Mutants of *Azotobacter chroococcum* Defective in Hydrogenase Activity

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Three classes of Hup⁻ mutants of *Azotobacter chroococcum* were obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis and screening by H³H uptake: (1) those with no discernible H₂-uptake or H₂-evolving activity, (2) those showing no uptake but some H₂ production and (3) those leaky for both activities. One mutant strain, MCD-124, expressed hydrogenase activity similar to the solubilized wild-type enzyme in O₂ sensitivity, sedimenting behaviour and pH optimum. All the other mutants were probably mutated in the hydrogenase structural or processing (methylene blue) genes rather than in genes for hydrogenase-linked respiratory proteins. Four mutants chosen from the first category were complemented for hydrogenase activity by conjugation with *Escherichia coli* carrying plasmid pHU1 containing *Rhizobium japonicum* hydrogenase genes. A pHU1 transconjugant of strain MCD-124, on the other hand, did not express any additional hydrogenase activity.

INTRODUCTION

Three main types of hydrogenases exist in micro-organisms: (1) the reversible hydrogenase which acts as an electron sink in anaerobes; (2) the ATP-dependent H₂ production by nitrogenase; and (3) the H₂-uptake hydrogenase activities (Hup) of H₂-oxidizing and N₂-fixing bacteria (see Yates & Walker, 1980). In the latter the uptake hydrogenase can recycle H₂ produced by nitrogenase through H₂-dependent respiration to produce ATP.

All hydrogenases are iron sulphur proteins (Adams *et al.*, 1981) and uptake hydrogenases are usually membrane-bound. The protein from *Azotobacter chroococcum* has been partially purified; it was estimated to have a molecular weight of ~60000 and exhibited a very high (>100) H₂ uptake to H₂-evolving activity ratio. It was also O₂-sensitive when solubilized (Van der Werf & Yates, 1978). In these respects it resembles the uptake hydrogenase of *Rhizobium japonicum* bacteroids (Arp & Burris, 1979).

Expression of hydrogenase activity in *A. chroococcum* is complex and is affected by O₂, H₂ and carbon and nitrogen sources (Partridge *et al.*, 1980; Lee and Wilson, 1943); it also requires nickel (Partridge & Yates, 1982). In most of these respects it again resembles hydrogenase from *R. japonicum* or *R. japonicum* bacteroids (Maier *et al.*, 1978, 1979; Klucas *et al.*, 1983).

In an attempt to understand the role and possible benefits of hydrogenase in N₂-fixing aerobes we have isolated Hup⁻ mutants of *A. chroococcum*. The present paper describes preliminary biochemical and genetic characterization of these mutants.

METHODS

Organisms and strains. Bacteria are described in Table 1. *A. chroococcum* MCD-1 is a derivative of a non-gummy variant (MCA-1) of *A. chroococcum* NCIMB 8033 (Robson *et al.*, 1984). *A. chroococcum* strains were grown routinely on Burk's N-free sucrose medium (Newton *et al.*, 1953) or on rich medium (RM) (Robson *et al.*, 1984) at 30 °C under air. Tetracycline (5 µg ml⁻¹) was added as required. *Escherichia coli* HB101 was grown in Luria-Bertani (LB) medium containing either tetracycline (10 µg ml⁻¹) or kanamycin (15 µg ml⁻¹) or both.

Abbreviation: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Table 1. *Bacterial strains and plasmids*

Strain	Genotype or phenotype	Reference or source
<i>Azotobacter chroococcum</i>		
MCA-1		
MCD-1	Sm ^r Nal ^r	Robson <i>et al.</i> (1984)
<i>Escherichia coli</i>		
HB101	F ⁻ <i>hsd-20</i> <i>r_B</i> <i>m_B</i> <i>recA13</i> <i>ara-14</i> <i>proA2</i> <i>lacY galK2 rpsL20 xyl-5 mtl-1 supE44</i>	Boyer & Roullard-Dussoix (1969)
Plasmids		
pRK2013	Km ^r Tra ⁺ . Helper plasmid	Figurski & Helinski (1979)
pHU1	Tc ^r Mob ⁺	Cantrell <i>et al.</i> (1983)

Plasmid pHU1 (Table 1), which carries some determinants for the uptake hydrogenase from *R. japonicum* in the wide host range vector plasmid pLAFR1 (Cantrell *et al.*, 1983), was a gift from Dr M. A. Cantrell and Professor H. J. Evans of Oregon State University, Corvallis, USA.

