Expression and content of terminal oxidases in *Azotobacter vinelandii* grown with excess NH_4^+ are modulated by O_2 supply

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The influence of the rate of O_2 supply to batch cultures on the contents of cytochromes *bd* and 'o' in NH_4^+ -grown *Azotobacter vinelandii* has been investigated. Difference spectra at room temperature (reduced + CO minus reduced) were recorded for whole cells of a wild-type strain and mutants which either lacked or over-produced the cytochrome *bd*-type terminal oxidase encoded by *cydAB*. A Tn5-B20 insertion in *cydB* in the former mutant also provided a means of monitoring *cydAB* gene expression from measurements of β -galactosidase activity. The content of cytochrome *d* in the wild-type, and the expression of *cydAB-lacZ*, in the mutant, increased as the O_2 supply was raised, suggesting that O_2 regulates *cydAB* expression even in the absence of diazotrophy. In a strain carrying a mutation in *cydR*, a regulatory gene upstream of *cydAB*, and which over-produces cytochrome *bd*, the responses to O_2 supply during growth at different O_2 supply rates were reversed. Changes in the content of a haemoprotein detectable in low temperature photodissociation spectra, and attributed to cytochrome b_{595} – the high-spin cytochrome *b* component of the cytochrome *bd* complex – followed the changes in cytochrome *d* levels. CO difference spectra of both the wild-type strain and the cytochrome *bd*-deficient mutant revealed a haemoprotein with spectral characteristics similar to cytochrome *o*, the levels of which increased as the O_2 supply was raised. These results are discussed with reference to previous reports of cytochrome changes in cells grown under N_2 -fixing conditions.

Keywords: *Azotobacter vinelandii*, cytochrome *bd*, cytochrome *o*-like oxidase, O_2 supply and oxidase content, *cyd* genes

INTRODUCTION

The obligately aerobic N_2 -fixing bacterium *Azotobacter* has one of the highest known rates of respiration and can alter its O_2 consumption to match a wide range of rates of O_2 supply. The initial biochemical and physiological work (Lisenkova & Khmel, 1967; Dalton & Postgate, 1969; Drozd & Postgate, 1970; Ackrell & Jones, 1971; Jones *et al.*, 1973; Haaker & Veeger, 1976;

Post *et al.*, 1983; Hill, 1992) and more recently the characterization of mutants (Ramos & Robson, 1985; Kelly *et al.*, 1990; Leung *et al.*, 1994) has indicated that such flexibility of respiratory activity has a role in maintaining suitable conditions *in vivo* for the O_2 -sensitive process of N_2 fixation.

Recent molecular studies have necessitated a reinterpretation of earlier schemes for the organization of electron-carrying pathways to oxygen (Haddock & Jones, 1977). The structural genes for cytochrome *bd* (*cydAB*) were first cloned by Kelly *et al.* (1990), allowing construction of Tn5 mutants affected in cytochrome *bd* synthesis and regulation. The demonstration that *Cyd*[−] mutants cannot fix N_2 in air was the first genetic evidence for the essential role of this oxidase in preventing O_2 inactivation of nitrogenase in the process termed 'respiratory protection'. Subsequent sequencing

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of the *cydAB* operon (Moshiri *et al.*, 1990) revealed striking similarities with the *Escherichia coli* cytochrome *bd*-type oxidase, in accord with spectral studies (Poole, 1983; D'mello *et al.*, 1994b). However, there is a remarkable difference between the *E. coli* and *A. vinelandii* oxidases with respect to regulation of synthesis. The *E. coli* cytochrome *bd*-type oxidase is synthesized maximally microaerobically (Cotter *et al.*, 1990; Fu *et al.*, 1991; Tseng *et al.*, 1996). Expression of the *cydAB* operon comprising the oxidase structural genes is affected by Fnr and ArcA/ArcB, although dissection of the direct or indirect roles of these regulators requires further study (Gennis & Stewart, 1996). Cytochrome *bd* levels are also elevated at low oxygen tensions in the diazotroph *Klebsiella pneumoniae* (Smith *et al.*, 1990). In contrast, the level of cytochrome *bd* in N_2 -fixing *A. vinelandii* oxidase is greatest under high aeration (Drozd & Postgate, 1970; Jones *et al.*, 1973; Haaker & Veeger, 1976), although one report describes the reverse situation (Ackrell & Jones, 1971). Accompanying the increase in the cytochrome *bd* level is a loss of energy conservation at 'Site I' (Jones *et al.*, 1973). Consistent with the majority of these observations is the finding that cytochrome *bd* in *A. vinelandii* has a low affinity for O_2 (apparent $K_m = 4.5 \mu M$; D'mello *et al.*, 1994a), yet exhibits a higher velocity for electron transport than does the alternative oxidase 'cytochrome *o*' (Hoffman *et al.*, 1979; D'mello *et al.*, 1994a). In marked contrast, the oxidase in *E. coli* has the highest affinity yet recorded ($K_m = 5 \text{ nM}$) for a terminal oxidase (D'mello *et al.*, 1996).

