JOURNAL OF BACTERIOLOGY, Nov. 1985, p. 823–830
0021-9193/85/110823-08$02.00/0

Vol. 164, No. 2

Nitrous Oxide Reduction by Members of the Family *Rhodospirillaceae* and the Nitrous Oxide Reductase of *Rhodopseudomonas capsulata*

ALASTAIR G. McEWAN,† ANTHONY J. GREENFIELD, HEINZ G. WETZSTEIN,‡ J. BARRY JACKSON, AND STUART J. FERGUSON§*

Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, United Kingdom

Received 11 February 1985/Accepted 3 June 1985

After growth in the absence of nitrogenous oxides under anaerobic phototrophic conditions, several strains of *Rhodopseudomonas capsulata* were shown to possess a nitrous oxide reductase activity. The enzyme responsible for this activity had a periplasmic location and resembled a nitrous oxide reductase purified from *Pseudomonas perfectomarinus*. Electron flow to nitrous oxide reductase was coupled to generation of a membrane potential and inhibited by rotenone but not antimycin. It is suggested that electron flow to nitrous oxide reductase branches at the level of ubiquinone from the previously characterized electron transfer components of *R. capsulata*. This pathway of electron transport could include cytochrome *c'*, a component hitherto without a recognized function. *R. capsulata* grew under dark anaerobic conditions in the presence of malate as carbon source and nitrous oxide as electron acceptor. This confirms that nitrous oxide respiration is linked to ATP synthesis. Phototrophically and anaerobically grown cultures of nondenitrifying strains of *Rhodopseudomonas sphaeroides*, *Rhodopseudomonas palustris*, and *Rhodospirillum rubrum* also possessed nitrous oxide reductase activity.

Rhodopseudomonas capsulata is frequently regarded as one of the most versatile procaryotes (20) because of its diverse modes of growth. It grows phototrophically under anaerobic conditions and aerobically in the dark with a wide variety of carbon sources. A third mode of growth has been known for several years. This is anaerobic growth in the dark with either trimethylamine-*N*-oxide (TMAO) or dimethyl sulfoxide (DMSO) as an added electron acceptor. Several recent lines of experimental evidence indicate that electron flow from NADH to TMAO or DMSO is via a proton-translocating respiratory chain (23, 28). The reductase for DMSO and TMAO is probably a single enzyme and is located in the periplasmic space (26). Another recent discovery concerning *R. capsulata* is that several strains possess a periplasmic respiratory nitrate reductase (24, 25).

In view of the recent findings concerning electron transport to TMAO, DMSO, and nitrate, we investigated whether *R. capsulata* might be able to use other electron acceptors. Nitrous oxide was tested because the recent introduction of an electrode for detecting N_2O (1, 2) permits a simple assay for N_2O reductase in bacterial cells, and as suggested elsewhere (12), the high solubility of N_2O in water could mean that this molecule is a readily available electron acceptor in natural environments. The present paper reports that all tested strains of *R. capsulata* can use N_2O as an electron transport-linked oxidant. Furthermore, anaerobic growth occurs in the dark with nonfermentable substrates and N_2O. Although we were primarily concerned with the characteristics of N_2O reduction in *R. capsulata*, the present paper also includes a preliminary survey of N_2O reduction by other members of the family *Rhodospirillaceae*.

Little is known about nitrous oxide reductases, although progress has been made recently in characterizing the enzyme from *Pseudomonas perfectomarinus* (39). The present paper reports that N_2O reductase from *R. capsulata* is a periplasmic protein and resembles the enzyme found in *Pseudomonas perfectomarinus*.

MATERIALS AND METHODS

R. capsulata Kb1 was obtained from B. A. Melandri, University of Bologna, Italy, and *R. capsulata* N22 was obtained from O. T. G. Jones, University of Bristol, United Kingdom. A nitrate-respiring mutant, *R. capsulata* N22DNAR$^+$ was isolated from strain N22 as described by McEwan et al. (24). *Rhodospirillum rubrum* S1, *Rhodopseudomonas sphaeroides* 2.4.1, and *Rhodopseudomonas palustris* PW5 were obtained from L. Slooten, Free University, Brussels, Belgium; O. T. G. Jones; and J.-H. Klemme, University of Bonn, Federal Republic of Germany; respectively. *R. capsulata* Kb1 and N22 were grown on RCV medium (31) under phototrophic conditions as described previously (8). The mutant of *R. capsulata* N22 that possesses a constitutive respiratory nitrate reductase activity, designated N22DNAR$^+$, was grown on RCV medium supplemented with 15 mM KNO_3 (24). The other species studied were grown in the same medium as used for *R. capsulata* except that it was supplemented with biotin (100 μg liter^{-1}) for *Rhodospirillum rubrum* or biotin (100 μg liter^{-1}), nicotinic acid (1 mg liter^{-1}), and yeast extract (0.1 g liter^{-1}) for *R. sphaeroides*. For the growth of *R. palustris*, the medium was the same as for *R. sphaeroides* except it was supplemented with *p*-aminobenzoic acid (0.2 mg liter^{-1}). Cells were grown phototrophically for 16 h at 30°C as described previously (8) except where indicated otherwise, then harvested, and washed in fresh growth medium.

The bacteriochlorophyll content of *Rhodospirillum*

* Corresponding author.
† Present address: Department of Microbiology, University of Illinois, Urbana, IL 61801.
‡ Present address: Bayer AG, 5090 Leverkusen, Federal Republic of Germany.
§ Present address: Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom.

rubrum, *R. capsulata*, *R. sphaeroides*, and *R. palustris* was measured as described by Clayton (7). The protein content of intact cells was determined by the method of Lowry et al. (19). Cells were boiled in 1 M NaOH for 10 min before the protein determination.

Cytoplasmic membrane potentials ($\Delta\psi$) in *R. capsulata* N22 and N22DNAR$^+$ were measured from the absorption band shift in the endogenous carotenoid pigments as described by McEwan et al. (24).

Absorption spectra were obtained with a Kontron Uvikon 810 spectrophotometer. Dual wavelength spectrophotometric measurements were made with a Perkin-Elmer 356 instrument.

Sodium dodecyl sulfate linear acrylamide gradient gels were run with the discontinuous buffer system of Laemmli (17) modified by the method of Douglas and Butow (10). Protein standards of molecular-weight range 14,300 to 116,000 were from Sigma Chemical Co. Molecular weights were calculated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Lambin (18).

