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Structural and functional analysis of aa₃-type and cbb₃-type cytochrome c oxidases of Paracoccus denitrificans reveals significant differences in proton-pump design

Jan-Willem L. de Gier, Mike Schepper, Willem M. Reijnders, Stefan van Dyck, Dirk Jan Slotboom, Antony Warne, Matti Saraste, Klaas Krab, Moshe Finel, Adrian H. Stouthamer, Rob J. M. van Spanning and John van der Oost

Introduction

In mitochondrial respiration the reduction of oxygen to water is catalysed by the aa₃-type cytochrome c oxidase. This membrane-bound protein complex is the last component of a linear respiratory pathway in which electrons from NADH and succinate are transferred, in a sequence of redox reactions, to oxygen. The aerobic respiration in bacteria, on the other hand, often proceeds via a more complex electron-transfer network. Alternative respiratory pathways enable a bacterium to adjust the composition and/or the efficiency of oxidative phosphorylation in response to changes in environmental conditions (Anraku and Gennis, 1987; van der Oost et al., 1994; Garcia-Horsman et al., 1994a; van Spanning et al., 1995a).

In addition to a number of alternative quinone-reducing dehydrogenases, the terminal oxidases appear to represent an important site of flexibility in respiratory networks. To date, the best-characterized bacterial respiratory system is the one in Escherichia coli. In this bacterium two distinct quinol oxidases (cytochromes b₅₆ and b₇₃) are expressed during aerobic growth, but a cytochrome c branch is absent (Anraku and Gennis, 1987). Paracoccus denitrificans, in turn, possesses three distinct terminal oxidases.

In the present study, a P. denitrificans Δaa₃/bb₃ mutant oxidase under certain conditions. Sequence alignment suggests that many residues that have been proposed to constitute the chemical and pumped proton channels in cytochrome aa₃ (and probably also in cytochrome bb₃) are not conserved in cytochrome bb₃. It is concluded that the design of the proton pump in cytochrome bb₃ differs significantly from that in the other oxidase types.
was used for the purification of an alternative cytochrome c oxidase: cytochrome cbb$_3$. Using the N-terminal amino acid sequence of one of its subunits, a genomic locus, ccoNOQP, was isolated that contained a gene cluster with four open reading frames. Sequence analysis of the flanking regions revealed the presence of a ccoGH cluster with four open reading frames. Sequence analysis of the acid sequence of one of its subunits, a genomic locus, downstream of which are homologous to HemN and FNR. Multiple oxidase mutants of $P$. denitrificans suggests that, besides cytochrome aa$_3$ and cbb$_3$, no additional cytochrome c oxidase is present. The currently generated set of oxidase mutants of $P$. denitrificans was used to demonstrate that all three terminal oxidases have the capacity to pump protons. It is discussed, however, that in the cbb$_3$-type cytochrome c oxidase the coupling between oxygen reduction and proton translocation may be less tight than in cytochromes aa$_3$ and bb$_3$/bo$_3$.

**Results**

**Purification of the cbb$_3$-type cytochrome c oxidase**

In a previous study, the simultaneous deletion of cytochromes aa$_3$ and bb$_3$ in $P$. denitrificans (Δaa$_3$/bb$_3$) has been reported. Analysis of this mutant revealed (i) the presence of an alternative cytochrome c oxidase, (ii) the presence of only protohaem IX (haem B) in membrane extracts, and (iii) an increased expression of cytochromes c and b. No quinol oxidase activity was detectable in this mutant (de Gier et al., 1994). Here, we report the isolation of a cb-type cytochrome c oxidase from membranes of the *Paracoccus* Δaa$_3$/bb$_3$ mutant. Both the activity and the subunit composition of the oxidase complex turned out to be rather unstable during the purification, as described in the **Experimental procedures**. As will be discussed below, a cytochrome c subunit with an apparent molecular mass of 45 kDa is lost during the purification of *Paracoccus* cytochrome cbb$_3$ (as described in the **Experimental procedures**).

Analysis of the purified cytochrome c oxidase by a Coomassie brilliant blue-stained SDS–PAGE gel indicates that it consists of two components with apparent molecular masses of 45 kDa and 30 kDa (Fig. 1A). Only the fast-migrating component has been identified as a cytochrome c because of the presence of covalently bound haem, as demonstrated by haem-stained SDS–PAGE (not shown). Sequencing of this 30 kDa subunit revealed the N-terminal sequence AILEKHKVLEKNATLLLVFSFLVVR. The optical spectrum of this oxidase preparation indicates that it involves a cb-type cytochrome (Fig. 1B). However, the relatively small amount of cytochrome c in this spectrum relative to a previous preparation (de Gier et al., 1994) can best be explained as the loss of a second haem.

**Isolation and characterization of the ccoNOQP cluster**

Based on the N-terminal sequence of the 30 kDa subunit, degenerate primers were designed (see the **Experimental procedures**, primers 171 and 172) and used to perform the polymerase chain reaction (PCR) on genomic DNA from the Δaa$_3$/bb$_3$ mutant. A PCR product of the expected size (0.1 kb) was obtained. Sequence analysis of the PCR product indicated that the DNA sequence corresponded to the N-terminal peptide of the 30 kDa subunit (not shown). With the PCR product as a probe, the complete ccoNOQP cluster could be cloned (Fig. 2A). Fragments were subcloned into M13mp18/19 for sequence analysis. The complete DNA sequence has been submitted to the GenBank database Data Library (Accession Number BankIt12589 U34353).

Four open reading frames (ORFs) in the isolated genomic locus share a high degree of homology with the ccoNOQP gene cluster from *Rhodobacter capsulatus* (Thöny-Meyer et al., 1994) and to the ffxNOQP operons of *Rhizobium meliloti* (Kahn et al., 1993; D. Kahn, 1993, EMBL Data Library Accession No. 221854), *Bradyrhizobium japonicum* (Preisig et al., 1993; Fig. 3) and *Azorhizobium caulinodans* (Mandon et al., 1994). As *P. denitrificans* is not known to be able to fix molecular nitrogen, the ccoNOQP rather than the ffxNOQP nomenclature...
Cytochrome cbb₃ of Paracoccus denitrificans

Fig. 2. A. Genetic organization and physical map of the P. denitrificans ccoNOQP region. Arrows represent individual genes. The positions of FNR-boxes are indicated by arrows. 

