The Beneficial Effect of Hydrogenase in *Azotobacter chroococcum* Under Nitrogen-fixing, Carbon-limiting Conditions in Continuous and Batch Cultures

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The growth of three hydrogenase minus (Hup^-) mutants of *Azotobacter chroococcum* was compared with that of the parent Hup^+ strain in batch or continuous cultures. All three mutants gave similar yields to the parent under N_2 -fixing conditions at an optimum dilution rate (D) of $0.1\ h^{-1}$ in sucrose-limited N_2 -fixing cultures. However, at higher D values the steady-state yields of sucrose-limited mutants were lower than those of the parent and washout occurred at lower D values. These observations were confirmed in carbon-limited mixed cultures where the parent strain outgrew the mutant at high D values. Such marked differences were not obtained in SO_4^{2-} - or O_2 -limited continuous cultures. In batch culture at low sucrose levels the mutants displayed a long division lag compared with the parent, particularly with dilute inocula. Non- N_2 -fixing (NH_4^+ -grown) conditions removed these differences. We suggest that one beneficial effect of hydrogenase is on the initiation of diazotrophic growth, particularly with restricted carbon/energy supply.

INTRODUCTION

Although hydrogen is a normal by-product of nitrogenase function (see Postgate, 1982), aerobic diazotrophs rarely evolve H_2 when fixing N_2 . Exceptions are some strains of *Rhizobium* and *Bradyrhizobium* (see Evans *et al.*, 1981) and *Azotobacter chroococcum* grown under certain physiological conditions (Yates *et al.*, 1981) or pre-treated with an inactivator of hydrogenase, acetylene (Walker *et al.*, 1981). H_2 evolution by nitrogenase represents a metabolic drain on the organisms since ATP is consumed and, presumably, wasted. H_2 production can use 25 to 80% of the electron flux to nitrogenase *in vivo* (Evans *et al.*, 1981; Yates *et al.*, 1981). Most diazotrophs possess a hydrogenase; in aerobes such as the azotobacters and rhizobia the enzyme is essentially unidirectional, catalysing the uptake of H_2 . It is believed to contribute to the metabolic efficiency of the diazotrophic system by scavenging H_2 produced during the nitrogenase function; possible benefits (Dixon, 1972) include sparing of electron sources for diazotrophy, recovery of ATP through H_2 -linked respiration, respiratory protection of nitrogenase against O_2 and protection of the enzyme from inhibition by H_2 (see Robson & Postgate, 1980).

Hydrogenase ought therefore to increase the metabolic efficiency of aerobic diazotrophy. This matter has been much studied in the legume symbiosis, in plants inoculated with either Hup^+ or Hup^- strains of rhizobia with conflicting results. Evans *et al.* (1985) estimated that only 61% of all experiments had shown a positive response (higher yields and nitrogen contents) to inoculation with Hup^+ over Hup^- strains.

These data are no doubt much influenced by the complexities of the legume symbiosis. A non-symbiotic diazotroph is, in principle, a physiologically simpler system with which to analyse the metabolic role of hydrogenase in diazotrophy. In the present work we compared the growth of Hup^- mutants of *Azotobacter chroococcum* with the Hup^+ parent strain in batch and continuous cultures under different nutrient limitations.

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METHODS

Growth of bacteria. *Azotobacter chroococcum* MCD-1 is a spontaneous streptomycin and nalidixic acid resistant mutant of a non-gummy variant of *A. chroococcum* NCIMB 8003 (Robson *et al.*, 1984). *A. chroococcum* MCD-105, 119 and 122 are uptake hydrogenase negative (Hup^-) mutants obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Yates & Robson, 1985). They were grown in batch culture on Burk's N-free medium (Newton *et al.*, 1953) with or without NH_4Cl (18 mM). Continuous cultures were grown in 1-litre glass vessels (Baker, 1968) with a working volume of approximately 450 ml under air at 30 °C. Burk's medium was added by a dual-feed system to avoid the use of chelating agents which inhibit hydrogenase synthesis (Partridge & Yates, 1982). The pH was maintained at 7.0 ± 0.05 by means of a pH controller (Uniprobe 706) and O_2 in solution was monitored by an O_2 electrode fitted through a side port. O_2 - and carbon-limited cultures contained 58.4 and 4.5 mM-sucrose respectively; for SO_4^{2-} -limited cultures, sulphates were replaced by chlorides and Na_2SO_4 was added to 50 μM . Nutrient limitations were confirmed by determining residual O_2 , sucrose or sulphate concentrations in the culture fluid and by showing that the yields increased only in response to additional limiting nutrient (Dalton & Postgate, 1969). NH_4^+ -utilizing, non- N_2 -fixing conditions were confirmed by an inability to reduce acetylene.

Yields were determined as dry weights in triplicate.

Batch cultures. Batch cultures of *A. chroococcum* were grown in 250 ml conical flasks containing Burk's medium (100 ml) on a rotary shaker (60 r.p.m.) under air at 30 °C. The flasks were fitted with a side tube to allow growth measurements by OD at 540 nm using a Klett spectrophotometer.

