species has not revealed the presence of any gene encoding a classical bc-type complex.

Here, we report oxygen reductase activities in DvH cells in the presence of various electron donors, including hydrogen. We show the presence of both a quinol oxidase bd and a cyt c oxidase in the membrane fraction of DvH, and report their spectroscopic and enzyme properties. The data provide new insights into the characteristics of the membrane-bound reductases, and specify their respective activities, haem composition and transcriptional levels in the cells. The atypical genomic organization of the cyt operon, and the occurrence of these reductases and their evolution in other anaerobic micro-organisms, are also discussed.

METHODS

Bacterial growth conditions. The sulfate-reducing bacterium DvH was grown anaerobically at 33 °C in liquid lactate/sulfate medium under nitrogen pressure in 10 ml (Hungate tubes) to up to 1 L flasks, which were inoculated at 10% (v/v) as described by Postgate (1984) and Voordouw et al. (1989). Growth was monitored by following OD600. Growth on solid medium E (Postgate, 1984) was performed on plates under gas exchange conditions in an anaerobic chamber (COY) with a 10% H2/90% N2 mixed gas atmosphere.

Cell and membrane preparation. Membranes were prepared from 2 L cultures of DvH cells collected at the beginning of the stationary phase (OD600 0.7–0.8). Cells were centrifuged at 6800 g at 4 °C for 20 min, and the pellet was washed with 0.1 M Tris/HCl, 0.15 M NaCl buffer (pH 7.5). This washing step was repeated at least three times to remove excess sulfide and to prevent any chemical reaction with O2. In case O2 respiration occurred with hydrogen as electron donor, cells were prepared in anaerobic buffer degassed with argon for all steps to prevent the inactivation of hydrogenases with O2. For preparation of membranes, cells were broken with two successive passages through a French press at 96 MPa in the presence of DNase (10 μg ml−1) in 0.1 M Tris/HCl, 0.15 M NaCl buffer (pH 7.5). Unbroken cells and debris were removed by two successive centrifugations for 20 min at 3000 g (4 °C), and the membrane fraction was obtained by two successive ultracentrifugations at 4 °C for 1 h at 149 000 g with a 70Ti rotor. Membranes were homogenized with a Potter device, frozen in liquid nitrogen and stored at −80 °C.

The protein concentrations of cell suspensions and membrane fractions were determined by the Bradford method with BSA as standard (Bradford, 1976). For whole cells, 1 M NaOH was added to disrupt the cells before protein quantification.

UV–visible optical spectroscopy. Absorption spectra of DvH membranes resuspended in 0.1 M Tris/HCl, 0.15 M NaCl buffer (pH 7.5) were obtained at 25 °C with a dual-wavelength DW2000 SLM-Amico spectrophotometer. A 2 nm slit width was used unless otherwise stated in the figure legends. Low-temperature optical absorption spectra were obtained at liquid nitrogen temperature (77 K) with cuvettes of 0.2 cm light path. Haem content was determined from the reduced (few grains of dithionite or ascorbate) minus oxidized difference spectrum using the following molar absorption coefficients for the a peak at 25 °C: 18 mM−1 cm−1 (haems c at 550 nm), 24 mM−1 cm−1 (haems b at 560 nm) and 18.8 mM−1 cm−1 (haem d at 630 nm).

Haem analysis by HPLC. Haemns were extracted from membranes with acetone/HCl (19:1), as in Lübben & Morand (1994), and recovered in a phase of ethyl acetate. Samples (corresponding to 0.3 mg protein) dissolved in acetonitrile were analysed using a C4 reversed-phase column (5 μm, 4.6 x 250 mm, 30 nm; Interchim). The solvent system consisted of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and acetonitrile (solvent B). The column was eluted with a linear gradient from 30 to 80% solvent B over 40 min at a flow rate of 1 ml min−1. The eluant was monitored using a diode array detector at 400 nm.

