Membrane-bound oxygen reductases of the anaerobic sulfate-reducing *Desulfovibrio vulgaris* Hildenborough: roles in oxygen defence and electron link with periplasmic hydrogen oxidation

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Cytoplasmic membranes of the strictly anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough contain two terminal oxygen reductases, a *bd* quinol oxidase and a *cc(b/o)o*3 cytochrome oxidase (*Cox*). Viability assays pointed out that single *Dbd* and *Dcox* and double *DbdDcox* deletion mutant strains were more sensitive to oxygen exposure than the WT strain, showing the involvement of these oxygen reductases in the detoxification of oxygen. The *Dcox* strain was slightly more sensitive than the *Dbd* strain, pointing to the importance of the *cc(b/o)o*3 cytochrome oxidase in oxygen protection. Decreased *O2* reduction rates were measured in mutant cells and membranes using lactate, NADH, ubiquinol and menadion as substrates. The affinity for oxygen measured with the *bd* quinol oxidase (*Km, 300 nM*) was higher than that of the *cc(b/o)o*3 cytochrome oxidase (*Km, 620 nM*). The total membrane activity of the *bd* quinol oxidase was higher than that of the cytochrome oxidase activity in line with the higher expression of the *bd* oxidase genes. In addition, analysis of the *DbdDcox* mutant strain indicated the presence of at least one *O2*-scavenging membrane-bound system able to reduce *O2* with menaquinol as electron donor with an *O2* affinity that was two orders of magnitude lower than that of the *bd* quinol oxidase. The lower *O2* reductase activity in mutant cells with hydrogen as electron donor and the use of specific inhibitors indicated an electron transfer link between periplasmic *H2* oxidation and membrane-bound oxygen reduction via the menaquinol pool. This linkage is crucial in defence of the strictly anaerobic bacterium *Desulfovibrio* against oxygen stress.

INTRODUCTION

Both physiological and phylogenetic studies of microbial diversity suggest that anaerobic and thermophilic microorganisms are the oldest forms of life on Earth, dating back to when the atmosphere was hot and reductive. The rise in atmospheric oxygen, which occurred about 2.3 gigayears ago, when oxygenic photosynthesis arose, changed the course of biological evolution. The main challenge for anaerobic forms of life was to protect themselves against damage induced by this strong oxidizer and eventually to use it as an energy source. Many microorganisms developed the capability to reduce oxygen to water. Systems dedicated to this reaction include the membrane-bound terminal haem-copper oxygen reductases (Castresana et al., 1994). A second unrelated family of enzymes is composed of the *bd*-type quinol oxidase enzymes and has been shown to be involved more in oxygen detoxification than in energy production (Poole & Hill, 1997). The quinol *bd* oxidase was also described for its reactivity towards nitric oxide (Giuffrè et al., 2012) and is associated with a peroxidase activity (Borisov et al., 2010). In the course of the evolutionary challenge to adapt to the

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Abbreviations: *DvH*, *Desulfovibrio vulgaris* Hildenborough; cyt., cytochrome; HQNO, 2-n-heptyl-4-hydroxyquinoline-N-oxide; HQQ, 1-hydroxy-2-dodecyl-4(1H) quinolone; *E*<sub>m</sub>, mid-point redox potential; *E*<sub>0</sub>, standard redox potential at 1 M, pH 7; DBH<sub>2</sub>, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol; DB, oxidized DBH<sub>2</sub>; KCN, potassium cyanide; CO, carbon monoxide; NO, nitric oxide; SRB, sulfate-reducing bacteria.

Three supplementary figures and one supplementary table are available with the online version of this paper.
presence of increasing \( \text{O}_2 \) levels, aerobic micro-organisms succeeded in using oxygen in complex high energy-yielding respiratory chains that include a terminal oxidase of the haem-copper superfamily (Pereira et al., 2001). Surprisingly, genome analysis indicates that some strictly anaerobic micro-organisms such as Geobacter, Pelobacter and Desulfovibrio species contain genes encoding such enzymes (Lamrabet et al., 2011). While their function is easily understandable in aerobic micro-organisms, the role of this enzyme in anaerobic forms of life is intriguing. Most of the Desulfovibrio species are especially interesting as both \( bd \)-type quinol oxidase and haem-copper oxidase are present together. Desulfovibrio species belong to the large and diverse group of the sulfate-reducing bacteria (SRB). SRB are one of the most studied organisms with regard to aerobic-tolerance mechanisms. SRB, which have been regarded for many years as strict anaerobes, are universally distributed in marine and fresh water sediments where sulfate reduction is the dominant bimineralization pathway. SRB gain energy for biosynthesis and growth by coupling oxidation of organic compounds or molecular hydrogen to reduction of sulfate into sulfide (Thauer et al., 2007).

Despite the anaerobic nature of this metabolic process, SRB activity is not confined to permanently anaerobic habitats. Abundance and metabolic activity of SRB in oxic zones of numerous biotopes are frequently evaluated to be higher than in neighbouring anoxic zones (Mußmann et al., 2005; Ravenschlag 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 subjected to regular periods of oxygen exposure (Ito et al., 2002; Kjeldsen et al., 2004). 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, viability and cell motility decreases (Marschall et al., 1993), and morphological changes (Sass et al., 1998a) and inhibition of sulfate reduction (Krekeler et al., 1998) are observed with time when cells are exposed to oxygen, revealing the oxygen toxicity.

