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Redox poise and oxygenation of cytochrome *bd* in the diazotroph *Azotobacter vinelandii* assessed *in vivo* using diode-array reflectance spectrophotometry

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A ferrous oxygenated form of cytochrome *d* is characteristic of all cytochrome *bd*-type oxidases so far examined, but its participation in enzyme turnover is unclear. It is relatively stable, occurs in aerated cell suspensions and predominates during enzyme preparation. In this study, diode-array reflectance spectrophotometry was used to assess the redox poise and oxygenation of cytochrome *bd in vivo*, in the aerobic diazotroph *Azotobacter vinelandii*. Mutants either lacking or overproducing the cytochrome *bd* oxidase were used to confirm the reliability of the optical configuration. Changes in absorbance attributed to cytochromes *b*, *c* and *d* were followed as the O_2 supply was altered either in suspensions of harvested cells or during steady-state growth. In washed cell suspensions, three states of cytochrome *d*, which differed in absorbance characteristics, were seen: (1) an oxygenated form that absorbs at 650 nm, (2) a form which has little absorbance at either 650 or 630 nm and (3) the reduced form that absorbs at 630 nm. The transition between states 2 and 3, but not 1 and 2, correlated with the changes in the redox states of cytochromes b_{595} and b_{560}. The dissolved O_2 concentration at which this transition occurred coincided approximately with the apparent O_2 affinity for the oxidase *in vivo* (approx. 5 μM). During steady-state growth, the cytochromes were partially reduced and the oxygenated form of cytochrome *d* was undetected. These *in situ* measurements support the view that an oxygenated form of cytochrome *d* (absorbing at 650 nm) in the one-electron-reduced cytochrome *bd*-type oxidase does not take part in enzyme turnover.

Keywords: *Azotobacter vinelandii*, cytochrome *bd*-type oxidase, O_2 supply and redox changes of cytochromes, diode-array spectrophotometry

INTRODUCTION

Members of the genus *Azotobacter* are obligately aerobic heterotrophs, which can fix N_2 in the free-living state. An understanding of how aerobic organisms can fix N_2 has long been a focus for investigation, because N_2 fixation catalysed by the nitrogenases is a strictly anaerobic process. Respiration in organisms capable of aerobic N_2 fixation has at least two functions: to provide adequate ATP for the energy-intensive process of N_2 fixation, and to remove excess O_2 as part of the mechanism to protect nitrogenase from inactivation by O_2. *Azotobacter vinelandii* has the ability to fix N_2 over a wide range of O_2 concentrations. This ability, although not fully understood, involves in part changes in the synthesis and activities of components of the respiratory chain and of those supplying electrons for respiratory activity (see reviews by Yates, 1988; Hill, 1992; Poole & Hill, 1997).

The respiratory chain of *Azotobacter vinelandii* is branched at both ends. Several dehydrogenases feed

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Abbreviations: DARS, diode-array reflectance spectrophotometry; DOC, dissolved oxygen concentration.

electrons into a quinone pool. Subsequently, the flow of electrons proceeds to two, or possibly three, different terminal oxidases, dependent upon the electron pressure, the prevailing oxidase concentrations and their affinities for O_2. Mutational and gene expression studies have revealed that the cytochrome *bd*-type quinol oxidase plays an essential role in the removal of O_2 under conditions of high O_2 input (Kelly *et al.*, 1990; Leung *et al.*, 1994; D'mello *et al.*, 1997; Wu *et al.*, 1997). It has a relatively high apparent K_m (low affinity) for O_2 (D'mello *et al.*, 1994a). The alternative terminal oxidase, of the haem-copper type (Yang, 1986; Leung *et al.*, 1994), has a higher apparent affinity for O_2 (D'mello *et al.*, 1994a), whereas a putative third oxidase may have an even higher apparent affinity (D'mello *et al.*, 1994a).

The cytochrome *bd*-type quinol oxidase of *A. vinelandii* is similar to that of *Escherichia coli*. The genes (*cydAB*) encoding the subunits (Green *et al.*, 1988; Moshiri *et al.*, 1991), and the structure and properties of the enzymes (Kita *et al.*, 1984; Kolonay *et al.*, 1994; Jünemann & Wrigglesworth, 1995) all share common features. One striking difference between them is their apparent K_m for O_2, the value, either *in vitro* or *in vivo*, being much lower for the *E. coli* than for the *A. vinelandii* enzyme (D'mello *et al.*, 1994a, 1996; Jünemann *et al.*, 1995). This feature probably reflects differences in the rate of electron donation to the enzyme complexes (Jünemann *et al.*, 1995). The *A. vinelandii* enzyme, like that of *E. coli*, contains one mole each of haem *d*, haem b_{595} (high spin) and haem b_{558} (low spin), although the α-peak of the low-spin *b* is quoted as 560 nm by Kolonay *et al.* (1994) and Jünemann & Wrigglesworth (1995).

Early studies of cytochrome *bd* in *A. vinelandii* revealed a spectral form of the oxidase with a distinctive absorption maximum at 650 nm (Kauffman & van Gelder, 1973). As in the pioneering work of Keilin (1966), this form was initially ascribed to the oxidized form of the enzyme, since it was generated by shaking suspensions of cells or membranes in air. However, low-temperature experiments with *E. coli* cells and membranes revealed that a form of the oxidase absorbing at 650 nm (d_{650}) was observed immediately after photolysing the CO-ligated species in the presence of O_2 and before redox changes could be observed by EPR (Poole *et al.*, 1983). On the basis of these experiments and re-analysis of earlier data, Poole *et al.* (1983) proposed that cytochrome d_{650} is an oxygenated or oxy-complex in which the haem *d* remains reduced. It was suggested (Poole *et al.*, 1983) that the oxidized form of the oxidase lacks distinct features in the red region of the spectrum and may be equated with the (d_x) form described by Kauffman & van Gelder (1973). Subsequent studies of membrane-bound and purified cytochrome *bd* from *A. vinelandii* and *E. coli* have led to a model for the catalytic cycle of O_2 reduction in which one (Kahlow *et al.*, 1991) or two forms of oxygenated cytochrome *d* are implicated (for a review see Jünemann, 1997).

