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Evidence for the role of soluble cytochrome *c* in the dissimilatory reduction of nitrite and nitrous oxide by cells of *Paracoccus denitrificans*

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The role of periplasmic cytochrome *c* in the denitrification pathway has been investigated using a wild-type and / or a cytochrome *c* deficient strain of *Paracoccus denitrificans*. The reconstitution experiments with the isolated proteins showed that bacterial cytochrome *c*-550 restored the electron transport from the cytoplasmic membrane to soluble nitrite reductase (cytochrome cd_1). In response to decreased aeration lasting 3 h, the HUUG25 strain synthesized nitrous-oxide reductase significantly starved of electrons from the respiratory chain and only very small amounts of soluble cytochrome *c*. The membrane-bound part of the respiratory chain catalyzing the reduction of soluble cytochrome *c* resembled an analogous region in wild-type cells kinetically and by its sensitivity to antimycin. In the periplasmic fraction obtained from anaerobically grown wild-type cells N_2O caused the reoxidation of endogenous cytochrome(s) *c* previously reduced by *N,N,N',N'* tetramethyl-*p*-phenylenediamine plus ascorbate. All these results indicate the involvement of soluble cytochrome(s) *c* as the electron donor(s) for the reduction of NO_2^- and N_2O in the periplasmic space of cells.

Introduction

When grown under denitrifying conditions, the bacterium *Paracoccus denitrificans* possesses two soluble respiratory enzymes localized in the periplasmic space [1,2]: nitrite reductase of cytochrome cd_1 type [3,4] and cuproprotein nitrous-oxide reductase [5]. Although early spectroscopic and inhibitory studies have already shown that both reductases are connected to the respiratory chain on the level of cytochrome(s) *c* [6,7], their immediate physiological donor remains to be established. Recently, Bosma et al. [8] have suggested that membrane-bound cytochrome *c* (M_r 14000) might play a role in the metabolism of NO_2^- and N_2O. Another possible candidate is belived to be soluble periplasmic cytochrome *c*, known as *c*-550, which is abundantly present in anaerobically grown cells (9,10] and in vitro reacts with cytochrome cd_1 [11]. However, it has been observed [12] that the inactivation of the cytochrome *c*-550 gene did not change the anaerobic growth of *P. denitrificans* in the presence of nitrate.

Existing uncertainties concerning the participation of various redox carriers in the reduction of NO_2^- and N_2O prompted us to investigate the two reactions on the level of subcellular fractions (membrane vesicles, periplasm, and purified cytochromes *c*-550 and cd_1) and their mixtures. In addition, we also focused our attention on another cytochrome-*c*-deficient respiratory mutant. This strain, referred to as HUUG25 or S1659, was previously shown to grow aerobically with the involvement of a respiratory chain insensitive to antimycin or myxothiazol [13]. On the other hand, the capacity of HUUG25 for anaerobic growth turned out to be very limited because, although nitrate reductase was functional, nitrite reductase was formed only as an inactive apoprotein lacking both *c*- and *d*-type haem [14]. A part of the work described in this paper therefore addresses itself to the possibility of using HUUG25

Abbreviations: BV, benzyl viologen; SDS-PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis; TMBZ, 3,3',5,5'-tetramethylbenzidine; TMPD, *N,N,N',N'*-tetramethyl-*para*-phenylenediamine.

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strain for the elucidation of the anaerobic electron-transfer pathway in *P. denitrificans*.

Materials and Methods

The wild-type strain of *P. denitrificans* (NCIB 8944) was obtained from the Czechoslovak Collection of Microorganisms as CCM 982 and the cytochrome-*c*-deficient mutant HUUG25 was kindly donated by Dr. H.W. van Verseveld (Vrije Universiteit Amsterdam, The Netherlands).