B. Insertion mutants have been generated by substituting part of the ccoNOQP operon with a kanamycin-resistance (KmR) cassette, deleting the 3' half of ccoN and the two 5' codons of ccoO.

has been adopted for these genes. The four ORFs have a GC-rich codon preference which is typical of P. denitrificans. The ccoNOQP genes are tightly clustered, with the exception of the ccoQ and ccoP genes which are 41 bases apart. As no transcription-termination signal was found here, the four genes are probably transcribed as a single operon, as has been demonstrated for R. meliloti (Batut et al., 1989). The proposed initiation codons of all four genes are six to eight bases downstream of putative Shine–Dalgarno sequences. A putative anaerobox (TTGAC-N₄-ATCAA) is located upstream of ccoN. Its sequence is very similar to the binding sites of FNR in E. coli and of FixK in R. meliloti and B. japonicum (TGAT-N₄-ATCAA; Spiro, 1994), and identical to the anaerobox found upstream of the A. caulinodans fixNOQP operon (Mandon et al., 1994).

Attempts to sequence the N-terminus of subunit I (CcoN, apparent molecular mass 45 kDa (Fig. 1A)) were unsuccessful, probably because of blocking of the first amino group. The N-terminal methionine of CcoN (Fig. 3A) has been chosen because the corresponding ATG codon is downstream of a Shine-Dalgarno sequence, and because no alternative in-frame initiation codons are present. Moreover, the size of the gene correlates with that of ccoN (Fig. 3A) in the aforementioned bacteria. The putative anaerobox is located at positions −110 and −124 of the chosen start codon. Based on sequence alignment with the subunits I of the well-characterized cytochromes aa₃ from Rhodobacter sphaeroides and P. denitrificans, as well as with the subunit I of cytochrome bo₃ from E. coli (Hosler et al., 1993), four histidines in the CcoN sequence (H271, H321, H322 and H409) are the most probable ligands of the binuclear catalytic centre formed by a penta-co-ordinated haem B and a copper atom, CuB (Fig. 3A). The ligands of the hexa-co-ordinated haem B are H411 and (probably) H122 in the Paracoccus CcoN (Fig. 3A).

CcoO has an N-terminal amino acid sequence that corresponds to the N-terminal sequence determined for the 30 kDa subunit (Fig. 3B). Apart from this hydrophobic, putative membrane-spanning fragment, CcoO contains a hydrophilic, probably periplasmic, domain with a Cx₂CHxnM motif which is the consensus for haem C binding (Fig. 3B). Apart from this motif, CcoO/FixO does not share much similarity with the available cytochrome c sequences (Moore and Pettigrew, 1990). CcoQ is a small protein with one hydrophobic region (Fig. 3C); apart from CcoQ and FixQ, no significant homologues have been found in sequence databases. CcoP appears to be a membrane-anchored di-haem cytochrome c. Its C-terminal hydrophilic domain, probably facing the periplasmic side of the plasma membrane, contains two motifs (Gx₃Fx₃Cx₃CHxnM) that are well conserved among soluble mono-haem cytochromes of photosynthetic bacteria and eucaryotic algae (Mandon et al., 1994). In contrast to the FixP/CcoP sequences from other sources, an N-terminal hydrophilic extension is present in the Paracoccus sequence (Fig. 3D), probably located at the cytoplasmic side of the membrane.

Flanking regions

Upstream of the ccoNOQP cluster of P. denitrificans is an ORF that potentially codes for a polypeptide of 278 amino acids (orf278 in Fig. 2A). It shares a rather high degree of homology (28% identical amino acids, not shown) with orf1277, which is located adjacent to the fixNOQP operon.
**Cytochrome cbb3 of Paracoccus denitrificans**

<table>
<thead>
<tr>
<th></th>
<th>Pd CcoO</th>
<th>Bj FixO</th>
<th>Pd CcoO</th>
<th>Bj FixO</th>
<th>Pd CcoO</th>
<th>Bj FixO</th>
<th>Pd CcoO</th>
<th>Bj FixO</th>
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<tr>
<td>A</td>
<td>MAILEKHVLYEKNATLILVVSFLVVTGIVEITPLFYQLNTIE</td>
<td>MFSWTRQVFEKNSIIILIVGIIIAIAGGIVEILEFYKSTIE</td>
<td>KVGGMRPYTPELKVGVIIYRVEGCVCHSMIRPMRDEEVERYG</td>
<td>KVGGMRPYTPELKVGVIIYRVEGCVCHSMIRPMRDEEVERYG</td>
<td>HYSLAEESMYCHFQWGSRTPGDLARVGRSDEWHLHLVDV</td>
<td>HYSLAEESMFCHPFQWGSRTPGDLARVGRSDEWHLHLVDV</td>
<td>QAVFESIMPKRYGF-LNRRQVDASNMQRLKTDALEGVYPYDGA</td>
<td>QAVFESIMPKRYGF-LNRRQVDASNMQRLKTDALEGVYPYDGA</td>
</tr>
<tr>
<td>B</td>
<td>123</td>
<td>132</td>
<td>174</td>
<td>176</td>
<td>216</td>
<td>218</td>
<td>173</td>
<td>175</td>
</tr>
<tr>
<td>C</td>
<td>MDYFSFLRELADS- - - - WVILLLLVVFLLTIVAFGRPFGAASS</td>
<td>MKAIIETDNLASGLVTITWTPVFVAIFALIAIAAFWEPRNKAAPF</td>
<td>RRRKHP</td>
<td>EALHPREE</td>
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<td>45</td>
<td>48</td>
<td>54</td>
</tr>
<tr>
<td>D</td>
<td>MAIYDDEHASPQNPDRNIERLEQADAENHAKAKI1AHPPPEAGDP</td>
<td>MTDKSESDFS</td>
<td>LHPVTPRPGATRVRDRKGGRRVVEVPSGSHSDGISREYDNPL</td>
<td>10</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>88</td>
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<tr>
<td>E</td>
<td>PRWLWTIFYAITWIVGVTLYIAAYPA1PLVNGATQ1LGLGQNYRSD</td>
<td>PRWVICFYLTVAIWAGYIVXPAWELSITNSNTGFGSSPS</td>
<td>89</td>
<td>31</td>
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<td>76</td>
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<tr>
<td>F</td>
<td>RWCQCHGSGAGGAFYPSPSDLIDNWLGGLTEEIHTTVMHGAR</td>
<td>177</td>
<td>119</td>
<td>221</td>
<td>163</td>
<td>265</td>
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<td>G</td>
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<td>251</td>
<td>251</td>
<td>1247-1260</td>
<td></td>
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</tr>
</tbody>
</table>

Fig. 3. Alignments of the *P. denitrificans* ccoNOQP-encoded polypeptides.

