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Changes in cytochrome composition of *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* grown under denitrifying conditions

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Electrophoretic and spectrophotometric techniques have been used to detect changes in the cytochrome composition of *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* during adaptation to denitrifying conditions. During photosynthetic growth with nitrate, the amounts of soluble cytochrome *c* were depressed relative to those found in cells grown without nitrate. This effect was associated with a loss of two soluble, CO- and NO-binding cytochromes, namely cytochrome *c'* and cytochrome *c*-551.5. When cells were grown under denitrifying conditions a 21 kDa *b*-type membrane cytochrome was also not detected, but there was an increase in the amount of another membrane 24 kDa *b*-type cytochrome. A new high potential ($E_{m,7}$ = 252 mV) soluble cytochrome, namely cytochrome *c*-552, was also synthesized under these conditions. The cytochrome composition of either membrane or soluble fractions of *Rps. sphaeroides* f.sp. *denitrificans* grown in light without nitrate, was very similar to that of the wild-type (non-denitrifying) *Rps. sphaeroides*.

Introduction

In common with many of the photosynthetic bacteria *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* can grown aerobically or photosynthetically in the presence of simple organic reductants. In addition this bacterium generates ATP via nitrate respiration [1], and denitrification involves three discrete enzymes: nitrate, nitrite and nitrous oxide reductases with dinitrogen gas as the end product [1,2]. Although nitrate does not serve as a nitrogen source for growth [3] some of the dinitrogen produced by denitrification can be incorporated into cell nitrogen via nitrogenase [4].

Nitrate, nitrite and nitrous oxide reductases of *Rps. sphaeroides* f.sp. *denitrificans* are present at low levels in cells not supplied with nitrate. Their synthesis and activities are enhanced in the presence of nitrate under anaerobic conditions [2].

Nitrate reductase from *Rps. sphaeroides* f.sp. *denitrificans* is a complex protein which probably contains cytochrome *c* ($E_{m,7}$ = +250 mV), and accepts electrons from NADH/FMN via a *b*-type cytochrome ($E_{m,7}$ = +175 mV) [3,5]. Nitrite reductase is a copper protein [6] which accepts electrons from reduced cytochrome c_2 [7]. This soluble cytochrome, reduced by the cytochrome b-c_1 complex also acts as an electron donor to bacteriochlorophyll in a reaction centre [8,9].

Urata and Satoh [10,11] have recently shown that the cytochrome b-c_1 complex involved in photosynthetic electron transfer also donates elec-

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; TMBZ, 3,3',5,5'-tetramethylbenzidine; DCIP, 2,6-dichlorophenolindophenol.

trons to the dissimilatory nitrite reductase via cytochrome c_2 ($E_{m,8} = +309$ mV). They also claim that electrons from the cytochrome b-c_1 complex first reduce NO_2^- to NO via a nitrite reductase and subsequently NO to N_2O via a nitric oxide reductase [11]. However, Satoh [12] has previously reported that NO inhibited the cytochrome b-c_1 complex of the photosynthetic electron transport system. Thus, the precise role of this complex in the denitrification of nitrate remains to be determined. The electron transport system involved in the reduction of N_2O in this bacterium is still not known. This paper reports on changes in cytochrome composition of *Rps. sphaeroides* f.sp. *denitrificans* during adaptation to denitrifying conditions.

Methods

Bacteria and growth conditions

Details of the denitrifying organism, growth conditions were as previously described [2]. Wild-type (non-denitrifying) *Rhodopseudomonas sphaeroides* 2.4.1. was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland. Photosynthetic cultures of the wild-type strain were grown in DL-malate medium as described by Ward et al. (1983). Soluble and 'bulk' membrane fractions were prepared as described by Barrett et al. [13].

Cytochrome purification

Soluble cytochromes were purified from soluble fractions (supernatant fraction 140 000 × g; 2 h) of *Rps. sphaeroides* f.sp. *denitrificans* cells grown photoheterotrophically with or without 20 mM nitrate, according to the method of Meyer and Cusanovich developed for the purification of cytochromes from *Rps. sphaeroides* wild type [14]. The five soluble c-type cytochromes (sphaeroides haem protein, c_2, c', c-551.5, c-554) reported in Ref. 14 were also separated from cells grown without nitrate, but only three of them (c_2, c-554 and *sphaeroides* haem protein) were detected in denitrifying cells. A new soluble cytochrome c-552 has been isolated from those cells (see Results). The cytochromes were adsorbed onto a DEAE-Sephacel column (3 × 15 cm) and were chromatographed using two gradients: first 1–20 mM Tris-HCl (pH 7.4) when only a small amount of *sphaeroides* haem protein was eluted, and then 20 mM Tris-HCl plus 0–200 mM sodium chloride. The cytochromes from cells grown without nitrate were eluted in sequence at the following concentrations of NaCl: *sphaeroides* heme protein (20 mM), cytochrome c_2 – oxidized and reduced forms (35–50 mM), cytochrome c' (60–90 mM) cytochrome c-551.5 (90–120 mM) and cytochrome c-554 (170–500 mM). A minor cytochrome c-551.5 did not form a sharp band on DEAE cellulose and was not well separated from a major cytochrome c'. Therefore, cytochrome c-551.5 fractions obtained from two separate preparations were pooled and rechromatographed on Sephacryl S-200 equilibrated with 100 mM NaCl in 20 mM Tris-HCl (pH 7.4). Cytochrome c-554 was partially eluted from DEAE-Sephacel with 20 mM Tris-HCl containing 170 mM NaCl. More of this cytochrome was obtained by soaking the DEAE-Sephacel in 20 mM Tris-HCl containing 500 mM NaCl for several h (cf. Ref. 14). The cytochromes from cells grown in the presence of nitrate were eluted from DEAE-Sephacel column (gradient 0–500 mM NaCl) in sequence at the following concentrations of NaCl: *sphaeroides* heme protein (20–30 mM), oxidized cytochrome c_2 (a minor fraction, 40–50 mM), reduced cytochrome c_2 (55–65 mM), traces of cytochromes c' and c-551.5 (detected only spectrophotometrically in reduced forms at A_{550}, 70–130 mM), cytochrome c-552 (320 mM) cytochrome c-554 (170–500 mM). A new cytochrome c-552 which was eluted from ion-exchange with 20 mM Tris-HCl containing 320 mM NaCl was contaminated with trailings of cytochrome c-554. These cytochromes, however, were easily separated by gel filtration chromatography (see Results). The various cytochrome fractions were desalted, adsorbed onto DEAE-Sephacel columns (1 × 7 cm) and eluted with 20 mM Tris-HCl (pH 7.4)/500 mM NaCl. Each concentrated cytochrome was then chromatographed on a Sephacryl S-200 column equilibrated with 100 mM NaCl in 20 mM Tris-HCl (pH 7.4). The native size of purified cytochromes were estimated by comparison with marker proteins on Sephacryl S-200, whereas the subunit size were estimated by both sodium and lithium dodecyl sulphate (SDS and LDS, respectively) polyacrylamide gel electro-

