Biochem. J. (1986) 238, 437–442 (Printed in Great Britain)

Nitrogen fixation in molybdenum-deficient continuous culture by a strain of *Azotobacter vinelandii* carrying a deletion of the structural genes for nitrogenase (*nifHDK*)

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Steady-state chemostat cultures of *Azotobacter vinelandii* strain CA11, carrying a deletion of genes encoding the structural polypeptides of nitrogenase *nifHDK*, were established in a simple defined medium chemically purified to minimize contamination by Mo. The medium contained no utilizable N source. Growth was dependent on N_2 (1.1×10^8 viable cells·ml^{-1} at $D = 0.176$ h^{-1}), and was inhibited by Mo (20 nM). DNA hybridization showed the deletion to be stable during prolonged (55 days) growth in the chemostat (132 doublings). Since batch cultures, using unsupplemented 'spent' chemostat medium, showed good growth (1.9×10^8 cells·ml^{-1}), no requirement for subnanomolar concentrations of Mo was found. The biomass yield, as the dilution rate (D) was varied, showed that the N content of the culture, protein and dry wt. increased as D was decreased, indicating that neither N_2 nor O_2 were limiting growth. The limiting nutrient was not identified. Substantial amounts of H_2 were evolved by the chemostat cultures, probably as the result of inhibition of O_2-dependent hydrogenase activity by nitrilotriacetic acid present in the medium. Over a range of D values approx. 50% of the electron flux through the alternative system was allocated to H^+ reduction. C_2H_2 was a poor substrate, being reduced at 0.14–0.1 times the rate of N_2 fixation, calculated from the N content of the cells. SO_4^{2-}-limited steady-state continuous cultures of strain UW136 (wild-type for *nifHDK*) had a 2-fold greater biomass in the presence of MoO_4^{2-} (1 μM). The significance of this finding for 'Mo-limited' continuous cultures [Eady & Robson (1984) Biochem. J. **224**, 853–862] is discussed.

INTRODUCTION

Nitrogenase, the well-characterized molybdoenzyme system which catalyses the reduction of N_2, comprises a MoFe protein ($\alpha_2\beta_2$ tetramer) encoded by *nifD* and *nifK*, and an Fe protein (γ_2 dimer) encoded by *nifH* (see Eady & Smith, 1979; Dixon, 1984). Some diazotrophs contain reiterated *nif* DNA sequences encoding *nifHDK* or *nifH* (Quinto *et al.*, 1985; Scolnik & Haselkorn, 1984; Jones *et al.*, 1984). Such reiterated sequences can be functional as in *Rhizobium phaseoli* (Quinto *et al.*, 1985) or, as in *Rhodopseudomonas capsulata*, normally silent but capable of activation by mutation (Scolnik & Haselkorn, 1984).

Azotobacter vinelandii has an alternative system for N_2 fixation in addition to conventional nitrogenase (Bishop *et al.*, 1980). Evidence for the alternative system was earlier based primarily on the observation that several classes of Nif$^-$ mutant strains of *A. vinelandii* underwent phenotypic reversal under Mo-deficient conditions. Since explanations other than the existence of an alternative N_2 fixation system were possible, deletion strains of *A. vinelandii* lacking *nifHDK* were constructed, and N_2 fixation under Mo-deficient conditions demonstrated (Bishop *et al.*, 1986). This work provided definitive proof for the existence of a nitrogenase complex in *A. vinelandii* other than that encoded by the *nifHDK* gene cluster.

In order to define the parameters of the alternative N_2 fixation system *in vivo*, in the absence of possible interference from the conventional system, a chemostat study of growth of a *nifHDK* deletion growing under Mo-deficient conditions was undertaken. We report here that the unusual pattern of substrate reduction by the alternative N_2 fixation system in steady-state cultures is similar to that of the wild type under Mo deficiency (Eady & Robson, 1984) but, contrary to the earlier study, no requirement for Mo could be established for diazotrophic growth of the deletion strain CA11.

