The Effect of Nutrient Limitation on the Competition between an H₂-uptake Hydrogenase Positive (Hup⁺) Recombinant Strain of *Azotobacter chroococcum* and the Hup[−] Mutant Parent in Mixed Populations

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Competition studies in continuous culture between a Hup[−] mutant of *Azotobacter chroococcum* and its presumed isogenic Hup⁺ recombinant showed that Hup activity benefited the organism under N₂-fixing, sucrose- or phosphate-limiting conditions but was ineffective or disadvantageous under O₂-, sulphate- or iron-limitation.

INTRODUCTION

The H₂-uptake hydrogenases of aerobic N₂-fixing bacteria are dimeric ($\alpha\beta$), nickel-containing, iron-sulphur, membrane-bound proteins which catalyse the oxidation of H₂ *in vivo* to produce ATP via the respiratory chain. They recycle H₂ produced by nitrogenase *in vivo* and may assist the organisms by: (a) producing ATP, (b) increasing the respiratory protection of nitrogenase and (c) preventing the H₂ from accumulating at the nitrogenase active site and inhibiting N₂ reduction (Dixon, 1972). Whether this recycling of H₂ by the nodule bacteroids formed by rhizobia is a significant contribution to the yield of agronomically important legumes has been researched extensively, but with contradictory results (Evans *et al.*, 1985, 1987; Sorensen & Windaele, 1986). Such contradictions arise partly because hydrogenase activities vary widely and in some legume nodules are insufficiently active to recycle all the H₂ produced by nitrogenase. Contradictory results have also been attributed to the complexity of the symbiotic system, non-standardized experimental designs (Evans *et al.*, 1987) and the dominance of major variables, such as water supply, in the field (Dilworth & Glenn, 1984).

Azotobacter chroococcum is a free-living aerobic nitrogen-fixing organism which possesses an H₂-uptake hydrogenase similar in biochemical, regulatory and genetic aspects to the *Bradyrhizobium japonicum* hydrogenase (Tibelius *et al.*, 1987; Sayavedra-Soto *et al.*, 1988) and, therefore, may be used as a more easily controlled model for the complex legume symbiotic system. Aguilar *et al.* (1985) showed that three different Hup[−] mutants of *A. chroococcum* (Yates & Robson, 1985) produced lower yields than the parent wild-type Hup⁺ organism at high dilution rates in N₂-fixing, sucrose-limited chemostat cultures. These mutants were also out-competed by the wild-type in mixed populations under the same conditions.

It was recognized that the three mutant strains mentioned above were not necessarily isogenic with the wild-type parent organism. The present paper is an extension of this work, involving a study of competition in a mixed population of a Hup[−] mutant of *A. chroococcum* and its Hup⁺ recombinant, two strains believed to be isogenic except for hydrogenase activity.

METHODS

Isolation of a Hup⁺ recombinant from the initial Hup[−] mutant. *A. chroococcum* strain MCD503, a Hup⁺ recombinant of *A. chroococcum* strain MCD103 (Hup[−]) (Yates & Robson, 1985) was obtained by conjugation in a tri-parental mating between *A. chroococcum* strain MCD103 (recipient), *Escherichia coli* strain HB101 : pKHT22 (donor) carrying *A. chroococcum* hup genes on the wide-host-range vector pLAFR1 (K. H. Tibelius & M. G. Yates,

unpublished) and *E. coli* HB101 carrying the helper plasmid pRK2013 (Figurski & Helinski, 1979) as described by Tibelius *et al.* (1987). Transconjugants were initially selected on RM agar (Robson *et al.*, 1984) containing tetracycline, streptomycin and nalidixic acid at 5, 10 and 20 $\mu\text{g ml}^{-1}$ respectively. The frequency of transfer was 3×10^{-3} . The recombinant strain MCD503 was isolated from one of the Hup⁺ transconjugants by selecting for the reappearance of tetracycline sensitivity with retention of the Hup⁺ phenotype. The absence of the vector was confirmed by agarose gel electrophoresis and hybridization experiments using ³²P-labelled pLAFR1 as a probe. These results indicated that the *hup* genes were incorporated into the chromosome by a double recombination event.

The *A. chroococcum* and *E. coli* strains were routinely grown in RM and Luria Bertani (LB) media respectively containing the relevant antibiotics.

Continuous culture. Chemostat cultures of mixed populations of *A. chroococcum* strains MCD103 and MCD503 were grown in a modified Burk's N-free medium containing sucrose (58.4 mM; Partridge & Yates, 1982). Fixed nitrogen was added as ammonium acetate (15 mM) when required. The chemostats were inoculated either directly from similar continuous cultures or from batch-grown cultures harvested during exponential growth and washed and resuspended in the substrate-limiting medium. Each culture was inoculated to a cell density of $2-3 \times 10^8$ to ensure that the initial steady state was achieved in the minimum possible time.