Less is known of the properties of the oxidase(s) terminating alternative route(s) to oxygen in *A. vinelandii*, although early work showed cytochrome(s) *c* to be involved (Wong & Jurtshuk, 1984; Yang, 1986 and references therein). K_m measurements reveal, in addition to cytochrome *bd*, two high affinity oxidase activities (D'mello *et al.*, 1994a). Assuming that a second oxidase might belong to the 'haem-copper' superfamily of terminal oxidases (Poole, 1994), in which the O_2 -reactive binuclear centre comprises a copper atom coupled with a haem A, B or O, we have cloned (with PCR primers designed to amplify a gene in this superfamily) a 0.55 kb fragment of such an oxidase gene from *A. vinelandii* (Leung *et al.*, 1994). Recombination of the fragment carrying a kanamycin-resistance marker with the chromosome resulted in a mutant lacking this oxidase, which has tentatively been called Cyo. Since this mutant (*cyo*) remained aerotolerant when fixing N_2 , the role of this oxidase is clearly different from that of cytochrome *bd* (Leung *et al.*, 1994). The mutant over-expresses the latter oxidase, particularly at lower oxygen tensions, presumably as compensation for the lack of the haem-copper-type oxidase. Recently, a gene fragment from *A. vinelandii* has been sequenced and shown to resemble *fixN* or *ccoN* encoding a *cb*-type cytochrome *c* oxidase (Cco) (Thöny-Meyer *et al.*, 1994). It is not known whether Cco and Cyo are distinct oxidases. Consequently, in this paper, we refer to the component with spectral features similar to established cytochrome *o*-

type oxidases having haem O (as in *E. coli*; Gennis & Stewart, 1996) as cytochrome *o*-like.

Here, we have investigated the effect of differing rates of O_2 supply on the cellular levels of the cytochrome *bd*-type and the cytochrome *o*-like oxidases in *A. vinelandii*, using mutants to clarify the spectral signals and to determine whether O_2 regulates *cyd* expression. To prevent any interaction between the regulation imposed by O_2 supply and diazotrophic growth, the medium contained an excess of ammonium acetate.

METHODS

Bacterial strains and growth conditions. *A. vinelandii* strain UW136, a rifampicin-resistant derivative of UW (Bishop *et al.*, 1980), and its kanamycin-resistant derivatives MK5, carrying *cydB::Tn5-B20*, and MK15, carrying *Tn5-B20* near the *cydAB* locus in a gene that we have named *cydR* (G. Wu, S. Hill, M. J. S. Kelly, G. Sawers & R. K. Poole, unpublished), were maintained in 50% (v/v) glycerol at -70°C . They were grown in Burke's medium with 2% (w/v) sucrose and 15 mM ammonium acetate (BSN) (Drozd & Postgate, 1970) and either kanamycin ($1 \mu\text{g ml}^{-1}$) for MK5 and MK15 or rifampicin ($10 \mu\text{g ml}^{-1}$) for UW136. Growth was in Erlenmeyer flasks (1 l) at 30°C with rotary shaking ($190 \pm 10 \text{ r.p.m.}$). The following descending range of volumes (ml) of the medium gave the ascending rates of O_2 supply ($\text{mmol } O_2 \text{ l}^{-1} \text{ h}^{-1}$, $K_L a$; Pirt, 1975) when estimated by the sulphite oxidation method as described by Gil *et al.* (1992): 400, 3; 300, 7; 200, 16; 100, 31; 50, 60; 25, 90. Cultures were inoculated (about 5% of the final culture volume) from an 18 or 24 h culture grown in the same medium (25 ml) contained in an Erlenmeyer flask (250 ml). Growth was followed by measuring OD_{600} in a Unicam SP600 spectrophotometer after appropriate dilution of the culture with medium. Protein was measured by the method of Markwell *et al.* (1978). Cultures were harvested either during mid-exponential or mid-linear phases of growth for the measurements described below.