The wild-type cells were grown anaerobically at 30°C for 22 h in 1-litre batch cultures on minimal medium containing 50 mM succinate and 10 mM nitrate [15]. The aerobic cultivation of the strains was carried out under vigorous aeration to the mid-exponential phase of growth; in this case nitrate and molybdate were discharged from the growth media. The absence of revertants in HUUG25 cultures was confirmed by Nadi staining [16]. The formation of denitrification enzymes in HUUG25 was induced by 3 h incubation of aerobically cultivated cells in anaerobic growth medium with nitrate (cell concentration 1 mg of dry weight per ml of medium) [14,17]. The preparation of periplasmic and membrane fractions, the isolation of nitrite reductase and the measurement of enzyme activities were done as described [17,18]. To solubilize membrane proteins, membrane vesicles were suspended to a concentration of 10 mg of protein per ml in 50 mM potassium phosphate (pH 7.3) containing 1% Thesit and the solution was homogenized for 40 min at 4°C and then centrifuged (40 min, 170000 × *g*).

The samples containing cytochromes *c* were dissociated in 62.5 mM Tris-HCl/2% sodium dodecyl sulphate/10% glycerol/1% mercaptoethanol (pH 6.8). Laemmli-type electrophoresis was conducted on sodium dodecyl sulphate polyacrylamide slab gels (180 × 160 × 1.5 mm) using a Bio-Rad apparatus. The gels, stained with Coomassie brilliant blue or with 3,3′,5,5′-tetramethylbenzidine [19], were scanned with an integrating video densitometer (model 620, Bio-Rad, U.S.A.); molecular-weight protein standards were: myosin (200000), phosphorylase *b* (92500), bovine serum albumin (69000), ovalbumin (46000), carbonic anhydrase (30000), trypsin inhibitor (21500) and lysozyme (14300). Kinetic and spectroscopic measurements were performed on a Shimadzu UV 3000 spectrophotometer connected with PC 20-II computer (Commodore, U.S.A.). The kinetic data were evaluated using the program EZ-FIT developed by F. Perrella [20].

Results

The reduction of nitrite by succinate requires the presence of cytochrome c-550

If some of membrane-bound components normally present in anaerobically grown cells of *P. denitrificans* could interact with periplasmic nitrite reductase, it should be possible to observe the reduction of nitrite in the mixture containing only succinate, membrane vesicles and purified nitrite reductase. However, this is at variance with our experience. The results given in Fig. 1 show that, in addition to nitrite reductase, the presence of another soluble haemoprotein, namely cytochrome *c*-550, is strongly required. The activities of nitrite reductase obtained in the reconstituted system depended hyperbolically on the amount of cytochrome *c*-550 added, a half-maximal saturation being achieved at its 1.5 μM concentration. An observed parallel rise in the activity of hydroxylamine reductase (Fig. 1) accords with our suggestion that the reduction of both NO_2^- and NH_2OH may be catalyzed by the same enzyme in *P. denitrificans* [17]. Both activities were strongly inhibited by antimycin, thus establishing that the electron flow through membrane-bound bc_1 complex was involved.

