University of Groningen

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

Gier, Jan-Willem L. de; Schepper, Mike; Reijnders, Willem N.M.; Dyck, Stef J. van; Slotboom, Dirk Jan; Warne, Antony; Saraste, Matti; Krab, Klaas; Finel, Moshe; Stouthamer, Adriaan H.

Published in:
Molecular Microbiology

DOI:
[10.1111/j.1365-2958.1996.tb02644.x](https://doi.org/10.1111/j.1365-2958.1996.tb02644.x)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1996

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Gier, J-W. L. D., Schepper, M., Reijnders, W. N. M., Dyck, S. J. V., Slotboom, D. J., Warne, A., Saraste, M., Krab, K., Finel, M., Stouthamer, A. H., Spanning, R. J. M. V., & Oost, J. V. D. (1996). Structural and functional analysis of aa3-type and cbb3-type cytochrome c oxidases of *Paracoccus denitrificans* reveals significant differences in proton-pump design. *Molecular Microbiology*, 20(6), 1247-1260.
<https://doi.org/10.1111/j.1365-2958.1996.tb02644.x>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

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,¹ Mike Schepper,¹
Willem N. M. Reijnders,¹ Stef J. van Dyck,¹
Dirk Jan Slotboom,^{1,2} Antony Warne,² Matti Saraste,²
Klaas Krab,¹ Moshe Finel,³ Adriaan H. Stouthamer,¹
Rob J. M. van Spanning^{1*} and John van der Oost^{1,4}

¹Department of Molecular and Cellular Biology,
BioCentrum Amsterdam, Vrije Universiteit, De Boelelaan
1087, NL-1081 HV Amsterdam, Netherlands.

²European Molecular Biology Laboratory,
Meyerhofstrasse 1, D-69012 Heidelberg, Germany.

³Department of Medical Chemistry, University of Helsinki,
Siltavuorenpenger 10, FI-00014 Helsinki, Finland.

⁴Present address: Department of Microbiology,
Wageningen Agricultural University, H. van Suchtelenweg
4, NL-6703 CT Wageningen, Netherlands.

Summary

In *Paracoccus denitrificans* the *aa₃*-type cytochrome *c* oxidase and the *bb₃*-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 *ccoNOQP*, and demonstrate that it encodes an alternative *cbb₃*-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 *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 *aa₃*, *bb₃* and *cbb₃* make up the complete set of terminal oxidases in *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^+/e^- coupling efficiencies of the *cbb₃*-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 *aa₃* (and probably also in cytochrome *bb₃*) are not conserved in cytochrome *cbb₃*. It is concluded that the design of the proton pump in cytochrome *cbb₃* differs significantly from that in the other oxidase types.

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 *bo₃* and *bd*) 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. An *aa₃*-type cytochrome *c* oxidase (Raitio *et al.*, 1987; 1990; Steinrück *et al.*, 1987; van Spanning *et al.*, 1990; van der Oost *et al.*, 1991), as well as a *bb₃/ba₃*-type quinol oxidase, hereafter called cytochrome *bb₃*, 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 *P. denitrificans* $\Delta aa_3/bb_3$ mutant

Received 13 September, 1995; revised 18 March, 1996; accepted 20 March, 1996. *For correspondence. E-mail spanning@bio.vu.nl; Tel. (20) 4447183; Fax (20) 4447123.

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 downstream of *ccoNOQP*. In the upstream region two open reading frames were found, the derived sequences 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 *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* ($\Delta aa_3/bb_3$) 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* $\Delta aa_3/bb_3$ 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

Fig. 1. A. SDS-PAGE of purified cytochrome *cbb*₃ (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 *cbb*₃ after reduction with Na-dithionite (approx. 0.01 mg protein ml⁻¹).

C-containing subunit which would co-migrate with the 45 kDa polypeptide, as demonstrated below.

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 $\Delta aa_3/bb_3$ 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 *fixNOQP* 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 *fixNOQP* nomenclature

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 (Km^R) 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* (TTGAT-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/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 *Rhodospirillum rubrum* 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, Cu_B (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 Cx₂CHx_nM 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 (Gx₃Fx₃Cx₂CHx_nM) 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 orf277, which is located adjacent to the *fixNOQP* operon