Cytochrome measurements and enzyme assays. For cytochrome measurements, organisms were harvested by centrifugation at $4000 g$ for 15 min, washed and resuspended in twice the pellet volume of 0.1 M potassium phosphate buffer (pH 7.0). The cell suspension was either kept on ice for a maximum of 2 h or stored overnight at -20°C before spectra were recorded. CO difference electronic spectra (reduced + CO minus reduced) at room temperature and photodissociation spectra (i.e. photolysed minus reduced + CO) at low temperature (about -100°C) were recorded in a Johnson Foundation SDB3 dual-wavelength scanning spectrophotometer as described previously (Leung *et al.*, 1994; D'mello *et al.*, 1994b). In brief, a suspension of cells was reduced with a few grains of sodium dithionite, bubbled with a fine stream of CO gas to form the carbon monooxy compounds of oxidases and other CO-reactive haemoproteins, then frozen in the dark. A baseline difference spectrum (reduced + CO minus reduced + CO) was recorded at -100°C . The sample was then photolysed *in situ* in the spectrophotometer using the focused beam of a 150 W projector bulb transmitted to the sample using a bifurcated fibre optic guide as shown in Jones & Poole (1985). The difference spectrum was again recorded, i.e. photolysed (= reduced) minus pre-photolysis (= reduced + CO).

To calculate cytochrome *d* content, an absorbance coefficient of $13.9 \text{ mM}^{-1} \text{ cm}^{-1}$ [642 nm (± 2 nm) peak minus 622 nm (± 2 nm) trough] was used; this was measured from CO

difference spectra shown in Jünemann & Wigglesworth (1995) and is very close to the value ($12.6 \text{ mM}^{-1} \text{ cm}^{-1}$) suggested for the *E. coli* oxidase by Kita *et al.* (1984b). For the cytochrome *o*-like haemoprotein, a value of $145 \text{ mM}^{-1} \text{ cm}^{-1}$ between 415 and 430 nm in CO difference spectra was used, as proposed by Kita *et al.* (1984a) for *E. coli* cytochrome *bo'*. The precision of the procedures for quantifying cytochromes was such that the coefficient of variance of measurements on a typical cell sample was in the range 7–17%.

For estimates of β -galactosidase specific activity, organisms were harvested from the culture (1 ml) by centrifugation at 4000 *g* for 15 min, resuspended in ice-cold Z buffer (Miller, 1972; 1 ml) and the activity was estimated in a suitable aliquot at 28 °C by the method of Miller (1972).

All measurements were made on three separate occasions over the entire range of $K_L a$ values. The pattern of change in each experiment was reproducible, whereas the value of each parameter at a particular $K_L a$ value showed some variation.

RESULTS AND DISCUSSION

Effects of O₂ supply on batch growth

Initially, the effect of varying the rate of O₂ supply on growth of *A. vinelandii* wild-type and mutant strains was determined in batch cultures supplied with ammonium acetate. A range of rates of O₂ supply was achieved by varying the culture volume but keeping the flask geometry and shaking rate constant. The resultant O₂ transfer rates were quantified by measurements of sulphite oxidation rates and gave a range of $K_L a$ values from 3 to 90 mmol O₂ l⁻¹ h⁻¹ (see Methods). Over this range, growth profiles of the wild-type, UW136, and of a strain lacking cytochrome *bd*, MK5, were exponential. As expected, the doubling times increased as the rate of O₂ supply decreased. At the higher $K_L a$ values, the doubling time of the Cyd⁻ mutant tended to be longer than that of the wild-type (data not shown). The growth of the *cydR* strain, MK15, which over-produced cytochrome *bd*, was exponential at the higher rates of O₂ supply, but at the lower rates (below 30 mmol O₂ l⁻¹ h⁻¹) it was linear (data not shown), indicating that cultures were O₂-limited. A severe O₂ limitation could account for the previously noted failure of this strain to grow under microaerobic conditions (Kelly *et al.*, 1990).

