Mutants of *Paracoccus denitrificans* Deficient in *c*-type Cytochromes

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Eight mutants of *Paracoccus denitrificans* deficient in *c*-type cytochrome were detected by their inability to catalyse the conversion of α -naphthol plus dimethyl-*p*-phenylenediamine to indophenol. From the properties of two of these mutants which completely lacked spectrophotometrically detectable *c*-type cytochrome, evidence was provided for the presence of a respiratory pathway which terminates in a cytochrome *o*-like oxidase. The presence of this pathway allows mutants lacking *c*-type cytochrome to grow aerobically, and also explains many of the non-mitochondrial features of the aerobic respiratory chain of the wild-type. None of the mutants had significantly lower levels of *b*-type cytochromes compared with the parental strain, but two mutants were deficient in cytochrome *aa*₃. All of the mutants could reduce nitrate, but four of them were unable to denitrify and these showed poor growth with nitrate as added terminal electron acceptor.

INTRODUCTION

The aerobic respiratory chain of *Paracoccus denitrificans* shows many similarities to the respiratory chain of mammalian mitochondria both in the types of respiratory carriers present and in its sensitivity to inhibitors (John & Whatley, 1975, 1977*a, b*). However, it also shows several features not normally found in mitochondria, some of which may be due to a terminal branching of the aerobic respiratory chain, as found in many other bacteria (see Haddock & Jones, 1977). In particular, it has been suggested that in *P. denitrificans* an *o*-type cytochrome functions as an alternative oxidase to cytochrome *aa*₃, accepting reducing equivalents either from the *b*-type (Haddock & Jones, 1977) or from the *c*-type (Knobloch *et al.*, 1971) cytochromes. However, direct evidence that *P. denitrificans* possesses more than one terminal oxidase has yet to be provided (Lawford *et al.*, 1976).

In the present paper we describe eight mutants of *P. denitrificans* that are deficient in *c*-type cytochromes but are able to grow aerobically at rates similar to those of the parent strain. Two of these mutants completely lack spectrophotometrically detectable *c*-type cytochrome, and their properties provide evidence for an alternative pathway which branches from the cytochrome *c*–cytochrome *aa*₃ pathway on the substrate side of the *c*-type cytochromes.

Mutants were detected by their failure to give a positive ‘Nadi reaction’, as described by Marrs & Gest (1973) for the isolation of respiratory mutants of *Rhodopseudomonas capsulata*. The Nadi reaction: α -naphthol + dimethyl-*p*-phenylenediamine + O₂ → indophenol blue + H₂O, is dependent on the presence of functional *c*-type cytochrome and cytochrome *c* oxidase. Cells containing both these components in an active form stain dark blue (see Keilin, 1966).

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METHODS

Abbreviations. HOQNO, 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide; DAD, diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine); DMPD, *N,N*-dimethyl-*p*-phenylenediamine; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

Bacteria. Strain J-1 is a spontaneous streptomycin-resistant mutant of *P. denitrificans* NCIB 8944. Strain J-1 was used as the parent strain for the Nadi-negative mutants (N1 to N8) and showed no significant difference from the wild-type in its growth characteristics and cytochrome content. All strains were grown at 30 °C on the minimal medium containing succinate as substrate as described by Burnell *et al.* (1975). Aerobic growth was obtained in 2 l Erlenmeyer flasks, containing 500 ml medium without KNO₃ agitated at 180 rev. min⁻¹ in a rotary shaker. Anaerobic growth was obtained in culture vessels filled with medium containing KNO₃ (10.1 g l⁻¹). Minimal agar was prepared by the addition of Oxoid purified agar (1.5%, w/v) and NaHCO₃ (0.1%, w/v, autoclaved separately) to the medium described above. Streptomycin-resistance was tested by streaking cells on to minimal agar containing streptomycin (500 mg l⁻¹, filter-sterilized). Membrane particles were prepared as described by John (1977) but from cultures grown aerobically to mid-exponential phase.

