The adaptation of *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* for growth under denitrifying conditions

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The adaptation of cells of *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* for growth under denitrifying conditions in the light has been studied. The presence of nitrate in photosynthetically grown bacterial cultures resulted in a drastic reduction of carotenoid and bacteriochlorophyll contents as well as a loss of one of the polypeptides of the light harvesting complex, resulting in colour changes. Denitrifying cells had high activities of nitrate, nitrite and nitrous oxide reductases. The polypeptides corresponding to subunits of these enzymes were separated by PAGE. Synthesis of these enzymes was studied by pulse-chase labelling techniques. Nitrate and nitrite reductases are constitutive enzymes and it is likely that copies of mRNA for synthesis of these enzymes are 'long lived' in the cells.

**INTRODUCTION**

The purple non-sulphur photosynthetic bacteria are remarkably versatile in that they can grow under either light/anaerobic or dark/aerobic conditions (Madigan et al., 1979). In addition to these two distinct growth regimes, the denitrifying phototrophic bacterium *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* can also generate ATP by nitrate respiration (Satoh et al., 1976; Satoh, 1981). Denitrification in this bacterium presumably involves a three (or four) step reduction of nitrate to dinitrogen, as in other denitrifying bacteria (Payne, 1973; Averill & Tiedje, 1982). The pathway from nitrate to nitrogen gas is summarized below (Payne, 1973).

\[
\begin{align*}
\text{NO}_3^- & \xrightarrow{R_1} \text{NO}_2^- & \xrightarrow{R_2} (\text{NO}) & \xrightarrow{R_3} \text{N}_2 \\
\end{align*}
\]

Thus far only nitrate reductase \((R_1)\) and nitrite reductase \((R_2)\) have been purified and partially characterized from *R. sphaeroides* f. sp. *denitrificans* (Satoh et al., 1976; Sawada & Satoh, 1980; Satoh, 1981; Kundu & Nicholas, 1983), but the activity of nitrous oxide reductase \((R_3)\) has been recently detected in this bacterium (Urata et al., 1982). Nitrous oxide reductase \((R_4)\) has been purified from *Paracoccus denitrificans* (Kristjanson & Hollocher, 1980) and various pseudomonads (Zumft & Matsubara, 1982; Matsubara & Zumft, 1982).

In general, the denitrifying enzymes are induced by nitrate under anaerobic conditions (Whatley, 1981), since oxygen suppresses the production of both nitrate and nitrite reductases (Stouthamer, 1976). However, in some micro-organisms anaerobic conditions alone are sufficient to induce nitrate reductase, but the amount of enzyme formed is increased when nitrate is present (Stouthamer, 1976).

**Abbreviations:** BV(H), benzyl viologen (reduced form); CM, cytoplasmic membranes fraction; LH, light-harvesting complex; RC, photochemical reaction center; RCH, RCM and RCL, high, medium and low molecular weight RC polypeptides.
With respect to cell morphology, structure of the photosynthetic membranes, photopigments and requirements for growth factors, *R. sphaeroides* f. sp. *denitrificans* is similar to *R. sphaeroides*. The main difference is in the capacity of the former species to denitrify nitrate to dinitrogen. The regulation of photosynthetic membrane assembly has been studied in *R. sphaeroides* (Kaplan 1978, 1981). The regulation of the synthesis and composition of the intracellular membrane (CM) is under at least two different levels of control, namely the partial pressure of oxygen and the intensity of incident light (Aagaard & Sistrom, 1972; Cohen-Bazire *et al.*, 1957; Kaplan, 1978, 1981; Chory & Kaplan, 1983).

In this paper, we report on the characterization of the protein composition and synthesis in the cell fractions of *R. sphaeroides* f. sp. *denitrificans* during its adaptation to denitrifying conditions, when nitrate, nitrite and nitrous oxide reductases are induced.