Effects of O₂ supply on the levels of components of the cytochrome *bd*-type oxidase

To estimate the content of the low-affinity cytochrome *bd*-type terminal oxidase in whole cells, we measured the difference in absorbance between 622 nm and 642 nm in the CO difference spectra (CO + reduced minus reduced) (see Methods). Features in this area of the spectrum relate specifically to cytochrome *d* (Poole, 1983). We have found this to be a more reliable assay than reduced minus oxidized difference spectra for cytochrome *d*, particularly in *A. vinelandii*, because of the difficulty of maintaining rapidly respiring cells or membranes in the oxidized state. For these estimates, cultures of strains UW136 and MK5 were harvested during mid-exponential growth and cultures of strain

Fig. 1. Effects of O₂ supply ($K_L a$) on the cytochrome *d* content in strains UW136 (wild-type) (●) and MK15 (○).

MK15 were harvested either during mid-exponential or mid-linear growth (see above). Consistent with most of the earlier reports (see Introduction), the cytochrome *d* content in the wild-type, UW136, increased slightly but significantly as the rate of O₂ supply was raised. The greatest increase occurred below 20 mmol O₂ l⁻¹ h⁻¹ (Fig. 1). Conversely, in strain MK15, which carries a mutation in a gene, *cydR*, close to *cydAB* (Kelly *et al.*, 1990; G. Wu, S. Hill, M. J. S. Kelly, G. Sawers & R. K. Poole, unpublished), the cytochrome *d* content markedly decreased with increase in O₂ supply. So, in MK15 there was a reversal in the apparent response of the cytochrome *d* content to the rate of supply of O₂ during growth. This strain was reported to over-produce cytochrome *d* (Kelly *et al.*, 1990), and did so at the lower rates of O₂ supply where growth was linear. However, at the higher rates of O₂ supply (at and above a $K_L a$ of 60 mmol O₂ l⁻¹ h⁻¹), when growth was exponential, the content approached that of the wild-type (Fig. 1). As expected, in strain MK5, which carries a mutation in *cydB*, cytochrome *d* was not detected during growth with any of the differing rates of O₂ supply.

The high-spin cytochrome *b*₅₉₅ component of the *bd*-type terminal oxidase has a relatively weak absorption in the reduced form at about 595 nm. It is assayed most readily spectroscopically in low-temperature photodissociation spectra (D'mello *et al.*, 1994b). In such measurements, a reduced CO-ligated sample is scanned at sub-zero temperatures, then photolysed to generate the photolysed (= reduced) minus pre-photolysis (= reduced + CO) difference spectra. Consequently, the photodissociation spectrum is expected to be inverted relative to the conventional CO difference spectrum (i.e. reduced + CO minus reduced). Cytochrome *b*₅₉₅ gives a trough at around 418 nm and peak at 437–440 nm. Both wavelengths are somewhat longer than for typical signals for cytochrome *o* (Poole, 1983; Poole *et al.*, 1994). Fig. 2 shows that the Soret region of the spectra was dominated by the cytochrome *b*₅₉₅ signal in those

Fig. 2. Soret region of the low temperature (-100°C) photodissociation spectra of whole cells of strains UW136 (wild-type) (a and b), MK15 (c and d) and MK5 (e and f). Cultures were grown at low (left) or high (right) O_2 supply rates. The $K_L a$ values ($\text{mmol O}_2 \text{ l}^{-1} \text{ h}^{-1}$) were (a) 15, (b) 60, (c) 3, (d) 31, (e) 7 and (f) 60. The protein concentrations (mg ml^{-1}) were (a) 17.6, (b) 13.6, (c) 8.6, (d) 9.7, (e) 24.0 and (f) 5.8.

cultures that had elevated levels of cytochrome *d*. In wild-type strain UW136, it was more pronounced in cells grown under high aeration (Fig. 2b) than in cells grown at low $K_L a$ values (Fig. 2a) whereas in cells of strain MK15, this pattern was reversed (Fig. 2c and d). The magnitudes of these signals are in accord with the changes shown in Fig. 1 for cytochrome *d*, lending support to the assignment to cytochrome b_{595} . However, we cannot rule out the possibility that cytochrome *o* makes some contribution to the short wavelength side of the peak and trough. As expected, in MK5 clear signals at these wavelengths were missing (Fig. 2e and f). The 435 nm peak in Fig. 2(f) and the weak troughs at lower wavelengths may arise from the cytochrome *o*-like oxidase. We have consistently failed to obtain clear photodissociation spectra that reveal cytochrome *o* in *A. vinelandii*, despite early evidence for its existence (Yang, 1986; Wong & Jurtschuk, 1984), identification of a fragment of a gene that might encode such an oxidase (Leung *et al.*, 1994) and CO difference spectra (see later, Fig. 4). A possible explanation is that the recombination of CO with the ferrous haem is too fast to be observable at the temperatures used and with the time resolution available.

