SHORT COMMUNICATION

A Method for Screening for Hydrogenase Negative Mutants of *Azotobacter chroococcum*

By J. R. POSTGATE,¹* C. D. PATRICIA PARTRIDGE,¹
R. L. ROBSON,¹ F. B. SIMPSON² AND M. G. YATES¹

¹ ARC Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, U.K.

² Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

(Received 14 December 1981)

Slow reduction of methylene blue under H₂, after treatment with NaF + EDTA, distinguished Hup[−] (uptake hydrogenase negative) from Hup⁺ colonies of *Azotobacter chroococcum* on plates.

INTRODUCTION

The isolation of Hup[−] mutants of *Rhizobium japonicum* defective in uptake hydrogenase (Maier *et al.*, 1978*a*) was greatly facilitated by the discovery of an associated phenotype, slow reduction of tetrazolium in tetrazolium-agar plates, which in its turn was discovered because naturally occurring Hup[−] strains of *R. japonicum* were available (Maier *et al.*, 1978*b*). Hup[−] mutants of *R. japonicum* are being used to evaluate the contribution of the uptake hydrogenase to the diazotrophic economy of the soya bean symbiosis (Evans *et al.*, 1980; Lepo *et al.*, 1981). The uptake hydrogenase of *Azotobacter chroococcum*, and by implication of other azotobacters, may contribute to its relatively high efficiency as a diazotroph, but quantitative assessment of its importance requires the preparation of isogenic Hup[−] mutants and their physiological comparison with the wild type or Hup⁺ revertants. No simple screening method was available for Hup[−] mutants of any kind, nor was any associated phenotype known comparable to the tetrazolium reaction of *R. japonicum*. However, mutagenesis of *A. chroococcum* with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) followed by screening of individual colonies for ³H₂ uptake yielded a number of Hup[−] mutants; this success has enabled us to set up mixtures of Hup[−] and Hup⁺ populations and to test various methods of screening colonies, smears or streaks of *A. chroococcum* for their Hup phenotype. Details of the mutants will be reported elsewhere; here we report a rapid procedure which is currently being used successfully by ourselves and which ought to be applicable to other *Azotobacteraceae*.

METHODS

Derivatives of *Azotobacter chroococcum* NCIB 8003 used in this work are listed in Table 1. Cultures for routine use were grown at 28 or 32 °C on BSNA agar, i.e. the sucrose medium of D. Burk (Newton *et al.*, 1953) modified to include 25% (w/v) nutrient agar; reserve stocks were held frozen in glycerol (20%, v/v) at −20 °C. Where appropriate, nalidixic acid was included at 20 µg ml^{−1}, streptomycin at 10 µg ml^{−1} and rifampicin at 50 µg ml^{−1}; all sub-strains retained the antibiotic resistance of their parents. Cultures in liquid media were grown in nominally 25 ml conical flasks containing 5 ml Burk's medium, shaken at 30 °C. Dilutions were made in Burk's medium with no carbon source, a diluent which avoids dilution shock (Billson *et al.*, 1970). The procedure finally used to distinguish Hup⁺ and Hup[−] phenotypes is described in the Results section.

Table 1. *Derivatives of Azotobacter chroococcum* NCIB 8003 used in this work

Strain	Phenotype
MCB1	Spontaneous, non-gummy variant of NCIB 8003. Does not 'clump' in liquid culture
MCC1 <i>nal str</i>	Derivative of MCB1 which developed successive spontaneous resistances to nalidixic acid and low level streptomycin
MCD1 <i>nal str</i>	Derivative of MCC1 cured of three of its five natural plasmids
MCD2 <i>nal str rif</i>	Spontaneous rifampicin-resistant mutant of MCD1

Colonies on agar plates were examined for dye reduction in a stream of H_2 . In early tests a plastic bag was used to expose the plates to H_2 but this was optically unsatisfactory and a 'screying box' for viewing the plates under H_2 was devised. This was essentially a shallow expanded-polystyrene box ($5 \times 19 \times 19$ cm) with the lid and one side removed. A 14 cm circular plastic window was taped with transparent tape and transparent polythene sheet over the top; attached to the side was a transparent polythene sleeve of reasonable length such that the plate could be introduced by hand, with the minimum of air, yet the sleeve could then be folded and held tight by a strong paper clip. A stream of H_2 (from a cylinder) passed across the plate through facing walls of the box; a disc of filter paper provided a white background for observing the stained colonies. The screying box was used at room temperature in a fume cupboard with care to avoid H_2 explosion.

