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Oxidation of methylamine by a *Paracoccus denitrificans* mutant impaired in the synthesis of the bc_1 complex and the aa_3-type oxidase

Evidence for the existence of an alternative cytochrome *c* oxidase in this bacterium

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A *Paracoccus denitrificans fbcC–ctaDII* double mutant strain impaired in the synthesis of both the bc_1 complex and the aa_3-type oxidase has been constructed. This mutant strain, which is still able to grow on methylamine as sole carbon and energy source, exhibits unimpaired oxygen consumption with succinate, methylamine and endogenous substrates as electron donors. From kinetic studies of the oxidation and reduction rates of cytochromes *c*, it can be concluded that *P. denitrificans* contains a second cytochrome *c* oxidase, different from the aa_3-type.

Methylamine oxidation; Alternative cytochrome *c* oxidase; Respiratory chain; *Paracoccus denitrificans*

1. INTRODUCTION

P. denitrificans is able to grow on methylamine as sole carbon and energy source. Methylamine dehydrogenase (MADH) oxidizes methylamine to formaldehyde and ammonia and the electrons released are passed to amicyanin [1]. Both MADH and amicyanin are located in the periplasm. It is still generally assumed that amicyanin donates electrons via cytochrome c_{550} to the aa_3-type oxidase [2,3]. This assumption is not in agreement with the findings that *P. denitrificans* strains lacking either cytochrome c_{550} or the aa_3-type oxidase are still able to grow on methylamine as sole carbon and energy source [4–6]. A possible explanation for the latter phenomenon might be the supposition that electrons can flow from amicyanin via different cytochromes *c* back through the bc_1 complex to the ubiquinone pool and subsequently to the terminal quinol oxidase. Another explanation might be found in the presence of a second cytochrome *c* oxidase apart from the aa_3-type oxidase. In other methylotrophic bacteria *o*- or *co*-type cytochrome *c* oxidases, which are involved in the oxidation of methanol, have been characterized [7,8]. Whether or not such cytochrome *c* oxidases are also present in *P. denitrificans* for the oxidation of methylamine is the subject of this paper. For this study a double mutant strain was constructed with mutations in the gene encoding the cytochrome c_1 of the bc_1 complex (*fbcC*) [9] and in the gene encoding the heme containing subunit I (*ctaDII*) of the aa_3-type oxidase [10]. As a result the synthesis of both the bc_1 complex and the aa_3-type oxidase was impaired.

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2. EXPERIMENTAL

2.1. *Organisms and growth conditions*

P. denitrificans cells of the wild-type and mutant strain were cultivated overnight in aerobic batch cultures at 30°C either with brain heart infusion broth (precultures) or with mineral salts medium containing 100 mM methylamine as the sole carbon and energy source as described previously [4]. When necessary, antibiotics were added to final concentrations of 40 μg of rifampin, 25 μg of kanamycin, 25 μg of streptomycin, and 100 μg of ampicillin per ml.

2.2. *DNA manipulations*

General cloning techniques were carried out essentially as described by Maniatis et al. [11]. Plasmid DNA was isolated by the cleared-lysate method and purified by using the Qiagen plasmid kit (Diagen GmbH, Düsseldorf, Germany). DNA fragments were purified from agarose gels by using GeneClean (Bio101, INC., San Diego, CA). The *P. denitrificans fbcC* deletion-*ctaDII* insertion mutant was constructed by means of gene replacement techniques as described previously [12], and verified by means of Southern blotting.

2.3. *Cytochrome studies*

Spectra were recorded by using a completely rebuilt Aminco/SLM DW-2 UV/Vis spectrophotometer. Spectra of dithionite-reduced whole-cell suspensions were recorded at 77 K in the dual-wave length mode, with the reference set at 578 nm. Prior to freezing by plunging of the cuvette in liquid nitrogen, argon was flushed over the 1 ml cell suspensions for 10 min, followed by the addition of 50 μl of a sodium dithionite solution of 100 mg per ml.

