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Regulation of Oxidative Phosphorylation: The Flexible Respiratory Network of *Paracoccus denitrificans*[1]

Rob J. M. Van Spanning,[2,3] Anthonius P. N. de Boer,[2] Willem N. M. Reijnders,[2] Jan-Willem L. De Gier,[2] Cécile O. Delorme,[2] Adriaan H. Stouthamer,[2] Hans V. Westerhoff,[2] Nellie Harms,[2] and John van der Oost[4]

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Paracoccus denitrificans is a facultative anaerobic bacterium that has the capacity to adjust its metabolic infrastructure, quantitatively and/or qualitatively, to the prevailing growth condition. In this bacterium the relative activity of distinct catabolic pathways is subject to a hierarchical control. In the presence of oxygen the aerobic respiration, the most efficient way of electron transfer-linked phosphorylation, has priority. At high oxygen tensions *P. denitrificans* synthesizes an oxidase with a relatively low affinity for oxygen, whereas under oxygen limitation a high-affinity oxidase appears specifically induced. During anaerobiosis, the pathways with lower free energy-transducing efficiency are induced. In the presence of nitrate, the expression of a number of dehydrogenases ensures the continuation of oxidative phosphorylation via denitrification. After identification of the structural components that are involved in both the aerobic and the anaerobic respiratory networks of *P. denitrificans*, the intriguing next challenge is to get insight in its regulation. Two transcription regulators have recently been demonstrated to be involved in the expression of a number of aerobic and/or anaerobic respiratory complexes in *P. denitrificans*. Understanding of the regulation machinery is beginning to emerge and promises much excitement in discovery.

KEY WORDS: Respiratory network; multiple oxidases; denitrification; gene regulation; FNR; *Paracoccus denitrificans*; *Escherichia coli*.

INTRODUCTION

The ultimate goal of living organisms is to contribute to a continued existence of their species by means of survival and reproduction. In unicellular organisms, the ability to survive depends on the cell's potential to adapt its metabolism to the available carbon and free-energy sources in its natural habitat, ensuring maintenance and growth. The more such an environment is subject to fluctuations in the supply of these substrates, the higher the demands that are made upon the potential of the cell to adjust its metabolic properties. A profound example of this flexibility is the process of bacterial respiration, a membrane-associated process that drives the free-energy transduction by oxidative phosphorylation. An intriguing question is how the cell manages to adjust its respiratory efficiency to environmental changes. The possession of a respiratory network rather than a linear pathway is the basis of a flexible energy-generating system. The adaptation may be achieved either by redirection of electron fluxes through existing branches within a respiratory network, or by specific regulation of the

[1] Dedicated to L. Fred Oltmann.

[2] Department of Microbial Physiology, Faculty of Biology, BioCentrum Amsterdam, Vrije Universiteit, De Boelelaan 1087, NL-1081 HV Amsterdam, The Netherlands.

[3] Corresponding author: Department of Microbiology, Biological Laboratory, BioCentrum Amsterdam, Vrije Universiteit, De Boelelaan 1087, NL-1081 HV Amsterdam, The Netherlands.

[4] Present address: Department of Microbiology, Wageningen Agricultural University, Hesselink van Suchtelenweg 4, NL-6703 CT Wageningen, The Netherlands.

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expression of certain branches, thereby changing the make-up of the network.

Studies on *Escherichia coli* have revealed that complex regulatory cascades control the composition of its relatively simple respiratory network in response to the different intra- and extracellular signals Spiro, 1994; Stewart, 1994; Unden *et al.*, 1994). Similar adaptive responses are required for the proper regulation of oxidative phosphorylation in all classes of bacteria, especially for those that live in relatively fast-changing environments. Moreover, since organisms in such habitats would obviously benefit from having a more comprehensive respiratory network, it is anticipated that their regulation may proceed via an even more sophisticated control mechanism than observed in *E. coli.*

In *Paracoccus denitrificans* the electron-transfer network involved in reduction of molecular oxygen is integrated with the network reducing nitrate and nitrite. For a number of compelling reasons, bacterial denitrification has drawn major attention. First, significant fluxes of nitric oxide, and nitrous oxide from soil to atmosphere have been reported (Ye *et al.*, 1994). These nitrogenous oxides may contribute to the initiation of "acid rain," they affect the ozone layer, and they may be partly responsible for global warming. Secondly, from the viewpoint of understanding the biology of the living cell, it is a challenging aim to find out how the denitrifying electron pathways are controlled and integrated in the cell's metabolism.

In the terrestrial ecosystem where bacterial denitrification takes place, the oxygen tension tends to be low. This situation brings the bacterium in the predicament of how to divide its electron fluxes between either the limiting amount of oxygen or to the more abundant nitrogen oxides for capture of free energy, where the latter route generates toxic intermediates. Imbalance of either the genetic or the enzymatic control of denitrification confronts the bacterium with the accumulation of these toxic compounds. Especially nitric oxide is highly reactive and interacts rapidly with metal-binding proteins like hemoproteins, iron-sulfur proteins, and copper proteins. Moreover, nitric oxide inhibits heme-copper oxidases where it readily binds to the high-spin ferrous heme in its reaction center (Kucera and Skaldal, 1990). Apparently nitric oxide is chemically related to molecular oxygen. The catalytic subunits of the NO reductase and the cbb_3-type oxidases are related structurally (Van der Oost *et al.*, 1994; Saraste and Castresana, 1994). This finding has stimulated the efforts to compare the properties of nitric oxide reductase and heme copper oxidases (the oxygen reductases) in terms of substrate reduction and proton pumping capabilities. Another question that should be addressed is to what extent nitric oxide binds to the oxidases, and modulates their activities. In mammals, nitric oxide has recently been recognized as being a key chemical signal in a variety of processes (Koshland, 1992). The molecular details of signal transduction via nitric oxide have not been established yet. It is possible that both in bacteria and mammals nitric oxide is directly involved in signal transduction by modulating the activity of oxidases.

For more than twenty years the characterization of electron transfer phenomena in *Paracoccus denitrificans* has been subject of research in our group (reviewed by Stouthamer, 1991). In the past decade, many redox enzymes from *P. denitrificans* have been cloned (genes and corresponding references are listed in Table I). The function of the individual enzymes was studied by means of directed mutagenesis. In this procedure, the gene encoding the redox enzyme of interest is disrupted in a gene-exchange experiment (Van Spanning *et al.*, 1990a). Next, the characteristics of the mutated strain were analyzed in terms of the kinetics and efficiency of respiration. An advanced version of this procedure allowed us to introduce unmarked multiple mutations in one and the same strain (Van Spanning *et al.*, 1991b). This was of particular importance for the unraveling of the *P. denitrificans* respiratory network that comprises a number of electron transfer routes that operate in parallel. During the course of our studies it has become evident that synthesis of most of the respiratory branches is under the control of highly integrative regulatory networks. Our working hypothesis is that these systems mediate the transfer of internal and external signals to the genome in order to accomplish finely tuned synthesis of the individual redox enzymes. By means of an integration of molecular genetics, biochemistry, physiology, and biomathematics, we aim at the unraveling of the interconnective regulatory networks that control gene expression and enzyme activity during aerobic respiration and denitrification.

METABOLIC FLEXIBILITY OF *Paracoccus denitrificans*

P. denitrificans is a Gram negative, facultative anaerobic bacterium that is found in soil, sewage, or sludge. During aerobic heterotrophic growth, its respiratory chain resembles the mitochondrial one (Fig.1A).

