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Periplasmic location of the terminal reductase in trimethylamine *N*-oxide and dimethylsulphoxide respiration in the photosynthetic bacterium *Rhodopseudomonas capsulata*

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(1) The trimethylamine *N*-oxide and dimethylsulphoxide reductase activities of *Rhodopseudomonas capsulata* have been shown to have a periplasmic location. Previous evidence for a cytoplasmic location was based on an observation of a soluble FMN-dependent NADH-trimethylamine *N*-oxide oxidoreductase activity in soluble total cell extracts (Cox, J.C., Madigan, M., Favinger, J.F., Gest, H. (1980) Arch. Biochem. Biophys. 204, 10–17). The latter activity has now been shown to arise from the combination of a cytoplasmic NADH-FMN oxidoreductase and the periplasmic dihydro: FMN-trimethylamine *N*-oxide oxidoreductase in such total extracts. A periplasmic location is consistent with the linking of trimethylamine *N*-oxide reductase to the proton-translocating electron-transport chain as concluded earlier (McEwan, A.G., Ferguson, S.J. and Jackson, J.B. (1983) Arch. Microbiol. 136, 300–305). (2) Observations on the relative dimethylsulphoxide and trimethylamine *N*-oxide reductase activities in extracts from cells grown on either of these electron acceptors, together with the identification of an identical electrophoretic mobillity of both activities on a polyacrylamide gel, suggest that a single enzyme is probably responsible for both activities in *Rps. capsulata*.

Introduction

The photosynthetic bacterium *Rhodopseudomonas capsulata* is capable of growing either phototrophically or aerobically in the dark. In addition, a third mode of growth has been demonstrated under anaerobic dark conditions in the presence of sugars and either trimethylamine *N*-oxide (TMAO) or dimethyl sulphoxide (DMSO) [1,2].

Several nonphotosynthetic bacteria have been shown to be capable of anaerobic growth in the presence of TMAO [3–5], and in these cases TMAO appeared to function as the terminal electron acceptor of a respiratory electron-transport chain which was linked to energy conservation [3,4]. In constrast, Cox et al. [6] reported that electron flow to TMAO in membrane vesicles from *Rps. capsulata* was not linked to energy conservation. From this and other evidence they concluded that a soluble enzyme system catalysed the anaerobic oxidation of NADH and reduction of TMAO. Consequently, in *Rps. capsulata* NADH-TMAO oxidreductase was proposed to function as a nonenergy-conserving electron sink and represented a system for maintenance of redox balance during the fermentation of sugars under dark anaerobic conditions. More recently, we showed that in intact cells of dark, anaerobically grown

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Abbreviations: TMAO, trimethylamine *N*-oxide; DMSO, dimethyl sulphoxide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta\psi$, cytoplasmic membrane potential.

Rps. capsulata a membrane potential ($\Delta\psi$) was generated during electron flow to TMAO or DMSO [7]. This TMAO- or DMSO-dependent $\Delta\psi$ was abolished by an uncoupler and was reduced by rotenone, indicating that electron flow to TMAO and DMSO was via a proton-translocating respiratory chain, of which the rotenone-sensitive NADH-ubiquinone oxidoreductase was a constituent. This observation was in apparent contradiction to the previous conclusions of Cox et al. [6].

In this paper we show that TMAO reductase and DMSO reductase activity is found in *Rps. capsulata* cells grown phototrophically as well as under anaerobic dark conditions and that TMAO and DMSO are probably substrates for the same enzyme. In addition, by cell fractionation the TMAO/DMSO reductase is shown to be a soluble enzyme located on the periplasmic face of the cell membrane. A NADH-FMN oxidoreductase activity, which was thought to be associated with reduction of TMAO [6] is shown to have a cytoplasmic location. These results are taken as further evidence that in *Rps. capsulata* electron flow to TMAO and DMSO is linked to energy conservation as in other bacteria [3,4].

