Reversed electron transfer through the bc_1 complex enables a cytochrome c oxidase mutant ($\Delta aa_3/cbb_3$) of *Paracoccus denitrificans* to grow on methylamine

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Abstract **In *Paracoccus denitrificans* four classes of redox proteins are involved in the electron transfer from methylamine to oxygen: methylamine dehydrogenase (MADH), amicyanin, cytochrome *c* and cytochrome *c* oxidase. MADH and its electron acceptor amicyanin are indispensable for growth on methylamine. At least three different cytochromes *c* and two types of cytochrome *c* oxidase, cytochromes aa_3 and cbb_3, have previously been proposed to participate in the electron transfer pathways from methylamine to oxygen. In this study, participation of both cytochrome *c* oxidases and of the quinol oxidase (cytochrome bb_3) has indeed been confirmed by analysis of a series of oxidase mutants. Interestingly, a *P. denitrificans* cytochrome *c* oxidase mutant ($\Delta aa_3/cbb_3$) retains the capacity to oxidise methylamine. It is demonstrated that the oxidation of the cytochrome *c* pool in this mutant does not proceed via an alternative cytochrome *c* oxidase, but rather via an 'uphill' electron transfer through the bc_1 complex to ubiquinone, driven by the membrane potential. The subsequent oxidation of ubiquinol proceeds via the only remaining terminal oxidase, the bb_3-type quinol oxidase.**

Key words: Respiration; Methylotrophic growth; Methylamine; Methanol; Cytochrome *c* oxidase; Cytochrome *c*; bc_1 complex; *Paracoccus*

1. Introduction

Paracoccus denitrificans is capable of methylotrophic growth, in which either methylamine or methanol is used as the only source of carbon and energy [1]. Methylamine dehydrogenase (MADH) catalyses the conversion of methylamine into formaldehyde and ammonia. Reduced MADH is oxidised by its specific electron acceptor amicyanin, which passes electrons to the cytochrome *c* pool [2]. Methanol is oxidised 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* [3]. 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 [2–5].

In a recent mutagenesis study, De Gier et al. [6] focussed on the downstream part of the respiratory chain of methylamine. It turned out that transfer of electrons from amicyanin to oxygen potentially proceeds via a network that includes several cytochromes *c* (at least cytochromes c_1 and c_{550}), and more than one cytochrome *c* oxidase (at least cytochrome aa_3). Apart from cytochrome aa_3, two distinct terminal oxidases are expressed in *P. denitrificans*: a bb_3-type quinol oxidase, and a cbb_3-type cytochrome *c* oxidases [7]. In the present study, a number of *P. denitrificans* oxidase mutants have been analysed for their capacity to grow on methylamine and methanol, in order to establish the role of the individual oxidases in methylotrophic metabolism.

2. Materials and methods

2.1. Organism and growth conditions

P. denitrificans wild type and mutants are listed in Table 1. Except for the cytochrome cbb_3 single/multiple mutants (see below), all strains have been described before [7]. For growth analysis, strains were streaked on minimal plates supplemented with either succinate (25 mM), methylamine (100 mM) or methanol (100 mM) as only carbon/energy source. When required, antibiotics were added to final concentrations of 60 mg/l of rifampicin, 50 mg/l of kanamycin, and inhibitors to final concentrations of 7 μM antimycin A or myxothiazol. Batch cultures were grown in conical flasks, vigourously shaken on a rotory shaker at 30°C, in minimal medium supplemented with 100 mM methylamine, as described previously [2].

2.2. DNA manipulations

General cloning techniques were carried out essentially as described by Ausubel et al. [8]. *P. denitrificans* cytochrome cbb_3 mutants were constructed by gene replacement with pGRPd1, as described before [9]. A pGRPd1-derived construct contains a cbb_3 operon that has been partly substituted by a KmR cassette (*ccoNO*::KmR), details of the cloning, sequencing and mutagenesis of the cbb_3 operon (*ccoNOQP*) will be published elsewhere [10]. This construct has been conjugated both to wild type Pd1222 and to cytochrome aa_3 mutant Pd9220 (ΔctaDI/ctaDII) [7], and the generated cytochrome cbb_3 single/multiple mutants were verified by means of Southern blotting (not shown).