SRB have developed adaptation strategies to protect against oxygen, including oxygen removal. The first evidence for a membrane-bound terminal oxygen reductase in SRB was reported by Lemos et al. (2001). The authors described a canonical \( bd \) quinol oxidase from membrane fractions of Desulfovibrio gigas. A cytochrome (cyt.) \( c \) oxidase encoding gene has also been found in Desulfovibrio vulgaris Miyazaki, downstream of the gene encoding cyt. \( \varepsilon_{53} \) (Kitamura et al., 1995). The presence of genes encoding both membrane-bound oxygen reductases in several Desulfovibrio isolates from salt-marsh sediments was shown by hybridization and PCR experiments (Santana et al., 2008). In addition to these membrane-bound oxygen reductases, two other systems account for oxygen reduction by Desulfovibrio species: a cytoplasmic reduction chain involving a rubredoxin oxygen oxidoeductase (Frazão et al., 2000; Santos et al., 1993; Wildschut et al., 2006) and a periplasmic reduction involving hydrogenases and cytochromes (Baumgarten et al., 2001; Fournier et al., 2004).

Recently, we showed the presence of both a \( bd \) quinol oxidase and a cyt. \( c \) oxidase in the membrane fraction of Desulfovibrio vulgaris Hildenborough (DvH) and reported their spectroscopic and biochemical properties (Lamrabet et al., 2011). We pointed out that the cyt. \( c \) oxidase does not contain \( a \) but instead \( o/b \)-type haems in subunit I as well as two \( c \)-type haems in subunit II (Lobo et al., 2008) and is therefore the first described \( cc(b/o)o\)-type haem-copper oxidase (Lamrabet et al., 2011). Relative transcription levels of the genes encoding the cyt. \( c \) oxidase or the \( bd \) quinol oxidase showed that the latter genes were more highly expressed than the former (about 35-fold) in lactate–sulfate medium under anaerobic conditions in mid-exponential phase, which was in agreement with the higher abundance of the \( bd \) oxidase in membrane fractions, as deduced from optical spectroscopy (Lamrabet et al., 2011).

In the present study, we describe the phenotypic analysis of single \( \Delta bd \), \( \Delta cox \) deletion mutants of the genes encoding the \( bd \) quinol oxidase and the \( cc(b/o)o\), cyt. \( c \) oxidase (\( cox \)), respectively, as well as the double deletion mutant \( \Delta bd\Delta cox \). Growth phenotypes and survival in oxidative stress as well as measurements of the oxygen reductase activities in both whole cells and membrane fractions are reported, allowing further insights into the function of these membrane-bound oxidases in DvH. In addition, we describe for the first time an electron transfer communication between the periplasmic hydrogenases/cytochrome system and the membrane-bound oxygen reduction.

**METHODS**

**Construction of the \( \Delta bd \) and \( \Delta cox \) deletion mutants.** The 500 bp region upstream from the \( bd \) genes (locus tags DVU3270, DVU3271) was PCR amplified with primers p243-f and p246-r (Table S1, available in Microbiology Online), cleaved with \( PstI \) and \( BamHI \), and ligated to similarly digested pNOT19 to generate pNOT243/246. The 500 bp region downstream from the \( bd \) genes was amplified with p245-f and p244-r (Table S1), digested with \( BamHI \) and \( KpnI \), and ligated to similarly cleaved pNOT243/246 to generate pNOT\( \Delta bd\). Insertion of the \( cat \) gene-containing \( BamHI \) fragment from pUC19Cm into pNOT\( \Delta bd\) gave pNOT\( \Delta bd\)Cm, and insertion of the 4.5 kb \( NotI \) fragment from pMOB2 gave pNOT\( \Delta bd\)CmMob containing the gene coding for the levansucrase. It was transferred to \( D. vulgaris \) by conjuguative with \( E. coli \) S17-1 on \( E \) plates containing chloramphenicol and kanamycin as described elsewhere (Fu & Vooroud, 1997). A selected single crossing-over integrant was grown in the presence of sucrose (which selected positively the double crossing-over event with the removal of the levansucrase gene since this latter led to the production of a toxic product for \( DvH \) cells in the presence of sucrose) and chloramphenicol to obtain the \( \Delta bd \) deletion mutant strain (\( D. vulgaris \) CBD100, Table 1).

The same strategy was used to obtain the \( \Delta cox \) deletion mutant strain, by using primers p248-f and p251-r to amplify the 500 bp region upstream of the \( cox \) genes (locus tags DVU1810–1816) and primers p267-f and p266-r to amplify the 500 bp region downstream of the \( cox \) genes (\( D. vulgaris \) COX100, Table 1).
The ΔbdΔcox double deletion mutant was obtained by taking the previously constructed Δbd mutant and deleting the cox genes, replacing them with the gene encoding antibiotic resistance to G418 sulfate (Table 1).