Materials and Methods

Rps. capsulata N22, a green mutant derived from a wild-type strain St. Louis, was obtained from Dr. O.T.G. Jones, of the University of Bristol. Phototrophic cultures were grown on RCV * medium [8] supplemented with 80 mM DMSO or 30 mM TMAO.

The basal medium for anaerobic dark cultures was 7Y-G medium [1] supplemented with 1 g/l NaCl, 100 μg/l biotin and 0.05% yeast extract. To this basal medium 20 mM fructose plus 30 mM TMAO or 20 mM glucose plus 80 mM DMSO were added aseptically from filter-sterilised stock solutions. Cells grown phototrophically in this medium were used to inoculate a 300 ml screw capped bottle which was filled completely. Anaerobic dark cultures were grown for 48 h at 30°C. The doubling time during exponential growth for cells grown under anaerobic dark conditions with sugar and TMAO or DMSO was 23 h. This compares with a doubling time of 4 h for phototropically grown cells of this strain of *Rps. capsulata* on RCV medium (Cotton, N.P.J. and Jackson, J.B., unpublished data).

After growth the turbidity of the culture at 650 nm was measured and the cells fractionated as described previously [17]. The results from fractionation experiments are expressed with reference to the entire culture from a 300 ml bottle.

TMAO and DMSO reductase activities were measured essentially as described by Jones and Garland [9], except that the absorbance decrease at 585 nm due to methyl viologen oxidation was monitored. DihydroFMN-TMAO and dihydroFMN-DMSO oxidoreductases were measured by following the absorbance increase at 450 nm. Photoreduced FMN was produced by illumination of FMN in the presence of EDTA [10]. NADH-FMN oxidoreductase was measured from the FMN-dependent absorbance decrease at 340 nm. Malate dehydrogenase was assayed as in Markwell and Lascelles [11]. In all of these assays the temperature was 30°C and the buffer used was 50 mM Tris-HCl (pH 8.0) which had been sparged with argon. The cuvettes (volume 1 ml) used were sealed with rubber stoppers (Arthur H. Thomas Co., Philadelphia, PA), and after addition of reagents the contents were sparged again to ensure anaerobiosis. Additions were made through the rubber stopper using syringes, followed by rapid hand mixing. In the assay for TMAO/DMSO reductase activity methyl viologen was fully reduced by titration with dithionite. A stable absorbance value was attained, indicating anaerobic conditions. The reaction was started by addition of DMSO or TMAO. No reduced methyl viologen oxidase activity was observed in the absence of DMSO or TMAO. For membrane potential determinations cells were harvested and washed as described previously [7]. Experiments were performed at room temperature in argon-sparged 10 mM sodium phosphate (pH 7.0)/7.5 mM $(NH_4)_2SO_4$/30 mM sodium malate. Anaerobiosis was maintained by continuous gassing with argon using a gas train constructed as described in a previous paper [12]. Membrane potentials were estimated from the absorbance change associated with the electrochromic carotenoid band shift on a

* RCV, for definition, see Ref. 8.

chopped dual wavelength spectrophotometer as described by McEwan et al. [7]. The wavelength pairs were 503–487 nm.

Protein concentrations were determined by the method of Lowry et al. [13].

Discontinuous electrophoresis of soluble total cell extracts was performed using 8% polyacrylamide slab gels under nondenaturing conditions at high pH as described by Davis [14]. Bromophenol blue was used as a marker. Gels were stained for TMAO or DMSO reductase activity essentially as described by Simokawa and Ishimoto [15]. Negatively stained bands were fixed with triphenyltetrazolium chloride [16].

Results

Location of DMSO/TMAO reductase in Rps. capsulata cells grown phototrophically or anaerobically in the dark

We have recently shown that in *Rps. capsulata* N22DNAR$^+$ the respiratory nitrate reductase is located on the periplasmic face of the cell membrane [17]. Using the same procedure, cells of *Rps. capsulata* strain N22 grown either (a) phototrophically with malate plus DMSO or TMAO or (b) anaerobically in the dark or with glucose and DMSO, were fractionated. Table I shows that from cells grown under all conditions TMAO reductase activity and DMSO reductase activity was released in a soluble form after treatment with lysozyme. Malate dehydrogenase used as a cytoplasmic marker was found almost exclusively in the pelleted fraction. Contamination of the soluble periplasmic fraction with malate dehydrogenase was low indicating that during lysozyme treatment there was little leakage of the cytoplasmic content.

