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Structural and functional analysis of aa\textsubscript{3}-type and cbb\textsubscript{3}-type cytochrome c oxidases of \textit{Paracoccus denitrificans} reveals significant differences in proton-pump design


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Summary

In \textit{Paracoccus denitrificans} the aa\textsubscript{3}-type cytochrome c oxidase and the \textit{bb}_{3}\textit{b}_{3}-type quinol oxidase have previously been characterized in detail, both biochemically and genetically. Here we report on the isolation of a genomic locus that harbours the gene cluster \textit{ccoNOQP}, and demonstrate that it encodes an alternative cbb\textsubscript{3}-type cytochrome c oxidase. This oxidase has previously been shown to be specifically induced at low oxygen tensions, suggesting that its expression is controlled by an oxygen-sensing mechanism. This view is corroborated by the observation that the \textit{ccoNOQP} gene cluster is preceded by a gene that encodes an FNR homologue and that its promoter region contains an FNR-binding motif. Biochemical and physiological analyses of a set of oxidase mutants revealed that, at least under the conditions tested, cytochromes aa\textsubscript{3}, \textit{bb}_{3}\textit{b}_{3}\textit{b}_{3} and cbb\textsubscript{3} make up the complete set of terminal oxidases in \textit{P. denitrificans}. Proton-translocation measurements of these oxidase mutants indicate that all three oxidase types have the capacity to pump protons. Previously, however, we have reported decreased H\textsuperscript{+}/e\textsuperscript{-} coupling efficiencies of the cbb\textsubscript{3}-type oxidase under certain conditions. Sequence alignment suggests that many residues that have been proposed to constitute the chemical and pumped proton channels in cytochrome aa\textsubscript{3} (and probably also in cytochrome \textit{bb}_{3}\textit{b}_{3}) are not conserved in cytochrome cbb\textsubscript{3}. It is concluded that the design of the proton pump in cytochrome cbb\textsubscript{3} differs significantly from that in the other oxidase types.

Introduction

In mitochondrial respiration the reduction of oxygen to water is catalysed by the aa\textsubscript{3}-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 \textit{Escherichia coli}. In this bacterium two distinct quinol oxidases (cytochromes \textit{bo}_{3} and \textit{bd}) are expressed during aerobic growth, but a cytochrome \textit{c} branch is absent (Anraku and Gennis, 1987). \textit{Paracoccus denitrificans}, in turn, possesses three distinct terminal oxidases. An aa\textsubscript{3}-type cytochrome c oxidase (Raitio et al., 1987; 1990; Steinrücke et al., 1987; van Spanning et al., 1990; van der Oost et al., 1991), as well as a \textit{bb}_{3}/\textit{ba}_{3}-type quinol oxidase, hereafter called cytochrome \textit{bb}_{3}, have been characterized previously (de Gier et al., 1994; Richter et al., 1994). In addition, the expression of an alternative cytochrome c oxidase has been suggested (Bosma, 1989; de Gier et al., 1992; 1994; Raitio and Wikström, 1994).

In the present study, a \textit{P. denitrificans} \textit{aa}_{3}/\textit{bb}_{3} mutant...
was used for the purification of an alternative cytochrome c oxidase: cytochrome cbb₃. 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 downstream of which are homologous to HemN and FNR.

Analysis of the oxygen consumption by single and multiple oxidase mutants of *P. denitrificans* suggests that, besides cytochrome aa₃ and cbb₃, 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 cbb₃-type cytochrome c oxidase the coupling between oxygen reduction and proton translocation may be less tight than in cytochromes aa₃ and bb₃/bo₃.

**Results**

**Purification of the cbb₃-type cytochrome c oxidase**

In a previous study, the simultaneous deletion of cytochromes aa₃ and bb₃ in *P. denitrificans* (Δaa₃/bb₃) 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* Δaa₃/bb₃ 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 cbb₃ (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 AILEKHVKIKNATLLLVFSLVVRI. 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

![Fig. 1. A. SDS–PAGE of purified cytochrome cbb₃ (approx. 7.5 μg of protein). The molecular mass standards are: 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa. B. Absorption spectrum of purified cytochrome cbb₃ after reduction with Na-dithionite (approx. 0.01 mg protein ml⁻¹).](https://example.com/fig1.png)
Cytochrome cbb₃ of Paracoccus denitrificans

Fig. 2. A. Genetic organization and physical map of the P. denitrificans cccoNOQP 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 cccoNOQP 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 cccoNOQP 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 (TTGAT-N₄-ATCAA; Spiro, 1994), and identical to the anaerobox found upstream of the A. caulino noans 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/fixN 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 Cx₂CHx₄M 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 (Gx₃Fx₃Cx₂CHx₄M) 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 cccoNOQP 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 orf177, which is located adjacent to the fixNOQP operon.
Fig. 3. Alignments of the *P. denitrificans* cooNOQP-encoded polypeptides.

