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Physiological characterisation of an *Azotobacter vinelandii nifU*-deletion mutant and its spontaneous Nif$^+$ revertants that over-produce cytochrome *bd*

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Abstract

A *nifU*-deletion mutant of *Azotobacter vinelandii* fixed N_2, but only under low atmospheric O_2 (2 kPa), whereas, under air, it reverted to Nif$^+$ at 5×10^{-8}. The revertant's O_2-tolerant nitrogenase activity, surprisingly, was not accompanied by an increased respiration rate, although, like *cydR* mutants, the revertants over-produce cytochrome *bd*. The introduction of a *cydR* mutation into the *nifU* mutant yielded transformants, of which 100% fixed N_2 in air. This is consistent with the revertant mutations residing in *cydR*. Inactivation of CydR (a Fnr-like transcriptional repressor) could lead to the up-regulation of a process (e.g. IscU activity in iron-sulfur cluster formation) that substitutes for NifU.

Keywords: Nitrogenase biosynthesis; *NifU* revertant; *Azotobacter vinelandii*; Cytochrome *bd*

1. Introduction

Nitrogenases are oxygen-sensitive proteins catalysing the ATP-dependent reduction of N_2 to NH_3. They carry three different types of Fe-containing metal centres [1–3]. Of the relatively large number of genes in *Azotobacter vinelandii* that encode the subunits of the nitrogenases and their maturation, three *nif* gene products, namely *nifS*, *nifU* and *nifV*, are required for all three nitrogenases [4]. Strains deleted for *nifU* or *nifS* are distinguished from other *nif* mutants in having low activities for the two nitrogenase components, suggesting roles for these gene products in activation or stabilisation of nitrogenase [5]. NifS catalyses the desulfurisation of cysteine to yield alanine and sulfur; the sulfur is present as protein-bound persulfide, which is the proposed sulfur donor in Fe-S cluster formation [6]. Whether NifU is involved in metal cluster formation is less clear: it may participate in core formation by mobilisation of Fe [7]. In *A. vinelandii*, homology, at the amino acid level, to the *orf6nifUS* gene cluster has been shown for the newly identified *iscSUA* (iron-sulfur cluster formation) gene products [8]. A function in iron-sulfur cluster formation has

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only been shown for IscS, but IscU is a strong candidate for a house-keeping replacement for NifU [8].

An important constituent of aerobic diazotrophy in *A. vinelandii* is respiration, which plays a part in preventing irreversible damage to nitrogenase by O_2 [9]. *A. vinelandii* possesses a complex and flexible respiratory chain, of which the cytochrome *bd*-type terminal oxidase is essential for diazotrophic growth under high O_2 input [9,10]. Mutants unable to make this terminal oxidase (*cydAB*$^-$) are obligate microaerobes when fixing N_2 [10]. We observed that on a N-free agar medium both *nifS* and *nifU* mutants of *A. vinelandii* grew much better in an atmosphere containing low O_2 (2 kPa) in N_2 than in air [11]. In the present work we investigated the aerobic physiology of spontaneous Nif$^+$ revertants arising from a *nifU*-deletion mutant. A preliminary report of some of this work has been published elsewhere [11].

2. Materials and methods

2.1. Bacterial strains, media and growth

A. vinelandii strains used were: DJ (wild-type) and DJ105 (carrying an in-frame deletion in *nifU*) [5], MV51 and MV52 (spontaneous Nif$^+$ revertants of DJ105), and MK5 (carrying *cydB*::Tn*5*-B20) and MK8 (carrying *cydR*::Tn*5*-B20) [10]. They were cultured aerobically at 30°C in Burk's medium (B) [10] containing either sucrose (2%) (BS) or sucrose (1%) plus sodium lactate (1%) (BSL) as carbon sources, and when required the fixed N sources of either urea (15 or 20 mM) or ammonium acetate (15 mM). Lactate was included in the sucrose-containing liquid medium because adding a carboxylic acid improved the diazotrophic growth of O_2-sensitive *Azotobacter chroococcum* mutants [12]; sterile HEPS (30 mM) was then added for extra buffering. For strains carrying Tn*5*-B20 kanamycin was used at a final concentration of 1 μg ml^{-1}. For solid media 1.5% bacteriological agar (Oxoid) was added, and when required plates were incubated under an atmosphere composed of O_2 (2 kPa) in N_2 in a perspex box as described earlier [10].