In periplasmic fractions from cells grown under all of the conditions described in Table I, both DMSO reductase and TMAO reductase activities were present. The rate of TMAO reduction always exceeded the rate of DMSO reduction. Table II shows the rate of reduced methyl-viologen oxidation by the periplasmic fraction from DMSO-grown cells with a range of electron acceptors. TMAO was the most effective substrate of those tested, ClO_3^- was a better substrate than DMSO, but the extracts were not active with NO_3^- as an acceptor.

These findings concerning the relative activities observed with various electron acceptors prompted consideration of whether a single enzyme might be responsible for reduction of DMSO and TMAO. Consequently, soluble fractions from *Rps. capsulata* were subjected to nondenaturing polyacrylamide gel electrophoresis. The gels were then stained for enzyme activity using reduced methyl viologen as a donor, as described in the methods. TMAO reductase or DMSO reductase each appeared as a colourless band against a blue background due to the reduced methyl viologen. The zymogram pattern was fixed using triphenyltetrazolium chloride which turned the background red. Fig. 1 shows that in a soluble extract (cytoplasmic and peri-

TABLE I

LOCALISATION OF TMAO/DMSO REDUCTASE IN *RPS. CAPSULATA*

TMAO reductase and DMSO reductase is expressed as μmol reduced methyl viologen oxidised per min. Malate dehydrogenase is expressed as μmol NADH oxidised per min. In each case the enzyme levels relate to the total activity in the whole of the three fractions derived from a 300 ml culture harvested at the A_{650} shown. Numbers in parenthesis are percentages of the total activity.

Growth conditions	A_{650}	Enzyme	Activities		
			Periplasmic fraction	Membrane fraction	Soluble fraction after sonicating spheroplasts (cytoplasmic fraction)
Phototrophic malate/TMAO	1.4	TMAO reductase	34.2 (94)	0.6 (2)	1.6 (4)
		Malate dehydrogenase	2.1 (12)	2.4 (14)	12.7 (74)
Phototrophic malate/DMSO	1.7	DMSO reductase	9.9 (91)	0.1 (1)	0.9 (8)
		Malate dehydrogenase	3.8 (13)	0.6 (2)	24.6 (85)
Anaerobic dark glucose/DMSO	0.7	DMSO reductase	46 (98)	0.1 (0)	0.8 (2)
		Malate dehydrogenase	0.8 (5)	0.7 (4)	15.6 (91)

TABLE II

RATES OF METHYL VIOLOGEN OXIDATION WITH DIFFERENT ELECTRON ACCEPTORS CATALYSED BY A PERIPLASMIC FRACTION OF *RPS. CAPSULATA* N22 GROWN UNDER ANAEROBIC DARK CONDITIONS ON GLUCOSE AND DMSO

In this experiment reduced methyl-viologen oxidation was measured from the absorbance decrease at 600 nm. Reduced methyl-viologen concentrations were determined $\varepsilon = 1.3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [9]. 5–10 μl periplasmic fraction was used in each assay.

Electron acceptor (5 mM)	Rate of reduced methyl-viologen oxidation (μmol/min per mg protein)
TMAO	393
DMSO	75
ClO_3^-	220
NO_3^-	0

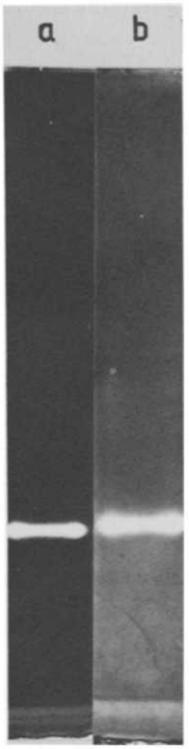

Fig. 1. Staining of a polyacrylamide gel for TMAO reductase and DMSO reductase activity. 80 μg soluble protein from *Rps. capsulata* was applied to each track on the gel. After electrophoresis individual tracks were cut from the gel and stained for either TMAO reductase (track a) or DMSO reductase (track b) as described in the Materials and Methods section.

