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The terminal oxidases of *Paracoccus denitrificans*

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Summary

Three distinct types of terminal oxidases participate in the aerobic respiratory pathways of *Paracoccus denitrificans*. Two alternative genes encoding subunit I of the aa_3-type cytochrome *c* oxidase have been isolated before, namely *ctaDI* and *ctaDII*. Each of these genes can be expressed separately to complement a double mutant (Δ*ctaDI*, Δ*ctaDII*), indicating that they are isoforms of subunit I of the aa_3-type oxidase. The genomic locus of a quinol oxidase has been isolated: *cyoABC*. This protohaem-containing oxidase, called cytochrome bb_3, is the only quinol oxidase expressed under the conditions used. In a triple oxidase mutant (Δ*ctaDI*, Δ*ctaDII*, *cyoB*::Km^R) an alternative cytochrome *c* oxidase has been characterized; this cbb_3-type oxidase has been partially purified. Both cytochrome aa_3 and cytochrome bb_3 are redox-driven proton pumps. The proton-pumping capacity of cytochrome cbb_3 has been analysed; arguments for and against the active transport of protons by this novel oxidase complex are discussed.

Introduction

In mitochondrial respiration the reduction of oxygen to water is catalysed by cytochrome *c* oxidase. This membrane-bound complex is the last component of the respiratory chain, in which electrons from a reductant (NADH, succinate) are transferred in subsequent redox reactions to oxygen (Babcock and Wikström, 1992). In bacteria aerobic respiration is often a more complex branched pathway. For many bacterial species more than one terminal oxidase has been described, catalysing the oxidation of either cytochrome *c* or quinol. The possession of alternative respiratory branches enables a bacterium to adapt its efficiency of oxidative phosphorylation to changes in the environmental conditions.

An extensively studied prokaryotic cytochrome *c* oxidase is cytochrome aa_3 from *Paracoccus denitrificans* and *Rhodobacter sphaeroides*. The primary sequences of its three subunits show strong homology to the mitochondrion-encoded eukaryotic cytochrome oxidase subunits I, II and III (Raitio *et al.*, 1987; 1990; Steinrücke *et al.*, 1987; Cao *et al.*, 1991; Shapleigh and Gennis 1992; Saraste, 1990). Subunit I of cytochrome aa_3 contains two haems A: one is in the low-spin (6-co-ordinate) configuration and the other is in the high-spin (5-co-ordinate) configuration. The catalytic site of oxygen reduction is formed by a binuclear centre of the high-spin haem A and a copper ion, Cu_B (Wikström *et al.*, 1981). Subunit II harbours another copper site, Cu_A (van der Oost *et al.*, 1992; Lappalainen *et al.*, 1993; von Wachenfeldt *et al.*, 1994), which is probably involved in the oxidation of the substrate cytochrome *c* (Hill, 1993). Like the mitochondrial cytochrome *c* oxidase (Wikström, 1977; Krab and Wikström, 1978), the bacterial cytochrome aa_3 is a redox-driven proton pump: the reduction of oxygen is coupled to the translocation of protons across the membrane (van Verseveld *et al.*, 1981; Solioz *et al.*, 1981; Hosler *et al.*, 1993).

The best-characterized prokaryotic quinol oxidase is cytochrome bo_3 from *Escherichia coli*. As the name suggests, a low-spin haem B and a high-spin haem O are located in the major subunit, in addition to Cu_B (Minghetti *et al.*, 1992). Like the cytochrome *c* oxidases, cytochrome bo_3 functions as a proton pump (Puustinen *et al.*, 1989). Sequence analysis has revealed that cytochrome bo_3 is closely related to cytochrome aa_3 (Saraste *et al.*, 1988; Chepuri *et al.*, 1990). However, in contrast to the aa_3-type cytochrome *c* oxidases the quinol-oxidizing cytochrome bo_3 does not contain a Cu_A site (Puustinen *et al.*, 1991; van der Oost *et al.*, 1992). An alternative type of quinol oxidase from *E. coli*, cytochrome *bd*, is not related to the haem–copper oxidases (Anraku and Gennis, 1987; Green *et al.*, 1988).

Apart from the aa_3-type cytochrome *c* oxidase (John and Whatley, 1975), *P. denitrificans* has been reported to express alternative terminal oxidases. Several ubiquinol oxidases (cytochrome b_o, Cox *et al.*, 1978; cytochrome *d*, Henry and Vignais, 1979; cytochrome a_1, van Verseveld *et al.*, 1983; cytochrome ba_3, Ludwig, 1992) and cytochrome *c* oxidases (cytochrome c_o, Bosma, 1989; cytochrome aa_3 isoenzyme, Raitio *et al.*, 1990) have

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been either demonstrated or suggested. However, in contrast to cytochrome aa_3 the alternative oxidases from *Paracoccus* have not been described in detail.

In this paper a molecular genetic approach is used to acquire more detailed information on the terminal oxidases from *Paracoccus*. A new oxidase locus (*cyoABC*) has been cloned and its product identified as a protohaem-containing quinol oxidase. Single and multiple mutants have been generated for cytochrome aa_3, the putative cytochrome aa_3 isoenzyme, and the quinol oxidase. For the first time expression of the *ctaDI* gene has been observed and the iso-cytochrome aa_3 has been characterized. After mutagenesis of the *cyoABC* locus no alternative quinol oxidase activity is detectable. In a triple oxidase mutant a *cb*-type cytochrome *c* oxidase has been analysed in more detail.

Results

Mutagenesis of cytochrome aa_3 *and iso-cytochrome* aa_3

The genomic loci encoding the aa_3-type cytochrome *c* oxidase (*ctaCBGE* and *ctaDII*), as well as the gene encoding a putative subunit I isoenzyme (*ctaDI*) have been isolated previously (Raitio *et al.*, 1987; 1990; Steinrücke *et al.*, 1987; van Spanning *et al.*, 1990). In the course of this study deletions have been generated in both subunits I (Δ*ctaDI*, Δ*ctaDII*) as shown in Fig. 1A, using a gene-replacement method as described by van Spanning *et al.* (1991). In this method the target gene is first inactivated by insertion of a kanamycin-resistance marker; the subsequent removal of this insertion cassette in a second recombination event results in an unmarked deletion.

Cloning and mutagenesis of a new oxidase locus

Degenerated oligonucleotides derived from conserved regions in subunit I of haem–copper oxidases have been designed as described by M. Lübben *et al.* (submitted). A polymerase chain reaction (PCR) has been performed on genomic DNA from the cytochrome aa_3 double mutant (Δ*ctaDI*, Δ*ctaDII*), in conditions essentially as before (M. Lübben *et al.*, submitted). A PCR product of the expected size has been cloned into an M13 derivative and sequenced. The predicted amino acid sequence turned out to be very similar to that of subunit I of cytochrome bo_3 from *E. coli* (Chepuri *et al.*, 1990). Using the PCR fragment as a probe, a genomic *Sph*I fragment of approx. 5 kb has been isolated (Fig. 1B).

Sequence analysis of the cyoABC *locus*

The genomic *Sph*I fragment has been subcloned and sequenced. Independently, the same locus has been isolated by O. Preisig and B. Ludwig (personal communication); their complete sequence has been submitted to the EMBL Data Library and they will publish their data elsewhere. Upstream of the subunit I gene (*cyoB*) a subunit II (*cyoA*) homologue was found, downstream of a *cyoB* subunit III-like gene (*cyoC*) (Fig. 1B). Screening of the data library revealed highest homology with *E. coli cyoABCDE* (Chepuri *et al.*, 1990) and *Bacillus subtilis qoxABCD* (Santana *et al.*, 1992), encoding the quinol oxidases cytochrome bo_3 and aa_3, respectively.