Growth and subsequent nitrogenase derepression was carried out in 10 ml of liquid medium contained in a 25-ml bottle and when required fitted with a Klett tube as a side arm. A cottonwool-stoppered glass tube was mounted in the screw-capped lid through which air flowed at 300 ml min^{-1} to provide a stream of bubbles in the medium. These bubbles provided a rate of O_2 supply of 45.2 mM O_2 l^{-1} h^{-1} when measured by the sulfite oxidation method, as described previously [13]. Following 18 h growth in BSL with excess urea, cells were harvested by centrifugation and, after pouring off the supernatant and wiping away residual liquid, they were resuspended in 10 ml of the N-free BSL medium to give an optical density (540 nm) of 45. After bubbling these suspensions for 3.5 h, aliquots were assayed for nitrogenase activity by C_2H_2 reduction. Initial experiments with the wild-type established that, immediately after resuspension in the N-free medium, C_2H_2 was not reduced, and prolonging the time for derepression did not lead to greater C_2H_2-reducing activity. Similar derepressed cultures were also harvested by centrifugation, washed and resuspended in B medium; then they were either stored on ice (for a maximum of 4 h) for measurements of O_2 uptake, or stored at 4°C (for a maximum of 48 h) for measurements of cytochrome *d* content. Experiments were performed at least twice, and results of assays are shown as means ± S.D.; occasionally, where a single result is shown, an experiment was performed once, as it represented a verification.

2.2. Assays

Nitrogenase (C_2H_2-reducing) activities in derepressed cultures were measured under a range of initial atmospheric O_2 concentrations. Samples (1 ml) of culture were injected into assay bottles (7 ml) containing 10 kPa C_2H_2 in Ar into which O_2 had been introduced by injection, to give after pressure equalisation at 30°C, the following atmospheric O_2 concentrations (kPa): 3, 6, 11, 23, 46, and 81. The assays were incubated for 30 min at 30°C with shaking (160 strokes min^{-1} of 4 cm) and terminated by injecting 0.2 ml of 30% trichloroacetic acid; then, 0.5-ml gas samples were analysed by gas chromatography as described previously [14]. O_2 uptake rates were measured in a Clark-type O_2 electrode (Rank, Bottisham, Cambridge, UK) at 30°C with a mixture of lactate and sucrose (17.8 and 11.6 mM respectively). Less than 10% of these rates occurred in

the absence of the added substrates. Cytochrome *d* contents were estimated from recordings of sodium-dithionite-reduced minus O_2-oxidised difference spectra obtained with washed cells. Spectra were recorded in a dual beam spectrophotometer (Shumatzu) with a spectral band width of 0.2 nm and a light path of 10 mm adjacent to the photomultiplier. Biomass was estimated from a determined relationship between optical density (540 nm) and bacterial protein. The latter was measured as previously described [14].

2.3. Transformations

DNA was prepared from donor strains and transformed into competent recipient cells using the methods described earlier [15]. Kan^R transformants were selected on BS containing ammonium acetate and kanamycin. Subsequently, Km^R clones were scored for Nif^+ on BS medium incubated in air at 30°C.

3. Results and discussion

3.1. Diazotrophic growth of a nifU mutant

The strain DJ105 carries an in-frame deletion in *nifU*. It is incapable of diazotrophic growth in air, although it synthesises low levels of the nitrogenase proteins when derepressed for N_2 fixation after growth on a fixed N source [5]. Microaerobic conditions favour diazotrophic growth of certain mutants of *Azotobacter* species. For example, *cydB* mutants of *A. vinelandii* and Fos− (inability to fix nitrogen on sugars) mutants of *Azotobacter chroococcum* were unable to grow on N-free solid medium in air, but they grew in an atmosphere of 2 kPa O_2 in N_2 [10,12]. Therefore we tested the *nifU* mutant (strain DJ105) for its ability to grow diazotrophically under this microaerobic condition. On the solid BS medium in air it produced very tiny white colonies, whereas in an atmosphere of 2 kPa O_2 in N_2 (see Section 2) it produced yellowish-brown colonies similar to those of the wild-type (strain DJ). The apparent 'leakiness' of the Nif− phenotype under the microaerobic condition might arise through the function of an enzyme with NifU activity, for example IscU [8]. Two possibilities could account for the restricted activity of such an enzyme under the microaerobic condition; either its activity might be O_2-sensitive or the overall demand for its activity might be less under the microaerobic condition than in air.