phoresis. Molecular characteristics determined for all these purified cytochromes were similar to those reported by Meyer and Cusanovich [14], except that the subunit sizes estimated by LDS electrophoresis (run at 4°C) were slightly different from those obtained by SDS electrophoresis (Table I in Ref. 14) and were as follows: *sphaeroides* heme protein (13.5 kDa), cytochrome c_2 (14 kDa), cytochrome *c'* (12 kDa), cytochrome *c*-551.5 (13 kDa), cytochrome *c*-552 (13.5 kDa), cytochrome *c*-554 (14 kDa). The index purity values (A_{280}/A_{Soret}) obtained for all cytochromes were similar to those reported by Meyer and Cusanovich [14].

Cytochromes *b* and *c* in soluble fractions and in membranes were assayed by difference spectra (dithionite reduced-minus-ferricyanide oxidized) recorded in a Lambda 5 Perkin Elmer spectrophotometer. The millimolar absorption coefficients used were: cytochromes *c*, $A_{551}-A_{540}$, $\Delta\epsilon = 20$ $mM^{-1} \cdot cm^{-1}$; cytochromes *b*, $A_{560}-A_{570}$, $\Delta\epsilon = 20$ $mM^{-1} \cdot cm^{-1}$ [15]. The haem content of purified cytochomes was determined by a pyridine haemochromogen method, using an absorbance coefficient of 31.8 $mM^{-1} \cdot cm^{-1}$ for the α-peak [16].

Spectroscopy

Reduced-minus-oxidized difference spectra of cytochrome fractions were recorded in a Lambda 5 Perkin-Elmer spectrophotometer in which an abscissa calibration was performed automatically at the sharp emission line at 656.1 nm emitted by the deuterium lamp. Cytochrome fractions for spectroscopy were prepared in 25 mM Mops (4-morpholinepropane sulfonic acid) buffer (pH 7.0), containing 100 mM KCl. Low-temperature (77 K) spectra of purified cytochromes (in 50%, w/v glycerol) were recorded in a Shimadzu multipurpose recording spectrophotometer, model MPS-50L fitted with low-temperature spectroscopy attachments. Wavelength-coordinate calibration in this spectrophotometer was routinely tested with holmium oxide and didymium glass filters (Unicam).

Determination of redox midpoint potentials

Oxidation-reduction potentials of purified cytochromes were measured in a stirred cuvette fitted with Pt-Ag|AgCl combination microelectrode (CMMRPt, Russell Laboratory, Scotland), under an atmosphere of oxygen-free nitrogen, using a dual-wavelength Aminco 2A spectrophotometer as described by Dutton [17]. The potential of the reference electrode (Ag|AgCl in saturated KCl) was taken as $E_h = +219$ mV [17]. The buffer, at pH 7.0, was 50 mM Mops containing 100 mM KCl, and the following mediators were added to the reaction mixture (25 μM each): *N,N,N',N'*-tetramethyl phenylenediamine; *N*-methyl phenazonium methosulphate; *N*-ethyl phenazonium methosulphate; 2-hydroxyl-1,4-naphthoquinone (all from Sigma) and anthraquinone 2-sulphonate (Aldrich Chemicals). Reductive titrations were carried out by injecting small amounts of sodium dithionite solution, and oxidative titrations by addition of small amounts of potassium ferricyanide solution. The redox titrations were analysed with a computer programme based on standard non-linear least-square-fitting procedures which calculate optimal parameters to give best fit between experimental and predicted points.

Electrophoresis

Details of sodium dodecyl sulphate polyacrylamide slab gel electrophoresis, sample preparation were as previously described [2]. Laemmli-type polyacrylamide gel electrophoresis of membrane and soluble fractions in the presence of lithium dodecyl sulphate was performed as described by Ward et al. [18]. Gels were analysed for haem-containing proteins with TMBZ/H_2O_2 (haem-dependent peroxidase activity) as described by Thomas et al. [19]. Samples to be analysed for fluorescence of the *c*-type cytochromes (from which Fe had been removed), were prepared and separated by gel electrophoresis as described by Ward et al. [18], except that SDS was substituted for LDS in the electrophoresis gel and buffer. The fluorescent bands in the gels were photographed with a Polaroid 665 film. The negative was then scanned with a double-beam recording and integrating densitometer (Chromoscan MK 11, Joyce Loebl). In each case when gel electrophoresis was performed the gels were calibrated with low-molecular-weight protein standards containing: phosphorylase *b* (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000),

trypsin inhibitor (20 100) and α-lactalbumin (14 400).

Isoelectric points of purified cytochromes were determined by focusing on polyacrylamide slab gel with Ampholines from pH 3.5 to 10 and pH 4 to 6 (LKB, Bromma, Sweden) using a modification of the methods of O'Farrell [20] and Iborra and Buchler [21] as described in Ref. 22.

Enzyme assays

Nitrate reductase (EC 1.7.99.4), nitrite reductase (EC 1.7.2.1) and nitrous oxide (N_2O) reductase activities were assayed spectrophotometrically by a modification of the method of Kristjansson and Hollocher [23] as described previously [2].

Identification of the products of nitrite reductase in vivo

Measurements were carried out under helium, using 6 ml sealed vials containing a suspension (2 ml) of cells in a growth medium (approx. 5 mg dry wt.) and nitrile (10 μmol per mg dry wt.). After incubating at 30°C, in light, samples of gas phase were analysed in a Shimadzu GC 9A gas chromatograph fitted with a column of Poropak Q and a gas sampling device for head space analyses.