A. CcoN of *P. denitrificans* (cytochrome cbb), FixN of *B. japonicum* (cytochrome cbb₃; Preisig et al., 1993), CtaDll of *P. denitrificans* (cytochrome *a*₁; 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.

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 *cbb*$_3$ 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 *ccNOQP* 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 (*Km*) 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 *ctaDI/ctaDII* double mutant Pd92.20 (*Δaa*$_3$), yielding Pd27.21 (*Δcbb*$_3$) and Pd93.12 (*Δaa*$_3$/*cbb*$_3$), respectively. Southern blot analysis of chromosomal DNA of selected *Km* 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 cytoperipheral membranes showed that the 30 kDa cytochrome *c* is absent, and the 45 kDa cytochrome *c* is only just detectable in the *Δcbb*$_3$ 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 *bc*$_1$ 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 *Δaa*$_3$ mutant and the *Δcbb*$_3$ mutant was only slightly inhibited, and that of the *Δaa*$_3$/*cbb*$_3$ mutant strain appeared to be unaffected.

The effect of the cytochrome *bc*$_1$ inhibitors on succinate oxidation in the *Δcbb*$_3$ mutant and in the *Δaa*$_3$ mutant was much less than in the wild type. Again, in the *Δaa*$_3$/*cbb*$_3$ 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 *Δcbb*$_3$ and the *Δaa*$_3$ mutant. In the *Δaa*$_3$/*cbb*$_3$ 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 *cbb*$_3$-type cytochrome *c* oxidase. The H$^+$/e$^-$ ratios of the *Δaa*$_3$ mutant and the *Δbb*$_3$/*aa*$_3$ mutant clearly show that the *cbb*$_3$-type cytochrome *c* oxidase does have the capacity to translocate protons. In Fig. 5, representative proton-translocation traces of the *Δbb*$_3$/*aa*$_3$ mutant are shown. The H$^+$/e$^-$ ratios of the *Δcbb*$_3$ mutant and the *Δaa*$_3$/*cbb*$_3$ mutant (not shown).

![Fig. 4. SDS–PAGE and subsequent haem staining of membrane fractions of the wild type (wt) and the cytochrome *cbb*$_3$ mutant (*Δcbb*$_3$) of *P. denitrificans* after microaerobic cultivation. The *c*-type cytochromes indicated are subunits of the *bc*$_1$ complex (*FbcC*), subunits of cytochrome *cbb*$_3$ (*CcoP*, 45 kDa; *CcoO*, 30 kDa), cytochrome *c*$_{552}$ (*CycM*), and a subunit of the NO reductase (*NorC*).]
Table 1. Oxygen-consumption measurements of whole-cell suspensions of different P. denitrificans strains. Pd1222 (wild type), Pd92.20 (Δaa3 mutant), Pd27.21 (Δcbb3 mutant), and Pd93.12 (Δaa3/cbb3 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</td>
<td>myx</td>
<td>AA</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td>1.5</td>
<td>0.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Δaa3 mutant</td>
<td></td>
<td>1.3</td>
<td>1.2</td>
<td>2.9</td>
</tr>
<tr>
<td>Δcbb3 mutant</td>
<td></td>
<td>1.4</td>
<td>1.2</td>
<td>2.7</td>
</tr>
<tr>
<td>Δaa3/cbb3 mutant</td>
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<td>2.2</td>
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<td>2.1</td>
</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 O2 per mg of protein per s). Inhibitors: antimycin A (AA), myxothiazol (myx).

mutant confirm that the aa3-type cytochrome c oxidase and the bb3-type quinol oxidase both translocate protons. Upon the addition of inhibitors of the bc1 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 Δaa3/cbb3 mutant. This suggests that ubiquinol apparently was preferentially oxidized by ubiquinol:cytochrome c oxidoreductase (bc1 complex) rather than by ubiquinol:oxygen oxidoreductase (cytochrome bb3).

With the artificial electron-donating couple ascorbate/TMPD, proton translocation was barely detectable in the absence of cytochrome aa3. 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 aa3 and cytochrome cbb3, 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 Δaa3/cbb3 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 (bc1 complex), cytochrome cbb3 and cytochrome c oxidase (cytochrome aa3) (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 bb3 (de Gier et al., 1994).