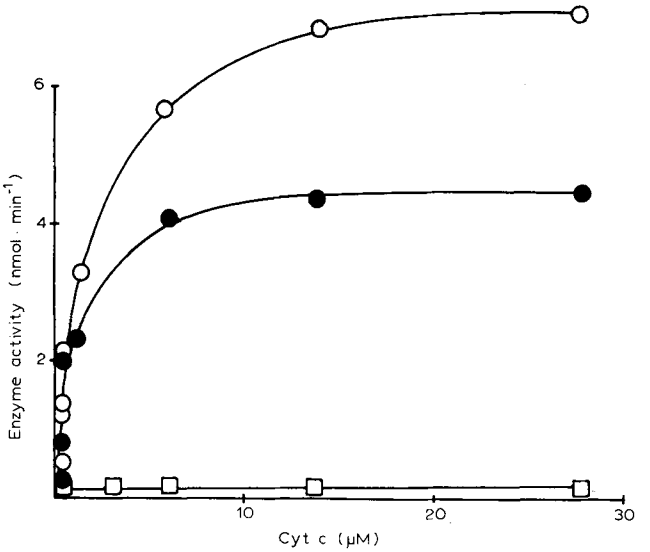

Fig. 1. Effect of bacterial cytochrome *c*-550 on nitrite (○, □) or hydroxylamine (●) consumption in the mixture of nitrite reductase and membranes from anaerobically grown *P. denitrificans*. The samples (0.5 ml) contained 0.22 mg of membrane protein and 0.12 mg of nitrite reductase in 2 ml of N_2-flushed 0.1 M sodium phosphate with 10 mM sodium succinate (pH 7.3, 30 °C) (○, ●, □) and 10 μg of antimycin (□). The reaction was started by the addition of 1 μmol NO_2^- or hydroxylamine. After 15 min incubation the remaining terminal acceptor was determined.

HUUG25 cells can form nitrous-oxide reductase starved of electrons from the respiratory chain

In experiments with HUUG25 strain we found that a decreased aeration of growing cultures led to the formation of nitrous-oxide reductase. Resulting activities, measured with either physiological (succinate) or artificial (reduced benzyl viologen) electron donors, are listed in Table I. Based upon these data it can be calculated that mutant-type cells previously exposed to decreased aeration exhibit an approx. 10-times lower

TABLE I

Rates of N_2O reduction in wild-type and mutant cells of P. denitrificans

Aerobically grown cells were previously adapted to decreased aeration by a 3 h incubation of their suspension (1 mg dry weight of cells per ml) in the growth medium with or without 10 mM NO_3^-.

Type of cell	BV oxidation by N_2O [a]	N_2O reduction by succinate [a]	Ratio of electron flows (BV/succinate) [b]
Wild-type adapted with NO_3^-	4.2	2.0	1.1
Wild-type adapted without NO^{-3}	4.2	1.3	1.6
HUUG25 adapted with NO_3^-	7.6	0.3	8.8
HUUG25 adapted without NO	3.8	0.26	7.3

[a] nmol s^{-1} (mg dry wt. of cells)$^{-1}$

[b] Calculated on the basis that oxidation of BV is a one-electron process, the reduction of N_2O requires two electrons.

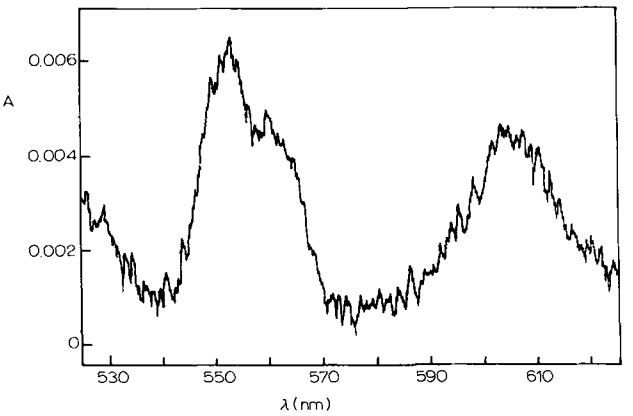

Fig. 2. Difference spectrum (ascorbate reduced minus ferricyanide oxidized) of HUUG25 membranes extracted by Thesit. The scanning speed and the data sampling pitch were 20 nm min^{-1} and 0.1 nm, respectively.

ratio of both activities (succinate/benzyl viologen) than similarly treated cells of the wild-type strain. These findings thus seem to indicate that the active nitrous-oxide reductase can be expressed in HUUG25, although the enzyme is significantly starved of electrons supplied by the respiratory chain.