Primary sequences have been analysed in order to obtain information on the metal-binding sites. In subunit I of haem–copper oxidases six invariant histidines are the established ligands for the redox centres, i.e. two haems

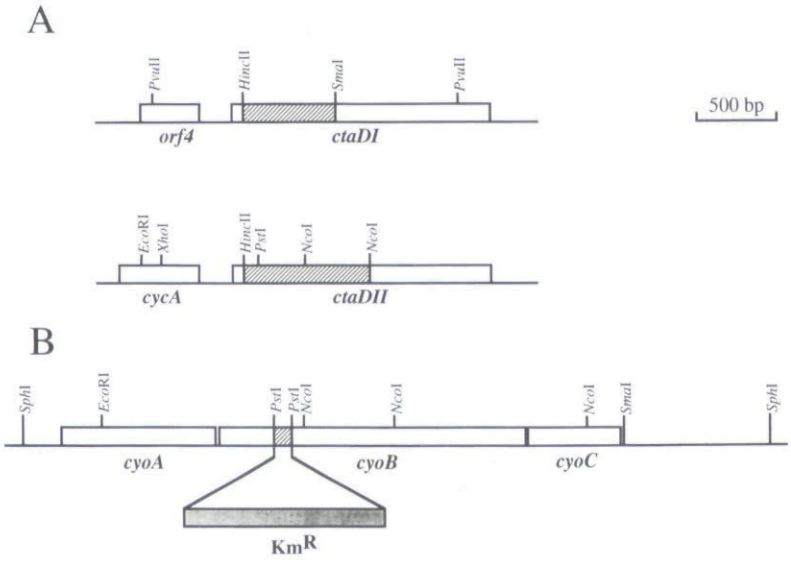

Fig. 1. Genomic oxidase loci of *P. denitrificans*.
A. Subunit I genes of iso-cytochrome aa_3 (*ctaDI*) and cytochrome aa_3 (*ctaDII*); the latter is located adjacent to the *cycA* gene, encoding cytochrome c_{550}.
B. The *cyoABC* locus, encoding the *b*-type quinol oxidase (cytochrome bb_3).
Sites of deletion and/or insertion mutagenesis are indicated.

A subunit I

	helix II	helix VI
P.d. CtaDI	...VVTY**H**GILMMFFVVIPALFG...	...HILWFFG**H**PEVYIIILPGFG...
P.d. CtaDII	...MITY**H**GVLMMFFVVIPALFG...	...HILWFFG**H**PEVYIIILPGFG...
B.s. CtaD	...VMTM**H**GTTMIFLAAMPLLFA...	...HLFWIFG**H**PEVYILILPAFG...
B.j. FixN	...LRPL**H**TSAVIFAFGGNVLIA...	...MFQWWYG**H**NAVGFFLTAGFF...
P.d. CyoB	...IFTA**H**GVIMIFFVAMPFITG...	...NLIWIWG**H**PEVYILILPLFG...
E.c. CyoB	...IFTA**H**GVIMIFFVAMPFVIG...	...NLIWAWG**H**PEVYILILPVFG...
S.a. SoxA	...ALTI**H**GWAAMIAFVPMAAAA...	...ILFWFYG**H**PVVYYVPFPLFG...
	102 121	277 296

	helix VII	helix X
P.d. CtaDI	...MAAIAFLGFIVWA**HH**MYTAG...	...YHDTYYIVA**HFH**YVMSLGAV...
P.d. CtaDII	...MAAIGILGFVVWA**HH**MYTAG...	...YHDTYYVVA**HFH**YVMSLGAV...
B.s. CtaD	...AIVLGFLGFMVWV**HH**MFTTG...	...FHDTYFVVA**HFH**YVIIGGVV...
B.j. FixN	...FWALIFLYIWAGP**HH**LHYTA...	...SHYTDWTIG**HVH**SGALGWVG...
P.d. CyoB	...TVCITVLSYLVWL**HH**FFTMG...	...LHNSLFLIA**HFH**NVIIGGVL...
E.c. CyoB	...TVCITVLSFIVWL**HH**FFTMG...	...LHNSLFLIA**HFH**NVIIGGVV...
S.a. SoxA	...IYLLAIGTMGVWV**HH**LQTWP...	...FHNSYYVVG**HFH**LMIWTLII...
	320 339	410 429

B subunit II

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P.d. CtaC     ...TATDVIHAWTIPAFAVKQDAVPGRIAQLWFSVD------QEGVYFGQCSELCGINHAYMPIVVKA...
B.s. CtaC     ...KASDVKHSFWIPSVGGKLDTNTDNENKFFLTFDSKRSKEAGDMFFGKCAELCGPSHALMDFKVKT...

P.d. CyoA     ...TSTSVMNAFYIPAMAGMIYAMPGMETKLNGVLN------HPGKYKGIASHYSGHGFSGMHFKAHA...
E.c. CyoA     ...TSNSVMNSFFIPRLGSQIYAMAGMQTRLHLIAN------EPGTYDGISASYSGPGFSGMKFKAIA...
                 166                                                          225
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Fig. 2. Alignment of metal-binding amino acid sequences of four putative membrane-spanning helices of subunit I (A), and the *C*-terminal periplasmic domain of subunit II (B). Ligands are in bold. *P. denitrificans* CtaDI, CtaDII, CtaC (Raitio *et al.*, 1987; 1990), *B. subtilis* CtaD, CtaC (Saraste *et al.*, 1990), *B. japonicum* FixN (Preisig *et al.*, 1993), *P. denitrificans* CyoB, CyoA (this study), *E. coli* CyoB, CyoA (Chepuri *et al.*, 1990), *Sulfolobus acidocaldarius* SoxB (Lübben *et al.*, 1992). Sequence numbering refers to *E. coli* proteins.

and a copper ion, Cu_B (reviewed by Hosler *et al.*, 1993). Alignment of *Paracoccus* CyoB sequences with corresponding fragments from several haem–copper oxidases clearly indicates similar metal-binding sites (Fig. 2A). A fourth redox centre resides in subunit II of cytochrome *c* oxidase, i.e. a binuclear copper centre, Cu_A (van der Oost *et al.*, 1992; Lappalainen *et al.*, 1993; von Wachenfeldt *et al.*, 1994). Five amino acids in subunit II (2 cysteines, 2 histidines and 1 methionine) have been identified as a copper ligand (Kelly *et al.*, 1993). In quinol-oxidizing members of the haem–copper oxidase family this copper centre is absent (Puustinen *et al.*, 1991; van der Oost *et al.*, 1992). In *Paracoccus* CyoA four of the five Cu_A ligands are substituted (Fig. 2B), indicating that the *cyoABC* genes code for a quinol oxidase.

Optical spectra, oxygen consumption

Deletion of the *Paracoccus ctaDI* gene, proposed to encode subunit I of cytochrome aa_3 (Raitio *et al.*, 1987), did not affect the cytochrome aa_3 biosynthesis. This result has led to the isolation of a second gene, *ctaDII* (Raitio *et al.*, 1990). Deletion of the latter gene (Δ*ctaDII*) results in a complete loss of cytochrome aa_3 absorption, with typical maxima at 445 nm and 605 nm (de Gier *et al.*, 1992; Raitio and Wikström, 1994).