Analysis of a recently generated Δaa3/bb3 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 cbb3-type oxidase has been cloned and sequenced. It has previously been demonstrated that no quinol oxidase activity remains in a Δbb3 mutant (de Gier et al., 1994), and here we show that a Δaa3/cbb3 double mutant does not
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., 1995), the presence of a binuclear centre typical of haem-copper oxidases and the absence of CuA. Moreover, metal analysis revealed the ratio of haemC:haemB:copper to be 3:2:1. 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 CuB, 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 CuA. 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 CuB) 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 bc₁ 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 anaerob box upstream of the fixNOOP and fixGHIS clusters, thereby activating their transcription (David et al., 1988; Batut et al., 1989; Kahn et al., 1989; Fischer, 1994). To date, no FixJ-like system in _P. denitrificans_ has been identified 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 flank ing 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-
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 bb\(_3\) (Fig. 6). On the other hand, a ratio of 3H+/e\(^{-}\) is the theoretical maximum when ubiquinol is oxidized via cytochrome bc\(_1\) and cytochrome aa\(_2\) (Fig. 6).

Measurements of the set of P. denitrificans mutants are in perfect agreement with the theoretical values (Table 2). Succinate oxidation by cytochrome bb\(_3\) in the Δaa\(_2\)/cbb\(_3\) mutant results in 2 H+/e\(^{-}\). In the conditions used for analysis of proton translocation (0.4–1.7 μM O\(_2\)), ubiquinol appears to be oxidized preferentially via the cytochrome c branch (bc\(_1\), complex, cytochrome c, cytochrome c oxidase) rather than by the quinol oxidase cytochrome bb\(_3\). This observation may be due to the low affinity for oxygen that has been measured for cytochrome bb\(_3\), (K\(_m\) 10–20 μM O\(_2\); de Gier, 1995). This may explain why, in the Δcbb\(_3\) mutant, electrons mainly flow to cytochrome aa\(_3\), resulting in a stoichiometry of 3H+/e\(^{-}\). The proton pumping capacity of cytochrome cbb\(_3\), as first reported by Raitio and Wikström (1994) in a Δaa\(_3\) mutant, has been confirmed here with the Δaa\(_3\)/bb\(_3\) mutant during electron transfer from succinate to oxygen (Table 2).

The conclusion that cytochrome cbb\(_3\) 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 Δaa\(_3\)/bb\(_3\) mutant, suggesting that cytochrome cbb\(_3\) 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 cbb\(_3\) 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 cbb\(_3\), a phenomenon that is not observed in the case of cytochromes aa\(_2\) and bb\(_3\), is not understood at present (see below).

The finding that all three terminal oxidases of Paracoccus (aa\(_3\), cbb\(_3\) and bb\(_3\)) 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) P. denitrificans, grown under a variety of conditions. The maximal growth efficiency is observed only under conditions in which cytochrome aa\(_3\) is expressed (reviewed by Stouthamer, 1991). To address the discrepancy between the latter physiological studies and the above-described proton-pumping capacity of cytochrome cbb\(_3\), 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 cbb\(_3\) is indeed lower than that of cytochrome aa\(_3\) (de Gier, 1995).

The latter conclusion would be consistent with the aforementioned observation that the H+/e\(^{-}\) stoichiometry of cytochrome cbb\(_3\) 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 Δaa\(_3\)/bb\(_3\) 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 aa\(_3\) (de Gier et al., 1994; Raitio and Wikström, 1994). This apparent variability in the H+/e\(^{-}\) ratio of cytochrome cbb\(_3\) 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 aa₃ and cytochrome bo₃, 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 aa₃, CtaDII, is used throughout) in both cytochromes aa₃ and bo₃ 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 CuB, 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 CuB ligand has recently been proposed to play a key role in the proton-translocation machinery. In the 'histidine cycle' model, a CuB 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 aa₃ (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 a₃, 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 imidazole, via imidazole, into imidazolium. In the latter state, the positively charged H325 moves away from the binuclear centre, and no longer ligates CuB. The two protons of H325 imidazolium would leave the system via the exit pathway, in which groups of the haem a₃ molecule and/or residues in the periplasmic loop XI–X may be involved (Table 3), and H325 imidazolium would return to its original position (for details, see Iwata et al., 1995).

As cytochrome cbb₃ has the capacity to pump protons (Raitio and Wikström, 1994; Table 2), comparison of its sequence with that of the much better characterized cytochrome aa₃ 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 aa₃-type and the bo₃-type oxidases, especially

### Table 3. Comparison of residues proposed to be involved in the chemical and the pumped proton channels in cytochrome aa₃ (Hosler et al., 1993; Fetter et al., 1995; Iwata et al., 1995), with counterparts in cytochrome cbb₃, based on the alignment presented in Fig. 3.