Fig. 3. Effects of O_2 supply ($K_L a$) on the expression of *cyd-lacZ* activity in MK5 (●) and in MK15 (○).

These results show that mutation of *cydR* dramatically increases (1) the level of cytochrome *d* assayed spectroscopically by its reaction with CO and (2) the level of a high-spin cytochrome, probably cytochrome b_{595} , assayed by photodissociation spectroscopy. If the *cydR* gene product were the only regulator of the *cydAB* operon, the levels of these components would be expected to be unregulated in *cydR* mutants. However, O_2 supply clearly regulates cytochrome *bd* levels when fixed N_2 is provided, suggesting the existence of additional regulators of *cydAB* transcription or post-translational modification to give the spectrally detectable forms.

Effect of O_2 supply on the expression of *cyd* genes

Jones *et al.* (1973) found that chloramphenicol largely abolished the rise in cytochrome *d* in *A. vinelandii* wild-type cells when the O_2 supply was increased to a N_2 -fixing culture. Thus, O_2 may affect *cydAB* gene expression. The mutations in strains MK5 and MK15 were generated by the insertion of Tn5-B20, which carries a transcriptional *lacZ* fusion. Therefore, the synthesis of β -galactosidase in these strains should give a measure of the expression of the mutated gene, provided that the transposon is correctly oriented, as is the case in these strains (Kelly *et al.*, 1990). However, the expression of genes mutated by fusion constructs is complicated by the possibility that the gene whose regulation is being investigated may have a critical role in growth under the chosen experimental conditions.

The levels of β -galactosidase were measured at the time of harvest for cytochrome estimations. In strain MK5, which carries a Tn5-B20 insertion in the structural gene *cydB*, the level of β -galactosidase increased as the rate of O_2 supply for growth was raised to $30 \text{ mmol O}_2 \text{ l}^{-1} \text{ h}^{-1}$ (Fig. 3). This pattern was similar to that seen in the wild-type strain, UW136, for the changes in cytochrome *d* level with increasing $K_L a$ (Fig. 1). Thus the apparent O_2 -

Fig. 4. Effect of O₂ supply ($K_L a$) on the cytochrome o-like haemoprotein in a cytochrome *bd*-deficient mutant (strain MK5) harvested from exponentially growing cultures. Spectra (a), (b) and (c) show the Soret region of the room temperature CO-difference spectra (reduced + CO minus reduced) of whole cells. The $K_L a$ values (mmol O₂ l⁻¹ h⁻¹) for growth were (a) 7, (b) 31 and (c) 60. The concentrations of protein (mg ml⁻¹) in the samples were (a) 11.6, (b) 10.0 and (c) 7.6. The changes in the content of the cytochrome o-like haemoprotein in cultures grown at various $K_L a$ values are shown in (d).

induced increase in the cytochrome *d* content in the wild-type can be accounted for, at least in part, by O₂ regulation of *cydAB* expression. Moshiri *et al.* (1991) have reported that the expression of *cydAB* in *A. vinelandii* increased upon the derepression of N₂ fixation and this elevation did not occur in a *ntxA* mutant, suggesting that the mechanism controlling expression was associated with the change in nitrogen status. Since the apparent O₂ regulation of *cydAB* expression reported here occurred in cultures supplied with excess NH₄⁺, the underlying mechanism must differ from that seen by Moshiri *et al.* (1991).

In strain MK15, the level of β -galactosidase was highest at the lowest rate of O₂ supply, and then declined as the rate of O₂ supply was increased (Fig. 3). The levels of cytochrome *d* (Fig. 1) and of cytochrome *b*₅₉₅ (Fig. 2) also decreased at higher rates of O₂ supply. The position of Tn5-B20 in strain MK15 has been mapped by restriction analysis to a position just upstream of *cydA* (Kelly *et al.*, 1990). More recent data indicate that it lies within a coding sequence called *cydR* that has close homology to *fmr*-like genes (G. Wu, S. Hill, M. J. S. Kelly, G. Sawers & R. K. Poole, unpublished). The results of the present work confirm that the insertion is in a transcribed region and suggest that the transcription is regulated by O₂, but in an opposite manner to that of *cydAB* expression in MK5 (Fig. 2). The Tn5-B20 mutation in MK15 clearly gives rise to the over-expression of cytochrome *d* (Fig. 1), and thus appears to be in a gene that influences the expression of cytochrome *d*.