**Table 1. Strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Antibiotic resistance</th>
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<tbody>
<tr>
<td>WT DvH</td>
<td><em>D. vulgaris</em> WT strain NCIMB 8303, isolated from clay soil near Hildenborough, UK (Postgate, 1984)</td>
<td>Natural resistance to kanamycin (50 μg ml⁻¹)</td>
</tr>
<tr>
<td>Δcox</td>
<td><em>D. vulgaris</em> COX100 deleted of the cox operon coding for the cc(b/o)o₃ cyt. oxidase, Suc' G418; this study</td>
<td>G418 (400 μg ml⁻¹), kanamycin (50 μg ml⁻¹)</td>
</tr>
<tr>
<td>Δbd</td>
<td><em>D. vulgaris</em> CBD100 deleted of the bd operon coding for the bd quinol oxidase, Suc' Tm' G418; this study</td>
<td>Thiampenicol (20 μg ml⁻¹) (analogue of chloramphenicol), kanamycin (50 μg ml⁻¹)</td>
</tr>
<tr>
<td>ΔbdΔcox</td>
<td><em>D. vulgaris</em> CC100 deleted of both the cox and bd operons, Suc' Tm' G418; this study</td>
<td>G418 (400 μg ml⁻¹), thiampenicol (20 μg ml⁻¹), kanamycin (50 μg ml⁻¹)</td>
</tr>
<tr>
<td>Δqrc</td>
<td><em>D. vulgaris</em> JW8151 deleted of the qrcABCD operon (Judy Wall, unpublished data)</td>
<td>G418 (400 μg ml⁻¹), kanamycin (50 μg ml⁻¹)</td>
</tr>
<tr>
<td>ΔqrcΔbd</td>
<td><em>D. vulgaris</em> deleted of both the qrcABCD and bd operons, Tm' G418; this study</td>
<td>G418 (400 μg ml⁻¹), thiampenicol (20 μg ml⁻¹), kanamycin (50 μg ml⁻¹)</td>
</tr>
<tr>
<td>E. coli S17-1</td>
<td>thi pro hsdR hsdM+ recA RP4-2 (Tc:: Mu, Km:: Tn7)</td>
<td></td>
</tr>
</tbody>
</table>

**Bacterial growth conditions.** The sulfate-reducing bacterium DvH was grown at 33 °C in liquid lactate/sulfate (medium C) under anaerobic conditions in 10 ml Hungate tubes up to 2 l flasks which were inoculated at 10% (v/v) as described previously (Postgate, 1984; Voordouw et al., 1989). Growth was monitored by following the optical density at 600 nm. Growth on solid medium E (Postgate, 1984; Voordouw et al., 1989) was performed on plates under gas exchange conditions in a Coy anaerobic chamber with a 10% H₂/90% N₂ mixed gas atmosphere. Deletion mutant strains were cultured in the same conditions as the WT strain in the presence of the appropriate antibiotic (Table 1); however, for growth analyses and viability tests, antibiotics were omitted.

**Viability tests after oxygen exposure.** Cultures of the WT and of the deletion mutants (Δbd, Δcox and ΔbdΔcox) of DvH were grown in anaerobic conditions to an OD₆₅₀ of 0.6 in medium C. A 500 μl sample of each culture was used to inoculate Erlenmeyer flasks containing 50 ml of fresh medium C previously equilibrated under atmospheric air conditions. Cultures were then shaken (150 r.p.m.) at 33 °C for 4, 8 and 24 h in air. The same experiment was also carried out at a lower, 0.1% O₂ concentration: cultures in Hungate tubes were bubbled for 8 and 24 h at 33 °C with a flux of 0.825 l h⁻¹ of 99.9% N₂ and 0.1% O₂ (40 μg ml⁻¹) using a gas mixer (Pegas 4000 MF, Columbus instruments). Aliquots of these cultures were taken at the times mentioned above and diluted serially to 10⁻⁵ in anaerobic medium C. A 100 μl sample of each dilution was spread on solid medium E plates. The cfu were counted after 5 days of anaerobic incubation at 33 °C in the Coy anaerobic chamber. The number of cfu ml⁻¹ was compared with that obtained for a sample not exposed to oxygen and experiments were carried out in triplicate.

**Cell and membrane preparations.** Cells and membranes were prepared as described previously (Lamrabet et al., 2011). In the case of O₂ reduction with hydrogen as an electron donor, cells were prepared in anaerobic buffer degassed with argon for all steps to prevent any inactivation of hydrogenases with O₂.

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

**Oxygen reduction, quinol and cyt. c oxidation measurements and determination of Kᵣ.** O₂ reductase activities on whole DvH cells or membrane fractions were measured polarographically with a Clark-type electrode in a stirred volume of 1.8 ml of 150 mM NaCl, 100 mM Tris/His buffer (pH 7.5) saturated with air (237 μM O₂ at 30 °C) with different electron donors: H₂, NA DH (3 mM), decyl-ubiquinol (DBH₂; 100 μM), menadiol (50 μM) or lactate (100 mM). In the case of H₂-dependent O₂ reductase activity in cells, the buffer was saturated with H₂ and small O₂ bursts were applied as described in Lamrabet et al. (2011) to induce O₂ reduction with a total O₂ concentration lower than 40 μM to prevent hydrogenase inactivation.

Quinol oxidase activity in DvH membranes was measured with DBH₂ (40 μM) as a substrate in 150 mM NaCl, 100 mM Tris/His/HCl buffer (pH 7.5) at 30 °C. Oxidation of quinol 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. DvH cyt. c₅₅₃ oxidation was monitored at the wavelength pair 553/540 nm and auto-oxidation with O₂ was subtracted for the determination of enzymatic activity. Extinction coefficients of 16 and 29 mM⁻¹ cm⁻¹ were used for calculations of oxidized DBH₂ (DB) and reduced cyt. c₅₅₃, respectively. Any abiotic or chemical reaction (especially with menadion) was subtracted to obtain the enzymatic activity. For the O₂ Kᵣ determination of the bd quinol oxidase, the quinol (in excess, 40 μM) oxidation was followed as described above with injection of increasing small amounts of O₂ contained in air-saturated water (258.5 μM O₂ at 25 °C). DBH₂ was used as a substrate and not menadion due to its high auto-oxidation rate. Kinetics data obtained were plotted with a Lineweaver–Burk representation for the determination of the Kᵣ value. For the O₂ Kᵣ determination of the cc(o/b)o₃ cyt. oxidase, the pre-reduced cyt. c₅₅₃ (16 μM) oxidation was followed at 553/540 nm after injection of increasing small amounts of O₂ contained in air-saturated water and data were plotted with a Lineweaver–Burk representation. For the low affinity O₂ measurement, this was followed directly on the oxygraph with O₂ reduction rate plotted against O₂ concentration and Kᵣ determined as described above.
RESULTS