plasmic) from cells grown anaerobically in the dark with fructose and TMAO only one band stained for TMAO reductase activity (track a). An electrophoresis gel of another sample of the same extract stained for DMSO reductase activity, showed one band in the same position as the TMAO reductase (Fig. 1, track b). Both bands migrated with a mobility $R_f = 0.73$ relative to bromophenol blue. Further experiments showed that fractions from cells grown on DMSO produced the same pattern as those from TMAO grown cells (data not shown). These observations with total cell extracts, taken together with the data in Table I, strongly suggest that in *Rps. capsulata* a single periplasmic enzyme is responsible for the reduction of both TMAO and DMSO.

NADH-FMN oxidoreductase and dihydro:FMN-TMAO / DMSO oxidoreductase differ in their cellular location

Cox et al. [6] showed that the soluble fraction obtained after French press treatment of *Rps. capsulata* cells possessed TMAO-dependent NADH oxidase in addition to methyl viologen-TMAO oxidoreductase activity. The NADH-dependent activity was markedly enhanced by FMN. These authors also showed that cell-free extracts from *Rps. capsulata* could link NADH oxidation to FMN reduction. It was proposed [6] that a flavodoxin-like protein accepted reducing equivalents from NADH, and that the dihydroFMN formed from this reaction was rapidly reoxidised by TMAO. The implication was that the operation of this NADH-oxidising system in the cytoplasm was responsible for the balanced fermentation. To test an alternative explanation, that NADH-dependent TMAO reduction in French press extracts arises from the combination of two enzyme activities which are normally independent and separate in intact cells, we performed the following experiments.

Using the procedure described above, cells which had been grown anaerobically in the dark with glucose and DMSO were fractionated. Table III shows that malate dehydrogenase was located predominantly in the cytoplasmic fraction which indicated that the fractionation of cells was suc-

cessful. DihydroFMN-TMAO oxidoreductase and dihydroFMN-DMSO oxidoreductase were found almost exclusively in the periplasmic fraction; the same location as the methyl viologen-TMAO/DMSO oxidoreductase. In contrast, NADH-FMN oxidoreductase was found exclusively in the cytoplasmic fraction; no activity could be detected in the periplasmic fraction.

Generation of a membrane potential in phototrophically grown Rps. capsulata

The results in Table I show that TMAO/DMSO reductase was present in cells grown phototrophically in the presence of TMAO or DMSO. In a previous publication [7] we showed that washed and harvested cells from an anaerobic, dark grown culture of *Rps. capsulata* responded to DMSO or TMAO addition with an uncoupler-sensitive red shift in the carotenoid absorption spectrum. This indicated generation of a membrane potential and was taken to be the result of proton translocation associated with respiratory electron flow to TMAO or DMSO. Fig. 2 shows measurements of membrane potentials in cells grown phototrophically with malate and TMAO. An uncoupler-sensitive carotenoid band shift was observed upon illumination or upon addition of TMAO, DMSO or ClO_3^-, but not of NO_3^-. The same result was observed when DMSO replaced TMAO during growth (data

TABLE III

LOCALISATION OF DIHYDRO:FMN-TMAO/DMSO OXIDOREDUCTASE AND NADH-FMN OXIDOREDUCTASE IN *RPS. CAPSULATA*

DihydroFMN-TMAO oxidoreductase and dihydroFMN-DMSO oxidoreductase is expressed as μmol dihydroFMN oxidised/min; malate dehydrogenase is expressed as μmol NADH oxidised/min; NADH-FMN oxidoreductase is expressed as μmol NADH oxidised/min. Numbers in parenthesis represent percentages of the total activity.