Oxygen consumption, quinol and cyt c oxidation measurements. O2 reductase activities on whole DvH cells or membrane
fractions were measured polarographically with a Clark electrode (Gilion oxigraph) in a stirred volume of 1.8 ml of 0.1 M Tris/HCl, 0.15 M NaCl buffer (pH 7.5) at 25 or 30 °C with different electron donors: H₂, NADH (3 mM), 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol (DBH₂; 100 μM), menadion (50 μM), ascorbate (10 mM) + horse heart cyt c (200 μM), lactate (200 mM), succinate (50 mM) or pyruvate (30 mM). As indicated in Fig. 1, addition of H₂ led to a lower amount of dissolved oxygen in the chamber.

Quinol oxidase activity in DvH membranes was measured with DBH₂ (40 μM) as substrate in 100 mM Tris/HCl, 150 mM NaCl buffer (pH 7.5) at 30 °C. Oxidation of quinol to quinone was followed in dual mode (at the wavelength pair 278/325 nm) with a stirred reaction cuvette equipped with a sample injection system in an Aminco DW2A spectrophotometer. Cyt 553 oxidation was monitored at the wavelength pair 553/540 nm. Molar absorption coefficients of 16 and 29 mM⁻¹ cm⁻¹ were used for calculations of oxidized DBH₂ (DB) and reduced cyt 553, respectively. Any abiotic or chemical reaction (especially with menadion) was subtracted to obtain the enzyme activity. For anaerobic conditions, cuvettes were bubbled with argon and sealed with a rubber stopper. All values reported in the tables are the mean of at least three experiments.

**RESULTS**

**DvH cells are able to consume oxygen with various substrates as electron donors**

Oxygen consumption rates by washed DvH cells were evaluated in the presence of various electron donors. In the presence of H₂ as electron donor, whole cells were able to consume oxygen only when the oxygen concentration in the oxygraph chamber was lower than about 100 μM (Fig. 1, Table 1). In addition, the lower the oxygen concentration, the higher the rate of oxygen consumption (Fig. 1a). Repeated introduction of small amounts of oxygen into the H₂-saturated oxygraph chamber resulted in high oxygen

![Fig. 1. O₂ consumption of DvH whole cells with hydrogen as electron donor. (a) DvH cells were added to the oxygraph chamber at 0.58 mg ml⁻¹ (final protein concentration) in 100 mM Tris/HCl, 150 mM NaCl buffer (pH 7.5) at 25 °C. Hydrogen, injected at different times as indicated by arrows, replaced O₂ in the chamber, as shown by the rapid abiotic drop of the O₂ level in the chamber. The enzymic phase is indicated by an asterisk. Addition of 1.38 mM KCN is indicated by an arrow. (b) Anaerobiosis was obtained by injection of H₂ gas, which replaced O₂ in the chamber. DvH cells were then added at 0.58 mg ml⁻¹, and pulses of O₂ were applied (arrows). The enzymic phase of O₂ consumption by the cells is indicated by an asterisk.](image-url)
consumption (Fig. 1b). At low oxygen concentration (about 40 μM), the oxygen consumption rate was \(230\pm30\) nmol min\(^{-1}\) (mg total protein\(^{-1}\)). It should be noted that the hydrogen-dependent oxygen consumption was not sensitive to KCN (Fig. 1a) but was completely inhibited in the presence of CO (Table 1).

The use of lactate (present in the growth medium) as substrate gave a value of \(48\pm4\) nmol O\(_2\) min\(^{-1}\) mg\(^{-1}\) (Table 1), and this activity was completely inhibited by CO and KCN (data not shown). When other substrates (NADH or DBH\(_2\)) were used as electron donors, the oxygen consumption rates were at least one order of magnitude lower than that observed in the presence of H\(_2\) (Table 1). In addition, in the presence of pyruvate, no oxygen consumption was detected (Table 1).

The data indicate that oxygen is efficiently consumed by whole cells, especially when molecular hydrogen is the electron donor. The high content of reactive oxygen species (ROS)-detoxifying enzymes in DvH cells prevents quantification of the amount of ROS generated during O\(_2\) consumption, and thus we cannot exclude that reduction of O\(_2\) leads partly to the production of ROS.

