THE VARIABLE CYTOCHROME CONTENT OF *PARACOCCUS DENITRIFICANS* GROWN AEROBICALLY UNDER DIFFERENT CONDITIONS

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1. Introduction

Paracoccus denitrificans possesses a remarkable number of mitochondrial-like features including the cytochrome composition of its aerobic electron transport chain [1,2]. A lively controversy exists, however, as to whether the number of potential sites of energy conservation operating in heterotrophically-grown aerobic cultures of *Pc. denitrificans* is 3, as in mitochondria (e.g. [3–6]), or 2, as in bacteria like *Escherichia coli* (e.g. [7–10]). A solution to this problem has been suggested, based upon changes in the relative activities of two terminal cytochrome oxidases, the mitochondrial-like cytochrome aa_3 or the *E. coli*-like cytochrome *o*, that would accept reducing equivalents from different donors in the electron transport chain [5]. Both of these oxidases have been identified in membranes derived from aerobically grown *Pc. denitrificans* using reduced + CO minus reduced difference spectra [11–13] although, to date, evidence for cytochrome *o* functioning as a terminal oxidase under these growth conditions is lacking [4,13]. In support of this idea [5], we present here spectral and potentiometric evidence to demonstrate that *Pc. denitrificans* can, following alterations to the aerobic growth conditions, synthesize either cytochrome aa_3 or cytochrome *o* separately as the predominant terminal oxidase and, in addition, that both oxidases can coexist in the cytoplasmic membrane.

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2. Materials and methods

2.1. *Cell types and growth conditions*

Three preparations of *Pc. demitrificans* were used in these experiments and were designated types 1, 2 and 3. Type 1 was *Pc. denitrificans* (NCIB 8944) obtained from the National Culture of Industrial Bacteria, Torry Research Station, Aberdeen AB9 8DG. Initially this strain contained spectrally-detectable cytochrome aa_3 but after prolonged subculturing on rich nutrient medium, a strain (type 1) was obtained which lacked cytochrome aa_3. Growth of this strain was achieved in 2 l conical flasks containing 600 ml growth medium (tryptone, 10 g/l; yeast extract, 5 g/l; KH_2PO_4, 3 g/l; K_2HPO_4, 12 g/l) agitated on a rotary shaker at 30°C. Cells were harvested in the early stationary phase of growth (24 h). Types 2 and 3 were *Pc. denitrificans* (ATCC 13543) obtained from The American Type Culture Collection, Rockville, Md. and grown aerobically in continuous culture in a minimal medium with succinate (0.5%, w/v) as carbon source at 30°C [6]; *Pc. denitrificans* type 2 was grown at a dilution rate of 0.26 h^{-1} and type 3 at 0.37 h^{-1}. The concentration of dissolved oxygen was maintained at > 50% satn.

2.2. *Harvesting and preparation of membrane particles*

The medium used throughout contained: 300 mM

KCl; 5 mM $MgCl_2$; 10 mM 2-(*N*-2-hydroxyethyl-piperazin-*N'*ethyl) ethane sulphonic acid (Hepes); KOH (pH 7.0). Cells were harvested, broken by sonication and membrane particles prepared by differential centrifugation as outlined in [14].

2.3. *Identification of cytochromes by potentiometric redox titration*

The principles in [15] were followed using apparatus constructed by Professor P. B. Garland. Redox potentials were measured with a platinum/calomel combination electrode (Russell pH Ltd, Auchtermuchty, Fife). Absorbance changes in the cytochromes occurring at differently poised E_h values were measured with a scanning single wavelength spectrophotometer, corrected for base-line anomalies using a split-momory transient recorder, and finally displayed on an $X-Y$ pen recorder.

The measuring chamber consisted of an optical cuvette (~6 ml) of 1 cm pathlength sealed with a tightly fitting Teflon bung into which was inserted the electrode, a gas line and a small hole to allow gas to vent and additions to be made. The contents of the cuvette (membrane particles, ~10 mg protein/ml) were stirred continuously and anaerobic conditions were maintained by flushing with ultra-pure argon pre-bubbled through Fiesers' solution. To study the effect of CO on the potentiometric redox titrations of the cytochromes the system was flushed with CO instead of argon.