Isolation of Nadi-negative mutants. Strain J-1 was mutagenized with ethyl methanesulphonate by the method of Paraskeva (1977). A 10 ml sample of an anaerobically grown culture of J-1, in late-exponential or early-stationary phase, was sedimented by centrifugation. The pellet was resuspended in 5.0 ml NaH₂PO₄/Na₂HPO₄ buffer (0.2 M, pH 8.0) containing 2% (v/v) ethyl methanesulphonate and incubated for 2 h at 30 °C. This treatment gave a survival rate of 20 to 60% (Paraskeva, 1977). A portion (0.2 ml) was then inoculated into 20 ml minimal medium and grown anaerobically to mid-exponential phase. Samples (0.1 ml) of a 1 × 10⁻⁸ dilution of this culture were then plated on to minimal medium solidified with agar and without nitrate. Colonies (150 to 200 per plate) were allowed to reach a diameter of 1.5 to 2.5 mm and then screened with Nadi reagent, as described by Marrs & Gest (1973). Colonies of J-1 began to stain dark blue within 15 s. Any colonies which failed to stain within 30 s of the addition of Nadi reagent were picked off with a wire loop and streaked on to fresh medium.

Assays. Difference spectra were measured with an Aminco Chance DW-2 double-beam spectrophotometer. The kinetics of cytochrome reduction were measured using the same instrument in the dual wavelength mode. Respiration rates were measured with a Clark-type oxygen electrode. The rates of nitrate and oxygen reduction were determined simultaneously using oxygen and nitrate-ion specific electrodes essentially as described by John (1977). The presence of nitrite in cultures was detected by adding a 1:1 mixture of sulphanilic acid (0.8% in 5 M-acetic acid) and 8-aminonaphthalene-2-sulphonic acid (0.8% in 5 M-acetic acid). A red colouration indicated the presence of nitrite. Protein was determined by the Folin method of Lowry.

RESULTS AND DISCUSSION

Nadi reaction

Eight Nadi-negative mutants (N1 to N8) were isolated from about 30000 colonies of J-1 screened with Nadi reagent. Colonies of N2, N5 and N7 showed no staining for at least 30 min after adding the reagent; colonies of N1, N3, N4, N6 and N8 began to stain between 30 and 60 s after adding the reagent, but stained to a much lower intensity than did colonies of J-1. All the Nadi-negative mutants grew under aerobic conditions at similar rates to J-1 with a doubling time of about 1.1 h.

Spectrophotometry

The Nadi reaction is catalysed by cytochrome *c* + cytochrome *c* oxidase (see Keilin, 1966). We expected, therefore, that some of the Nadi-negative mutants would be deficient in *c*-type cytochrome or in cytochrome *aa*₃. Reduced minus oxidized difference spectra obtained both at room temperature (data not shown) and at 77 K (Fig. 1) revealed that the mutants all contained decreased levels of *c*-type cytochrome compared with J-1 (N6 was unstable and low-temperature spectra were not obtained for this mutant). At 77 K, the α -absorption maxima characteristic of a cytochrome *c* (547 nm) and a cytochrome *c*₁ (550 nm) were both absent from difference spectra of N2 and N7 (Fig. 1). In spectra of the other Nadi-negative mutants (N1, N3, N4, N5 and N8), the relative heights of these two absorption maxima

Fig. 1. Low-temperature dithionite-reduced minus oxidized spectra of membrane particles from aerobically grown mutants of *P. denitrificans*. Particles were suspended in 50 mM-Tris/phosphate (pH 7.3) containing 250 mM-sucrose. The suspension in the sample cuvette was reduced with a few crystals of sodium dithionite and read against a similar suspension in the reference cuvette without additions. Spectra were recorded at 77 K. Particle protein concentrations (mg ml^{-1}) were: J-1, 3.5; N1, 4.1; N2, 3.3; N3, 2.7; N4, 2.6; N5, 3.6; N7, 3.5; N8, 3.2.

were the same as in J-1 ($\Delta A_{550-540}/\Delta A_{547-540} = 1.5$). This suggests that in the mutants isolated in the present work the genetic lesion responsible for the Nadi-negative phenotype affects the levels of both *c*-type cytochromes (*c* and *c*₁) to a similar extent.