**In silico analysis of the membrane-bound oxygen reductases of DvH**

Genome analysis of DvH (Heidelberg et al., 2004) shows the presence of two gene clusters, DVU3270–DVU3271 and DVU1810–DVU1816, encoding a quinol oxidase \(bd\) and a haem-copper cyt \(c\)-type oxidase, respectively (Fig. 2). The first gene, DVU3271 or cydA (Fig. 2b), encodes subunit I of the quinol oxidase \(bd\), predicted to comprise nine transmembrane helices of 48 kDa, and which likely binds two \(b\)-type haems coordinated by the conserved axial ligand residues H\(_{139}/E_{375}\) for one \(b\) haem (equivalent to haem \(b_{595}\) in *Escherichia coli*) and H\(_{183}/M_{325}\) for the second \(b\) haem (equivalent to haem \(b_{558}\) in *E. coli*). DVU3270 or cydB encodes subunit II, predicted to comprise eight transmembrane helices 38 kDa, and which contains one \(d\)-type haem.

The cyt \(c\) oxidase-encoding cluster is composed of seven genes (DVU1816–1810) (Fig. 2a), organized as a putative multi-cistronic unit (cox operon). DVU1815 encodes coxl (one of the four structural subunits of the cyt \(c\) oxidase), which is the hydrophobic and catalytic subunit containing a low-spin haem and a binuclear centre formed by a Cu\(_{B}\), and a high-spin haem where the reduction of O\(_2\) takes place. DVU1812 encodes the catalytic subunit II (CoxII), which contains the Cu\(_A\) centre with two copper atoms ligated to the conserved residues H\(_{135}/C_{170}/E_{172}/C_{174}/H_{178}\) and M\(_{181}\). Sequence analysis revealed that this subunit contains two binding consensus sequences of the CXCH type (C\(_{216}/I_{220}\) and C\(_{317}/L_{321}\)), and might also bind two \(c\)-type haems. This possibility has been recently confirmed by Lobo et al. (2008). The third gene, DVU1814, encodes CoxIII, the transmembrane subunit of 23 kDa without any cofactor, and DVU1813, the fourth structural subunit (CoxIV), a small non-redox protein of 11 kDa with three predicted transmembrane helices. In

**Table 1. Oxygen consumption activity in DvH cells**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>O(_2) consumption (nmol min(^{-1}) mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_2)*</td>
<td>230 (O(_2) concentration (&lt;\sim 40) μM), ND (O(_2) concn (&gt;\sim 100) μM)</td>
</tr>
<tr>
<td>Lactate</td>
<td>48</td>
</tr>
<tr>
<td>Ascorbate+cyt(c)</td>
<td>3.5</td>
</tr>
<tr>
<td>DBH(_2)</td>
<td>3</td>
</tr>
<tr>
<td>NADH</td>
<td>5</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.5</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>ND</td>
</tr>
</tbody>
</table>

*This activity was completely inhibited by CO. When present, the low activities due to abiotic and chemical reactions were subtracted.