N_2O solutions were prepared by gassing an argon-sparged solution of 10 mM sodium phosphate (pH 7) for 15 min with N_2O. The N_2O solution was stored on ice in an airtight Hungate tube, and its concentration was taken to be 58 mM (2). An acetylene solution was prepared by the same procedure. At 0°C, the concentration of acetylene in solution is 76 mM (2). N_2O reduction by cells was measured in a Hansatech-type or Rank-type Clark electrode with a silver cathode as described by Alefounder and Ferguson (2). N_2O reductase activity of fractionated periplasmic proteins was determined from the N_2O-dependent rate of oxidation of dithionite-reduced methyl viologen essentially as described by Kristjansson and Hollocher (16).

Cytochrome *c'* and N_2O reductase were obtained from a 20-liter culture of *R. capsulata* N22DNAR$^+$ that was grown for 24 h. The cells were harvested at mid-exponential phase of growth (A_{650} = 0.7), and a periplasmic fraction was released by treatment with lysozyme as described by McEwan et al. (25). This periplasmic fraction did not contain detectable malate dehydrogenase, a cytoplasmic marker. Contaminating particulate material was removed by centrifugation twice at 26,000 × *g* for 30 min at 4°C, and phenylmethylsulfonyl fluoride was added to 0.1 mM. The periplasmic fraction (760 ml) was pumped at a rate of 40 ml h^{-1} onto a DEAE-Sepharose CL-6B column (gel bed, 0.8 by 12 cm) that had been preequilibrated with 20 mM Tris hydrochloride (pH 8.0). The column was washed with 30 ml of 20 mM Tris hydrochloride (pH 8.0). A solution of 30 ml of 20 mM Tris hydrochloride–80 mM NaCl (pH 8.0) was then applied to the column, followed by a linear gradient from 80 to 300 mM NaCl in 600 ml of 20 mM Tris hydrochloride (pH 8.0). Fractions (7 ml) were collected. The second fraction collected after the step increase in NaCl concentration had a visible absorption spectrum similar to that reported by Bartsch (4) for cytochrome *c'*. After elution with the linear gradient of fractions up to number 45, material still bound to the column was blue colored. This material was eluted at higher applied concentrations (200 to 220 mM) of NaCl and collected in fractions 50 to 55. It was found to possess N_2O reductase activity and to be free of periplasmic nitrate and TMAO reductase. This preparation of N_2O reductase was used without further purification.

The cytochrome *c'* obtained by the above procedure was further purified by dialysis against 20 mM Tris hydrochloride (pH 8), followed by chromatography with the above-mentioned DEAE-Sepharose CL-6B column that had been equilibrated with 20 mM Tris hydrochloride (pH 8.0). A 40 to 100 mM NaCl gradient in 20 mM Tris hydrochloride (pH 8.0) was used to develop the column. Material eluting upon application of approximately 60 mM NaCl had absorption spectra identical to those published for cytochrome *c'* (4) under both oxidizing and reducing conditions. Examination of this material by lithium dodecyl sulfate-polyacrylamide gel electrophoresis at 4°C showed that the major polypeptide stained for heme and had a molecular weight of 14,000, in agreement with previous estimates for the subunit molecular weight of cytochrome *c'* from *R. capsulata* (4).

The protonophore 2',5-dichloro-3-*t*-butyl-4'-nitrososalicylanilide (S13) was a gift from P. John, University of Reading, United Kingdom.

RESULTS

Nitrous oxide reduction by intact cells of *R. capsulata*. Figure 1 shows that when washed cells of *R. capsulata* N22, a green mutant derived from the wild-type strain St. Louis, were added to growth medium under dark conditions, there was a period of aerobic respiration that was recorded with a Clark-type electrode that detects O_2 and N_2O (2). Upon exhaustion of dissolved oxygen, N_2O was added. N_2O was reduced at a rate (Table 1) that was slightly faster than the maximum rate of oxygen reduction (64 nmol of O mg of protein^{-1} min^{-1}). A short period of illumination strongly but incompletely inhibited N_2O reduction (Fig. 1). Addition of the protonophore S13 slightly stimulated N_2O reduction and prevented the inhibition of N_2O respiration by light. The proton motive force generated during photosynthetic electron transport has been shown to inhibit electron transport pathways to either oxygen or nitrate (9, 21, 22). In these cases, the proton motive force generated by light-driven electron transport was sufficiently large to exert a thermodynamic backpressure on the proton-translocating respiratory pathways of electron flow. A similar explanation would account for the inhibition of nitrous oxide reduction by light and its relief by the protonophore S13, provided that the electron flow to N_2O is linked to proton translocation. Figure 2 shows that addition of N_2O to a dark anaerobic suspension of *R. capsulata* resulted in the generation of a membrane potential as judged by the uncoupler-sensitive change in the absorption spectrum of the endogenous carotenoids. Thus, electron flow to N_2O was coupled to proton translocation across the cytoplasmic membrane. Also shown in Fig. 2 are the carotenoid band shifts which were observed during illumination or during a period of aerobic respiration. The carotenoid absorption changes can be taken as linear indicators of the magnitude of the $\Delta\psi$ generated (33). Thus, during illumination, oxygenation or N_2O reduction values of $\Delta\Psi$ were in the ratio 100:64:68 relative to the base line established after addition of a high concentration of uncoupler.

The data presented above show that *R. capsulata* N22 has a high N_2O reductase activity and that the $\Delta\psi$ generated during electron flow to N_2O was comparable in size to the $\Delta\psi$ generated during aerobic respiration. This suggested that *R. capsulata* might be capable of anaerobic dark growth with N_2O as terminal electron acceptor. Anaerobic dark growth of *R. capsulata* N22 was observed with malate as carbon source (Fig. 3). The doubling time for cells growing exponentially under these conditions was 10 h, compared with the <4 h normally observed during phototrophic growth of this organism with malate as carbon source in RCV medium (N. P. J. Cotton and J. B. Jackson, unpublished observations). These observations show that electron transport to