Protein content of cell fractions were determined by the method of Bradford [24], using bovine serum albumin as a standard. Nitrite was measured as described by Nicholas and Nason [36].

Results

Effects of growth conditions on cytochrome c and b levels and nitrate and nitrite reductase activities of cells

Both soluble and 'bulk' membrane fractions of photoheterotrophically grown cells of *Rps. sphaeroides* f.sp. *denitrificans* contained higher amounts of *c*-type cytochromes than those of cells shown in light, in the presence of nitrate. The amount of membrane *b*-type cytochromes, however, was not markedly changed by the adaptation of cells to a denitrifying mode of growth (Table I). Low basal activities of nitrate and nitrite reductases were found in photoheterotrophically grown cells, but their activities markedly increased in cells grown in light in the presence of nitrate.

Characterization of soluble cytochromes

Cytochromes in the soluble fraction of denitrifying cells were fully reduced by ascorbate/DCIP and gave no CO-difference spectra (dithionite-reduced plus CO minus dithionite-reduced). Some cytochromes in the soluble fraction from cells grown without nitrate in light, complexed with CO, since a trough was observed at approx. 550 nm in CO-difference spectra. Some of the soluble *c*-type cytochromes in non-denitrifying cells had low redox potentials, since dithionite reduced minus ascorbate/DCIP reduced difference spectra showed a peak at approx. 550 nm. The pronounced CO-binding was only observed when soluble fractions from non-denitrifying cells were reduced with dithionite. The ascorbate/DCIP reduced fractions did not produce CO-binding effects, hence the *c*-type cytochrome responsible for these observations had a lower redox potential from that recorded with ascorbate/DCIP thus lower than approx. +100 mV.

In addition to cytochromes c_2 and c', the major soluble electron transfer components in *Rhodopseudomons sphaeroides* (wild type) [8] were the three minor soluble *c*-type cytochromes (*sphaeroides* haem protein, *c*-551.5 and *c*-554) which have been well characterized. The same five soluble cytochromes have been isolated from cells of *Rps. sphaeroides* f.sp. *denitrificans* grown photoheterotrophically without nitrate. Their molecular properties were very similar to those reported recently [14]. However, only *sphaeroides* haem protein, c_2, *c*-554, as well as a new cytochrome *c*-552, were detected in cells grown in the presence of 20 mM nitrate. Neither cytochrome c' (a high-spin CO-binding haem protein, spectrum a in Fig. 1) nor cytochrome *c*-551.5 (a low-spin CO-binding haem protein, spectrum b in Fig. 1) were present in denitrifying cells.

Potentiometric titration at $A_{551}-A_{540}$ of those major cytochromes indicated the difference in their redox midpotentials (cf. Ref. 14): cytochrome c' had an E_m value of +11 mV at pH 7.0, whereas cytochrome *c*-551.5 (spectrum b in Fig. 1) had a much lower potential of −247 mV, at 7.0. Cytochrome c_2 (spectrum d in Fig. 1), in our hands had a potential at pH 7.0 of +292 mV, which is similar to that previously reported [10,14]. A new *c*-type cytochrome found only in the soluble frac-

TABLE I

EFFECTS OF GROWTH CONDITIONS ON THE AMOUNTS OF *c*- AND *b*-TYPE CYTOCHROMES AND DENITRIFYING ENZYMES ACTIVITIES IN *RPS. SPHAEROIDES* F.SP. *DENITRIFICANS*

Cytochromes were quantified from difference spectra; dithionite-reduced minus ferricyanide-oxidized, as described in Methods. BVH, reduced benzyl viologen

Growth conditions (anaerobic in light)	Cytochrome content ($n = 4$) (nmol per mg protein ± SD)			Washed cells, BVH-linked NO_3^- and NO_2^- reductase activities; (nmol BVH oxidised per min per mg dry wt. ± SD) substrate added (1 mM); $n = 6$	
	soluble *c*-type	'bulk' membrane			
		c-type	*b*-type	NO_3^-	NO_2^-
Without nitrate	1.20 ± 0.10	0.30 ± 0.05	0.41 ± 0.07	14 ± 5	31 ± 7
With nitrate (20 mM)	0.50 ± 0.06	0.14 ± 0.06	0.38 ± 0.06	954 ± 56	435 ± 24

tion of denitrifying cells, namely cytochrome *c*-552 (spectrum in Fig. 1) had a potential at pH 7.0 of +252 mV. After storage for a few days at 4°C the reduced α-peak of this cytochrome shifted to a shorter wavelength, viz. 550.8 nm. However, neither the relative intensities of the absorption peaks, nor the redox midpotential were affected by this change. The redox reactions were completely reversible and the Nernst equation gave an *n*-value of 1.0 for all these cytochromes. The isoelectric points, determined in polyacrylamide gels, in the presence of 6 M urea, were 5.5 (cytochrome c_2), 4.8 (cytochrome *c'*), 4.3 (cytochrome *c*-551.5), 4.2 (cytochrome *c*-552) and 4.1 (cytochrome *c*-554). The native size of cytochrome *c*-552, estimated by comparison with other marker proteins on Sephacryl S-200, was 13.5 kDa and the subunit size, estimated by the SDS/LDS polyacrylamide gel methods was also 13.5 kDa. This cytochrome was easily separated from a minor cytochrome *c*-554 by gel filtration, since the native size of the latter one was 44 kDa. The separation of these cytochromes by focusing on polyacrylamide gel with a pH gradient from 4 to 6 is shown in Fig. 2.

Cytochromes *c'* and *c*-551.5 complex with CO and their CO-binding difference spectra recorded at 77 K are shown in Fig. 1. The reduced form of both cytochromes also complex with NO under anaerobic conditions and their NO-difference spectra (not shown) are similar to those recorded in the presence of CO (Fig. 1). Purified cytochrome *c*-552 did not complex with either CO or NO, and the reduced form of this cytochrome had a spectrum typical of a low-spin *c*-type cytochrome (Fig. 1).