Effects of O₂ supply on the levels of the cytochrome o-like oxidase

The number and nature of haems in the cytochrome o-like oxidase complex of *A. vinelandii* are unclear. Before the description of haem O, the difference spectra were interpreted as revealing both haem C and a CO-binding haem B (Yang, 1986). Like *E. coli* (Poole, 1983), the distinctive feature in the CO difference spectrum of *A. vinelandii* cells is a peak at 415–416 nm and a trough at 430–431 nm, which may be due to the shift in absorbance to lower wavelengths upon binding of CO to the ferrous form of the cytochrome o-like haemoprotein (Wong & Jurtshuk, 1984). However, the signals in this region can be influenced by the cytochrome *b*₅₉₅ component of the cytochrome *bd*-type oxidase (Fig. 2), particularly in mutants such as MK15 that over-produce this oxidase. Therefore, we did not attempt to determine the level of the cytochrome o-like protein in cultures of this strain. The Soret region was chosen for detailed examination and quantification because of the high Soret (γ)/ α,β ratio reported for *E. coli* cytochrome *o* (Poole *et al.*, 1994).

Fig. 4 shows CO difference spectra recorded with intact cells of the cytochrome *bd*-deficient mutant in which, therefore, neither cytochrome *d* nor cytochrome *b*₅₉₅ makes a spectral contribution. At high $K_L a$ values, the spectra exhibited a marked peak at 413–415 nm and

trough at 432 nm (Fig. 4c), typical of cytochrome *o* difference spectra in other bacteria (Poole, 1983). These signals decreased and broadened upon lowering the $K_L a$ for growth (Fig. 4a), probably due to the additional presence of uncharacterized haem proteins. Fig. 4(d) shows the changes in the concentration of the cytochrome *o*-like haemoprotein measured from such spectra of cells grown at a range of $K_L a$ values. When the O_2 supply was raised from 4 to 60 mmol O_2 l^{-1} h^{-1} , the cytochrome *o*-like haemoprotein increased about four-fold.

CO difference spectra of the wild-type strain showed similar cytochrome *o*-like features (not shown) but the possibility of a contribution from cytochrome b_{595} , particularly at higher $K_L a$ values where the cytochrome *bd* levels were highest (Fig. 1), precluded reliable quantification. Nevertheless, the magnitude of the Soret signal was generally lower in the wild-type strain than in strain MK5 and also increased at higher $K_L a$ values (not shown). The mutant apparently compensates for the lack of the cytochrome *bd*-type oxidase by increased production of the cytochrome *o*-like haemoprotein. An analogous compensation has been observed in an *A. vinelandii* mutant lacking the cytochrome *o*-like haemoprotein in which the level of cytochrome *bd* was greater than in the wild-type during stationary phase (Leung *et al.*, 1994).

These results indicate that the content of the cytochrome *o*-like haemoprotein, like that of cytochrome *d*, is regulated by O_2 supply in a positive manner, at least in mutant MK5, where the cytochrome *o* quantification is most reliable. Earlier estimates (Ackrell & Jones, 1971; Haaker & Veeger, 1976) of the content of a cytochrome *o*-like haemoprotein, measured by room temperature CO difference spectra in membranes prepared from N_2 -fixing cultures of the wild-type, showed that the level increased when the O_2 supply for growth was lowered. The difference between these earlier results and ours may reside in the nitrogen source for growth (ammonium acetate in this work, not N_2). Understanding the possible contributions of both nitrogen status and oxygen status to the regulation of components of the branched respiratory chains of *A. vinelandii* will require further study.