Table I. *Paracoccus denitrificans* Wild Type and Mutants Analyzed for Growth on Minimal Medium Plates Supplemented with Succinate (succ.), Methylamine (MA), or Methanol (MeOH)[a]

Disrupted gene(s)	Mutated gene-product(s)	Growth on succ.	Growth on MA	Growth on MeOH	Reference(s)
None	None	+	+	+	Van Spanning *et al.*, 1990b
cycA	c_{550}	+	+	+	Van Spanning *et al.*, 1990b
cycM	c_{552}	+	+	+	Turba *et al.*, 1995; Van der Oost *et al.*, 1995a
fbcC	c_1	+	+	+	Gerhus *et al.*, 1990; De Gier *et al.*, 1995b
mauB	MADHα	+	−	+	Van der Palen *et al.*, 1995d
mauA	MADHβ	+	−	+	Van der Palen *et al.*, 1995d
mauC	Amicyanin	+	−	+	Van Spanning *et al.*, 1990a
moxF	MDHα	+	+	−	Harms *et al.*, 1987
moxJ	moxJ	+	+	−	Van Spanning *et al.*, 1991a
moxG	c_{551i}	+	+	−	Van Spanning *et al.*, 1991a
cycB	c_{553i}	+	+	+	Ras *et al.*, 1991
fydA	FALDH	+	−	−	Ras *et al.*, 1995
ctaDII, ctaDI	aa_3 iso-aa_3	+	+	+	Van der Oost *et al.*, 1995a
qoxB	bb_3	+	+	+	De Gier *et al.*, 1994
ccoN	cbb_3	+	+	+	Van der Oost *et al.*, 1995a
cycA, moxG, cycB	c_{550}, c_{551i}, c_{553i}	+	+	−	Van Spanning *et al.*, 1991b
cycA, fbcC	c_{550}, c_1	+	+	+	De Gier *et al.*, 1995b
cycA, ctaDII	c_{550}, aa_3	+	+	+	De Gier *et al.*, 1995b
fbcC, ctaDII	c_1, aa_3	+	+	+	De Gier *et al.*, 1995b
cycA, fbcC, ctaDII	c_{550}, c_1, aa_3	+	−	−	De Gier *et al.*, 1995b
cycA, cycM	c_{550}, c_{552}	+	+	+	Van der Oost *et al.*, 1995a
fbcC, cycM	c_1, c_{552}	+	+	+	Van der Oost *et al.*, 1995a
ctaDII, cycM	aa_3, c_{552}	+	+	+	Van der Oost *et al.*, 1995a
cycA, ctaDII, cycM	c_{550}, aa_3, c_{552}	+	+	+	Van der Oost *et al.*, 1995a
fbcC, ctaDII, cycM	c_1, aa_3, c_{552}	+	+	+	Van der Oost *et al.*, 1995a
cycA, fbcC, cycM	c_{550}, c_1, c_{552}	+	−	−	Van der Oost *et al.*, 1995a
ccoN, ctaDII, ctaDI	cbb_3, aa_3, iso-aa_3	+	+	+	Van der Oost *et al.*, 1995a

[a] Subunits of methylamine dehydrogenase (MADH) and methanol dehydrogenase (MDH) are indicated by α and β. FALDH refers to formaldehyde dehydrogenase. All cytochromes are mentioned in the text.

Both in mitochondria and in bacteria, generation of the transmembrane electrochemical potential for protons (protonmotive force) is achieved by redox-driven proton translocation across the cytoplasmic membrane. This involves a specific set of redox protein complexes: the respiratory electron transfer chain. Electrons from NADH flow to oxygen via the NADH dehydrogenase, quinones, the cytochrome bc_1 complex, a cytochrome *c*, and the terminal aa_3-type cytochrome *c* oxidase (Fig. 1A). Although such a relatively simple system may suit a mitochondrion, which is in the comfortable position of operating in a constant environment, it does not serve all purposes for a free-living bacterium.

P. denitrificans is a bacterium that, in its natural environment, has to deal with ever-fluctuating conditions in terms of the availability of carbon and free-energy sources, and of terminal electron acceptors. It manages to survive by displaying a large metabolic flexibility. This flexibility is the consequence of its (genetic) potential to adapt its metabolism to the prevailing growth condition, especially at the level of its respiratory network. Under aerobic conditions, respiration in which oxygen is the terminal electron acceptor has priority: it generates the most ATP per electron. At atmospheric oxygen tensions, *P. denitrificans* synthesizes an oxidase with a relatively low affinity for oxygen. In contrast, under oxygen limitation a high-affinity oxidase appears (see below).

Under anoxic or nearly anoxic conditions and in the presence of nitrate, the oxidative phosphorylation proceeds via anaerobic respiration. This is the only biological process that converts nitrate to atmospheric nitrogen (denitrification), and it is a key process in the earth's nitrogen cycle. Denitrification involves the