Examination of CO- and NO-difference spectra of soluble fractions grown photosynthetically with

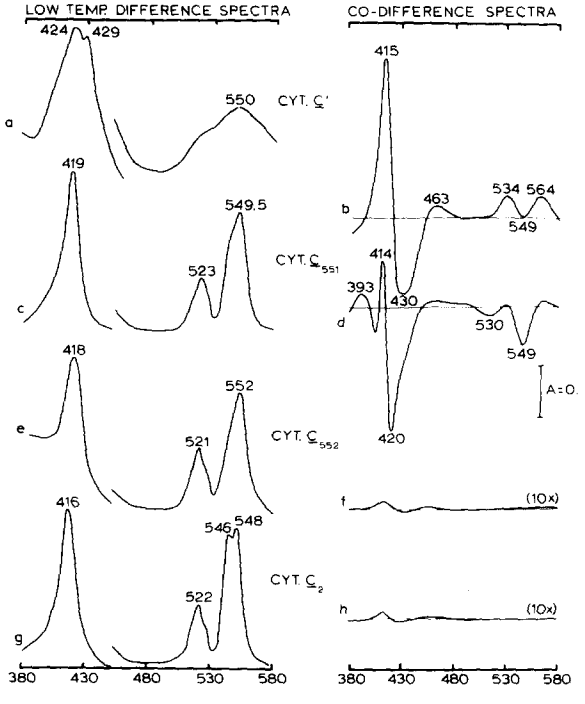

Fig. 1. Low temperature (77 K) difference spectra of purified soluble *c*-type cytochromes. (a, b) cytochrome *c'*; (c, d) cytochrome *c*-551.5; (e, f) cytochrome *c*-552; (g, h) cytochrome c_2. (a), (c), (e) and (g) dithionite reduced-minus-ferricyanide oxidized spectra; (b), (d), (f) and (h) dithionite reduced plus CO-minus-dithionite reduced spectra. Note that spectra (f) and (h) are shown at a 10-fold gain.

nitrate at different stages of growth showed that CO-binding declined dramatically over the early exponential phase of growth (approx. 30% present at 6–7 h of growth; CO-binding effect was almost not detectable after 10 h growth). This CO-complexing reaction coincided with a massive accumulation of nitrite in the medium (up to 5 mM) from 20 mM nitrate supplied initially. In cells harvested at the late exponential phase of growth,the contents of the CO- and NO-binding soluble cytochromes c were reduced by 90%. However, these cells also contained very small amounts of CO-binding *sphaeroides* haem protein which may account for the feeble CO-complexing reaction in denitrifying cells. The soluble fractions prepared from denitrifying cells did not contain any nitrite. If present its subsequent reduction to NO by sodium dithionite would of course interfere with the CO-binding properties of the reacting cytochromes.

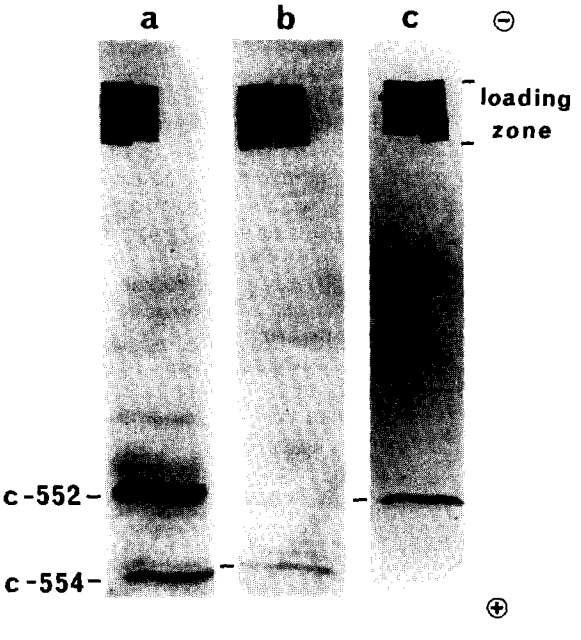

Fig. 2. Separation of cytochromes *c*-554 and *c*-552 by focusing on 5% w/v, polyacrylamide gel with a pH gradient from 4 to 6. Cytochromes were purified from a soluble fraction of cells grown in the presence of nitrate as described in Methods. (a) Fraction containing both cytochromes eluted from DEAE-Sephacel column (40 μg protein), (b) cytochrome *c*-554 and (c) cytochrome *c*-552 purified by gel filtration method (15 μg protein).

Detection of soluble and membrane cytochromes by haem-staining after LDS-electrophoresis

Substitution of lithium dodecyl sulphate for the sodium salt employed in Laemmli-type electrophoresis allows solubilization and electrophoresis to be conducted at 4°C. Staining for peroxidase activity under mildly acid conditions detects both *b*- and *c*-type cytochromes [18,25]. The application of this technique to soluble and 'bulk' membrane preparations from cells grown under various conditions, is shown in Fig. 3.

Soluble cytochromes

At least eight positively stained cytochrome bands (1–9) were detected in soluble fractions of *Rps. sphaeroides* (Fig. 3g), and in *Rps. sphaeroides* f.sp. *denitrificans* grown without nitrate (Fig. 3h). Since the separation of the cytochromes was performed under rather mild conditions (presence of LDS and low temperature) the haem-staining proteins correspond to both native and denatured forms of the six soluble cytochromes described recently (cf. Table I in Ref. 14). Thus, bands 1 (44 kDa) and 6/8 (14–12 kDa) represent various forms of cytochrome *c*-554; bands 2 (25 kDa) and 6/8 of cytochrome *c'*; bands 3 (21 kDa), 6/8 (14–12 kDa) and 9 (9 kDa) of cytochrome *c*-551.5; band(s) 6/8 of cytochrome c_2 and *sphaeroides* haem protein, and band 5 (17 kDa) of cytochrome *b*-558 (Table II and Fig. 4). The individual cytochromes purified from the soluble fraction gave the same separation pattern on LDS gels-haem staining (Fig. 4). In soluble fractions of *Rps. sphaeroides* f.sp. *denitrificans* grown with nitrate however, only three positively stained bands were found (1, 5 and 6/8) and bands corresponding to either cytochrome *c'* or cytochrome *c*-551.5 were not detected (Fig. 3i). This phenomenon is well seen in gels loaded with smaller protein samples which usually give much better separation of small molecular weight polypeptides (Fig. 3, k–m). Again, two of the three major soluble cytochromes *c'* and *c*-551.5 were not detected in the gel. Only trace amounts of these cytochromes were obtained during the fractionation of the soluble fraction from cells grown under denitrifying conditions (see Methods). Since the new cytochrome *c*-552 has an apparent molecular weight identical to that of cytochrome c_2 (13.5 kDa, Fig. 4) only one band appears in the