A. CcoN of *P. denitrificans* (cytochrome cbb,); FixN of *B. japonicum* (cytochrome cbb3; Preisig et al., 1993), CtoDI of *P. denitrificans* (cytochrome aa3; Raitio et al., 1990), and CyoB of *E. coli* (cytochrome bo3; Chepuri et al., 1990). Transmembrane helices are shaded and conserved residues discussed in the text are indicated.

B. CcoO of *P. denitrificans* and FixO of *B. japonicum* (Preisig et al., 1993). Identical residues are boxed.

C. CcoQ of *P. denitrificans* and FixQ of *B. japonicum* (Preisig et al., 1993). Identical residues are boxed.

D. CcoP of *P. denitrificans* and FixP of *B. japonicum* (Preisig et al., 1993). Identical residues are boxed.

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of *B. japonicum*. No homologue has yet been reported in other members of the Rhizobiaceae family. As no mutants are available, a link between this gene product and cytochrome *ccb*3 remains to be demonstrated. Further upstream, two genes are located that encode homologues of *E. coli* HemN and FNR (Fig. 2A). In the latter bacterium both polypeptides play an important role during oxygen limitation: HemN catalyses an oxygen-independent bypass in haem biosynthesis (Plunkett et al., 1993), and FNR is a transcription activator of genes involved in anaerobic metabolism (Spiro and Guest, 1990).

Without exception, the gene cluster *fixGHIS* is located downstream of the *fixNOQP* genes of the Rhizobiaceae family (Kahn et al., 1993; Preisig et al., 1993; Mandon et al., 1994) and *ccoNOQP* genes of *R. capsulatus* (Thöny-Meyer et al., 1994). In *R. meliloti*, a number of transposon insertions in *fixGH* have been isolated which had a ‘fix-minus’ phenotype (Kahn et al., 1989). Also, in *Paracoccus* at least part of this cluster is located adjacent to the *ccoNOQP* genes. Two genes homologous to *fixG* and *fixH* (Fig. 2A) have been found downstream of *ccoNOQP* but, for the reasons given above, these genes are designated *ccoG* and *ccoH*. In all instances an anaerobox is located in the *fixG* and in the *ccoG* promoter regions (Fig. 2A), suggesting expression of this oxidase type at low oxygen tensions. The *ccoG* product is a ferredoxin-like protein, with two typical 4Fe4S-binding motifs. *FixGHIS* has been suggested to be a polypeptide complex consisting of, at least, a redox protein (FixG) and a cation pump (FixI) (Kahn et al., 1989).

**Mutagenesis of ccoNO**

Insertion mutants were generated by substituting part of the *ccoNOQP* operon by a kanamycin-resistance (KmR) cassette, deleting the 3’ half of *ccoN* and the two 5’ codons of *ccoO* (Fig. 2B). The *ccoNOQP* operon was deleted in wild-type *Paracoccus* (Pd1222) and in the *ctaDl/ctaDlII* double mutant Pd92.20 (Δaa3), yielding Pd27.21 (Δccbb3) and Pd93.12 (Δaa3/ccbb3), respectively. Southern blot analysis of chromosomal DNA of selected KmR clones confirmed the appropriate recombination (not shown). Analysis of the mutant strains generated, grown on minimal medium with succinate, did not show significant spectroscopic changes relative to their respective parent strain. SDS–PAGE and subsequent haem staining of cytoplasmic membranes showed that the 30 kDa cytochrome *c* is absent, and the 45 kDa cytochrome *c* is only just detectable in the Δccbb3 mutant strains (Fig. 4).

**Oxygen consumption**

The oxygen-consumption rates in cell suspensions of wild type and oxidase mutants were measured polarographically, with endogenous as well as exogenous substrates (succinate or ascorbate with *N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD)*). The oxidation rates are shown in Table 1. In separate assays the electron flow to oxygen via cytochrome *c* reductase (the *bc1* complex) was inhibited by addition of either antimycin A or myxothiazol. The endogenous respiration of the wild type was clearly affected by these inhibitors. In contrast, the endogenous respiration of the Δaa3 mutant and the Δccbb3 mutant was only slightly inhibited, and that of the Δaa3/ccbb3 mutant strain appeared to be unaffected.

The effect of the cytochrome *bc1* inhibitors on succinate oxidation in the Δccbb3 mutant and in the Δaa3 mutant was much less than in the wild type. Again, in the Δaa3/ccbb3 mutant the succinate oxidation appeared to be unaffected by these inhibitors. More or less equal values of ascorbate/TMPD oxidation indicated the presence of cytochrome *c* oxidase(s) in the wild type, as well as in the Δccbb3 and the Δaa3 mutant. In the Δaa3/ccbb3 mutant, the endogenous respiration was not enhanced at all upon the addition of ascorbate/TMPD, indicating that no cytochrome *c* oxidase was expressed.

**Proton translocation**

The proton-pumping capacity of the set of oxidase mutants was measured by using the oxygen-pulse method as described elsewhere (Raitio and Wikström, 1994; de Gier et al., 1994). In the proton-translocation assay, succinate was used as the electron-donating substrate and either oxygen or potassium ferricyanide was used as the terminal electron acceptor (Table 2). The main goal was to study the proton-pumping capacity of the *ccbb3*-type cytochrome *c* oxidase. The H+/e− ratios of the Δaa3 mutant and the Δbb3/aa3 mutant clearly show that the *ccbb3*-type cytochrome *c* oxidase does have the capacity to translocate protons. In Fig. 5, representative proton-translocation traces of the Δbb3/aa3 mutant are shown. The H+/e− ratios of the Δccbb3 mutant and the Δaa3/ccbb3 mutant were measured.