The amount of *b*-type cytochrome in the aerobically grown mutants was similar to, or slightly below, that in J-1 (Fig. 1). The two α -maxima characteristic of *b*-type cytochromes, at around 557 nm and 563 nm, were detectable in 77 K spectra of all strains (Fig. 1). The level of cytochrome *aa*₃, indicated by an absorption maximum at about 604 nm, was greatly decreased only in N2 (Fig. 1).

The distribution of cytochromes among the mutants, as revealed by difference spectrophotometry (Fig. 1), was not affected either by replacing dithionite with succinate as reductant, or by using intact cells instead of membrane particles.

Succinate-reduced minus succinate-reduced + antimycin + H_2O_2 spectra were also determined (Fig. 2) to examine the absorption maxima characteristic of *c*-type cytochromes in isolation from the maxima characteristic of the *b*-type cytochromes (Lawford *et al.*, 1976). These spectra revealed two significant differences between mutants N2 and N7 and the parent strain J-1. Firstly, the absence of *c*-type cytochrome from N2 and N7 was confirmed, and secondly, the characteristic antimycin-dependent, oxygen-induced reduction of *b*-type cytochrome (Lawford *et al.*, 1976; John & Papa, 1978), indicated by a trough at 556 nm, was found with J-1, but was absent from the mutants N2 and N7 (Fig. 2).

CO difference spectra obtained at room temperature (Fig. 3) show that the cytochrome *o*-like pigment (see John & Whatley, 1977*b*) found in wild-type *P. denitrificans* (absorption

Fig. 2. Low-temperature succinate-reduced minus succinate-reduced + antimycin + H_2O_2 spectra of membrane particles from aerobically grown cells. Particles were suspended in 50 mM-Tris/phosphate (pH 7.3) containing 250 mM-sucrose. The sample suspension was incubated with 5 mM-sodium succinate for 15 min at room temperature. The reference suspension was incubated with 5 mM-sodium succinate and antimycin [$50 \mu\text{g}$ (mg protein) $^{-1}$] for 15 min at room temperature, then re-oxidized by the addition of 3 μl catalase (Sigma C-100) and 6 mM- H_2O_2 . Spectra were recorded at 77 K. Particle protein concentrations were as in Fig. 1.

maxima at around 420 nm and 575 nm, minima at around 432 nm and 560 nm) was present at higher levels in cells of N2 and N7 than in J-1.

The spectrophotometric evidence is consistent with a scheme (Fig. 4) in which aerobic respiration in mutants N2 and N7 is mediated by an alternative oxidase, possibly cytochrome *o*, which accepts reducing equivalents from a donor on the substrate side of the *c*-type cytochromes. Based on studies of methanol-grown *P. denitrificans*, Van Verseveld & Stouthamer (1978) have recently reached a similar conclusion regarding both the location of the branch point in the aerobic respiratory chain and the relative KCN sensitivities of the two alternative oxidases. Methanol is thought to be oxidized via a soluble *c*-type cytochrome which interacts with the aerobic respiratory chain at the level of the membrane-bound *c*-type cytochrome or cytochrome aa_3 (Van Verseveld & Stouthamer, 1978). In agreement with this view, the Nadi-negative mutant N7, unlike the wild-type, is unable to grow with methanol as sole carbon source (J. C. Willison, unpublished observation). A similar scheme to that of Fig. 4 has been proposed for *Rhodopseudomonas capsulata* (Marrs & Gest, 1973; Zannoni *et al.*, 1976).

Additional evidence for the proposed respiratory scheme

Additional evidence for this scheme is provided by the following observations and considerations.