**Fig. 2.** Gene clusters encoding the cyt\(c\) oxidase (a) and quinol oxidase \(bd\) (b) of DvH.
Fig. 3. Occurrence and operon organization of the haem-copper oxidase in different phylogenetic branches. For clarity, the size of the arrows is not proportional to the size of the corresponding genes. Cyan, scoI; red, coxI; orange, coxIII; green, coxIV; olive green, coxII; light green, protohaem IX farnesyltransferase; pink, putative uncharacterized membrane-bound protein. The red rectangle highlights the Desulfovibriales and the Desulfuromonadales in the Deltaproteobacteria for which the coxII gene (olive green, indicated by an asterisk) is located after the coxIV gene, in contrast to all other organisms, in which the coxII gene is located before coxI. The figure was created with String 8.3 (Jensen et al., 2009).
addition to these genes encoding the structural subunits, the *cox* operon also includes genes involved in the maturation of the cyt oxidase. The first gene (*scoI* or *DVU1816*) encodes a protein involved in the maturation of the copper CuA centre in subunit II (Cobine *et al.*, 2006), and *DVU1811* encodes a protohaem IX farnesyltransferase of 31 kDa that adds the farnesyl group onto haem b to produce haem o. Finally, *DVU1810* encodes a hypothetical membrane-bound protein with four putative transmembrane helices, sharing weak homology with the membrane domain of a cyt c biogenesis protein (CcdA) found in *Bacillus pseudofirmus* OF4. This protein is only present in the Desulfuvibionales (Fig. 3), and not in the Desulfuromonadales (*Geobacter* and *Pelobacter* species), which do not contain two cyt c-type haems in subunit II. Thus, one might hypothesize that *DVU1810* is important for the maturation of the dihaemic subunit II.

Analysis of the occurrence of this *cox* gene cluster in bacteria indicated that the gene encoding subunit II (*coxII*) in *Desulfovibrio* as well as in *Geobacter* and *Pelobacter* (both belonging to the Desulfuromonadales class of the Deltaproteobacteria) is located downstream of the gene encoding subunit IV (*coxIV*) (Figs 2a and 3). This gene organization contrasts with that found in all other sequenced genomes available, where *coxII* is the first gene of the *cox* operon or the second gene after *scoI* when present (Fig. 3). *Desulfovibrio* species belong to a class of the Deltaproteobacteria that contains both aerobic and anaerobic bacteria. Note that the Myxococcales (exemplified by *Anaeromyxobacter dehalogenans* and *Myxococcus xanthus*) do not exhibit this reorganization of the *cox* gene cluster, and *coxII* is located just after *scoI* at the beginning of the *cox* operon (Fig. 3). This suggests that relocation of the *coxII* gene occurred in a common, strictly anaerobic ancestor of Desulfuvibionales and Desulfuromonadales in the Deltaproteobacteria. A cyt c-type haem is present in subunit II of the *caao* cyt c oxidase in aerobic respiratory chains present, for example, in *Bacillus subtilis* (Laureus *et al.*, 1991). The second cyt c-type haem in subunit II only found in cyt c oxidase from Desulfuvibionales would originate from an internal duplication of this cyt c domain, as suggested by the high sequence similarity between these two cyt c domains. Due to the fact that only one cyt c domain is present in subunit II in Desulfuromonadales, relocation of *coxII* within the *cox* operon would have occurred before duplication of the cyt c domain (Fig. 3).

**Table 2.** Enzyme activities in membranes of DvH

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Consumption or oxidation (nmol min^{-1} mg^{-1})</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2$ consumption</td>
<td>19</td>
<td>100% CO</td>
</tr>
<tr>
<td>NADH</td>
<td>14</td>
<td>100% CO, 100% 1.7 mM KCN</td>
</tr>
<tr>
<td>Ascorbate + cyt c</td>
<td>38</td>
<td>100% CO, 100% 2 mM KCN, 80% 25 mM HQNO</td>
</tr>
<tr>
<td>DBH$_2$</td>
<td>310</td>
<td>70% 60 µM HDQ</td>
</tr>
<tr>
<td>Menadiol</td>
<td>28</td>
<td>80% 3 mM KCN, 100% CO</td>
</tr>
<tr>
<td>Cyt c$_{553}$ oxidation</td>
<td></td>
<td>95% CO, 80% 3 mM KCN, 80% 40 µM HQNO</td>
</tr>
<tr>
<td>Cyt c$_{553}$ reduced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinol oxidation</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>
Oxygen reduction and various enzyme activities in membrane fractions of DvH