HUUG25 and wild-type cells differ in the content of the periplasmic cytochrome c-550 but not in the kinetics of its reduction

In order to identify the respiratory component that limits the low nitrous-oxide reductase activity of the HUUG25 strain, a more detailed analysis of membrane and periplasmic fractions was performed. It has been established that the aerobically growing mutant can synthesize at least some of membrane-bound cytochromes *c* normally present in the wild-type strain. Two lines of evidence support this conclusion: the appearance of a component with an absorption maximum at 552 nm in the difference spectrum (ascorbate-reduced minus ferricyanide-oxidized) of the HUUG25 extract (Fig. 2) and the distinguishing of four major polypeptides with apparent molecular masses of 14, 25, 46 and 68 kDa by SDS-PAGE followed by haem-staining (Fig. 3). Quantitative evaluation of the scans A and B in Fig. 3 shows that the specific content of the two low-molecular-mass cytochromes *c* in HUUG25 was significantly lower than in the wild-type strain (7.6% for 14 kDa and 22% for 25 kDa species). The 46 kDa cytochrome *c* synthesized in the wild-type strain under denitrifying conditions of growth (C) was found in decreased amount (27%) in the aerobically grown mutant (A) as well. Further analysis revealed an at least 100-fold-diminished content of soluble cytochromes *c* in periplasmic fractions obtained from the aerobically grown or anaerobically adapted mutant cells (not shown).

Bolgiano et al. [21] have recently demonstrated that membrane vesicles prepared from HUUG25-type cells are able to reduce both mammalian and *Paracoccus* cytochromes *c* similarly to the wild-type membranes. Our own results (Fig. 4) confirm this observation and extend it by pointing out that the activities of both membrane preparations have very similar kinetic parameters (with respect to cytochrome *c*) and are comparably sensitive to the bc_1 inhibitor antimycin. The cytochrome *c*-reductase activity of HUUG25 strain enabled us to reconstitute the antimycin-sensitive nitrite-reducing system analogous to that in Fig. 1 by adding

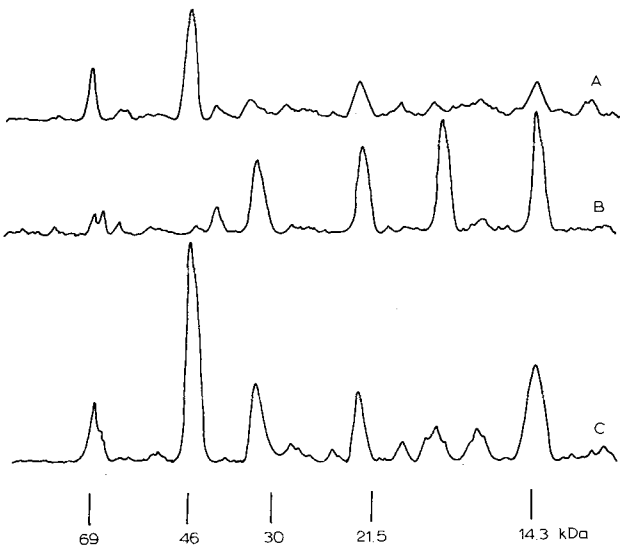

Fig. 3. Densitometer scans of SDS-PAGE of membrane vesicles from aerobically grown HUUG25 (lane A, 50 μg protein) or wild-type cells (lane B, 26 μg protein) and from anaerobically grown wild-type cells (lane C, 29 μg protein). Covalently bound haem was stained with TMBZ.