MATERIALS AND METHODS

Bacterial strains and media

Azotobacter vinelandii strain UW136 (Bishop *et al.*, 1977) referred to as UWrif^r by Eady & Robson (1984) is wild type for the structural genes coding for nitrogenase (*nifHDK*). Strain CA11 contains a 5.25 kbp *Bgl*II deletion which removes both *nifD* and *nifK* plus approximately two-thirds of *nifH* (Bishop *et al.*, 1986) and, except for the deletion, is isogenic with strain UW136. Both strains were maintained on agar plates containing modified Burk's medium (Strandberg & Wilson, 1968) supplemented by 29 mM-ammonium acetate. Chemostat medium routinely had the following composition (g/l): KH_2PO_4, 0.2; K_2HPO_4,$3H_2O$, 0.8; $MgSO_4$,$7H_2O$, 0.2; $CaCl_2$,$2H_2O$, 0.09; sucrose, 20. High purity $Fe_2(SO_4)_3$ (Johnson Matthey Chemicals, Royston, Herts. SG8 5HE, U.K.), 240 mg (final concn. 30 μM) and trisodium nitrilotriacetate (Na_3NTA), 2.87 g (final concn. 522 μM) were mixed in approx. 20 ml of water and

Abbreviations used: NTA, nitrilotriacetate; c.f.u., colony-forming units; (k)bp, (kilo)base-pairs.

added to the bulk media (minus phosphate) shortly before autoclaving. The phosphate and bulk media were autoclaved separately and mixed when cool.

Medium used for SO_4^{2-}-limitation experiments was modified by substituting high purity Fe_2O_3 for $Fe_2(SO_4)_3$, converted to $FeCl_3$ by dissolving 96 mg in 1 ml of boiling concentrated HCl. The $FeCl_3$ was mixed with 20 ml of water containing 2.87 g of Na_3NTA and was added to 20 litres of bulk medium before autoclaving (final concn. 60 μM-Fe^{3+} and 522 μM-NTA). Appropriate amounts of SO_4^{2-} were added to the medium as Na_2SO_4 after filter sterilization.

Medium was routinely prepared in 20 litre batches, and purified to remove trace Mo contamination as described previously (Eady & Robson, 1984), except that bulk medium was made up as a 10× (2 litres) concentrated solution in order to facilitate extraction by dichloromethane. Double-glass-distilled water was used to bring the volume up to 20 litres after the final extraction step.

Experimental procedures

All procedures including chemostats, cleaning of glassware, measurement of growth, viability, yield, N analyses, and assays for nitrogenase and hydrogenase were carried out essentially as described by Eady & Robson (1984).

DNA–DNA hybridization

Preparation of genomic DNAs, *Sma*I digestions, electrophoresis of DNA in 0.8% agarose and transfer to Genescreen (New England Nuclear) have been described previously (Jones *et al.*, 1984). Hybridization to a *Sma*I digest of genomic DNA of *A. vinelandii* was conducted at 42 °C for 16 h in 1% SDS and 50% formamide using a ^{32}P-labelled probe of a 6.2 kbp *Sma* I fragment containing *nifHDK* from *A. vindelandii* (Bishop *et al.*, 1986) as described by the suppliers of Genescreen.