2.3. Analysis of mutants

Cells were harvested in the late exponential phase of the growth curve. Polarographic analysis of the oxygen consumption in cell suspensions was performed as described before [6,7], using methylamine (100 mM) or ascorbate (0.4 mM) with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD; 0.1 mM) as exogenous substrate. The bc_1 complex was inhibited by addition of antimycin A (7 μM, dissolved in dimethylformamide (DMF)), or myxothiazol (7 μM, dissolved in DMF). Piericidin

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Abbreviations: FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; DMF, dimethylformamide.

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A (5 $\mu g \cdot ml^{-1}$, dissolved in DMF) was used to block the oxidation of endogenous NADH, and carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone (FCCP; 5 μM, dissolved in DMF) as uncoupler. Protein concentrations were determined with the BCA reagent (Pierce).

3. Results and discussion

It has been recognized before that a complex catabolic network is potentially involved in methylotrophic grow of *P. denitrificans* [1,6,11]. The polypeptide components that participate in the oxidation of methylamine and methanol include: (i) the redox proteins that are involved in the respiratory electron transfer to oxygen; and (ii) the dehydrogenases that catalyse the step-wise conversion of C_1 substrate, via formaldehyde and formate, to carbon dioxide. Both the oxidation of formaldehyde and formate yields one molecule of NADH [1,12].

In this study the role of different terminal oxidases in methylotrophic metabolism has been investigated. In *P. denitrificans*, three distinct oxidases are expressed: a bb_3-type quinol oxidase, and two cytochrome *c* oxidases, cytochromes aa_3 and cbb_3 [7]. The three oxidases have been cloned, and insertion/deletion mutants have been generated [7,10]. All strains tested (wild type, Δaa_3, Δcbb_3, $\Delta aa_3/cbb_3$, and Δbb_3), had the capacity to grow on minimal plates supplemented with succinate (Table 1). When the bc_1 complex is specifically blocked by antimycin A or myxothiazol, however, the cytochrome bb_3 mutant does not grow on succinate (Table 1). This confims the earlier report by De Gier et al. [7], that cytochrome bb_3 is the only quinol oxidase that is expressed in succinate-grown cells of *P. denitrificans*.

A $\Delta aa_3/bc_1$ mutant of *P. denitrificans* has previously been reported to retain the capacity to grow on C_1 substrates. This observation has led to the conclusion that an alternative cytochrome *c* oxidase is expressed in *Paracoccus* [11]. Cytochrome cbb_3 has been suggested to be the second type of cytochrome *c* oxidase [7], and the genomic locus that encodes this oxidase has indeed been isolated [10]. A *P. denitrificans* mutant has been generated in which both types of cytochrome *c* oxidase have been mutated ($\Delta aa_3/cbb_3$). Interestingly, this mutant is able to grow on methylamine as well as on methanol, just like the wild type and all strains in which a single type of oxidase was mutated (Table 1). There are three possible explanations for this phenomenon: (i) the operation of an unidentified alternative cytochrome *c* oxidase; (ii) auto-oxidation of the cytochrome *c* pool; or (iii) reversed electron transfer from cytochrome *c* to ubiquinone, through the bc_1 complex.

The electron transfer pathway in the $\Delta aa_3/cbb_3$ mutant, from methylamine to oxygen, has been identified by analysis of the oxygen consumption (Fig. 1). Oxygen consumption by endogenous substrate has been eliminated by addition of piericidin A, an inhibitor of the proton-translocating type of NADH dehydrogenase [13]. Apparently, this type of NADH dehydrogenase is the only type expressed under these conditions in *P. denitrificans*. In the wild type strain (Fig. 1), and in the single mutants (not shown), the oxidation of methylamine proceeds in the presence of the uncoupler FCCP, indicating that both cytochrome aa_3 and cytochrome cbb_3 operate during methylamine oxidation. In the $\Delta aa_3/cbb_3$ mutant, however, the methylamine oxidation was completely blocked upon the addition of FCCP (Fig. 1). This indicates that no alternative cytochrome *c* oxidase is expressed in this mutant and, moreover, that the oxidation of the cytochrome *c* pool in the absence of cytochrome *c* oxidase proceeds via an 'uphill' pathway driven by the protonmotive force. Involvement of the bc_1 complex has been demonstrated by the effect of the specific inhibitors an-