The single, double, and triple oxidase mutants generated in this study have been analysed spectroscopically using whole cells, grown in batch culture on minimal medium with succinate (Fig. 3). As expected, the cytochrome aa_3 double mutant (Δ*ctaDI*, Δ*ctaDII*) has a

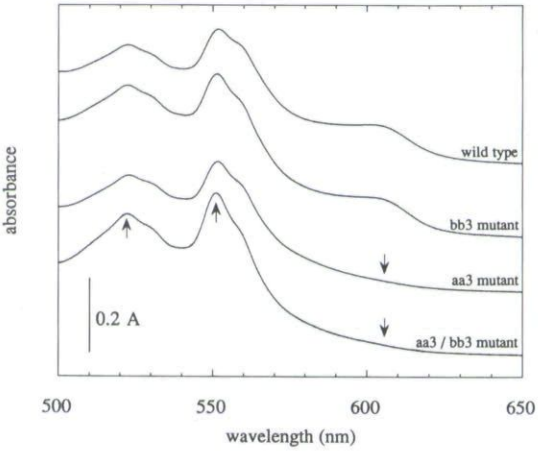

Fig. 3. Optical spectra of cells of *Paracoccus*. Cells were grown in batch cultures on minimal medium with succinate, and harvested during late-exponential growth. The figure presents absolute spectra of dithionite-reduced cell suspensions: wild type (Pd1222), cytochrome bb_3 mutant (Pd2621), cytochrome aa_3 double mutant (Pd9220), and the triple oxidase mutant (Pd9311).

Table 1. Oxygen-consumption measurements on whole-cell suspensions of different *P. denitrificans* strains: Pd1222 (wild type), Pd9220 (aa_3 double mutant), Pd2621 (bb_3 mutant), and Pd9311 (aa_3/bb_3 triple mutant).

Substrate	Endogenous			Succinate			Ascorbate/ TMPD
Strain Inhibitor	–	AA	myx	–	AA	myx	–
Wild type	1.2	0.9	0.9	3.8	1.0	1.0	4.3
aa_3 double mutant	1.2	1.2	1.2	3.8	3.2	3.3	3.1
bb_3 mutant	1.1	0	0	3.6	0	0	4.3
aa_3/bb_3 triple mutant	1.2	0	0	3.7	0	0	3.4

Values are the means of three independent assays (nmol O_2 mg protein^{-1} s^{-1}). Inhibitors: antimycin A (AA), myxothiazol (myx).

cytochrome aa_3-minus phenotype. The spectrum of the *cyoB* mutant (*cyoB*::Km^R), on the other hand, did not differ significantly from that of the wild type. In the optical spectrum of the triple mutant (Δ*ctaDI*, Δ*ctaDII*, *cyoB*::Km^R) a clear increase of cytochrome *b* and *c* is observed (Fig. 3).

The oxygen consumption of wild-type and mutant cells has been measured polarographically, both without supplemented substrate (endogenous) and after addition of succinate (Table 1). In separate assays the electron flow via cytochrome *c* reductase has been inhibited by addition of either antimycin A or myxothiazol. The endogenous respiration of the wild type and the aa_3 double mutant is only slightly affected by these inhibitors. The inhibition becomes relatively more pronounced when succinate is used as substrate. In contrast, the oxygen consumption is blocked completely in the *cyoB* mutant and in the triple mutant. This is in agreement with the analysis of the primary structure, and implies that CyoABC encodes a quinol oxidase. In addition, the oxidation of the artificial electron-donating couple ascorbate and *N, N, N′, N′*-tetramethyl-*p*-phenylenediamine (TMPD) has been assayed; more or less equal values indicate the presence of cytochrome *c* oxidase(s) in all strains.

Mutant complementations

All three oxidase mutants have been complemented using an expression vector and the triple oxidase mutant as a host. Although the *lac* promoter is thought to be inactive in *Paracoccus*, the orientation of the oxidase genes in the expression constructs is such that they are encoded by the complementary strand, indicating that transcription has been under the control of the native promoters. An interesting observation was that all three complementation constructs, but not the vector alone, gave rise to a decline of the *bc* peak (Fig. 4).

The plasmid-encoded expression of CtaDII was demonstrated by the restoration of the cytochrome aa_3 optical spectrum, with characteristic maxima at 445 nm and 605 nm (not shown). For the first time the spectrum of cytochrome aa_3 with subunit I expressed from *ctaDI* has been observed (Fig. 4); it is obvious that it concerns a cytochrome aa_3 isoenzyme, as originally proposed by Raitio *et al.* (1990).

The quinol oxidase has been complemented using the *Sph*I fragment shown in Fig. 1B. In this case the effect was most clearly demonstrated by analysis of the oxygen consumption. As stated above, the respiration of the triple mutant was inhibited completely by antimycin A and myxothiazol (Table 1). In the CyoABC-complemented clone, however, the direct oxidation of ubiquinol (independent of cytochrome *c* oxidase) is restored (not shown).

Haem analysis

Haems have been extracted from wild-type and mutant cells and subsequently analysed by high-performance liquid chromatography (HPLC) reversed-phase chromatography (Table 2). In extracts of the wild type and the quinol oxidase mutant, haem B (protohaem) and haem A are present abundantly. In the cytochrome aa_3 double mutant and in the triple mutant the haem B quantity is not altered significantly; in contrast, haem A is just detectable in the absence of cytochrome aa_3 (the concentration decreased 10–15-fold). At least under the cultivation conditions used, no haem O is detected in membranes of *Paracoccus*. This implies that the cloned quinol oxidase is a *b*-type cytochrome, and that the alternative cytochrome *c* oxidase is a *cb*-type cytochrome.

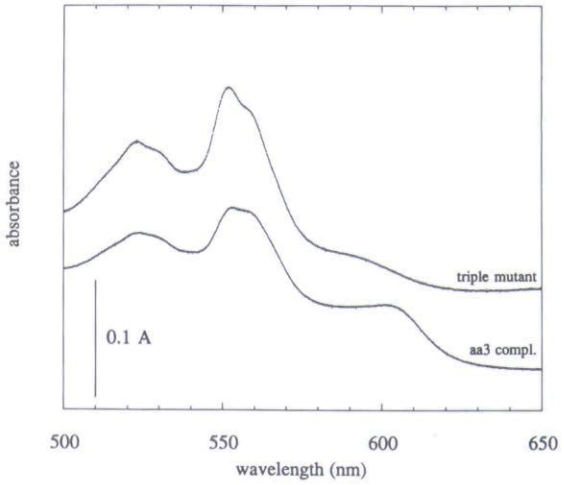

Fig. 4. Optical spectra of cells of *Paracoccus*. The figure presents absolute spectra of dithionite-reduced cell suspensions (as described in legend Fig. 3) of the triple oxidase mutant Pd9311, and triple oxidase mutant after complementation with CtaDI: Pd9311(pEG.ctaDI).

Table 2. Haem analysis by reversed-phase HPLC (Deltapak C18 column, Waters) of different *P. denitrificans* strains: Pd1222 (wild type), Pd9220 (aa_3 double mutant), Pd2621 (bb_3 mutant), and Pd9311 (aa_3/bb_3 triple mutant).

	Haem		
Strain	B	O	A
Wild type	++	–	++
aa_3 double mutant	++	–	±
bb_3 mutant	++	–	++
aa_3/bb_3 triple mutant	++	–	±

Haems were extracted from isolated membranes and eluted on an acetonitrile gradient in 0.05% trifluoroacitic acid, as described by Sone and Fujiwara (1991). Relative quantities of extracted haem: ++, abundantly present; ±, just detectable; –, not detectable.