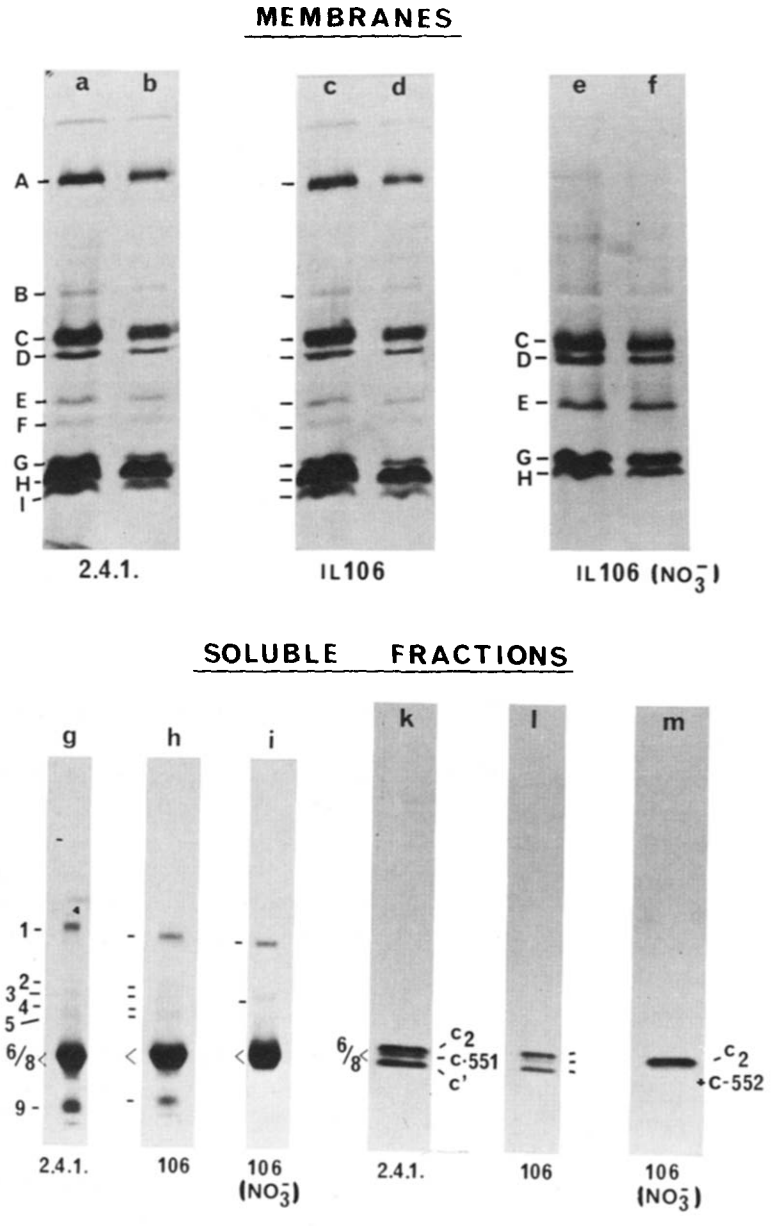

Fig. 3. LDS-polyacrylamide gradient gel electrophoresis (10–15% w/v) of membrane and soluble fractions from *Rps. sphaeroides* wild-type 2.4.1 (a, b, g, k) and *Rps. sphaeroides* f.sp. *denitrificans* IL106 (c–f, h, i, l, m). The photograph shows the pattern of haem-containing proteins found after LDS-solubilization of 'bulk' membranes (a–f) or soluble fractions (g–m) prepared from cells grown under the following conditions: (a) and (b) membranes from the 2.4.1 strain grown photosynthetically (80 and 40 μg protein); (c) (d) membranes from the IL106 strain grown photosynthetically (80 and 40 μg protein); (e) (f) membranes from the IL106 strain grown in light in the presence of 20 mM nitrate (80 and 40 μg protein); (g) (k) soluble fraction from the 2.4.1 strain (120 and 40 μg protein); (h) (l) soluble fraction from the IL106 strain (110 and 30 μg protein), (i) (m) soluble fraction from the IL106 strain grown in light with nitrate (110 and 30 μg protein).

gel representing both cytochromes in the soluble fraction from denitrifying cells (Fig. 3, m).

Membrane cytochromes

Nine positively stained cytochrome bands (A-I) were detected in membrane fractions of photoheterotrophically grown cells of *Rps. sphaeroides* f.sp. *denitrificans*, but only six of them were detected in membranes of cells grown in the presence of nitrate. Under these conditions the high-molecular weight haem-staining protein (band A), and 40 kDa and 21 kDa *b*-type cytochromes (bands B and F) were not present. There was, however, an increase in the amount of another 24 kDa *b*-type cytochr(band E). Since the breakage of cells of *Rps. sphaeroides* by either sonication or French pressure cell, resulted in the formation of sealed membrane vesicles, which contain entrapped soluble *c*-type cytochromes, we consider that the presence of low-molecular-weight cytochromes (bands

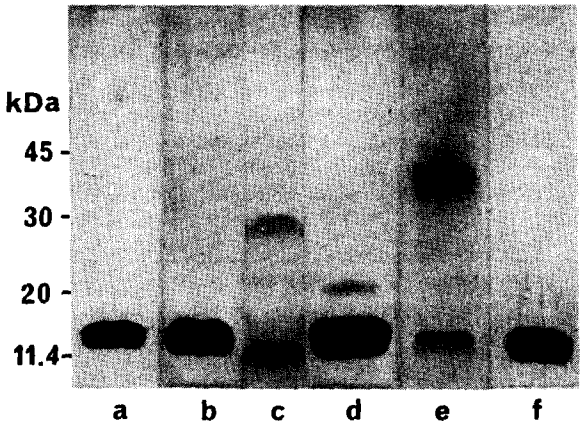

Fig. 4. LDS-polyacrylamide gradient gel electrophoresis (10–13%, w.v) of purified soluble cytochromes. (a) *sphaeroides* haem protein, (b) cytochrome c_2, (c) cytochrome *c'*, (d) cytochrome *c*-551.5, (e) cytochrome *c*-554, (f) cytochrome *c*-552. The photography shows the pattern of haem-staining protein after LDS solubilization (20 μg protein each).