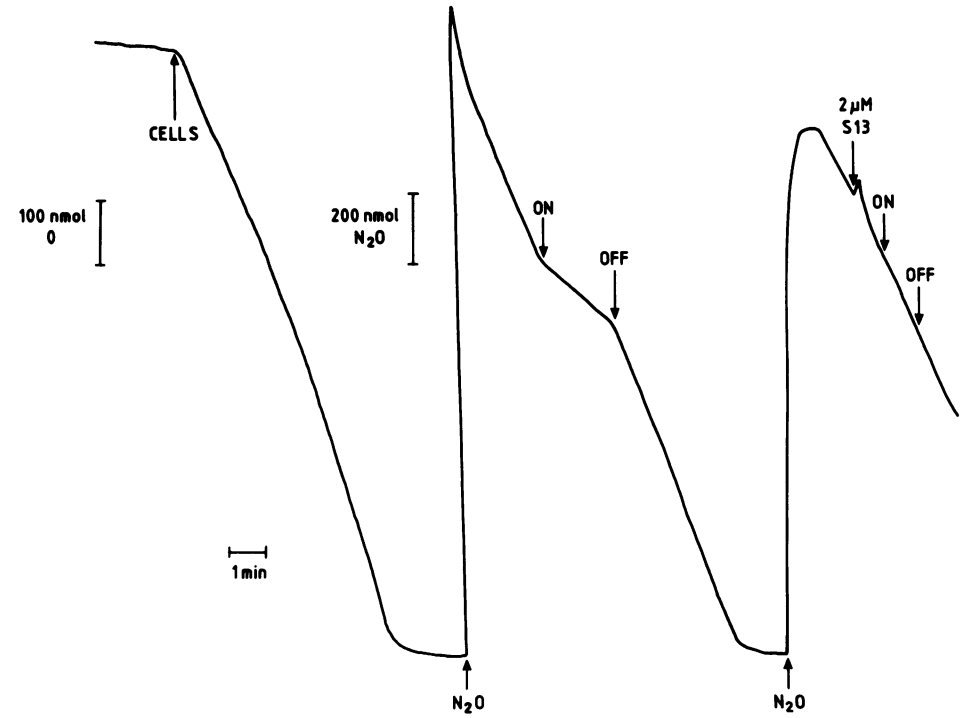

FIG. 1. N_2O reduction by cells of *R. capsulata* N22. A culture grown phototrophically on RCV medium to late-log phase was harvested, washed, and resuspended in RCV medium (pH 6.8). Cells (final bacteriochlorophyll concentration, 20 μM; bacteriochlorophyll-to-protein ratio was 70 nmol per mg) were added to a reaction chamber that was fitted with a silver cathode Clark-type electrode and thermostatted at 30°C by a water jacket. The rate of oxygen and N_2O reduction was followed under dark conditions except when, as indicated, illumination was provided from a 150W quartz halogen lamp with filtering through one layer of Wratten 88A gelatin filter. S13 was added from a 2 mM stock solution in methanol. Because the silver cathode electrode is less sensitive to nitrous oxide than to oxygen, the amplification on the strip chart recorder was increased by a factor of 10 during the anaerobic period before addition of nitrous oxide.

N_2O must be linked to oxidative phosphorylation. There was no growth under dark anaerobic conditions in the absence of added N_2O.

Information concerning the pathway of electron flow to N_2O was obtained by studying the effects of known inhibitors of electron transfer reactions in *R. capsulata*. Rotenone, an inhibitor of NADH-ubiquinone oxidoreductase, gave substantial inhibition of N_2O reduction in strain N22 (Table 1). In contrast, antimycin A, an inhibitor of the ubiquinol-cytochrome $c_2$oxidoreductase in *R. capsulata*, had only a slight effect (Table 1). Antimycin A is an effective inhibitor of photosynthetic electron transport in intact cells of *R. capsulata* N22; at a concentration of 1 μM it produced greater than 85% inhibition of the rate of cyclic electron transport (J. F. Myatt and J. B. Jackson, unpublished observations). The same concentration of antimycin, added to an equivalent quantity of cells grown under identical conditions, only reduced the rate of electron flow to N_2O by 18% (Table 1). Thus, the relatively small inhibitory effect of antimycin upon N_2O reduction cannot be attributed to a failure of this inhibitor to reach its specific site of action in cells but can be explained by the recognized secondary inhibitory effects of antimycin outside its site of action within the ubiquinol-cytochrome c_2 oxidoreductase complex. In the nonphotosynthetic bacterium *Paracoccus denitrificans*, which is closely related to *R. capsulata* according to some criteria (13), N_2O receives electrons from cytochrome *c* (2, 5). Hence, with most physiological substrates reduction of N_2O by *P. denitrificans* is strongly inhibited by antimycin, and ascorbate plus *N*, *N*, *N'*, *N'*-tetramethyl-*p*-phenylenediamine (TMPD) is a very effective electron donor, via cytochrome *c*, for N_2O reduction (2). Table 1 shows that addition of ascorbate plus TMPD to rotenone-inhibited cells of *R. capsulata* elicited only a slight restoration of the rate of reduction of N_2O. However, upon illumination of cells under these conditions a high rate of N_2O reduction, similar to the uninhibited rate of N_2O reduction in the dark, was observed (Table 1). This suggests that electrons passed from TMPD into the electron-transfer chain at the level of cytochrome c_2 had to be driven by the photosynthetic reaction center to ubiquinone before they

TABLE 1. Effect of light, uncouplers, and respiratory inhibitors on the rate of N_2O reduction in *R. capsulata* N22[a]

Additions and conditions (concn)	Rate of N_2O reduction (nmol of N_2O reduced mg of protein^{-1} min^{-1})	% Change in original rate
None	83	0
None, light	30	−64
S13 (2 μM)	90	+9
S13 (2 μM), light	80	−3
Rotenone (25 μM)	10	−88
Rotenone (25 μM) Ascorbate (1 mM) TMPD (100 μM)	20	−76
Rotenone (25 μM) Ascorbate (1 mM) TMPD (100 μM) light	84	+2
Antimycin A (1 μM)	69	−18

[a] Experiments were performed as described in the legend to Fig. 1 under dark conditions unless indicated otherwise.