Partial purification of alternative cytochrome c *oxidase*

From membranes of the triple mutant (Δ*ctaDI*, Δ*ctaDII*, *cyoB*::Km^R), grown on minimal medium with succinate, the alternative cytochrome *c* oxidase has been extracted. Partial purification of the oxidase complex has been accomplished by column chromatography, and the resulting preparation oxidizes both TMPD and horse-heart cytochrome *c* at high rates (M. Saraste and J. van der Oost, unpublished). The optical spectrum shows maxima at 551 nm and 559 nm, indicating the presence of the cytochromes *c* and *b* (Fig. 5).

Proton translocation

The coupling of respiratory electron (e^-) transfer to the translocation of protons (H^+) across the bacterial cytoplasmic membrane drives the oxidative phosphorylation. The efficiency of the latter phenomenon in the different mutants has been studied by determination of the $H^+/2e^-$ stoichiometry. A cell suspension is supplemented with substrate, either ascorbate and TMPD or succinate; a subsequent oxygen pulse induces the proton efflux, which is measured as acidification of the medium (Fig. 6, Table 3).

Ascorbate donates electrons via TMPD to cytochrome *c* (van Verseveld *et al.*, 1981) (Fig. 7). When ascorbate is oxidized to dehydro-ascorbate one 'scalar' proton is released per two electrons; when a proton-pumping cytochrome *c* oxidase is operative, a maximum of two 'vectorial' protons is translocated to the medium in addition (Wikström *et al.*, 1981). In the wild type and the quinol oxidase mutant an $H^+/2e^-$ ratio of 2.0–2.4 is measured upon ascorbate oxidation; in the aa_3 double mutant and the triple oxidase mutant an $H^+/2e^-$ ratio of 1.1–1.2 (Table 3) is found. Apparently, vectorial proton translocation is detected only in the presence of cytochrome aa_3, which is an established proton pump (van Verseveld *et al.*, 1981); in its absence, however, only the scalar proton release is measured. This observation is confirmed by analysis of the triple oxidase mutant, in which cytochrome aa_3 is reintroduced by complementation with either CtaDII or CtaDI. The induced increase of the $H^+/2e^-$ ratios (2.1–2.2) also indicates that both isoforms of cytochrome aa_3 are proton-pumping cytochrome *c* oxidases (Table 3).

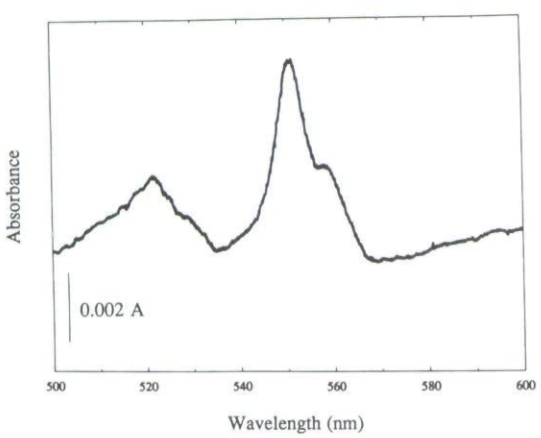

Fig. 5. Absolute absorbance spectrum of the alternative cytochrome *c* oxidase (cytochrome cbb_3) complex after reduction with Na-dithionite; the oxidase has been extracted from membranes of the triple oxidase mutant Pd9311.

Table 3. Proton-translocation measurements on whole-cell suspensions of different *P. denitrificans* strains. $H^+/2e^-$ ratios are avarages ± standard deviation.

Strain	succinate O_2 ($H^+/2e^-$)	succinate $Fe(CN)_6^{3-}$ ($H^+/2e^-$)	ascorbate O_2 ($H^+/2e^-$)
Wild type(s)			2.40 ± 0.11
aa_3 double mutant(s)			1.21 ± 0.16
bb_3 mutant(s)	5.15 ± 0.21	3.99 ± 0.11	2.01 ± 0.18
aa_3/bb_3 triple mutant(s)	4.15 ± 0.14	4.05 ± 0.10	1.12 ± 0.10
aa_3/bb_3 triple mutant, *ctaDI* compl.(s)			2.20 ± 0.36
aa_3/bb_3 triple mutant, *ctaDII* compl.(s)			2.12 ± 0.10
Wild type, aerobic/ methanol-limited (m/ch)			2.81 ± 0.14
Wild type, aerobic/O_2-limited (s/ch)			1.21 ± 0.35
Wild type, anaerobic/ NO_3-limited (s/ch)			1.19 ± 0.07

Cells were cultivated as aerobic batch cultures, in minimal medium with either succinate (s) or methanol (m); data are compared with analyses from chemostat-cultured cells (ch) of *P. denitrificans* NCIB 8944 (wild type) with limitations as indicated, reported by van Verseveld *et al.* (1981; 1983). The electron donor was either succinate or ascorbate; anaerobic suspensions were pulsed with oxygen (O_2) or ferricyanide ($Fe(CN)_6^{3-}$). A control pulse with ferricyanide of the ascorbate mixture routinely resulted in $H^+/2e^- = 1.1 \pm 0.1$, both in the presence and in the absence of cells (not shown). The assay was as described in the legend to Fig. 5; for details see the *Experimental procedures*.

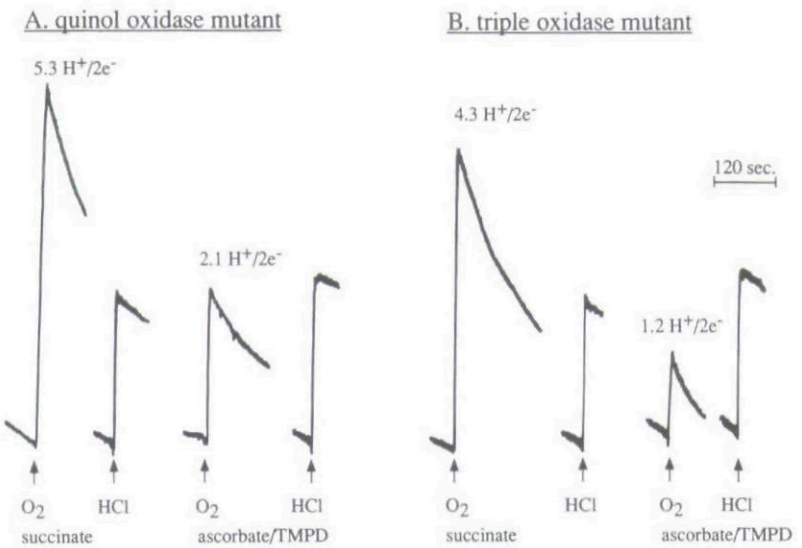

Fig. 6. Respiration-driven proton translocation in cells of *Paracoccus*. Anaerobic cell suspensions were pulsed with oxygen (O_2) by injection of air-saturated KCl. Traces were calibrated by injecting an anaerobic solution of 1 mM HCl. The calculated $H^+/2e^-$ ratio is shown for each oxygen pulse.