**METHODS**

**Organism and growth conditions.** Rhodopseudomonas sphaeroides f. sp. *denitrificans* strain IL106 was kindly supplied by Dr T. Satoh, Department of Biology, Faculty of Science, Tokyo Metropolitan University, Tokyo 158, Japan. Cells were grown (5% v/v, inoculum) in a liquid culture (Satoh *et al.*, 1976) with modifications as described previously (Kelley *et al.*, 1982). DL-malate (21.4 mM) and (NH₄)₂HPO₄ (at the concentrations indicated) were included as carbon and nitrogen sources, respectively. Denitrifying cultures were grown in a medium containing DL-malate supplemented with KNO₃ (20 mM) and (NH₄)₂HPO₄ (at the concentration indicated). Cells were grown phototrophically at 30 °C under anaerobic conditions in screw-cap bottles completely filled with culture medium illuminated with 5000 lx (7.35 W m⁻²). Cell growth was monitored by measuring the OD of the cell suspension at 660 nm. Dry weights were determined as described previously (Hillmer & Gest, 1975).

**Protein labelling with radioisotopes.** In vivo labelling of cell proteins of *R. sphaeroides* f. sp. *denitrificans* with [³⁵S]sulphate (37.5 GBq mmol⁻¹, New England Nuclear) was achieved as follows. Preculture of strain IL106 grown phototrophically for 24 h (5000 lx) with 2 mM (NH₄)₂HPO₄ was used to inoculate (5% v/v) a series of flasks containing 60 ml mineral salt medium without sulphate but supplemented with carrier-free [³⁵S]sulphate (30 µCi; 1-11 MBq).

The concentration of carrier sulphate introduced with the inoculum was 60 µM, which was essential for the growth of *R. sphaeroides* f. sp. *denitrificans*.

For radioactive pulse-chase labelling of the ‘nitrate-induced’ proteins, 10 µCi of [¹⁴C]-labelled protein hydrolysate (2.07 GBq milliatom⁻¹, Amersham) was added to each of two 20 ml anaerobic cell suspensions (approximately 0.8 mg dry wt ml⁻¹). At the same time, KNO₃ (20 mM) was added to one of these flasks. After 10 and 30 min incubation, respectively (30 °C, in the light), 4 ml Casamino acids (casein, acid hydrolysate, 2.07 GBq milliatom⁻¹) were added to the cell suspension 20 min before the radioactive precursor. The preparation of radioactive soluble extracts was carried out as described by Michalski *et al.* (1983).

**Preparation of cell fractions.** Soluble and intracellular membrane (CM) fractions were prepared from the [³⁵S]-labelled cell pellet as follows: the resuspended cells (10 mM-Tris/HCl buffer, pH 7.5) were disrupted by passage (twice) through a French pressure cell at 25000 psi (approximately 0.17 GPa). Then deoxyribonuclease I (200 µg ml⁻¹) was added and the crude extracts were incubated at room temperature (20 °C) for 10 min. The crude extract was centrifuged at 15000 g for 20 min at 4 °C. The supernatant was then recentrifuged at 14400 g for 90 min at 4 °C (S₁₄₄ x ₉₀). The supernatant was then centrifuged again at 144000 g for 90 min and this supernatant was designated the soluble fraction (S₁₄₄ x ₉₀). The membrane pellet from the first high-speed centrifugation (P₁₄₄ x ₆₀) was resuspended in 10 mM-Tris/HCl buffer, pH 7.5 and centrifuged at 144000 g for 90 min. The washed pellet resuspended in the same buffer was designated cytoplasmic membrane (CM) fraction.

**Cytochrome c₂ preparation.** The soluble cell-free extract (S₁₄₄ x ₆₀) was heat treated at 70 °C for 5 min and centrifuged at 20000 g for 15 min. The supernatant after precipitation with 50% (w/v) saturated (NH₄)₂SO₄ was dialysed overnight against 25 mM-Tris/HCl buffer pH 7.4, loaded on to a DEAE-cellulose column (20 x 100 mm) and eluted with a linear NaCl gradient (0 to 0.2 M) in 25 mM-Tris/HCl buffer pH 7.4. A strong cytochrome c₅₅₀ (cyt. c₂) band was eluted at about 100 mM-NaCl.

**Electrophoresis.** Discontinuous electrophoresis was performed in the presence of 0-1% (w/v) SDS using either cylindrical or slab polyacrylamide gels according to Laemmli & Favre (1973). The CM fraction was solubilized as described by Chory & Kaplan (1983) by treating with 2% (w/v) SDS/5 mM-dithiothreitol/50 mM-Tris/HCl buffer (pH 6.8) at 100 °C for 2 min.