Characterization of the deletion mutant strains

The quinol bd oxidase is encoded by an operon containing the two genes cydA (DVU-3271) and cydB (DVU-3270) whereas the cyt. c oxidase cc(b/o)o3 is encoded by an operon (DVU1816 to DVU1810) containing the four genes encoding the structural subunits I–IV and three additional genes involved in its biogenesis (Lamrabet et al., 2011). Effects of the gene deletions on growth under anaerobic conditions were evaluated for each deletion mutant strain (Fig. S1). The WT and the Δbd mutant had similar specific growth rate constants (μ of 0.133 h⁻¹ and 0.130 h⁻¹, respectively) as determined on a semi-logarithmic representation (not shown). These strains also had a similar final cell density (OD₆₀₀ of 0.9 ± 0.05 SD, n=5 and 0.89 ± 0.04 SD, n=5, respectively). The Δcox deletion mutant had a slightly lower specific growth rate constant (μ of 0.116 h⁻¹) and a lower final cell density (OD₆₀₀ of 0.74 ± 0.03) in comparison with the WT strain, even though lactate was completely consumed at stationary phase by both strains (data not shown). Surprisingly, the ΔbdΔcox double deletion strain had a specific growth rate constant and final cell density similar to those of the WT strain (μ of 0.130 h⁻¹ and OD₆₀₀ of 0.9 ± 0.05, respectively). For unknown reasons, the deletion of the bd genes in the Δcox mutant restored a WT growth phenotype under anaerobic conditions. These data show that the deletion of the genes encoding the two membrane-bound oxidases has no effect or only weak effects on growth parameters under anaerobic conditions.

Optical spectroscopy of the membranes in the deletion mutants showed that the Δbd and ΔbdΔcox deletion mutants strains, devoid of the bd oxidase, were missing d-type haem absorbing at 630 nm, whereas the Δcox mutant devoid of the cc(b/o)o3 cyt. oxidase exhibited a slightly higher quantity of haem d (35 % more) compared to the WT strain (Fig. S2).

In order to test whether the deletion of the genes encoding one oxidase would be compensated by increased expression of the other oxidase, the mRNA expression level was measured by Real Time quantitative PCR (RT-qPCR) of the corresponding genes in the single deletion mutant strains under anaerobic conditions. No significant changes in the expression of the coxl gene were detected in the Δbd mutant (Fig. S3); however, a twofold increase of cydA gene (coding for the bd quinol oxidase subunit I) expression was detected in the Δcox strain. This correlates with the slightly higher content of haem d in the Δcox strain (Fig. S2).

Effect of oxygen exposure on survival of terminal oxidase deletion mutants

In order to understand the in vivo function of each terminal oxidase, the viability of each deletion mutant strain was evaluated after exposure to either low (0.1 % O₂) or high (air exposure, 21 % O₂) oxygen concentration. When the cells were exposed to a low oxygen concentration (0.1% O₂, corresponding to 1.25 μM of dissolved O₂, Fig. 1a), no decrease in survival (ability to make colonies on plates when switched to anaerobic conditions) of the WT strain was observed while the deletion mutant strains had a lower survival rate. After 24 h of exposure, the deletion mutant strains exhibited 30–70% survival: the Δcox deletion mutant strain was slightly more affected than the Δbd strain and the ΔbdΔcox strain was the most affected.

WT strain exhibited quite a strong resistance to air exposure (228 μM O₂ at 33 °C) with 80 % and 50 % of the cells being able to make colonies on plates under anaerobic conditions after 8 h and 24 h of air exposure, respectively. However, survival in air was lower than for cells exposed to 0.1 % O₂ (Fig. 1a, b), as expected. The single and double deletion mutant strains were more strongly affected with less than 50 % of cells being able to make colonies after 4 h of air exposure, conditions which gave 90 % survival of the WT. Survival of the deletion mutant strains was even lower after 8 h. After 24 h of air exposure, none of the deletion mutant strains was able to make colonies on plates under anaerobic conditions, except the Δbd deletion strain which had a 3 % survival. The WT strain still exhibited 50 % survival under these conditions. It appears that the Δcox deletion mutant strain was slightly more affected than the Δbd strain (Fig. 1a, b).

These data show that both the bd quinol oxidase and the cyt. c oxidase have the important function of protecting the cells from damage at low or high oxygen concentrations.

Oxygen reduction activities in whole cells

Measurements of oxygen reduction in washed whole cells indicate the in vivo rate at which the WT and deletion mutant strains detoxify the oxidizing agent O₂. The WT strain had an activity of 38 nmol O₂ min⁻¹ (mg protein)⁻¹ with lactate as the electron donor (Fig. 2). This activity was strongly decreased in the Δbd, Δcox and ΔbdΔcox deletion mutant strains (14, 8 and 7 nmol O₂ min⁻¹ (mg protein)⁻¹, respectively). A similar pattern was obtained with cells in growth medium C that contained lactate as an electron donor and sulfate as an electron acceptor: relative to the WT strain (41 nmol O₂ reduced min⁻¹ mg⁻¹), a strong decrease in O₂ reduction was observed in the Δbd, Δcox and ΔbdΔcox strains (8, 7 and 6 nmol O₂ reduced min⁻¹ mg⁻¹, respectively). Results of O₂ reduction with NADH as a substrate showed in Fig. 2 that the Δcox strain exhibits a decrease of about 40 %, and the ΔbdΔcox and Δbd strains exhibit a decrease of between 50 and 60 % compared to the WT strain.