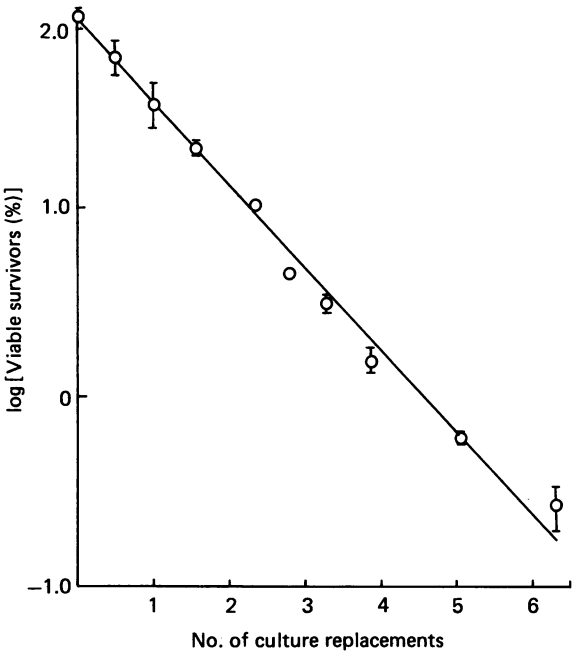

Fig. 1. Dependence on N_2 as N source for growth of *A. vinelandii* strain CA11 in chemostat culture in Mo-deficient medium

A steady-state chemostat culture of strain CA11 ($D = 0.097\ h^{-1}$) was established under air in chemically purified 'N-free' Mo-deficient medium. At zero time the air passing over the culture was replaced with an Ar/O_2 (4:1) mixture. The effect on the population was determined by measuring viable counts expressed as c.f.u. in culture samples withdrawn at timed intervals. The solid line indicates the theoretical dilution of a non-dividing though viable population. ○ indicates viable organisms expressed as a percentage of initial population at time zero (1.9×10^8 c.f.u./ml).

RESULTS

Continuous culture of *A. vinelandii* strain CA11 in Mo-deficient medium

A continuous culture of the *nifHDK* deletion strain CA11 was established in purified N-free medium containing no added Mo. The medium contained ferric nitrilotriacetate (0.5 mM), added to avoid problems, encountered early in this study, of instability of steady-states assigned to Fe limitation (characterized by a yellow-green colouration of the culture), so it was necessary to ensure that NTA was not acting as a nitrogen source and that N_2 was the sole nitrogen source. The inflowing air supplied to the steady-state was changed from air to Ar/O_2 (79:21) and over 6.3 replacement times (65 h) the population density decreased to 0.27% that of the initial steady-state population (Fig. 1), following closely the theoretical washout rate for non-growing organisms and indicating that the N_2-to-Ar shift did not result in the death of the population.

Before restoring air to the culture, NH_4Cl (final concn. 3.85 nM) was added to the chemostat vessel and the medium pump was turned off. The resulting dense growth of this batch culture indicated that the Ar/O_2 gas mixture was not toxic. N_2 fixation was also established by ^{15}N enrichment experiments *in situ* in the chemostat as described by Eady & Robson (1984). After 2 h incubation under air containing $^{15}N_2$ the isotopic composition of the cells showed a 3.21 atom% ^{15}N excess. These experiments show that growth of strain CA11 was N_2-dependent.

Stability of the Nif$^-$ phenotype of strain CA11 maintained in continuous culture

Periodically samples were removed and tested for growth on solid N-free agar medium containing Na_2MoO_4 (1 μM). In one steady-state the Nif$^-$ phenotype remained stable for 81 days. Occasional Nif$^+$ colonies were observed but these are pseudorevertants which express the alternative N_2 fixation system in the presence of repressive levels of Mo or W (Bishop *et al.*, 1980). In addition, Southern blot hybridization analysis (using a 6.2-kbp *Sma*I fragment containing *nifHDK* from *A. vinelandii* as a probe) of DNA (digested with *Sma*I), isolated from strain CA11 after 55 days in continuous culture showed the persistence of the 5.25-kbp deletion carried by the inoculum.

Growth parameters of strain CA11 in continuous culture

A number of steady-states of strain CA11 growing on Mo-deficient medium was established at various dilution rates. Fig. 2 shows the changes that occurred in cell