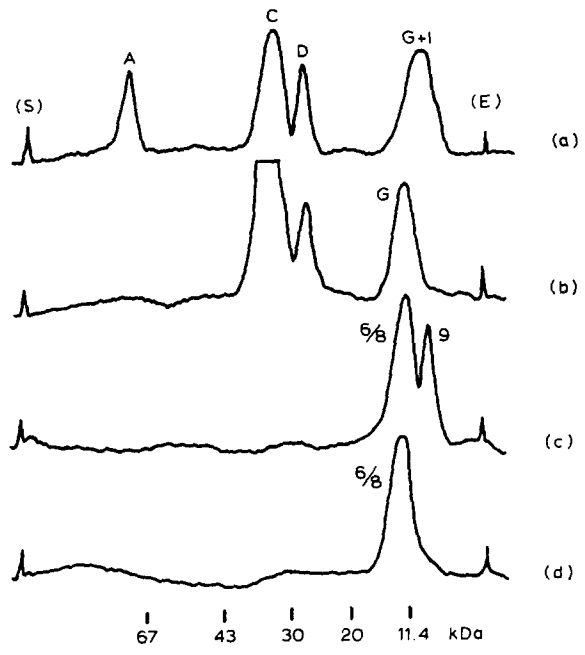

Fig. 5. Red-fluorescent bands given on LDS gels (10–15%, w/v) of membrane and soluble fractions from *Rps. sphaeroides* f.sp. *denitrificans*. The figure shows a densitometer scan of a photographic negatives exposed for red-fluorescence from LDS-polyacrylamide gel electrophoretograms (a) and (b) membranes (approx. 450 μg protein) from cells grown without and with nitrate, (c) and (d) soluble fractions (150 μg protein) from cells grown without and with nitrate. Band assignments (A, C, D, G. and I; membrane cytochromes; 6/8 and 9, soluble cytochromes) were based on comparison of the fluorescent gel data with results from haem-staining after LDS electrophoresis. S, start of resolving gel; E, gel ends.

G and I) in membrane fractions were those of soluble cytochromes c_2 and *c'* in non-denitrifying cells, and c_2 and *c*-552 in cells grown with nitrate.

Detection of membrane cytochromes c by fluorescence of demetallated cytochrderivatives after SDS-electrophoresis

Wood [26] has described a method for the removal of haem iron from *c*-type cytochromes to yield strongly fluorescent demetallated porphyrin derivatives. Excitation of the electrophoretically separated porphyrin derivatives with near ultraviolet light allows their detection by red-fluorescence.

Comparison of results from SDS-fluorescent gel electrophoresis (Fig. 5) and from LDS electrophoresis followed by haem-staining enabled us to designate components resolved on gels to either cytochromes *b* or *c* (Table II).

Comparison of the membrane cytochrcomposition in Rps. sphaeroides f.sp. denitrificans with that of wild-type Rps. sphaeroides

Ward et al. [18] have analysed the cytochrome composition of *Rps. sphaeroides* grown aerobically, photosynthetically and with dimethyl sulphoxide as an electron acceptor using LDS/haem staining and SDS-fluorescent gel techniques. *Rps. sphaeroides* f.sp. *denitrificans* is considered to be closely related to wild-type *Rps. sphaeroides* [1]; thus the cytochrome composition of these two strains was compared by LDS electrophoresis (Fig. 3, lanes a, b, g and k for a wild-type). Since accurate molecular-weight estimates for the membrane cytochromes of the wild-type strain have been published [14,18], values for these components from the denitrifying strain were derived from these data (Table II). Molecular-weight estimates for haem-staining bands from LDS gel data were slightly lower than those derived from SDS-gel data (Table II). Minor changes in apparent molecular weight associated with changes in solubilization temperature have been reported [18,27]. As shown in Fig. 3 and Table II four *b*-type (one soluble) and seven *c*-type cytochromes

TABLE II

ELECTROPHORETICALLY SEPARATED POLYPEPTIDES OF DENITRIFYING *RPS. SPHAEROIDES* F.SP *DENITRIFICANS* THAT EXHIBIT HAEM-DEPENDENT PEROXIDASE ACTIVITY

The results are a summary of several gel electrophoretic experiments. The apparent molecular weights of numbered proteins were determined by LDS polyacrylamide gel electrophoresis run at 4°C (see Methods), except where indicated by an asterisk (*). This was achieved by comparison of their relative electrophoretic mobilities with protein markers specified in Methods. Treatment of the membranes with SDS at 100°C resulted in a small change in the electrophoretic mobility of the low molecular weight components*. n.d., not determined. SHP, *sphaeroides* haem protein.

Band	Apparent molecular mass (kDa)	Haem-staining activity	Red fluorescence after Fe^{2+} displacement from haem	Assignment
Soluble fraction				
1	44	+ + +	n.d.	*c*-554 (native)
2	25	+ +	n.d.	*c'* (native)
3	21	+	n.d.	*c*-551.5 (native)
4	18/17 *	+	n.d.	*c*-551.5
5	17/15 *	+	n.d.	*b*-558
6/8	12–14	+ + + +	+ + +	mainly: c_2, *c'*, SHP, *c*-552
9	9	+ +	n.d.	*c*-551.5
Membranes				
A	~ 90	+ + +		*b*-c_1 complex
B	40	+		*b* (*b* of *b* c_1)
C	33	+ + +	+ + +	*c* (c_1)
D	31	+ + +	+ +	*c*
E	24	+ +		*b*
F	21	+		*b*
G	13.5/14.5 *	+ + +	+ +	*c* (c_2, *c*-552)
H	12.5	+ + +	+ +	*b*
I	11.0/12.5 *	+ + +	+ + +	*c'*

(including five soluble ones) were detected in both bacterial strains (cf. Refs. 14 and 18). In addition, LDS-solubilized membrane preparations from photoheterotrophically grown cells of both strains gave an intense haem-staining band of high molecular weight (approx. 90 kDa, band A Fig. 3, lanes a–d) which was not found in membranes from cells of *Rps. sphaeroides* f.sp. *denitrificans* grown in light in the presence of nitrate (Fig. 3, lanes e and f).