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REFERENCES

- Ackrell, B. A. C. & Jones, C. W. (1971). The respiratory system of *Azotobacter vinelandii* 2. Oxygen effects. *Eur J Biochem* **20**, 29–35.
- Bishop, P. E., Jarlenski, D. M. L. & Hetherington, D. R. (1980). Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*. *Proc Natl Acad Sci USA* **77**, 7342–7346.
- Cotter, P. A., Chepuri, V., Gennis, R. B. & Gunsalus, R. P. (1990). Cytochrome *o* (*cyoABCDE*) and *d* (*cydAB*) oxidase genes in *Escherichia coli* are regulated by oxygen, pH, and the *fnr* gene product. *J Bacteriol* **172**, 6333–6338.
- Dalton, H. & Postgate, J. R. (1969). Effect of oxygen on growth of *Azotobacter chroococcum* in batch and continuous culture. *J Gen Microbiol* **54**, 463–473.
- D'mello, R., Hill, S. & Poole, R. K. (1994a). Determination of the oxygen affinities of terminal oxidases in *Azotobacter vinelandii* using deoxygenation of oxyleghaemoglobin and oxymyoglobin: cytochrome *bd* is a low-affinity oxidase. *Microbiology* **140**, 1395–1402.
- D'mello, R., Palmer, S., Hill, S. & Poole, R. K. (1994b). The cytochrome *bd* terminal oxidase of *Azotobacter vinelandii*: low temperature photodissociation spectrophotometry reveals reactivity of cytochromes b_{595} and *d* with both carbon monoxide and oxygen. *FEMS Microbiol Lett* **121**, 115–120.
- D'mello, R., Hill, S. & Poole, R. K. (1996). The cytochrome *bd* quinol oxidase in *Escherichia coli* has an extremely high oxygen affinity and two oxygen-binding haems: implications for regulation of activity *in vivo* by oxygen inhibition. *Microbiology* **142**, 755–763.
- Drozd, J. & Postgate, J. R. (1970). Effects of oxygen on acetylene reduction, cytochrome content and respiratory activity of *Azotobacter chroococcum*. *J Gen Microbiol* **63**, 63–73.
- Fu, H.-A., Iuchi, S. & Lin, E. C. C. (1991). The requirement of ArcA and Fnr for peak expression of the *cyd* operon in *Escherichia coli* under microaerobic conditions. *Mol Gen Genet* **226**, 209–213.
- Gennis, R. B. & Stewart, V. (1996). Respiration. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 217–261. Edited by F. C. Neidhardt, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & H. E. Umberger. Washington, DC: American Society for Microbiology.
- Gil, A., Kroll, R. G. & Poole, R. K. (1992). The cytochrome composition of the meat-spoilage bacterium *Brochothrix thermosphacta*: identification of cytochrome a_3 - and *d*-type terminal oxidases under various conditions. *Archiv Microbiol* **158**, 226–233.
- Haaker, H. & Veeger, C. (1976). Regulation of respiration and nitrogen fixation in different types of *Azotobacter vinelandii*. *Eur J Biochem* **63**, 499–507.
- Haddock, B. A. & Jones, C. W. (1977). Bacterial respiration. *Bacteriol Rev* **41**, 47–99.
- Hill, S. (1992). Physiology of nitrogen fixation in free-living heterotrophs. In *Biological Nitrogen Fixation*, pp. 87–134. Edited by G. Stacey, R. H. Burris & H. J. Evans. New York, London: Chapman & Hall.
- Hoffman, P. S., Morgan, T. V. & Der-Vartanian, D. V. (1979). Respiratory chain characteristics of mutants of *Azotobacter vinelandii* negative to tetramethyl-*p*-phenylenediamine oxidase. *Eur J Biochem* **100**, 19–27.
- Jones, C. W. & Poole, R. K. (1985). The analysis of cytochromes. *Methods Microbiol* **18**, 285–328.
- Jones, C. W., Brice, J. M., Wright, V. & Ackrell, B. A. C. (1973). Respiratory protection of nitrogenase in *Azotobacter vinelandii*. *FEBS Lett* **29**, 77–81.
- Jünemann, S. & Wrigglesworth, J. M. (1995). Cytochrome *bd* oxidase from *Azotobacter vinelandii* – purification and quantitation of ligand binding to the oxygen reduction site. *J Biol Chem* **270**, 16213–16220.
- Kelly, M. J. S., Poole, R. K., Yates, M. G. & Kennedy, C. (1990). Cloning and mutagenesis of genes encoding the cytochrome *bd* terminal oxidase complex in *Azotobacter vinelandii*: mutants

deficient in the cytochrome *d* complex are unable to fix nitrogen in air. *J Bacteriol* 172, 6010–6019.