A

Pd CcoN	1	-----MLDTIKLIAAGTIAVLAAIAANYARPDDLAYLVNALIIMLAAGIMFLRVLRQMGNEQPA	
Bj FixN	1	MSQPSISKSMITIGESGLAVFAATAFLCVIAAAKALDAPFAFHAALSAAASVAAVFCIVNRYFERPA	
Pd CtaD	1	-----MADAHVH	
Ec CyoB	1	-----MFGKLSLDAVPFHEPIVMVTIAGIILGGLALVG	
TM I			
Pd CcoN	60	LEPHPETQYMDVVVR---AGVIATAFWGVVGVFLVGVVIAFQIAFPALNLS-----	
Bj FixN	68	ALPPAEINGRPNYNGMPIKFSFMMFWGIAGFLVGLIIASQLAWPALNFDL-----	
Pd CtaD	8	GHGDHHDTRGFFTRWFMSTNHKDIGILYLFITAGIVGLISVCFTVYMRMELQHPGVQYMCLEGARLIA	
Ec CyoB	34	LITYFGKWTYLVKEWLTSVDHKLIGIMYIIIVMLLRGFAADAIMMRSSQAL-----A	
TM II			
Pd CcoN	104	-----ITMGYNFGKLRPLHTSAVIFAFGGNLIATSFYVVQRTSAAR-----	TM III
Bj FixN	116	-----PWISFGRLRPLHTSAVIFAVGGNLIATSFYVVQKSCRVR-----	-----LWGGNAAWFVFWG
Pd CtaD	75	DASAECTPNGLHWNVMITYHGVLMFFVVIPALFGGFGNYFMPLHIGAPDMAFPRLNLSYWMYVCG	
Ec CyoB	87	SAGEAGFLPPHHYDQIFTAGHVMIFVAMPFVIG-LMNLVVLQIGARDVAFPLNLSFWFTVVG	
H N D N			
94 113 124 131			
TM IV			
Pd CcoN	164	YQLFIVLA-----ATGYILGATQSK-----EYAEPEWYVDWVLTVVVVVYLAFLGTILK	
Bj FixN	173	YNFFILVA-----GTGYLLGVTQSK-----EYAEPEWYADLWLTIVVVVYLLVFLATIIK	
Pd CtaD	142	VALGVASLLAPGGNDQMGSGVGVVLYPPLST--TEAGYSMDLATFAVHVSAGSSILGAINIITTFIN	
Ec CyoB	153	VILNVNLSLGVGE----FAQTGWLAYPPLSGIEYSPGVGVVYWIWSLQLSGIGTTLTGINFVTLK	
N T			
199 203			
TM V			
Pd CcoN	214	RKEP-HIYVANWFYLSFIVTIAMHLIVNNLAIPVSLFGSKSVQLF-----	TM VI
Bj FixN	223	RKEP-HIFVANWFYLAFTVIAVLHLGNNPALPVSAFGSKSYVAW-----	SGVQDAMTQWW
Pd CtaD	207	MRAPGMTLFKVPFLFAWSVFTAWLILSLPLVLAGAITMLLMDRNFQTFQFDPAGGGDPVLYQHILWF	
Ec CyoB	216	MRAPGMTMFKMPVFTWASLCANVLIASFPILTITVALLTLDRLGTHFTNDMGGNMMMYINLIWA	
TM VII			
Pd CcoN	269	YGHNAVGFFLTAGFLGMMYFIPKQAEPRVSYKLSIIHFWALIFLYIWAGPHHLHYTALPDWASTL	TM VIII
Bj FixN	278	YGHNAVGFFLTAGFLAIMYFIPKRAERPIYSYRLSIIHFWALIFLYIWAGPHHLHYTALPDWTQTL	
Pd CtaD	274	FGHPEVYIIILPGFGIISHVISTF-AKKPIFGYLPVLMAMAAIGILGFVVAHMYTAGMSLTQOAY	
Ec CyoB	282	WGHPEVYIIILPVFGVFSEIAATF-SRKRLFGYTSLVWATVCITVLSFIVWLHFFFTMGAGANVNAF	
H E Y S HH			
276 278 280 291 325 326			
TM IX			
Pd CcoN	336	GMVFSIILWMPWSWGGMINGLMTLSGAWDKLRDPIIRMMVVAVGFYGMATFEGPMMSIKAVNFVSHY	
Bj FixN	345	GMTFSIMLWMPWSWGGMINGLMTLSGAWDKLRDPIVLRMLVSVAFYGMSTFEGPMMSIKVNSLSHY	
Pd CtaD	340	FMLATMTIAVPTGIKVSFIATMWGGSIEFKTP--MLWAFGFLFLFTVGGVTVGVLSQAPLDRVYHD	
Ec CyoB	348	FGITTMIIALPTGVKIFNWLFTMYQGRIVFHS--MLWTIGFIVTFSVGGMTGVLLAVPGADFVLHN	
T T K L D			
344 351 354 393 399			
TM X			
Pd CcoN	403	TDWTIGHVHSGALGWNGMITFGALYYLVPRWLGR-ERLYSTGLVSNHFWLATIGLVLYAASMWVSGI	TM XI
Bj FixN	412	TDWTIGHVHSGALGWVGFVSFGALYCLVPWAWNR-KGLYSLKLVNWHFWATLGIVLYISAMWVSGI	
Pd CtaD	405	TYVVVAHFHYVMSLGAVFGIFAGVYY--WIGKMSGRQYPEWAGQLHFWMMFIGNLIFFPQHFLGR	
Ec CyoB	413	SLFLIAHFHNVIIGGVVFGCFAHMTY--WWPKAFGFKLNETWGRKAFWFWIIGFFVAFMPLYALGF	
H H			
411 413			
TM XII			
Pd CcoN	469	MEGLMWREVDAQGFVLNADFVAAKFPNMNVVRALGGVLYLFFALIMCYNLWATVAKQPKTQSTAAA	
Bj FixN	478	LQGLMWRAVTSGLFLEYSFIETVEAMHFFYIIRAAGGGLFLIGALIMAYNLWMTVRVGEAEVQMPVA	
Pd CtaD	469	Q-GMPRRYIDYVPE----FAYWNNISSIGAYISFASFLFFIGIVFTLFGAKRVNPNVWNEHADT	
Ec CyoB	477	M-GMTRRLSQIDP----QFHTMLMIAASGAVLIALGILCLVIQMYVSIRDQNRDLTGDPWGGRT	
Pd CcoN	536	VPAE	
Bj FixN	545	LQPAE	
Pd CtaD	530	LEWTLPSPPPEHTFETLPKREDWDRAHAH	
Ec CyoB	539	LEWATSSPPPFYNFAVVPVHERDAFWEMKEKGEAYKKPDHYEEIHMPKNSGAGIVIAAFSTIFGFA	