Determining the percentage of the Hup⁺ strain in a mixed population. (a) *Scrying.* This is a modification of the methods described by Postgate *et al.* (1982) and by Hoagland *et al.* (1983). Bacteria were plated on Burk's sucrose agar to give a final number of no more than 150 colonies per plate. When the colonies had grown (after 2 d) they were counted and lifted by filter paper (Whatman no. 1) and then stained by dipping the filter paper in a solution of methylene blue (1 mM), NaF (150 mM), EDTA (4 mM), iodoacetic acid (200 μM) and sodium malonate (200 μM) in 50 mM-sodium phosphate buffer, pH 8.0, in a Petri dish lid for 10 min. After removing from the stain the filter papers were exposed to H₂ in a scrying box (a perspex box with a water-sealed lid containing access and exit ports for H₂). White colonies within 30 min denoted the Hup⁺ phenotype; Hup⁻ colonies appeared blue-black.

(b) *Tritium uptake.* Bacterial colonies were transferred individually to Burk's-sucrose-agar-filled U-cavity plates containing 96 cavities per plate (Sterilin) and, after 48 h growth at 30 °C, were exposed to 10% (v/v) H₂ containing 200 MBq tritium in air in a 3.5 l jar for 20 min at room temperature. The agar blocks were transferred by toothpicks to 3 ml scintillation vials containing 20% (v/v) LUMAX (Lumac BV, Holland) as the scintillant and the radioactivity incorporated was measured in a Beckman 1750 scintillation counter. Hup⁻ colonies scored less than 100 c.p.m. above background (200–300 c.p.m.); active (Hup⁺) colonies scored 2000–20000 c.p.m. (Yates & Robson, 1985).

RESULTS

Sucrose-limited cultures. Mixed populations of sucrose-limited N₂-fixing MCD103 (Hup⁻) and MCD503 (Hup⁺) were dominated by the Hup⁺ recombinant strain. In three experiments with starting ratios of 35:65, 7:93 and 1:99 of MCD503:MCD103 in the mixed population the Hup⁺ strain became dominant within the wash-out time (Fig. 1a). Moreover, the increase in the percentage of the Hup⁺ phenotype corresponded to a decrease in H₂ evolution by the culture and a concomitant increase in Hup activity (Fig. 1b). Since NH₄⁺-grown cultures, in which nitrogenase is repressed and which therefore do not produce H₂, showed no clear dominance pattern by either strain (data not shown) it is evident that H₂ recycling by Hup is sufficiently important to guarantee dominance of the Hup⁺ strain during N₂-fixation.

Phosphate limitation. Two experiments with phosphate-limited N₂-fixing cultures yielded similar results: in both cases MCD503 (Hup⁺) dominated and the Hup⁻ mutant disappeared (was washed out) within 11 doubling times, approximately half as fast as the dominance rate under carbon limitation (Fig. 1c).

Oxygen limitation. In four separate experiments, oxygen-limited mixed populations of MCD103 and MCD503 were dominated by the Hup⁻ mutant. In two experiments, with starting

Fig. 1. Phenotypic distribution in a mixed population of *A. chroococcum* strains MCD503 (Hup⁺) and MCD103 (Hup⁻) grown in N₂-fixing continuous cultures under air at 30 °C. Each point is a mean of 12 determinations (12 plates as described in Methods). (a) Carbon-limited. The discontinuous line represents the increase in Hup⁺ at a constant dilution rate (5–6 generations). (b) Carbon-limited. Concomitant appearance of H₂-uptake hydrogenase activity (○) and decrease in H₂ evolution by the culture (●) in a continuous culture containing an initial ratio of *A. chroococcum* MCD503:MCD103 of 1:99 grown at $D = 0.1 \text{ h}^{-1}$. The culture was ~100% Hup⁺ within 5 d. (c) Phosphate-limited. (d) O₂-limited. (e) Sulphate-limited. (f) Iron-limited.

ratios of 22 : 78 and 28 : 72 of MCD503 : MCD103, the Hup⁺ strain was washed out. However, in two other experiments, initially 39 and 78% MCD503 (Hup⁺), the proportion of Hup⁺ dropped more slowly than the washout rate and then stabilized at a low value of <20% Hup⁺ (Fig. 1 d).

Iron and sulphate limitation. A very slow decline to zero of the Hup⁺ strain occurred over a period of 50 doubling times under sulphate limitation and a rather rapid decline, again to 0% Hup⁺, occurred under iron limitation (Fig. 1 e, f).

Cross-feeding experiments. The possibility that any of the above effects is due to cross-feeding of a required activator (excreted by one strain and taken up by the other) for the synthesis or loss of hydrogenase, rather than the effect of competition, is most unlikely since in the 'scrying' test the Hup⁺ and Hup⁻ colonies are randomly distributed. In addition, if the Hup⁺ or Hup⁻ phenotype depended on cross-feeding then one strain would always appear to dominate, whereas our experiments showed that both the strain domination and the stability of mixed populations depended on the experimental conditions. Nevertheless, a cross-feeding experiment in which Hup⁺ and Hup⁻ strains were streaked alternately as the sides of squares, leaving 0.5 cm gaps between the ends of each streak, revealed no cross-feeding on sucrose- or phosphate-limited media.