![Fig. 4. SDS–PAGE and subsequent haem staining of membrane fractions of the wild type (wt) and the cytochrome *ccbb3* mutant (Δccbb3) of *P. denitrificans* after microaerobic cultivation. The *c*-type cytochromes indicated are subunits of the *bc1* complex (FbcC), subunits of cytochrome *ccbb3* (CcoP, 45 kDa; CcoO, 30 kDa), cytochrome *ccbb*2 (CycM), and a subunit of the NO reductase (NorC).](image-url)
mutant confirm that the \( \Delta aa_3 \)-type cytochrome \( c \) oxidase and the \( bb_3 \)-type quinol oxidase both translocate protons. Upon the addition of inhibitors of the \( bc_1 \) complex, the \( H^+/e^- \) ratio drops from 3 to 2 (not shown). This is consistent with the decreased ratio of \( 2H^+/e^- \) that is detected only in the \( \Delta aa_3/cbb_3 \) mutant. This suggests that ubiquinol apparently was preferentially oxidized by ubiquinol:cytochrome \( c \) oxidoreductase (bc, complex) rather than by ubiquinol:oxygen oxidoreductase (cytochrome \( bb_3 \)).

With the artificial electron-donating couple ascorbate/TMPD, proton translocation was barely detectable in the absence of cytochrome \( aa_3 \). However, at elevated TMPD concentrations (final concentration: 2–3 mM) some proton translocation was observed, albeit never more than 50% of the theoretical maximum (not shown). The oxygen-consumption experiments indicate that, in the absence of cytochrome \( aa_3 \) and cytochrome \( cbba \), no electron flow from ascorbate to oxygen is detectable in succinate-grown cells of \( P. \) denitrificans. Accordingly, no proton extrusion was observed with ascorbate/TMPD in the \( \Delta aa_3/cbb_3 \) mutant.

**Discussion**

\( P. \) denitrificans contains a respiratory network in which electrons are transferred from specific dehydrogenases to different types of terminal oxidases. One electron transfer route closely resembles the mitochondrial respiratory chain in which ubiquinol, reduced by either NADH or succinate, is oxidized by a supercomplex consisting of cytochrome \( c \) reductase (bc, complex), cytochrome \( c_552 \) and cytochrome \( c \) oxidase (cytochrome \( aa_3 \)) (Berry and Trumpower, 1985; Trumpower, 1991). In addition, it has been demonstrated that \( Paracoccus \) expresses a distinct respiratory branch in which electrons are directed to a second terminal oxidase, the quinol-oxidizing cytochrome \( bb_3 \) (de Gier et al., 1994).

Analysis of a recently generated \( \Delta aa_3/bb_3 \) mutant revealed that the aerobic respiration proceeds via an alternative cytochrome \( c \) oxidase (de Gier et al., 1994). In the present study, the allocated gene cluster of this \( cbb_3 \)-type oxidase has been cloned and sequenced. It has previously been demonstrated that no quinol oxidase activity remains in a \( \Delta bb_3 \) mutant (de Gier et al., 1994), and here we show that a \( \Delta aa_3/cbb_3 \) double mutant does not

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**Table 1.** Oxygen-consumption measurements of whole-cell suspensions of different \( P. \) denitrificans strains: Pd1222 (wild type), Pd92.20 (\( \Delta aa_3 \) mutant), Pd27.21 (\( \Delta cbb_3 \) mutant), and Pd93.12 (\( \Delta aa_3/cbb_3 \) mutant).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Endogenous</th>
<th>Succinate</th>
<th>Ascorbate/TMPD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AA myx</td>
<td>AA myx</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>1.5</td>
<td>0.9</td>
<td>2.4</td>
<td>0.6</td>
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<tr>
<td>( \Delta aa_3 )</td>
<td>1.3</td>
<td>1.2</td>
<td>2.9</td>
<td>2.6</td>
</tr>
<tr>
<td>( \Delta cbb_3 )</td>
<td>1.4</td>
<td>1.2</td>
<td>2.7</td>
<td>1.9</td>
</tr>
<tr>
<td>( \Delta aa_3/cbb_3 )</td>
<td>2.2</td>
<td>2.2</td>
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</tr>
</tbody>
</table>

Rates after addition of exogenous substrate have been corrected by subtraction of the corresponding endogenous rates. Values are the means of three independent assays (nmol \( O_2 \) per mg of protein per s). Inhibitors: antirnycin A (AA), myxothiazol (myx).

---

**Table 2.** Proton-translocation measurements of whole-cell suspensions of different \( P. \) denitrificans strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Succinate</th>
<th>Succinate</th>
<th>Succinate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( O_2 )</td>
<td>Fe(CN)(^{3-})</td>
<td>Fe(CN)(^{3-})</td>
</tr>
<tr>
<td>Wild type</td>
<td>2.53 ± 0.13</td>
<td>1.96 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>( \Delta aa_3 )</td>
<td>2.96 ± 0.15</td>
<td>1.94 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>( \Delta bb_3 )</td>
<td>2.97 ± 0.12</td>
<td>1.96 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>( \Delta aa_3/bb_3 )</td>
<td>2.95 ± 0.12</td>
<td>1.98 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>( \Delta cbb_3 )</td>
<td>3.01 ± 0.14</td>
<td>1.94 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>( \Delta aa_3/cbb_3 )</td>
<td>1.98 ± 0.11</td>
<td>1.97 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

\( H^+/e^- \) ratios are averages ± standard deviation (n=8). Cells were cultivated as aerobic batch cultures in minimal medium with succinate. The electron donor was succinate; anaerobic suspensions were pulsed with either oxygen \( (O_2) \) or ferricyanide \( (Fe(CN)_{5}^{3-}) \).
express cytochrome c oxidase activity (Table 1) (van der Oost et al., 1995). Hence, it is concluded that, at least under a variety of tested conditions, these three types form the complete set of terminal oxidases expressed in _P. denitrificans_.