Mutagenesis. *A. chroococcum* MCD1, grown to approximately 10^8 organisms ml⁻¹, was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG, 20 µg ml⁻¹) for 10 min to a survival of 10 to 50%, collected on sterile cellulose nitrate filter discs (Sartorius), washed with PEM buffer (Robson *et al.*, 1984) to remove MNNG and out-grown on the discs on Burk's sucrose nutrient agar (4:1, v/v) containing 0.1% yeast extract (BSYNA) for 24 h. Single colonies obtained on BSYNA 48 h after serial dilution of bacterial lawns were transferred to Burk's sucrose agar (approx. 0.5 ml) in Sterilin U-cavity plates and grown for 2 d.

Selection of *Hup*⁻ mutants. Colonies grown on U-cavity plates were exposed for 10 min to H³H + H₂ (10%, v/v, containing 25.9 MBq H³H) in air (3.4 litre) at room temperature. Tritiated protons released into the agar blocks were counted in a Beckman scintillation counter with 20% LUMAX in toluene as the scintillant. The vials were shaken and equilibrated in the dark for 1 h before counting.

Hydrogenase assay. H₂-uptake activity was measured anaerobically with methylene blue as the electron acceptor essentially as described by Pedrosa *et al.* (1982), with either 0.1 M-sodium phosphate buffer, pH 8.0, or 0.1 M-citric acid/sodium phosphate buffer, pH 5.5. Other electron acceptors, except O₂, were tested at 8.2 mM at the pH optimum of the particulate (pH 8.0) or solubilized (pH 5.5) enzyme (Van der Werf & Yates, 1978). O₂-dependent H³H uptake was measured in 1 ml culture portions (pH 6.5) in 8 ml serum bottles sealed with a suba seal under air plus 10% H³H + H₂ at 30 °C.

H₂ evolved by hydrogenase was measured in crude extracts under anaerobic conditions with sodium dithionite (20 mM) and methyl viologen (2 mM) in 0.1 M-sodium phosphate buffer, pH 6.5. The H₂ produced was measured by gas chromatography on a molecular sieve MS 5A column with a thermal conductivity detector (Pye Unicam 204).

Crude extracts for either H₂-uptake or H₂-evolution measurements were obtained by resuspending bacteria (5 ml; 2 mg protein ml⁻¹) in 0.1 M-sodium phosphate buffer, pH 8.0, and disrupting them in an Aminco pressure cell from 26 MPa. Extracts were stored in liquid nitrogen.

Protein was measured by the Folin reagent with bovine serum albumin as the standard.

DNA analysis by gel electrophoresis. Small scale analysis of plasmids from *E. coli* and *A. chroococcum* was by alkaline lysis followed by electrophoresis on horizontal gels of 0.7% (w/v) agarose at 4 °C and 80 V for 16 h in TAE buffer (Robson *et al.*, 1984).

Genetic techniques. *E. coli* HB101(pHU1) and HB101(pRK2013) were mated overnight at 30 °C on filter discs on RM medium agar containing glucose (0.5%, w/v) and NH₄Cl (5 mM). Transconjugants [*E. coli* HB101(pHU1, pRK2013)] carrying both plasmids were isolated and maintained on LB containing tetracycline (10 µg ml⁻¹) and kanamycin (25 µg ml⁻¹).

All salts were purchased from BDH and all biochemicals, electron acceptors, antibiotics and MNNG from Sigma. ⁶³Ni²⁺ and H³H were obtained from Amersham.

RESULTS

Table 2 shows the hydrogenase activities of the parent, MCD-1 (*Hup*⁺), and strains defective in hydrogenase activity (*Hup*⁻) obtained after MNNG mutagenesis. Originally, 24 mutants were obtained from 1850 colonies; a frequency of 1.3×10^{-2} . Eight of these failed to survive after sub-culturing. Of the remaining 16, the frequency of reversion was less than 5×10^{-4} , the limit of the screening technique for *Hup*⁺ revertants.

The surviving mutants can be divided into three subgroups on the basis of relative activities (Table 2): (1) those with very low activities in agar blocks (<200 c.p.m.) or in O₂- or methylene blue-dependent H₂-uptake assays and with no detectable H₂-evolving activity; (2) those with

Table 2. *Hydrogenase activities of parent and Hup⁻ mutants of A. chroococcum*

Assay procedures are described in Methods. All activities of the mutants represent the highest values obtained from at least two assays.