Redox mediators were added to final conc. 10 μM and were: potassium ferricyanide, E_m +430 mV; quinhydrone, E_m +280 mV (BDH Chemicals Ltd, Poole, Dorset); 1,2-naphthoquinone-4-sulphonate, E_m +215 mV; 1,2-naphto-quinone, E_m +143 mV (Koch Light Laboratories Ltd, Colnbrook, Bucks); trimethylhydroquinone, E_m +115 mV (K and K Labs Ltd, Plainview, New York); phenazine methosulphate, E_m + 80 mV; 2-methyl-1, 4-naphthoquinone, E_m +10 mV (Sigma Chemical Co. Ltd, Poole, Dorset); duroquinone, E_m −5 mV; and 2-hydroxyl-1, 4-naphthoquinone, E_m −145 mV (K and K Labs). Reductive titrations were accomplished with traces of 0.1 M ascorbic acid (+500 mV to +50 mV) or 0.1 M sodium dithionite (+50 mV to −200 mV) and oxidative titrations with traces of 0.1 M potassium ferricyanide. Titration curves were obtained firstly using successive reductions followed by an oxidative cycle. Wavelength pairs used to follow the redox changes in the cytochromes were: cytochrome *b*, 560–575 nm; cytochrome *c*, 550–540 nm; and cytochrome aa_3, 605–625 nm.

2.4. *Other techniques*

Low temperature difference spectroscopy and protein estimations were performed as in [16,17], respectively.

3. Results

Low temperature reduced minus oxidised difference spectra of membrane particles from the three *Pc. denitrificans* cell types grown heterotrophically under aerobic conditions are shown in fig.1. The

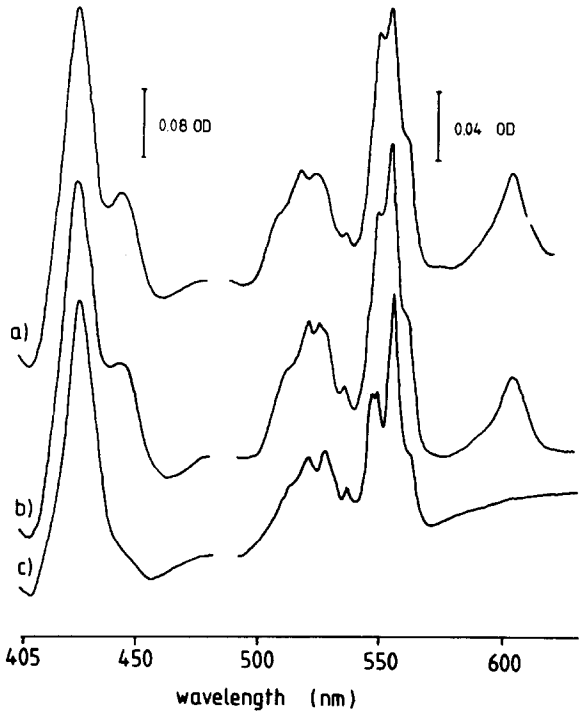

Fig.1. Low temperature difference spectra of membrane particles from *Pc. denitrificans*. Spectra were recorded at 77 K after the following additions had been made to the test and reference cuvettes: sodium dithionite (~1 mM) reduced minus H_2O_2 (2 mM) oxidised. (a) Type 3 particles; (b) type 2 particles; (c) type 1 particles. Protein was 12 mg/ml (trace a,b) or 9.5 mg/ml (trace c).