B

Pd CcoO	1	MAILEKHKVLEKNATLLVFSFLVVTIGGIIVEIAPLFYLNNTIE	44
Bj FixO	1	MSFWTRHQVFEEKNSIILIVGILLVIAIGGLVEITPLFYLKSTIE	44
Pd CcoO	45	KVQGMRPYTPLELKGRDIYVREGCYVCHSQMIRPM-RDEV ERYG	87
Bj FixO	45	KVDGVRPYTPLELALGRNVYVREGCYLCHSQMIRPLRDEV- ERYG	87
Pd CcoO	88	HYSLAAESMYDHPFQWGSKRTPDLARVGRYSDEWHLDHLVDP	131
Bj FixO	88	HFSLAAESMF DHPFQWGSKRTPDLARVGA KYSD DWHVTHL TNP	131
Pd CcoO	132	QAVVPESEIMPKYGF-LLNRQVDASNMQRLKTDALG-GVPYDDA	173
Bj FixO	132	RAIIVPQSVMGPYFELSA TEVD PDTIADHMR TLR TVGV P YTD DQI	175
Pd CcoO	174	MIAAAGEDFRVQAAPDADASGLEERYPG-AQQRNFDRRPG-VSE	215
Bj FixO	176	ANASADLKAQADPDNAGADAFNKRYAKAVVRNFD-GKTGTPT-E	217
Pd CcoO	216	MDALIAYLQVLGTMVDFSTFEPPDNR	241
Bj FixO	218	MDALIAYLOMLGTLVDFKIYNEKANLR	244

C

Pd CcoQ	1	MDRYSFLRELADSS---WVLLLVVFFLGTIVFAFRPGFAAAAS	40
Bj FixQ	1	MKAAILTLDNLASGLVTTIWTPIVFAIFLAIIAYAFWPRNKAAFD	44
Pd CcoQ	41	RRGRKHLPL	48
Bj FixQ	45	EAAHLPLREE	54

D

Pd CcoP	1	MADTDDEHASPQNPDNRIELERQAADAEAHKAKILAHPPPEAGGDP	44
Bj FixP	1	MTDHSSEFDS-----	9
Pd CcoP	45	LHPPVTPRPGATRVVRDRKGGRRVVEVPSTGHSWDGIEEYDNPL	88
Bj FixP	10	-----VSGKTTTGHEWDGIEKELNTPL	30
Pd CcoP	89	PRWWLWTFYATIVWGVLYLIAYPAPIPLVNGATQGLLGQNYRSDV	132
Bj FixP	31	PRWWVICFYLTIVWATGYWIVYPAWPLISSNTTGLFGYSSRADV	74
Pd CcoP	133	AAEIQRFNANAPIQAKLVETPLEEIAADPELANYTANAGAAIF	176
Bj FixP	75	AVEELANLEKIRGDKMAALGAASLADVEKDPALLALARAKGKTVF	118
Pd CcoP	177	RTWCACQCHGSGAGGATGYPSLLDNDWLWGGTLEEIHTTVMHGIIR	220
Bj FixP	119	GDNCAFCCHGSGGAGAKGFENLNDNDWLWGGTLDQIMQTIQFGAR	162
Pd CcoP	221	DPKADADTRYSEMPRFGIDGLLENAQISQVVNHVLELGLPHDAA	264
Bj FixP	163	SGHAKTHEGQMLAFGKDGV LKGDEIVTVANYVRSLSGLPTRKGY	206
Pd CcoP	265	LAAEGVEVFADNCSSCHAEDGTGDRAQGAPDLTDVWLYGSDPA	308
Bj FixP	207	DAAKGKEIFEVENCVACHGDGKGKGNQEMGAPNLTDKIWLYGSDPA	250
Pd CcoP	309	TITRIVRDGPFGVMPAWTGRLLSEADIVAVAAAYVHSLGGGE	348
Bj FixP	251	ALIETISQGRAGVMPAWEGRLLDPSTIKAMAVYVHSLGGGK	290