Two-dimensional slab gel electrophoresis of [³⁵S] or [¹⁴C]-labelled proteins was carried out using a modification of the methods of O'Farrell (1975) and Iborra & Buchler (1976) as described by Hallenbeck *et al.* (1982). The location
of electrophoretically separated proteins was determined by fluorography (Bonner & Laskey, 1974) using intensifying screens Cronex Hi-plus, Du Pont.

Cytocrome c and protein complexes containing bacteriochlorophyll were separated on SDS-polyacrylamide (13-5%, w/v) rod gels and detected by their red-fluorescence in near-UV light, as described by Wood (1981). The fluorescent bands in the gels were photographed with a Polaroid 665 film. The camera lens was covered by a Zuiko no. 25 red filter. The negative was then scanned with a double beam recording and integrating densitometer (Chromoscan MKII, Joyce Loebel).

Bacterial proteins separated by electrophoresis were calibrated against low and high molecular weight protein standards (Pharmacia electrophoresis calibration kits). The proteins were stained with Coomassie brilliant blue R250.

Analysis of pigments. Bacteriochlorophyll a was extracted by acetone/methanol (7:2, v/v) from whole cell suspensions and quantified using an extinction coefficient value of 76 mm⁻¹ cm⁻¹ at 770 nm (Cohen-Bazire et al., 1957).

Carotenoid pigments were extracted from whole-cell suspensions with acetone/methanol (1:1, v/v) (Cogdell et al., 1976). Individual carotenoids were separated by TLC on Eastman Kodak, silica gel sheets with benzene/chloroform (1:1, v/v) as the solvent. The coloured carotenoid-containing bands were scraped off the plate, and carotenoids were eluted with diethyl ether and determined in a Lambda 5, Perkin-Elmer spectrophotometer, using the extinction coefficients for individual compounds cited by Cogdell et al. (1976).

Enzyme assays. Nitrate reductase (EC 1.7.99.4), nitrite reductase (EC 1.7.2.1) and nitrous oxide (N₂O) reductase activities were assayed spectrophotometrically by a modification of the method of Kristjansson & Hollocher (1980) described for nitrous oxide reductase. The cuvettes (3 ml) fitted with rubber stoppers, containing 2-5 ml 200 mm-potassium phosphate buffer, pH 7.4 and 0-4 mm benzyl viologen, were made anaerobic with argon, as were all reagents and syringes used in the assay. The suspension of washed cells was injected first into the cuvettes followed by approximately 10 µl sodium dithionite (Na₂S₂O₄) solution (10 mg in 1 ml 1% w/v, sodium bicarbonate) resulting in an A₅₅₀ of 1·8 to 1·9. The absorption base line was then monitored for 4 min.

The enzyme reactions were initiated by injecting either potassium nitrate (1 mM) or potassium nitrite (1 mM) or N₂O-saturated water (0·5 mm-N₂O, final concentration) and activity rates were followed at 550 nm. The solubility of N₂O in water was taken to be 25 mm at 1 atm (101·32 kPa) 25 °C and the extinction coefficient of reduced benzyl viologen as 10·4 mm⁻¹ cm⁻¹ (Kristjansson & Hollocher, 1980). Specific activities calculated from the initial rates of benzyl viologen oxidation were related to dry weight of cells.

ATP Determination. ATP was determined in washed cell suspensions by the firefly luciferin luciferase bioluminescence technique (Stanley & Williams, 1969) as described (Kelley et al., 1982).

Protein contents of cell fractions were determined by the method of Bradford (1976), using bovine serum albumin as a standard protein.

RESULTS

ATP contents of washed cells incubated under various conditions

The isolation of denitrifying strains of the Rhodopseudomonas f. sp. denitrificans of the Rhodosporillaceae (Satoh et al., 1976; Klemme et al., 1980) indicates that they utilize respiration to nitrate, under anaerobic conditions, as an alternative terminal electron acceptor to O₂ for ATP production (Kelley et al., 1982). Cellular ATP contents were determined in washed cells prepared from cultures grown photosynthetically under denitrifying conditions (Fig. 1). The highest concentration of ATP produced was recorded in cells incubated anaerobically in light. Amounts of ATP similar to those in light were found in cells respiring in the dark to either O₂ or nitrate. In the absence of a respiratory terminal acceptor in the dark, the endogenous ATP levels were rapidly depleted. Thus, in cells of R. sphaeroides f. sp. denitrificans, ATP generated during respiration to nitrate seems to be sufficient to meet their energy requirements.