Succinate-dehydrogenase catalyses the oxidation of succinate and the reduction of ubiquinone. Subsequently, ubiquinol is oxidized either by a quinol oxidase (cytochrome bb_3; $H^+/2e^- = 4.0$), or by cytochrome *c* reductase (cytochrome bc_1; $H^+/2e^- = 4.0$). When electrons flow from the latter complex, via cytochrome *c*, to a proton pumping cytochrome *c* oxidase the energy-transducing efficiency increases (cytochrome aa_3; $H^+/2e^- = 6.0$). In the actual experiments with succinate the same tendency is observed with cells of the aa_3 double mutant and the triple oxidase mutant: in the presence of cytochrome aa_3 the $H^+/2e^-$ stoichiometry is 5.0–5.3, but in the cytochrome aa_3 mutants it never exceeds an $H^+/2e^-$ ratio of 4.0–4.3 (Table 3). As an internal control, the proton efflux associated with the succinate oxidation has been measured with ferricyanide as terminal electron acceptor.

Discussion

P. denitrificans contains a number of alternative respiratory pathways, branching at the level of ubiquinol. One route closely resembles the mitochondrial respiratory chain in which ubiquinol is oxidized by the so-called supercomplex consisting of cytochrome *c* reductase (cytochrome bc_1), cytochrome c_{552} and cytochrome *c* oxidase (cytochrome aa_3) (Berry and Trumpower, 1985). In a commonly expressed alternative pathway ubiquinol is oxidized directly by a quinol oxidase called cytochrome b_o by Cox *et al.* (1978).

When *Paracoccus* is cultivated under microaerobic or anaerobic conditions, the expression level of cytochrome aa_3 and, to some extent, that of cytochrome c_{552} appears significantly lower (Bosma *et al.*, 1987a). Instead of the

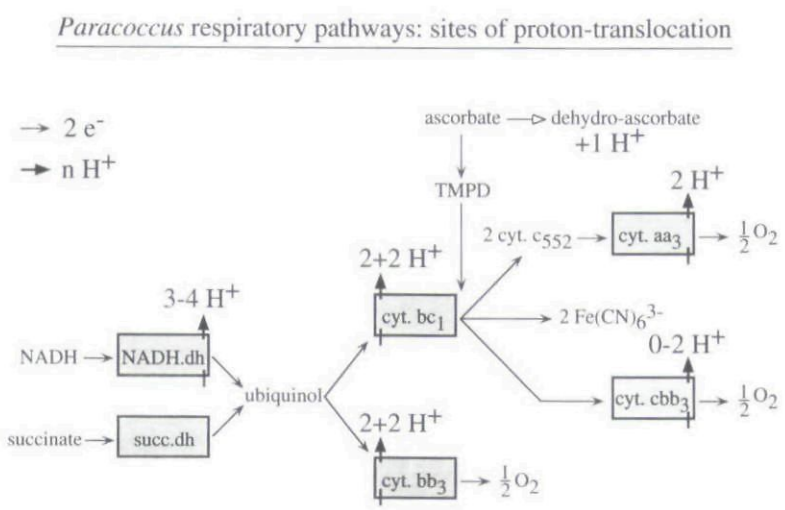

Fig. 7. Respiratory pathways in *Paracoccus*. Indicated here is the theoretical stoichiometry of the respiration-driven proton translocation ($H^+/2e^-$), detectable in the medium, for each of the contributing respiratory complexes: NADH-dehydrogenase (NADH.dh), succinate dehydrogenase (succ.dh), cytochrome *c* reductase (cyt.bc_1), cytochrome c_{552} (cyt.c_{552}), aa_3-type cytochrome *c* oxidase (cyt.aa_3), cbb_3-type cytochrome *c* oxidase (cyt.cbb_3) and the bb_3-type quinol oxidase (cyt.bb_3). Ascorbate (with TMPD) is an artificial reductant of cytochrome *c*, and ferricyanide ($Fe(CN)_6^{3-}$) an oxidant. The chemical conversion of ascorbate is indicated (open arrow) to show generation of the 'scalar' proton. For discussion, see text.

aforementioned supercomplex, a quinol-oxidizing complex has been isolated from such oxygen-limited cells in which cytochrome bc_1 is associated with an alternative *cb*-type cytochrome *c* oxidase, called cytochrome c_o by Bosma (1989).

Ubiquinol oxidase

A new oxidase locus (*cyoABC*) has been isolated from a *P. denitrificans* genomic library. The predicted protein sequences share a high degree of homology with subunits I, II and III of the quinol-oxidizing cytochrome bo_3 from *E. coli* (Fig. 2, Chepuri *et al.*, 1990). In subunit I (CyoB) the six conserved histidines are present, indicating that it concerns a haem–copper oxidase (Saraste, 1990; Hosler *et al.*, 1993). As in *E. coli* CyoA, subunit II of this *Paracoccus* oxidase has substituted four out of five residues that have been identified as Cu_A ligands (Kelly *et al.*, 1993). This suggests that CyoABC is a quinol oxidase (Saraste *et al.*, 1991). Moreover, the oxygen consumption of the *cyoB* mutant was completely inhibited by either antimycin A or myxothiazol, whereas the wild-type *Paracoccus* strain was hardly affected by these compounds (Table 1). This confirms that the *cyoABC* locus codes for a quinol oxidase.

The *E. coli cyo* operon is a cluster of five genes: *cyoABCDE*. The *cyoD* gene product is thought to be a fourth subunit (Minghetti *et al.*, 1992), which may be absent from the *Paracoccus* quinol oxidase complex. The *cyoE* product is proposed to be a protohaem IX farnesyl transferase, involved in the conversion of haem B into haem O (Saiki *et al.*, 1992). Deletion of the latter gene in *E. coli* resulted in an oxidase with two B haems, however, without oxidase activity (Hill *et al.*, 1992; Saiki *et al.*, 1992).

In well-aerated, succinate-grown cells of *Paracoccus* the contribution of the quinol oxidase to the endogenous respiration varies: it is 75% in wild-type cells and 100% in the cytochrome aa_3 double mutant. Apparently, relatively more electrons flow via the cytochrome *c* branch when succinate is added as a substrate (Table 1).

Haem analysis indicates that wild-type membranes, with quinol oxidase activity, contain haem B and haem A; in the cytochrome aa_3 double mutant haem B and only traces of haem A could be detected (Table 2). Unlike haem A and haem B, haem O has never been detected in membrane extracts from the *Paracoccus* strains grown under these conditions. We conclude that the CyoABC quinol oxidase is a cytochrome with two protohaems. This implies that the hydroxyethylfarnesyl side-chain, which is present in haems A and O but not in haem B (Wu *et al.*, 1992), is not essential for catalytic activity or for proton translocation. Previously this oxidase has been referred to as cytochrome *o* or b_o (Cox *et al.*, 1978; Puustinen *et al.*, 1989); we propose to call it cytochrome bb_3, according to the classical terminology of the mitochondrial cytochrome *c* oxidase in which the O_2-binding haem is denoted by the subscript 3 (Puustinen and Wikström, 1991).

An alternative quinol oxidase complex, cytochrome ba_3, has been purified from a *Paracoccus* cytochrome *c* reductase mutant (Ludwig, 1992). The genomic locus of cytochrome ba_3 has been isolated (O. Richter and B. Ludwig, personal communication), and comparison of these data with the partial sequences as presented in Fig. 2 revealed that cytochrome ba_3 and cytochrome bb_3 are encoded by the same set of genes (*cyoABC*). As far as we are aware, the cytochrome bc_1 mutant is the only *Paracoccus* strain in which this mixed haem oxidase is detectable. Hence, it may be more likely that the genotype rather than the growth conditions gives rise to the observed variable haem incorporation into the CyoB apo-cytochrome. Similar variability has recently been reported for the cytochrome *c* oxidase from the thermophilic bacterium PS3 (Sone and Fujiwara, 1991), and for quinol oxidases from *E. coli* and *Acetobacter aceti* as well (Puustinen *et al.*, 1992; Matsushita *et al.*, 1992).