Enzyme	Activities	
	Periplasmic fraction	Cytoplasmic fraction
DihydroFMN-TMAO oxidoreductase	9.7 (98)	0.2 (2)
DihydroFMN-DMSO oxidoreductase	5.7 (98)	0.1 (2)
NADH-FMN oxidoreductase	0	0.43 (100)
Malate dehydrogenase	2.8 (6)	41 (94)

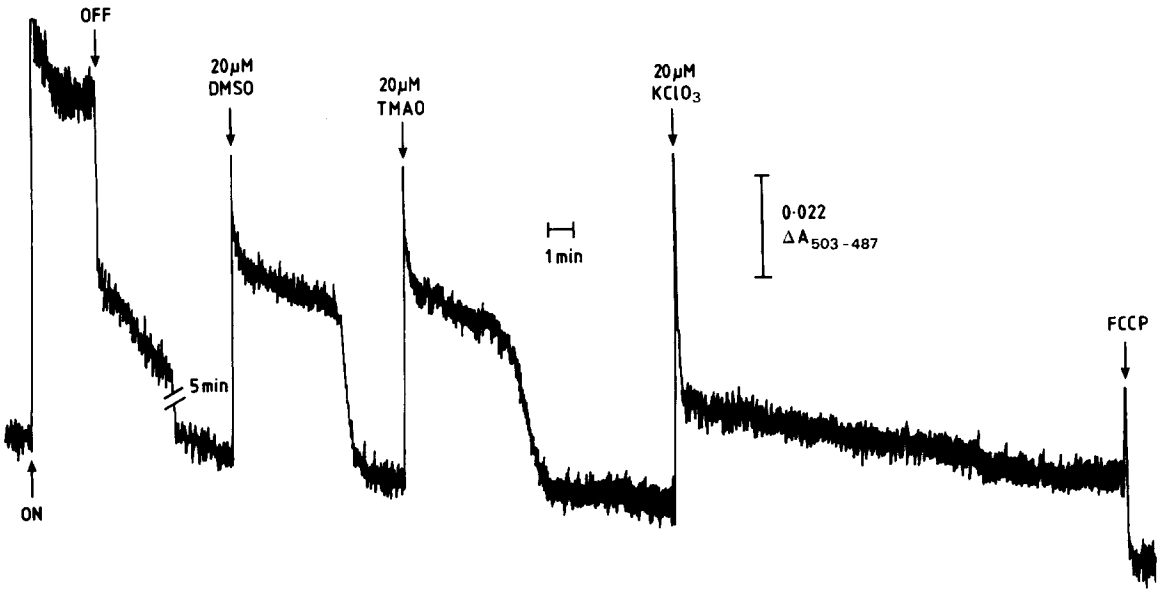

Fig. 2. Carotenoid band shifts in *Rps. capsulata* N22 during illumination or upon addition of TMAO, DMSO or ClO_3. Bacteria were added to a final concentration of 20 μM bacteriochlorophyll. Residual oxygen was removed by the bacterial respiration and the anaerobic suspension was left to equilibrate for 10 min. Addition of KNO_3 (not shown) or the same volume of water as introduced with solutions of the electron acceptor or KNO_3 (not shown) did not induce carotenoid band shifts.

not shown). These bandshifts were reversed upon exhaustion of substrate or upon addition of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) which showed that these bandshifts were genuinely in response to $\Delta\psi$ generation. Since the carotenoid band shift is a linear indicator of $\Delta\psi$ [18], it follows that the $\Delta\psi$ values achieved during illumination or during DMSO, TMAO and ClO_3^- reduction were in the ratio 100 : 60 : 56 : 32. These values were measured 1 min after illumination or addition of an electron acceptor. The baseline, $\Delta\psi = 0$, was taken to be the carotenoid absorption after the addition of a high concentration of FCCP.