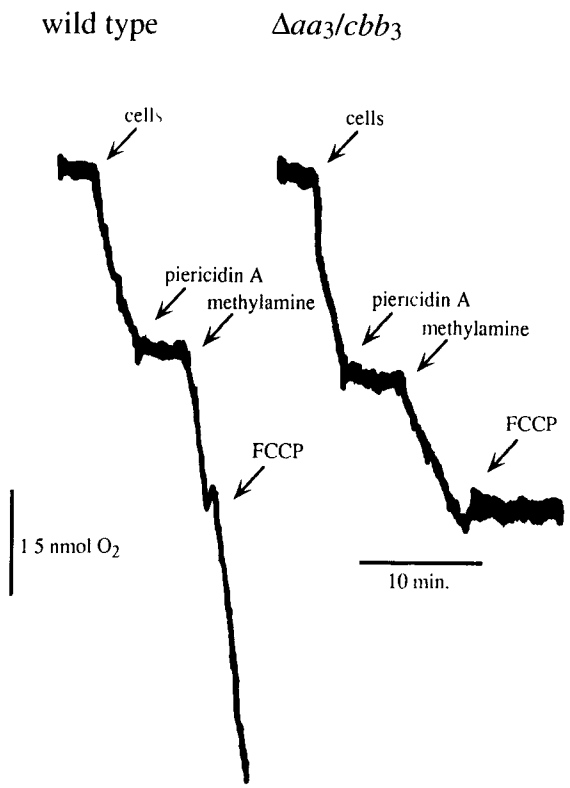

Fig. 1. Oxygen consumption analysis of cell suspensions of the *P. denitrificans* wild type and the cytochrome *c* oxidase mutant ($\Delta aa_3/cbb_3$). Piericidin A was added to block the oxidation of endogenous NADH. After addition of this inhibitor, the oxidation of methylamine can be measured directly. FCCP is a protonophore (uncoupler) that dissipates the membrane potential.

Table 1
P. denitrificans wild type and oxidase mutants analysed for growth on minimal plates with either succinate, methylamine (MA) or methanol (MeOH) as carbon/energy source

substrate [inhibitors] strain	succinate	MA	MeOH	succinate [AA/myx]	MA [AA/myx]	MeOH [AA/myx]
wild-type	+	+	+	+	+	+
Δaa_3	+	+	+	+	+	+
Δcbb_3	+	+	+	+	+	+
$\Delta aa_3/cbb_3$	+	+	+	+	–	–
Δbb_3	+	+	+	–	–	–

When indicated, plates have been supplemented with antimycin A or myxothiazol [AA/myx]. +, growth, –, no growth.