3.2. Diazotrophic growth of spontaneous revertants

Amongst the tiny white colonies on the solid BS medium in air, the *nifU* mutant produced occasional larger yellowish-brown colonies at a frequency of about 5×10^{-8}. The morphology of these colonies and of the cells was indistinguishable from the wild-type, except that these colonies were slightly greenish. Since the *nifU* mutation is a deletion [5], these apparent Nif^+ revertants carry suppressor mutations: two were isolated (strains MV51 and MV52) and studied in more detail. Unlike the *nifU* mutant, which failed to grow in liquid N-free medium (BSL) when bubbled with air (see Section 2), MV51 and MV52 grew with mean doubling times of 5.3 ± 0.3 and 4.6 h, respectively. These rates were slower than the wild-type, which had a mean doubling time of 3.4 ± 1.3 h. When the fixed-N source urea was added to the BSL medium, growth rates of the

Table 1
Influences of atmospheric O_2 concentration on nitrogenase activities expressed as percentages of the highest in *A. vinelandii* strains after derepression in N-free medium, ±S.D.

Strain	Atmospheric O_2 concentration (kPa)					
	3	6	11	23	46	81
Wild-type	26	35	78 ± 2	100	68 ± 20	4
nifU mutant	64 ± 32	100	90 ± 5	65 ± 7	nt	nt
Revertant MV51	17	51	77 ± 4	100	77 ± 34	12 ± 5
Revertant MV52	nt	nt	75 ± 5	100	59 ± 8	nt

Activities (nmol C_2H_2 reduced mg protein^{-1} min^{-1}) of 100% were as follows: for wild-type 132 ± 22, for *nifU* mutant 20 ± 3, for revertants MV51 64 ± 14, and MV52 43 ± 6. nt indicates not tested. Where a single result is shown, the experiment was performed once.

nifU mutant and strains MV51 and MV52 were like that of the wild-type (data not shown). Thus, the effect of the suppressor mutations appeared to be associated with the ability to grow diazotrophically in air.

3.3. Whole-cell nitrogenase activities in the nifU mutant and its revertants

In order to investigate the O_2 tolerant diazotrophy in the *nifU* revertants MV51 and MV52, their whole-cell nitrogenase activities were compared with those of the *nifU* mutant and the wild-type. The strains were grown and derepressed for nitrogenase in air and then assayed for C_2H_2-reducing activity under a range of atmospheric O_2 concentration (see Section 2 and Table 1). The highest activity for each strains occurred under a particular atmospheric O_2 concentration (Table 1). In the *nifU* mutant the highest nitrogenase activity (nmol C_2H_4 produced min^{-1} mg $protein^{-1}$) was 20 ± 3, and less than a fifth of that of 137 ± 24 in the wild-type. Moreover, the atmospheric O_2 concentration under which this activity was observed was 6 kPa, almost four times lower than that of 23 kPa for the wild-type (Table 1). These data are consistent with the inability of this mutant to grow diazotrophically in air. The highest nitrogenase activities in the revertants were 67 ± 15 for MV51 and 43 ± 6 for MV52 and thus were two to three times greater compared to the activity of the *nif* mutant, although they were a third to one half lower than in the wild-type. Significantly, for MV51 and MV52, the atmospheric O_2 concentration under which these activities were observed was the same as that of 23 kPa for the wild-type (Table 1), and thus it was nearly four times greater than that of 6 kPa for the *nifU* mutant. These data are consistent with the revertants' ability to grow diazotrophically in air, and suggested, moreover, that the suppressor mutation had in some way changed the *nifU* mutant's physiology so that O_2 was effectively kept away from nitrogenase.