Mixed populations. The percentage of Hup^+ bacteria in mixed populations of the parent strain and a Hup^- mutant was determined by a modification of the 'scrying' technique (Postgate *et al.*, 1982; Haugland *et al.*, 1983). The mixed culture was serially diluted to 2×10^4 cells ml^{-1} and plated on Burk's sucrose agar containing 25% of the normal Ca^{2+} level. The developed colonies were absorbed on to filter paper (Whatman no. 1), soaked in a solution of 50 mM-sodium phosphate buffer, pH 8.0, methylene blue (1 mM), NaF (150 mM), EDTA (4 mM), iodoacetic acid (200 μM) and malonic acid (200 μM) for 20 min and then exposed to H_2 for 20 min. White spots represented Hup^+ colonies.

RESULTS

Continuous cultures of individual populations

Yields of all three Hup^- mutants and the Hup^+ parent strain in N_2 -fixing, carbon-limited continuous cultures (Fig. 1) increased upon increasing the dilution rate from 0.05 to 0.1 h^{-1} , consistent with the report of Dalton & Postgate (1969). The yield of the parent Hup^+ strain remained constant (0.3 mg dry wt ml^{-1}) from $D = 0.1$ to 0.2 h^{-1} and then declined slightly to 0.25 mg dry wt ml^{-1} at $D = 0.27 \text{ h}^{-1}$. At higher dilution rates washing out occurred. The three Hup^- mutants gave similar optimum yields to the wild-type at $D = 0.1 \text{ h}^{-1}$. The steady-state yields decreased sharply at D values above a narrow optimum range which differed among the three mutants and were considerably lower than those of the parent strain at dilution rates above 0.1 h^{-1} . A progressive change from high to low dilution rates increased the steady-state yields to a similar maximum at the same optimum D value (data not shown). Yields under carbon limitation in the presence of NH_4^+ showed no clear differences between parent and mutant strains: yields increased slightly from low D values to an optimum and then declined with increasing dilution rate to D values of 0.35 h^{-1} and the cells washed out.

Yields of O_2 -limited, N_2 -fixing cultures of the Hup^+ parent and MCD-122 declined similarly between D values of 0.05 and 0.2 h^{-1} (data not shown). Yields of SO_4^{2-} -limited cultures of the wild-type and all three mutants were similarly affected across a range of D values from 0.1 to 0.2 h^{-1} although small differences were observed at lower and higher dilution rates (data not shown). This pattern of yields corresponds to the theoretical for the two classes of nutrient limitation.

Batch cultures

Flask cultures of the three Hup^- mutants, when inoculated from exponential phase cultures and shaken under air from the time of inoculation, showed longer division lags than the parent Hup^+ strain, a phenomenon particularly pronounced at higher dilutions in medium containing 10 mM-sucrose (Table 1). Once growth had started, doubling times of all four strains were similar. Similar results were obtained with 20 or 30 mM-sucrose. However, with 58.4 mM-sucrose

Fig. 1. Effect of nutrient limitations on the steady-state yields of parent (*Hup*⁺) and *Hup*⁻ strains of *A. chroococcum* in carbon-limited continuous culture. Filled symbols, N_2 -fixing; open symbols, NH_4^+ -grown. $\blacklozenge$, $\lozenge$, MCD-1 (*Hup*⁺); $\bullet$, $\circ$, MCD-105; $\blacktriangle$, $\triangle$, MCD-119; $\blacksquare$, $\square$, MCD-122. The results are the mean of dry weights in triplicate at each steady-state.

Table 1. *Effect of serial dilution on the growth of A. chroococcum Hup*⁺ *and Hup*⁻ *strains in N*₂-fixing culture

Inocula were made from N_2 -fixing batch cultures shaken under air in exponential phase containing 5×10^8 cells ml^{-1} . Each culture was diluted 10^2 -, 10^3 - and 10^4 -fold and 1 ml was inoculated into N-free Burk's medium (100 ml) containing 10 mM-sucrose in a 250 ml conical flask with a side tube for measuring growth as described in Methods. Growth initiation time (zero time for MCD-1 and 10^{-2} inoculum) was determined by extrapolated growth curves. The data are from a single experiment. The figures in parentheses are the specific growth rates (μ) for each culture.

Strain	Hup	Serial dilution . . .	Relative lag (h)		
			10^{-2}	10^{-3}	10^{-4}
MCD-1	+		0	2 (0.168)	35
MCD-105	-		1	3 (0.170)	42
MCD-119	-		3	34 (0.100)	42
MCD-122	-		2	37 (0.158)	∞

(the original concentration of this carbon source in Burk's medium) the differences in division lags were not observed. Differences in division lags at 10 mM-sucrose were not observed with NH_4^+ under air or under diazotrophic conditions with $N_2 + 5\%$ (v/v) O_2 .