**Characterization of cytochrome cbb₃**

The products of the isolated _P. denitrificans_ ccoNOQP gene cluster share a high degree of homology with the cytochrome c oxidase of _R. capsulatus_ (Thöny-Meyer et al., 1994) and the 'high-affinity' oxidase from several members of the Rhizobiaceae (Kahn et al., 1993; Preisig et al., 1993; Mandon et al., 1994) (Fig. 3). Although the cbb₃-type oxidase apparently represents a distant member of the haem-copper oxidase family (van der Oost et al., 1994; Saraste and Castresana, 1994), its cytochrome b subunit (CcoN) still shares some typical features with the catalytic subunit (subunit I) of these oxidases. Six histidines that are invariant in the available CcoN/FixN sequences have been aligned with the conserved histidine ligands of the redox centres of subunit I of cytochromes aa₃ and bo₃. The alignment shown in Fig. 3A suggests that H271, H321, and H322 are the most probable ligands of Cu₆, H409 of the penta-co-ordinated haem B, and H411 and H122 of the hexa-co-ordinated haem B in _P. denitrificans_ CcoN.

Garcia-Horsman et al. (1994b) have recently reported a thorough biochemical analysis of the cbb₃-type oxidase from _R. sphaeroides_ and have demonstrated the presence of a binuclear centre typical of haem–copper oxidases and the absence of Cu₆. Moreover, metal analysis revealed the ratio of haemC:haemB:copper to be 3:2:1. This agrees very well with the sequence data of cytochrome cbb₃: two haems B and a copper ion are located in subunit I (CcoN), and three haems C reside in the two cytochrome c subunits (one in CcoO and two in CcoP). Bosma (1989) has reported a 30 kDa cytochrome c associated with the alternative oxidase from _P. denitrificans_ with an _E_m₇_ value of 322 mV. Gray et al. (1994) purified cytochrome cbb₃ from _R. capsulatus_ and reported a 28 kDa cytochrome c (CcoO) with an _E_m₇_ value of 320 mV, a 32 kDa cytochrome c (CcoP) with an _E_m₇_ value of 265 mV, and a low-spin cytochrome b with an _E_m₇_ value of 385 mV. These data may indicate that CcoO is the direct electron donor of the redox centres (haems B and Cu₆) in CcoN. Indeed, the purified 2 subunit CcoNO complex (Fig. 1) retains TMPD oxidase activity. Apparently CcoO is less tightly bound to the cytochrome cbb₃ core and may play a role as electron carrier from the _bc_ complex to the CcoO–CcoN complex, which would be in agreement with the reported midpoint potential values.

**Expression**

Comparison of membrane proteins from the wild type and from the Δcbb₃ mutant of _P. denitrificans_ by haem-stained SDS–PAGE (Fig. 4) identified the 30 kDa and 45 kDa cytochromes c as the CcoO and CcoP subunits of cytochrome cbb₃, respectively. Previously, Bosma (1989) performed a detailed analysis of the expression of c-type cytochromes in chemostat-grown cells of _P. denitrificans_, using the same haem analysis (reviewed by van Spanning et al., 1995a). Bosma demonstrated that both the 30 kDa (CcoO) and the 45 kDa (CcoP) cytochromes c were abundantly present in membrane fractions when cells were grown in either nitrate-limited (anaerobically) or oxygen-limited (microaerobically) conditions. In succinate-limited (aerobic) cultures, on the other hand, the 45 kDa cytochrome c was absent and the 30 kDa cytochrome c was barely detectable (Bosma, 1989). It was concluded that the cbb₃-type oxidase plays an important role at decreased oxygen tensions.

In the promoter regions of all presently known cco/fixNOQP and cco/fixGH(IS) clusters is a sequence motif that closely resembles the binding site of the well-known anaerobic/microaerobic transcription activator FNR (Spiro and Guest, 1990). In _R. meliloti_, the 'oxygen state' is sensed by the haem protein FixL. In the absence of oxygen, this protein is autophosphorylated (FixL-P). Subsequently, the phosphate is transferred from the sensor to the regulator, FixJ, inducing its activation (FixJ-P). The latter modification results in transcription activation of fixK, the gene that encodes an FNR homologue. FixK, in turn, binds at the anaerobox upstream of the fixNOQP and fixGHIS clusters, thereby activating their transcription (David et al., 1988; Batut et al., 1989; Kahn et al., 1989; Fischer, 1994). To date, no FixLJ system has been demonstrated in any non-nitrogen-fixing organism. However, a FixK-like protein, NNR, has been identified in _P. denitrificans_ as a transcription activator of nitrite reductase and nitric oxide reductase, two polypeptide complexes that are involved in anaerobic respiration (van Spanning et al., 1995b). Mutagenesis of the _nnr_ gene, however, did not affect expression of cytochrome cbb₃. Here we show that in the flanking region of the cco locus of _Paracoccus_, a gene is located that potentially codes for a second transcription regulator: fnRP (Fig. 2A). Unlike NNR and FixK, this FNR homologue has an N-terminal cysteine cluster which, in the case of _E. coli_ FNR, has been demonstrated to be involved in the binding of iron. The redox state of this iron ion determines whether the regulator is active or not (Spiro and Guest, 1990).

**Energy conservation**

Cytochromes aa₃ and bb₃ from _P. denitrificans_ are proton-
pumping terminal oxidases (van Verseveld et al., 1981; Solioz et al., 1981; Puustinen et al., 1989). Analysis of proton translocation in cell suspensions of the Paracoccus oxidase mutants offers the unique opportunity to measure proton translocation of individual oxidases in whole-cell suspensions. During succinate oxidation, a minimum H+/e\⁽⁻⁾ stoichiometry of 2 is theoretically expected when ubiquinol is oxidized directly by cytochrome bb₃ (Fig. 6). On the other hand, a ratio of 3H+/e\⁽⁻⁾ is the theoretical maximum when ubiquinol is oxidized via cytochrome bcₑ and cytochrome aa₃ (Fig. 6).

Measurements of the set of Paracoccus mutants are in perfect agreement with the theoretical values (Table 2). Succinate oxidation by cytochrome bb₃ in the Δaa₃/cbb₃ mutant results in 2 H+/e\⁽⁻⁾. In the conditions used for analysis of proton translocation (0.4–1.7 μM O₂), ubiquinol appears to be oxidized preferentially via the cytochrome c branch (bcₑ complex, cytochrome c, cytochrome c oxidase) rather than by the quinol oxidase cytochrome bb₃. This observation may be due to the low affinity for oxygen that has been measured for cytochrome bb₃ (Kₘ 10–20 μM O₂; de Gier, 1995). This may explain why, in the Δcbb₃ mutant, electrons mainly flow to cytochrome aa₃, resulting in a stoichiometry of 3H+/e\⁽⁻⁾. The proton pumping capacity of cytochrome cbb₃, as first reported by Raitio and Wikström (1994) in a Δaa₃ mutant, has been confirmed here with the Δaa₃/bb₃ mutant during electron transfer from succinate to oxygen (Table 2).