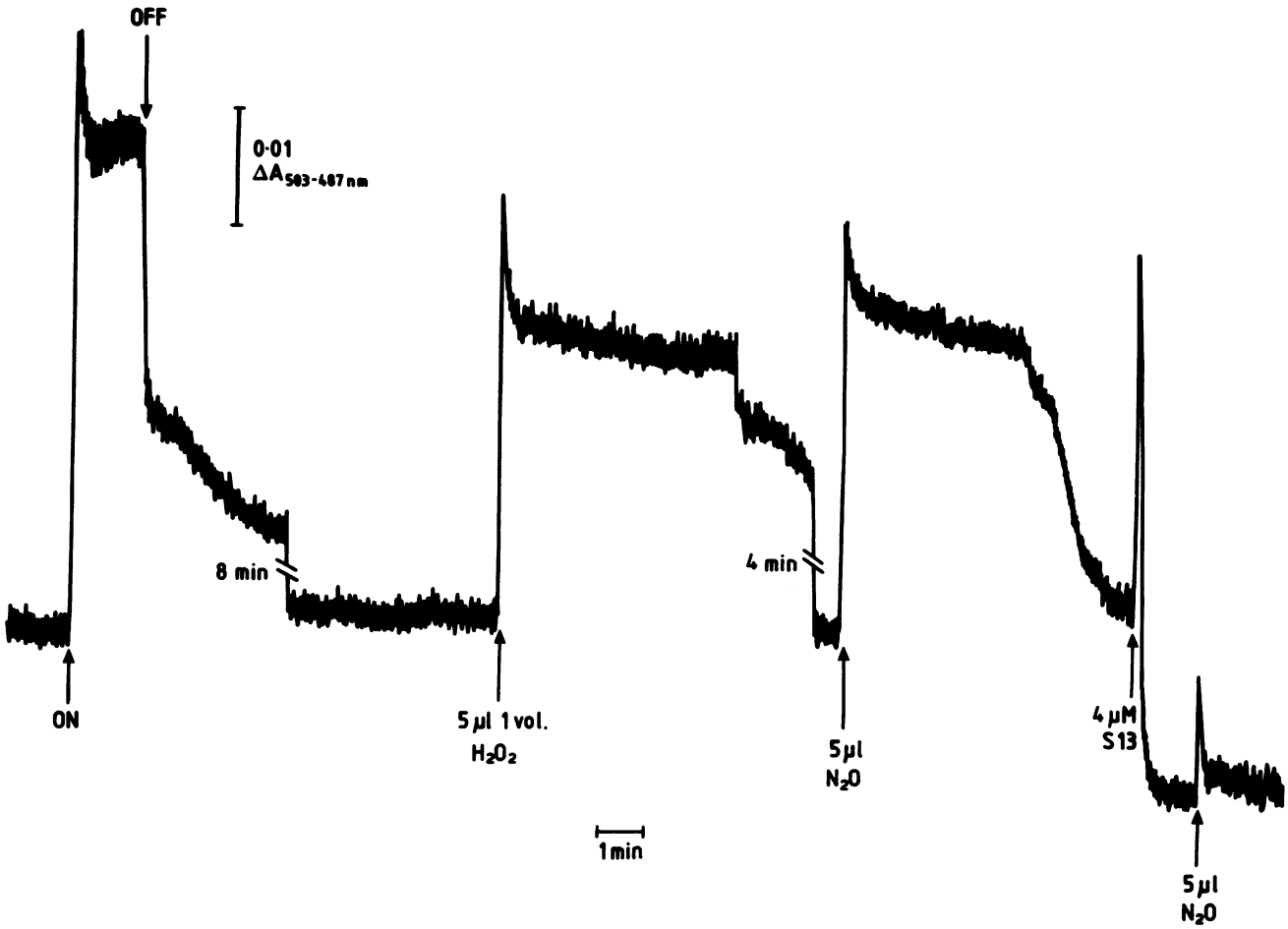

FIG. 2. Generation of a membrane potential, as judged by the carotenoid band shift, during illumination and after addition of either oxygen or N_2O to *R. capsulata* N22. Bacteria were added to a final bacteriochlorophyll concentration of 20 µM in 2.5 ml of argon-sparged RCV medium (pH 6.8) contained in a cuvette. Residual oxygen was removed by bacterial respiration, and the anaerobic suspension was left to equilibrate for 10 min. N_2O was added from an anaerobic N_2O-saturated solution.

could be transferred to the pathway of electron flow leading to N_2O reductase. The reduction of N_2O was completely inhibited by addition of 1 mM acetylene, a known inhibitor of N_2O reductases (34).

Properties of nitrous oxide reductase. Only fractions 50 to 55 from the ion-exchange chromatography (see Materials and Methods) of *R. capsulata* periplasmic proteins contained N_2O reductase activity. Examination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of a pool of these fractions revealed a single polypeptide of approximate molecular weight 76,000 (Fig. 4). Heavier loading of a gel with 40 µg of protein indicated very minor contamination by other polypeptides. The spectra of these pooled fractions under both reducing (dithionite present) and oxidizing (ferricyanide present) conditions are shown in Fig. 5, together with a spectrum of the material as it was obtained, partially reduced, from the ion-exchange column. These spectra are identical to those recently reported by Zumft and Matsubara (39) for a protein isolated from *Pseudomonas perfectomarinus* which they concluded to be the N_2O reductase of that bacterium.

The use of dithionite in conjunction with methyl viologen as the reductant for N_2O reductase from *R. capsulata* resulted in a rapid loss of enzyme activity just as has been reported for the enzyme from *Pseudomonas perfectomarinus* (39). This problem prevented the measurement of N_2O reductase activity with precision, but the initial rate was of the order of 1 µmol of N_2O reduced min^{-1} mg of $protein^{-1}$, which is similar to the activity reported by Kristjansson and Hollocher (16) and Snyder and Hollocher (30) for an N_2O reductase preparation from *P. denitrificans* but greater than the activity reported by Zumft and Matsubara (39) for the enzyme from *Pseudomonas perfectomarinus*.

Evidence of a role for cytochrome *c'* in the electron transport pathway to nitrous oxide reductase. Cytochrome *c'* is an abundant protein in *R. capsulata* (4) and, as our data confirm, is located in the periplasmic space. A physiological role for cytochrome *c'* has never been established. The electron transfer pathway from ubiquinol to N_2O reductase is uncharacterized but, as shown in Table 1, does not apparently involve the antimycin-sensitive ubiquinol-cytochrome c_2 oxidoreductase. Hence, we tested whether cytochrome *c'* might have a role in the transfer of electrons to N_2O reductase. Figure 6 shows that a sample of reduced cytochrome *c'* was oxidized upon addition of both N_2O and N_2O reductase. Oxidation did not occur upon addition of air-saturated water, N_2O alone (Fig. 6), or N_2O reductase