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

A. CcoN of *P. denitrificans* (cytochrome *cbb*₃), FixN of *B. japonicum* (cytochrome *cbb*₃; Preisig *et al.*, 1993), CtaDII of *P. denitrificans* (cytochrome *aa*₃; Raitio *et al.*, 1990), and CybB of *E. coli* (cytochrome *bo*₃; 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 *ccoNOQP* genes of *R. capsulatus* (Thöny-Meyer *et al.*, 1994). In *R. meliloti*, a number of transposon insertions in *fixGHI* 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*^R) 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 (Δ *aa*₃), yielding Pd27.21 (Δ *cbb*₃) and Pd93.12 (Δ *aa*₃/*cbb*₃), respectively. Southern blot analysis of chromosomal DNA of selected *Km*^R 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 Δ *cbb*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 Δ *aa*₃ mutant and the Δ *cbb*₃ mutant was only slightly inhibited, and that of the Δ *aa*₃/*cbb*₃ mutant strain appeared to be unaffected.

The effect of the cytochrome *bc*₁ inhibitors on succinate oxidation in the Δ *cbb*₃ mutant and in the Δ *aa*₃ mutant was much less than in the wild type. Again, in the Δ *aa*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 Δ *cbb*₃ and the Δ *aa*₃ mutant. In the Δ *aa*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 Δ *aa*₃ mutant and the Δ *bb*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 Δ *bb*₃/*aa*₃ mutant are shown. The *H*⁺/*e*⁻ ratios of the Δ *cbb*₃ mutant and the Δ *aa*₃/*cbb*₃

Fig. 4. SDS-PAGE and subsequent haem staining of membrane fractions of the wild type (wt) and the cytochrome *cbb*₃ mutant (Δ *cbb*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 (Δaa_3 mutant), Pd27.21 (Δcbb_3 mutant), and Pd93.12 ($\Delta aa_3/cbb_3$ mutant).

Strain	Substrate inhibitor	Endogenous			Succinate			Ascorbate/TMPD
		–	AA	myx	–	AA	myx	
Wild type		1.5	0.9	0.9	2.4	0.6	0.5	3.7
Δaa_3 mutant		1.3	1.2	1.1	2.9	2.6	2.7	2.5
Δcbb_3 mutant		1.4	1.2	1.3	2.7	1.9	1.7	2.6
$\Delta aa_3/cbb_3$ mutant		2.2	2.2	2.2	2.1	2.1	2.1	0.0

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 *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*₁ 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 *cbb*₃, 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*₁ 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

Fig. 5. Proton translocation in whole cells from strain Pd93.11 ($\Delta aa_3/bb_3$) with succinate as substrate. An anaerobic cell suspension was pulsed with equal volumes (10 μ l) of air-saturated water (258 μ M O₂), ferricyanide (1.0 mM FIC) and acid (1.0 mM HCl) to calibrate the system. The H⁺/e[−] ratio with oxygen is 3.0 and with ferricyanide the H⁺/e[−] ratio is 2.0.

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

Strain	Succinate O ₂ (H ⁺ /e [−])	Succinate Fe(CN) ₆ ^{3−} (H ⁺ /e [−])
Wild type	2.93 ± 0.13	1.96 ± 0.09
Δaa_3 mutant	2.96 ± 0.15	1.94 ± 0.18
Δbb_3 mutant	2.97 ± 0.12	1.96 ± 0.12
$\Delta aa_3/bb_3$ mutant	2.95 ± 0.12	1.98 ± 0.16
Δcbb_3 mutant	3.01 ± 0.14	1.94 ± 0.13
$\Delta aa_3/cbb_3$ mutant	1.98 ± 0.11	1.97 ± 0.09

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 (O₂) or ferricyanide (Fe(CN)₆^{3−}).