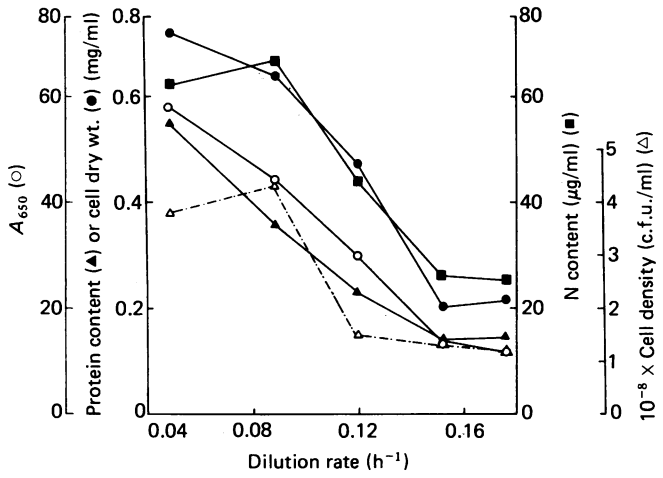

Fig. 2. Relation of cell concentrations and cellular composition to dilution rate in continuous cultures of *A. vinelandii* strain CA11

Cultures were grown on Mo-deficient 'N-free' medium, and the parameters characterizing steady-states at various dilution rates were determined as described in the Materials and methods section.

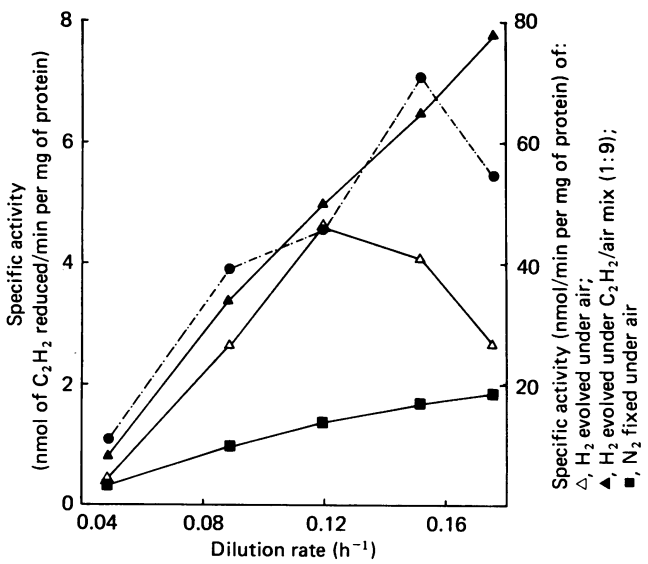

Fig. 3. Pattern of substrate reduction by nitrogenase in relation to dilution rate in continuous culture of *A. vinelandii* strain CA11

Cultures were grown in Mo-deficient 'N-free' medium and the substrate reduction pattern of steady-state cultures was determined *in situ*, at different dilution rates. Nitrogenase activity was assayed either from the rate of reduction of added acetylene or from the rate of evolution of H_2 from the culture. The rate of N_2 reduction was calculated from the N content of the organisms and the dilution rate of the culture.

density (measured as c.f.u. and A_{650}), dry weight of organisms, protein, and total N content over a range of D from 0.049 h^{-1} to 0.176 h^{-1}. The yield of organisms showed a marked increase with decreasing growth rate. This increase is unlikely to be due to poly-β-hydroxybutyrate accumulation associated with O_2 limitation because dry weight and protein content increased in parallel. Microscopic examination of the organisms also showed normal morphology over a range of D from 0.089 h^{-1} to 0.176 h^{-1} with little indication of polymer accumulation. The increased yield at low dilution rates is not due to 'cryptic growth' since the viability determined as c.f.u. did not decrease markedly. The parallel increase of N content·ml^{-1} indicates that cultures were not intrinsically N_2-limited, as defined by Dalton & Postgate (1969). When the medium inflow was stopped, a steady-state population continued to grow as a batch culture. The nature of the limiting nutrient is discussed later.