RESULTS

To test various screening procedures, portions of 18 to 24 h flask cultures of various mutants were mixed with an equal volume of a culture of strain MCD1 (used as Hup⁺ wild type), diluted 10^{-5} and 0.1 ml portions spread on agar plates. Viable populations were about 10^8 ml⁻¹. A variant of the tetrazolium procedure which is effective with *R. japonicum* was sought. Tetrazolium at 5 or 10 μ g ml⁻¹ in BSNA delayed growth of colonies and decreased the viable count; resistance to this inhibition was not found among survivors. Tetrazolium at 2 μ g ml⁻¹ was not inhibitory and gave rise to light pink colonies, but no differences were seen with the mixed populations. This approach was therefore abandoned.

We then sought a procedure based on dye reduction involving maximum access of dye to hydrogenase combined with inhibition of other dyestuff reductases. Preliminary experiments with suspensions of *A. chroococcum* and *A. vinelandii* strain OP had shown that decolorization of methylene blue (MB; 0.1 mM) under H_2 was accelerated 7 to 10-fold in the presence of 1 M-NaF. The concentration of NaF could be lowered to 100 mM if EDTA (1 to 10 mM) were also present. Acceleration of the H_2 -MB reaction occurred both with culture samples and with cells washed and resuspended in the medium free of carbon source in the pH range 6.4 to 7.0. In a representative experiment, whole culture or washed cell samples from a 48 h culture of *A. chroococcum* in liquid Burk's medium took more than 20 min to decolorize 0.1 mM-MB at 30 °C under H_2 or N_2 ; when supplemented with NaF (100 mM) + EDTA (10 mM), the samples decolorized MB under H_2 in 3 to 4 min compared with more than 20 min under N_2 .

We therefore tested a procedure in which plates carrying colonies were stained with MB + EDTA + NaF at the optimal pH for hydrogenase activity (pH 8.0) and observed in the screying box under a stream of H_2 . Colonies decolorizing rapidly (to a yellow colour) were presumptive Hup⁺; colonies decolorizing slowly or not at all were presumptive Hup⁻.

The following procedure has proved very successful with 18 Hup⁻ mutants of strain MCD1, most of which are not siblings. A 9 cm Petri dish culture is incubated until all colonies are 0.5 to 2 mm diameter; this takes 3 to 5 d at 32 °C. Then 10 to 15 ml of a solution containing 0.2 mM-MB + 150 mM-NaF + 4 mM-EDTA in 50 mM-sodium phosphate,

pH 8.0, is gently poured on the plate so as not to disturb the colonies. After 2 to 5 min staining, according to the colony density, the plate is gently tilted, the MB solution removed gently with a pipette and drained at the edge with filter paper. The uniformly stained plate is introduced into the scrying box with a minimum of air. Within 60 to 90 s presumptive Hup⁺ colonies decolorize; within 4 to 5 min some of the Hup⁻ colonies begin to change. The plate may be removed, exposed to air to re-oxidize the colonies and the procedure repeated.

The above procedure worked excellently with smears, patches or 'streaked-out' populations of *A. chroococcum*; with smears or patches the plates need only be incubated for 24 h before screening. Uniformity of staining with MB was very important. Colonies of strain MCB1 and its derivatives sometimes revert to the gummy phenotype of NCIB 8003; such colonies stained badly and interfered with the test. All 18 available Hup⁻ mutants conformed to pattern; two were slow at taking up MB and required the longer staining time to give unambiguous results. Comparable procedures using tetrazolium or benzyl viologen in place of MB were not successful.

For isolation of presumptive Hup⁺ or Hup⁻ types we originally made replicas of the plates to be screened, but we found this to be unnecessary. Despite the toxicity of MB, NaF and EDTA to *A. chroococcum*, colonies after screening included sufficient viable cells for the clone to be recovered with a loop or sterile toothpick. We have used this fact to prove the method: in a definitive experiment, the rifampicin-resistant derivative MCD2 was used as the wild type and mixed with four distinct Hup⁻ mutants. After screening, the positions of four Hup⁺ and four Hup⁻ colonies were marked on a sketch map of each plate and the colonies were 'patched' with sterile toothpicks on BSNA and BSNA + 50 µg rifampicin ml⁻¹. After incubation for 36 h the BSNA plate was screened with MB and compared with the BSNA + rifampicin plate. Correlation of Hup⁺ with rapid MB reduction by the patch and resistance to rifampicin was absolute in most instances with but two errors of judgement made in the 32 isolations: once a Hup⁺ Rif^r strain was picked as Hup⁻; once a Hup⁻ Rif^r strain was picked as Hup⁺.