A form of cytochrome *d* absorbing at 650 nm, presumably an oxygenated form, has been observed in many other bacteria including *Klebsiella pneumoniae* (Smith *et al.*, 1990) and *Photobacterium phosphoreum* (Konishi *et al.*, 1986). In all cases, the form is remarkably stable (compared with the oxygenated complex of haem-copper type oxidases; e.g. Poole *et al.*, 1979). However, nothing is known about the physiological significance of this form and whether it exists in growing cells. Therefore we have devised a modified diode-array spectrophotometer to investigate the effects of O_2 supply on the redox poise and oxygenation *in vivo* of cytochromes *b*, *d* and *c* of *A. vinelandii*. A preliminary summary of some of this work has been published elsewhere (Kavanagh *et al.*, 1995).

METHODS

Bacteria, culture and preparations of cell suspensions. *A. vinelandii* strain UW136 (a rifampicin-resistant derivative of strain UW; Bishop *et al.*, 1980) and its kanamycin-resistant derivatives MK5 (carrying *cydB*::Tn*5*-B20) and MK8 (carrying *cydR*::Tn5-B20) (Kelly *et al.*, 1990; Wu *et al.*, 1997) were maintained on Burke's medium with 2% sucrose and 15 mM ammonium acetate (BSN; Kelly *et al.*, 1990) and subcultured monthly. Batch cultures were grown in 50 ml BSN in Erlenmeyer flasks (250 ml) and incubated at 30 °C in air without shaking for 6 h and then on a rotary shaker (140 r.p.m.) for 17 h. Cultures were inoculated (2% for UW136 and MK8; 4% for MK5) from cultures grown in an enriched Burke's-type medium (RM; Robson *et al.*, 1984) that had been incubated as above.

Sucrose-limited chemostat cultures of strain MK8 were grown at a dilution rate of 0·1 h^{-1} in N-free Burke's medium containing sucrose (0·5%), nitrilotriacetic acid (0·5 mM) and kanamycin (1 $\mu g\ ml^{-1}$) contained in a 1 l New Brunswick Bioflo III fermenter with a working volume of 500 ml and modified as described below for diode-array spectrophotometry. Growth was maintained at pH 7, at 30 °C and at dissolved O_2 concentrations (DOC) of 12 or 24 μM O_2 as indicated. Due to the high respiratory activity of this strain, a DOC of 24 μM O_2 was the highest we could easily achieve when using an agitation rate of about 500 r.p.m. and 80–90% O_2 in N_2 in place of air. Cultures were inoculated (2%) from the RM culture grown as described above, and achieved a steady-state sucrose limitation even at 12 μM O_2, which is below the O_2 level at which Kelly *et al.* (1990) were able to grow this strain on plates. This difference probably resides in the more favourable rate of O_2 transfer provided by the chemostat environment. Cells from batch cultures (50 ml) were harvested by centrifugation (15 min at 2000 *g*) at 4 °C, washed in 50 ml Burke's medium without carbon and fixed nitrogen sources and resuspended in 10 ml of the same medium to give a five fold concentration. The cells were stored at 4 °C.

Biomass was estimated by protein content, which was calculated from the relationship of OD_{540} to bacterial protein (Smith *et al.*, 1988).

Modifications to a Hewlett Packard HP 8452A diode-array spectrophotometer and Clark-type O_2 electrode for recording absorption spectra in bacterial samples. Although oxidases (and all cytochromes) have intense and characteristic absorbance bands, their study *in vivo* is not trivial. The major experimental obstacle is that the absorbance bands are superimposed on, and therefore masked by, the scattering by the cells themselves of the measuring light from the sample.

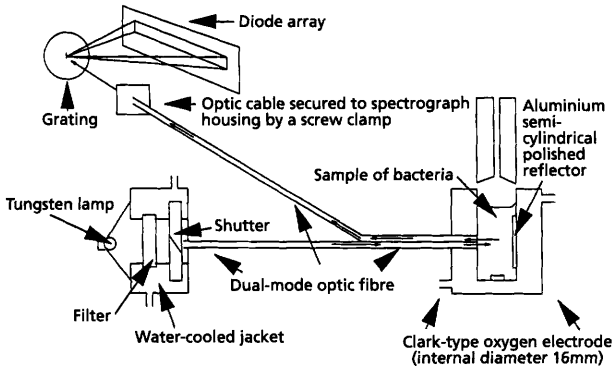

Fig. 1. Diagram of light path in modifications to the Hewlett Packard diode-array spectrophotometer and the Clark-type O_2 electrode chamber for recording absorption spectra in bacterial samples retained in the O_2 electrode chamber. The light source was a tungsten projector lamp (Philips type 6423, 15 V, 150 W), mounted in the back of a water-cooled aluminium frame and powered by a highly stable DC source (0–15 V) fitted with a 10-turn potentiometer for accurate control of light intensity. Within the frame were mounted a light filter with a transmission range 475–980 nm (Oriel Scientific), an electromagnetically operated shutter (Magnetic), which was controlled through the existing shutter drive circuit of the spectrophotometer and the larger afferent arm of the bifurcated optic fibre. The efferent limb of the bifurcated optic fibre was abutted on to the window of the spectrograph and secured to the housing by a screw clamp. To deliver and return light to and from the bacterial sample, the dual-mode end of the optic fibre was mounted to penetrate the water jacket and inner wall of the perspex Clark-type O_2 electrode chamber (Rank Bros), so as to be opposite a thin polished aluminium sheet, acting as reflector, that was placed against the inside wall of the chamber.

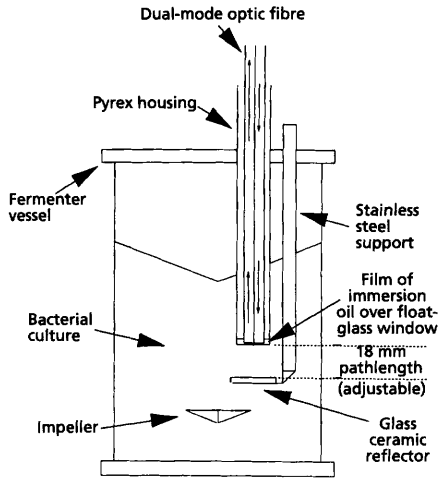

Fig. 2. Light path for monitoring cytochrome redox poise in a fermenter. A pyrex glass dip tube with a float glass end window and carrying, after autoclaving, the dual-mode end of the bifurcated fibre-optic cable resting in a drop of microscope immersion oil, was mounted in the head plate of the fermenter next to a stainless steel support on which was secured a glass ceramic reflector (Macor). The 'macor' reflector material reflects light uniformly, its thermal coefficient of expansion is suitable for repeated autoclaving *in situ* in the fermenter, and it is non-wettable and non-porous, which minimized microbial growth on its surface.