Strain	Hup	Hydrogenase activity (H ³ H + H ₂ uptake)			Percentage H ₂ -evolving activity‡	Secondary phenotype
		'U-cavity' agar blocks (c.p.m.)*	O ₂ -dependent†	Methylene blue- dependent†		
MCD-1	+	35907	228	855	100	
MCD-105	—	185	0.3	0.6	0	Fast growth
MCD-108	—	133	0.1	0.4	0	
MCD-110	—	178	0.15	0	0	
MCD-112	—	173	0.1	0.7	0	Slow growth
MCD-115	—	211	0	0.2	0	
MCD-116	—	288	0	0.1	0	
MCD-117	—	175	0.1	0.4	0	
MCD-119	—	118	0	0	0	Acid ⁺
MCD-122	—	286	0.2	0	0	
MCD-102	—§	209	0.7	0.4	4	
MCD-103	—§	142	0.1	0	3	
MCD-104	—§	197	0.2	0.7	2	
MCD-106	—§	153	0.1	0.2	3	
MCD-113	—	543	6.1	3.7	2	
MCD-118	—	300	1.7	2.9	7	Mb uptake ⁻ Acid ⁻
MCD-124	—	1056	3.0	0.2	40	
Blank		64	—	—	0	

* The blank value indicates H³H + H₂ absorption by bacteria-free agar.

† Cultures were grown to 3×10^8 cells ml⁻¹. Blank assay values (without bacteria) were subtracted in each case.

Methylene blue-dependent activities were measured at pH 8.0.

‡ 100% H₂-evolving activity was 3.5 ± 0.5 nmol (mg protein)⁻¹ min⁻¹.

§ H₂-uptake activity is zero but there is some H₂-evolving activity.

|| Both H₂-uptake and H₂-evolving activities are leaky.

equally low H₂-uptake activities but discernible H₂ evolution; and (3) those with significant H₂-uptake and H₂-evolving activities. Because of the high sensitivity of the H³H-uptake assay it was difficult to decide whether low positive H³H-uptake values reflected low hydrogenase activity or non-specific absorption by bacteria. Thus emphasis was placed on failure to observe H₂-evolving capacity when designating a Hup⁻ strain. Mutant MCD-124 contained a soluble hydrogenase with optimal methylene blue-dependent H₂-uptake activity at pH 5.5. This was usually <10% of the parent Hup⁺ strain but it varied considerably and in one culture reached 60% of the wild-type activity.

Secondary phenotypes were observed for some of the mutants. MCD-105 apparently grew more rapidly than the parent strain on Burk's sucrose agar (colonies were more easily discerned after 24 h). MCD-112 grew slowly on agar (5 to 6 d for colonies to become easily visible compared with 2 d for MCD-1) or in liquid culture. Mutant MCD-119 produced more acid than MCD-1 in N-free Burk's medium; batch cultures of *A. chroococcum* progressively acidify during growth (pH 7.4 to pH 6.3 in 2 d), while MCD-119 cultures were usually pH 6.1 at this stage. Under similar circumstances cultures of mutant MCD-124, which produced less acid than the parent strain, were pH 6.5 to 6.6. Mutant MCD-118 apparently failed to absorb methylene blue; methylene blue-dependent hydrogenase activity was 15% of the parent in crude extracts but almost negligible in whole cells.

All the mutants obtained were defective in both O₂- and methylene blue-dependent hydrogenase activity, indicating that the mutations were probably in the hydrogenase genes rather than any other gene coding for proteins involved in a hydrogenase-linked respiratory chain. A possible exception, mutant MCD-124, is discussed below.

Table 3. *Reactions of hydrogenases from A. chroococcum with electron acceptors*

Assay conditions are described in Methods. Crude supernatants from Burk's sucrose grown cells were used (mg protein per assay: MCD-1, 0.22; MCD-124, 0.24). The electron acceptors were used at a final concentration of 8.2 mM. Methyl viologen, phenazine methosulphate and brilliant cresyl blue were dissolved in water, the others in dimethyl sulphoxide (DMSO).

Electron acceptor	Redox potential, E_0 (mV)	Percentage hydrogenase activity*		
		MCD-1 at pH 8.0	MCD-1 at pH 5.5	MCD-124 at pH 5.5
Methyl viologen	-339	ND	17	11
NAD	-320	ND	16	8
Anthraquinone sulphonic acid	-225	6	21	51
Indigo carmine	-125	6	20	35
Tetrazolium salt	-80	7	11	8
2,5-Hydroxybenzoquinone	-60	10	4	6
Methylene blue	+11	100	100	100
Methylene blue + DMSO	+11	101	93	62 ± 15
Janus green	+41	12	63	10
Brilliant cresyl blue	+48	168	75	18
Thionine	+64	73	66	33
Phenazine-methosulphate	+80	27	46	47
2,4-Dimethylbenzoquinone	+180	51	52	13