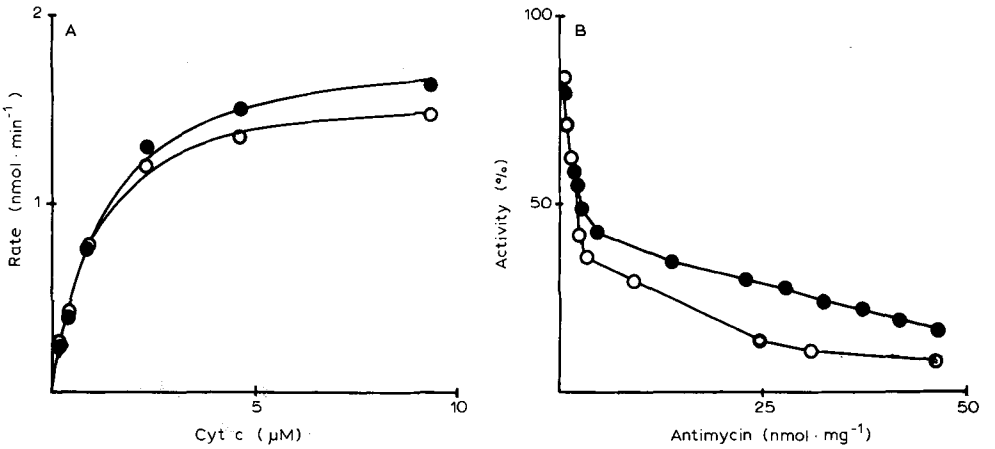

Fig. 4. Succinate-cytochrome *c* reductase activity of wild-type (○) or mutant (●) membranes: dependence on horse-heart cytochrome *c* concentration (panel A) and inhibition by antimycin (panel B). The reaction mixture (2 ml, 30 °C) contained 0.1 M sodium phosphate (pH 7.3), 10 mM sodium succinate and 1 mM KCN to inhibit terminal oxidases; in experiments B, 6 μM horse-heart cytochrome *c* was present. The curves depicted in panel A correspond to the optimalized values of kinetic parameters calculated by the non-linear regression (○, $K_m = 1.1 \pm 0.2$ μM, $V_{max} = 1.69 \pm 0.08$ nmol s^{-1} mg^{-1}; ●, $K_m = 1.3 \pm 0.4$ μM, $V_{m_x} = 1.90 \pm 0.20$ nmol s^{-1} mg^{-1}).

the bacterial cytochromes cd_1 and *c*-550 to the mutant membranes (not shown).

Reoxidation of endogenous cytochrome c in periplasmic fraction of anaerobically grown wild-type cells by nitrous oxide

The results described thus far suggest that the low rate of N_2O reduction in HUUG25 strain may be a consequence of the decreased amount of a soluble component which participate in the electron transport to the periplasmic nitrous-oxide reductase. To ascertain the presumed role of soluble cytochrome *c*, the periplasmic fraction prepared from the anaerobically-grown wild-type cells of *P. denitrificans* was reduced by ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (which reacts with cytochrome(s) *c* but not with nitrous-oxide reductase [22]) and then nitrous oxide was added. From the time-course of absorbance $A_{550-540}$ shown in Fig. 5 it follows that N_2O caused a transient reoxidation of cytochrome *c* present and that these redox changes did not take place in the presence of azide, a known inhibitor of N_2O reductase [22]. The time interval between redox transitions in Fig. 5 allowed to estimate the specific activity of N_2O reductase as 0.9 nmol N_2O s^{-1} (mg of protein)$^{-1}$. When the same sample was assayed with reduced benzyl viologen as an artifical electron donor for nitrous-oxide reductase [22], a 5-times greater value of specific activity (10.2 nmol benzyl viologen (i.e., 5.1 nmol N_2O) s^{-1} (mg of protein)$^{-1}$) was obtained.

Discussion

The data presented here are compatible with the idea that the membrane-bound redox proteins in the denitrifying cells of *P. denitrificans* do not reduce nitrite and nitrous-oxide reductases directly but only via another periplasmic protein, probably cytochrome *c*-550. This is in accord with the earlier suggestion that a major role of cytochrome *c*-550 may be in transferring electrons to and from the periplasmic enzymes [23,24].