Scattering is most intense, and thus most problematic, at the lowest wavelengths. Routinely, these difficulties are overcome by two approaches. First, the sample cuvette is located close to the light detector so that as much of the scattered beam as possible is detected by the photomultiplier, diode array or other detector. Very few commercially available spectrophotometers offer this facility and are therefore unsuitable for observing cytochrome absorbance *in vivo*. Second, advantage is taken of the substantial absorbance changes that occur upon oxidation and reduction of the sample without major changes in the light scattering. Thus, a difference spectrum (most commonly reduced minus oxidized) reveals the cytochrome spectral features without observation of the light-scattering signal (Poole & Bashford, 1987). Computing a derivative of the absolute spectrum (see below) also aids analysis by virtue of a marked narrowing of the absorbance bands. Further experimental difficulties arise if the sample is not easily contained in a cuvette suitable for measuring transmitted light. These have been generally overcome by the availability of fibre-optic light guides to bring the measuring beam to the sample and the reflected signal from the sample to the detector. For example, such light guides, in conjunction with UV/visible spectrophotometers, have been used to monitor the cytochrome content in steady-state growth of the N_2 fixer *Azorhizobium caulinodans* (Pronk *et al.*, 1993) and in conjuction with light-emitting diodes to estimate the oxygenation of leghaemoglobin in intact birdsfoot trefoil (*Lotus corniculatus*) nodules (Denison & Layzell, 1991). In this study, we harnessed fibre-optic light guides to a diode-array spectrophotometer. The latter has the advantage, when compared to a UV/visible spectrophotometer, of being able to make simultaneous measurements over a wide range of wavelengths.

We used an HP 8452A instrument (470–1100 nm). The modifications made for the purpose of *in situ* cytochrome analysis, using a bifurcated optic fibre to carry light to and from the sample (retained in a Clark-type O_2 electrode chamber), are indicated in Fig. 1. Summaries of these modifications have appeared earlier (Cavinato *et al.*, 1990, 1992); technical information can be provided by the authors on request. The sample (7 ml) of bacterial suspension was stirred in the O_2 electrode chamber, maintained at 30 °C by water circulation, and where indicated, flushed with a stream of either air or N_2. Power settings for the light source were adjusted, in the absence of the bacterial sample, to give maximum light intensity but within the recommended limits of the supplied Hewlett Packard software. Blanks were then recorded. The values of absorption of these, and subsequently those from the samples of bacteria, were converted to the second-order derivative with a smoothing factor of five by the supplied Hewlett Packard software. The negative value of the second-order derivative of absorbance was used to report the signal intensity at a particular wavelength (see Results). The wavelengths used for reporting the changes in signal intensity when varying the O_2 supply were those giving maximum deflection in the second-order derivatives when the sample of cells was under anaerobiosis. They varied in different samples by 2 or 4 nm.

Oxystat for controlling DOC in the Clark-type O_2 electrode chamber. The maintenance of desired DOCs in the Clark-type O_2 electrode chamber was achieved by the equipment previously described by Kavanagh & Hill (1990), which uses feedback control of the magnetic stirrer by the electrode current. In earlier work, O_2 detection was by photoemission

from a photobacterium. In the present work, the DC polarizing voltage required for the Clark-type O_2 electrode was provided by the amplifier incorporated into this previously described equipment. A gas space retained above the sample in the Clark-type O_2 electrode chamber was flushed with either air or N_2 to bring the DOC near the required value, at which point the controller was switched on.

Additions to a New Brunswick Bioflo fermenter for *in situ* spectral analysis. The equipment for light delivery to, and collection from, the bacterial chemostat culture in a New Brunswick Bioflo fermenter is shown in Fig. 2. The path length of light could be adjusted to give optimum signals. Blanks were recorded before the fermenter was filled with medium or during growth by removing the optic fibre and placing it in a duplicate dip tube and reflector mounted outside the fermenter.

RESULTS AND DISCUSSION

Detection of cytochromes in washed suspensions of *A. vinelandii* by diode-array reflectance spectrophotometry (DARS)

To investigate the influence of O_2 status on the redox poise of cytochrome *bd*, and in particular the occurrence of the stable oxygenated form of cytochrome *d* in *A. vinelandii* ,we employed DARS. Initially, we tested the efficacy of the modified diode-array spectrophotometer by measuring the cytochrome spectra of N_2-sparged suspensions of bacterial cultures within the chamber of a Clark-type O_2 electrode chamber (Fig. 1; Methods). Three strains of *A. vinelandii* known to synthesize different amounts of cytochrome *d* (Kelly *et al.*, 1990) were grown in batch culture with excess NH_4^+ until stationary phase, harvested, washed and resuspended in buffer as described in Methods. Strain MK5 carries a Tn*5* insertion in *cydB* and synthesizes no cytochrome *bd* (Kelly *et al.*, 1990; Moshiri *et al.*, 1991). Strain MK8 has a Tn*5* insertion in *cydR*, encoding an Fnr-like transcriptional repressor of the *cydAB* operon and thus overproduces this oxidase (Wu *et al.*, 1997; D'mello *et al.*, 1997). Fig. 3 shows the second-order derivatives of spectra obtained from N_2-sparged suspensions of these three strains. The second-order derivatives provided a means of observing absorption spectra in these turbid suspensions (see Methods). Absorption bands that appear positive (peaks) in the raw data appear as negatives (troughs) of narrower band width in the second-order derivatives (Butler & Hopkins, 1970). In the absence of O_2, when the cytochromes are reduced, cytochrome *d* has a distinctive absorbance at 630 nm. This absorbance in the second-order derivative appears as a trough at 630 nm in the wild-type (strain UW136) and is more pronounced in strain MK8, but is missing in strain MK5 (Fig. 3).