Patterns of substrate reduction by nitrogenase in chemostat cultures

Substantial rates of H_2 evolution were exhibited by steady-state populations of strain CA11, increasing with dilution rate up to 0.12 h^{-1} where the activity was 46 nmol of H_2 evolved/min per mg of protein (Fig. 3). These high rates of H_2 evolution were probably due to the inhibition of uptake hydrogenase by NTA (Partridge & Yates, 1982) since O_2-dependent uptake hydrogenase activity (measured in samples removed from a different steady state) decreased from 252 to 3.2 nmol H_2 evolved/min per mg of protein when the NTA concentration in the medium was increased from 18 to 522 μM. Concomitant with this inhibition of uptake hydrogenase activity, the rate of H_2 evolution increased from 4.4 to 23.2 nmol of H_2 evolved/min per mg of protein.

As observed previously with strain UW136, H_2 evolution was not inhibited by C_2H_2 (10%, v/v) (Eady & Robson, 1984). In the case of strain CA11, at higher dilution rates in the presence of 522 μM-NTA a more than 2-fold stimulation of the rate of H_2 evolution to 78 nmol of H_2 evolved/min per mg of protein in the presence of C_2H_2 was observed (Fig. 3). H_2 evolution was attributed to the presence of a nitrogenase since the activity was repressed by the addition of 29 mM-NH_4Cl to the medium reservoir.

Substrate reduction patterns by Mo-deficient continuous cultures of strain UW136 indicated that the rate of C_2H_2 reduction under represented the rate of N_2 fixation (Eady & Robson, 1984). Fig. 3 shows that a similar pattern was obtained with strain CA11. Over the range of D = 0.049 h^{-1} to 0.176 h^{-1}, the rate of C_2H_2 reduction determined *in situ* underestimated the N_2 fixation rate, calculated from D and the N content of the cells by 7–10-fold. Over this range of D the specific activity for N_2 reduction increased from 3.9 to 19 nmol of N_2 reduced/min per mg of protein at D = 0.176.

The proportion of electrons allocated to H^+ reduction as the percentage of the total electron flux through nitrogenase is shown in Table 1 for different steady-state populations. Within the mid-range of D values investigated approx. 50% of the total electron flux through nitrogenase was allocated to the reduction of H^+. This proportion decreased at higher and lower dilution rates.

Effect of Mo on diazotrophic growth of strains CA11 and UW136

Since a previous study (Eady & Robson, 1984) indicated that *A. vinelandii* strain UW136 had an absolute requirement for Mo when growing on N_2, it was

Table 1. Electron allocation to proton reduction by nitrogenase in chemostat cultures of *A. vinelandii* strain CA11

The rate of N_2 fixation was calculated from the N content of the organisms and D for different steady-states; H^+ reduction was measured from the H_2 content of the effluent air from the chemostat headspace and the air flow rate.

	Rates of substrate reduction (nmol/min per mg of protein)			Allocation of total flux of electrons to H^+ reduction (%)	H_2 evolved/N_2 reduced
D(h^{-1})	N_2	$N_2 \times 3$	H^+		
0.049	3.47	10.4	3.5	25.2	1
0.089	9.96	29.9	26.3	46.8	2.6
0.120	13.53	40.6	45.6	52.9	3.4
0.152	16.80	50.4	40.7	44.7	2.4
0.176	17.97	53.9	26.5	33.0	1.47

of interest to see whether this requirement could be established for strain CA11. Continuous cultures of strain UW136 (wild-type with respect to *nifHDK*) and strain CA11 fed with Mo-deficient medium from the same reservoir were tested for the effect of low concentrations of Na_2MoO_4 on growth under N_2-fixing conditions. The D for the strain UW136 and CA11 cultures was adjusted to 0.194 h^{-1} and 0.198 h^{-1} respectively, corresponding to replacement times of 5.125±0.75 h. At steady-state the cell densities were 7×10^7 c.f.u./ml for strain UW136 and 3×10^7 c.f.u./ml for strain CA11. Two sequential additions (at 24 h intervals) of Na_2MoO_4 to give 10 nM and 20 nM final concentration were made to the medium reservoir. The culture density of strain UW136 increased from $A_{540} = 0.94$ to 1.24 while that for strain CA11 decreased from $A_{540} = 0.65$ to 0.115 over 47 h: growth of strain CA11 was inhibited by nanomolar concentrations of Mo, while growth of strain UW136 was stimulated.