* Values are percentages of the hydrogenase activity with methylene blue in water as the electron acceptor. All values were single values obtained with the same crude protein extract. A second experiment with a different protein extract gave similar results (<10% error for all activities above 25% excepting the DMSO effect on MCD-124 hydrogenase). The 100% activity rates in nmol (mg protein)⁻¹ min⁻¹ for each sample were: MCD-1 at pH 8.0, 330; MCD-1 at pH 5.5, 159; MCD-124 at pH 5.5, 109. MCD-124 hydrogenase activity was not tested with the electron acceptors at pH 8.0 because the 100% activity with methylene blue was too low (~50 d.p.m. above background) for comparative accuracy.

ND, Not determined.

All the Hup⁻ mutants were Nif⁺; they all grew normally on N-free Burk's sucrose medium and reduced C₂H₂ as effectively as the parent with the exception of the slow-growing mutant MCD-112.

Nickel is essential for hydrogenase expression in *A. chroococcum* (Partridge & Yates, 1982). However, all the mutants absorbed ⁶³Ni²⁺ as readily as the parent.

Biochemical complementation studies. Maier & Mutaftschiev (1982) observed that extracts of soybean bacteroids inoculated with two Hup⁻ strains of *R. japonicum* yielded hydrogenase activity when incubated together for several hours. No hydrogenase activity was obtained in similar experiments with *A. chroococcum* Hup⁻ mutant extracts mixed in groups, or all together, and incubated anaerobically at 30 °C and pH 7.5 for 16 h in the presence of chloramphenicol (30 µg ml).

H₂-uptake activity in MCD-124. Hydrogenase activities of MCD-124 varied widely from batch to batch of organisms but were generally lower than those of the parent: 0 to 60% for methylene blue-dependent H₂ uptake and 40% for H₂ evolution. This enzyme differed from the uptake hydrogenase of MCD-1 in the following aspects. (1) It remained (70 to 100%) in the supernatant after centrifuging the crude extract at 100 000g for 90 min whereas 60 to 100% of the hydrogenase activity of MCD-1 sedimented under the same conditions. (2) The pH optimum for H₂ uptake in crude extracts was 5.5 whereas that of the wild-type was 8.0. (3) It was apparently more sensitive to oxygen; O₂-dependent H³H uptake was at a maximum below 2% (v/v) O₂ in MCD-124 compared with 40% (v/v) O₂ in MCD-1, although exposure of crude extracts to air for 45 min led to only a slightly greater loss of hydrogenase activity in MCD-124 than in MCD-1 extracts (36 and 26% respectively). (4) Hydrogenase activity in MCD-124 was more sensitive to dimethyl sulphoxide (DMSO) than was that of the wild-type *A. chroococcum*, since 10% (v/v) DMSO inhibited hydrogenases in crude extracts from MCD-124 by 23 to 53% and from MCD-1 by 0 to 7% at pH 5.5 (Table 3).

Fig. 1. Agarose gel electrophoresis of plasmids from *A. chroococcum* strain MCD-1, the pHU1 transconjugants of Hup⁻ mutants of *A. chroococcum* and *E. coli* HB101(pHU1, pRK2013). Plasmid isolation and electrophoresis are described in Methods. 1, MCD-1; 2, MCD-105(pHU1); 3, MCD-115(pHU1); 4, MCD-117(pHU1); 5, MCD-122(pHU1); 6, MCD-124(pHU1); 7, *E. coli* HB101(pHU1, pRK2013). pHU1 is reported to be 46.9 kb in length (Haugland *et al.*, 1984).

Other electron acceptors. Table 3 shows how other electron acceptors substituted for methylene blue in the H₂-uptake assay with particulate and soluble preparations from *A. chroococcum* MCD-1 and MCD-124 respectively. The best electron acceptors for both hydrogenase preparations were those with mid-point potentials between +11 mV (methylene blue) and +80 mV (phenazine methosulphate). There were some major differences between the preparations: indigo carmine (E_0 -125 mV) and anthraquinone sulphonic acid (E_0 -225 mV) were much better electron acceptors for the soluble hydrogenase from MCD-124, despite the presence of DMSO, than for the particulate enzyme. On the other hand, brilliant cresyl blue was a good electron acceptor for the particulate enzyme from MCD-1 at pH 8.0 and 5.5 but not for hydrogenase in MCD-124.