(i) Cytochrome aa_3 was reduced by substrate on anaerobiosis much more slowly in particles of N7 ($t_{\frac{1}{2}}$ about 90 s) than in particles of J-1 ($t_{\frac{1}{2}} < 1$ s) (Fig. 5).

Fig. 3. Room-temperature CO difference spectra of cells of J-1, N2 and N7, harvested in the exponential phase (a) and the stationary phase (b) of aerobic growth. Cells were suspended in 0.2 M- Na_2HPO_4 (pH 8.0). A suspension of cells ($125 \text{ mg dry wt ml}^{-1}$) was fully reduced with a few crystals of dithionite, and the sample suspension bubbled with CO for 2 min.

Fig. 4. The aerobic respiratory chain of *P. denitrificans*: Q-10, ubiquinone-10; cyt, cytochrome. The dashed line indicates the deletion in mutants N2 and N7.

(ii) Rates of NADH-dependent respiration were lower with particles of N7 than with particles of J-1, although both types of particle gave similar rates of succinate-dependent respiration (Table 1). In these experiments the particles from both N7 and J-1 were determined to be at least 95% inside-out (see Burnell *et al.*, 1975), and thus the difference in the rates of NADH oxidation is not attributable to a difference in the accessibility of the NADH dehydrogenase to NADH. These differences in respiratory rates between N7 and J-1 suggest that reducing equivalents from succinate are preferentially channelled into the alternative pathway of aerobic respiration, which is the major pathway of respiration in N7, while reducing equivalents from NADH are preferentially channelled into the cytochrome *c*-cytochrome *aa*₃ pathway (Fig. 4).

Fig. 5. The reduction of cytochrome aa_3 in membrane particles from aerobically grown J-1 (a) and N7 (b). Particles were suspended to a concentration of $1 \text{ mg protein ml}^{-1}$ in a medium containing $50 \text{ mM-Tris/acetate (pH 7.3)}$, $5 \text{ mM-magnesium acetate}$, $5 \text{ mM-carbonyl cyanide } p\text{-trifluoromethoxyphenylhydrazine}$, $18 \text{ mM-H}_2\text{O}_2$ and $3 \mu\text{l catalase (Sigma C-100)}$. NADH (8 mM), sodium succinate (10 mM) or dithionite (few crystals) were added where indicated, and the reduction of cytochrome aa_3 was followed at 607 nm using a reference wavelength of 630 nm . An upward deflection of the trace indicates reduction.

Table 1. Rates of O_2 uptake with different substrates by membrane particles from aerobically grown *P. denitrificans* strains J-1 and N7

The reaction medium contained $50 \text{ mM-Tris/acetate (pH 7.3)}$, $5 \text{ mM-magnesium acetate}$, $30 \text{ mM-ammonium acetate}$ and $0.17 \mu\text{g gramicidin ml}^{-1}$. Substrate concentrations were: NADH, 2 mM ; sodium succinate, 10 mM ; sodium ascorbate, 20 mM ; TMPD, 0.1 mM ; DAD, 0.1 mM . The rates of oxygen uptake with ascorbate were corrected for the rate of oxygen uptake due to the autoxidation of ascorbate in the absence of particles. The particles used in the two experiments were prepared from different cultures.

Expt no.	Strain	Rate of O_2 uptake [$\mu\text{mol O min}^{-1} (\text{mg protein})^{-1}$]			
		NADH	Succinate	Ascorbate + TMPD	Ascorbate + DAD
1	J-1	6.42	2.61	1.14	0.218
	N7	1.36	2.35	< 0.001	< 0.001
2	J-1	6.40	1.55	1.46	—
	N7	2.50	2.35	< 0.001	—

(iii) As expected from the absence of *c*-type cytochrome, particles of N7 failed to oxidize ascorbate at measurable rates with either TMPD or DAD as mediators (Table 1).