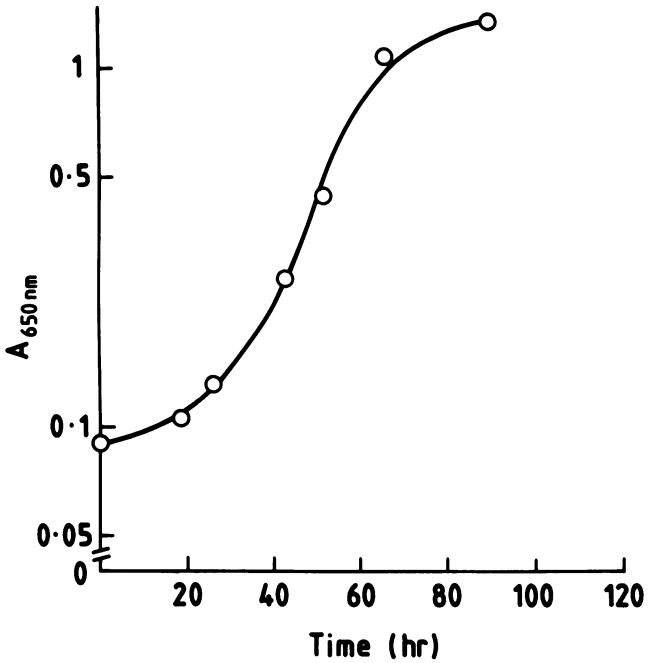

FIG. 3. Growth of *R. capsulata* N22 under dark and anaerobic conditions with a nonfermentable carbon source and N_2O as an electron acceptor. Cells were grown in Hungate tubes containing RCV medium at 30°C. This medium was sparged with argon and then nitrous oxide (final N_2O concentration was assumed to be saturating, i.e., 58 mM). Growth was followed from the increase in turbidity at 650 nm.

alone (data not shown). The wavelength pair chosen (Fig. 6) corresponds to the largest difference in absorbance between the oxidized and reduced forms of cytochrome *c'*. Oxidation of reduced cytochrome *c'* by the combination of N_2O and N_2O reductase could also be observed by following the increase in A_{635}, which is a characteristic absorbance maximum of the oxidized form. Indeed, addition of nitrous oxide and its reductase to reduced cytochrome *c'* resulted in the reappearance of the characteristic spectrum of oxidized cytochrome *c'* over the wavelength range 380 to 650 nm. Control experiments showed that there was no contribution to the spectral change from either dithionite (at the concentrations used in these experiments) or its oxidation products.

Is N_2O reductase widespread among the family *Rhodospirillaceae*? The discovery of an N_2O reductase activity in *R. capsulata* raised the question of whether other members of the *Rhodospirillaceae* have the same enzyme. We found that *Rhodospirillum rubrum* S1, *R. sphaeroides* 2.4.1, *R. palustris* PW5, and *R. capsulata* Kb1, a strain which is unrelated to strain N22, all possessed N_2O reductase activity when grown under phototrophic conditions (Table 2). No attempt was made in this preliminary survey to determine whether conditions could be found in which the N_2O reductase rates for *R. sphaeroides*, *R. palustris*, and *Rhodospirillum rubrum* are as high as for *R. capsulata*. Growth of these three bacteria on N_2O has not yet been tested. Harvested and washed cells from phototrophic cultures of the mutant *R. capsulata* N22DNAR$^+$ grown on nitrate as the sole source of nitrogen were also capable of N_2O reduction (Table 2). In all cases, N_2O reduction was inhibited during a period of illumination and completely blocked by 1 mM acetylene.

DISCUSSION

The data in this paper establish that nitrous oxide respiration is a characteristic of *R. capsulata*. With nonfermentable carbon sources, the extent and the rate of dark, anaerobic growth are considerably higher with N_2O than with the previously recognized terminal electron acceptors TMAO and DMSO (29) and nitrate (A. G. McEwan, J. B. Jackson, and S. J. Ferguson, unpublished observations). In these cases, it has been suggested that the $\Delta\psi$ generated during TMAO and nitrate respiration might be capable of supporting only a very low rate of ATP synthesis (23, 25). However, this is clearly not the case for N_2O respiration. The $\Delta\psi$ generated during electron flow to N_2O was comparable in size to the $\Delta\psi$ generated during aerobic respiration and was sufficient to support growth under anaerobic dark conditions. N_2O respiration has usually been considered an exclusive property of denitrifying bacteria, although recent reports have shown that it may be more widespread (12). From our results, it appears that several species of the *Rhodospirillaceae* are capable of N_2O respiration. Only one strain of *R. sphaeroides* (27) and two strains of *R. palustris* (15) are reported to have the capacity for denitrification. It is interesting that *R. capsulata* N22DNAR$^+$ and *R. palustris* PW5, which both respire with nitrate (15, 24), may fail to be

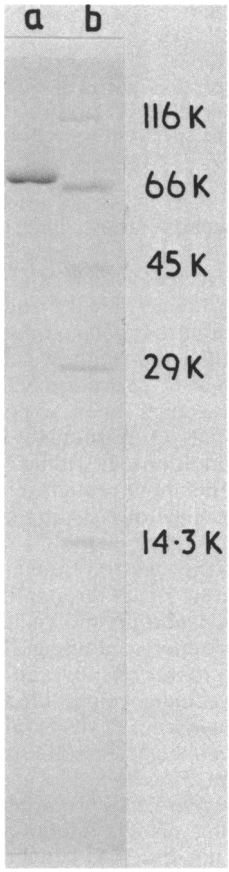

FIG. 4. Analysis of nitrous oxide reductase by polyacrylamide gel electrophoresis under denaturing conditions. Lane a, Nitrous oxide reductase (20 μg of protein); lane b, 5 μg of protein of each of the following standards: β-galactosidase (116,000 [116K]), bovine serum albumin (66,000 [66K]), ovalbumin (45,000 [45K]), carbonic anhydrase (29,000 [29K]), and lysozyme (14,300 [14.3K]).