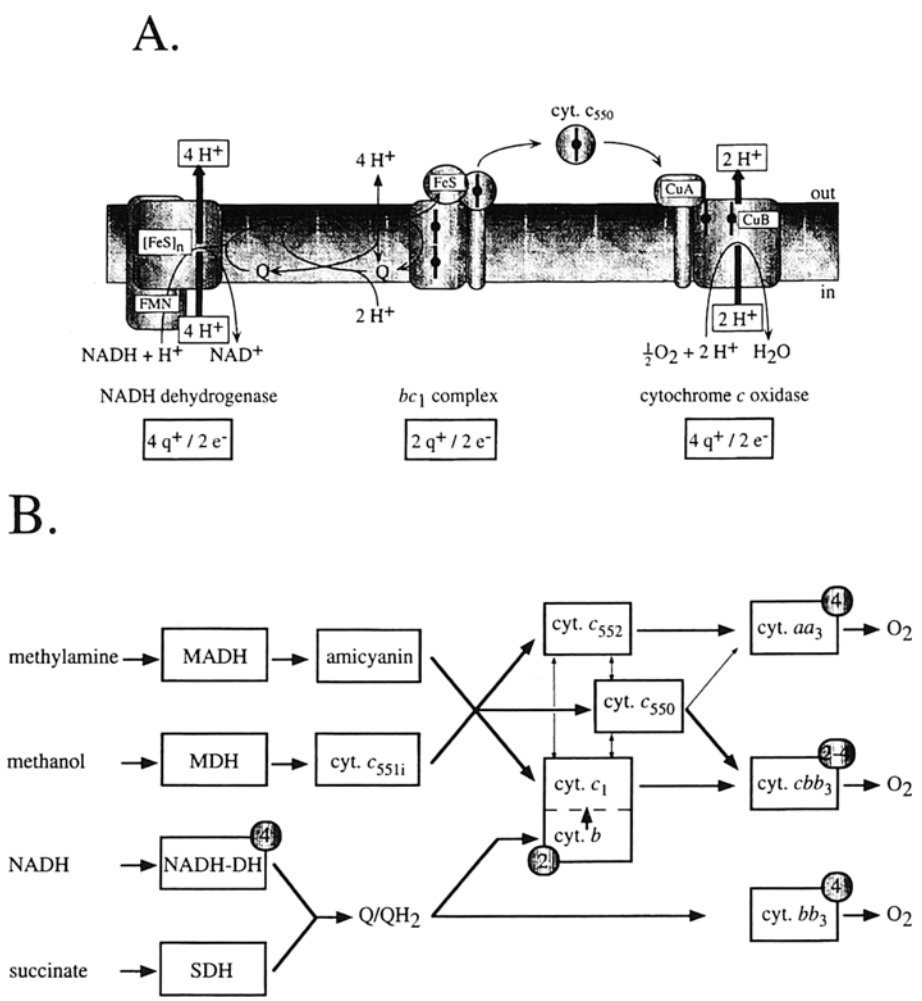

Fig. 1. The aerobic respiration. (A) Cartoon of the respiratory chain as it is found in the mitochondrial inner membrane and in the cytoplasmic membrane of many aerobic bacteria: NADH dehydrogenase, ubiquinone (Q), the bc_1 complex, cytochrome c_{550}, and the aa_3-type cytochrome *c* oxidase. Pumped protons (H^+) are boxed, scalar protons are not. Charge separation ($q^+/2e^-$) sites are indicated. (B) The aerobic respiratory network of *Paraccoccus denitrificans*. MADH, MDH, NADH-DH, and SDH refer to the dehydrogenases of methylamine, methanol, NADH, and succinate, respectively. All cytochromes (cyt) are mentioned in the text. In circles the $q^+/2e^-$ stoichiometry is indicated, as discussed in the text.

concerted action of four reductases that catalyze the reduction of nitrate via nitrite, nitric oxide, and nitrous oxide to dinitrogen (Stouthamer, 1991; Ferguson, 1994). Since nitrite and nitric oxide are toxic, denitrification is potentially lethal to its host. The intriguing question arises of how the organism manages to balance the electron fluxes so as to prevent accumulation of the toxic intermediates, yet allow the flow of electrons that is essential for its free energy metabolism.

Interestingly, there are several indications of a certain link between the aerobic and the anaerobic respiration. Apart from sharing a number of redox enzymes, it has been demonstrated in *E. coli* that the expression of both processes is controlled by a single transcription regulator, FNR (see below). Moreover, sequence alignments suggest an evolutionary relation between nitrous oxide reductase and cytochrome *c* oxidase (Viebrock and Zumft, 1987), as well as between nitric oxide reductase and cytochrome *c* oxidases (Van der Oost *et al.*, 1994, Saraste and Castresana, 1994).

THE AEROBIC RESPIRATORY NETWORK

Unlike the mitochondrial respiratory chain, *Paracoccus denitrificans* contains a number of alternative

respiratory pathways in which electrons are transferred from specific dehydrogenases to different types of terminal oxidases (Fig. 1B). This respiratory network enables *Paracoccus* to respond to a variety of environmental changes.

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), cytochome c_{552}, and cytochrome *c* oxidase (cytochrome aa_3) (Berry and Trumpower, 1985). In addition, however, it has been demonstrated that *Paracoccus* expresses two distinct respiratory branches, in which electrons are directed to three distinct types of heme-copper oxidases: a cytochrome *c* oxidase (cbb_3) and a quinol oxidase (bb_3) (Van der Oost *et al.*, 1994; De Gier *et al.*, 1994, 1995a) (Fig. 1b). The gene clusters that encode the three terminal oxidases have been cloned, and single/multiple mutants have been generated (De Gier *et al.*, 1994, 1995a). Because a bb_3 mutant did not exhibit quinol oxidase activity, and a aa_3/cbb_3 double mutant did not express cytochrome *c* oxidase activity, it is concluded that this is the complete set of oxidases of *P. denitrificans* (De Gier *et al.* 1994, 1995a).

P. denitrificans is also capable of autotrophic growth in the presence of oxygen. During methylotrophic growth, either methylamine or methanol is used as the only source of carbon and free energy (Harms and Van Spanning, 1991). It has been recognized before that a complex catabolic network is involved in methylotrophic growth of *P. denitrificans*. The components that participate in the oxidation of methylamine and methanol include redox proteins that are involved in the respiratory electron transfer to oxygen, as well as dehydrogenases that catalyze the stepwise conversion of C_1 substrate to carbon dioxide (Harms and Van Spanning, 1991; De Gier *et al.*, 1992, 1995b). Except for the genes that encode the formate dehydrogenase complex, all structural genes of the components that participate in the C_1 catabolism of *P. denitrificans* have been cloned (Table I). Mutations have now been introduced in all these genes. From the phenotypes of the current set of mutants, the *in vivo* respiratory network of *P. denitrificans* during methylotrophic growth has been deduced (De Gier *et al.*, 1995b; Van der Oost *et al.*, 1995a) (Fig. 1B).

Methylamine dehydrogenase (MADH) catalyzes the conversion of methylamine into formaldehyde and ammonia. Reduced MADH is oxidized by its specific electron acceptor amicyanin, which passes electrons on to the cytochrome *c* pool (Van Spanning *et al.*, 1990b). Methanol is oxidized to formaldehyde by methanol dehydrogenase (MDH). Again, a specific electron acceptor, cytochrome c_{551i}, shuttles electrons from the dehydrogenase to a number of cytochromes *c* (Van Spanning *et al.*, 1991b). Mutagenesis of the individual genes that encode either one of these dehydrogenases or their electron acceptors, results in the inability to grow on the corresponding substrate (Harms *et al.*, 1987, Van der Palen *et al.*, 1995) (Table I, Fig. 1B).

In a subsequent mutagenesis study, De Gier *et al.* (1995b) focused on the downstream part of the respiratory chains of methylamine and methanol. It was demonstrated that the respiratory network of both methylamine- and methanol-grown cells includes cytochromes c_{550} and c_1 and at least one additional *c*-type cytochrome, as well as cytochrome aa_3 and at least one additional cytochrome *c* oxidase. This was deduced from the observation that a triple mutant (Δc_1, c_{550}, aa_3) was not capable of using the C_1 substrates, whereas the double mutants of the same set of cytochromes all retained the capacity to grow methylotrophically (Table I, De Gier *et al.*, 1995b). In addition, the latter study demonstrated that, apart from cytochromes c_{550} and c_1, at least one additional cytochrome *c* is capable of mediating electron transfer between amicyanin and (exclusively) the aa_3-type oxidase (Fig. 1B).

Recently, the set of *P. denitrificans* mutants has been extended to include inactivations of cytochrome c_{552} and the cbb_3-type cytochrome *c* oxidase (Van der Oost *et al.*, 1995a). It has been demonstrated that the unidentified electron carrier from amicyanin to cytochrome *c* oxidase is cytochrome c_{552}: a triple mutant (Δc_1, c_{550}, c_{552}) was not capable of methylotrophic growth, unlike the corresponding double mutants (Table I). Hence, the cytochromes c_1, c_{550}, and c_{552} make up the complete set of potential electron acceptors of amicyanin (Fig. 1B).

The $\Delta aa_3/c_1$ mutant can still grow on C_1 substrates, suggesting the presence of an alternative cytochrome *c* oxidase (De Gier *et al.*, 1992). A likely candidate has recently been described in *P. denitrificans:* cytochrome cbb_3 (De Gier *et al.* 1994, 1995b). Indeed, no oxidation of the artificial substrate TMPD was measured in a $\Delta aa_3/cbb_3$ mutant, indicating that no alternative cytochrome *c* oxidase is expressed in this mutant (De Gier, *et al.*, 1995a, Van der Oost *et al.*, 1995a).