To further dissect the contributions of the different membrane-bound oxygen reductases in oxygen consumption activity, activities were measured in DvH membrane fractions prepared from cells cultured in lactate/sulfate medium under anaerobic conditions. When the decyl-ubiquinol DBH2 was added to the oxygraph chamber in the presence of membranes, oxygen was consumed at a rate of $38 \pm 4 \, \text{nmol min}^{-1} \,(\text{mg protein})^{-1}$, and KCN totally inhibited this activity (Fig. 4a, Table 2). A value of $310 \pm 24 \, \text{nmol O}_2 \, \text{min}^{-1} \,(\text{mg protein})^{-1}$ was obtained with menadiol (reduced vitamin K3) as electron donor (Table 2). Menadiol is a better substrate because it is a naphthoquinol like the endogenous menaquinol 6 present in DvH (Weber et al., 1970). In contrast to the hydrogen-dependent oxygen consumption on whole cells, the oxygen consumption activities in membranes with quinol was linear from O$_2$ saturation (250 $\mu$M) to O$_2$ depletion, and did not increase at low oxygen concentration (Fig. 4a). Addition of superoxide dismutase did not modify the rate of O$_2$ consumption, but catalase alone was able to lower the rate by about 15% using NADH, DBH2 and lactate as substrates (data not shown), showing that membranes are able to produce H$_2$O$_2$ at a low level. Oxidation of DBH2 was followed spectrophotometrically at 278/325 nm. Fig. 4(b) shows that membranes were unable to oxidize DBH2 in the absence of oxygen. DBH2 oxidation started when air was introduced into the cuvette (Fig. 4b). The oxidation rate of DBH2 was $30 \pm 3 \, \text{nmol min}^{-1} \,(\text{mg protein})^{-1}$ (Table 2). Note that the control experiment without membranes showed a very slow oxidation of quinol that was subtracted from the reported value (Table 2). Ubiquinol oxidation was inhibited by CO (Fig. 4b) as well as by KCN and 2-n-heptyl-4-hydroxyquinoline-N-oxide (HQNO) (Table 2). The data show that DvH membranes are able to consume oxygen with ubiquinol and menaquinol as substrates, and that quinol oxidation is oxygen-dependent. Activity inhibition patterns of CO, KCN and HQNO (Table 2) indicate that the quinol oxidase $bd$ is involved in oxygen reduction, as the reaction was inhibited in the presence of the quinol analogue HQNO [or HDQ (1-hydroxy-2-dodecyl-4(1H) quinolone), results not shown], whereas the cyt $c$ oxidases are sensitive to CO and KCN, but are not known to be sensitive to HQNO. Indeed, we measured a specific cyt $c_{553}$ oxidase activity of 28 nmol min$^{-1}$ mg$^{-1}$ that was inhibited by KCN and CO (Table 2).

The use of other substrates is reported in Table 2. NADH- and ascorbate-reduced cyt $c$ were efficient substrates for oxygen consumption by the membranes, while no activity

![Fig. 5. Spectral and HPLC analyses of DvH membranes.](image-url)

Notes: (a) Spectrum recorded at room temperature of the membrane fraction of DvH reduced with dithionite. The sample was suspended at 1 mg ml$^{-1}$ in 100 mM Tris/HCl, 150 mM NaCl buffer (pH 7.5). Note that the $\alpha$ and $\beta$ band region is amplified fivefold ($\times 5$) in comparison with the Soret region for a better visualization of the cyts. (b) Difference spectrum between ascorbate-reduced minus ferricyanide-oxidized sample of the DvH membrane fraction. The spectrum was recorded at liquid nitrogen temperature (77 K) with a slit of 0.1 nm and a light path of 0.2 cm. (c) Reversed-phase HPLC chromatograms of the membrane-bound haems in Desulfovibrio (DvH), E. coli and yeast (S. cerevisiae). Retention times for haems $b$, $a$ and $o$ are 13.5, 22.3 and 27 min, respectively. Haems were detected at 400 nm.
was detected when hydrogen, lactate, succinate or pyruvate was used (results not shown).