During kinetic measurements, the reduction level of cytochromes *c* as a function of time was recorded at 551.2 nm with the reference set at 578 nm. Cuvettes were thermostated at 20°C.

For both types of experiment cells were cultivated in mineral salts medium with 100 mM methylamine as the sole carbon and energy source. They were harvested at the end of the exponential phase.

washed once with phosphate buffered saline pH 7.0 and finally concentrated to an OD_{660} = 50 as measured on a Philips PU8720 spectrophotometer.

2.4. *Protein analyses*

Protein concentrations were determined as described by Lowry [13].

3. RESULTS AND DISCUSSION

3.1. *Construction and analysis of the* fbcC-ctaDII *double mutant strain Pd9214*

The *fbcC* deletion mutant strain Pd2431 was constructed and described earlier. By means of spectral analysis and analysis of heme containing proteins by gel electrophoresis, it was shown that this mutant lacks the c_1-heme as well as the *b*-heme containing moieties of the bc_1 complex [14]. This strain was used to introduce a second mutation in the *ctaDII* gene. The cloning of this gene has been described previously [4,10]. The *ctaDII* gene was interrupted at the internal *Pst*I site by insertion of the kanamycin resistance gene of Tn*903* [15]. The mutated gene was cloned into suicide vector pGRPd1. Following conjugation, a mutant strain Pd9214 was isolated in which the wild-type *ctaDII* gene was exchanged for the mutated one. Southern analysis of chromosomal DNA revealed that the gene replacement had taken place properly (results not shown).

Both on plates and in liquid mineral medium, Pd9214 was able to grow with methylamine as sole carbon and energy source.

Spectra of dithionite reduced whole-cell suspensions of the wild-type strain Pd1235 and the mutant strain Pd9214, both grown on methylamine, were recorded at 77 K (see Fig. 1). From the comparison of these spectra it can be concluded that the strain Pd9214 lacks both *b*- and the *c*-hemes of the bc_1 complex and the *a*-hemes of the aa_3-type oxidase. Because of the absence of these hemes, the mutant must be devoid of normally functioning bc_1 and aa_3-type oxidase complexes.

3.2. *Kinetic studies on the reduction and oxidation of cytochromes* c

In order to study the effect of the impaired synthesis of the bc_1 and aa_3-type oxidase complexes on the electron transfer to oxygen, the kinetics of reduction and oxidation during electron transfer through the respiratory chain were studied at the level of cytochromes *c* in whole-cell suspensions. These suspensions contained either cells of the wild-type Pd1235 or the mutant strain Pd9214, both grown on methylamine. Typical results are illustrated in Fig. 2. After anaerobiosis, the reduction levels of cytochromes *c* in the suspensions of both the wild-type cells (traces A and B) and the mutant cells (traces C and D) reached (almost) the anaerobic state level at point 1. During the traces A and C the reduction was driven by endogenous substrate as well as sodium succinate. During the traces B and D, in addition to these two electron donors, 1 mM methylamine was present as an extra electron donor. At the points 1, oxygen was generated in the suspensions by the addition of 20 μl of a 0.3% H_2O_2 solution. Apparently, the catalase activity in the cells is sufficiently high to convert the peroxide into water and oxygen within the time needed for the addition and mixing. In all suspensions the cytochromes *c* present reached aerobic steady state levels of reduction at the points indicated by 2. The lengths of these aerobic steady states (distances between points 2 and 3) are determined by the oxygen consumption rates of the suspensions in the presence of the available electron donors. Repeated additions of H_2O_2 do not influence these rates substantially, indicating that the peroxide does not harm the respiratory system essentially (results not shown). Upon oxygen exhaustion (points 3), cytochromes *c* were reduced again to the anaerobic state levels at points 4 by the electron donors still present.