Link between periplasmic H₂ oxidation and membranous oxygen reduction

When molecular hydrogen was used as electron donor in whole cells, a high oxygen reduction rate of 570 nmol
O₂ min⁻¹ (mg protein)⁻¹ was determined at oxygen concentrations below 40 µM. This activity was completely inhibited at a high oxygen concentration due to the rapid inactivation of periplasmic hydrogenase. This high activity was probably due to the coupling of periplasmic hydrogenases and low redox potential soluble c-type cyt. exhibiting reductase activity (Baumgarten et al., 2001; Fournier et al., 2004; Lamrabet et al., 2011). H₂-dependent oxygen reduction activity was totally inhibited by CO (an inhibitor of hydrogenases and oxygen reductases). More surprisingly, this activity was also partially inhibited by n-heptyl-4-hydroxyquinoline-N-oxide (HQNO; 25–30 % inhibition at 30 µM) and potassium cyanide (KCN, 35 % inhibition at 3 mM) (Fig. 3), suggesting the involvement of the membrane-bound terminal bd quinol oxidase which is sensitive to both HQNO and KCN. It should be noted that some hydrogenases are also sensitive to KCN (Adams et al., 1980; Shima & Ataka, 2011) but not to the quinone analogue inhibitor HQNO. This H₂-dependent oxygen reductase activity was also measured in the various deletion mutant strains. This activity decreased by only 20 % in the Δcox mutant while it decreased by 40–50 % in the ΔbdΔcox and Δbd strains (Fig. 3). These data pointed out that the absence of the bd quinol oxidase lowers the H₂-dependent O₂ reduction in whole cells. Altogether, these data showed that membrane-bound oxygen reduction by the bd quinol oxidase is coupled with periplasmic H₂ oxidation by hydrogenases. This coupling requires a membrane-bound quinone reduction system which is likely reduced by soluble and low redox potential periplasmic cyt. c.

Fig. 1. Cell survival of DvH WT and deletion mutant strains after exposure to oxygen. (a) Exposure to continuous flushing with 0.1 % O₂ at 33 ºC for 8 and 24 h. (b) Air exposure (21 % O₂) at 33 ºC for 4, 8 and 24 h. Percentage survival is indicated relative to the number of c.f.u. at time 0 of exposure for each strain (100 % survival). Data are mean values ± SD of five independent experiments.

Fig. 2. O₂ reduction rates of whole cells of DvH. Enzymatic activities were measured with washed cells in Tris/HCl 0.1 M, NaCl 0.15 M buffer (pH 7.5) at a saturating air concentration (237 µM O₂ at 30 ºC). Rates are reported in nmol min⁻¹ mg⁻¹ with indicated substrates. Error bars represent the standard deviation of 2–5 experiments.
Oxygen reduction, quinol and cyt. c553 oxidation in membrane fractions

O2 reduction in membrane fractions with NADH as an electron donor indicated that the double mutant ΔbdΔcox exhibited only about 20% of the activity of the WT strain (Fig. 4). A significant NADH-O2 reductase activity, inhibited by KCN and CO, was also measured in whole cells, and this activity was also lowered in the double mutant strain (Fig. 2). Since genes coding for a complex I (NADH-quinone oxidoreductase) are absent in the genome of DvH, this NADH-O2 reductase activity likely involves an as yet uncharacterized type II FAD-containing NADH dehydrogenase whose gene is present in the genome of DvH. Importantly, the O2 reductase activity was never nil, in either whole cells or in membrane preparations of the double mutant strain, indicating the presence of one or several alternative systems in the DvH membranes able to reduce O2.

Use of an ubiquinol-type substrate (DBH2) for the bd quinol oxidase indicated that the Δcox strain retained about 100% of the WT activity (Fig. 4) while the Δbd and ΔbdΔcox strains, both devoid of bd quinol oxidase, exhibited a decrease of 40 and 70% in DBH2-dependent O2 reductase activity, respectively. The quinol-dependent O2 reductase activity was inhibited by CO, by KCN, and by quinol analogue inhibitors such as HQNO and HDQ (Table 2; Lamrabet et al., 2011). Similar results were obtained when monitoring spectrophotometrically the oxidation of DBH2 with O2 (data not shown).

Using the menaquinol analogue menadiol as an electron donor, O2 reduction in the WT strain was tenfold higher than that measured with DBH2 as an electron donor (357 versus 36 nmol O2 min⁻¹ mg⁻¹, Fig. 5). Menadiol is structurally closer to the endogenous menaquinol 6 in DvH (Weber et al., 1970) than ubiquinol DBH2. Menadiol is a stronger reducer than DBH2 (E0 = +80 mV). The menadiol-dependent O2 reductase activity was lower in the Δbd and ΔbdΔcox strains (49% and 37% of the WT value, respectively) and similar to the WT in the Δcox strain (Fig. 5a).

In the presence of oxygen, membranes of the Δbd strain did not exhibit a decrease of the cyt. c553 oxidase activity (Fig. 4), whereas membranes of both Δcox and ΔbdΔcox strains, which are devoid of the cyt. c oxidase, exhibited a 40–50% decrease in this activity. The remaining cyt. c553 oxidase activity indicates the presence of one or several alternative membrane-bound complexes, in addition to the cyt. c oxidase cc(b/o)0, that are also able to oxidize reduced cyt. c553.