Kita, K., Konishi, K. & Anraku, Y. (1984a). Terminal oxidases of the *Escherichia coli* aerobic respiratory chain. I. Purification and properties of cytochrome *b*₅₆₂-*o* complex from cells in early exponential phase of aerobic growth. *J Biol Chem* 259, 3368–3374.

Kita, K., Konishi, K. & Anraku, Y. (1984b). Terminal oxidases of the *Escherichia coli* aerobic respiratory chain. II. Purification and properties of cytochrome *b*₅₅₈-*d* complex from cells grown with limited oxygen and evidence of branched electron-carrying system. *J Biol Chem* 259, 3375–3381.

Leung, D., van der Oost, J., Kelly, M. J. M., Saraste, M., Hill, S. & Poole, R. K. (1994). Mutagenesis of a gene encoding a cytochrome *o*-like terminal oxidase of *Azotobacter vinelandii*: a cytochrome *o* mutant is aero-tolerant during nitrogen fixation. *FEMS Microbiol Lett* 119, 351–358.

Lisenkova, L. L. & Khmel, I. A. (1967). Effect of cultural conditions on cytochrome content of *Azotobacter vinelandii* cells. *Mikrobiologia* (translated) 36, 762–767.

Markwell, M. A. K., Haas, S. M., Bieber, L. L. & Talbert, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 87, 206–210.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Moshiri, F., Chawla, A. & Maier, R. J. (1990). Cloning, characterisation, and expression in *Escherichia coli* of the genes encoding the cytochrome *d* oxidase complex from *Azotobacter vinelandii*. *J Bacteriol* 173, 6230–6241.

Moshiri, F., Smith, E. G., Taormino, J. P. & Maier, R. J. (1991). Transcriptional regulation of cytochrome *d* in nitrogen-fixing *Azotobacter vinelandii*. *J Biol Chem* 266, 23169–23174.

Pirt, S. J. (1975). *Principles of Microbe and Cell Cultivation*. Oxford: Blackwell Scientific Publications.

Poole, R. K. (1983). Bacterial cytochrome oxidases: a structurally and functionally diverse group of electron transfer proteins. *Biochim Biophys Acta* 726, 205–243.

Poole, R. K. (1994). Oxygen reactions with bacterial oxidases and globins: binding, reduction and regulation. *Antonie Leeuwenhoek* 65, 289–310.

Poole, R. K., Salmon, I. & Chance, B. (1994). The high-spin cytochrome *o*' component of the cytochrome *bo*-type quinol oxidase in membranes from *Escherichia coli*: formation of the primary oxygenated species at low temperatures is characterized by a slow 'on' rate and low dissociation constant. *Microbiology* 140, 1027–1034.

Post, E., Kleiner, D. & Olze, J. (1983). Whole cell respiration and nitrogenase activities in *Azotobacter vinelandii* growing in oxygen controlled continuous culture. *Arch Microbiol* 134, 68–72.

Ramos, J. L. & Robson, R. L. (1985). Isolation and properties of mutants of *Azotobacter chroococcum* defective in aerobic nitrogen fixation. *J Gen Microbiol* 131, 1449–1458.

Smith, A., Hill, S. & Anthony, C. (1990). The purification, characterization and role of the *d*-type cytochrome oxidase of *Klebsiella pneumoniae* during nitrogen fixation. *J Gen Microbiol* 136, 171–180.

Thöny-Meyer, L., Beck, C., Preisig, O. & Hennecke, H. (1994). The *ccoNOQP* gene cluster codes for a *cb*-type cytochrome oxidase that functions in aerobic respiration of *Rhodobacter capsulatus*. *Mol Microbiol* 14, 705–716.

Tseng, C.-P., Albrecht, J. & Gunsalus, R. P. (1996). Effect of microaerophilic cell growth conditions on expression of the aerobic (*cyoABCDE* and *cydAB*) and anaerobic (*narGHJI*, *frdABCD* and *dmsABC*) respiratory pathway genes in *Escherichia coli*. *J Bacteriol* 178, 1094–1098.

Wong, T.-Y. & Jurtshuk, P. (1984). Activation studies by phospholipids on the purified cytochrome *c*₄:*o* oxidase of *Azotobacter vinelandii*. *J Bioenerg Biomembr* 16, 477–489.

Yang, T. (1986). Biochemical and biophysical properties of cytochrome *o* of *Azotobacter vinelandii*. *Biochim Biophys Acta* 848, 342–351.

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