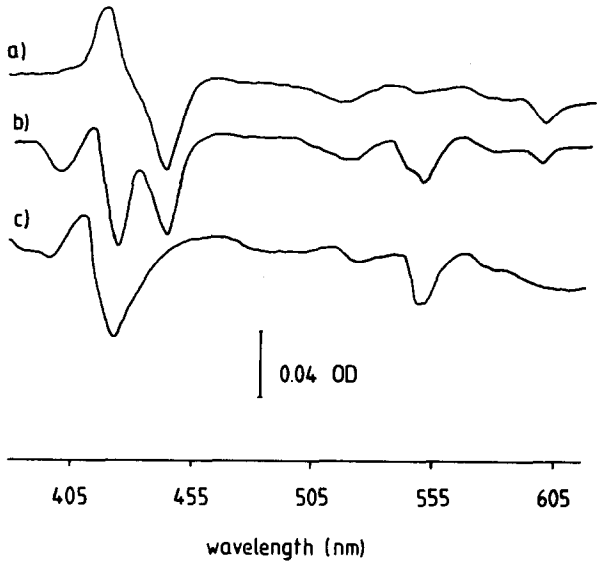

Fig.2. Low temperature CO difference spectra of membrane particles from *Pc. denitrificans*. Membrane particles (18.0 mg protein/ml) were reduced with 2 mM sodium dithionite and the test sample bubbled with O_2-free CO for 5 min. Spectra were recorded at 77 K. (a) Type 3 particles; (b) type 2 particles; (c) type 1 particles.

data indicate minimally the presence of two *c*-type cytochromes with absorption maxima at 546 nm and 549 nm, two *b* type cytochromes with absorption maxima at 556 nm and 562 nm and cytochrome aa_3 with an absorption maximum at 605 nm in membrane particles from type 2 and type 3 cells in accordance with [4]. Membrane particles from type 1 cells (trace c), however, differ in lacking the absorption maxima at 445 nm and 605 nm indicating the absence of cytochrome aa_3 from this preparation and also in the relative absorption of the various *b*- and *c*-type cytochromes.

The difference in the cytochrome oxidase content of the various *Pc. denitrificans* cell types is clearly illustrated in the reduced + CO minus reduced difference spectra reproduced in fig.2. In membrane particles from type 1 cells (trace c) the predominant terminal oxidase is cytochrome *o* (trough at 428 nm) a *b*-type cytochrome capable of reacting with CO when in the reduced form according to the original Castor and Chance terminology [18], whereas in preparations from type 3 cells (trace a) the predominant terminal oxidase is cytochrome aa_3 (trough at 445 nm). Membrane particles derived from type 2 cells (trace b) give spectra indicating the presence of both cytochrome *o* and cytochrome aa_3.

Table 1
Mid-point potentials of the different cytochromes of heterotrophically grown *Pc. denitrificans* under aerobic conditions

Cyto-chromes	Cell type 1		Cell type 2		Cell type 3	
	−CO	+CO	−CO	+CO	−CO	+CO
b	260 (29)	250 (34)	240 (19)	255 (22)	245 (20)	230 (29)
	120 (40)	n.d. (0)	135 (30)	n.d. (0)	n.d. (0)	n.d. (0)
	60 (14)	75 (27)	40 (29)	65 (51)	75 (33)	50 (49)
	−20 (18)	−50 (37)	−60 (18)	−65 (22)	−60 (47)	−50 (21)
c	285 (72)	300 (78)	285 (73)	325 (80)	320 (81)	325 (80)
	190 (27)	225 (21)	175 (25)	205 (19)	210 (19)	225 (19)
a	n.d.	n.d.	350 (66)	360 (100)	355 (63)	350 (100)
a_3	n.d.	n.d.	250 (35)	n.d. (0)	245 (37)	n.d. (0)

Mid-point potentials are expressed in mV and were obtained as described in section 2. The numbers in parentheses indicate the % contribution of each individual component to the total *b*-, *c*-, or *a*-cytochrome absorbance change. n.d. indicates not detectable. Potentiometric redox titrations were performed in the absence (−) or presence (+) of CO

When the cytochrome content of membrane particles derived from the different cell types is analyzed thermodynamically by potentiometric redox titration the following points emerge (table 1):

(i) In appropriate membrane particles (e.g., from type 2 cells), 4 distinct *b*-type cytochromes can be identified with different mid-point redox potentials in contrast to the 2 that have been identified spectrally and kinetically [4,19] or the 3 characterized from the fourth-order finite difference analysis of low temperature spectra [20];