In order to characterize the membrane-bound O2 reductase activity remaining in the ΔbdΔcox strain, we measured this activity at different O2 tensions (Fig. 5). At high oxygen concentration (air-saturated, 237 µM dissolved O2 at 30 °C), the Δcox strain was not affected in its menaquinol-dependent oxygen reductase activity (Fig. 5a), whereas the Δbd and ΔbdΔcox strains exhibited only 30–40% of this activity compared to the WT (Fig. 5a). Similar results were obtained with the ubiquinol analogue DBH2 as the
substrate (Fig. 4). Interestingly, at oxygen concentrations below 30 μM, no significant oxygen reductase activities were detected in either the Δbd or the ΔbdΔcox strains, whereas more than 75% of the oxygen reductase activity was still present in the Δcox strain (Fig. 5b), suggesting the existence of one or several uncharacterized membrane-bound O₂ reduction systems only active at high O₂ concentration and using menaquinol as substrate.

**Kinetic parameters of the oxygen reduction systems in DvH membranes**

In order to further characterize the different membrane-bound O₂ reduction systems, we measured their respective affinity for O₂. The newly discovered membrane-bound system(s) only effective at high O₂ concentration was characterized in the ΔbdΔcox strain and a $K_m$ value of 28 ± 6 μM for O₂ was determined (Table 2).

![Graph](http://mic.sgmjournals.org)  
**Fig. 5.** O₂ reductase activities with menadion as an electron donor in the membrane fraction measured either at air saturation [237 μM O₂ at 30 °C, (a)] or below 30 μM O₂ (b). 100% activity corresponded to 357 nmol O₂ min⁻¹ (mg protein)⁻¹ in the WT strain at air saturation measured at 30 °C in Tris 0.1 M, NaCl 0.15 M, pH 7.5. Chemical reduction of O₂ by menadion was subtracted and experiments were carried out at least in duplicate (+/− SD).

**Table 2.** Enzymatic characteristics of the three oxygen reduction membrane-bound systems

<table>
<thead>
<tr>
<th>Membrane-bound system</th>
<th>$K_m$ O₂ (μM) (+/−sd)</th>
<th>$V_{max}$ (nmol O₂ min⁻¹ mg⁻¹) and $K_{cat}$ (s⁻¹)</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>bd quinol oxidase</td>
<td>0.30 ± 0.07†</td>
<td>38 (DBH₂)</td>
<td>80 % HQNO or HDQ (50 μM), 100 % KCN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>357 (Menadiol)</td>
<td>(2 mM), 100 % CO, 80 % NO</td>
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<td></td>
<td></td>
<td>$K_w$ 30 s⁻¹</td>
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<tr>
<td>cc(o/b)O₂ cyt. c oxidase</td>
<td>0.62 ± 0.04‡</td>
<td>94 nmol $e_{553}$ min⁻¹ mg⁻¹</td>
<td>100 % CO, 80 % NO, 80 % KCN (2 mM)</td>
</tr>
<tr>
<td>Low O₂ affinity scavenging system(s)</td>
<td>28 ± 6§</td>
<td>10 (DBH₂)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>80 (Menadiol)</td>
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</table>

*Kₘ* value can be calculated when the amount of enzyme detected in the membranes is known (see Lamrabet et al., 2011) with 0.22 nmol (mg protein)⁻¹ for the bd quinol oxidase.

†Values were determined with membranes of the Δcox strain (0.084 mg ml⁻¹) and DBH₂ (40 μM) as a substrate.

‡Values were determined with membranes of the Δbd strain (0.12 mg ml⁻¹) and reduced cyt. $e_{553}$ (16 μM) as a substrate.

§Values were determined with membranes of the ΔbdΔcox strain.
The rather high affinity of the \(bd\) quinol oxidase for \(O_2\) impedes its precise measurement with a classical Clark-type electrode. However, determination of its \(K_m\) value was possible by following quinol (DBH\(_2\)) oxidation spectrophotometrically in anaerobic conditions with subsequent injections of small amounts of dissolved \(O_2\). This experimental protocol gave a \(K_m\) value of 0.30 \(\mu M\) for \(O_2\), which is two orders of magnitude lower than that for the newly discovered system(s). Using reduced cyt. \(c_{553}\) as the substrate for the \(cc(o/b)o_3\) cyt. \(c\) oxidase, a \(K_m\) for \(O_2\) of 0.62 \(\mu M\) (Table 2) was measured, indicating a twofold weaker affinity towards \(O_2\) in comparison with the \(bd\) quinol oxidase. This \(K_m\) value in the lower micromolar range was similar to values reported for classical haem-copper cyt. oxidases present in aerobic micro-organisms (Poole, 1983). Concerning the levels of enzymatic activities in the membrane of \(DvH\), the total activity of the \(bd\) quinol oxidase using menadion as the substrate [357 nmol \(O_2\) min\(^{-1}\) (mg protein\(^{-1}\))] was found to be about 15 times higher than that of the \(cc(b/o)o_3\) cyt. \(c\) oxidase in terms of \(O_2\) reduced: 94 nmol \(c_{553}\) min\(^{-1}\) mg\(^{-1}\) is equivalent to about 23.5 nmol \(O_2\) min\(^{-1}\) mg\(^{-1}\) when considering that four electrons are needed to reduce \(O_2\) into water (Table 2).

The data suggest that there are two pathways for quinol-dependent oxygen reduction: a major high affinity pathway involving \(bd\) quinol oxidase and a minor low affinity pathway involving one or several unidentified systems.