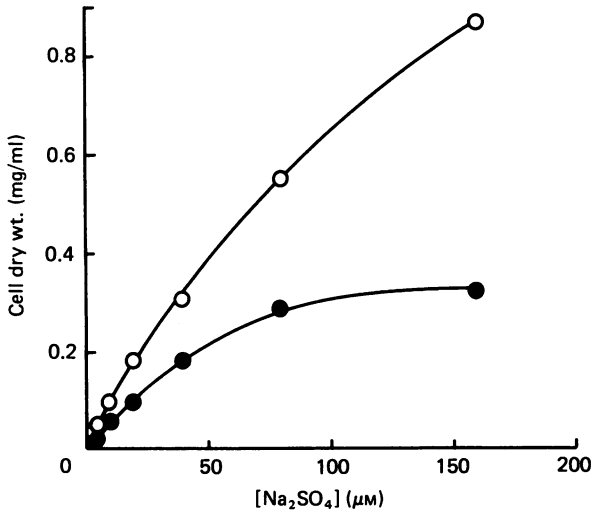

Fig. 4. Effect of Mo on biomass concentration of *A. vinelandii* strain UW136 in sulphate-limited continuous cultures

Cultures were grown in either Mo-deficient 'N-free' medium (●), or on the same medium supplemented with 1 μM-Na_2MoO_4 (○) at the SO_4^{2-} concentrations indicated. Samples were removed from different steady-state populations (D = 0.14 h^{-1}) for determination of the dry weight.

To test the possibility that subnanomolar levels of Mo might be necessary for diazotrophic growth of strain CA11, an outgrowth experiment, using 'spent' chemostat medium biologically scavenged of residual contaminating Mo, was done. Mo input from the organisms themselves was minimized by using low inoculation volume (1%, v/v) to give a culture density of 4.2×10^5 c.f.u./ml of organisms derived from a Mo-deficient steady-state chemostat culture. Filter-sterilized 'spent' chemostat medium was inoculated and cultures were incubated at 30 °C with vigorous shaking; the population increased to 1.9×10^8 c.f.u./ml over 44 h. On subculture of this batch culture with similar 'spent' medium the population increased from 1.9×10^6 c.f.u./ml to 6.4×10^8 c.f.u./ml over 48 h. In similar experiments, Eady & Robson (1984) reported 5×10^8 c.f.u./ml with NH_4^+ as a source of fixed N for strain UW136. Since 'spent' chemostat medium supports growth to a similar extent with N_2 or NH_4^+ as N source, it is not limiting with respect to a nutrient essential for N_2 fixation. The low Mo content of such media suggests that there is no Mo requirement for diazotrophic growth of strain CA11.

Effect of SO_4^{2-}-limitation on diazotrophic growth of strain UW136 in chemostat culture in the presence and absence of added Mo

A range of sulphate-limited steady-states of strain UW136 were established in Mo-deficient medium, and in the same medium supplemented with Na_2MoO_4 (1 μM). As shown in Fig. 4, the yield of organism was approx. 2-fold greater at all concentrations of SO_4^{2-} tested, in the presence of MoO_4^{2-}. These data indicate that when N_2 fixation is occurring via the conventional nitrogenase system (in the presence of Mo) a more effective utilization of SO_4^{2-} occurs, which results in an increase in biomass. The significance of these observations in relation to the evidence presented for Mo-limited growth of strain UW136 by Eady & Robson (1984) is discussed below.

DISCUSSION

Definitive evidence is now available for an alternative N_2 fixation system in *A. vinelandii* (Bishop *et al.*, 1986). Data reported here (Fig. 1) show unequivocally that growth of the strain deleted for conventional nitrogenase genes in continuous culture in Mo-deficient medium depends on N_2 as N source. Steady-state cultures also

incorporated $^{15}N_2$ in agreement with results obtained with other Nif$^-$ mutant strains (Bishop *et al.*, 1980, 1985).

