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 *o*-, *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 thus of the *cc(o/b)o₃* type, a type not previously described. The monohaem cytochrome *c*₅₅₃ 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.

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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

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Abbreviations: cyt, cytochrome; DBH₂, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol; DB, oxidized DBH₂; DvH, *Desulfovibrio vulgaris* Hildenborough; *E*_m, midpoint redox potential; HDQ, 1-hydroxy-2-dodecyl-4(1H) quinolone; HQNO, 2-n-heptyl-4-hydroxyquinoline-*N*-oxide; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; SRB, sulfate-reducing bacteria.

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.*, 1993), morphological changes (Sass *et al.*, 1998a) and inhibition of sulfate reduction (Krekeler *et al.*, 1997) are observed with time when cells are exposed to oxygen, showing that oxygen is toxic to SRB.

SRB might have developed adaptation strategies to protect them against oxygen, including oxygen removal. The ability to reduce oxygen is widespread among SRB (Dannenbergh *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 oxidoreductase (Frazão *et al.*, 2000; Santos *et al.*, 1993; Wildschut *et al.*, 2006) and a periplasmic reduction involving hydrogenases and cytochromes (cyts) (Baumgarten *et al.*, 2001; Fournier *et al.*, 2004). The first evidence for a membrane-bound terminal oxygen reductase in SRB was reported by Lemos *et al.* (2001). Those authors described a canonical quinol oxidase *bd* from membrane fractions of *Desulfovibrio gigas* grown in fumarate/sulfate medium. A *cyt c* oxidase-encoding gene has also been found in *Desulfovibrio vulgaris* Miyazaki, downstream of the gene encoding *cyt c*₅₅₃ (Kitamura *et al.*, 1995). In *D. vulgaris* Hildenborough (DvH), it has been shown that the *cyt c* oxidase, named *ccaa*₃, 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 *cox* 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-Aminco 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 α 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. Haems were extracted from membranes with acetone/HCl (19:1), as in Lübken & 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 × 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 (Gilson oxygraph) 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), menadiol (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 *c*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 *c*₅₅₃, respectively. Any abiotic or chemical reaction (especially with menadiol) 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.

RNA isolation and quantitative real-time PCR (qRT-PCR). For RNA quantification, DvH cultures were grown under anaerobic conditions in lactate/sulfate medium at 33 °C to mid-exponential phase (OD₆₀₀ ~0.4). Cultures were split into two parts: for experiments with O₂ stress, 0.1–100 % O₂ was then added at a gas flux of about 100 ml min⁻¹ for 1 h in cultures of 40 ml. The desired O₂ concentration was obtained by mixing pure nitrogen gas with filtered air (from 0.1 to 21 % O₂) or with pure O₂ (100 % O₂) in a dual-valve gas mixer (Aalborg). O₂ concentration was measured precisely with a Mettler-Toledo M700 recorder with an O₂ module ppb 4700 and a calibrated Inpro 6900 O₂ probe. After oxygen exposure, 8 ml of three independent cultures was withdrawn. Cells were then washed with 100 mM Tris/HCl, 150 mM NaCl buffer (pH 7.5), and frozen to -80 °C before RNA extraction. The second part of the culture that corresponded to the control experiments (untreated cells in triplicate) was kept under anaerobiosis without any addition of oxygen for 1 h. Cells were then harvested to proceed with RNA isolation. Isolation of total RNA, cDNA synthesis and qRT-PCR were performed as previously described (Brioukhanov *et al.*, 2010), except that the last step of cDNA purification on a YM-30 centrifugal filter was omitted. Primers were designed to specifically amplify a fragment of about 200 bp of each targeted gene. Primers used for qRT-PCR were: 1815dir (*coxI* gene or *DVU1815*), 5'-GAGCACGGCTGTACTACTCA-3'; 1815rev, 5'-CCCC-TGTTGTACCAGCATCT-3'; 3271dir (*cydA* gene or *DVU3271*), 5'-CGGTGAGGAAGTTGTGCGAGT-3'; 3271rev, 5'-GGACAAGGTGAG-CAAGAAGG-3'; 1817dir (*cyf* gene or *DVU1817*), 5'-CCGCACTC-TACAAGTCATGC-3'; 1817rev, 5'-TATTTCCTTGACGGCGTTGGT-3'; 16Sdir (16S RNA), 5'-GAGCATGCTGATCTCGAATTACTA-3'; 16Srev, 5'-CCTAGGGCTACACAGTACTACAA-3'. The Relative Expression Software Tool (REST) was used to calculate the relative expression of each gene in each condition (Pfaffl *et al.*, 2002), with the 16S RNA gene as reference for normalization. Quantification was performed in duplicate or triplicate for each cDNA preparation.