NH₄⁺-grown conditions. In two experiments with NH₄⁺-grown sucrose-limited cultures, the percentage of MCD503 changed from 40% to 49% in 18 generations and from 44% to 38% in 20 generations respectively. An NH₄⁺-grown sulphate-limited culture dropped from 62% to 49% MCD503 in 14 generations whereas O₂-limited NH₄⁺-dependent cells dropped from 84% to 74% MCD503 in 18 generations. Thus neither O₂- nor sucrose-limited NH₄⁺-grown cultures showed the dominance patterns exhibited by the N₂-grown cells under these limitations: under sulphate-limitation the slow decline was apparently similar to that with N₂ as nitrogen source despite the fact that nitrogenase (a major source of sulphur utilization) was not synthesized.

Recycling of H₂. The Hup⁺ strain absorbs H₂ produced by the nitrogenase of the Hup⁻ strain. In a mixed population the H₂ was decreased by 10% at 7% Hup⁺ and by >90% at 60% Hup⁺ when compared with a Hup⁻ control.

DISCUSSION

There seems no doubt from the above results that hydrogenase activity can be either beneficial or disadvantageous to *Azotobacter*, depending on the growth conditions: carbon- or phosphate-limitations both encourage the Hup⁺ strain to dominate, whilst O₂-, sulphate- or iron-limitations encourage the Hup⁻ strain.

The occurrence of this dominance pattern under N₂-fixing conditions but not with NH₄⁺, when nitrogenase and hence H₂ production are absent, strongly suggests that the recycling of this H₂ produced by nitrogenase is the underlying reason, rather than the presence or otherwise of synthesized hydrogenase protein as a membrane constituent. The removal of H₂ from the environment of nitrogenase may contribute to the cause of dominance, but it is not likely to be a major factor otherwise Hup⁺ strains would surely dominate under all conditions.

Under sucrose-limitation H₂ recycling is therefore most likely to benefit the organism by providing additional electrons for ATP synthesis and also augmenting respiratory protection of nitrogenase. This recycling provides only a small percentage (<3%) of the total electron flow through the respiratory chain (Walker *et al.*, 1981). Nevertheless it appears to be sufficient to give the Hup⁺ strain a significant advantage over the Hup⁻ strain under these conditions. Uptake of the H₂ produced by the Hup⁻ strain should be an additional benefit to the Hup⁺ strain; however, this source of energy becomes relatively less significant as the percentage of Hup⁺ approaches 100. This may account for the apparent slowing down of the domination rate at high percentages of the Hup⁺ strain (Fig. 1 a).

Under O₂-limitation, H₂ recycling competes with carbon-substrate-dependent respiration and, if the former process is less well coupled to energy transduction than the latter, then Hup activity would be disadvantageous. Drevon & Salzac (1984) and Sorenson & Wyndaele (1986) both used this argument to explain how hydrogenase activity might be disadvantageous in soybean and pea nodules respectively. Laane *et al.* (1979) measured P/O ratios in *Azotobacter*

vinelandii particles and found that H₂ and NADH were equally efficient and calculated that the oxidation of both substrates supported three energy transducing sites. The results in two of the four experiments reported here when stable mixed populations were obtained under O₂ limitation are consistent with these findings, but the initial decrease in the Hup⁺ population in these two experiments and the complete loss of the Hup⁺ strain in the other two experiments are more consistent with the hypothesis of Drevon & Salzac (1984). A possible explanation for this apparent inconsistency is that the cultural conditions select mutant strains that are more efficient in H₂ respiration in such long-term experiments.

In phosphate-limited *A. chroococcum* cultures, where respiration rates are low, both the nitrogenase activity and the enzyme itself are particularly sensitive to O₂ (Dalton & Postgate, 1969; Lees & Postgate, 1973). H₂ recycling, including the uptake of H₂ released by nitrogenase activity in the Hup⁻ population, would be of advantage to the Hup⁺ population by aiding respiratory protection of nitrogenase if, as claimed by Ackrell *et al.* (1972), NADH or tricarboxylic acid cycle intermediate oxidation is the rate-limiting step in respiration. Alternatively, H₂ respiration may proceed via a physically distinct respiratory chain as proposed for *Azospirillum brasilense* (Pedrosa *et al.*, 1982).

Since hydrogenases are iron-sulphur proteins, hydrogenase synthesis would compete for these limiting nutrients with the synthesis of more vital iron-sulphur proteins such as nitrogenase and hence hydrogenase synthesis might be a disadvantage under sulphate- or iron-limiting conditions. Both limitations were disadvantageous to the Hup⁺ strain but at very different rates, the loss of the Hup⁺ strain under iron limitation being much faster. Moreover the decline rate of the Hup⁺ strain under sulphate-limitation was similar under N₂- or NH₄⁺-grown conditions; presumably factors other than nitrogenase synthesis determine this behaviour.

These results show that N₂-fixing *A. chroococcum* benefits by recycling the H₂ produced by nitrogenase under some but not all metabolic conditions. If this system is an acceptable model for N₂-fixing legume nodule bacteroids then the effect on plant yield of recycling H₂ produced by nitrogenase will depend upon the metabolic limitation within the bacteroid.

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