The conclusion that cytochrome cbb₃ does translocate protons, however, is in conflict with the previous measurements by de Gier et al. (1994). In the latter study an H+/e\⁽⁻⁾ stoichiometry of 2 has been measured during succinate oxidation of the Δaa₃/bb₃ mutant, suggesting that cytochrome cbb₃ does not pump protons. The main technical difference is that, in the experiment presented here, HEPES (0.5 mM) rather than glycylglycine (1.5 mM) has been used as buffer. The 'decoupling' of cytochrome cbb₃ in glycylglycine (de Gier et al., 1994) has been confirmed by analysis of cells from a single batch in both buffers (not shown). The apparent buffer sensitivity of cytochrome cbb₃, a phenomenon that is not observed in the case of cytochromes aa₃ and bb₃, is not understood at present (see below).

The finding that all three terminal oxidases of Paracoccus (aa₃, cbb₃ and bb₃) couple the reduction of oxygen to the translocation of protons with maximal efficiency (1 H+/e\⁽⁻⁾) also does not agree with analyses of growth efficiency in chemostat cultures of (wild-type) Paracoccus denitrificans, grown under a variety of conditions. The maximal growth efficiency is observed only under conditions in which cytochrome aa₃ is expressed (reviewed by Stouthamer, 1991). To address the discrepancy between the latter physiological studies and the above-described proton-pumping capacity of cytochrome cbb₃, we are currently cultivating a number of Paracoccus oxidase mutants under well-defined conditions. Preliminary data from a comparison of the growth yields of these mutants suggest that the energy-transducing efficiency of cytochrome cbb₃ is indeed lower than that of cytochrome aa₃ (de Gier, 1995).

The latter conclusion would be consistent with the aforementioned observation that the H+/e\⁽⁻⁾ stoichiometry of cytochrome cbb₃ is not a fixed number, and may be sensitive to, for example, buffer composition (de Gier et al., 1994; Table 2). Moreover, proton pumping analysis of the Δaa₃/bb₃ mutant with ascorbate/TMPD as substrate, either in HEPES or in glycylglycine, results in H+/e\⁽⁻⁾ values that never exceed 50% of the ratio that is measured in the presence of cytochrome aa₃ (de Gier et al., 1994; Raitio and Wikström, 1994). This apparent variability in the H+/e\⁽⁻⁾ ratio of cytochrome cbb₃ might be the result of certain structural deviations of subunit I within the regions that have been demonstrated to be important for the coupling between oxygen reduction and proton pumping (see below).

Residues involved in proton translocation

A major challenge of cytochrome oxidase research is elucidation of the proton-translocation mechanism at a...
molecular level. This requires the identification of residues that are involved in the transfer of consumed (scalar) and/or pumped (vectorial) protons. Among the highly conserved residues in subunits I of cytochrome aa$_3$ and cytochrome bo$_3$, a large number of polar residues have been substituted by means of site-directed mutagenesis (reviewed by Hosler et al., 1993). An aspartic acid in the domain between transmembrane helices II and III (loop II–III) has recently been proposed to participate in the influx of protons into the oxidase core (Garcia-Horsman et al., 1995; Fetter et al., 1995). Substitution of this conserved aspartate with an asparagine (D124N; numbering for P. denitrificans cytochrome aa$_3$, CtaDII, is used throughout) in both cytochromes aa$_3$ and bo$_3$ results in decoupling of proton pumping from electron transfer. In addition, substitution of two conserved asparagine residues (N113, N131) results in a decrease in the H$^+$/e$^-$ ratio. Interestingly, proton pumping is recovered in a double mutant of E. coli CyoB, D124N/N131D (Garcia-Horsman et al., 1995). It has been suggested that this cytoplasmic loop plays an important role as part of the entry to the pumped proton pathway, and moreover, that there should be a second channel, the chemical proton pathway. Residues that may be part of the latter pathway are a tyrosine (Y280) in helix VI, as well as two threonines (T344, T351) and a lysine (K354) in helix VIII. Non-polar substitutions for each of these residues result in a severe loss of oxidase activity (Hosler et al., 1993). Both T351 and K354 have been proposed to participate in a proton-conducting channel towards the binuclear centre (Hosler et al., 1993; Fetter et al., 1995). Spectroscopic analyses suggest that T344 is in close proximity to the binuclear centre. Y280 may be a ligand to Cu$_B$, at least during part of the catalytic cycle of oxygen reduction (Hosler et al., 1993). In addition, one of the invariant histidines that has been identified as a Cu$_B$ ligand has recently been proposed to play a key role in the proton-translocation machinery. In the ‘histidine cycle’ model, a Cu$_B$ co-ordinating histidine has been proposed to move back and forth in the binuclear ‘pocket’, thereby passing two protons from the inside to the outside (Morgan et al., 1994).

Analysis of the recently resolved structure of P. denitrificans cytochrome aa$_3$ (Iwata et al., 1995) supported many of the conclusions that were based on previous studies of the E. coli and R. sphaeroides oxidases (Hosler et al., 1993; Fetter et al., 1995; Garcia-Horsman et al., 1995). Indeed, two suggested proton channels have been envisaged in the structural model (Iwata et al., 1995). A pathway for consumed protons, from the cytoplasmic surface to the oxygen-binding site, appears to be made up of S291, K354, T351, the hydroxyl group of haem a$_3$, and, ultimately, Y280, which may donate a proton to oxygen (Fig. 3A, Table 3). With the exception of K354, all residues in this channel appear to be connected by hydrogen bonds, either directly or via putative solvent molecules. A second series of hydrophilic residues may constitute the pathway for pumped protons. A gate is formed by D124, N119, and T203; subsequently, a number of hydrophilic residues, again with additional putative solvent molecules, may form a pathway to E278 (Table 3). Beyond E278 the proton pathway is less clear. However, as proposed before (Morgan et al., 1994), the structural model suggests that one of the histidine ligands, H325, may not be in a fixed position, but rather may switch between different conformational states. One option is that two subsequent protons, approaching the catalytic site via E278, convert the H325 imidazolate, via imidazole, into imidazolium. In the latter state, the positively charged H325 moves away from the binuclear centre, and no longer ligates Cu$_B$. The two protons of H325 imidazolium would leave the system via the exit pathway, in which groups of the haem a$_3$ molecule and/or residues in the periplasmic loop XI–X may be involved (Table 3), and H325 imidazolide would return to its original position (for details, see Iwata et al., 1995).