<table>
<thead>
<tr>
<th>Residue</th>
<th>aa₃ Location</th>
<th>cbb₃ Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemical channel</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a₃-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>
<tr>
<td><strong>Pump channel</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D399</td>
<td>Loop-I/X</td>
<td>N</td>
</tr>
<tr>
<td>L393-carbonyl</td>
<td>Loop-I/X</td>
<td>M</td>
</tr>
<tr>
<td>a₂-propionate</td>
<td>Haem</td>
<td>a₂-propionate</td>
</tr>
<tr>
<td>a₂-formyl</td>
<td>Haem</td>
<td>No formyl</td>
</tr>
<tr>
<td>H325</td>
<td>TM-VII</td>
<td>H</td>
</tr>
<tr>
<td>E278</td>
<td>TM-VI</td>
<td>A</td>
</tr>
<tr>
<td>N113</td>
<td>TM-II</td>
<td>X</td>
</tr>
<tr>
<td>N131</td>
<td>Loop-II/III</td>
<td>X</td>
</tr>
<tr>
<td>N199</td>
<td>TM-IV</td>
<td>V</td>
</tr>
<tr>
<td>T203</td>
<td>TM-IV</td>
<td>T</td>
</tr>
<tr>
<td>D124</td>
<td>Loop-II/III</td>
<td>X</td>
</tr>
</tbody>
</table>

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 cbb₃ 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-termini 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 as serines, but a glycine is found at the position of the conserved lysine (Fig. 3A, Table 3). In conclusion, comparison of the primary structures suggests that many residues that have been proposed to constitute the chemical and pumped proton channels in cytochrome aa3 (and probably also in cytochromes bo3 and bb3) are not conserved in cytochrome cbb3. These proton-conducting pathways are undoubtedly an essential component of the proton-translocation 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 100 ml of 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 (1.5 h, 150,000 × g). Membranes were solubilized in 20 mM Bis-Tris propane (pH 7.3), with 1.0% (w/v) dodecyl maltoside and 0.15 mM PMSF (30 min, 4 °C). The suspension contained approx. 10 mg protein per ml and was cleared by 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 (Lübken 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 Chelating–Sepharose FF (Pharmacia) column, saturated with Cu2+. A linear ammonium chloride gradient (0.0–1.0 M) in 20 mM Tris (pH 8.0), 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>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. denitrificans</td>
<td>Pd1222</td>
<td>RifR, enhanced conjugation frequencies, (rmp m')</td>
<td>de Vries et al. (1989)</td>
</tr>
<tr>
<td>Pd92.20</td>
<td>Pd1222 derivative, ΔcduA</td>
<td>de Gier et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>Pd39.11</td>
<td>Pd1222 derivative, ΔcduA</td>
<td>de Gier et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>Pd27.21</td>
<td>Pd1222 derivative, ΔcduA</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>Pd93.12</td>
<td>Pd1222 derivative, ΔcduA</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>Plasmid</td>
<td>pUC19</td>
<td>AmpR lacZ</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>M13mp18/19</td>
<td>pUC18/19 mcs, lacZ</td>
<td>Sanger et al. (1980)</td>
<td></td>
</tr>
<tr>
<td>pPK2020</td>
<td>TcR pPK2013 Km: Tn10 oriV</td>
<td>Ditta et al. (1985)</td>
<td></td>
</tr>
<tr>
<td>pGPRpd1</td>
<td>TcR pPK2013 Km: Tn10 oriV (colE1) AmpR oriT SmR</td>
<td>van Spanning et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>pCoo1</td>
<td>pUC19 derivative, cccNOQP</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pCoo2</td>
<td>pUC19 derivative, cccNOQP</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pCoo3</td>
<td>pUC19 derivative, cccNOQP</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pCooNO: KmR</td>
<td>pCoo1 derivative, cccNO: KmR</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pRT27.21</td>
<td>pGRPd1 derivative, cccNO: KmR</td>
<td>This work</td>
<td></td>
</tr>
</tbody>
</table>
eluted with 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 30 kDa subunit of the Paracoccus cbcA 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/A]CTNGA[A/G]CA[T/C]-3'; and 172 (antisense), 5'-AAGAATTC[A/G/T]ATNGNGAANCNACNAG[A/G]AA9'.

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 SEQUENCER 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 OD660 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 C25 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.

**Acknowledgements**

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