CO- and NO-binding by membrane cytochromes

CO- and NO-difference spectra of membranes obtained from cells of *Rps. sphaeroides* f.sp. *denitrificans* grown in light without nitrate (Fig. 6a and b) indicate that sof the *c*- and *b*-type membrane cytochromes complex with CO and NO (troughs are observed at 550 and 560 nm). However, when the cells were grown in light, in the presence of nitrate, the amount of CO-binding membrane cytochromes, especially the *b*-types, was markedly reduced (Fig. 6c). Moreover, the membranes obtained from these cells did not contain the NO-binding *b*-type cytochrome(s) (Fig. 6d). These results indicate that the 40 and/or 21 kDa *b*-type cytochromes, which are not synthesized in cells grown under denitrifying conditions (Fig. 3), are the ones that bind NO.

Production of NO during denitrification

Under denitrifying conditions nitrate is normally reduced to dinitrogen via nitrite and nitrous oxide (N_2O). It has also been shown that N_2O is a product of nitrite reductase in vivo [6]. However, when the activity of N_2O reductase was completely inhibited by C_2H_2 (10%, v/v) the reduction of nitrite in vivo [10 μmol per mg dry wt.) resulted in a rapid accumulation of N_2O (3 μmol/h

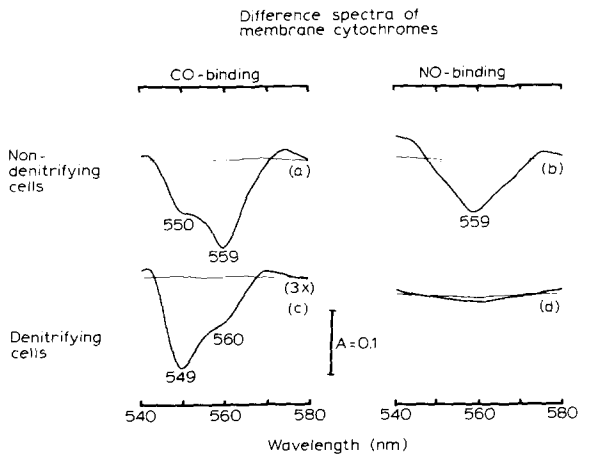

Fig. 6. CO- and NO-binding by membrane cytochromes in *Rps. sphaeroides* f.sp. *denitrificans* (a, c) dithionite reduced plus CO-minus-dithionite reduced difference spectra; (b, d) dithionite reduced plus NO-minus-dithionite reduced difference spectra. Membrane fractions were obtained from cells grown photoheterotrophically without nitrate (a, b) and with 20 mM nitrate (c, d). Note that spectrum (c) is shown at a 3-fold gain. NO-difference spectra of membrane fractions were recorded under anaerobic conditions (after sparging with argon) in 1 cm glass cuvettes sealed with rubber septa.

per mg dry wt.) followed by the production of nitric oxide (NO) (approx. 0.1 μmol/h per mg dry wt.). The cytochromes, which are not synthesized in cells grown with nitrate, complex with NO. Thus, it is likely that their production would affect the utilization of NO should it be produced during denitrification by *Rps. sphaeroides* f.sp. *denitrificans*. It is not established, however, whether NO is a physiological intermediate in denitrification in this bacterium.

Discussion

Soluble fractions from non-denitrifying cells of *Rps. sphaeroides* f.sp. *denitrificans* contained five *c*-type cytochromes [14] two of which (cytochromes *c'* and *c*-551.5) were not detected in the corresponding fractions from denitrifying cells (Fig. 3). These cytochromes purified to homogeneity have isoelectric points of 4.8 and 4.3, respectively. These characteristics and redox potential data indicate that they are very similar to those purified from wild-type *Rps. sphaeroides*. In our hands cytochrc_2 had a lower redox potential ($E_{m,7}$ = 292 mV) than that reported recently ($E_{m,8}$ = 309 mV in Ref. 10 and $E_{m,7}$ = 356 mV in Ref. 14). Both cytochrome *c'* and cytochrome *c*-551.5 were found to be CO- and NO-binding *c*-type cytochromes and were not detected in denitrifying cells. Low-potential (−250 mV at pH 7.0, cf. Ref. 14) cytochrome *c*-551.5 is similar to cytochrc_3 isolated from sulphate-reducing bacteria [8,31]. All the cytochromes c_3 preparations from photosynthetic bacteria are about 20 kDa, but the molecular weight per haem ranges from 10 to 14 kDa [8]. Thus, these cytochromes c_3 have similar properties to those of the cytochrome *c*-551.5 described in this paper (see Methods, Fig. 4 and Table II).

Membranes from denitrifying cells contained *c*-type cytochromes, designated bands C, D and G (Fig. 3), with molecular weights of 33, 31 and 14 kDa, respectively. By analogy with other photosynthetic bacteria, band C is probably associated with cytochrc_1; the molecular weight of this compound from the wild-type strain was found to be 34 kDa [18]. Cytochrome *b* of the cytochrome *b*-c_1 complex in the wild-type strain has an apparent molecular weight of this compound from the wild-type strain was found to be 34 kDa [18]. Cytochrome *b* of the cytochrome *b*-c_1 complex in the wild-type strain has an apparent molecular weight of 43 kDa [28], but smaller values for this cytochrhave also been reported (21 kDa, Ref. 18; 16 kDa, Ref. 29). Since *Rps. sphaeroides* f.sp. *denitrificans* has no membrane cytochromes of this molecular weight (40 kDa) it is likely that denitrifying cells contain only trace amounts of this cytochrome, since the electrophoretic separation of membranes from these cells did not show a high-molecular band associated with the cytochrome *b*-c_1 complex (Fig. 3 and Ref. 18). Although an aggregated form of *b*-c_1 complex was not detected in the gels (except cytochrome c_1) it is likely that this complex is synthesized in denitrifying cells. The identification of the cytochr*b* in gels, however, is very difficult, since this cytochrin particular is present in various aggregation states [29]. The very pronounced band C may contain more than one cytochrome, thus the estimation of the amount of cytochrome c_1 associated with this band could be erroneous. The membrane proteins were separated by preparative LDS-electrophoresis (6 mm thick gels were used