The aforementioned observations concerning the aerobic respiratory routes are summarized in Fig. 1B. There are only few uncertainties left. Mutagenesis of the NADH dehydrogenase (NDH-1) appears to be lethal (Yano, Van Spanning, Stouthamer, and Yagi, unpublished results), and piericidinA seems to inhibit the endogenous respiration almost completely (Van der Oost *et al.*, 1995a). This suggests that only the proton-translocating type of NADH dehydrogenase (NDH-1) is expressed in *P. denitrificans*. *In vitro* studies have provided evidence for electron transfer between the bc_1 complex and cytochrome c_{552} (Turba, 1994), and between cytochrome c_{550} and the aa_3-type oxidase (Van Wielink *et al.*, 1989; Lappalainen *et al.*, 1995). Together with the clustering of the corresponding *cycA* (c_{550}) and *ctaDII* (aa_3) genes on the *Paracoccus* chromosome, and the finding that deletion of *cycA* affects the expression of cytochrome aa_3 (Van Spanning *et al.*, 1990a), this contributes to the belief that the latter cytochromes are physiological redox partners. Based on the redox midpoint potentials of the three components of the cytochrome *c* pool of *P. denitrificans* (at pH 7.0: E'_o cyt. c_1 = *178 mV;* E'_o cyt. c_{550} = 226 mV; E'_o cyt. c_{552} = 245 mV) (Bosma, 1989), direct electron transfer between these proteins may be expected as well. A fourth *c*-type cytochrome that is induced during methylotrophic growth, cytochrome c_{553i}, is not involved in the oxidation of methylamine or methanol (Ras *et al.*, 1991) (Table I); its physiological role remains to be identified. Based on sequence similarity with MDH, it is anticipated that this cytochrome is the electron acceptor of a second type of methanol dehydrogenase.

EFFICIENCY OF FREE-ENERGY TRANSDUCING

During respiratory electron transfer the redox free energy is converted into an electrochemical gradient across the cytoplasmic membrane, the yield of which is expressed as the number of charges (protons or electrons) translocated across the membrane per two electrons transferred from reductant to oxidant ($q^+/2e^-$). The distinct charge separation sites that will be discussed below are depicted in Fig. 1A, including their proton translocation efficiency ($H^+/2e^-$) Although various values have been reported for the NADH dehydrogenase, we have adopted the assessment by Walker (1992): 4 $H^+/2e^-$ are translocated across the membrane to the periplasm (4 $q^+/2e^-$). Via operation of the Q-cycle, the oxidation of ubiquinol by the bc_1 complex gives rise to the translocation of two electrons from the periplasmic to the cytoplasmic side of the membrane (2 $q^+/2e^-$). The third site of charge separation concerns the terminal oxidase(s). Apart from two scalar protons consumed in the reduction of oxygen, all types of heme-copper oxidase have the capacity to pump two additional protons to the periplasmic space (4 $q^+/2e^-$) (however, see discussion below).

In anaerobically grown cells of *P. denitrificans*, in which the aa_3-type cytochrome *c* oxidase is not detectable, two proton-translocating sites have been reported during the oxidation of NADH (Boogerd *et al.*, 1981; Van Verseveld *et al.*, 1983; Stouthamer, 1991). The two sites have been identified as the NADH dehydrogenase (4 $q^+/2e^-$) and either the quinol oxidase cytochrome bb_3 (4 $q^+/2e^-$, Puustinen *et al.*, 1989) or a cytochrome *c*-branch, involving the bc_1-complex (2 $q^+/2e^-$) and a nonproton pumping cytochrome *c* oxidase (2 $q^+/2e^-$; see discussion below): in total 8 $q^+/2e^-$ (Fig. 1B). Methylotrophic growth on methanol as well as methylamine has been reported to induce cytochrome aa_3 (Van Verseveld *et al.*, 1981; Harms and Van Spanning, 1991). Oxidation of NADH in such cells involves an additional charge-separating site: NADH dehydrogenase (4 $q^+/2e^-$), cytochrome bc_1 (2 $q^+/2e^-$, Trumpower, 1991) and cytochrome aa_3 (4 $q^+/2e^-$; Van Verseveld *et al.*, 1981): in total 10 $q^+/2e^-$ (Fig. 1B). Calculated growth yields and proton translocation efficiencies ($H^+/2e^-$) of *Paracoccus* cultivated in chemostats suggest that cytochrome aa_3 is a proton-translocating cytochrome *c* oxidase, whereas the alternative cytochrome *c* oxidase is not (reviewed by Stouthamer, 1991).

Analysis of proton translocation in cell suspensions of the generated *Paracoccus* oxidase mutants offers the unique opportunity to measure proton-translocation efficiency of individual oxidases in whole cell suspensions. During succinate oxidation, a minimum $q^+/2e^-$ stoichiometry of 4 is expected theoretically when ubiquinol is oxidized directly by cytochrome bb_3 (Fig. 1B). On the other hand, when ubiquinol is oxidized via cytochrome bc_1 and cytochrome aa_3 the maximal $q^+/2e^-$ stoichiometry is 6 (Fig. 1B). Measurements of the set of *P. denitrificans* mutants are in perfect agreement with the theoretical values (De Gier *et al.*, 1995a). Analysis of a Δaa_3, bb_3 mutant with succinate as electron donor and oxygen as acceptor indicated 4 $H^+/2e^-$ (De Gier *et al.*, 1994). This suggests that cytochrome cbb_3 does not translocate protons, and

hence, has a lower charge/electron yield: 2 $q^+/2e^-$. It is concluded 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 (always) couple the reduction of oxygen to the vectorial translocation of protons across the membrane (De Gier *et al.*, 1994).

The latter conclusion is in conflict with the measurements presented by Raitio and Wikström (1994). The latter authors have analysed a Δaa_3 mutant, and reported a $H^+/2e^-$ stoichiometry of 6 with succinate as substrate and oxygen as electron acceptor, indicating that the alternative cytochrome *c* oxidase (cbb_3) does have the capacity to pump protons (4 $q^+/2e^-$). The main technical difference with the aforementioned experiments is the buffer composition: HEPES (0.5 mM) rather than glycylglycine (1.5 mM) has been used in the latter case. A consensus between the two groups has been reached in a study in which both buffer systems are compared. The conclusion is that in the HEPES system, all oxidases from *P. denitrificans* have the capacity to pump protons (De Gier *et al.*, 1995a). 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).

Although it is now generally accepted that all terminal oxidases of *Paracoccus* (aa_3, cbb_3, and bb_3) have the capacity to pump protons ($2H^+/2e^-$), this finding does not agree with analyses of chemostat cultures of (wild type) *P. denitrificans*, grown under a variety of conditions. To address this matter, the set of oxidase mutants has been cultivated in the pH-auxostat. The principle of the pH-auxostat cultivation, with Na-succinate as carbon/energy source, is that the growth-dependent alkalization of the culture medium induces the inlet of (succinate-containing) acid medium, thereby decreasing the culture pH to its original level (Pronk, 1995). Calculation of the growth yield (YO_2) confirms previously drawn conclusions by Stouthamer (1991) with respect to the $q^+/2e^-$ yield of cytochrome cbb_3. A single route strain of the quinol-oxidizing cytochrome bb_3 (Δaa_3, cbb_3) has been used as a theoretical minimum in the yield of oxidative phosphorylation: oxidation of NADH should give rise to 8 $q^+/2e^-$ (Fig. 1B). The theoretical maximum of a single route strain of the cytochrome aa_3, which is not yet available, should be 10 $q^+/2e^-$. Whereas the wild type is significantly higher, the cbb_3 single route mutant is only slightly more efficient than the bb_3 single route strain. It is concluded that under these cultivation conditions, maximum growth (respiratory electron flux) due to "unlimited" supply of substrates (succinate and oxygen), the free-energy transduction efficiency of the cytochrome cbb_3 pathway is significantly decreased, at least when compared to the cytochrome aa_3 pathway (De Gier *et al.*, 1995c). This is consistent with the lower $q^+/2e^-$ yield we observed in proton translocation in glycylglycine (see above).