Continuous cultures of mixed populations

The yield kinetics observed in continuous carbon-limited diazotrophic cultures predict that the *Hup*⁺ phenotype will outgrow the *Hup*⁻ type at higher dilution rates. Mixed cultures were therefore initiated by mixing approximately 200 ml of *Hup*⁺ and *Hup*⁻ strains taken from carbon-limited continuous cultures growing at steady-states ($D = 0.1 h^{-1}$). Volumes were adjusted to account for the small differences in cell density. These large volumes were used to establish steady-states rapidly. Samples plated at the time of mixing showed that cultures had approximately equal numbers of viable cells of each type. The behaviour of the mixed populations is illustrated in Fig. 2. In two instances (MCD-122 + MCD-1 and MCD-105 + MCD-1) the *Hup*⁻ mutant tended to out-compete the *Hup*⁺ strain, but at higher dilution rates the *Hup*⁺ phenotype took over in all cases. Mixtures of MCD-105 and MCD-1 differed from the others in that, on three occasions, the mutant dominated completely at lower dilution rates and attempts to establish mixtures failed because the wild-type washed out. The fourth

Fig. 2. Take-over by Hup⁺ phenotype at high dilution rates in carbon-limited continuous cultures. Inocula were mixtures from carbon-limited individual populations at $D = 0.1 \text{ h}^{-1}$. Each steady-state was analysed for the proportions of Hup⁺ and Hup⁻ strains by the 'scrying' technique (see Methods) using four replicates at three different serial dilutions (10^{-4} , 10^{-5} , 10^{-6}). A state was designated steady when the cell density remained constant for five generations, i.e. ($5 \times 1 \text{ D}$) h. However we recognize that the proportion of Hup⁺ to Hup⁻ mutants may have continued to change during this time, as indicated by the MCD-1:MCD-105 mixture sampled twice at $D = 0.26 \text{ h}^{-1}$. Points at $D = 0$ signify the ratio of phenotypes at the initiation of the chemostat culture. ●, MCD-1:MCD-105; ■, MCD-1:MCD-119; ▲, MCD-1:MCD-122.

attempt is shown in Fig. 2: the proportion of MCD-1 declined to 8% at $D = 0.21 \text{ h}^{-1}$ but then increased to 77% by $D = 0.31 \text{ h}^{-1}$. MCD-1 remained preponderant (>90%) when the mixed cultures with MCD-119 or MCD-122 were returned abruptly to a low dilution rate (0.06 h^{-1}) but MCD-105 recovered to such an extent that MCD-1 represented only 0.5% of the population within 11 generations at $D = 0.07 \text{ h}^{-1}$. All three mutants survived at higher D values in mixed cultures with MCD-1 than in pure culture.

Mixed cultures of Hup⁻ mutants and the parent type grown in the presence of NH_4^+ did not show consistent dominance patterns; instead, if one partner became dominant the other washed out. Out of six attempts to establish mixed cultures in Burk's sucrose (58.4 mM) plus NH_4^+ (18 mM) the mutant washed out in four and the wild-type in two cases.

DISCUSSION

The mutants examined here were obtained by MNNG mutagenesis which can induce multiple mutations and were therefore not necessarily isogenic with the parent strain MCD-1. However, they grew as rapidly as MCD-1 under most conditions tested and it is therefore unlikely that they possessed any new metabolic characters affecting growth behaviour other than the lack of hydrogenase activity. They had different secondary phenotypes and were presumed not to be siblings. Their frequency of reversion was less than 5×10^3 , the limit of the 'scrying' technique (Yates & Robson, 1985). For these reasons they were considered adequate for use in this study.

Hydrogenase benefits *A. chroococcum* under N_2 -fixing but not NH_4^+ -utilizing conditions, which suggests that this occurs through recycling H_2 produced by nitrogenase. The benefit was only observed at high growth rates or with sparse populations, which implies that hydrogenase assists the organism either by protecting against inhibition of nitrogenase by O_2 or by providing extra energy through ATP synthesis rather than by protecting nitrogenase against inhibition of N_2 reduction by H_2 . These conditions mimic those which a cell encounters on transfer of batch culture: high $p\text{O}_2$, separation from neighbours, conditions favouring rapid growth. We conclude

that hydrogenase can be crucially important to the initiation of diazotrophic growth or the maintenance of steady-states under stress (carbon limitation) at high growth rates but otherwise has little effect on the steady-state efficiency of diazotrophy in *A. chroococcum*.

The ability of the parent Hup⁺ strain to out-compete the Hup⁻ mutants at high *D* values and, alternatively, to be dominated by the mutants at low *D* values may be because of the different 'coupling' efficiencies to ATP synthesis of H₂- and carbon-dependent respiration under different conditions. Laane *et al.* (1979) showed that, in *Azotobacter vinelandii* particles, H₂ oxidation yielded higher P/O ratios at low O₂ and lower P/O ratios at high O₂ concentrations than did malate oxidation.

If our results can be extended to legume symbiosis then hydrogenase activity would benefit carbon-limited but not O₂-limited bacteroids. The physiological status of bacteroids may change according to the plant's environmental regime of temperature, illumination, hydration, etc. Such a situation would explain the contradictory reports regarding the benefits of hydrogenase in symbiotic systems.

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