**Spectrophotometric and HPLC analyses of DvH membranes**

The spectrum of the membrane fraction reduced with dithionite revealed two absorbance peaks centred at 553 and 630 nm in the \( \alpha \) region of the visible spectra, specific for \( c \)- and \( d \)-type haems, respectively (Fig. 5a). The abundance of \( c \)-type haems in the membranes precluded the direct observation of \( b/o \)-type haems with a strong reductant such as dithionite. To better visualize the \( b/o \)-type haems present in the membrane fractions, samples were reduced with a weaker reducing agent (ascorbate) that does not reduce the numerous low midpoint redox potential (\( E_m \)) \( c \)-type cys in DvH. In addition, spectra were recorded at liquid nitrogen temperature over a small bandwidth. Under these conditions, a shoulder around 560 nm was visible, typical of the presence of \( b/o \)-type haems at 521 and 528 nm, respectively (Fig. 5b). Non-covalently bound haems were extracted from the membranes of DvH with acetone/HCl (Lübben & Morand, 1994) and reversed-phase HPLC showed that DvH does not contain \( a \)-type haem (present in the \( aa_3 \) cyt oxidase of *Saccharomyces cerevisiae*) but \( b \)- and \( o \)-type haems as in the \( bo_3 \) oxidase of *E. coli* (Fig. 5c). Additional data on pyridine-haemochrome spectra (not shown) confirmed the absence of \( a \)-type haem and the presence of \( b \)- and \( o \)-type haems in DvH.

Haem quantification from the dithionite-reduced fraction revealed that \( c \)-type haems were in the majority \( 2.2 \text{ nmol (mg protein)}^{-1} \); \( d \)- and \( b/o \)-type haems were present at 0.22 and 0.8 nmol (mg protein)\(^{-1} \), respectively. The spectroscopic and biochemical data presented above show that cyt \( c \) oxidase does not contain \( a \)-type haem in its subunit I but does contain \( b/o \)-type haems.

Binding of CO induces a shift in the maximum absorption of the haem groups present in the binuclear centre of the cyt \( c \) oxidase where oxygen reduction takes place (Bickar et al., 1984). In Fig. 6(a), the spectrum in the presence of CO (dotted line) exhibited a shoulder around 569 nm in comparison with the spectrum in the absence of CO (solid line). This bathochromic effect due to the binding of CO was reversed by bubbling the sample with O\(_2\) (dashed line, Fig. 6a). The difference spectrum obtained between a reduced sample in the presence of CO minus a reduced sample in the absence of CO (Fig. 6b) revealed a typical sigmoidal signal exhibiting a peak at 569 nm and a trough at 553 nm. The middle of this sigmoid was centred at 560 nm, which corresponded to \( o/b \)-type haems. The effects of CO binding on the \( \beta \) band absorbance of haems \( o/b \), with a red shift of the 530 nm absorbance, are also seen in Fig. 6(b). No sigmoid could be detected in the region around 605 nm in the difference spectrum (Fig. 6b), in
agreement with the absence of \(a\)-type haem in the membrane fraction. Fig. 6(b) shows a red shift from 630 to 640 nm in the presence of CO, characteristic of \(d\)-type haem. This indicated that CO was able to bind to the binuclear centre composed of haem \(b\) and haem \(d\) of the quinol oxidase \(bd\) and inhibited its activity (Table 2, Fig. 4b).

**Variations of gene expression levels**

To define the relative expression of the two different membrane-bound oxygen reductases, we measured the expression level of the genes corresponding to cyt \(c\) oxidase, the small soluble cyt \(c_{553}\) and the quinol oxidase \(bd\). We used primers in the \(coxI\) (DVU1815), \(cyf\) (DVU1817) and \(cydA\) (DVU3271) genes (Fig. 2) to detect their mRNA expression levels by qRT-PCR. When cells were grown under anaerobic conditions in lactate/sulfate medium and harvested in the mid-exponential phase, \(cyf\) was about 50-fold more highly expressed than \(coxI\) (Fig. 7a). Interestingly, \(cydA\) was about 36-fold more highly expressed than \(coxI\). Unexpectedly, these data showed that despite the anaerobic conditions of growth, both oxidases were expressed and that the \(cydAB\) genes were more highly expressed than the \(cox\) genes.