In the quinol oxidase mutant and in the triple mutant, no alternative quinol oxidase activity could be demonstrated (Table 1). Moreover, extensive attempts to generate a cytochrome *c* reductase/quinol oxidase double mutant have not succeeded (not shown), which indeed might indicate that the *Paracoccus* genome contains only one quinol oxidase locus. Previously, the existence of two more terminal oxidases was deduced from optical spectra of whole cells. First, a cyanide-tolerant oxidase with a high affinity for oxygen and an absorbance maximum at 629 nm has been called cytochrome *d* by Henry and Vignais (1979). However, cytochrome bb_3 might have been responsible for the reported cyanide-tolerant respiration of *Paracoccus*; in a membrane preparation of the cytochrome aa_3 double mutant a K_i for cyanide of approx. 250 μM has been determined, whereas the triple mutant is extremely sensitive to cyanide (J.-W. L. de Gier and J. van der Oost, unpublished). The observed absorbance band may have originated from the nitrite reductase cytochrome cd_1 (Timkovitch *et al.*, 1982). Second, an oxidase that is expressed microaerobically, with an absorbance maximum at 590 nm, has been called cytochrome a_1 by van Verseveld *et al.* (1983). It is not unlikely, however, that the observed cytochrome belongs to another class of enzymes, e.g. the CO-binding catalase-peroxidase cytochrome b_{590} (Appleby and Poole, 1991), which was originally proposed to be the high-affinity oxidase (cytochrome a_1) from nitrogen-fixing *Bradyrhizobium japonicum*. Indeed, a cytochrome b_{595}-associated peroxidase activity has been observed in periplasmic fractions of *Paracoccus* (J. Ras, unpublished).

aa_3-*type cytochrome* c *oxidase*

Expression of the *Paracoccus* cytochrome aa_3 has been studied extensively. When *Paracoccus* is growing on succinate, either microaerobically or anaerobically, cytochrome aa_3 is undetectable or barely detectable in the optical spectrum (Bosma, 1989; Stouthamer, 1991). In contrast, this cytochrome *c* oxidase plays an active role during autotrophic growth on the C_1 substrates methanol and methylamine (reviewed by Harms and van Spanning 1991). On the other hand, a cytochrome aa_3 mutant of *Paracoccus* (Willison *et al.*, 1981) is able to grow on methanol (Harms *et al.*, 1985), and a cytochrome *c* reductase/cytochrome aa_3 double mutant of *Paracoccus* is able to grow on methylamine (de Gier *et al.*, 1992). This suggests the presence of an alternative cytochrome *c* oxidase (see below).

The genomic loci encoding the cytochrome aa_3 isoenzymes have been isolated previously (reviewed by van der Oost *et al.*, 1991). In the triple oxidase mutant (Δ*ctaDI*, Δ*ctaDII*, *cyoB*::Km^R) we show that it is possible to restore the optical spectrum of cytochrome aa_3 by complementation with either CtaDI or CtaDII (Fig. 4). This has been confirmed by Western blotting, using antibodies raised against the three-subunit cytochrome aa_3 from *Paracoccus* (not shown). No spectral/immunological evidence has been found so far for expression of CtaDI in the Δ*ctaDII* mutant. The successful expression *in trans* might indicate that a repression of the *ctaDI* promoter activity has been (partially) overcome by the elevated copy number. The physiological conditions under which the expression of the cytochrome aa_3 isoenzyme is induced remain unknown.

cb-*type cytochrome* c *oxidase*

Cells of wild-type *Paracoccus* grown anaerobically/microaerobically on succinate are still able to oxidize ascorbate/TMPD although no aa_3-type cytochrome *c* oxidase is detectable in the optical spectrum (van Verseveld *et al.*, 1983). The latter authors have reported 'the presence of a cytochrome oxidase with a very high affinity for oxygen and a low affinity for CO'. This high-affinity cytochrome *c* oxidase has been characterized in more detail by Bosma (1989), who partially purified it from anaerobically grown cells. The alternative cytochrome *c* oxidase was isolated as a complex with cytochrome *c* reductase, but without cytochrome c_{552}. Spectroscopic analysis revealed that it concerns a *cb*-type oxidase complex, consisting of a cytochrome *b*, a 30 kDa cytochrome *c* and perhaps a loosely membrane-associated 45 kDa dihaem cytochrome *c* (Bosma *et al.*, 1987b; Bosma 1989).

In the present study a triple oxidase mutant (Δ*ctaDI*, Δ*ctaDII*, *cyoB*::Km^R) has been generated, in which an alternative cytochrome *c* oxidase has been studied *in vivo*. The optical spectrum of this mutant indicates the absence of cytochrome aa_3 and an increase in the *bc* pool (Fig. 3). This increased amount of cytochromes *b* and *c* turned out to be reversible: upon complementation of the triple mutant *in trans* with either one of the three oxidases, the absorbance peak decreased to a wild-type level (Fig. 4). The alternative cytochrome *c* oxidase has recently been partially purified from the membranes of the triple mutant; the optical spectrum indicates the presence of cytochromes *c* and *b* (Fig. 5). It is tempting to assume that an expression induction/repression of this *cb*-type oxidase is responsible for the observed increase/decrease in the absorbance spectra of the triple mutant (Figs 3 and 4). Analysis of the haems extracted from membranes of the triple mutant only indicates the presence of haem B (Table 2). Haem A and haem O are absent; haem C is not extracted, owing to its covalent binding. Oxygen-consumption measurements indicate that respiration in the triple mutant could be inhibited completely at the level of cytochrome bc_1 (Table 1). It may be that the alternative cytochrome *c* oxidase is the only remaining terminal oxidase in this mutant.

Recently, we obtained additional genetic information on this *cb*-type oxidase. Using peptide sequences from the aforementioned purified cytochrome *cb* complex, a DNA fragment has been isolated from a *P. denitrificans* genomic library; preliminary sequence analysis indicates the presence of a new oxidase locus (M. Saraste and J. van der Oost, unpublished). The isolated gene cluster is very similar to *fixNOQP* from *B. japonicum* (Preisig *et al.*, 1993). The genes from *Bradyrhizobium* have been demonstrated to encode an alternative oxidase that is expressed microaerobically or anaerobically; this is in good agreement with the finding that the gene cluster is located adjacent to the genes of the oxygen-sensing regulators FixJL. The predicted protein sequences suggest that FixN is the haem- and copper-binding subunit I homologue, FixO a monohaem cytochrome *c*, and FixP a dihaem cytochrome *c* (Preisig *et al.*, 1993). The alignment of the *Bradyrhizobium* FixN sequence (Fig. 2A) suggests that all six invariant histidines, typical of the haem–copper oxidase superfamily, are present in the *cb*-type cytochrome *c* oxidase; it probably concerns a *b*-type haem–copper oxidase (Table 2, see below) with associated cytochrome(s) *c*. In accordance with classical cytochrome oxidase terminology (Puustinen and Wikström, 1991), we propose to name this *cb*-type oxidase cytochrome cbb_3.