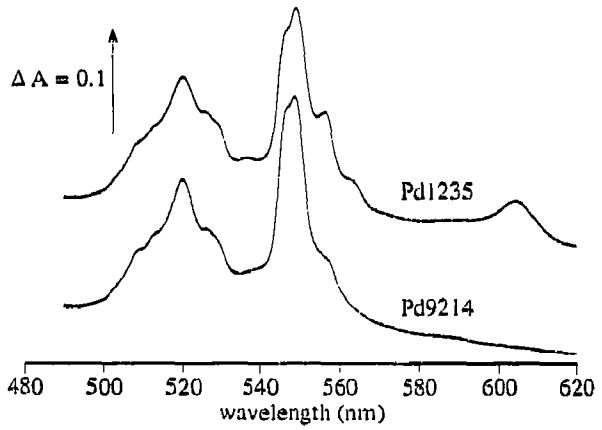

Fig. 1. Light absorption spectra of dithionite-reduced whole-cell suspensions of the wild-type strain Pd1235 (A) and the mutant strain Pd9214 (B). Cells were grown in batch cultures on minimal medium with methylamine as sole carbon and energy source. The spectra were recorded at 77 K.

For conclusions about the electron transfer routes, especially the oxygen consumption rates (steady states 2 to 3) and the reduction rates of cytochromes *c* (slopes of 3 to 4) are of importance. On the basis of at least 4 independent assays, mean oxygen consumption rates per mg protein have been calculated from the steady state lengths described above. The results are given in Table I. There were no essential differences in the oxygen consumption rates between the wild-type strain and the mutant strain. Supposing that the wild-type respiratory chain contains at least two functioning oxidases, a terminal quinol oxidase and an aa_3-type cytochrome *c* oxidase, while in the mutant strain the latter oxidase is missing, it should be concluded from this similarity that the oxygen consumption rate is (almost entirely) controlled by the dehydrogenase activities. This conclusion is also supported by the observation that the oxygen reduction rate was strongly enhanced by the addition of methylamine. Very surprisingly, also in this phenomenon the mutant did not stay behind the wild-type strain.

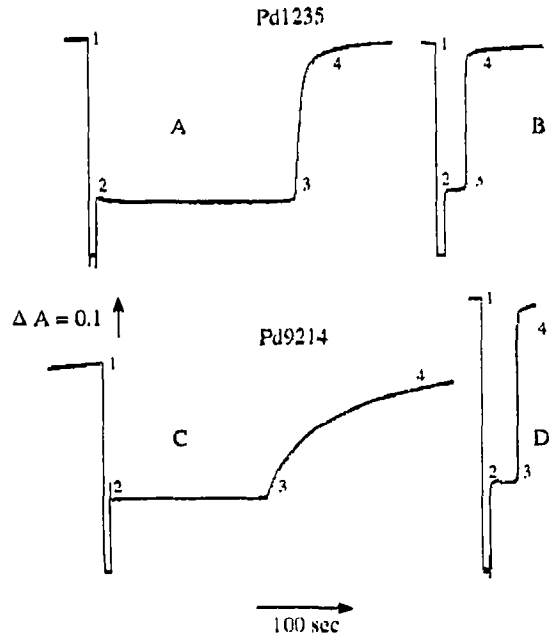

Fig. 2. Reduction and oxidation kinetics of cytochromes *c* in whole-cell suspension of the wild-type Pd1235 (curves A and B) and the mutant strain Pd9214 (curves C and D). With curves A and C the suspensions contained endogenous substrates and 50 mM sodium succinate as electron donors, with curves B and D, besides those, also 1 mM methylamine is present as an electron donor. At the points marked by '1' the suspensions were supplied with 20 μl 0.3% H_2O_2. Protein concentrations were 8.9 mg per ml in the wild-type suspension and 10.5 mg per ml in the mutant strain suspension.