(ii) One of these *b*-type cytochromes, with a mid-point potential of about +120 mV to +135 mV, is absent from membranes derived from type 3 cells (table 1) and is not present when the redox titrations with membrane particles from type 1 or type 2 cells (table 1, fig.3) are performed in the presence of CO suggesting that this component corresponds to cytochrome *o*;

(iii) Two thermodynamically distinct *c*-type cytochromes can be identified in particles from all cell types in accordance with the spectral data (fig.1) but at variance with the kinetic data [4];

(iv) Cytochrome aa_3, present in membrane particles, from type 2 and 3 cells, titrates as 2 components with mid-point potentials of about +250 mV and +350 mV in accordance with previous data [21], and the +250 mV component can be identified with cytochrome a_3 since it disappears when the titration is performed in the presence of CO (table 1, fig.4).

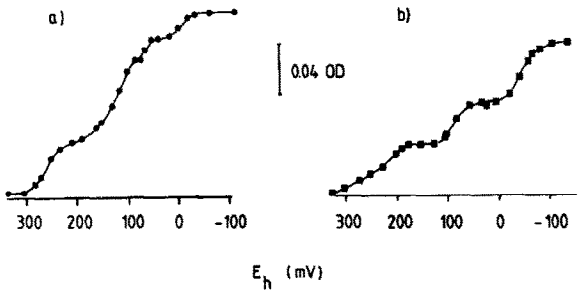

Fig.3. Redox titration of *b*-type cytochromes from type 1. *Pc. denitrificans*. The suspension analysed contained 5 ml membrane particles (18 mg protein/ml) in 10 mM Hepes, 150 mM KCl, 5 mM $MgCl_2$ buffer, pH 7.0, at 22°C. The mediators used and reductive and oxidative titrations were performed as described in section 2. Absorbance was determined as the difference in absorbance between 560 nm and 575 nm. The starting voltage was +460 mV (adjusted with $K_3Fe(CN)_6$). Trace (a) in the absence of CO; (b) after bubbling the particles with O_2-free CO for 15 min.

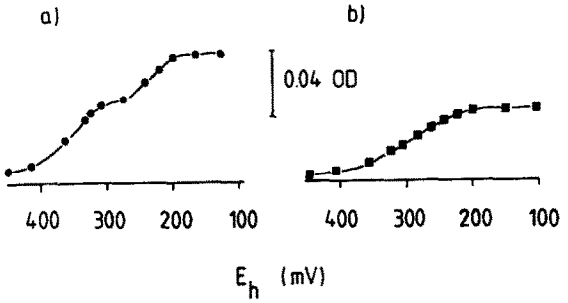

Fig.4. Redox titration of cytochrome aa_3 from type 2 *Pc. denitrificans*. The suspension analysed contained 5 ml membrane particles (18 mg protein/ml) in 10 mM Hepes, 150 mM KCl, 5 mM $MgCl_2$ buffer, pH 7.0, at 22°C. Conditions were as for fig.3 except that absorbance was determined as the difference in absorbance between 605 and 625 nm. (a) Minus CO; (b) after bubbling with O_2-free CO for 15 min.

4. Discussion

The data presented here indicate that the components of the electron transport chain(s) of heterotrophically and aerobically grown *Pc. denitrificans* can alter significantly. Thus the terminal cytochrome oxidase can be:

1. Cytochrome *o* in type 1 cells grown in rich nutrient medium;
2. Cytochrome aa_3 in type 3 cells grown in minimal medium in continuous culture;
3. Both cytochrome *o* and aa_3 can be present simultaneously for example in type 2 cells.

Since it is likely that cytochrome *o* and cytochrome aa_3 terminate electron transport chains with different efficiencies of energy conservation [22] it is clearly important to determine which, or if both, of these oxidases are functioning in cells grown under particular conditions. In the absence of this knowledge measurements of the number of potential sites of oxidative phosphorylation or the site-specific stoicheiometry of respiration driven proton translocation [3–10] remain equivocal.

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