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 Δbb_3 mutant (de Gier *et al.*, 1994), and here we show that a $\Delta aa_3/cbb_3$ 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 *cbb*₃

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 *cbb*₃-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 *aa*₃ and *bo*₃. The alignment shown in Fig. 3A suggests that H271, H321, and H322 are the most probable ligands of Cu_B, 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 *cbb*₃-type oxidase from *R. sphaeroides* and have demonstrated the presence of a binuclear centre typical of haem-copper oxidases and the absence of Cu_A. 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 *cbb*₃: 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 *cbb*₃ 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 Cu_B) in CcoN. Indeed, the purified 2 subunit CcoNO complex (Fig. 1) retains TMPD oxidase activity. Apparently CcoP is less tightly bound to the cytochrome *cbb*₃ core and may play a role as electron carrier from the *bc*₁ 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 Δcbb_3 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 *cbb*₃, 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 *cbb*₃-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 *cbb*₃. 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 *aa*₃ and *bb*₃ 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 *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*₃ (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 $\Delta aa_3/cbb_3$ mutant results in $2H^+/e^-$. In the conditions used for analysis of proton translocation ($0.4\text{--}1.7\text{ }\mu\text{M O}_2$), 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 $10\text{--}20\text{ }\mu\text{M O}_2$; 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 $\Delta aa_3/bb_3$ 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 $\Delta aa_3/bb_3$ 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

cytochrome *cbb*₃, a phenomenon that is not observed in the case of cytochromes *aa*₃ 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 ($1H^+/e^-$) 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 $\Delta aa_3/bb_3$ 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

Fig. 6. Aerobic respiratory pathways in *P. denitrificans*. The proton-translocation ratios (H^+/e^- ; protons per electron detectable in the medium) and the charge-separation ratios (q^+/e^-) for each of the contributing respiratory complexes are shown. NADH-dehydrogenase is represented by NADH.dh, succinate dehydrogenase by succ.dh, cytochrome reductase by cyt. *bc*₁, cytochrome *c*552 by cyt. *c*552, *aa*₃-type cytochrome *c* oxidase by cyt. *aa*₃, *cbb*₃-type cytochrome *c* oxidase by cyt. *cbb*₃, and *bb*₃-type quinol oxidase by cyt. *bb*₃.

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, N199, 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 imidazole 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.

<i>aa</i> ₃	Location	<i>cbb</i> ₃	Consensus
Chemical channel			
<i>a</i> ₃ -hydroxyl	Haem	No hydroxyl	—
Y280	TM-VI	G	—
T351	TM-VIII	S	+
K354	TM-VIII	G	—
S291	TM-VI	M	—
Pump channel			
D399	Loop-IX/X	N	+
L393-carbonyl	Loop-IX/X	M	+
<i>a</i> ₃ -propionate	Haem	<i>b</i> ₃ -propionate	+
<i>a</i> 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'.

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 *bo₃*-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 ($\Delta aa_3/bb_3$) 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 (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 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 4. Strains and plasmids.

Strain/Plasmid	Relevant characteristics	Reference/Source
<u>Strain</u>		
<i>E. coli</i>		
TG1	<i>supE hsdD5 thi</i> $\Delta(lac^- proAB)$ F' (<i>traD36 proAB lacI^q lacZAM15</i>)	Sambrook <i>et al.</i> (1989)
HB101	F [−] <i>hsdS20</i> (<i>r_B m_B</i>) <i>lacY1 proA2 recA13</i>	Boyer <i>et al.</i> (1969)
S17.1	Sm ^R <i>pro</i> (<i>r_B m_B</i>) RP4-2, integrated (Tc::Mu) (Km::Tn7)	Simon <i>et al.</i> (1983)
<i>P. denitrificans</i>		
Pd1222	Rif ^R , enhanced conjugation frequencies, (<i>r_B m_B</i>)	de Vries <i>et al.</i> (1989)
Pd92.20	Pd9218 derivative, $\Delta actA/DI$ $\Delta actA/DII$	de Gier <i>et al.</i> (1994)
Pd26.21	Pd1222 derivative, <i>qoxB</i> ::Km ^R	de Gier <i>et al.</i> (1994)
Pd93.11	Pd9220 derivative, $\Delta actA/DI$ $\Delta actA/DII$ <i>cyoB</i> ::Km ^R	de Gier <i>et al.</i> (1994)
Pd27.21	Pd1222 derivative, <i>ccoNO</i> ::Km ^R	This work
Pd93.12	Pd9220 derivative, $\Delta actA/DI$ $\Delta actA/DII$ <i>ccoNO</i> ::Km ^R	This work
<u>Plasmid</u>		
pUC19	Amp ^R <i>lacZ</i> '	Yanisch-Perron <i>et al.</i> (1985)
pUC4KIXX	Km ^R (Tn5)	Pharmacia
M13mp18/19	pUC18/19 mcs, <i>lacZ</i> '	Sanger <i>et al.</i> (1980)
pRK2020	Tc ^R pRK2013 Km::Tn10	Ditta <i>et al.</i> (1985)
pGRPd1	<i>oriV</i> (colE1) Amp ^R <i>oriT</i> Sm ^R (Tn1831)	van Spanning <i>et al.</i> (1990)
pCco1	pUC19 derivative, <i>ccoNOQP</i>	This work
pCco2	pUC19 derivative, <i>ccoNAorfN</i>	This work
pCco3	pUC19 derivative, <i>ccoNOQPGH</i> '	This work
pCcoNO::Km ^R	pCco1 derivative, <i>ccoNO</i> ::Km ^R	This work
pRTd27.21	pGRPd1 derivative, <i>ccoNO</i> ::Km ^R	This work