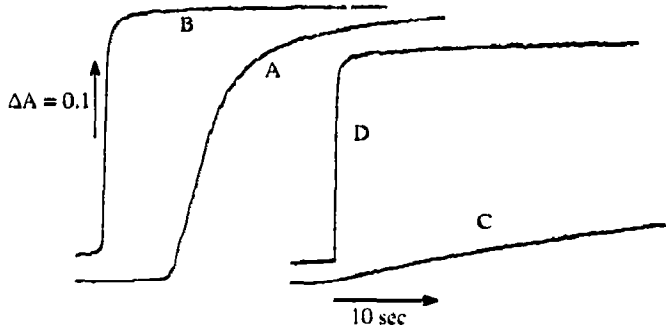

Fig. 3. Reduction rates of cytochromes *c* upon shifts from the aerobic steady state to the anaerobic state in suspensions of the wild-type strain Pd1235 with either endogenous substrate plus succinate (curve A) or endogenous substrate plus succinate and methylamine (curve B) and in suspensions of the mutant strain Pd9214 with either endogenous substrate plus succinate (curve C) or endogenous substrate plus succinate and methylamine (curve D).

It has previously been shown that the blue copper protein amicyanin functions as an indispensable intermediate between MADH and the respiratory chain [1]. A direct reduction of ubiquinone by reduced amicyanin is inconceivable on mechanistic and thermodynamic grounds. In wild-type cells reversed electron flow through cytochromes *c* and the bc_1 complex to ubiquinone and the quinol oxidase to oxygen would be possible on the expense of the proton motive force. In the mutant strain however this route is impossible.

In spite of the defectiveness of the bc_1 complex in the mutant strain, a slow electron transfer from endogenous substrate and succinate to the present cytochromes *c* occurred. For a further analysis of this electron flow, the curves 3 to 4 in Fig. 2 were expanded as shown in Fig. 3. Obviously, the reduction rate of cytochromes *c* by endogenous substrates and succinate in the mutant Pd9214 is relatively very slow. Assuming a provisional molecular extinction coefficient for cytochromes *c* of 23 $mM^{-1} \cdot cm^{-1}$ [16], estimates can be made of the initial reduction rates of cytochromes *c* under the different circumstances. Those estimates are given in Table II.

On thermodynamic grounds it is very unlikely that the rate of the backflow of electrons from cytochromes *c* to ubiquinone would not be substantially lower than the rate found for the opposite direction. It is evident that such a very low activity in the mutant strain (<0.05 nmol cytochrome *c* per mg protein per second) cannot maintain the at least fifty-fold higher rate of electron flow from methylamine to oxygen, a rate which can be deduced from Table I (4 × (1.32–0.37)nmol 'electrons' per second). As a consequence, the quinol oxidase cannot be the most important terminal oxidase in this electron flow. Therefore, in the absence of the aa_3-type oxidase, the presence of an alternative cytochrome *c* oxidase has to be accepted unavoidably.

Table I

Oxygen consumption rates (nmol O_2 per mg protein per second) of the wild-type strain Pd1235 and the mutant strain Pd9214 with either endogenous substrate plus 50 mM sodium succinate or endogenous substrate plus 50 mM sodium succinate plus 1 mM methylamine

Strain	Electron donors	
	Endogenous substrate 50 mM sodium succinate	Endogenous substrate 50 mM sodium succinate 1 mM methylamine
Pd1235	0.32	1.16
Pd9214	0.37	1.32

Table II

Initial cytochromes *c* reduction rates (nmol cytochromes *c* per mg protein per second) of the wild-type strain Pd1235 and the mutant strain Pd9214 with either endogenous substrate plus 50 mM sodium succinate or endogenous substrate plus 50 mM sodium succinate plus 1 mM methylamine

Strain	Electron donors	
	Endogenous substrate 50 mM sodium succinate	Endogenous substrate 50 mM sodium succinate 1 mM methylamine
Pd1235	4.7	23
Pd9214	<0.05	23

Previously, upon the characterization of purified proteins with oxidase activity, the existence of a *co*-type cytochrome *c* oxidase in *P. denitrificans* has been suggested [17]. The results described in this paper form the first hard evidence for the presence of such an oxidase, leaving the question about the nature of the hemes involved, however, unsolved. Preliminary results (not shown) indicate that the alternative cytochrome *c* oxidase in *P. denitrificans* can operate also in the oxidation route from methanol to oxygen, albeit less efficient than with methylamine as electron donor.

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