3.4. Respiratory characteristics of the nifU mutant and its revertants

The O_2-tolerant diazotrophy of *A. vinelandii* is attributed in part to the removal of O_2 by the cytochrome *bd*-type oxidase, which is termed respiratory protection [9,10]. Colonies of strains that over-produce the cytochrome *bd*-type oxidase (*cydR* mutants) are slightly greenish in colour like those of the *nifU* revertants (strains MV51 and MV52). We therefore compared the cytochrome *d* content of the *nifU* mutant, MV51 and MV52 with that of the wild-type. Spectral analyses of harvested cells from the derepressed cultures showed that the cytochrome *d* content of MV51 and MV52 was three to four times greater than the level found in either the *nifU* mutant or the wild-type (Fig. 1 and Table 2). A *cydR* mutant (strain MK8) also produced an elevated cytochrome *d* content under these conditions (Table 2), as it does when growing diazotrophically (S. Edwards, S. Hill and R.K. Poole, unpublished). Spectral analysis (by a procedure described earlier [16]) of cells harvested from aerobic ammonia-assimilating batch cultures showed that the cytochrome *d* content was 12 times greater in the revertant, MV51, compared to the *nifU* mutant and the wild-type. Thus, the raised cyto-

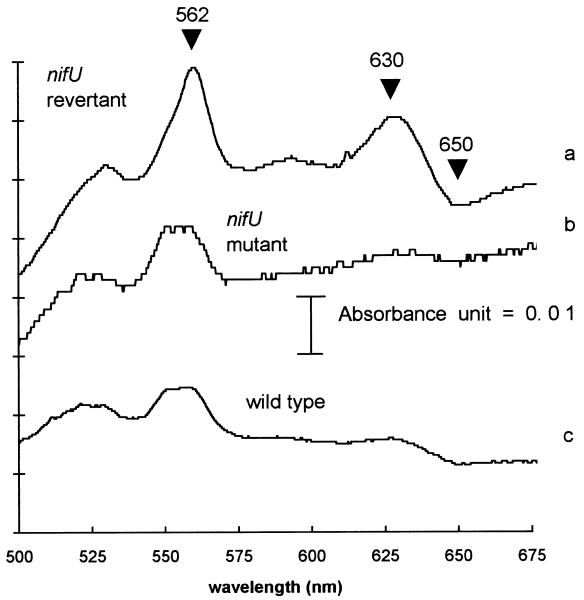

Fig. 1. Dithionite-reduced minus oxidised difference spectra of whole cells of *A. vinelandii* strains. Cells were derepressed for nitrogenase, harvested, washed and resuspended as described in Section 2. The spectra are shown for the following strains at protein concentration (mg ml^{-1}) of: (a) revertant MV51 0.57, (b) *nifU* mutant 0.57, and (c) wild-type 0.62. Traces b and c have been displaced and attenuated to facilitate display.

Table 2
Cytochrome *d* content and potential rate of O_2 uptake in washed cells of wild type and mutant strains after derepression in N-free medium, ± S.D.

Strain number	Relevant genotype	Relative cytochrome *d* content[a]	Potential O_2 uptake rate (μmol O_2 min^{-1} mg $protein^{-1}$)
DJ	wild-type	1[a]	2.22 ± 0.62
DJ105	*nifU*	0.6 ± 0.2	2.43 ± 0.71
MV51	*nifU*, revertant	4.4 ± 2.1	3.19 ± 0.50
MV52	*nifU*, revertant	3.4	3.59
MK8	*cydR*	6.2	2.09
MK5	*cydB*	–	0.19 ± 0.01

One unit equals 170 ± 30 pmol mg $protein^{-1}$ of cytochrome *d* using the extinction coefficient of 27 cm^{-1} mM^{-1} (630–650 nm) for the reduced-minus-oxidised spectrum [18].

chrome *d* level in the revertant strain MV51 was not confined to diazotrophy; this is also a property of *cydR* mutants (e.g. strain MK8) [10,16,17]. The spectra of MV51 (Fig. 1) and MV52 (data not shown) also showed a raised level of absorbance at 562 nm compared to the wild-type (Fig. 1); this absorbance is, in part, due to another component of the cytochrome *bd*-type oxidase (cytochrome b_{562}). Thus, over-production of the whole cytochrome *bd*-type oxidase probably occurred in the revertants MV51 and MV52, as it does in *cydR* mutants [10,16,17].