The narrowing of band widths afforded by the second-order-derivative analysis gave a clear resolution of cytochromes *c* (552 nm) and *b* (560 nm). The scans of UW136 and MK8 (Fig. 3) also show differences in the relative sizes of the signals at 552 nm and at 560 nm. The ratio of the depths of these troughs (552 nm : 560 nm) is lower in MK8 than in UW136. A similar difference was

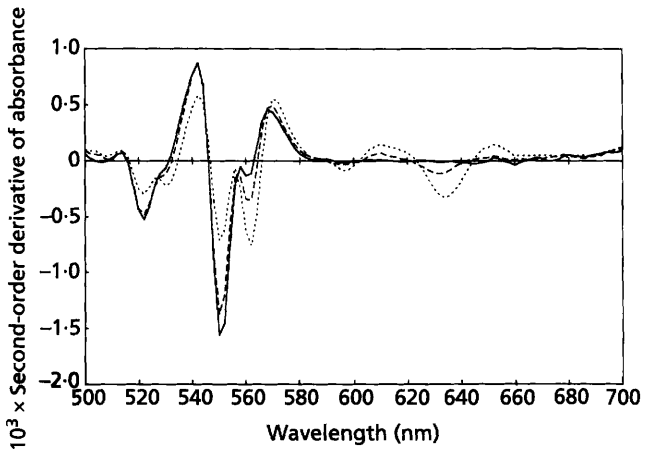

Fig. 3. Second-order derivatives of absorption spectra of suspensions of *A. vinelandii* strains stirred under N_2. Spectra of strains UW136 (dashed line), MK8 (dotted line) and MK5 (solid line) were recorded using the optics shown in Fig. 1. Batch cultures were grown, harvested and resuspended as described in Methods to give protein concentrations (mg ml^{-1}) of 1·9 for UW136, 1·4 for MK8 and 2·0 for MK5. The suspensions were stored for 2 d at 4 °C, and when placed in the Clark-type O_2 electrode chamber, were supplemented with sucrose (4·2 mM), and then stirred under N_2.

found in the reduced minus oxidized difference spectra of these strains; in MK8 the ratio of cytochromes *c* to *b* (A_{552}:A_{560}) was lower than in UW136 (Kelly *et al.*, 1990). From this initial feasibility study, we concluded that the optics associated with the electrode chamber could distinguish the reduced forms of cytochromes *d*, *b* and *c*.

Two aspects of this application of diode-array spectrophotometry limit the quantification of bacterial cytochromes to relative values rather than absolute ones. First, the path length is unknown due to light scattering by the bacteria. However, we found an apparent proportionality of signal intensity (e.g. at 560 and 630 nm), when measured from the line drawn through zero to the bottom of the trough, with biomass concentrations (up to about 0·7 mg protein ml^{-1}) in N_2-sparged suspensions of UW136 or MK8 (data not shown). Because the intensity of a signal in a second-order derivative spectrum is influenced by the natural bandwidth of the chromophore (Butler & Hopkins, 1970) and because these bandwidths cannot be assumed to be equal, two *different* species in the same spectrum cannot be compared. However, these data demonstrate that the trough depth for the *same* species indicates relative amounts.

Furthermore, in second-order derivatives of absorbance spectra, the depth of a trough is influenced not only by the sharpness of the original peak but also by the accompanying positive shoulders of a closely occurring trough (Butler & Hopkins, 1970). The latter is the case with the troughs at 552 and 560 nm, which are attributed to cytochromes *c* and *b* respectively. Therefore caution

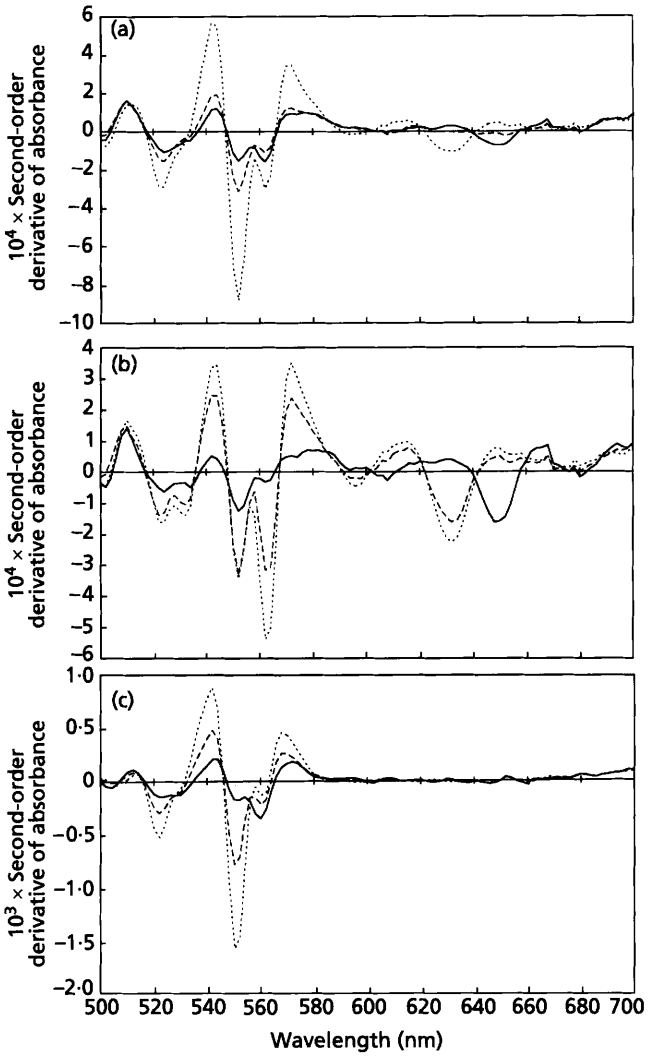

Fig. 4. Second-order absorption spectra showing transitions between fully oxygenated and fully reduced cytochromes in *A. vinelandii* strains. Suspensions of strains UW136 (a), MK8 (b) and MK5 (c) (grown, resuspended to give protein concentrations of 0·7, 0·7 and 2·0 mg ml^{-1} respectively and stored as indicated in Methods) were placed in the Clark-type O_2 electrode chamber. They were supplemented with sucrose (4·2 mM), and spectra were recorded, first when the suspensions were stirred under air (solid line) and then when the gas supply was changed to N_2; a series of spectra were taken during the subsequent 2 min until the cytochromes became fully reduced (dotted line). For clarity only the fully oxidized spectrum, the fully reduced spectrum and one partially reduced representative spectrum (dashed line) are shown.

is required when interpreting the sizes of signals, as such overlaps could obscure isosbestic points.