**DISCUSSION**

In the present study, we characterized the *in vivo* and *in vitro* roles of the \(bd\) quinol oxidase and the \(cc(b/o)o_3\) cyt. \(c\) oxidase by studying single and double deletion mutants of these two membrane-bound enzymes.

Cell viability data have shown that deletion mutant strains devoid of one or both of the terminal membrane-bound oxygen reductases exhibit a higher sensitivity to oxygen, the \(AbdAcox\) strain being the most sensitive. Santana (2008) reported that the \(Abd\) strain was more sensitive than the WT strain after exposure to air for 18 h. In our present study, the \(Dcox\) strain was even more sensitive than the \(Abd\) strain, showing the importance of the cyt. oxidase enzyme. These results show that the two membrane-bound oxygen reductases play a crucial role in cellular survival under conditions of \(O_2\) stress.

The \(DvH\) cyt. oxidase first described as a \(ccat_5\) (Lobo et al., 2008) and later as a \(cc(b/o)o_3\) oxidase (Lamrabet et al., 2011) is a proton pump with conserved D and K channels (Lobo et al., 2008). It would thus be more energetically effective in establishing a proton-motive force than the \(bd\) quinol oxidase which causes proton extrusion by quinol oxidation and not by a transmembrane movement of protons. This may partially explain the importance of the \(cc(b/o)o_3\) cyt. oxidase in response to oxygen stress in \(DvH\) since ATP produced in the presence of \(O_2\) may be used for repair of \(O_2\) sensitive enzymes and for cell maintenance.

We have shown that deletion mutant strains exhibited lower \(O_2\) reductase activities in both whole cells and membrane fractions, with the lowest activities found for the double mutant strain \(AbdAcox\). With DBH\(_2\) or a menaquinol analogue (menadion) as electron donor, the decrease of the quinol-oxygen reductase activity in strains devoid of the \(bd\) quinol oxidase shows the importance of this enzyme in the oxidation of the menaquinol pool in \(DvH\). Conversely, the decrease of cyt. \(c_{553}\) oxidase activity in strains devoid of the \(cc(o/b)o_3\) oxidase points out the importance of this enzyme in the oxidation of the relatively high redox potential monohaematochrome \(c_{553}\) (\(K_{m,0} + 40\) mV; Hagen, 1989) encoded by the cyf gene located upstream of the \(cox\) operon (Heidelberg et al., 2004; Kitamura et al., 1995; Lamrabet et al., 2011). Although the cyt. \(c_{553}\) is a bona-fide substrate for the cyt. oxidase (Lamrabet et al., 2011; Lobo et al., 2008), the remaining 50% of the \(c_{553}\) oxidase activity in the \(Dcox\) strain reveals a possible interaction of cyt. \(c_{553}\) with other membrane-bound oxidoreductase complexes.

The remaining \(O_2\) reductase activity of cells and membranes of the double mutant strain at high oxygen concentration amounts to between 20–30% of the total activity in the WT strain. An *in silico* search failed to reveal a third membrane-bound oxygen reductase in the \(DvH\) genome. However, membrane-bound and soluble redox proteins in \(DvH\) exhibit lower redox mid-point potentials than their homologues in aerobic organisms, as exemplified by the multihaem \(c\)-type cyt. with \(E_{m,0} = -320\) mV (Hagen, 1989) and the menaquinone pool which exhibits a low standard redox potential \(E_{0,0} = -80\) mV compared to the 160 mV higher value for ubiquinone in aerobic organisms \(E_{0,0} = +80\) mV. Chemical reactions as well as unspecific and diverted electron transfer catalysed by redox proteins, from low redox potential electron donors to the high redox potential electron acceptor \(O_2\) \(E_{0,0} = +820\) mV, will thus be enhanced, since the Gibbs free energy \(\Delta G\) values for these electron transfer reactions are more negative. The remaining \(O_2\) reductase activity involves one or several unidentified systems able to reduce \(O_2\) with low affinity \((K_{m,0}, 28\) \(\mu M\)). They are likely to be menaquinol-oxidizing complexes, diverted from their primary function, donating electrons to \(O_2\) under oxidative stress conditions. Possible candidates are Qmo, Dsr (Pires et al., 2006; Zane et al., 2010) or other as-yet- unidentified complexes. A typical periplasmic example of a reaction diverted from its primary function is the hydrogenase/cyt. \(c\) couples which are able to very efficiently reduce \(O_2\) (Baumgarten et al., 2001; Fournier et al., 2004; Krekeler et al., 1998; Lamrabet et al., 2011). These systems could act as \(O_2\)-scavenging systems when cells are exposed to high oxygen concentration and thus participate in the overall cell defence against oxidative stress.