There is no reason to believe that *P. denitrificans* expresses more than one type of NADH dehydrogenase (NDH-1), and it expresses only one type of bc_1 complex (Kurowski and Ludwig, 1987). Although it cannot be ruled out that variable stoichiometry of proton translocation may occur at the level of the NADH dehydrogenase (Walker, 1992), or the bc_1 complex (Murphy and Brand, 1987), we conclude that cytochrome cbb_3 is the most likely site of the observed drop in free-energy transduction efficiency, implying a submaximal coupling between oxygen reduction and proton translocation under these conditions (De Gier *et al.*, 1995c). This conclusion is supported by the aforementioned observation that the $H^+/2e^-$ stoichiometry at the level of cytochrome cbb_3, in the Δaa_3, bb_3 mutant, appears buffer-dependent when succinate is the electron-donating substrate (De Gier *et al.*, 1994). Moreover, rather low (2–3) $H^+/2e^-$ values are measured with TMPD as substrate (De Gier *et al.*, 1994; Raitio and Wiklström, 1994). This apparent variability in the $q^+/2e^-$ yield of cytochrome cbb_3 is not well understood at present. It has been observed, however, that certain regions in subunit I that are believed to be important for the coupling between oxygen reduction and proton pumping in cytochrome aa_3 and cytochrome bo_3, e.g., the loop between helix II and III (Garcia-Horsman *et al.*, 1995) as well as helix VIII (Svensson *et al.*, 1995; Hosler *et al.*, 1992; Fetter *et al.*, 1995; Thomas *et al.*, 1994), are not very well conserved in cytochrome cbb_3 (Van der Oost *et al.*, 1994).

REGULATED EXPRESSION OF AEROBIC RESPIRATORY BRANCHES

After mapping the respiratory pathways, the next question was whether *P. denitrificans* can adjust the hardware of this network to changes in its environment. Before the application of molecular genetics, the qualitative and quantitative analysis of the composition of the respiratory network of *P. denitrificans* was mainly performed by means of optical spectroscopy, and specific analysis of cytochromes *c* after separation by

Table II. Relative Quantification of Membrane-Bound Respiratory Complexes of *P. denitrificans*, Cultivated Either in Chemostat with Indicated Limitation (Bosma, 1989), or in Batch (Van Spanning *et al.*, 1990a; Van Spanning, 1991c)[a]

			No-red	c_{552}	cbb_3	cbb_3	bc_1	aa_3
Strain	Substrate	Limitation	NorC 14 kDa	CycM 22 kDa	CcoO 30 kDa	CcoP 45 kDa	FbcC 55 kDa	CtaDII 605 nm
Wild type	Succinate/O_2	Succinate	−	++	±	−	++	+
Wild type	Succinate/O_2	O_2	±	+	++	++	++	+
Wild type	Succinate/O_2/NO_2-	O_2/NO_2-	++	+	++	++	++	−
Wild type	Succinate/NO_3-	NO_3-	++	+	++	++	++	−
Wild type	Methanol	Methanol	+	++	+	+	++	++
Wild type	Methylamine	Methylamine	+	++	+	+	++	++
Wild type	Succinate/O_2		−	+	+	+	++	+
Δbc_1	Succinate/O_2		−	±	++	++	−	+
Δc_{550}	Succinate/O_2		−	+	+	+	++	±

[a] Quantities of cytochromes *c* are based on estimations of membrane fractions after SDS-PAGE and staining for covalently bound heme (apparent molecular mask (kDa) is indicated); the expression of cytochrome aa_3 has been deduced from the absorbance peak at 605 nm in the optical spectrum. (− Absent, ± just detectable, + present, ++ abundantly present.)

SDS-PAGE (Bosma, 1989) (Table II). The expression level of cytochrome aa_3, and to some extent of cytochrome c_{552}, was significantly lower during cultivation of *Paracoccus* under microaerobic or anaerobic conditions than during aerobic growth (Bosma, 1989) (Table II). When the dissolved oxygen tension (DOT) exceeds 1%, the expression of cytochrome aa_3 appears at a constant level (De Gier *et al.*, 1995c). During autotrophic growth on methanol, cytochrome aa_3 is expressed abundantly (Van Verseveld *et al.*, 1983) (Table II). Cytochrome cbb_3, on the other hand, is expressed predominantly (but not exclusively) at lower O_2 concentrations (Bosma, 1989; De Gier *et al.*, 1995a). In an alternative pathway that appears to be expressed at a wide range of oxygen concentrations, ubiquinol is oxidized directly by the bb_3-type quinol oxidase (Fig. 1B).

With the recently generated set of *P. denitrificans* oxidase mutants, differential expression of the individual respiratory pathways can be analyzed more accurately. Initially, several mutants have been cultivated in the pH-auxostat, at a range of dissolved oxygen tensions (DOT). In the setup we have used, all medium components are present in excess and the culture grows at its maximum specific rate (μ_{max}). The μ_{max} of the cytochrome bb_3 single route strain (Δaa_3, cbb_3) decreased significantly during a shift from 10% to 1% DOT. In the Δcbb_3 mutant a minor decrease in μ_{max} has been observed at 1% DOT, indicating that cytochrome aa_3 functions relatively well under these conditions. Only when cytochrome cbb_3 is present at 1% DOT are wild type growth rates observed. The available data suggest that at low aeration the operation of cytochrome cbb_3, and to some extent cytochrome aa_3, is essential for sustaining the μ_{max} at its wild type level (De Gier *et al.*, 1995c). These findings are in agreement with the relatively high affinity for oxygen that is expected for cytochrome cbb_3 (Preisig and Hennecke, personal communication).

CONTROL MECHANISMS

An important signal in the regulation of the aerobic respiratory components is, obviously, the availability of oxygen. Oxygen concentration can be sensed either directly, as has been described for the *fixLJ*/*fixK* cascade (Fischer, 1994; Batut and Boitard, 1994; Preisig *et al.*, 1993), or indirectly, as has been suggested for both the Fnr- and the ArcAB-mediated regulation (Spiro and Guest, 1990; Unden *et al.*, 1994).