Influence of O_2 supply on cytochrome redox state in washed suspensions

Fig. 4 shows the second-order derivatives of a series of repeated scans recorded when suspensions of washed cells of strains UW136 (Fig. 4a), MK8 (Fig. 4b) or MK5 (Fig. 4c) were stirred in the presence of added sucrose (approx. 4·2 mM), first under a stream of air, and then under a stream of N_2 in the Clark-type electrode. To obtain changes over a sufficiently long time period (from 2 to 4 min) so that sequential scans showed the intermediate redox states (of which, for clarity, only one is shown in each panel of Fig. 4), suspensions were stored for about 2 d at 4 °C; changes that occurred in suspensions used earlier were too rapid to be resolved. During the storage of these suspensions, no change was observed in cytochrome complement, when viewed under either N_2 or air. Since storage was in the absence of carbon and energy sources, the slowing of redox changes probably arose from a decline in rate of electron donation.

Certain spectral changes visible in UW136 (Fig. 4a) and particularly in MK8 (Fig. 4b) can be unambiguously attributed to components of the cytochrome *bd* complex. These are at 630 nm (reduced cytochrome *d*), 650 nm (oxygenated cytochrome *d*) and 595 nm (high-spin cytochrome b_{595}). A proportion of the change at 560 nm must also be due to the low-spin cytochrome *b* component of this oxidase complex. These assignments are unaltered by a redistribution of electron flux from ubiquinol that might occur when an oxidase with higher affinity for oxygen takes more of the electron flux at low oxygen concentration. As the O_2 supply declined, the oxygenated (650 nm) form was diminished and the reduced (630 nm) form appeared. There is a clear isosbestic point for cytochrome *d* at 642 nm. The reduction of cytochrome b_{595} is also just recognizable as the appearance of a small signal at 595 nm as the O_2 supply declined; as expected, this signal is more marked in the spectra of MK8 than of UW136. The increase in the signal intensity at 552 nm is attributed to the reduction of cytochromes *c*, and that at 560 nm is in part attributed to reduced low-spin cytochrome b_{560}. The increases in absorbance at 595 and 560 nm accompanying the decline in O_2 supply indicate that initially, under air, the cytochrome *bd* complex in strains UW136 and MK8 was in the oxygenated state (d_{650}). This is presumably the stable form of the oxidase – species 7 in the scheme of Jünemann (1997), in which cytochromes b_{560} and b_{595} are oxidized (and their reduction is observed in the experiment), whereas cytochrome *d* must be reduced, since only in this state can it bind oxygen to give the 650 nm form. As expected, the scans for MK5 (Fig. 4c) showed no signal at 650 nm, as this strain does not make cytochrome *bd*. The only increase in signal that occurred upon the decrease in O_2 supply was at 552 nm, attributed to reduction of cytochromes *c*.

Having observed the one-electron-reduced oxygenated form of cytochrome *bd*, we analysed the sequence of scans for changes in redox poise as the O_2 supply declined. Fig. 5 shows the data for the complete sequences of scans. Here the changes in signal intensities at wavelengths near 552 nm (attributed to cytochromes *c*), 560 nm (attributed to cytochrome b_{560} and other *b*-type cytochromes), 595 nm (attributed to cytochrome b_{595}), 630 nm (attributed to the deoxygenated cytochrome d^{2+}) and 650 nm (attributed to the oxygenated

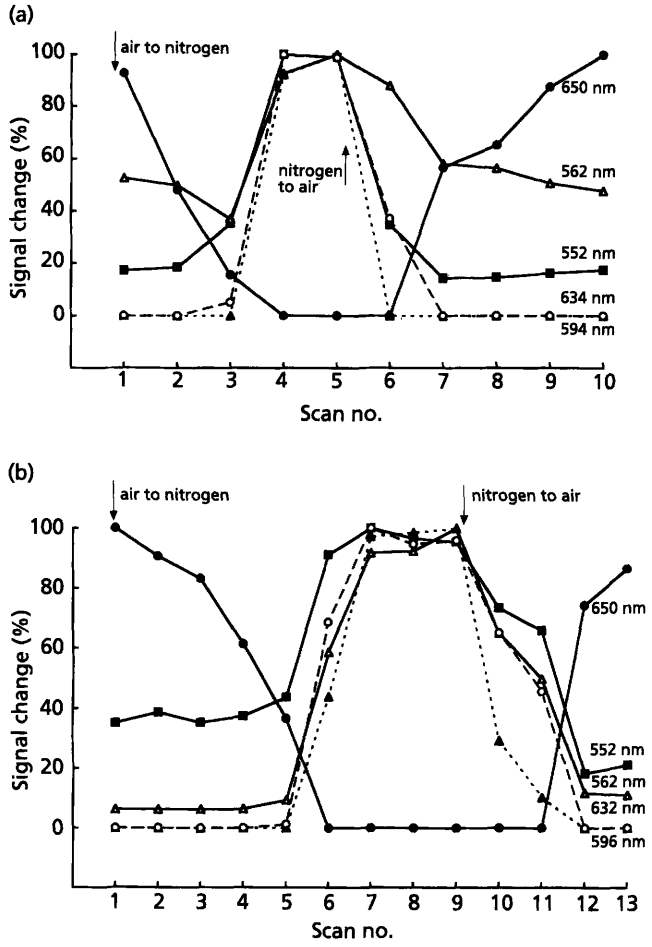

Fig. 5. Changes in cytochrome signal intensity at various wavelengths in suspensions of *A. vinelandii* strains during a decline and then an increase in O_2 supply. A series of spectra of strains UW136 (a) and of MK8 (b) were taken (as described in the legend to Fig. 4) over a time period of about 4 min. The gas supply was changed from air to N_2 just before the first scan shown. Spectra were recorded until no further change was seen, when the gas supply was changed back to air (after scan 5 in a and scan 9 in b); recording continued until there was no further change. Signal sizes at the wavelengths shown are shown as percentages of the maximum deflection recorded during the series. Signal sizes at 594/596 nm (▲) and 632/634 nm (○) partly overlap in (a) and (b). These maxima for the measurement from baseline to trough in the second-order derivatives of absorbance were, for UW136 and MK8 respectively, at wavelengths of: 552 nm, $8{\cdot}9\times10^{-4}$ and $3{\cdot}6\times10^{-4}$; 560 nm, $3{\cdot}0\times10^{-4}$ and $5{\cdot}4\times10^{-4}$; 595 nm, $0{\cdot}13\times10^{-4}$ and $0{\cdot}47\times10^{-4}$; 630 nm, $1{\cdot}0\times10^{-4}$ and $2{\cdot}4\times10^{-4}$; 650 nm, $0{\cdot}77\times10^{-4}$ and $1{\cdot}6\times10^{-4}$. The scans showing partially reduced cytochromes in Fig. 4 were scan 3 in (a) and 6 in (b).