We then studied the effects of \(O_2\) on the expression of the two oxidase genes. Fig. 7(b) shows that exposure to high oxygen tension (21 and 100 %) for 1 h induced a repression of \(cydA\) expression (15- and sevenfold, respectively). The \(coxI\) gene was also downregulated, but to a lesser extent (about threefold under both conditions). At low oxygen tension (0.1 and 1 %), no significant effect on \(cydA\) expression was detected while expression of \(coxI\) was downregulated only slightly (Fig. 7b). These data indicate that expression of the two oxidase-encoding operons is differently regulated.

**DISCUSSION**

In whole cells of DvH, we measured a high \(O_2\) reductase activity with hydrogen as substrate which corresponded to up to 230 nmol \(O_2\) min\(^{-1}\) mg\(^{-1}\). This activity from hydrogen to oxygen involves periplasmic hydrogenases and cytochromes \(c\) with low \(E_m\) (Baumgarten et al., 2001; Fournier et al., 2004). This finding is strengthened by the complete inhibition of oxygen consumption in the presence of CO, which is a well-known inhibitor of hydrogenases (Vincent et al., 2005), and by the absence of oxygen consumption in the membrane fraction in the presence of \(H_2\) (results not shown). This oxygen reductase activity is of the same order as that measured in aerobic organisms. However, in contrast to the case of oxygen consumption in strictly aerobic organisms, the hydrogen-dependent oxygen reductase activity in DvH is inhibited by high oxygen concentration (>100 \(\mu\)M), and becomes higher as the concentration of \(O_2\) is lowered. On membrane fractions, we measured oxygen reductase activities of 14, 19 and 38 nmol \(O_2\) min\(^{-1}\) mg\(^{-1}\) with ascorbate + cyt \(c\), NADH and the ubiquinol analogue DBH\(_2\) as electron donor, respectively, and a higher value of 310 nmol \(O_2\) min\(^{-1}\) mg\(^{-1}\) with menadiol, a naphthoquinol analogue of the endogenous menaquinol 6 in DvH. Oxidation of quinol in membranes was revealed in the presence of oxygen, showing that in membranes of DvH, oxidation of the quinol pool is linked directly to the terminal acceptor oxygen by the quinol oxidase \(bd\) inhibited by HQNO (and HDQ, data not shown) and/or indirectly by an as-yet-unknown system (the \(bc_1\) complex is absent in DvH). Note that the membrane \(O_2\)-respiring system is active both at high and low oxygen levels, as known for the quinol and cyt \(c\) oxidases enzymes of the \(bd\) and haem-copper superfamilies. However, the relative affinities of these enzymes for \(O_2\) vary from very high, as exemplified by the FixNOQP-type oxidases \([cbb_3]\) oxidases with \(K_m\) for \(O_2\)
sequences (C216ISCH220 and C317LSCH321) are present in c species. Two additional consistent with the anaerobic lifestyle of cox the oxidase bd haem content of the two oxidases. Absorption spectra of 
Spectroscopic characterization allowed us to specify the haem content of the two oxidases. Absorption spectra of DvH, they are replaced by a/b-type haems. In addition, the absence in the genome of the haem a synthase gene (ctaA gene or haem a mono-oxygenase), and the absence of a proto- haem IX farnesyltransferase-encoding gene DVU1811 in the cox operon (Fig. 2a), strengthen this idea, which is also consistent with the anaerobic lifestyle of Desulfovibrio species. Two additional c-type haem binding consensus sequences (C216ISCH220 and C317LSCH321) are present in subunit II of cyt c oxidase.

Overall, our data therefore suggest that this cyt c oxidase is not of the cca_4-type, as proposed recently by Lobo et al. (2008), but rather is a novel cc(o/b)_3-type oxidase that is not known to have been reported so far, rendering it unique.