A comparison of cells grown photosynthetically with and without nitrate

Satoh et al. (1976) have reported that photosynthetically grown cells of R. sphaeroides f. sp. denitrificans were yellowish green, whereas those grown with nitrate became greenish brown. In our experiments, however, cells grown photosynthetically under denitrifying conditions were markedly less coloured than those grown without nitrate, although the growth rates were identical up to late-exponential phase (18 h). The absorption spectra of intact cells showed a marked decrease of bacteriochlorophyll a absorption bands at 800 and 850 nm in the denitrifying cultures (not shown) and this was confirmed by analysis of the individual pigments

R. sphaeroides f. sp. denitrificans
Fig. 1. ATP production in washed cells from a denitrifying culture of *R. sphaeroides f. sp. denitrificans* grown under various conditions. Washed cells were prepared from denitrifying cultures grown photosynthetically as described in Methods. The reaction mixture (1 ml) contained washed cells (2 mg dry wt) and DL-malate (27 mM). Where indicated, vials were covered with foil and black plastic to exclude light. The vials were fitted with rubber septa, gassed with argon (20 min) and then incubated in a Warburg bath at 30 °C and illuminated with 5000 lx where indicated. At time intervals, the reaction was terminated with 1 ml 5% (w/v) perchloric acid, the mixture was immediately chilled and ATP was determined as described in Methods. Treatments: light/anaerobic (A); dark/anaerobic with 20 mM-nitrate (0); dark/aerobic, 20% (v/v) O₂ in argon (●); dark/anaerobic (□).

Table 1. *Effects of nitrate on bacteriochlorophyll a and carotenoid contents of *R. sphaeroides f. sp. denitrificans* grown with low (2 mM) and high (12 mM) concentrations of ammonium chloride*

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Cells grown with 2 mM-NH₄⁺</th>
<th>Cells grown with 12 mM-NH₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−NO₃ / +NO₃ (20 mM)</td>
<td>−NO₃ / +NO₃ (20 mM)</td>
</tr>
<tr>
<td>Carotenoids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurosporene</td>
<td>0.01 ± 0.001</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>Spheroidene</td>
<td>1.04 ± 0.09</td>
<td>0.66 ± 0.04</td>
</tr>
<tr>
<td>Spheroidenone</td>
<td>0.76 ± 0.08</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Total</td>
<td>1.81 ± 0.06</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td>Bacteriochlorophyll a</td>
<td>3.15 ± 0.24</td>
<td>1.98 ± 0.21</td>
</tr>
<tr>
<td>Bacteriochlorophyll a/carotenoids ratio</td>
<td>1.74</td>
<td>2.64</td>
</tr>
</tbody>
</table>

extracted from the cells. The pigment composition of *R. sphaeroides f. sp. denitrificans* (Table 1) is similar to that reported for *R. sphaeroides* when both were grown in light (Cogdell et al., 1976). However, the amounts of two major carotenoids, spheroidene and spheroidenone, as well as bacteriochlorophyll a were found to be drastically depleted (by approximately 35, 85 and 45%, respectively) in cells grown with nitrate. Various concentrations of ammonium ion had no effect on pigment composition. Thus, these data indicate that one of the responses of cells of *R. sphaeroides f. sp. denitrificans* to denitrifying conditions is a loss of photosynthetic pigment.

*Fluorescent gel techniques for characterizing membrane proteins of cells grown photosynthetically with and without nitrate*

Haem-containing polypeptides can be detected on SDS-PAGE gels by their red fluorescence in UV light (Katan, 1976; Wood, 1981). The bacteriochlorophyll is related in structure to the haems and, like many other porphyrins, fluoresces on excitation (Amesz, 1968). We have used
Fig. 2. Red-fluorescent bands of CM extracts and purified cytochrome c₂ from *R. sphaeroides* f. sp. *denitrificans*. The figure shows a SDS-polyacrylamide-gel photograph and densitometrograms of a negative. The CM extracts (20 µg protein) from cells grown photoheterotrophically with 20 mhl-nitrate (A) and without nitrate (B) were prepared as described in Methods. Lanes C and D, purified cytochrome c₂, 1 and 2 nmol, respectively. S, Start of resolving gel; F, dye front.