(iv) Antimycin and KCN were more effective as respiratory inhibitors for NADH oxidation by J-1 than for succinate oxidation by J-1 or either succinate or NADH oxidation by N7 (Fig. 6). Conversely, the inhibitor HOQNO proved to be most effective with succinate oxidation by N7 and least effective with succinate oxidation by J-1 (Fig. 6). These results extend to aerobically grown cells the previous suggestion (John & Papa, 1978) that antimycin and HOQNO have different sites of action in the respiratory chain of anaerobically grown *P. denitrificans*.

FIG. 6. The effect of antimycin (a), HOQNO (b) and KCN (c) on oxygen uptake by membrane particles from aerobically grown J-1 and N7. Reaction conditions were as for Table 1. In (c), particles were pre-incubated for 2 min with each concentration of KCN before the addition of substrate. Strain J-1 with succinate (●), NADH (○) and ascorbate plus TMPD (△). Mutant N7 with succinate (■) and NADH (□).

Table 2. *Properties of the Nadi-negative mutants of P. denitrificans*

	Nadi reaction*	Cytochrome content†			Anaerobic growth‡	Denitrification§
		<i>c</i> -type	<i>b</i> -type	<i>aa</i> ₃		
Parent strain (J-1)	+	+	+	+	+	+
Mutants N1	±	(±)	+	+	±	—
N2	—	—	+	(±)	±	—
N3	±	+	+	+	+	+
N4	±	±	+	+	+	+
N5	—	±	+	+	±	—
N6	±	NM	+	+	+	+
N7	—	—	+	±	±	—
N8	±	±	+	+	+	+

* Staining of colonies 10 min after addition of Nadi reagent: +, strong staining; ±, weak staining; —, no staining.

† Cytochrome content of membrane particles relative to J-1, as determined from low-temperature spectra: +, similar to J-1; ±, 10 to 50% of the level of J-1; (±), < 10% of the level of J-1; —, not detectable; NM, not measured.

‡ Growth in minimal medium plus nitrate: +, growth similar to J-1; ±, poor growth compared with J-1.

§ Gas production during anaerobic growth with nitrate as terminal electron acceptor: +, presence; —, absence.

Denitrification

Mutants N1, N2, N5 and N7 were unable to denitrify, as indicated by the absence of observable gas production from nitrate by anaerobically growing cultures of these mutants, which reached a lower turbidity than did cultures of those strains which produced gas. The inability to denitrify is at least partly explained if electron transport to nitrite involves the *c*-type cytochromes (Lam & Nicholas, 1969), but it is also possible that the Nadi-negative mutants are deficient in nitrite reductase, which contains haem *c* (Lam & Nicholas, 1969; Newton, 1969). All the Nadi-negative mutants reduced nitrate to nitrite as indicated by the presence of nitrite in anaerobically grown cultures. A nitrate electrode was used to measure nitrate reduction in cells which were grown aerobically in the presence of nitrate to late-

exponential phase, then incubated unshaken for 45 min to induce nitrate reductase. All the mutants reduced nitrate at similar rates to J-1. Nitrate reduction was rapidly and reversibly inhibited by oxygen, as in wild-type cells (John, 1977). This shows that the mechanism by which oxygen inhibits nitrate reduction operates in the virtual absence of electron transport from the *c*-type cytochromes to oxygen.

The properties of the Nadi-negative mutants are summarized in Table 2.

Conclusions

The scheme in Fig. 4 explains a number of the non-mitochondrial features of aerobic respiration in *P. denitrificans*. These include the observations that (i) succinate respiration is less sensitive than NADH respiration to antimycin (Scholes & Smith, 1968; John & Whatley, 1970), (ii) in coupled respiratory particles from anaerobically grown cells, the P:O ratio is 1.5 with NADH as substrate, but only 0.5 with succinate (John & Whatley, 1970), and (iii) the respiratory control ratios in coupled membrane particles from anaerobically grown cells are lower with succinate than with NADH (John & Whatley, 1977*a*).

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