The stability of the Mo-dependent Nif$^-$ phenotype of strain CA11 is shown by its inability to grow on Mo-sufficient media and by the persistence of the *nifHDK* deletion after prolonged growth in continuous culture. This is an important observation since *A. vinelandii* can contain up to 40 chromosomes per cell (Sadoff *et al.*, 1979) and growth on N_2 might otherwise have been attributable to amplification of a chromosome not carrying the deletion.

The use of strain CA11 simplifies interpretation of physiological data concerning the involvement of Mo in nitrogen fixation, since complications arising from the altered properties of the conventional system operating under Mo-deficiency are avoided. From the yield data presented here it is evident that Mo, when added at a nanomolar levels, prevents the alternative system from functioning and stimulates the conventional system. Although a role for Mo in regulating expression of conventional *nif* genes has been questioned (Shah *et al.*, 1984), the rate of synthesis and steady-state level of MoFe protein in *A. vinelandii* strain UW136 is very low during Mo starvation, in contrast to the rate of synthesis of Fe protein (Eady & Robson, 1984) and Fe protein activity (Premakumar *et al.*, 1984). In addition, studies on *nif* transcription have shown that low levels of Mo repress the *nifH*-hybridizing mRNA transcripts which are observed in *A. vinelandii* under Mo-deficient conditions (Jacobson *et al.*, 1985).

The similarity of the unusual substrate reduction pattern of nitrogenase in Mo-deficient continuous cultures of strain UW136 (Eady & Robson, 1984) and the data reported here for strain CA11 (Fig. 3) suggest that in medium which has been chemically purified to remove contaminating Mo, only the alternative system for N_2 fixation is functional. This is supported by our data which show that chemostat cultures of strains CA11 and UW136 fed from the same reservoir of Mo-deficient medium have similar biomass.

C_2H_2 is a poor substrate for the alternative system in comparison with N_2. This is unlikely to be due to an altered apparent K_m for C_2H_2 (see Eady & Robson, 1984).

When uptake hydrogenase is inhibited by NTA, substantial amounts of H_2 are evolved under air. The rate of H_2 evolution was stimulated by C_2H_2 (Fig. 3), in contrast to its effect on the conventional system where C_2H_2 inhibits H_2 evolution. The stimulation is unlikely to arise from inhibition of uptake hydrogenase since this activity is low, consequent on inhibition by NTA, and may arise from the inhibition of N_2 reduction by C_2H_2 directing electrons to H^+ reduction.

A chemostat study of mutant strains of *Azotobacter chroococcum* lacking uptake hydrogenase activity showed that the minimum stoichiometry of H_2 evolved: N_2 reduced by nitrogenase was 1 (Aguilar *et al.*, 1984). This ratio increased to 2 as the D value was increased to 0.17 h^{-1}. In contrast, our data for strain CA11 gave a ratio of 3.3 at intermediate D values, where approx. 50% of the electron flux through nitrogenase was allocated to H^+ reduction (Table 1). Recycling of H_2 by uptake hydrogenase is likely to play an important role in the economical use of energy during N_2 fixation under Mo-deficient conditions. We do not know whether the cultural conditions in our chemostat were optimal for the alternative system but, if the difference in efficiency of the two systems is real, it might provide a physiological explanation why the alternative nitrogen fixation system is repressed by low levels of Mo.

We have not attempted to determine the limiting nutrient under the conditions of our SO_4^{2-}-sufficient continuous cultures. Our data exclude O_2- and N_2-limitation; a comparable dependence of yield versus D can occur with a trace-metal limitation (Pirt, 1975) though this may not necessarily have a direct effect on N_2 fixation.