Upstream the cbb_3 operon, an *fnr*-like gene is located (Van der Oost *et al.*, 1995b) (*fnrA*, Fig. 2A). The deduced amino acid sequence of the *Paracoccus* homologue closely resembles *E. coli* FNR with respect to its C-terminal helix-turn-helix motif that is responsible for the specific interaction with target promoters, and its N-terminal cysteine cluster that binds an iron ion, the redox state of which determines the regulator to be active (Fe^{2+}) or inactive (Fe^{3+}). Because of the presence of an FNR-box in the promoter region of the cbb_3 operon (*ccoNOQP*), it is anticipated that its

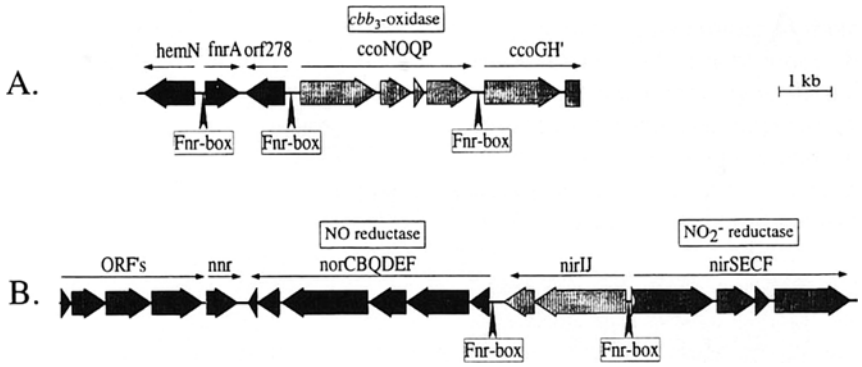

Fig. 2. Genomic loci that encode respiratory complexes in *Paracoccus denitrificans*. (A) The gene clusters that encode the cbb_3-type cytochrome *c* oxidase (*ccoNOQP*) and its flanking regions. The *fnrA* gene codes for a transcription regulator (De Gier *et al.*, 1995a; Van der Oost *et al.* 1995b). (B) The gene clusters that encode the cd_1-type nitrite reductase (*nirS*) (De Boer *et al.*, 1994), the nitric oxide reductase (norCB) (De Boer *et al.*, 1995). Adjacent the *nor* cluster a second transcription regulator has been identified: *nnr* (Van Spanning *et al.*, 1995).

expression is positively controlled by this regulator. In contrast, the location of an FNR-box very close to the start-codon of the cytochrome bb_3 operon (20 bp upstream; Richter *et al.*, 1994) suggests that the expression of cytochrome bb_3 is negatively controlled (Van der Oost *et al.*, 1995b).

Rather than oxygen, the redox state of respiratory intermediates may be the signal activating FNR in *Paracoccus* (De Gier *et al.*, 1995c). As in *E. coli*, FNR links the regulation of aerobic and anaerobic respiration in *P. denitrificans:* preliminary results indicate that apart from cytochrome cbb_3, also nitrate reductase requires FNR for transcription activation (Van der Oost *et al.*, 1995b) (see below).

In conclusion, the expression of both cytochrome bb_3 and cbb_3 appears to respond to the prevailing redox state, which is often low at high aeration and high during oxygen limitation. As suggested for *E. coli*, it may be that the FNR signal is a respiratory redox couple (NAD^+/NADH, Q/QH_2) that, in its reduced state, brings about the reduction of the FNR iron ion ($Fe^{3+} \rightarrow Fe^{2+}$), leading to its activation (Unden *et al.*, 1994). No anaeroboxes are located upstream the cytochrome aa_3 loci. At present, no transcription regulator has been identified that is responsible for the expression of this oxidase. The induction of cytochrome aa_3 under methylotrophic conditions (Table II), when the reduction level of the cytochrome *c* pool is expected to be relatively high, suggests that expression of this oxidase responds to the redox state of the cytochrome *c* pool. This might also explain the repression of cytochrome aa_3 in the Δc_{550} mutant (Van Spanning *et al.*, 1990a) (Table II).

THE ANAEROBIC RESPIRATORY NETWORK

When the molecular oxygen concentration drops below a critical level, induction of an anaerobic respiratory network enables *P. denitrificans* to survive (Fig. 3a). The first reduction step in the process of denitrification is catalyzed by nitrate reductase (Nar), which converts nitrate to nitrite: $NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$. This membrane-bound enzyme resembles the dissimilatory nitrate reductase of *E. coli* to a great extent (Ballard and Ferguson, 1988; Stewart, 1988). The enzyme consists of three different subunits (α, β, and γ) and it is suggested that during nitrate reduction electrons from ubiquinol are passed to the *b*-type hemes in the γ-subunit and subsequently via iron-sulfur centers in the β-subunit to the molybdenum cofactor, which is located in the α-subunit (Fig. 3A). The reduction of nitrate is achieved at the latter catalytic center. Both the α- and the β-subunits are soluble and facing the cytoplasm, while the γ-subunit is a transmembrane protein. In *E. coli* the α-, β-, and γ-subunits are encoded by *narGHI* genes, respectively (Sodergren and DeMoss, 1988; Blasco *et al.*, 1989, 1990). Recently, part of the *Thiosphaera pantotropha nar* operon has been cloned (Berks *et al.*, 1995). The cytochrome *b* of the nitrate reductase complex has

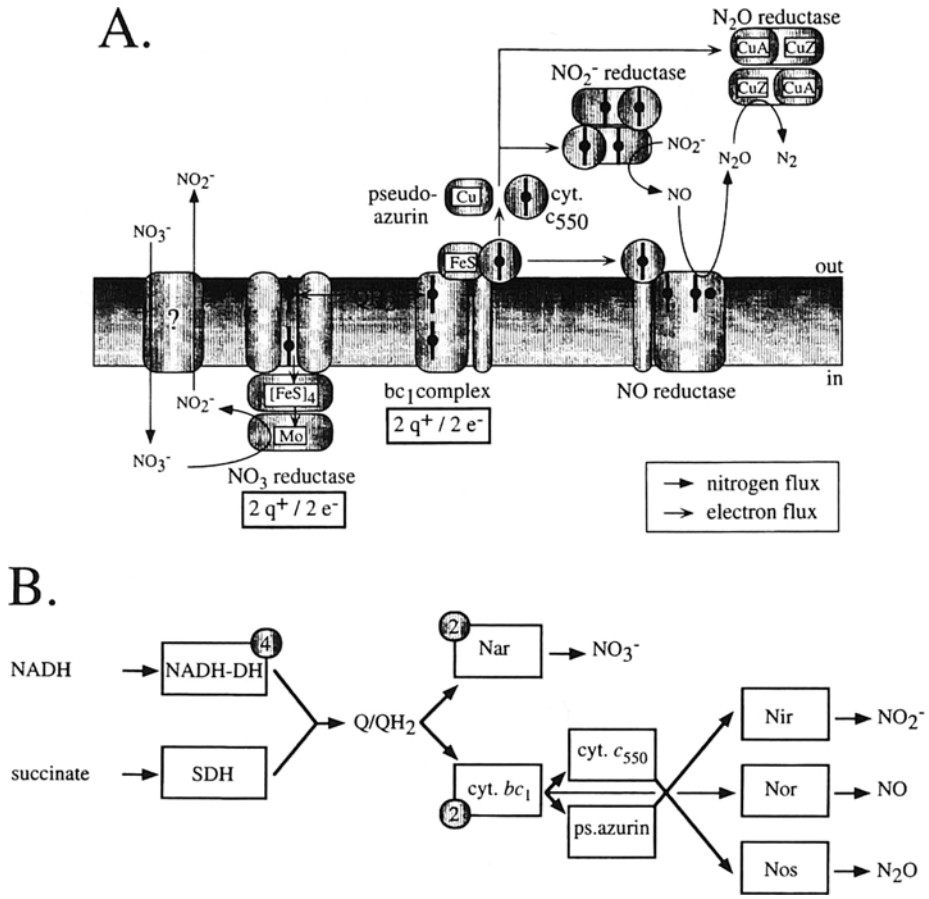

Fig. 3. The anaerobic respiration. (A) Cartoon of the polypeptide complexes involved in denitrification, located in the cytoplasmic membrane and the periplasm of many (facultative) anaerobic bacteria. Charge separation ($q^+/2e^-$) occurs at the level of nitrate reductase, and the bc_1 complex. (B) The anaerobic respiratory network of *Paraccoccus denitrificans*. Nar, Nir, Nor, and Nos refer to reductases of nitrate, nitrite, nitric oxide, and nitrous oxide, respectively. Apart from cytochrome c_{550}, pseudoazurin may be an alternative electron carrier from the bc_1 complex to both Nir and Nos. In analogy with the cbb_3-type oxidase (Fig. 1b), no additional electron carier may be required for reduction of Nor. The $q^+/2e^-$ stoichiometry is indicated in circles, as discussed in the text.