Discussion

Cox et al. [6] have shown that whilst inverted membrane vesicles from *Escherichia coli* were able to generate a pH gradient upon addition of NADH and TMAO no such gradient was formed in vesicles from *Rps. capsulata.* In *E. coli* the situation is not in dispute; the TMAO-reductase is membrane-bound [6,19] and respiratory electron flow from NADH to TMAO is believed to drive proton translocation [4]. The failure to observe ΔpH formation during TMAO reduction by vesicles from *Rps. capsulata* [6] was taken as evidence against an energy-conserving role for this reaction in this organism. This is in apparent contraction with our experiments [7] in which we demonstrated that in intact cells of *Rps. capsulata* a membrane potential was generated during TMAO (or DMSO) reduction. Our explanation for the data of Cox et al. is that the TMAO-reductase activity is lost during vesicle preparation. This is supported by our finding that TMAO reductase is a soluble enzyme which is located in the periplasmic space of the bacterium. Also in the experiments of Cox et al. it should be pointed out that more than 98% of the TMAO reductase activity was soluble (they believed it to be a cytoplasmic enzyme) and less than 2% was found in the membrane (vesicle) fraction. Our view is that there was probably insufficient enzyme associated with the membranes of the correct orientation to catalyse sufficient rates of proton translocation to generate a pH gradient.

Our evidence that the DMSO/TMAO reductase is located in the periplasmic space is strengthened by the clear-cut data regarding the cytoplasmic marker enzyme, malate dehydrogenase (Table I). Cox et al. supplied two pieces of information supporting a cytoplasmic location. (a) The enzyme was soluble although this clearly does not argue against a periplasmic location. (b) In French press extracts NADH could serve as an electron donor especially in the presence of FMN. Since reactions involving NADH are only likely in the cytoplasm of the cell it was reasonable to assume that this was also where TMAO reductase was located. However, an alternative view is supported by the data of Table III. The FMN-dependent, NADH-TMAO oxido-reductase catalysed by whole cell extracts is a mixture of two enzyme activities: an NADH-FMN oxido-reductase (diaphorase) located in the cytoplasm and an dihydroFMN-TMAO (or DMSO) oxido-reductase located in the periplasm. The function of the diaphorase activity is not known. Alef and Klemme [20] previously reported that an NADH oxidase could be coupled to NO_3^- reduction in *Rps. capsulata* strain AD2 in the presence of FMN. We have shown previously that the NO_3^- reductase of *Rps. capsulata* AD2 is also a periplasmic enzyme [17]. It is conceivable that the NADH-FMN oxidoreductase is the physiological electron donor to TMAO reductase (and NO_3^- reductase), but this seems unlikely in view of the spatial separation of the two enzymes across the cytoplasmic membrane. The rotenone sensitivity of respiratory electron flow to TMAO/DMSO in intact cells [7] is a strong indication that the NADH-ubiquinone oxidoreductase is the NADH-oxidising component of the TMAO reducing pathway.

The pathway of electron flow from ubiquinol to TMAO is unknown, but antimycin insensitivity of electron transport to TMAO appears to rule out the involvement of the ubiquinol-cytochrome *c* oxidoreductase [7]. Further, we have failed to observe TMAO-dependent oxidation of the water-soluble quinone duroquinol by periplasmic extracts (unpublished data), a result which suggests that ubiquinol is probably not the direct donor to TMAO/DMSO reductase. However, it is known that when *Rps. capsulata* is grown under anaerobic dark conditions in the presence of DMSO dramatic changes in the cytochrome complement of the cell membrane are observed [21]. More specifically in

Rps. capsulata [21] and in *Rps. sphaeroides* grown under similar conditions [22], two cytochromes not clearly analogous to any described before have been observed. In *Rps. capsulata* a *b*-type cytochrome ($E_{m,7.0} = 0$ mV) and a loosely bound *c*-type cytochrome ($E_{m,7.0} = +134$ mV) were described [16]. The mid-point redox potentials of the trimethylamine/TMAO couple and the dimethylsulphide/DMSO couple are +130 mV [22] and +160 mV [24], respectively. Hence, at a reasonable poise of donor and acceptor couples these cytochromes described by Zannoni and Marrs [21] could be mediators of electron flow to TMAO and DMSO. Interestingly, the DMSO-dependent oxidation of a *b*-type cytochrome has been reported in *Rps. sphaeroides* [25].