A simple calculation based on the known content of approx. 600 nmol per g dry wt. of anaerobically grown *P. denitrificans* [10], on the previously determined intracellular volume (2.7 ml per g dry wt. of cells) [25] and on the fact that the periplasmic space comprises approx. 20% of the total cell volume in Gram-negative bacteria [26] implies that the cytochrome *c*-550 concentration in the periplasmic space should be at least

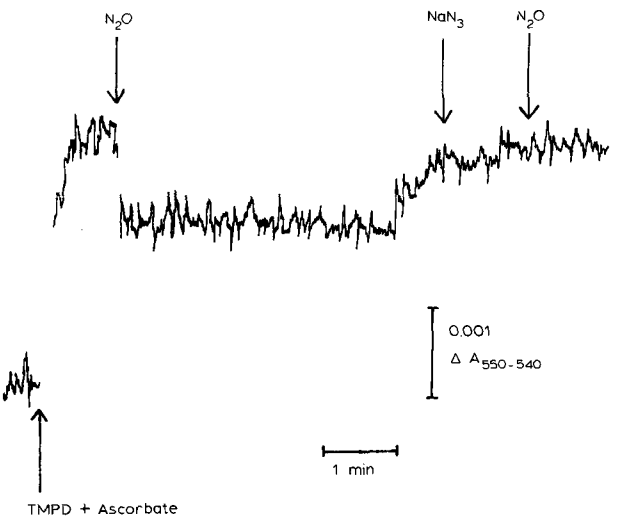

Fig. 5. Oxidation of endogenous cytochromes *c* by nitrous oxide. The stoppered cuvette contained the solution of periplasmic proteins from anaerobically grown *P. denitrificans* (1.8 mg ml^{-1}) in 3 ml of 0.1 M sodium phosphate (pH 7.3, 30 °C); the anaerobiosis was ensured by gassing with nitrogen. The additions were: TMPD (0.3 mM), ascorbate (6.7 mM), N_2O (1.2 μmol) and azide (1 mM).

10^{-4}–10^{-3} M. This value considerably exceeds the estimated concentration of 10^{-6} M saturating the electron transport to nitrite reductase in the reconstituted nitrite-reducing system (Fig. 1). It is therefore probable that the surplus of cytochrome *c*-550 in cells may be necessary to overcome the much smaller diffusion rate of proteins in periplasm in comparison with free water solutions [27]. The periplasmic pool of cytochrome *c*-550 might also bind reactive nitric oxide generated from nitrite and in this way prevent deleterious effects of NO on the cellular metabolism (Kučera, I., unpublished data).

Although the experiments in this work (Figs. 1, 4, 5, Table I) provide arguments in favour of cytochrome c-550 being a redox carrier in bacterial denitrification, they in fact do not exclude the possible involvment of two or more electron-transfer pathways between cytoplasmic membrane and periplasmic reductases. The existence of such an alternative route would explain why Van Spanning el al. [12] observed the anaerobic growth of a mutant of *P. denitrificans* fully depleted of cytochrome *c*-550 and why the electron-transfer rate in our reconstituted system (not containing the unknown additional carrier) was lower than in living cells. In this context it is notable that two pathways from NADH to nitrous oxide were shown to operate in the *Rhodobacter* species [28].

A surprising outcome of this work is the detection of cytochromes *c* in membranes from the 'cytochrome *c* deficient' HUUG25 strain of *P. denitrificans* (Figs. 2, 3). This finding, together with the observed sensitivity of the cytochrome *c*-reductase activity of mutant membranes to antimycin (Fig. 4) appears to be in conflict with the previous suggestion that ubiquinone or cytochrome *b* can act instead of membrane-bound cytochrome c_1 as the electron donor for soluble cytochrome *c* [21]. Contrary to the above-mentioned authors, we are therefore forced to conclude that the bc_1 segment containing intact binding sites for cytochrome *c* and/or antimycin persists in the respiratory chain of HUUG25. According to this view, the observed inability of mutant-type cells to denitrify [14] may be connected with the lack of periplasmic cytochromes *c*-550 and cd_1 rather than with any changes in the cytoplasmic membrane.

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