Fluorescent gel techniques to characterize polypeptides containing bacteriochlorophyll in cytoplasmic membranes (CM). The CM proteins prepared from photosynthetically grown cells gave two distinct bands of red fluorescence (Fig. 2, lane B). The apparent molecular weights of these two proteins were similar to the low-molecular-weight polypeptides (approximately 10000) which are associated with the light-harvesting (LH) complexes (Cohen & Kaplan, 1981). Moreover, only one of those bands was present in the CM extract from cells grown under denitrifying conditions (Fig. 2, lane A). No fluorescent bands were found when protein extracts were washed with acetone/methanol (7:2, v/v) mixture (which removes bacteriochlorophyll and carotenoids; Wood, 1981) before electrophoresis (not shown).

**Protein composition of the cell-fractions obtained from cells grown with and without nitrate**

Cells were grown photosynthetically with and without nitrate in the presence of [³⁵S] sulphate. The cell fractions were then prepared as described in Methods and their proteins separated by one- and two-dimensional PAGE. The eleven major polypeptides separated are shown in Figs 3 and 4. About six of the proteins underwent a biosynthetic derepression under denitrifying conditions (Fig. 3a, b, c; lanes B, D, F), whereas some of them were not synthesized at all in the presence of nitrate, most notably the LH proteins (Fig. 3c, lane F). Some of the molecular features of these proteins are summarized in Table 2. At least five proteins synthesized under denitrifying conditions can be recognized as subunits of nitrate, nitrite and nitrous oxide reductases, as judged by their molecular weights (Table 2). Trace amounts of these proteins were
Fig. 3. Autoradiograms of SDS-PAGE (a and b, 10–12% w/v, linear gradient; c, 17% w/v) of $^{35}$S-labelled cell fractions of *R. sphaeroides* f. sp. *denitrificans* (a, cell-free extract; b and c, CM fraction, all approximately $10^5$ c.p.m. per gel). Cell fractions were obtained from $^{35}$S-labelled cells grown photoheterotrophically without nitrate (A, C, E) and with 20 mM-nitrate (B, D, F) as described in Methods. Molecular weights of numbered protein bands were determined by comparison of their relative electrophoretic mobilities with protein markers of known molecular size (kDal): 1, 100–112; 2, 70; 3, 45; 4, 58; 5, 60; 6, approximately 20; 7, 35–2; 8 and 9, approximately 18 and 15, respectively; 10 and 11, $\leq 10$. RCH, RCM and RCL correspond to reaction centre polypeptides with apparent molecular weights of 28, 24 and 22 kDal, respectively.

present in cell fractions of non-denitrifying cells (Figs 3 and 4). The synthesis of two proteins, namely membrane heat-labile protein (Chory & Kaplan, 1983) and one of the light-harvesting complex polypeptides (Cohen & Kaplan, 1981), was repressed in cells grown with nitrate (Fig. 3b and c; lanes D, F).

The composition of photosynthetic reaction centre (RC) polypeptides (RCH, RCM and RCL see Fig. 3) was not affected by growth with nitrate.

The polypeptide number 6, which is most probably cytochrome c$_3$, as judged by its migration in two-dimensional electrophoresis (Meyer *et al.*, 1971) was found only in the soluble protein fraction obtained from denitrifying cells (Fig. 4b).
**R. sphaeroides f. sp. denitrificans**

Fig. 4. Two-dimensional gel electrophoresis patterns of \(^{35}\)S-labelled soluble protein fractions of cells grown photoheterotrophically without nitrate (a) and with 20 mM-nitrate (b). Numbers correspond to the protein bands as indicated in Fig. 3 and Table 2.