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 (pRK2020) 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 cbb₃* complex (CcoO) (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'-AAAA-GAATTC[A/G/T]ATNCGNGNAACNACNAG[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 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 rotenon, 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.

Acknowledgements

We thank M. Wikström and H. V. Westerhoff for critical reading of the manuscript. This research was supported by the Netherlands Foundation for Chemical Research, with financial aid from the Netherlands Organization for the Advancement of Science. J.v.d.O. had a Fellowship of the Royal Netherlands Academy of Science. EMBO supported visits of J.W.d.G. to the laboratories of M. Wikström and M. Saraste.

References

- Anraku, Y., and Gennis, R.B. (1987) The aerobic respiratory chain of *Escherichia coli*. *Trends Biochem Sci* **12**: 262–266.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.C., and Struhl, K. (1993) *Current Protocols in Molecular Biology*. New York: John Wiley and Sons, Inc.
- Batut, J., Daveran-Mingot, M., David, M., Jacobs, J., Garnierone, A.M., and Kahn, D. (1989) *fixK*, a gene homologous with *fmr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. *EMBO J* **8**: 1279–1286.
- Berry, E.A., and Trumpower, B.L. (1985) Isolation of ubiquinol oxidase from *Paracoccus denitrificans* and resolution into cytochrome *bc₁* and cytochrome *c-aa₃* complexes. *J Biol Chem* **260**: 2458–2467.
- Bosma, G. (1989) Growth-condition-dependent synthesis of electron transfer components in *Paracoccus denitrificans*. PhD Thesis, Vrije Universiteit, Amsterdam, Netherlands.
- Boyer, H.W., and Roulland-Dussoix, D. (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **41**: 459–472.
- Chepur, V., Lemieux, L.J., Au, C.-T., and Gennis, R.B. (1990) The sequence of the *cyo* operon indicates substantial structural similarities between the cytochrome *o* ubiquinol oxidase of *Escherichia coli* and the *aa₃*-type family of cytochrome *c* oxidases. *J Biol Chem* **265**: 11185–11192.
- David, M., Daveran, M.-L., Batut, J., Dedieu, A., Domergue, O., Ghai, J., Hertig, C., Boistard, P., and Kahn, D. (1988) Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. *Cell* **54**: 671–683.
- Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. (1985) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci USA* **77**: 7347–7351.
- Fetter, J.R., Qian, J., Shapleigh, J., Thomas, J.W., Garcia-Horsman, A., Schmidt, E., Hosler, J., Babcock, G.T., Gennis, R.B., and Ferguson-Miller, S. (1995) Possible proton relay pathways in cytochrome *c* oxidase. *Proc Natl Acad Sci USA* **92**: 1604–1608.
- Fischer, H.M. (1994) Genetic regulation of nitrogen fixation in *Rhizobia*. *Microbiol Rev* **58**: 352–386.
- Garcia-Horsman, A., Barquera, B., Rumbley, J., Ma, J., and Gennis, R.B. (1994a) The superfamily of haem-copper respiratory oxidases. *J Bacteriol* **176**: 5587–5600.
- Garcia-Horsman, A., Berry, J., Shapleigh, J., Alben, J.O., and Gennis, R.B. (1994b) A novel cytochrome *c* oxidase from