The data of Fig. 4 indicate that SO_4^{2-}-limited chemostat cultures of strain UW136 growing on N_2 on Mo-deficient media respond to the addition of MoO_4^{2-} with an approx. 2-fold increase in cell yield. Since, under our conditions, a SO_4^{2-}-limited steady-state of strain UW136 has no nutritional advantage over strain CA11 under Mo-deficiency, these two situations correspond to diazotrophic growth by strain UW136 using the alternative and conventional nitrogenases respectively. The effect of Mo could be attributed either to an increase in efficiency of SO_4^{2-} uptake and utilization or to an increased rate of N_2 fixation as the conventional system becomes functional. It is interesting to note that a Mo-uptake system associated with diazotrophy of *Clostridium pasteurianum* has been shown to co-transport SO_4^{2-} (Elliot & Mortenson, 1975). The difference in yield is unlikely to be a consequence of a greater SO_4^{2-}-requirement for the alternative system. As discussed by Tempest (1976), data for a single dilution rate can be misleading if there is a differential yield as a function of D, as is found for SO_4^{2-}-limited continuous cultures of *A. chroococcum* growing on N_2 as against assimilating NH_4^+ (Hill *et al.*, 1972).

The establishment of steady-state chemostat populations of strain UW136 growing on Mo-deficient media which responded to the addition of Mo by increased yield led Eady & Robson (1984) to suppose that they were Mo-limited, i.e. that there was a Mo-requirement for growth. Additional support for this suggestion was the observation that, in outgrowth experiments using 'spent' chemostat media biologically scavenged to remove Mo, a 25-fold lower population was attained when Mo was not added. However, data presented here for strain UW136 show that SO_4^{2-}-limited chemostats responded to the addition of MoO_4^{2-} at the SO_4^{2-} concentrations used by these workers for continuous culture (20 μM) and batch culture (50 μM). The stimulation of growth of chemostat and batch cultures by added Mo reported by Eady & Robson (1984) can be explained as being due to a partial relief of SO_4^{2-}-limitation. The question as to what is the limiting nutrient in such cultures is vexed, since SO_4^{2-}-limited diazotrophic cultures of strain UW136 growing in our medium respond to the addition of MoO_4^{2-}, SO_4^{2-} or NH_4^+ by increasing growth yield. In this strain there are two routes for N_2 fixation, and depending upon the Mo status of the organism, one or other, sometimes possibly both, can function. However, in the case of the deletion strain CA11, MoO_4^{2-} prevents growth when added at nanomolar concentrations and in SO_4^{2-}-sufficient 'spent' chemostat medium no differential limitation for final growth yield on N_2 compared with NH_4^+ as N source is apparent. This would suggest that Mo is not required for the alternative N_2 fixation system and by inference is not involved in diazotrophic growth

of this strain. Thus, the question as to whether Mo is absolutely required for diazotrophy remains open.

In *A. chroococcum* the second copy of the *nifH* gene (termed *nifH**), which is unlinked to the main *nif* gene cluster, has been sequenced and found to be 88% homologous with *nifH* (Kennedy *et al.*, 1985; Robson *et al.* 1986). Running off the same putative promoter, and 124 bp downstream from *nifH**, is a DNA sequence containing a ferredoxin-like sequence (Robson *et al.*, 1986). Since a DNA fragment from the *A. chroococcum* ferredoxin gene hybridizes to a mRNA transcript (which also hybridizes to *nifH*) present in N_2-grown strain CA11 (Jacobson *et al.*, 1985), these gene products are likely to be involved in diazotrophic growth under Mo-deficient conditions. Support for this suggestion is provided by the presence of Fe protein activity in extracts of N_2-grown strain CA11 (Bishop *et al.*, 1986) and the finding that the *nifM* product, which activates conventional Fe protein polypeptide, is required for growth under both Mo-sufficient and Mo-deficient conditions (Kennedy *et al.*, 1985).

We thank Professor J. R. Postgate for critical reading of the manuscript, Dr. R. Robson for useful discussion, Miss Beryl Scutt for preparation of the typescript, Dr. R. L. Richards for ^{15}N analyses, and Mr. C. Macdonald for N analyses. P.E.B. wishes to thank the U.S. Department of Agriculture – Agricultural Research Service for the ARS fellowship awarded to him.

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Received 25 November 1985/2 April 1986; accepted 7 May 1986