In addition, a *b*-type cytochrome *c* oxidase has been described in *Rhodobacter capsulatus* (Hüdig *et al.*, 1987). Regulation has been demonstrated at two levels: the expression as well as the activity of this oxidase respond to oxygen and light. Upon a shift from phototrophic conditions to chemotrophic conditions (microaerobically) the

activity of this oxidase increases fourfold. Garcia-Horsman *et al.* (1994) have purified a *cb*-type cytochrome *c* oxidase from a *Rhodobacter sphaeroides* cytochrome aa_3 mutant. The oxidase consists of three subunits: two cytochromes *c* and a cytochrome *b*. Metal and haem analyses indicate that this cbb_3-type oxidase contains haem C, haem B, and copper in a ratio of 3:2:1. The presence of a binuclear centre has been demonstrated, including a high-spin haem B and a Cu_B (Garcia-Horsman *et al.*, 1994).

Proton translocation

Analysis of proton translocation by the generated set of *Paracoccus* oxidase mutants has been performed in order to determine whether or not the alternative cytochrome *c* oxidase is a proton-pumping oxidase. During succinate oxidation, when ubiquinol is oxidized directly by cytochrome bb_3, a minimum $H^+/2e^-$ stoichiometry of 4 is expected theoretically; when ubiquinol is oxidized via cytochrome bc_1 ($H^+/2e^- = 4$) and cytochrome aa_3 ($H^+/2e^- = 2$) the maximal $H^+/2e^-$ stoichiometry is 6. An obvious consequence of the deletion of cytochrome aa_3 is a decreased $H^+/2e^-$ stoichiometry (Table 3). When ferricyanide is used as terminal electron acceptor instead of oxygen, equal values are obtained because ferricyanide is reduced directly by the cytochrome *c* pool (Table 3, Fig. 6). A similar tendency is observed when ascorbate/TMPD is used as substrate, donating electrons at the level of cytochrome *c*. Apart from the scalar proton ($H^+/2e^- = 1$), an additional acidification is expected in the case of a proton-translocating cytochrome *c* oxidase, such as cytochrome aa_3 ($H^+/2e^- = 2$); the maximal $H^+/2e^-$ stoichiometry would be 3. Again, in the absence of cytochrome aa_3 only the scalar ascorbate proton is measured (Table 3). When cytochrome aa_3 is present, an intermediate value of 2.0–2.4 is commonly detected. This indicates that, unlike cytochrome aa_3 (van Verseveld *et al.*, 1981) and cytochrome bb_3 (Puustinen *et al.*, 1989), the cbb_3-type cytochrome *c* oxidase does not couple the reduction of oxygen to the active transport of protons across the membrane, at least under these conditions (see below).

The proton translocation data as presented in this study are consistent with the absence of a proton-pumping oxidase in cytochome aa_3 deletion strains. However, Raitio and Wikström (1994) recently presented experimental data that strongly suggest that the alternative cytochrome *c* oxidase is able to pump protons. These authors report an $H^+/2e^-$ ratio of 6, with whole cells (wild-type, cytochrome aa_3 double mutant), succinate as substrate, and oxygen as electron acceptor. With ascorbate/TMPD as the electron-donating system, on the other hand, hardly any pumping was observed ($H^+/2e^- = 1.2$–1.5). The buffer composition of the proton translocation assay as presented by the latter authors is the main difference in the method used in this study (HEPES (0.5 mM) and glycylglycine (1.5 mM), respectively). Whereas the proton translocation linked to the cytochrome aa_3 activity is easily detectable when the cell suspension is buffered by either glycylglycine or HEPES, proton translocation by an alternative oxidase, which is likely to be cytochrome cbb_3, is apparently only detectable in the HEPES-buffered cell suspension.

It has been reported previously that maximal rates of oxidative phosphorylation in *Paracoccus* were measured only in the presence of cytochrome aa_3, e.g. after growth on methanol but not after microaerobic growth (Boogerd *et al.*, 1981; van Verseveld *et al.*, 1983) (Table 3). Stouthamer (1991) has recently reviewed the results obtained by his coworkers over the last decade, on *Paracoccus* cultivated in chemostats under a wide variety of growth conditions. Calculations of growth yields and proton-translocation rates (assayed in glycylglycine buffer) led him to suggest that cytochrome aa_3 is a proton-translocating cytochrome *c* oxidase, unlike the alternative cytochrome *c* oxidase. In view of the aforementioned variations of the cbb_3-catalysed proton translocation, it can be imagined that the *in situ* energy-transducing efficiency of this oxidase might also vary under different external conditions. Chemostat experiments with the available set of oxidase mutants are intended to address this point.

Understanding the nature of the differences observed in the proton pumping behaviour of the cbb_3-type oxidase as compared to, for example, the aa_3-type oxidase will be the goal of future experiments. With current knowledge, it is anticipated that cytochrome cbb_3 is indeed capable of pumping protons, perhaps only under specific external conditions. A detailed study of this 'distant' member of the haem–copper oxidase family will contribute substantially to a model of the redox-driven proton pump.

Experimental procedures

Bacterial strains, plasmids, and growth conditions

The strains of *P. denitrificans* and *E. coli*, as well as the plasmids, that were used in this study are listed in Table 4. Cells of the wild-type *P. denitrificans* (Pd1222) and the mutant strains were cultivated in aerobic batch cultures (0.5 l bottles with 100 ml of culture, on a rotary shaker at 30°C), either with brain–heart infusion broth (BHI) or minimal medium supplemented with 25 mM succinate, as described previously (van Spanning *et al.*, 1991). *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}).

DNA manipulations

General cloning techniques were carried out as described by

Table 4. Strains and plasmids.

Strain/Plasmid	Relevant characteristics	Reference
Strain		
E. coli		
TG1	*supE, hsdD5, thi,* Δ*(lac⁻ proAB),* F′, (*traD36 proAB lacI*^{q} *lacZ*ΔM15)	Sambrook *et al.* (1989)
HB101	F⁻, *hsdS20,* ($r_B^-m_B^-$), *lacY1, proA2, recA13*	Boyer *et al.* (1969)
S17.1	Sm^R, *pro,* ($r_B^-m_B^+$), RP4-2, integrated (Tc::Mu) (Km::Tn*7*)	Simon *et al.* (1983)
P. denitrificans		
Pd1222	Rif^R, enhanced conjungation frequencies, ($r_B^-m_B^+$)	De Vries *et al.* (1989)
Pd2521	Pd1222 derivative, *ctaDI*::Km^R	This work
Pd2541	Pd2521 derivative, Δ*ctaDI*	This work
Pd9218	Pd2541 derivative, Δ*ctaDI*, *ctaDII*::Km^R	This work
Pd9220	Pd9218 derivative, Δ*ctaDI*, Δ*ctaDII*	This work
Pd2621	Pd1222 derivative, *cyoB*::Km^R	This work
Pd9311	Pd9220 derivative, Δ*ctaDI*, Δ*ctaDII*, *cyoB*::Km^R	This work
Plasmid		
pUC18	Amp^R, *lacZ′*	Yanisch-Perron *et al.* (1985)
pUC4K	Km^R (Tn*903*)	Pharmacia
pUC4KISS	Km^R (Tn*903*)	Pharmacia
M13mp18	pUC18 mcs, *lacZ′*	Sanger *et al.* (1980)
pEG400/401	*IncP*, Sm^R, Spec^R, pUC12/13 mcs, *lacZ′*	Gerhus *et al.* (1990)
pRK2020	Tc^R, pRK2013 Km::Tn*10*	Ditta *et al.* (1985)
pGRPd1	*oriV* (colE1), Amp^R, *oriT*, Sm^R, (Tn*1831*)	Van Spanning *et al.* (1990)
pRVS1	*oriV* (colE1), Amp^R, *oriT*, Sm^R (Tn*1831*), Tn*5*p, *lacZ*	Van Spanning *et al.* (1991)
pMR3	pUC18 derivative, *ctaDI*	M. Raitio, unpublished
pMR3.Km^R	pMR3 derivative, *ctaDI*::Km^R	This work
pMR3D	pMR3 derivative, Δ*ctaDI*	This work
pUC.*cyoABC*	pUC18 derivative, *cyoABC*	This work
pUC.*cyoABC*.Km^R	pUC.*cyoABC* derivative, *cyoB*::Km^R	This work
pRT2521	pGRPd1 derivative, *ctaDI*::Km^R	This work
pRT2541	pRVS1 derivative, Δ*ctaDI*	This work
pRT2321	pGRPd1 derivative, *ctaDII*::Km^R	This work
pRT2341	pRVS1 derivative, Δ*ctaDII*	This work
pRT2621	pRVS1 derivative, *cyoB*::Km^R	This work
pEG.*ctaDII*	pEG400 derivative, *ctaDII*	This work
pEG.*ctaDI*	pEG401 derivative, *ctaDI*	This work
pEG.*cyoABC*	pEG401 derivative, *cyoABC*	This work