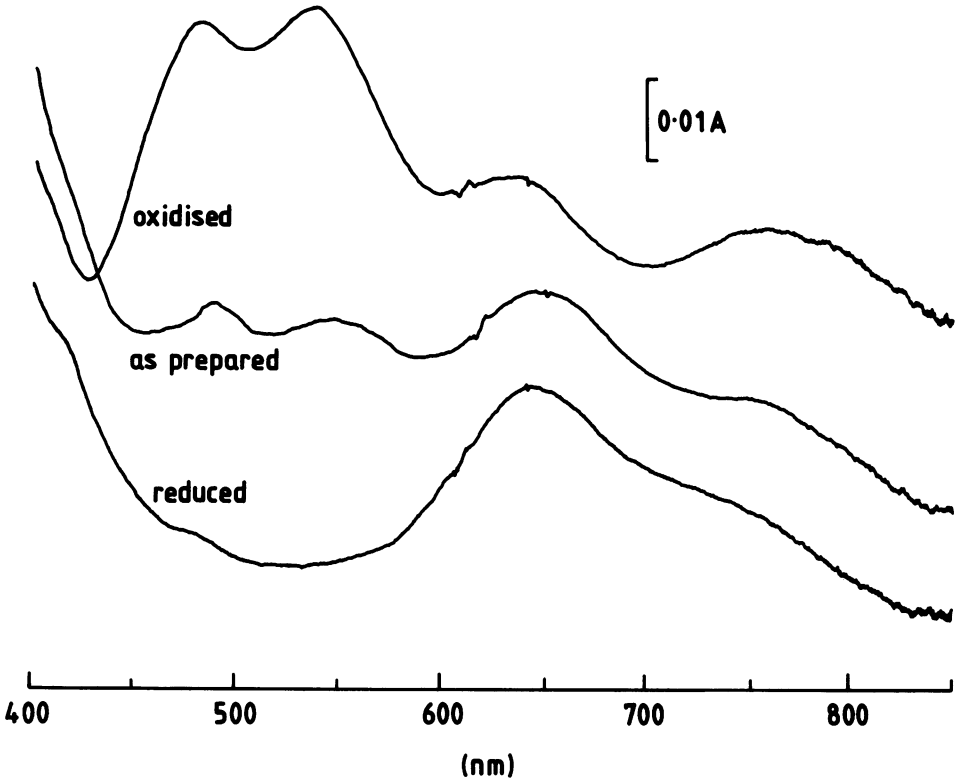

FIG. 5. Visible absorption spectra of nitrous oxide reductase from *R. capsulata* N22DNAR$^+$. The sample contained 1.9 mg of protein ml^{-1} in 50 mM Tris hydrochloride (pH 8) at 30°C. Oxidation or reduction was achieved by titration with a solution of $K_3Fe(CN)_6$ or dithionite until no further change in the spectrum was observed.

denitrifiers only because they lack dissimilatory nitrite reductase.

A striking feature of the present observations is that in *R. capsulata* N22 grown anaerobically and phototrophically in the absence of added nitrogenous oxides, the N_2O reductase activity is greater than the oxidase activity. Furthermore, the laboratory strain of *R. capsulata* N22 which we used has never, to our knowledge, been exposed to nitrogenous oxides. Evidently, N_2O reductase is constitutive for anaerobic growth conditions. It will be of value to determine in future work whether N_2O reductase activity is enhanced by growth in the presence of N_2O and depressed by aerobic growth.

Little is known of the electron transport pathways to N_2O or the molecular nature of its terminal reductase. The N_2O reductase from *R. capsulata* is shown here to be periplasmic and resemble the copper-containing N_2O reductase from *Pseudomonas perfectomarinus* (39), although we estimate a higher subunit molecular weight (76,000 compared with 62,000). N_2O reductase from *P. denitrificans* is also periplasmic (3, 6) but has been suggested (30) to be distinct from the enzyme identified in *Pseudomonas perfectomarinus*. The work with *P. denitrificans* has prompted consideration that the copper-containing protein identified in *Pseudomonas perfectomarinus* is not the N_2O reductase (30), but recent work has confirmed the association of N_2O reductase with the copper protein from *Pseudomonas perfectomarinus* (38). In view of the evidence in this paper that the antimycin-sensitive ubiquinol-cytochrome c_2 oxidoreductase of *R. capsulata* is not involved in electron flow to N_2O reductase, whereas in *P. denitrificans* this electron flow pathway is clearly implicated (2, 5), it is possible that the molecular nature of N_2O reductase differs between *R. capsulata* and *P. denitrificans*. The denitrifying strain of *R. sphaeroides* (27) resembles *R. capsulata* rather than *P. denitrificans* because electron flow to N_2O is also insensitive to antimycin (K. Chohan and S. J. Ferguson, unpublished observations).

It is not only the insensitivity to inhibition by antimycin of N_2O reduction that argues against a role for the ubiquinol-cytochrome c_2 oxidoreductase in N_2O reduction but also the observation of light-dependent electron transfer from ascorbate via TMPD and cytochrome c_2 to N_2O reductase. This resembles the photooxidase activity observed in many of the *Rhodospirillaceae* (29) which is a light-dependent oxygen uptake in the presence of TMPD and ascorbate. In *R. capsulata*, this electron-transport pathway is believed to proceed via cytochrome c_2 and the photosynthetic reaction center to ubiquinone and then through cytochrome b_{260} to oxygen (35). Electron transport from ubiquinol to cytochrome b_{260} does not involve the ubiquinol-cytochrome c_2 oxidoreductase (35), and photooxidase activity is therefore antimycin insensitive. Light-driven electron transport from TMPD and ascorbate to N_2O is catalyzed by a similar pathway to ubiquinone, followed by an antimycin-insensitive and uncharacterized pathway of electron flow to nitrous oxide reductase.

The results of the experiment shown in Fig. 6 indicate that cytochrome c' is a component on the electron transport pathway to N_2O reductase. This indication will require confirmation by identification of a physiological reductant for cytochrome c' and measurements of cytochrome c' oxidation when N_2O is added to intact cells. Such measure-

ments with cells would need to allow for the suggestion that cytochrome *c'* (midpoint potential at pH 7.0, 0 mV) when bound to the bacterial membrane has the spectroscopic characteristics of a b-type cytochrome (4) and thus could be the cytochrome b_0 ($E_{m7.0}$ = 0 mV) of uncertain function that has been observed in chromatophores (37). Although a physiological role for cytochrome *c'* has not been demonstrated previously, a function in electron transfer reactions is consistent with the identification of a possible binding site for an oxidoreductase within the high-resolution crystallographic structure of cytochrome *c'* (32). The simplest interpretation of Fig. 6 is that cytochrome *c'* is the reductant for N_2O reductase in *R. capsulata*. This would contrast with *P. denitrificans*, in which cytochrome *c* (equivalent to cytochrome c_2 in *R. capsulata*) is probably the reductant, and cytochrome *c'* has not been reported. Such a difference in the reductants for the N_2O reductases would be consistent with the possible distinction between the two reductases discussed earlier. Other possible roles for cytochrome *c'* in periplasmic electron transport in *R. capsulata* should also be considered in the context of the uncharacterized pathways of