The gene DVU1817, encoding cyt c_553 (cyf), is located just upstream of the cox operon and is about 50 times more expressed at the mRNA level than the coxl gene (DVU1815). The presence of a 442 bp intergenic region separating cyf and scoI further suggests that they may not be in the same transcriptional unit (Fig. 2a). The proximity of the cyf gene to the cox operon, the conservation of this organization among Desulfovibrio species, and the reported c_553 oxygen reductase activity (Lobo et al., 2008) suggest that cyt c_553 is the electron donor for the cyt oxidase. Indeed, we measured a cyt c_553 oxidase activity in membranes of 28 nmol min \(^{-1}\) mg \(^{-1}\), a value that is about 200-fold lower than that reported recently by Lobo et al. (2008) (with a surprisingly high value of 5 \(\mu\)mol O\(_2\) min \(^{-1}\) mg \(^{-1}\): higher than values obtained in aerobic organisms). However, our data are in the same range as the oxygen reductase activities reported for Desulfovibrio species (Lemos et al., 2001; Ozawa et al., 1997; Santana, 2008). It remains to be elucidated how this soluble cyt c_553 is reduced, as no bc_1-type complex is present in the genome of Desulfovibrio species. The alternative bc_1 complex ‘Qrc’ was proposed recently to function in the reverse mode as a menaquinone reductase enzyme (Venceslau et al., 2010). The quinol oxidase bd uses menaquinol as reduced substrate, and the menaquinone pool can be replenished with electrons via membrane enzymes such as type II NADH dehydrogenase, and other menaquinone-reducing enzymes present in Desulfovibrio such as the Qrc (Venceslau et al., 2010) and Tmc complexes (Pereira et al., 2006) (Fig. 8).

The relative expression levels of the genes encoding the cyt oxidase (c) and the quinol oxidase bd (cydA) show that the latter gene cluster is more highly expressed (about 35-
fold) than the former in lactate/sulfate medium. This is in agreement with the higher abundance of the oxidase bd in the membrane fractions, as deduced from optical spectroscopy and the higher menaquinol oxygen reductase activity compared with the cyt c oxidase activity (310 versus 14 nmol O₂ min⁻¹ mg⁻¹, respectively). It is interesting to note that despite the absence of O₂ during anaerobic growth conditions, the genes encoding both terminal oxidases are expressed and might reflect a protective mechanism when Desulfovibrio species are transiently exposed to oxidative conditions. Exposure of cells to oxygen shows that expression of the genes encoding both oxidases is regulated in a dose-dependent manner, and that the extent of this regulation is quantitatively different for the two gene clusters. At low O₂ concentrations (0.1 %), both operons are expressed, and no important variation of expression was observed compared with anaerobic conditions. Under these conditions, the bd genes are still much more highly expressed than the cog genes. It should be noted that 0.1 % O₂ in the gas phase corresponds to 1.25 µM dissolved O₂ in the growth medium, which is about the upper limit for the viability of DvH cells (Johnson et al., 1997). The higher expression level of the bd oxidase genes correlates with the higher affinity of this enzyme for O₂ compared with the classical cyt c oxidase.

The genomes of Desulfovibrio species sequenced so far (DvH, RCH1, DP4, Miyazaki, Desulfovibrio desulfuricans, Desulfovibrio magnetis, Desulfovibrio fructosovorans, Desulfovibrio piger, Desulfovibrio sp. FW1012B), as well as those of Geobacter (Methé et al., 2003) and Pelobacter species, contain a cyt c oxidase and a quinol oxidase bd, despite their strictly anaerobic form of life. The common anaerobic ancestor of the Desulfovibrionales and Desulfuromonadales in the Deltaproteobacteria would thus have possessed these two membrane-bound enzymes. Their presence allows these bacteria to cope with transient exposure to oxygen in their biotopes. Although Desulfovibrio species are not able to respire O₂ or use it as a sole terminal acceptor for growth, the proton motive force generated by the quinol oxidase bd and the cyt c oxidase activity could contribute at least partially to energy production in these organisms, in addition to their role in detoxifying traces of oxygen.

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