Ausubel *et al.* (1992). Conjugations were performed as described previously (de Vries *et al.*, 1989). The matings of *Paracoccus* host strains were performed either directly with *E. coli* S17-1 transformed with 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'.

P. denitrificans mutant strains were constructed by gene-replacement techniques as described previously (van Spanning *et al.*, 1991). First, a kanamycin-resistance cassette from pKISS (Boehringer) was inserted in the *Sma*I site of pMR3, a pUC18 derivative carrying a *ctaDI*-containing genomic *Bam*HI fragment (a generous gift of Dr M. Raitio, Fig. 1A). From the resulting construct (pMR3.Km) a *Pvu*II fragment was ligated in the *Sma*I site of pGRPd1 (pRT2521), which was transformed to *E. coli* S17.1 and conjugated to *P. denitrificans* Pd1222 to give Pd2521. Exconjugants were selected for kanamycin and rifampicin resistance and streptomycin sensitivity; genomic Southern blots were used to confirm the mutations (not shown).

From the *ctaDI* gene a *Sal*I–*Sma*I fragment was deleted (Fig. 1A); sticky ends were filled in using Klenow DNA polymerase fragment and religated (pMR3Δ). The in-frame deleted locus was cloned as a *Pvu*II fragment in the *Sma*I site of the suicide vector pRVS1 (pRT2541), which was transformed to *E. coli* S17.1 and conjugated to the insertion mutant Pd2521. *Paracoccus* colonies were initially selected by rifampicin-, kanamycin- and streptomycin resistance. Subsequent restreaking revealed two distinct phenotypes: (i) integrants turned blue on plates supplemented with Xgal (pRVS1 carries the β-galactosidase gene) and are streptomycin- and kanamycin resistant, and (ii) double recombinant colonies stayed

white and had lost streptomycin- and kanamycin resistance. The deletions in the latter clones (Pd2541) were verified by genomic Southern blotting (not shown). From the *ctaDI* deletion mutant, a *ctaDI/ctaDII* double deletion mutant was derived, using the same methods. As an intermediate an insertion mutant (Pd9218) was generated by conjugation with a pGRPd1 derivative (pRT2321), carrying a *Bam*HI–*Sph*I fragment of the *ctaDII* locus with the Km-resistance gene from pUC4K inserted in the *Pst*I site; in the subsequently derived *ctaDII* deletion mutant (Pd9220) the *Hin*cII–*Nco*I fragment was removed after a double recombination event with pRT2341 (Fig. 1A).

The degenerated primers used in the PCR experiment were: #151 (5′-GCGCGGAATTC-CAT/CGGG/CGTG/CATT-CATGATT/CTTT/CTT) and #152 (5′-GCGCGGAATTC-TAG/CACT/CTCG/CGGA/GTGG/CCCA/GAAA/GAACCA). Using genomic DNA from the cytochrome aa_3 double mutant and these primers a PCR was performed under conditions essentially like those described before (M. Lübben *et al.*, submitted); in this case the annealing temperature was 37°C. A PCR product of the expected size was digested with *Eco*RI (sites introduced in primers, underlined) and cloned into M13mp18. Using the PCR fragment as a probe, a clone was isolated (pUC.*cyoABC*) that appeared to contain the complete oxidase locus. This plasmid was digested with *Eco*RI, removing the *Pst*I site from the pUC multiple cloning site, and religated. Subsequently the internal *Pst*I fragment was substituted for the kanamycin-resistance box from pUC4KISS. The *Eco*RI–*Sph*I insert was cloned in pRVS1, transformed to *E. coli* S17.1, and conjugated to the wild-type *Paracoccus* Pd1222 and the *ctaDI/ctaDII* double deletion mutant (Pd9220). Conjugants were selected by kanamycin resistance; colonies of integrants turned blue on plates supplemented with Xgal, but colonies of double recombinants remained white. The insertions in the latter clones were verified by genomic Southern blotting (not shown).

Optical spectroscopy

Spectra of whole-cell suspensions were recorded at room temperature on an Aminco/SLM DW2 UV/Vis Spectrophotometer, essentially as decribed previously (de Gier *et al.*, 1992).

Oxygen-consumption analysis

Oxygen consumption by bacterial cell suspensions was analysed polarographically at 30°C in a biological oxygen monitor, Model 53 (Yellow Springs Instrument Co.). Succinate (25 mM), or ascorbate (0.4 mM) and TMPD (0.1 mM) were added as external substrates; electron flow via cytochrome *c* reductase was inhibited by antimycin A (6 μM) or myxothiazol (6 μM). Assays were repeated at least three times and were found to be reproducible within 10%. No auto-oxidation of ascorbate/TMPD was detected when solutions were prepared freshly.

Proton-translocation analysis

Measurement of H^+ translocation in intact cells was performed essentially as described by Boogerd *et al.* (1981). Cells from batch cultures (minimal medium with succinate) were harvested, washed, and resuspended in 1.5 mM glycylglycine buffer (pH 7.0), 100 mM KSCN, 50 mM KCl (approx. 25 mg dry weight cells ml^{-1}), incubated at 25°C, and bubbled with nitrogen. In the reaction vessel, 150 μl of cell suspension was mixed with 3.1 ml of buffer. With succinate (25 mM) as electron donor, rotenone (30 μM) was added and incubated for 30 min in order to inhibit endogenous respiration. With ascorbate/TMPD (0.4 mM/0.1 mM) antimycin A (6 μM) was added (final concentrations); in the presence of the quinol oxidase, rotenone (30 μM) was added as well. The anaerobic cell suspension was pulsed with oxygen (O_2) by injecting 20 μl of air-saturated KCl (9.4 ng atoms of oxygen), or with ferricyanide by injecting 20 μl of an anaerobic solution of 1 mM $K_3Fe(CN)_6$. Traces were calibrated by injecting 20 μl of an anaerobic solution of 1 mM HCl.

Haem analysis

The analysis of the haem composition after extraction from membranes was performed as described by Sone and Fujiwara (1991). Haems were extracted from isolated membranes of different *P. denitrificans* strains, separated by reversed-phase HPLC (Deltapak C18 column, Waters) on an acetonitrile gradient in 0.05% trifluoroacitic acid.

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