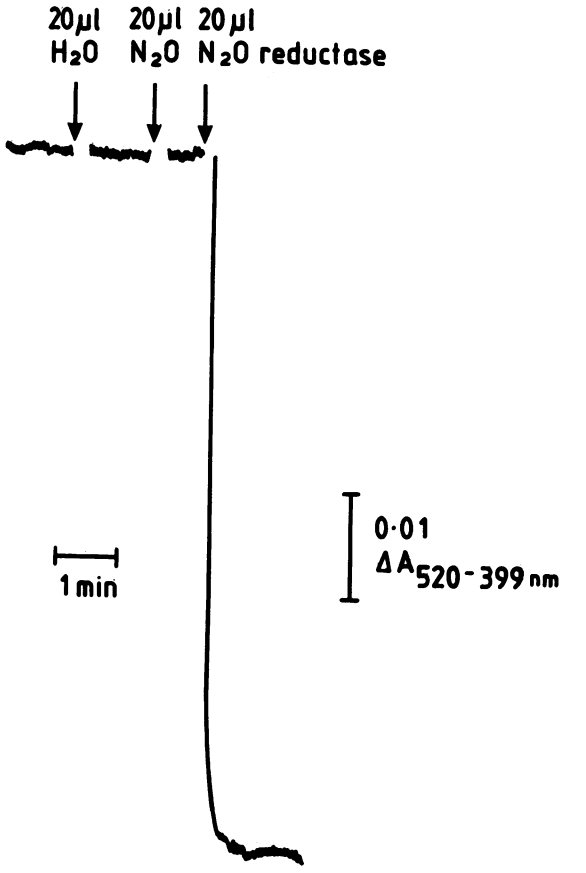

FIG. 6. Oxidation of reduced cytochrome *c'* by nitrous oxide in the presence of nitrous oxide reductase. Cytochrome *c'* (final concentration, 0.08 mg of protein ml^{-1}) was aded to 2.5 ml of argon-sponged Tris hydrochloride (pH 8) in a cuvette which was fitted with a stopper and stainless-steel entry ports to allow continuous gassing with argon and thus ensure anaerobiosis. The contents of the cuvette were stirred magnetically. Cytochrome *c'* was reduced by titration with a 25 mM solution of dithionite. Subsequent additions of air-saturated water, a saturated aqueous solution of nitrous oxide, and a solution of nitrous oxide reductase (1.9 mg of protein ml^{-1}) were made as shown.

TABLE 2. The use of N_2O as an electron acceptor in four species of the *Rhodospirillaceae*

Species	Rate of N_2O reduction (nmol of N_2O reduced mg of protein^{-1} min^{-1})[a]
Rhodopseudomonas capsulata N22	83
Rhodopseudomonas capsulata N22DNAR$^+$	156
Rhodopseudomonas capsulata Kb1	36
Rhodopseudomonas sphaeroides 2.4.1	5
Rhodopseudomonas palustris PW5	8
Rhodospirillum rubrum S1	7

[a] The rate of N_2O reduction in the dark was measured as described in the legend to Fig. 1.

electron transfer to the periplasmic nitrate and TMAO reductases (23–26). Electron transport in *R. capsulata* is evidently more complex than has been realized from studies in chromatophores (36).

The physiological role of respiratory electron flow in *R. capsulata* under anaerobic conditions is not certain. Although growth occurs under anaerobic dark conditions with N_2O, TMAO-DMSO, or NO_3^- as electron acceptors, in the case of TMAO-DMSO (29) and NO_3^- (A. G. McEwan, J. B. Jackson, and S. J. Ferguson, unpublished observations), significant growth requires the presence of fermentable carbon sources. The major energy-generating pathways in members of the *Rhodospirillaceae* are thought to involve photosynthetic electron transport and aerobic respiration. Thus, an additional physiological role for the pathways of anaerobic electron flow is worth considering. Photosynthetic electron transport in members of the *Rhodospirillaceae* operates as a closed cycle. In chromatophores, it is known that cyclic electron transport can function efficiently only at redox potentials at which a proportion of the early electron acceptors are oxidized (11). Observations in intact cells suggest that redox buffering occurs to maintain these acceptors (quinones) close to the optimal redox poise for photosynthesis. In both *Rhodospirillum rubrum* (14) and *R. capsulata* (37), there is evidence which suggests that antimycin-insensitive electron flow to oxygen via the cyanide-insensitive alternative oxidase fulfills this function and thus permits efficient photosynthesis. We suggest that antimycin-insensitive pathways of electron flow to N_2O, TMAO-DMSO, and NO_3^- may have a similar redox poising function under certain physiological conditions. Evidence for such a role will be reported in a forthcoming paper.

ACKNOWLEDGMENTS

This work was supported by a grant from the U.K. Science and Engineering Research Council to S.J.F. and J.B.J. H.G.W. was supported by a Royal Society European Science Exchange Fellowship under agreement with the Deutsche Forschungsgemeinschaft. S.J.F. was a Nuffield Foundation Science Research Fellow.

LITERATURE CITED

1. **Albery, W. J., W. N. Brooks, S. P. Gibson, M. W. Heslop, and C. E. W. Hahn.** 1979. An electroanalytical method for the determination of N_2O. Electrochim. Acta **24**:107–108.
2. **Alefounder, P. R., and S. J. Ferguson.** 1982. Electron transport-linked nitrous oxide synthesis and reduction by *Paracoccus denitrificans* monitored with an electrode. Biochem. Biophys. Res. Commun. **104**:1149–1155.
3. **Alefounder, P. R., A. J. Greenfield, J. E. G. McCarthy, and S. J. Ferguson.** 1983. Selection and organisation of denitrifying