to allow for loading of larger membrane samples). The high molecular weight (Band A in Fig. 3) component was then extracted at 4°C from the gel slices with 1 ml of the electrophoresis running buffer containing 0.1% w/v LDS, and characterized by LDS-electrophoresis on 11–20% w/v gradient, 2 mm thick slab gels. The separation of this sample gave a single high-molecular weight, haem-staining band on the gel (Fig. 7, lanes a and d). However, when the sample was pre-heated in boiling water for 90 s it gave rise to four additional polypeptides with apparent molecular weights of 38, 29, 23 and 10 kDa (Fig. 7, lane b). Subsequently, two of the separated proteins (38 and 30 kDa) were identified as cytochromes by a haem-staining procedure (Fig. 7, lane e). Thus, this electrophoretic separation pattern closely resembles the polypeptide composition of a highly purified cytochrome b-c_1 complex isolated from *Rps. sphaeroides* [28].

The cytochrome b-c_1 complex has been isolated from denitrifying cells of *Rps. sphaeroides* f.sp. *denitrificans*, but no quantitative data for this complex was given [10]. This complex also had approx. 40% lower electron-transfer activity (cytochrome c_2 reduction) than a similar preparation from wild-type cells of *Rps. sphaeroides* [32]. Furthermore, it has been shown [33] that the presence of nitrite in photosynthetically grown cultures of *Rps. sphaeroides* f. sp. *denitrificans* resulted in a drastic reduction of the contents of both photopigments and the reaction-centre complexes, as well as a loss of 12 kDa subunit of the B-870 light-harvesting complex. This in turn may also reflect a lower concentration of cytochrome b-c_1 complexes in these bacteria.

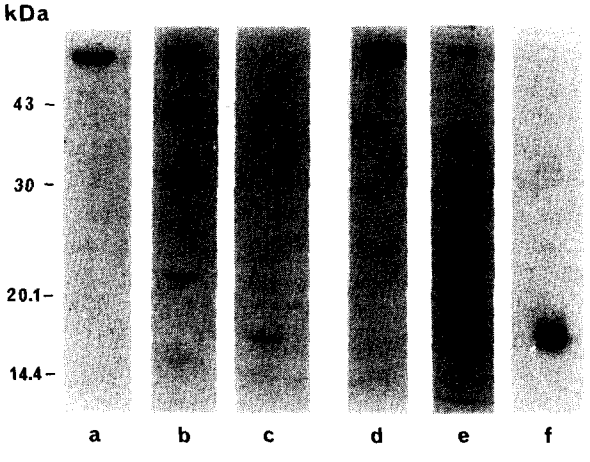

Fig. 7. Polypeptide composition of high molecular weight haem-containing protein complex (band A in Fig. 3). Lanes (a–c) and (d–f) represent gels stained with Coomassie Blue and with TMBZ/H_2O_2, respectively. (a) and (d) band A protein extracted from LDS-gel (Fig. 4) with electrophoresis running buffer and re-run on 11–20% (w/v) LDS-gel, (b) and (c) band A protein extracted as in (a) and (d), then heated for 90 s at 100°C and run in similar LDS-gel; (c) and (f) purified cytochrome c_2.

Urata and Satoh [10,12] have reported that the cytochrome b-c_1 complex is involved in an electron transfer to nitrite reductase. It has been shown, however, that NO (produced during denitrification, cf. Ref. 12 and this article) inhibits the photosynthetic cyclic electron transfer through the cytochrome b-c_1 region as well as the reduction of nitrite and NO in both intact cells and chromatophores, with succinate as an electron donor [10,11]. Thus, the precise role of this complex in denitrification linked electron transfer remains to be determined. When cells were grown under denitrifying conditions a 21 kDa CO- and NO-binding b-type cytochrome was not detected, but there was an increase in the amount of another 24 kDa of the b-type. Yokota et al. [5] have shown that a membrane cytochr$b$-560 ($E_{m,7}$ + 175 mV) reduced with NADH and FMN can be oxidized by nitrate in chromatophore membranes. Thus, it is likely that the 24 kDa cytochrome b (its synthesis enhanced in denitrifying cells) is the cytochrinvolved in electron transfer to nitrate reductase. On the other hand, it is possible that the 21 kDa CO- and NO-binding membrane cytochrome b (band F in Fig. 3 and Table II), not detected in denitrifying cells, corresponds to cytochrome o. This cytochris usually present during photoheterotrophic growth so that the cells can utilize traces of oxygen [8,30]. Denitrifying cells contained substantial amounts of a new soluble c-type cytochrome, namely cytochrome c-552. Its molecular size (13.5 kDa), spectral properties (α-peak 551–552 nm) and redox potential (252 mV) are almost identical to those of cytochrome c which donates electrons to nitrate reductase in *Rps. sphaeroides* f. sp. *denitrificans* [3,5]. A functional association between a soluble cytochr$c$ and a nitrate reductase has been also shown in halo-

tolerant *Micrococcus* grown anaerobically in the presence of nitrate. In this bacterium reduced cytochrome *c*-554 (I) compared with other cytochrome components was the most effective electron donor for nitrate reductase [35]. These cytochromes of *c*-type are known to link cytochrome *b* to the molybdenum-active centre of nitrate reductase [3,5,35].

A hierarchy of respiratory control is established in sfacultative anaerobes whereby terminal electron acceptors of more positive redox potential (hence, more favourable energetically) repress electron/hydrogen transfer systems of more negative potentials [34]. Thus in *Escherichia coli*, fumarate dehydrogenase is repressed by nitrate, and nitrate reductase is repressed by oxygen [34]. According to this model, when nitrate and nitrite (which have positive redox potentials) are terminal electron acceptors in *Rps. sphaeroides* f.sp. *denitrificans* the synthesis of proteins that function with acceptors of more negative redox potentials, e.g., cytochromes *c'* and *c*-551.5 is repressed in denitrifying cells.

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