- Rhodobacter sphaeroides* that lacks Cu_A. *Biochemistry* **33**: 3113–3119.
- Garcia-Horsman, A., Puustinen, A., Gennis, R.B., and Wikström, M. (1995) Proton transfer in cytochrome *bo₃* ubiquinol oxidase of *Escherichia coli*: second-site mutations in subunit I that restore proton pumping in the mutant Asp-135 → Asn. *Biochemistry* **34**: 4428–4433.
- de Gier, J.W.L. (1995) The terminal oxidases of *Paracoccus denitrificans*. PhD Thesis, Vrije Universiteit, Amsterdam, Netherlands.
- de Gier, J.W.L., van Spanning, R.J.M., Oltmann, L.F., and Stouthamer, A.H. (1992) Oxidation of methylamine by a *Paracoccus denitrificans* mutant impaired in the synthesis of the *bc₁* complex and the *aa₃*-type oxidase. Evidence for the existence of an alternative cytochrome *c* oxidase in this bacterium. *FEBS Lett* **306**: 23–26.
- de Gier, J.W.L., Lübben, M., Reijnders, W.N.M., Tipker, C.A., Slotboom, D.J., van Spanning, R.J.M., Stouthamer, A.H., and van der Oost, J. (1994) The terminal oxidases of *Paracoccus denitrificans*. *Mol Microbiol* **13**: 183–196.
- Gray, K.A., Grooms, M., Myllykallio, H., Moomaw, C., Slaughter, C., and Daldal, F. (1994) *Rhodobacter capsulatus* contains a novel *cb*-type cytochrome *c* oxidase without a Cu_A centre. *Biochemistry* **33**: 3120–3127.
- Hosler, J.P., Ferguson-Miller, S., Calhoun, M.W., Thomas, J.W., Hill, J., Lemieux, L.J., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M.M.J., Babcock, G.T., and Gennis, R.B. (1993) Insight into the active-site structure and function of cytochrome oxidase by analysis of site-directed mutants of bacterial cytochrome *aa₃* and cytochrome *bo*. *J Bioenerg Biomembr* **25**: 121–136.
- Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. *Nature* **376**: 660–669.
- Kahn, D., David, M., Domergue, O., Daveran, M.-L., Ghai, J., Hirsch, P.R., and Batut, J. (1989) *Rhizobium meliloti fixGHI* sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. *J Bacteriol* **171**: 929–939.
- Kahn, D., Batut, J., Daveran, M.-L., and Fourment, J. (1993) Structure and regulation of the *fixNOQP* operon from *Rhizobium meliloti*. In *New Horizons in Nitrogen Fixation*. Palacios, R., Mora, J., and Newton, W.E. (eds). Dordrecht: Kluwer Academic Publishers, p. 474.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Lübben, M., Arnaud, S., Castresana, J., Warne, A., and Saraste, M. (1994) The purified SoxABCD quinol oxidase complex of *Sulfolobus acidocaldarius* contains a novel haem. *Mol Microbiol* **13**: 327–335.
- Mandon, K., Kaminski, A., and Elmerich, C. (1994) Functional analysis of the *fixNOQP* region of *Azorhizobium caulinodans*. *J Bacteriol* **176**: 2560–2568.
- Moore, G.R., and Pettigrew, G.W. (1990) *Cytochromes c: Evolutionary, Structural and Physicochemical aspects*. Berlin: Springer-Verlag.
- Morgan, J.E., Verkovsky, M.I., and Wikström, M. (1994) The histidine cycle: a new model for proton translocation in the respiratory haem-copper oxidases. *J Bioenerg Biomembr* **26**: 599–608.
- van der Oost, J., Haltia, T., Raitio, M., and Saraste, M. (1991) Genes coding for cytochrome *c* oxidase in *Paracoccus denitrificans*. *J Bioenerg Biomembr* **23**: 257–267.
- van der Oost, J., de Boer, A.P.N., de Gier, J.-W.L., Zumft, W.G., Stouthamer, A.H., and van Spanning, R.J.M. (1994) The haem-copper oxidase family consists of three distinct types of terminal oxidases and is related to nitric oxide reductase. *FEMS Microbiol Lett* **121**: 1–10.
- van der Oost, J., Schepper, M., Stouthamer, A.H., Westerhoff, H.V., van Spanning, R.J.M., and de Gier, J.W.L. (1995) Reversed electron transfer through the *bc₁* complex enables a cytochrome *c* oxidase mutant ($\Delta aa_3/cbb_3$) of *Paracoccus denitrificans* to grow on methylamine. *FEBS Lett* **371**: 267–270.
- Plunkett, G., Burland, V.D., Daniels, B.L., and Blattner, F.R. (1993) Analysis of *E. coli* genome III. DNA sequence of the region from 87.2 to 89.2 minutes. *Nucl Acids Res* **21**: 3391–3398.
- Preisig, O., Antamatten, D., and Hennecke, H. (1993) Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. *Proc Natl Acad Sci USA* **90**: 3309–3313.
- Puustinen, A., Finel, M., Virkki, M., and Wikström, M. (1989) Cytochrome *o* (*bo*) is a proton pump in *Paracoccus denitrificans* and *Escherichia coli*. *FEBS Lett* **249**: 163–167.
- Raitio, M., and Wikström, M. (1994) An alternative cytochrome oxidase of *Paracoccus denitrificans* functions as a proton pump. *Biochim Biophys Acta* **1186**: 100–106.
- Raitio, M., Jalli, T., and Saraste, M. (1987) Isolation and analysis of the genes for cytochrome *c* oxidase in *Paracoccus denitrificans*. *EMBO J* **6**: 2825–2833.
- Raitio, M., Pispä, J.M., Metso, T., and Saraste, M. (1990) Are there isoenzymes of cytochrome *c* oxidase in *Paracoccus denitrificans*? *FEBS Lett* **206**: 154–156.
- Richter, O.M.H., Tao, J.S., Turba, A., and Ludwig, B. (1994) A cytochrome *ba₃* functions as a quinol oxidase in *Paracoccus denitrificans*, purification, cloning and sequence comparison. *J Biol Chem* **269**: 23079–23086.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Sanger, F., Nicklen, S., and Coulson, A. (1977) DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463–5467.
- Sanger, F., Coulson, R., Barrel, B.G., Smith, J.H., and Roe, B.A. (1980) Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J Mol Biol* **143**: 161–178.
- Saraste, M., and Castresana, J. (1994) Cytochrome oxidase evolved by tinkering with denitrification enzymes. *FEBS Lett* **341**: 1–4.
- Simon, R., Priefer, U., and Pühler, A. (1983) Vector plasmids for *in vivo* and *in vitro* manipulations of Gram-negative bacteria. In *Molecular Genetics of the Bacteria-plant Interaction*. Pühler, A. (ed.). Berlin: Springer-Verlag, pp. 98–106.
- Soloz, M., Carafoli, E., and Ludwig, B. (1981) The cytochrome *c* oxidase of *Paracoccus denitrificans* pumps protons in a reconstituted system. *J Biol Chem* **257**: 1579–1582.
- van Spanning, R.J.M., Wansell, C.W., Harms, N., Oltmann, L.F., and Stouthamer, A.H. (1990) Mutagenesis of the gene