As cytochrome cbb$_3$ has the capacity to pump protons (Raitio and Wikström, 1994; Table 2), comparison of its sequence with that of the much better characterized cytochrome aa$_3$ is of particular interest. From the alignment presented in Fig. 3A, it is obvious that the sequences from CcoN/FixN show some significant deviation from both the aa$_3$-type and the bo$_3$-type oxidases, especially

Table 3. Comparison of residues proposed to be involved in the chemical and the pumped proton channels in cytochrome aa$_3$ (Hosler et al., 1993; Fetter et al., 1995; Iwata et al., 1995), with counterparts in cytochrome cbb$_3$, based on the alignment presented in Fig. 3.

<table>
<thead>
<tr>
<th>aa$_3$ Location</th>
<th>cbb$_3$ Location</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical channel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a$_3$-hydroxyl</td>
<td>Haem</td>
<td>No hydroxyl</td>
</tr>
<tr>
<td>Y280</td>
<td>TM-Vi</td>
<td>G</td>
</tr>
<tr>
<td>T344</td>
<td>TM-VIII</td>
<td>S</td>
</tr>
<tr>
<td>K354</td>
<td>TM-VIII</td>
<td>G</td>
</tr>
<tr>
<td>S291</td>
<td>TM-VI</td>
<td>M</td>
</tr>
</tbody>
</table>

| Pump channel | | |
| D399 | Loop-IX/X | N | + |
| L393-carboxyl | Loop-IX/X | M | + |
| a$_3$-propionate | Haem | a$_3$-propionate | + |
| a$_3$-formyl | Haem | No formyl | — |
| H325 | TM-VII | H | + |
| E278 | TM-VI | A | — |
| N113 | TM-II | X | — |
| N131 | Loop-II/III | X | — |
| N199 | TM-IV | V | — |
| T203 | TM-IV | T | — |
| D124 | Loop-II/III | X | — |

In transmembrane helix II (TM-II) and the interconnecting loop between helices II and III (loop II–III) a reliable alignment is not possible; in this case the cbb$_3$ residues are marked ‘X’.

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with respect to the residues that have been predicted to be part of either one of the two proton channels (Table 3). Although alignment of the oxidase sequences at their N-terminals is difficult, it is clear that the invariant aspartate residue in loop II–III of cytochrome aa₃ is not conserved in cytochrome cbb₃ (Table 3). In helix VI of cytochrome cbb₃, the tyrosine (Y280) and the glutamate (E278) that are conserved in all aa₃- and b₀₃-type oxidases (and appear to be essential in the aforementioned proton pump model) are substituted by a glycine and an alanine, respectively. In helix VIII, the threonines are conserved in all aa₃- and b₀₃-type oxidases (and probably also in cytochromes b₀₂ and b₂₃) that are not conserved in cytochrome cbb₃. These proton-conducting pathways are undoubtedly an essential component of the proton-translocase machinery. Hence, it is concluded that the design of the proton pump in cytochrome cbb₃ differs significantly from that present in the other oxidase types. Although site-directed mutagenesis would provide more definitive evidence, it is tempting to assume that these deviations in cytochrome cbb₃ might correlate with its apparently variable H⁺/e⁻ coupling.

### Experimental procedures

**Bacterial strains, plasmids and growth conditions**

The strains of *P. denitrificans* and *E. coli* as well as the plasmids used in this study are listed in Table 4. Cells of wild-type *P. denitrificans* (Pd1222) and mutants were cultivated in aerobic batch cultures (0.5 l bottles with 10 mM culture, on a rotary shaker at 30°C), with either brain–heart infusion (BHI) broth or minimal medium supplemented with 25 mM succinate, as described previously (van Spanning et al., 1990). For microaerobic cultivation, cultures were not shaken. *E. coli* strains were cultivated in YT medium at 37°C. When appropriate, antibiotics were added: rifampicin (Rif, 40 mg l⁻¹), streptomycin (Sm, 50 mg l⁻¹), tetracycline (Tc, 12.5 mg l⁻¹), kanamycin (Km, 50 mg l⁻¹) and ampicillin (Amp, 100 mg l⁻¹).

### Purification of the cbb₃-type cytochrome c oxidase from *P. denitrificans*

Cells from succinate-grown Pd93.11 (Δaa₃/bb₃) were harvested in the late log phase, and resuspended in 20 mM Bis-Tris propane (pH 7.3), 2 mM EDTA and 0.5 mM PMSF. Membranes were prepared by passage of the cell suspension twice through a French Pressure Cell and subsequent centrifugation (30 min, 150,000 × g). The solubilized membrane proteins were applied to a Q-Sepharose HP (Pharmacia) column in 20 mM Bis-Tris propane (pH 7.3), with 0.03% (w/v) dodecyl maltoside, and bound protein was eluted with a linear NaCl gradient (0.0–1.0 M). Oxidase activity was measured spectrophotometrically with TMPD as described previously (Lübben et al., 1994). Fractions with cytochrome c oxidase activity eluted at 0.3 M NaCl. Pooled fractions were applied onto a sucrose gradient, as described previously (Gray et al., 1994). Fractions with TMPD oxidase activity were applied to a Chromab-Sepharose FF (Pharmacia) column, saturated with Cu²⁺. A linear ammonium chloride gradient (0.0–1.0 M) in 20 mM Tris (pH8.0), 0.5 M NaCl, 15 mM PMSF and 0.03% (w/v) dodecyl maltoside was used to elute bound proteins. Cytochrome c oxidase activity eluted at 0.3 M ammonium chloride. As a last purification step the active fractions were loaded onto a Q-Sepharose HP column (Pharmacia) in 20 mM Bis-Tris propane (pH 7.3), with 0.03% (w/v) dodecyl maltoside, and bound protein was ultracentrifugation (30 min, 150,000 × g). The solubilized membrane proteins were applied to a Q-Sepharose HP (Pharmacia) column in 20 mM Bis-Tris propane (pH 7.3), with 0.03% (w/v) dodecyl maltoside, and bound protein was eluted with a linear NaCl gradient (0.0–1.0 M). Oxidase activity was measured spectrophotometrically with TMPD as described previously (Lübben et al., 1994). Fractions with cytochrome c oxidase activity eluted at 0.3 M NaCl. Pooled fractions were applied onto a sucrose gradient, as described previously (Gray et al., 1994). Fractions with TMPD oxidase activity were applied to a Chromab-Sepharose FF (Pharmacia) column, saturated with Cu²⁺. A linear ammonium chloride gradient (0.0–1.0 M) in 20 mM Tris (pH8.0), 0.5 M NaCl, 15 mM PMSF and 0.03% (w/v) dodecyl maltoside was used to elute bound proteins. Cytochrome c oxidase activity eluted at 0.3 M ammonium chloride. As a last purification step the active fractions were loaded onto a Q-Sepharose HP column (Pharmacia) in 20 mM Bis-Tris propane (pH 7.3), with 0.03% (w/v) dodecyl maltoside, and bound protein was