cytochrome d^{2+}) are expressed as percentages of the maximum observed at that wavelength for the sequence. In both strains UW136 (Fig. 5a) and MK8 (Fig. 5b), upon changing the gas supply to the chamber from air to N_2, the level of oxygenated cytochrome d^{2+} (signal at 650 nm) started to decline well before the reduction of cytochromes b_{560} and b_{595} (increases in the signals near 595 nm and 560 nm) were discernible. In Fig. 5(a) for example, the level of oxygenated cytochrome d^{2+} (signal at 650 nm) had declined to about 15% of its initial value before significant change had occurred in the reduction of cytochromes b_{560} and b_{595} (increases in signals at 594 and 634 nm). Thus, the decrease occurring at 650 nm was apparently due to those associated with the one-electron-reduced oxygenated cytochrome *bd*. Subsequently, the reduction of cytochromes *c*, b_{560} and b_{595} (increase in signals at 552, 562 and 594 nm respectively) and the appearance of the deoxygenated cytochrome d^{2+} (increase in signal at 634 nm) occurred almost in parallel (Fig. 5a). The changes in the reduction of b_{595} (signal near 595 nm) are clearer in the data for strain MK8 (Fig. 5b). In the data for scan 3 of strain UW 136 (Figs 4a and 5a) the sizes of signals attributed to cytochrome d^{2+}, in both the oxygenated and the deoxygenated forms (signals at 650 and 634 nm), indicated that a large proportion of cytochrome *d* appeared to be in a form that did not absorb at either of these wavelengths. This poise was quite difficult to record, but was observed on two other occasions with this strain. A somewhat similar poise was seen in strain MK8 (scan 5 in Fig. 5b). Since each series of measurements was recorded in a single sample in which the total cytochrome *d* content was constant and since both the 630 nm and 650 nm signals were weak, we conclude that we were observing a species of cytochrome *d* that absorbed at neither 630 nor 650 nm.

When the N_2 supply was replaced by air, there was a general reversal in the redox and oxygenation changes in both strains UW136 and MK8 (Fig. 5). The oxidation of cytochromes *c*, b_{560} and b_{595} (decline in signals near 552, 560 and 590 nm, respectively) and disappearance of the deoxygenated form of cytochrome d^{2+} (signal near 630 nm) all started well before the oxygenated cytochrome d^{2+} (signal at 650 nm) was discernible. During this decline, scans were recorded for strains UW136 (scan 6 in Fig. 5a) and MK8 (scan 11 in Fig. 5b), where more than 50% of the cytochrome *d* could not be accounted for at either 630 or 650 nm. Again cytochromes b_{560} and b_{595} appeared to be oxidized when the cytochrome d^{2+} was oxygenated, indicating that the signal at 650 was due to the oxygenated one-electron-reduced cytochrome *bd*.

These changes in signal sizes with alterations in O_2 supply demonstrate that the transformations in redox states of cytochromes b_{595} and b_{560} are not closely coupled to the transitions of cytochrome *d* between the oxygenated one-electron-reduced species absorbing at 650 nm and a species which seems not to absorb significantly at either 630 or 650 nm. In contrast, they do appear to be coupled to the appearance and disappearance of the reduced cytochrome *d* (absorbing at 630 nm). Kauffman & van Gelder (1973) came to the same conclusion from following changes in absorbance between 600 and 700 nm during transitions in redox states of *A. vinelandii* phosphorylating particles.

Electron transfer from quinol in the *E. coli* enzyme occurs via haem b_{558} to the other two haems which

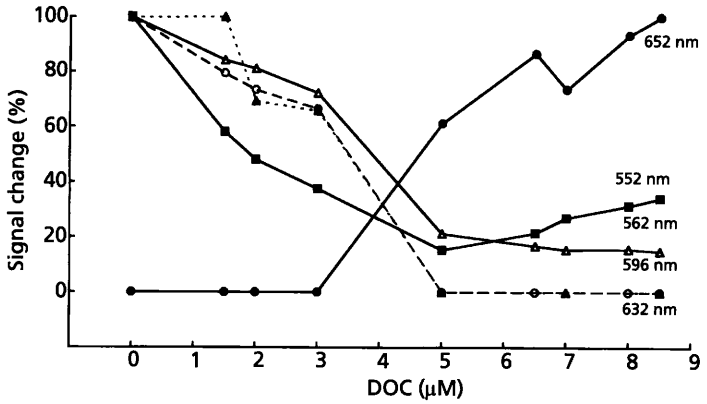

Fig. 6. Correlation of signal sizes at selected wavelengths with the DOC in a suspension of *A. vinelandii* strain MK8. The suspension (0·44 mg protein ml^{-1}) was prepared for spectroscopy as described in the legend to Fig. 4, and was stirred under N_2 or under air in the Clark-type O_2 electrode chamber using the optics shown in Fig. 1. The stirring rate was automatically controlled to maintain a series of rising preset DOC values (see Methods), each for about 4 min, during which time the spectra were recorded. The data are plotted as a percentage of the maximum deflection as described in the legend to Fig. 5, and are shown with the maximum signal for the following wavelengths: 552 nm (■), $3·2 \times 10^{-4}$; 562 nm (△), $4·0 \times 10^{-4}$; 596 nm (▲), $2·7 \times 10^{-5}$; 632 nm (○), $1·5 \times 10^{-4}$; and 652 nm (●), $1·0 \times 10^{-4}$.

probably reside in a common pocket where oxygen is reduced (Hill *et al.*, 1993). Spectral data on ligand binding to the *A. vinelandii* enzyme have confirmed that the haem b_{595} has a lower affinity for O_2 than does haem *d* (D'mello *et al.*, 1994b; Jünemann & Wrigglesworth, 1995), and that O_2 can migrate from the oxygenated ferrous complex of cytochrome *d* to the haem b_{595} (D'mello *et al.*, 1994b). Flash-flow rapid kinetics with the three-electron-reduced *E. coli* enzyme have shown (Hill *et al.*, 1994) that although O_2 rapidly binds to cytochrome *d* (giving a form that absorbs at 650nm) it rapidly decays to a species identified by Kahlow *et al.* (1991) as oxy-ferryl (d_{680}). Moreover, steady-state kinetics (Jünemann *et al.*, 1995; Jünemann & Rich, 1996) of the *A. vinelandii* enzyme suggest that the oxygenated one-electron-reduced enzyme (d_{650}) develops when reducing equivalents are exhausted; further, upon restoration of electron donation, the very short-lived oxygenated three-electron-reduced enzyme (d_{650}), observed by Hill *et al.* (1994), provides re-entry into the fast cycle of enzyme turnover. An earlier scheme of Kahlow *et al.* (1991) includes the oxygenated cytochrome d_{650} within enzyme turnover, but does not differentiate between the one- and three-electron-reduced oxygenated forms of the enzyme.