<table>
<thead>
<tr>
<th>Protein band/spot number</th>
<th>Cell fraction</th>
<th>Molecular size (kDa)</th>
<th>1-D PAGE*</th>
<th>2-D PAGE*</th>
<th>pI†</th>
<th>Possible function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. CM/Soluble</td>
<td>75</td>
<td>75</td>
<td>4.8</td>
<td>Nitrate reductase, subunit</td>
<td>Satoh (1981)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. CM/Soluble</td>
<td>45</td>
<td>45</td>
<td>4.95</td>
<td>Nitrite reductase, subunit</td>
<td>Sawada et al. (1978); Kundu &amp; Nicholas (1983)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. CM/Soluble</td>
<td>58</td>
<td>56</td>
<td>5.3</td>
<td>Glutamine synthetase, subunit</td>
<td>Michalski &amp; Nicholas (1983)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. CM/Soluble</td>
<td>60</td>
<td>60</td>
<td>6.3</td>
<td>Nitrous oxide reductase, subunit</td>
<td>Kristjansson &amp; Holocher (1981); Zumft &amp; Matsubara (1982); Matsubara &amp; Zumft (1982)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. CM/Soluble</td>
<td>~20</td>
<td>~20</td>
<td>4.0</td>
<td>Cytochrome c₃</td>
<td>Meyer et al. (1971); Chory &amp; Kaplan (1983)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. CM/Soluble</td>
<td>35</td>
<td>—</td>
<td>—</td>
<td>Heat-labile protein</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. CM/Soluble</td>
<td>&lt;18</td>
<td>—</td>
<td>—</td>
<td>?</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. CM</td>
<td>&lt;15</td>
<td>—</td>
<td>—</td>
<td>?</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. CM</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 1-D or 2-D PAGE, one- or two-dimensional PAGE.
† pI, Isoelectric point reported in this paper.

**Activities of denitrifying enzymes in cells grown under various conditions**

The activities of nitrate, nitrite and nitrous oxide reductases could be measured in washed cells by using reduced benzyl viologen as an electron donor. High activities for the three enzymes
Fig. 5. Two-dimensional gel electrophoresis patterns of soluble protein fraction of 'pulse-chase' labelling of cells with $^{14}$C-labelled amino acids for 10 min without nitrate (a) and with 20 mM-nitrate (b). Experimental details are given in Methods. The numbers for the proteins are the same as in Fig. 3 and Table 2. x and y are high molecular weight polypeptides.

Table 3. Activities of denitrifying enzymes in $R$. sphaeroides f. sp. denitrificans grown without and with nitrate, and in various concentrations of ammonium chloride

Cells were grown in mineral salt medium containing DL-malate supplemented with various concentrations of ammonia and nitrate as indicated. Since nitrate, nitrite and nitrous oxide reductases operate in a reaction sequence it should be noted that only $\text{N}_2\text{O}$ assay represents the single enzyme activity. The oxidation of reduced benzyl viologen (BVH) by nitrate and nitrite represents the three- or two-step reaction sequence.

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>[nmol BVH oxidized min$^{-1}$ (mg dry wt)$^{-1}$ ± SD; n = 4]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells grown with:</td>
<td>NO$_3^-$ (1 mM)</td>
</tr>
<tr>
<td>2 mM-NH$_3^-$</td>
<td>24.0 ± 2</td>
</tr>
<tr>
<td>2 mM-NH$_2$</td>
<td>666.0 ± 36</td>
</tr>
<tr>
<td>20 mM-NH$_3^-$</td>
<td>36.0 ± 5</td>
</tr>
<tr>
<td>12 mM-NH$_3^-$</td>
<td>854.3 ± 55</td>
</tr>
</tbody>
</table>

were recorded in the cells grown under denitrifying conditions (Table 3). Small activities for these enzymes were, however, detected in cells grown without nitrate. Ammonium salts did not appear to affect the activities of the denitrifying enzymes (Table 3).

Protein biosynthesis induced by nitrate

The synthesis of 'nitrate-induced' proteins was studied by following the incorporation of $^{14}$C-labelled amino acids into soluble protein fraction of washed cells grown with and without
Fig. 6. Effects of rifampicin (lanes B and E) and chloramphenicol (lanes C and F) on the 'pulse-chase' labelling of cell soluble proteins with 14C-labelled amino acids. Cells were pulse-labelled for 30 min without nitrate (lanes A–C) and with 20 mM-nitrate (lanes D–F). The cell-free extracts were then prepared, and radioactive proteins were separated by SDS-PAGE and stained with Coomassie blue (a) and autoradiographed (b) as described in Methods. The designation of the protein bands is the same as in Fig. 3 and Table 2.

nitrate, in pulse-labelling experiments (10 and 30 min). After a 10 min pulse labelling, three major polypeptides (numbers 1, 2 and 3) were synthesized in cells grown with and without nitrate (Fig. 5a and b). These polypeptides were located on two-dimensional electrophoretograms in identical positions to subunits of nitrate and nitrite reductases (Fig. 4; Table 2). These results indicate that these enzymes are constitutive. During the nitrate induction experiment (10 min pulse labelling), two additional groups of high molecular weight proteins (designated as X and Y) appeared (Fig. 5). Since they were not detected in two-dimensional electrophoretograms of 35S-labelled soluble cell protein (Fig. 4), it is possible that they represent precursors of high molecular weight membrane proteins synthesized in the cytoplasm prior to their insertion into membranes.