been proposed to consist of a homo-dimer that sandwiches the two hemes (Van der Oost *et al.*, 1995c) (Fig. 3A).

Two possible systems for transport of nitrate to the cytoplasm have been suggested, (i) a proton-nitrate symport system and (ii) a nitrate/nitrite antiporter (Boogerd *et al.*, 1984). The latter system does not require free energy, and in addition, toxic nitrite would be removed from the cytoplasm. It has been suggested that *narK* in *E. coli* encodes the antiporter since a mutation in this gene prevents nitrate transport (Stouthamer, 1991). However, recent work demonstrated that NarK is a nitrite-extrusion system and not involved in nitrate transport (Rowe *et al.*, 1994).

Apart from the membrane-bound nitrate reductase, also a periplasmic respiratory enzyme has been characterized in different denitrifying organisms including *P. denitrificans* and the closely related species *Thiosphaera pantotropha* (Bell *et al.*, 1990; Berks *et al.*, 1994; Sears *et al.*, 1993). The latter enzyme is soluble and appears to consist of two subunits, one with a *c*-type heme and one with a molybdenum center (Breton *et al.*, 1994). The enzyme has been suggested to be essential for aerobic denitrification. The genes encoding soluble nitrate reductase from *T. pantotropha* have been cloned by the group of Richardson (personal communication).

The second step in denitrification is carried out by nitrite reductase (Nir) which converts nitrite into gaseous nitric oxide: $NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$. Two types of nitrite reductase have been identified in denitrifying bacteria: (i) a homo-dimer with *c*

and d_1 type hemes (Fig. 3A) (Stouthamer, 1991), and (ii) a homo-trimer with two distinct copper centers (not shown) (Adman, 1991). *P. denitrificans* possesses the cd_1 type nitrate reductase (de Boer *et al.*, 1994) (Fig. 3A). Electrons for the reductase are supplied via the bc_1 complex and cytochrome c_{550}. A cytochrome c_{550} mutant is not capable of reducing nitrite when grown in copper-deficient medium (Van Spanning, unpublished). In the presence of copper, a parallel route from the bc_1 complex to nitrite/reductase involves the small blue copper protein pseudoazurin (Moir and Ferguson, 1994) (Fig. 3A,B). It is generally believed that electrons from cytochrome c_{550} or pseudoazurin are passed via heme *c* to the d_1 type heme where the reduction of nitrite to nitric oxide takes place. The *nirS* gene encoding cd_1 type nitrate reductase has been cloned from *Pseudomonas stutzeri* (Smith and Tiedje, 1992), *Ps. aeruginosa* (Silvestrini *et al.*, 1989), and recently from *P. denitrificans* (de Boer *et al.*, 1994). The *P. denitrificans nirS* gene is located in a gene cluster (Fig. 2B), the products of which have been suggested to be involved in the processing of the unique d_1 heme. The nature and organization of the *nir* clusters of the *Pseudomonads* is rather different from that of *P. denitrificans* (de Boer *et al.*, 1994).

The third step in denitrification is the reduction of nitric oxide to nitrous oxide ($2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$), catalyzed by the membrane-bound enzyme nitric oxide reductase (Nor) (Heiss *et al.*, 1989; Carr and Ferguson, 1990; Zumft *et al.*, 1994). Nor receives electrons via the bc_1 complex but it is not known which electron carriers, if any, mediate this transport. The enzyme consists of two subunits, the smaller with *c*-type heme, the larger one with probably two *b*-type hemes and a nonheme iron (Fig. 3A). The proposed redox center composition is based on the finding that the Nor complex is structurally related to the cbb_3-type cytochrome *c* oxidase that is found in *Rhizobiaceae, Rhodobacter,* and *P. denitrificans* (van der Oost *et al.*, 1994; Saraste and Castresana, 1994). It has been suggested that the cbb_3-type oxidase and nitric oxide reductase have a common ancestor and that they preceded "the more sophisticated" members of the heme copper oxidases: the aa_3-type cytochrome *c* oxidase and the bo_3- or bb_3-type quinol oxidase. Despite the similarity between nitric oxide reductase and the heme-copper oxidases, little is known about the catalytic core or the mode of action of the former enzyme. Recently, the gene cluster encoding nitric oxide reductase of *P. denitrificans* has been cloned and sequenced (De Boer *et al.*, 1995) (Fig. 2B). The *norB* and *norC* genes encoding the two subunits of the enzyme are highly similar to their counterparts in *Ps. stutzeri.* The *norE* gene encodes a hydrophobic protein, with sequence homology to subunit III of the aa_3-type cytochrome *c* oxidase, supporting the evolutionary link between the Nor complex and the heme-copper oxidases. NorBC mutants of *P. denitrificans* do not survive denitrification, apparently due to the accumulation of the highly toxic nitric oxide.

The final step in denitrification, the reduction of nitrous oxide to dinitrogen, is carried out by the periplasmic N_2O reductase (Nos): $N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$. This enzyme is a homo-dimer and contains two copper centers, center A and center Z (Zumft *et al.*, 1992). Center A has been demonstrated to be dinuclear (Kroneck *et al.*, 1988). Based on sequence alignment and spectroscopic comparison, the latter copper center has been suggested to be related to the electron entry site of the aa_3-type cytochrome *c* oxidase: Cu_A (Scott *et al.*, 1989; Van der Oost *et al.*, 1992). As in cytochrome aa_3, it has been suggested that the Cu_A-like center in nitrous oxide reductase guides the electrons to center Z, where nitrous oxide is reduced to dinitrogen. The electron transfer pathway to the N_2O reductase probably involves the bc_1 complex, and either cytochrome c_{550} or pseudoazurin (Fig. 3A,B). The gene (*nosZ*) encoding nitrous oxide reductase from *P. denitrificans* has been cloned and sequenced (Hoeren *et al.*, 1993).