The redox state of cytochrome *d* giving the near-featureless species between 630 and 650 nm is unclear, but the oxidized form (Fe^{III}) was proposed to have such properties in the scheme of Poole *et al.* (1983). In this case, the oxidase would be in the redox state b_{560}^{3+}, b_{595}^{3+}, d^{3+}. The resolution of our spectra was insufficient to observe changes at 680 nm (see Fig. 4b), which would identify the oxy-ferryl species of cytochrome *d* (Kahlow *et al.*, 1991; Hill *et al.*, 1994). In this case, the oxidase would be in the redox state b_{560}^{3+}, b_{595}^{3+}, $d^{4+}O^{2-}$, which corresponds to intermediate 3 in the scheme of Jünemann (1997). In our experiments, three different redox states of the cytochrome *bd*-type oxidase were apparent: initially under air, the one-electron-reduced oxygenated form, b_{560}^{3+}, b_{595}^{3+}, $d^{2+}O_2$, (intermediate 7 in the Jünemann, 1997, scheme), then as the O_2 supply declined, the unidentified species indicated above (either b_{560}^{3+}, b_{595}^{3+}, d^{3+} or b_{560}^{3+}, b_{595}^{3+}, $d^{4+}O^{2-}$) changing to the fully reduced form b_{560}^{2+}, b_{595}^{2+}, d^{2+}(unligated) (intermediate 6 in the Jünemann, 1997, scheme). These changes were reversed when the O_2 supply was re-introduced. In the catalytic cycle proposed by Jünemann *et al.* (1995), the stable one-electron-reduced oxygenated species of cytochrome *d* (absorbing at 650 nm) does not contribute to enzyme turnover associated with O_2 reduction. This species is referred to by Jünemann (1997) as intermediate 7 or the 'oxygenated (as prepared)' form to indicate its occurrence in preparations of the purified oxidase complex. It enters the cycle slowly, by way of reduction, to form the very short-lived oxygenated reduced enzyme (b_{560}^{2+}, b_{595}^{2+}, $d^{2+}O_2$), and only re-forms when reducing power becomes exhausted. Our observations are consistent with a slow rate of reduction of the one-electron-reduced oxygenated form, suggesting that this form is not an intermediate of turnover *in vivo*.

Relationship of cytochrome redox state in washed suspensions with DOC

To determine the DOC at which these redox changes were occurring, an oxystat was attached to the O_2 electrode. The changes in DOC during the transitions depicted in Figs 4 and 5 occurred within the range 0–10 μM, but at a rate too fast to correlate satisfactorily the prevailing DOC with the redox states of the cytochromes. The oxystat controlled the stirring rate to maintain preset DOC values in the suspension (see Methods). A suspension of strain MK8, which had been sparged with N_2, was stirred in air and second-order-

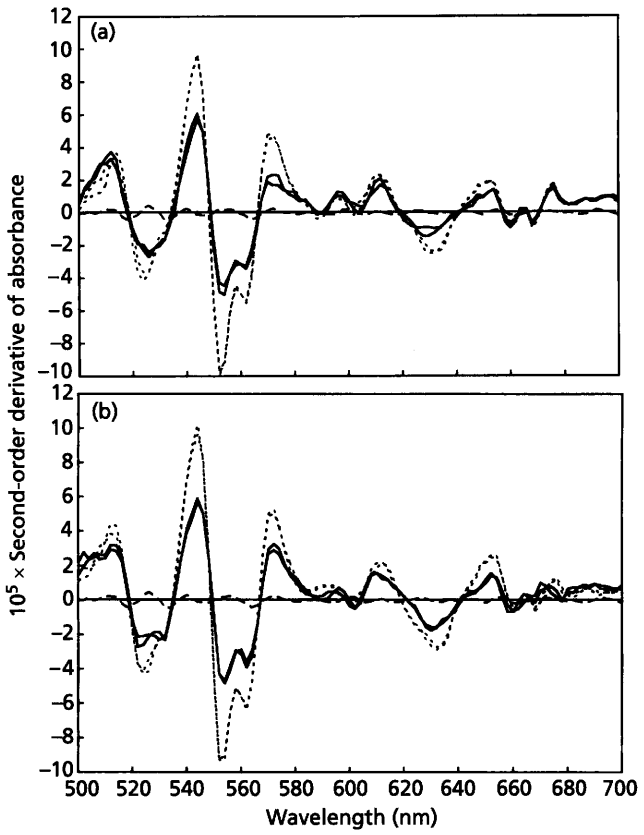

Fig. 7. Spectra of *A. vinelandii* strain MK8 during growth in the modified New Brunswick Bioflo fermenter. Sucrose-limited N_2-fixing steady states (0·13 mg protein ml^{-1}) were established as described in Methods and maintained at either 12 μM (a) or 24 μM (b) DOC. Spectra were recorded during steady-state growth (solid lines) and after the O_2 supply was turned off (dotted lines) as described in the text. Two representatives of repeated scans and the blank (dashed lines) are shown.

derivative spectra were recorded while the DOC was maintained at various increasing levels, each held for about 4 min. Fig. 6 shows the changes, at representative wavelengths, that occurred in the signal intensities; they are shown as percentages of the maximum deflection observed at a particular wavelength. Initially, the signals attributed to cytochromes *c*, b_{560}, b_{595} and *d* (at 552, 562, 596 and 632 nm respectively), started to decline when the DOC was hardly above zero. The 'featureless' spectrum of cytochrome *d*, where the signals at 632 and 652 nm were smallest, occurred near a DOC of 4 μM (Fig. 6). Unfortunately, maintenance of the DOC between 2 and 4 μM was difficult to achieve. At 5 μM DOC, cytochromes *c* (signal at 552 nm), b_{560} (signal at 562 nm) and b_{595} (signal at 596 nm) appeared to be largely oxidized, whereas the signal at 652 nm indicated that not all the cytochrome *d* could be accounted for in the oxygenated form. Above 5 μM DOC, the signal attributed to the oxygenated cytochrome *d*, in this case the one-electron-reduced cytochrome *bd*, continued to increase and this increase appeared to parallel a slight reduction in cytochromes *c* (increase in the 552 nm signal). Because the changes in redox state of these cytochromes took place close to the limit of sensitivity of the Clark-type O_2 electrode, the precise DOC at which they occurred could not be assessed. Nevertheless, the range appeared to be close to the value for the apparent K_m for O_2 for the *bd*-type oxidase of 5 μM which has been found in similar suspensions of whole cells of strains UW136 and MK8 (D'mello *et al.*, 1994a).