After pulse labelling for 30 min (Fig. 6) four major proteins (1, 2, 3 and 4) were found in cells grown with and without nitrate (Fig. 6, lanes A and D). After nitrate induction, however, the amounts of the proteins synthesized were markedly greater than those of the controls. The synthesis of high molecular weight polypeptides (designated X and Y, Fig. 5) also occurred in nitrate-induced cells (Fig. 6, lane D). Protein 5 with a molecular size of 60 kDa is most probably a subunit of the nitrous oxide reductase synthesized de novo (see also Figs 3, 4 and Table 2).

Since these four proteins seemed to be synthesized at different rates in denitrifying cells, the effects of rifampicin and chloramphenicol on their production were studied. Thus, proteins 2 and 3 (presumably subunits of nitrate and nitrite reductases, respectively) were synthesized in cells grown with or without nitrate even in the presence of 50 mM-rifampicin (Fig. 6, lanes B and E). Proteins were not synthesized, however, in pulsed cells preincubated with chloramphenicol (30 mM, Fig. 6, lanes C and F). Although the three denitrification enzymes were rapidly synthesized in cells exposed to nitrate for 10 or 30 min, there were no differences in the enzyme activities in the presence or absence of nitrate at these times.
DISCUSSION

*Rhodopseudomonas sphaeroides* f. sp. *denitrificans*, a phototrophic, denitrifying subspecies of *R. sphaeroides* can be readily grown anaerobically, either in light or in the dark, with nitrate. This bacterium derives ATP for growth, as do other denitrifiers, from the reduction of nitrate via nitrite to dinitrogen (Satoh *et al.*, 1976; Kelley *et al.*, 1982).

We have examined some of the changes which occur in cells grown with nitrate in light. Under denitrifying conditions cells contained about a half of the bacteriochlorophyll and carotenoid content of those grown without nitrate (Table 1). The synthesis of one of the polypeptides of the light-harvesting complex (designated as 15A by Chory & Kaplan, 1983) was depressed by nitrate (Figs 2 and 3c), but there was a marked increase in amounts and activities of the denitrification enzymes (Figs 3 and 4; Table 3). It is clear (Fig. 1) that the amount of ATP generated during respiration to nitrate (denitrification) was sufficient to meet the energy requirements of the cells, thus, in the presence of nitrate when *de novo* synthesis of denitrification enzymes occurred (notably nitrate reductase for ATP formation) the photosynthetic ATP-production was less effective. Kelley *et al.* (1982) found that light-dependent formation of ATP in denitrifying cells was about 40% lower than that of cells grown without nitrate.

The synthesis of denitrification enzymes is induced by nitrate under anaerobic conditions (Stouthamer, 1976; Whatley, 1981). We found, however, that nitrate, nitrite reductases, and probably nitrous oxide reductase were constitutive, since they were detected in cells grown without nitrate (Figs 3–6; Table 3). The inclusion of nitrate, however, in the culture medium markedly stimulated the production and activities of these enzymes. The synthesis of nitrate and nitrite reductases in cells grown with or without nitrate was not inhibited by rifampicin.

Even in the presence of this antibiotic their synthesis was increased by nitrate (Fig. 6). Since rifampicin is a powerful inhibitor of microbial RNA polymerases (Wehrli *et al.*, 1968) our results indicate that copies of mRNA for both enzymes are 'long lived' in cells and may be very quickly translated in response to nitrate.

It has been postulated (Reed, 1969; Sawada *et al.*, 1978) that a *c*-type cytochrome (presumably cytochrome *c*~3~) is the physiological electron donor for nitrite reductase and that denitrification is linked to the photosynthetic electron transport chain via key cytochromes. We observed a derepression of synthesis of small molecular weight polypeptides in denitrifying cells (most probably cytochrome *c*~3~, Figs 4b and 6).

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REFERENCES