Concerning the specific membrane-bound systems, the measured \(V_{max}\) and \(K_m\) for \(O_2\) for \(cc(o/b)o_3\) cyt. \(c\) oxidase and for \(bd\) quinol oxidase indicate that the latter exhibits a higher affinity for \(O_2\) \((K_{m,0}, 0.3\) \(\mu M\)) than the former \((K_{m,0}\)
0.62 μM). In the membranes of DvH, the total enzymatic activity (nmol O₂ min⁻¹ mg⁻¹) of the bd quinol oxidase using menadiol as substrate is more than an order of magnitude higher than that of the cc(o/b)₀₃ cyt. c oxidase. This may be related partly to the 30-fold higher expression level of the cydA mRNA in comparison with the coxI gene in anaerobic and O₂ stress conditions (Lamrabet et al., 2011). The bd quinol oxidase is found in aerobic or microaerophilic organisms and is also present in strictly anaerobic bacteria and archaea such as Bacteroides fragilis (Baughn & Malamy, 2004) and Methanosarcina acetivorans (Brochier-Armanet et al., 2009). It is involved in removing traces of oxygen in Azotobacter vinelandii (Poole & Hill, 1997) and Klebsiella pneumoniae (Juty et al., 1997) where the bd oxidase protects the O₂-labile nitrogenase. Excepting the FixN-type of cyt. oxidase (cbb₃ oxidase), which exhibits a very high affinity towards O₂ (Kₘ of below 50 nM) in microaerophilic organisms (Pitcher et al., 2002), the haem-copper cyt. oxidases exhibit generally a weaker affinity towards O₂ in the micromolar range in comparison with the submicromolar range for bd quinol oxidases. In DvH, Kₘ values indicate that bd quinol oxidase could function at lower O₂ tension than the cyt. oxidase; however, the latter may be more effective in conservation of energy into a proton gradient. Altogether our data point out the importance of both the cc(o/b)₀₃ cyt. c oxidase and the bd quinol oxidase; both exhibit a Kₘ for O₂ ≤0.6 μM, which is below the maximum O₂ concentration tolerated by DvH during continuous growth. Thus, both oxidases are functional during oxygen stress and should contribute to the production of energy necessary for cell survival.

Due to the lack of bc₁ complex genes in the genome of DvH, the electron donor for the periplasmic cyt. c₅₅₃ remains uncertain. One possibility could be that the MFlc complex (corresponding to the Qrc complex, genes DVU0692–DVU0695 in DvH) described in Rhodothermus marinus or Chloroflexus aurantiacus (Pereira et al., 2007; Yanyushin et al., 2005) serves as an alternative bc₁ complex (Gao et al., 2010; Refojo et al., 2010, 2013). However, analysis of both Δqrc and ΔqrcΔbd deletion strains argues against the involvement of the Qrc complex as an alternative bc₁ complex (unpublished data), in line with the reverse menaquinone reductase activity reported (Venceslau et al., 2010).

The high rate of the Knallgas reaction (O₂ reduction with H₂ as electron donor, >500 nmol O₂ min⁻¹ mg⁻¹) was shown to be inhibited partially by KCN and HQNO. Although [Fe] hydrogenase can be inhibited by KCN, this inhibitor has little effect on [Ni–Fe] and [Ni–Fe–Se] hydrogenases (Adams et al., 1980; Shima & Ataka, 2011).

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**Fig. 6.** Model of oxygen reduction and detoxification in the cytoplasmic membrane of Desulfovibrio vulgaris Hildenborough including electron transfer connecting the hydrogenases/c-type cyt. system in the periplasm to the membrane-bound oxygen reductase. Periplasmic soluble hydrogenases (Hyd) reduce low redox potential c-type cyt. c₅₅₃. This system scavenges itself O₂ very efficiently at low O₂ tension and also donates electrons to at least one transmembrane complex (MKred.TMC) able to reduce the menaquinone pool (MK/MKH₂). Menaquinol is oxidized by the bd quinol oxidase (bd), reducing O₂ into water. The membrane-bound cc(o/b)₀₃ c oxidase (Cox; Lamrabet et al., 2011) receives electrons from the soluble monohaemic cyt. c₅₅₃ and reduces O₂ into water. A third unidentified membrane-bound system ('Low O₂ affinity') reduces O₂ at high O₂ tension and corresponds probably to a diverted reactivity of a low redox potential membrane system towards the high redox potential O₂ molecule. The dashed arrows indicate putative sites of energy conservation through the creation of a proton-motive force in the presence of oxygen. Ndh, type II NADH dehydrogenase.
However HQNO and HDQ, quinol-like inhibitors of membrane-bound oxidoreductase complexes, have no reported effect on hydrogenase activity. Since HQNO partially inhibits H₂-dependent O₂ reduction, this overall activity involves the bd quinol oxidase, which is sensitive to this inhibitor. This hypothesis is confirmed by the analysis of single and double deletion mutants, which showed that the absence of the bd quinol oxidase leads to a lower O₂ reduction rate with H₂ as electron donor. Our work clearly shows, for the first time to our knowledge, a link between periplasmic H₂ oxidation by hydrogenases/c-type cytosolic and membranous reduction of O₂ by the bd quinol oxidase. Periplasmic c-type cytochrome bd is likely to reduce a membrane-bound complex (Fig. 6: MKred.TMC) able to reduce the menaquinone pool, which is in turn oxidized by the bd quinol oxidase. Candidates for this membrane-bound complex are the Qrcs (Venceslau et al., 2010), the HmC complex (Dolla et al., 2000) or another as-yet-unidentified c-type menaquinone oxidoreductase complex. This electron pathway from H₂ to the membrane-bound terminal oxygen reductase represents a source of energy for cell survival and maintenance when exposed to oxygen stress, since both the periplasmic oxidation of H₂ into H⁺ and the functioning of the bd quinol oxidase establish a proton-motive force used for ATP synthesis. ATP formation in Desulfovibrio desulfuricans and D. vulgaris with H₂ and O₂ as electron donor and acceptor (Cypionka, 2000) was sensitive to the uncoupler carbonylcyanide m-chlorophenylhydrazone (Fitz & Cypionka, 1989), although this O₂ reduction and its associated energy yield were not associated with cell growth. Future studies will aim to quantify the energy gain of ΔDH from the detoxification of O₂ with the two membrane-bound O₂ reductases.

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