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

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.

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

Table 1. Oxygen consumption activity in DvH cells

ND, Not detectable.

Substrate	O ₂ consumption (nmol min ⁻¹ mg ⁻¹)
H ₂ *	230 (O ₂ concentration <~40 µM), ND (O ₂ concn >~100 µM)
Lactate	48
Ascorbate + cyt <i>c</i>	3.5
DBH ₂	3
NADH	5
Succinate	1.5
Pyruvate	ND

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

consumption (Fig. 1b). At low oxygen concentration (about 40 µM), the oxygen consumption rate was 230 ± 30 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 ± 4 nmol O₂ min⁻¹ mg⁻¹ (Table 1), and this activity was completely inhibited by CO and KCN (data not shown). When other substrates (NADH or DBH₂) were used as electron donors, the oxygen consumption rates were at least one order of magnitude lower than that observed in the presence of H₂ (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 O₂

consumption, and thus we cannot exclude that reduction of O₂ 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 H₁₈/E₃₇₅ for one *b* haem (equivalent to haem *b*₅₉₅ in *Escherichia coli*) and H₁₈₃/M₃₂₅ 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 *coxI* (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 O₂ takes place. *DVU1812* encodes the catalytic subunit II (CoxII), which contains the Cu_A centre with two copper atoms ligated to the conserved residues H₁₃₅, C₁₇₀, E₁₇₂, C₁₇₄, H₁₇₈ and M₁₈₁. Sequence analysis revealed that this subunit contains two binding consensus sequences of the CXXCH type (C₂₁₆ISCH₂₂₀ and C₃₁₇LSCH₃₂₁), 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

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 Desulfovibrionales 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).

Fig. 4. Quinol oxidase activities of DvH membranes. (a) O₂ consumption of DvH membranes (0.25 mg ml⁻¹) with ubiquinol (DBH₂) (110 μM) as substrate in 100 mM Tris/HCl, 150 mM NaCl buffer (pH 7.5) at 30 °C. KCN was added at 2 mM. (b) Decyl-ubiquinol (DBH₂) oxidation measured spectrophotometrically (in dual mode at the wavelength pair 278/325 nm). Membranes of DvH (0.042 mg ml⁻¹) were incubated at 30 °C in 100 mM Tris/HCl, 150 mM NaCl buffer (pH 7.5) in anaerobiosis, and 40 μM quinol was added. Addition of air is indicated by an arrow.

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 Cu_A 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 Desulfovibrionales (Fig. 3), and not in the Desulfuromonadales (*Geobacter* and *Pelobacter* species), which do not contain two *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 Desulfovibrionales and Desulfuromonadales in the Deltaproteobacteria. A *c*-type haem is present in subunit II of the *caa₃* cyt *c* oxidase in aerobic respiratory chains present, for example, in *Bacillus subtilis* (Lauraus *et al.*, 1991). The second *c*-type haem in subunit II only found in cyt *c* oxidase from Desulfovibrionales 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

Substrate	Consumption or oxidation (nmol min ⁻¹ mg ⁻¹)	Inhibition
O ₂ consumption		
NADH	19	100 % CO
Ascorbate + cyt <i>c</i>	14	100 % CO, 100 % 1.7 mM KCN
DBH ₂	38	100 % CO, 100 % 2 mM KCN, 80 % 25 μM HQNO
Menadiol	310	70 % 60 μM HDQ
Cyt <i>c</i> ₅₅₃ oxidation		
<i>c</i> ₅₅₃ reduced	28	80 % 3 mM KCN, 100 % CO
Quinol oxidation		
DBH ₂	30	95 % CO, 80 % 3 mM KCN, 80 % 40 μM HQNO

Fig. 5. Spectral and HPLC analyses of DvH membranes. (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 α and β 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 .