From the substrate specificities in cell-free extracts (Table II) it appears that the TMAO/DMSO reductase and NO_3^- reductase of *Rps. capsulata* are distinct enzymes. In a previous paper [26] we reported that in a NO_3^- respiring mutant of *Rps. capsulata*, N22DNAR$^+$, a $\Delta\psi$ was generated during ClO_3^- reduction which was ascribed to the NO_3^- reductase. However, the observation that ClO_3^- reduction but not NO_3^- reduction occurs in TMAO/DMSO grown N22 cells and that a ClO_3^--dependent but not a NO_3^--dependent $\Delta\psi$ is generated in N22 (Fig. 2) suggests the ClO_3^- reductase activity might be a property of the TMAO/DMSO reductase.

Though our previous results [7] and those reported in this paper show that electron flow to TMAO and DMSO in *Rps. capsulata* is via respiratory electron transport, this may not mean that oxidative phosphorylation is the major generator of ATP during anaerobic dark growth. It has been observed [27,22] that both *Rps. capsulata* and *Rps. sphaeroides* are capable of anaerobic dark growth in the presence of nonfermentable substrates plus DMSO. In these cases ATP synthesis must have been via oxidative phosphorylation. However, in *Rps. capsulata* cell yields are much higher when glucose replaces malate [27]. This may indicate that under anaerobic dark conditions substrate level phosphorylation during sugar fermentation is the major generator of ATP, as suggested by Gest and co-workers [6,28]. In this case, electron flow to TMAO may indeed function as a redox sink for the maintenance of redox balance during fermentation. However, this pathway is clearly linked to $\Delta\psi$ generation via a proton-translocating respiratory chain. The rate of ATP synthesis in phototrophically grown *Rps. capsulata* decreases sharply with small decreases in $\Delta\psi$ [29] and therefore it is possible that $\Delta\psi$ generated during electron flow to TMAO and DMSO may be sufficient to support only very low rates of ATP synthesis, as suggested previously [7]. The $\Delta\psi$ generated during electron flow to DMSO or TMAO may contribute significantly in the energy economy of the cell under dark conditions even if it were incapable of driving substantial ATP synthesis. $\Delta\psi$ is involved in many cellular processes including solute transport. Direct demonstration of ATP synthesis by electron flow from NADH to TMAO in vesicle preparations would be extremely difficult because the NADH-oxidising site and the TMAO-reducing site are located on opposite sides of the membrane. In addition, preparation of membrane vesicles usually results in an almost complete loss of periplasmic enzymes.

The demonstration of a TMAO/DMSO reductase activity in phototrophically grown cells suggests that electron flow to TMAO or DMSO may have a role during photosynthetic growth as well as under anaerobic dark conditions. It has been suggested that under certain conditions branched respiratory electron transport may assume importance in the maintenance of the optimal redox poise of the photosynthetic electron transport system in both *Rhodospirillum rubrum* [30] and *Rps. capsulata* [21]. We suggest that, in addition to the alternative oxidase cytochrome *b*-260 [21], the NO_3^- reductase and TMAO/DMSO reductase may be of importance in the disposal of excess reducing equivalents in order that balanced growth and optimal photophosphorylation proceeds. Electron flow to the alternative oxidase, NO_3^- reductase and TMAO/DMSO reductase branches at the level of ubiquinone and does not involve the ubiquinol-cytochrome *c* oxidoreductase. However, it is known that redox components such as ubiquinol are shared between respiratory and photosynthetic electron transport chains [31]. Clearly, further work is required in order to understand the interaction between these respiratory chains and the cyclic electron transport chain and to understand their role during phototrophic

growth of the *Rhodospirillaceae*. A final point to emerge from the present work is that TMAO and DMSO reductase activities are most probably catalysed by a single enzyme in *Rps. capsulata*.

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