Redox poise of cytochromes during steady-state growth

In washed cells supplied with sucrose, the one-electron-reduced oxygenated species was observed at DOCs above 3 μM (Fig. 6). To determine whether this species occurred during growth, we established a carbon- and energy-limited chemostat fed with excess O_2. Under conditions of O_2 excess, the cytochrome *bd*-type oxidase plays an important role in removing O_2, particularly during diazotrophy. To estimate the redox poise of cytochromes under these conditions, a N_2-fixing sucrose-limited chemostat of MK8 growing at 12 μM O_2, was established in a fermenter that was fitted to deliver and to collect light for analysis by DARS as shown in Fig. 2.

Upon achieving a steady state, spectra were recorded. Examples of two repeated spectra are shown as second-order derivatives in Fig. 7(a) and show a trough at 630 nm. To determine whether cytochrome *d* was in the fully reduced state, the stirrer was turned off and the gas supply to the culture was changed to N_2. When the O_2 electrode registered zero, repeated scans were recorded over a period of not more than 5 min. The stirrer and gas supply were then reinstated slowly so as to prevent the DOC from rising above the set point. A comparison of the scans taken when the DOC was zero with those taken during steady-state growth (Fig. 7a) revealed that cytochromes *d*, *c* and b_{560} were partially reduced during sucrose-limited growth at 12 μM O_2. Furthermore, there was no negative deflection at 650 nm, indicating that there appeared to be little, if any, cytochrome *d* in the oxygenated form during steady-state growth. To determine the effect of increasing the O_2 supply, spectra were also recorded during sucrose-limited growth at 24 μM O_2. Representatives of the data are shown in Fig. 7(b). As found with the population growing at 12 μM O_2, there was no evidence that the oxygenated species of cytochrome *d* (anticipated trough at 650 nm) was present, and the cytochromes were again partially reduced during steady-state growth.

The quality of the spectra was insufficient to compare the amount of cytochromes present in the populations at different DOCs, but there appeared to be no gross changes upon a doubling in the DOC. The finding that, during steady-state sucrose-limited growth at both 12 and 24 μM O_2, the average redox state of cytochromes *in vivo* appears to be partially reduced suggests that the rate of electron supply to the respiratory chain and the rate of oxidase activity are regulated to maintain a low but sufficient level of O_2 for energy conservation. This

finding is similar to that reported for succinate-limited N_2-fixing growth of *Azorhizobium caulinodans* maintained at DOCs between 0·1 and 3·5% (Pronk *et al.*, 1993). Cytochromes *c* and *b* were partially reduced in such cultures. These authors used a dual-beam spectrophotometer with fibre optics to deliver and collect light through the wall of the fermenter. The fermenter had to be encased in a light-proof box. In comparison, our application of DARS has two advantages: (1) encasing the sample in a light-proof box is unnecessary, and (2) spectral acquisition is very fast so that transient changes over a wide absorbance range can be recorded.

Conclusions and further opportunities

Our study of the effect of O_2 status on the cytochrome oxygenation and redox poise of the *A. vinelandii* cytochrome *bd*-type oxidase *in vivo* using DARS has revealed the following features. First, when the O_2 supply was varied to washed cells supplied with sucrose, three different redox forms of the oxidase were sufficiently long-lived for presumptive identification. They were (1) the relatively stable one-electron-reduced oxygenated form (b_{560}^{3+}, b_{595}^{3+}, $d^{2+}O_2$), (2) a form where cytochrome *d* apparently had no absorbance at either 650 or 630 nm but cytochromes *b* were oxidized (b_{560}^{3+}, b_{595}^{3+}) and (3) the fully reduced form (b_{560}^{2+}, b_{595}^{2+}, d^{2+}). Changes in cytochrome *d* from the form with no absorbance at either 650 or 630 nm to the reduced form with an absorbance at 630 nm appeared to correlate with the reduction of cytochromes b_{595} and b_{560}. These changes were relatively rapid. It is possible that the 'featureless' cytochrome *d* is the ferric form ('d_x') rather than the oxy-ferryl species ($d^{4+}O^{2-}$). Unfortunately, our equipment was not sensitive enough at 680 nm to detect the latter. These changes, we suggest, are consistent with the scheme of Jünemann *et al.* (1995) and Jünemann (1997), where the oxygenated form of cytochrome *d* in the one-electron-reduced enzyme does not contribute to the enzyme turnover. Second, changes in the redox poise of cytochrome *d* occurred at a DOC which appeared to coincide approximately with the apparent O_2 affinity for the oxidase in similar washed cell suspensions (D'mello *et al.*, 1994a). This may indicate that the build-up of a relatively stable intermediate occurs at the DOC supporting half maximum turnover for a given rate of electron donation. Third, during steady-state growth in N_2-fixing populations, cytochromes *d*, *b* and *c* were partially reduced, which is consistent with the ability of *A. vinelandii* to show an immediate, as well as an adaptive, response of respiration to sudden increases in O_2 supply (see Poole & Hill, 1997; Hill, 1992; and references therein). In such growing populations we could not detect the oxygenated form of cytochrome *d* (absorbing at 650 nm).

We have not exploited all the opportunities for application of DARS. With regard to *A. vinelandii* physiology, comparisons of the redox poise of cytochromes during steady-state growth of N_2-fixing populations with those assimilating NH_4^+ should yield some further insight into the role of respiration during diazotrophy. The effect of respiratory inhibitors on cytochrome redox state in membrane preparations could also be studied with the modification to the Clark-type O_2 electrode. For transitions of cytochrome redox state in whole cells occurring near the limit of sensitivity of the Clark-type O_2 electrode, a more sensitive O_2 probe is required. A possibility is a time-shared arrangement to allow for the measurement and control of O_2 by the photoemission of a photobacterium (Kavanagh & Hill, 1990) as well as the analysis of light absorbance by DARS.

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