We have constructed mutant strains with single or combined mutations in the genes encoding cytochromes c_1, c_{550}, and c_{552} (Van Spanning, unpublished). These mutant strains were analyzed for effects on (i) the expression of the denitrification enzymes and (ii) the electron flow to either of the periplasmic reductases. For this purpose, the strains were cultured in batch and the production of nitrite and gaseous N-oxides was determined. In addition, *in vivo* kinetics of electron transport to the reductases were monitored. The c_1 mutant was only able to donate electrons to nitrate reductase (Fig. 3B). The routes to the three remaining reductases were completely blocked, indicating that the bc_1 complex is an essential gate to these denitrification reactions (Fig. 3B). When the c_{550} or the c_{552} single or double mutants were grown on mineral medium with nitrate, little or no effects on the growth properties were observed as compared to the wild type. However, when the c_{550} mutant strain was grown in copper-deficient medium, it accumulated nitrite (Van Spanning, unpublished). Apparently, nitrite reductase reduces the bc_1 complex either through cytochrome c_{550} or through a copper-requiring carrier, probably pseudoazurin (Moir and Ferguson, 1994) (Fig. 3B).

CONTROL OF DENITRIFICATION: ENZYME ACTIVITY AND GENE EXPRESSION

Present evidence suggests that all the periplasmic reductases are insensitive to molecular oxygen. In addition, they are fully active aerobically, provided that there is an adequate supply of electrons (Ferguson, 1994). The same appears to be true for the membrane-bound nitrate reductase. However, denitrifying cultures from *P. denitrificans* immediately stop reducing nitrate when oxygen is added to the medium (John, 1977). Little is known about the background of this regulation. It has been suggested that oxygen has a (direct/indirect) control on the putative nitrate/nitrite antiporter (Ferguson, 1994). Nevertheless, complete denitrification under aerobic conditions might be achieved by the set of periplasmic reductases. Although the relative yield of free energy ($q^+/2e^-$) by denitrification is less than obtained by oxidative phosphorylation (Fig. 1B,3B), the stand-by operation of the denitrification route might be of particular importance in environments where oxygen tensions tend to be low. Aerobic denitrification has clearly been demonstrated for *T. pantotropha* (Robertson and Kuenen, 1984), but controversy exists on the corresponding capacity of *P. denitrificans*. Of course, a prerequisite for aerobic denitrification is that expression of the reductases is not repressed by oxygen.

Generally, respiratory pathways with higher energy-transducing efficiency tend to repress pathways of lower efficiency. A good example of such a hierarchial control is the expression of aerobic and anaerobic enzymes by a so-called aerobic/anaerobic switch. From the extensive studies on regulatory phenomena in *E. coli*, it is known that expression of many aerobic and anaerobic metabolic routes is under the control of a complex regulatory system that achieves the fine-tuned regulation of expression, aiming at the optimal amount of enzyme during a certain growth condition (Iuchi and Lin, 1991; Stewart, 1994; Unden *et al.*, 1994). Since *E. coli* is not able to denitrify (it can only reduce nitrate), the control of denitrification is still relatively poorly understood. This is a very challenging question to be addressed.

Expression of the genes involved in denitrification appears to differ significantly among the denitrifying bacteria. In *Ps. stutzeri* expression of nitrate, nitrite, and nitric oxide reductase requires both anoxia and the presence of a nitrogenous oxide (Körner and Zumft, 1989). The control of expression appears to reside in more than one interconnective regulatory system, one being addressed through a homologue of the *E. coli* transcription factor FNR, which activates gene expression in response to anoxia (Spiro and Guest, 1990). The control of the anaerobic respiration in *P. denitrificans* appears to be different. The formation of the reductases for nitrite and nitrous oxide occurring in response to oxygen limitation, did not require nitrate to be present (Kucera *et al.*, 1984; Boublikova *et al.*, 1985). Yet, the gene clusters encoding nitrite and nitric oxide reductases are both preceded by potential FNR binding sites (De Boer *et al.*, 1994) (Fig. 2B). Adjacent to these two loci, a gene encoding an FNR homologue was identified (Van Spanning *et al.*, 1995). Disruption of the gene, designated *nnr* (*n*itrite and *n*itric oxide reductases *r*egulator), resulted in the inability of the mutant strain to synthesize nitrite reductase. The expression of nitric oxide reductase was dramatically decreased, and only just detectable (Van Spanning *et al.*, 1995). Like FixK from *Rhizobium meliloti* (Kahn *et al.*, 1993), NNR from *P. denitrificans* lacks the N-terminal cysteine cluster, that has been demonstrated to be the Fe-binding site in FNR of *E. coli* (see above). The control of NNR on denitrification appears to be exclusively at the level of expression of nitrite and nitric oxide reductases, where it may act as a fine-tuning regulator. In addition, adjacent to the *nir* gene cluster a gene is located, *nirI*, that activates the expression of nitrite reductase (De Boer *et al.*, 1995) (Fig. 2B). In the *nor* gene cluster, a gene is present that shares some homology with *nirQ* (De Boer *et al.*, 1995), which has been proposed to be a regulator protein in *Ps. stutzeri* (Jüngst and Zumft, 1992). In a *norQ* mutant, the synthesis of the nitric oxide reductase proceeds, but the complex appears to be inactive (De Boer *et al.*, 1995).

Apart from the anaeroboxes in *nir*- and *nor*-promoters, potential FNR boxes have been identified in the promoter regions of nitrous oxide reductase (Hoeren *et al.*, 1993), the cbb_3-type oxidase (De Gier *et al.*, 1995a), and the bb_3 type oxidase (Richter *et al.*, 1994). However, expression of the latter oxidoreductases, as well as the membrane-bound nitrate reductase, was not affected by the NNR mutation (Van Spanning *et al.*, 1995). Hence, it was anticipated that additional regulators were involved in the regulation of aerobic and anaerobic respiration in *P. denitrificans*.

Indeed, the gene of a second type of FNR-like transcription regulator (*fnrA*) has been identified recently, in the region upstream the cbb_3 operon (Van der Oost *et al.*, 1995b) (Fig. 2A; see above). Like *E.*

coli FNR, the *fnrA* product does have the cysteine cluster, suggesting that it responds to changes in the redox state of, probably, some respiratory intermediate. Mutagenesis of the *fnrA* gene affects both the expression of cbb_3 and the capacity to reduce nitrate (Van der Oost *et al.*, 1995b). The situation that more FNR homologues, with discrete regulatory functions, operate in parallel has also been suggested for *Shewanella putrefaciens* (Saffarini and Nealson, 1993), *Ps. stutzeri* (Cuypers and Zumft, 1993), and *B. japonicum* (Fischer, 1994). In *E. coli* expression of the *nar* operon requires both FNR and the NarL protein. Nitrate (or nitrite) from the environment is sensed by NarX which transmits the signal by phosphorylation of NarL, which is the transcriptional activator of a two-component signal regulatory system (Stewart, 1994).

CONCLUDING REMARKS

The recently isolated regulatory proteins from *Paracoccus denitrificans* suggest that complex and probably interconnective regulatory networks control the expression and the activity of the enzymes that participate in the aerobic and the anaerobic respiration. The bacterial cell seems capable of a flexible response to changes in its growth conditions. To achieve this flexibility, the cell does not just reroute electrons through a preexisting redox network, but it actively adjusts the infrastructure of this network.

An intriguing question to be addressed is how the signal-transducing routes are organized, either as individual pathways (cascades) or as a network. The ultimate goal of the regulation machinery is to achieve a balanced electron flow to the different terminal oxidases and/or reductases, during which the accumulation of highly toxic (oxygen and/or nitrogen) intermediates must be kept at a minimum, whereas the efficiency of the oxidative phosphorylation should be maximal. Future research aims at gaining further insight into the regulation of bacterial respiration, by integration of molecular genetics, biochemistry, physiology, and biomathematics.

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