<table>
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<tr>
<th>Strain/Plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Plasmid</td>
<td></td>
</tr>
<tr>
<td>E. coli TG1</td>
<td>supE hsdD5 thy Δlac ClaAB proA proAB lacZ lacZM15 F' traD26 recA13 lac157 intel proA2</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td>HB101</td>
<td>F' hsdS20 (5 m6) lacY1 proA2 recA13</td>
<td>Boyer et al. (1979)</td>
</tr>
<tr>
<td>S17.1</td>
<td>SmR pro (5 m6) RP4-2, integrated (Tc::Mu) (Km::Tn7)</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>P. denitrificans</td>
<td></td>
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<tr>
<td>Pd1222</td>
<td>RifR, enhanced conjuction frequencies, (5 m6)</td>
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<td>Pd9218 derivative, ΔctaDI ΔctaDI</td>
<td>de Gier et al. (1994)</td>
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<td>Pd1222 derivative, qoxB::KmR</td>
<td>de Gier et al. (1994)</td>
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<td>Pd9220 derivative, ΔctaDI ΔctaDI cycB::KmR</td>
<td>de Gier et al. (1994)</td>
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<td>Pd27.21</td>
<td>Pd1222 derivative, ccoNO::KmR</td>
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<tr>
<td>Pd93.12</td>
<td>Pd9220 derivative, ΔctaDI ΔctaDI ccoNO::KmR</td>
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<td>pUC19</td>
<td>AmpR lacZ'</td>
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<td>pUC4KXX</td>
<td>KmR (Tn5)</td>
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<td>pRK2020</td>
<td>TcR pRK2013 Km::Tn10 (colEI) AmpR oriV SmR</td>
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<td>pGRDp1 derivative, ccoNOQP</td>
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<td>pCo03</td>
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<tr>
<td>pCooNO::KmR</td>
<td>pCoo1 derivative, ccoNO::KmR</td>
<td>This work</td>
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<td>pGRDp1 derivative, ccoNO::KmR</td>
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</tr>
</tbody>
</table>
eluted at a linear NaCl gradient (0.0-1.0 M). Active fractions eluted at 0.3 M NaCl. The purified oxidase was analysed by SDS-PAGE and spectrophotometrically (Laemmli, 1970; de Gier et al., 1994). Haem staining was performed as described by Thomas et al. (1976).

**DNA manipulation**

General cloning techniques were carried out as described by Ausubel et al. (1993). Conjugations were performed as described previously (de Vries et al., 1989). The matings of Paracoccus host strains were performed either directly with an E. coli S17.1 derivative that carried the plasmid of interest or via a triparental mating using any E. coli strain transformed with the plasmid of interest, in combination with E. coli HB101(pRK2200) containing the 'helper plasmid'. Mutagenesis was performed using pGRPd1 as described by van Spanning et al. (1990).

The PCR was performed using Super-Taq polymerase (HT Biotechnology Ltd.), essentially as described by de Gier et al. (1994). Primers were synthesized according to the amino acid sequence of the N-terminal peptide of the 30kDa subunit of the Paracoccus cb2 complex (CooO) (nos 171 and 172). These were degenerate primers with a bias for G:C at the third position of a codon. The sequences of these primers are as follows: 171 (sense), 5'-AAAAAAGTCTGCNAT[T/C]CTNGA[A/G]CA[T/C]-3'; and 172 (antisense), 5'-AAAAGAATTCA[G/A]TATCGNGNACACNAG[A/G]AA-3'.

DNA sequencing was performed using the dideoxy method (Sanger et al., 1977) on fragments subcloned into M13mp18/19 (Sanger et al., 1980) with the Taq dye dideoxy terminator cycle sequencing kit, and the Taq dye primer cycle sequencing kit (Applied Biosystems). Sequence analysis was carried out on a 370A DNA sequencer (Applied Biosystems). Editing of the sequences was performed with the SEQUENCHER 2.1 Program (Gene Codes Corp.).

**Oxygen-consumption analysis**

Oxygen consumption by bacterial cell suspensions was performed polarographically with a Clark-type oxygen electrode, as described by de Gier et al. (1994).

**Proton translocation**

Proton translocation was studied by using the oxygen-pulse method in intact cell suspensions (de Gier et al., 1994; Raitio and Wikström, 1994). Cells were harvested in the late log phase from succinate batch cultures, washed twice and resuspended in 150 mM KCl (final OD680 is 100), and diluted four times in reaction medium (pH 7.4) in the stirred anaerobic reaction vessel (3.0 ml). The buffer contained 100 mM KCl, 100 mM KSCN, 0.5 mM HEPES (pH 7.4) and 30 μM rotenone, as described by Raitio and Wikström (1994). Succinate was used as the electron donor (final concentration 2.5 mM). The reactions were started with small pulses (5-20 μl) of air-saturated water (containing 258 μM O2 at 25°C) or 1.0 mM potassium ferricyanide, made anaerobic with argon. Pulses were calibrated with 1.0 mM HCl or 0.5 mM oxalic acid, made anaerobic with argon. Controls with the protonophore FCCP were routinely included to ascertain if the observed proton ejection was due to proton translocation.

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