The model mixtures tested so far are not typical of the practical situation because the populations were weighted heavily towards Hup⁻ mutants. The method was therefore used to isolate another Hup⁻ mutant. NTG-mutagenized cells were placed on 10 BSNA plates at approximately 200 colonies per plate. After 3 and 4 d at 32 °C, five plates were screened and approximately 30 potential Hup⁻ colonies were patched on to BSNA. Of these, only 50% survived and some developed a gummy phenotype which prevented methylene blue from staining. After further screening with MB + NaF + EDTA, and measurement of hydrogenase activity in batch culture, one clone with very low (but not zero) hydrogenase activity was identified.

Maier *et al.* (1978*a*) used a procedure involving reduction of phenazine methosulphate (PMS) followed by photo-oxygenation to enrich populations with Hup⁻ mutants of *R. japonicum*. In two tests we have obtained only marginal (10 to 30%) enrichment of Hup⁻ mutants in artificially mixed populations and we conclude that the PMS procedure is not useful with *A. chroococcum*.

DISCUSSION

Screening for Hup⁻ phenotype in a species which does not have naturally Hup⁻ strains presents a difficult problem but the unexpectedly frequent occurrence of NTG-induced Hup⁻ mutants in our strain has enabled us to devise a reasonably rapid screening procedure which, while it is not quantitatively absolute, enables us to recognize the Hup⁻ phenotype.

Since Hup⁻ mutants were prepared with NTG some may carry second mutations, and we already know that two mutants stain poorly with MB while another produces much acid. All such types have been tested with our MB procedure and such secondary phenotypes do not alter the efficiency of the method. The fact that the colonies retain viable bacteria

after screening, thus allowing recovery of the uncontaminated colony phenotype despite the plate being flooded with liquid, is surprising, if convenient. We suggest that organisms at the surface of the colony, or in free suspension, are indeed killed but that the short-term exposures used do not allow sufficient penetration of the colony to occur for those inside to be effectively sterilized.

J. R. Postgate and F. B. Simpson thank Profesor H. J. Evans for the hospitality of his laboratory at Oregon State University, where the effects of NaF and EDTA were discovered.

REFERENCES

- BILLSON, S., WILLIAMS, K. & POSTGATE, J. R. (1970). A note on the effect of diluents on the determination of viable numbers of Azotobacteriaceae. *Journal of Applied Bacteriology* **33**, 270–273.
- EVANS, H. J., EMERICH, D. W., RUIZ-ARGUESO, T., MAIER, R. J. & ALBRECHT, S. L. (1980). Hydrogen metabolism in the legume–*Rhizobium* symbiosis. In *Nitrogen Fixation*, vol. 2, pp. 69–86. Edited by W. E. Newton & W. H. Orme-Johnson. Baltimore: University Park Press.
- LEPO, J. E., HICKOK, R. E., CANTRELL, M. A., RUSSELL, S. A. & EVANS, H. J. (1981). Reversible hydrogen uptake-deficient mutants of *Rhizobium japonicum*. *Journal of Bacteriology* **146**, 614–620.
- MAIER, R. J., POSTGATE, J. R. & EVANS, H. J. (1978*a*). *Rhizobium japonicum* mutants unable to use hydrogen. *Nature, London* **276**, 494–495.
- MAIER, R. J., CAMPBELL, N. E. R., HANUS, F. J., SIMPSON, F. B., RUSSELL, S. A. & EVANS, H. J. (1978*b*). Expression of hydrogenase activity in free-living *Rhizobium japonicum*. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 3258–3262.
- NEWTON, J. W., WILSON, P. W. & BURRIS, R. H. (1953). Direct determination of ammonia as an intermediate in nitrogen fixation by *Azotobacter*. *Journal of Biological Chemistry* **204**, 445–451.