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 $310 \pm 24 \text{ nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ 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 O₂ saturation ($250 \mu\text{M}$) to O₂ depletion, and did not increase at low oxygen concentration (Fig. 4a). Addition of superoxide dismutase did not modify the rate of O₂ 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 H₂O₂ 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 $28 \text{ nmol min}^{-1} \text{ mg}^{-1}$ 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

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 α 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 cyts 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 (Fig. 5b). *d*-Type haem was detected by its absorbance centred at 630 nm. Note that the β band also showed specific absorptions for *c*- and *b*-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 β 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

Fig. 6. CO binding to DvH membranes. (a) Spectra of DvH membranes reduced with dithionite (solid line), with dithionite and bubbled with CO (dotted line), and with dithionite bubbled with CO and reversed with pure O_2 (dashed line). Note that the α and β band region is amplified threefold ($\times 3$) in comparison with the Soret region for a better visualization of the cyts. (b) Difference spectrum between the dithionite-reduced sample bubbled with CO minus the dithionite-reduced sample. Spectra were recorded at room temperature with a slit of 2 nm. Specific wavelengths are indicated by arrows.

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 O₂ 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 O₂ reductase activity with hydrogen as substrate which corresponded to up to 230 nmol O₂ 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 H₂ (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 µM), and becomes higher as the concentration of O₂ is lowered. On membrane fractions, we measured oxygen reductase activities of 14, 19 and 38 nmol O₂ min⁻¹ mg⁻¹ with ascorbate + cyt *c*, NADH and the ubiquinol analogue DBH₂

Fig. 7. Expression levels of the *coxI*, *cydA* and *cyf* genes determined by qRT-PCR. (a) Expression ratios relative to the *coxI* gene (*DVU1815*) in cells harvested at the mid-exponential phase grown on lactate/sulfate under anaerobic conditions. (b) Ratio of gene expression in cells grown on lactate/sulfate in mid-exponential phase and exposed to variable concentrations of O₂ for 1 h to that of the control without O₂.

as electron donor, respectively, and a higher value of 310 nmol O₂ 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 *bc₁* complex is absent in DvH). Note that the membrane O₂-respiring system is active both at high and low oxygen levels, as known for the quinol and cyt *c* oxidase enzymes of the *bd* and haem-copper superfamilies. However, the relative affinities of these enzymes for O₂ vary from very high, as exemplified by the *FixNOQP*-type oxidases [*cbb₃* oxidases with *K_m* for O₂

<10 nM (Pitcher *et al.*, 2002)], to much weaker in the micromolar range for the *aa₃*-type cyt *c* oxidases in aerobic organisms. The quinol oxidases *bd* exhibit high affinity for O₂ (about 10–100 nM), and have been shown to protect oxygen-labile nitrogenase in *Azotobacter vinelandii* during nitrogen fixation (Jünemann, 1997).

Spectroscopic characterization allowed us to specify the haem content of the two oxidases. Absorption spectra of the oxidase *bd* revealed haem *d* at 630 nm, which is red-shifted to 640 nm in the presence of CO. For cyt *c* oxidase, spectroscopic and HPLC data showed the absence of *a*-type haems usually present in aerobic organisms. In DvH, they are replaced by *o/b*-type haems. In addition, the absence in the genome of the haem *a* synthase gene (*ctaA* gene or haem *o* mono-oxygenase), and the presence 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 *caa₃*-type, as proposed recently by Lobo *et al.* (2008), but rather is a novel *cc(o/b)o₃*-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 *coxI* 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 μmol O₂ 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 *bc₁*-type complex is present in the genome of *Desulfovibrio* species. The alternative *bc₁* 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 (*coxI*) and the quinol oxidase *bd* (*cydA*) show that the latter gene cluster is more highly expressed (about 35-

Fig. 8. Model of oxygen reduction and detoxification in cells of DvH. The cytoplasmic detoxification system includes the NADH rubredoxin-oxidoreductase (NRO), the small iron-containing rubredoxin (Rd) and the flavodiiron-containing protein rubredoxin-oxygen-oxidoreductase (ROO), which reduces O₂ to water. The periplasmic system is composed of soluble hydrogenases which reduce low-redox-potential *c*-type cyts, which in turn detoxify O₂. The membrane-bound system is composed of the quinol oxidase *bd* and the cyt *c* oxidase *cc(o/b)o₃*, which reduce O₂ to water and are likely involved in energy production. Hyd, hydrogenase; cyt, low-redox-potential cyts; Ndh, type II NADH dehydrogenase; Q/QH₂, menaquinone/menaquinol pool; Qred.TMC, menaquinone-reducing transmembrane complexes (Qrc, TMC, etc.). This figure summarizes results of the present study and previous studies, and is adapted from Dolla *et al.* (2007).

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 *cox* 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 magneticus*, *Desulfovibrio fructosovorans*, *Desulfovibrio piger*, *Desulfovibrio* sp. FW1012B), as well as those of *Geobacter* (Méthé *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 Desulfovibriales 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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