electron-transfer pathways in *Paracoccus denitrificans*. Biochim. Biophys. Acta **724**:20–39.
4. **Bartsch, R. G.** 1978. Cytochromes, p. 249–280. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
5. **Boogerd, F. C., H. W. van Verseveld, and A. H. Stouthamer.** 1980. Electron transport to nitrous oxide in *Paracoccus denitrificans*. FEBS Lett. **113**:279–284.
6. **Boogerd, F. C., H. W. van Verseveld, and A. H. Stouthamer.** 1981. Respiration-driven proton translocation with nitrite and nitrous oxide in *Paracoccus denitrificans*. Biochim. Biophys. Acta **638**:181–191.
7. **Clayton, R. K.** 1963. Towards the isolation of a photochemical reaction center in *Rhodopseudomonas sphaeroides*. Biochim. Biophys. Acta **73**:312–323.
8. **Cotton, N. P. J., A. J. Clark, and J. B. Jackson.** 1981. The effect of venturicidin on light and oxygen-dependent electron transport, proton translocation, membrane potential development and ATP synthesis in intact cells of *Rhodopseudomonas capsulata*. Arch. Microbiol. **129**:94–99.
9. **Cotton, N. P. J., A. J. Clark, and J. B. Jackson.** 1983. Interaction between the respiratory and photosynthetic electron transport chains in intact cells of *Rhodopseudomonas capsulata* mediated by membrane potential. Eur. J. Biochem. **130**:581–587.
10. **Douglas, M., and R. A. Butow.** 1976. Variant forms of mitochondrial translation products in yeast; evidence for location of determinants on mitochondrial DNA. Proc. Natl. Acad. Sci. USA **73**:1083–1086.
11. **Dutton, P. L., J. B. Jackson.** 1972. Thermodynamic and kinetic characterization of electron transfer components in situ in *Rhodopseudomonas sphaeroides* and *Rhodospirillum rubrum*. Eur. J. Biochem. **30**:495–510.
12. **Ferguson, S. J.** 1985. Microbial nitrous oxide metabolism and the nitrous oxide electrode, p. 217–223. *In* R. K. Poole and C. S. Dow (ed.), Microbial gas metabolism—mechanistic, metabolic and biotechnological aspects. Academic Press, Inc., New York.
13. **Gibson, J., E. Stackebrandt, L. B. Zablen, R. Gupta, and C. R. Woese.** 1979. A phylogenetic analysis of the purple photosynthetic bacteria. Curr. Microbiol. **3**:59–64.
14. **Gimenez-Gallego, G., S. de Valle-Tascon, and J. M. Ramirez.** 1976. A possible physiological function of the oxygen-photoreducing system of *Rhodospirillum rubrum*. Arch. Microbiol. **109**:119–125.
15. **Klemme, J.-H., I. Chyla, and M. Preuss.** 1980. Dissimilatory nitrate reduction by strains of the facultative phototrophic bacterium *Rhodopseudomonas palustris*. FEMS Microbiol. Lett. **9**:137–140.
16. **Kristjansson, J. K., and T. C. Hollocher.** 1980. First practical assay for soluble nitrous oxide reductase of denitrifying bacteria and a partial kinetic characterization. J. Biol. Chem. **255**:704–707.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lambin, P. C.** 1978. Reliability of molecular weight determination of proteins by polyacrylamide gradient gel electrophoresis in the presence of sodium dodecyl sulphate. Anal. Biochem. **85**:114–125.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Madigan, M. T., and H. Gest.** 1979. Growth of the photosynthetic bacterium *Rhodopseudomonas capsulata* chemoautotrophically in darkness with H_2 as the energy source. J. Bacteriol. **137**:524–530.
21. **McCarthy, J. E. G., and S. J. Ferguson.** 1982. Respiratory control and the basis of light-induced inhibition of respiration in chromatophores from *Rhodopseudomonas capsulata*. Biochem. Biophys. Res. Commun. **107**:1406–1411.
22. **McEwan, A. G., N. P. J. Cotton, S. J. Ferguson, and J. B. Jackson.** 1984. The inhibition of nitrate reduction by light in *Rhodopseudomonas capsulata* is mediated by the membrane potential, but the inhibition by oxygen is not, p. 449–452. *In* C. Sybesma (ed.), Advances in photosynthesis research, vol. 2. Martinus Nijhoff-Dr. W. Junk Publishers, The Hague, The Netherlands.
23. **McEwan, A. G., S. J. Ferguson, and J. B. Jackson.** 1983. Electron flow to dimethylsulphoxide or trimethylamine-N-oxide generates a membrane potential in *Rhodopseudomonas capsulata*. Arch. Microbiol. **136**:300–305.
24. **McEwan, A. G., C. L. George, S. J. Ferguson, and J. B. Jackson.** 1982. A nitrate reductase activity in *Rhodopseudomonas capsulata* linked to electron transfer and generation of a membrane potential. FEBS Lett. **150**:277–280.
25. **McEwan, A. G., J. B. Jackson, and S. J. Ferguson.** 1984. Rationalisation of properties of nitrate reductases in *Rhodopseudomonas capsulata*. Arch. Microbiol. **137**:344–349.
26. **McEwan, A. G., H.-G. Wetzstein, J. B. Jackson, and S. J. Ferguson.** 1985. Periplasmic location of the terminal reductase in trimethylamine N-oxide and dimethylsulphoxide respiration in the photosynthetic bacterium *Rhodopseudomonas capsulata*. Biochim. Biophys. Acta **806**:410–417.
27. **Satoh, T., Y. Hoshino, and H. Kitamura.** 1976. *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* a denitrifying strain as a subspecies of *Rhodopseudomonas sphaeroides*. Arch. Microbiol. **108**:265–269.
28. **Schultz, J. E., and P. F. Weaver.** 1982. Fermentation and anaerobic respiration by *Rhodospirillum rubrum* and *Rhodopseudomonas capsulata*. J. Bacteriol. **149**:181–190.
29. **Smith, L., and P. B. Pinder.** 1978. Oxygen-linked electron transport and energy conservation, p. 707–718. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
30. **Snyder, S. W., and T. C. Hollocher.** 1984. Nitrous oxide reductase and the 120,000 MW copper protein of N_2-producing denitrifying bacteria are different entities. Biochem. Biophys. Res. Commun. **119**:588–592.
31. **Weaver, P. F., J. D. Wall, and H. Gest.** 1975. Characterisation of *Rhodopseudomonas capsulata*. Arch. Microbiol. **105**:207–216.
32. **Weber, P. C., A. Howard, N. H. Xuong, and F. R. Salemme.** 1981. Crystallographic structure of *Rhodospirillum molischianum* ferricytochrome c′ at 2.5 Å resolution. J. Mol. Biol. **153**:399–424.
33. **Wraight, C. A., R. J. Cogdell, and B. Chance.** 1978. Ion transport and electrochemical gradients in photosynthetic bacteria, p. 471–511. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
34. **Yoshinari, T., and R. Knowles.** 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Commun. **69**:705–710.
35. **Zannoni, D.** 1982. ATP synthesis coupled to light-dependent non-cyclic electron flow in chromatophores of *Rhodopseudomonas capsulata*. Biochim. Biophys. Acta **680**:1–7.
36. **Zannoni, D., P. Jasper, and B. Marrs.** 1978. Light-induced oxygen reduction as a probe of electron transport between respiratory and photosynthetic components in membranes of *Rhodopseudomonas capsulata*. Arch. Biochem. Biophys. **191**:625–631.
37. **Zannoni, D., and B. L. Marrs.** 1981. Redox chain and energy transduction in chromatophores from *Rhodopseudomonas capsulata* cells grown anaerobically in dark on glucose and dimethylsulphoxide. Biochim. Biophys. Acta **637**:96–106.
38. **Zumft, W. G., C. L. Coyle, and K. Frunzke.** 1985. The effect of oxygen on chromatographic behavior and properties of nitrous oxide reductase. FEBS Lett. **183**:240–244.
39. **Zumft, W. G., and T. Matsubara.** 1982. A novel kind of multicopper protein as terminal oxidoreductase of nitrous oxide respiration in *Pseudomonas perfectomarinus*. FEBS Lett. **148**:107–112.