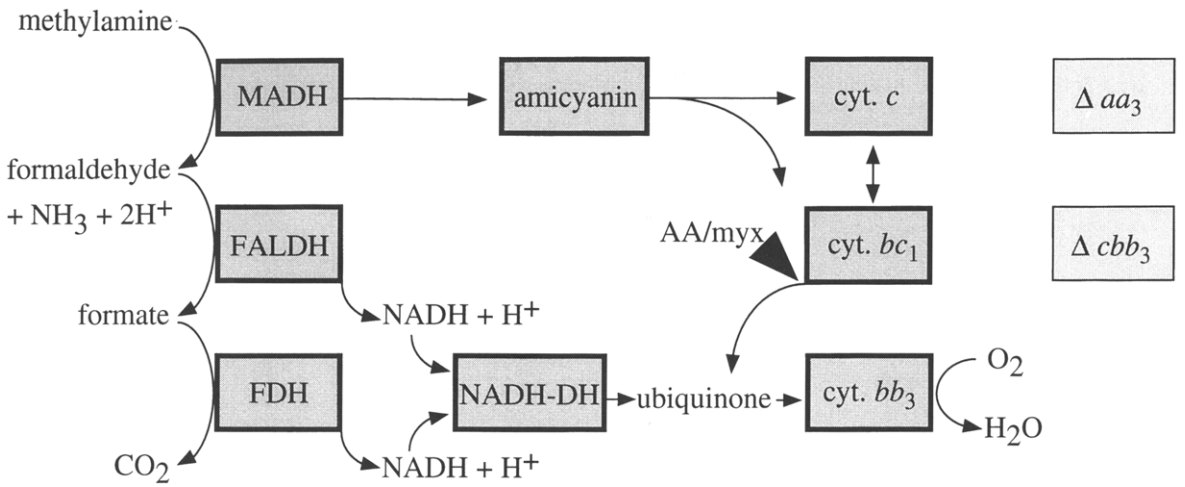

Fig. 2. Electron transfer routes from methylamine to oxygen in the $\Delta aa_3/cbb_3$ mutant of *P. denitrificans*. MADH, methylamine dehydrogenase; FALDH, formaldehyde dehydrogenase; FDH, formate dehydrogenase; NADH-DH, NADH dehydrogenase; cyt. *c*, cytochrome c_{550} and probably cytochrome c_{552} [6]. Inhibition of the methylamine oxidation by antimycin A or myxothiazol (AA/myx) is indicated.

timycin A or myxothiazol: (i) complete inhibition of the oxygen consumption (not shown); and (ii) no growth on methylamine plates (Table 1). In contrast, the oxidation of methylamine was not affected in the wild type, the Δaa_3 mutant and the Δcbb_3 mutant (Table 1). The latter indicates that both cytochrome *c* oxidases are potentially involved in methylamine oxidation. The observation that growth of the Δbb_3 mutant was completely inhibited by antimycin A or myxothiazol (Table 1), indicates that also during methylotrophic conditions no alternative quinol oxidase is expressed by *Paracoccus*. When ascorbate/TMPD was used as electron donating couple in the oxygen consumption assay (in the presence of piericidin A), the effect of FCCP was similar: no inhibition in wild type and single mutants, but complete blockage of respiration in the $\Delta aa_3/cbb_3$ mutant (not shown). The fact that uphill electron transfer through the bc_1 complex proceeds in the presence of piericidin A, when NADH oxidation is completely blocked, suggests a continued generation of protonmotive force. This would be in agreement with the assumption that reversal of the Q cycle requires 1 charge per electron, whereas the subsequent oxidation of ubiquinol by the proton translocating bb_3-type oxidase yields 2 charges per electron [7,10].

Reversed electron transfer through the mitochondrial bc_1 complex has been demonstrated in vitro, either from cytochrome *c* to NAD^+ [14], or from ascorbate to ubiquinone [15]. In addition, reversed electron flow has previously been reported to occur in chemolithotrophs, like *Nitrobacter* [16]. In this bacterium, NO_2^- is oxidised by nitrite oxidase, and a protonmotive force is generated upon the transfer of electrons, via cytochrome *c* and an aa_3-type cytochrome *c* oxidase, to oxygen. *Nitrobacter* probably possesses a bc_1(-like) complex and an NADH dehydrogenase. In order to generate NADH, the protonmotive force is used to reverse the electron flow from nitrite has been proposed via nitrite oxidase, the bc_1 complex, ubiquinone and the NADH dehydrogenase to NAD^+ [17]. As far as we are aware, the present study is the first direct demonstration of reversed electron transfer through the bc_1 complex in vivo.

Fig. 2 is a schematic representation of the oxidation of methylamine in the *P. denitrificans* cytochrome *c* oxidase mutant ($\Delta aa_3/cbb_3$). All cytochrome oxidase mutants tested in this study behave similarly on methylamine- or methanol-supplemented minimal plates, indicating that the downstream, branched part of the respiratory pathways of methylamine and methanol oxidation consists of the same set of cytochromes *c* and terminal oxidases. It is concluded that during methylotrophic growth of *P. denitrificans* the three terminal oxidases are potentially involved. Moreover, the bc_1 complex is indispensible in both the Δbb_3 mutant and the $\Delta aa_3/cbb_3$ mutant, catalysing the reduction or the oxidation of the cytochrome *c* pool, respectively.

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