To determine whether the greater level of cytochrome *bd*-type oxidase in strains MV51 and MV52 provided improved O_2 removal, rates of O_2 uptake were measured in the cells derepressed for nitrogenase activity. For comparison, O_2 uptake rates were measured in derepressed cells of a *cydB* mutant (strain MK5), which does not make the cytochrome *bd*-type oxidase [10], and of MK8 (*cydR*). Despite their higher cytochrome *bd* content, the respiratory rates in the MV51 and MV52 were not markedly greater than those in either the *nifU* mutant or the wild-type (Table 2). Thus, the effect of the suppressor mutation on the organism's physiology does not apparently involve an increase in the rate of O_2 removal when nitrogenase is derepressed. Similarly, the *cydR* mutant, MK8, did not display an elevated respiratory activity compared to the wild-type (Table 2) [19]. The lack of correlation between the cytochrome *bd* content and the respiratory rate suggests that factors other than the oxidase content appear to limit respiration. Liu et al. [20] also concluded that *A. vinelandii* contains more terminal oxidases than it requires for normal growth. A significantly lower rate of O_2 uptake than the wild-type was observed in MK5 (*cydB*) (Table 2), which is consistent with earlier work [10]. This mutant, like the *nifU* mutant, fails to grow on N-free medium in air, but will do so microaerophilically [10]. The markedly higher potential rate of O_2 uptake by the *nifU* mutant compared to that of the *cydB* mutant (Table 2) suggests that the obligate microaerobic habit of the *nifU* mutant, when fixing N_2, is due to some factor other than poor O_2 removal by respiration.

3.5. Correction of the nifU mutant's inability to grow diazotrophically in air by introducing a cydR mutation

The *cydR* mutants of *A. vinelandii* carry mutations in a gene lying about 1 kb upstream of the genes encoding the subunits of the cytochrome *bd*-type terminal oxidase (*cydAB*) [10,17]. The product of the *cydR* gene negatively regulates the expression of *cydAB* [17]. Insertions of Tn*5*-B20 into the *cydR* gene results in elevation of *cydAB* expression and of cytochrome *bd*-type oxidase synthesis [10,13,17]. The raised cytochrome *d* level in MV51 and MV52 suggested that the suppressor mutation in these strains might lie within the *cydR* gene. If this is the case then the introduction of a *cydR* mutation into the *nifU* mutant should correct its inability to grow diazotrophically in air. The *nifU* mutant was transformed with DNA prepared from a *cydR* mutant carrying *cydR*::Tn*5*-B20 (strain MK8). Kanamycin-resistant clones were selected on BS medium containing ammonium acetate and were scored for their ability to grow on the N-free BS medium in air. Significantly, all the Km^R transformants tested (156) grew as well

as the wild-type on N-free medium, indicating that the introduction of the *cydR* mutation had corrected the diazotrophic phenotype. To demonstrate that the raised cytochrome *d* level, which accompanied the reversion to Nif^+ in strains MV51 and MV52, was due to *cydAB* expression, DNA from a strain carrying *cydB*::Tn*5*-B20 (strain MK5) [10] was used to transform these revertants. None of the Km^R transformants tested (70 and 60, respectively) grew diazotrophically in air. Taken together, these results are consistent with the suppressor mutations lying within the *cydR* gene.

In conclusion, the *nifU*-deletion mutant DJ105, which was 'leaky' for diazotrophic growth under low O_2, produced spontaneous revertants in air. The revertants showed an improved tolerance towards O_2 for nitrogenase activity. Their cytochrome *bd* contents, like those in strains carrying mutations in *cydR* (e.g. MK8), were higher than in the wild-type, but these increases were not accompanied by enhanced potential respiratory activities. Thus, besides O_2 removal, the cytochrome *bd*-type oxidase may have another function in aerotolerant diazotrophy. A further phenotype shared by the revertant MV51 (unpublished data) and *cydR* mutants [10] is their unexplained poor growth under 2 kPa O_2. Taken together, these phenotypic similarities and the ability of a *cydR* mutation to correct the aerobic Nif^- phenotype of the *nifU* mutation are consistent with the revertant mutations residing within *cydR*. The product of *cydR* is likely to be a pleiotropic regulator, as it shows considerable homology to Fnr of *Escherichia coli* [17], which regulates the expression of a number of catabolic processes [21]. If CydR, in addition to its repressive role in *cydAB* expression, regulated the expression of genes associated with Fe metabolism, then a *cydR* mutation might relieve repression of a house-keeping process (i.e. IscU), to replace NifU [8].

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