- encoding cytochrome *c*₅₅₀ of *Paracoccus denitrificans* and analysis of the resultant physiological effects. *J Bacteriol* **172**: 986–996.
- van Spanning, R.J.M., de Boer, A.P.N., Reijnders, W.N.M., de Gier, J.W.L., Delorme, C.O., Stouthamer, A.H., Westerhoff, H.V., Harms, N., and van der Oost, J. (1995a) Regulation of oxidative phosphorylation: the flexible respiratory network of *Paracoccus denitrificans*. *J Bioenerg Biomembr* **27**: 499–512.
- van Spanning, R.J.M., De Boer, A.P.N., Reijnders, W.N.M., Spiro, S., Stouthamer, A.H., Westerhoff, H.V., and van der Oost, J. (1995b) Nitrite and nitric oxide reduction in *Paracoccus denitrificans* is under the control of NNR, a regulatory protein that belongs to the FNR family of transcriptional activators. *FEBS Lett* **360**: 151–154.
- Spiro, S. (1994) The FNR family of transcriptional regulators. *Antonie van Leeuwenhoek* **66**: 1–14.
- Spiro, S., and Guest, J.R. (1990) FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. *FEMS Microbiol Lett* **75**: 399–428.
- Steinrücke, P., Steffens, G.C., Pankus, G., Buse, G., and Ludwig, B. (1987) Subunit II of cytochrome *c* oxidase from *Paracoccus denitrificans* — DNA sequence, gene expression and the protein. *Eur J Biochem* **167**: 431–439.
- Stouthamer, A.H. (1991) Metabolic regulation including anaerobic metabolism in *Paracoccus denitrificans*. *J Bioenerg Biomembr* **23**: 163–185.
- Thomas, P.E., Ryan, D., and Levin, W. (1976) An improved staining procedure for the detection of peroxidase activity of cytochrome *p*450 on sodium dodecyl polyacrylamide gels. *Anal Biochem* **75**: 168–176.
- Thöny-Meyer, L., Beck, C., Preisig, O., and Hennecke, H. (1994) The *ccoNOQP* gene cluster codes for a *cb*-type cytochrome oxidase that functions in aerobic respiration of *Rhodobacter capsulatus*. *Mol Microbiol* **14**: 705–706.
- Trumpower, B.L. (1991) The three-subunit cytochrome *bc*₁ complex of *Paracoccus denitrificans*. Its physiological function, structure, and mechanism of electron transfer and energy transduction. *J Bioenerg Biomembr* **23**: 241–255.
- van Verseveld, H.W., Krab, K., and Stouthamer, A.H. (1981) Proton pump coupled to cytochrome *c* oxidase in *Paracoccus denitrificans*. *Biochim Biophys Acta* **635**: 525–534.
- de Vries, G.E., Harms, N., Hoogendijk, J., and Stouthamer, A.H. (1989) Isolation and characterization of *Paracoccus denitrificans* mutants with increased conjugation frequencies and pleiotropic loss of a (nGATCn) DNA-modifying property. *Arch Microbiol* **152**: 52–57.
- Yanisch-Perron, C., Vierra, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. *Gene* **33**: 103–119.