Genetic complementation. Plasmid pHU1, containing determinants for hydrogenase from *R. japonicum* (see Methods), was introduced into Hup⁻ strains of *A. chroococcum* by conjugation with *E. coli* HB101 (pHU1, pRK2013). Transconjugants were shown to contain plasmid pHU1 by agarose gel electrophoresis (Fig. 1). Four Hup⁻ strains (MCD-105, 115, 117 and 122) were all restored to Hup⁺ with hydrogenase activity at optimum rates comparable to that of the parent strain, MCD-1 (Table 4). In MCD-124(pHU1), on the other hand, no increased activity was observed at pH 5.5 or 8.0 over that present in MCD-124.

Plasmid patterns. Plasmid patterns in all the Hup⁻ mutants tested were identical to that of the parent MCD-1. This is contrary to the situation in *R. japonicum* where Hup⁺ strains contain no discernible plasmids but Hup⁻ strains or mutants do contain plasmids (Cantrell *et al.*, 1983).

DISCUSSION

Hup⁻ mutants of *A. chroococcum* were obtained at an approximate frequency of 10⁻² following exposure to MNNG. This unexpectedly high frequency has several possible explanations. (1) The growth conditions used to outgrow the survivors following mutagenesis

Table 4. *Effect of pHU1 on hydrogenase activity of Hup⁻ mutants of A. chroococcum*

Activities were tested with whole cells grown in RM plus antibiotics as described in Methods. The activities of the mutants without plasmids at pH 8.0 are given in Table 2; their activities (excepting MCD-124) at pH 5.5 were zero. The figures in parentheses are from a second growth experiment.

Strain	Electron acceptor ...	Hydrogenase activity [nmol H ³ H absorbed (mg protein) ⁻¹ min ⁻¹]		
		Methylene blue		O ₂
		pH 8.0	pH 5.5	
MCD-105(pHU1)		144 (189)	57 (87)	69 (102)
MCD-115(pHU1)		207 (163)	119 (90)	116 (96)
MCD-117(pHU1)		379 (179)	185 (94)	153 (89)
MCD-122(pHU1)		339 (230)	225 (142)	121 (74)
MCD-124(pHU1)		3 (4)	7.2 (14)	4 (5.5)
MCD-124		2.5 (1.3)	9.2 (46)	1.5 (2.1)
MCD-1 (Wild-type)		180 (173)	165 (103)	94 (61)

may have enriched for this phenotype. (2) There are relatively few genes specific for hydrogenase but they were more susceptible to mutagenesis than the bulk of the genome. (3) A substantial number of genes may be required for *hup* expression. The genome of *Azotobacter* is approximately 2000 kbp (Robson *et al.*, 1984) and so a frequency of mutagenesis of 1% might indicate that in the order of 20 kbp code for genes for *hup*. Haugland *et al.* (1984) have reported that Tn5 insertions which affect hydrogenase activity in the plasmid pHU1 cover a region of at least 15.5 kb, which compares well with the above estimate for *A. chroococcum*. In similar conditions of mutagenesis, presumptive Nif⁻ (nitrogen fixation⁻) mutants are also obtained at a frequency of 1 to 2% and it can be expected that at least in the order of 17 genes are required for Nif in *Azotobacter* by analogy with *Klebsiella pneumoniae* (Jones *et al.*, 1984). (4) *hup* genes may be plasmid borne as in other organisms (Brewin *et al.*, 1980; Friedrich & Friedrich, 1983) and MNNG may induce curing of the plasmid at high frequency. This explanation is unlikely because the Hup⁻ mutants have the same indigenous plasmid pattern as the parent strain MCD-1. Moreover, when the indigenous plasmids found in *A. chroococcum* MCA-1 were cured, hydrogenase activity was unaffected (Robson *et al.*, 1984). However, the possibility that *hup* genes are present on a very large plasmid that is undetected by alkaline lysis cannot be excluded. The *hup* genes in *R. japonicum* may be on a large undetected plasmid which breaks down to give detectable plasmids and the loss of the Hup⁺ phenotype (Cantrell *et al.*, 1982).

Three classes of Hup⁻ mutants were designated by this study: those with barely detectable hydrogen uptake and no H₂-evolving activity; those with some H₂-evolving and little or no H₂-uptake activity; those with low but discernible H₂-uptake and H₂-evolving activity. Whether these categories reflect truly different phenotypes or merely lack of assay sensitivity will be determined by further genetic analysis. However, one mutant, MCD-124, exhibited relatively high, though variable, activities and was shown to contain a largely soluble hydrogenase, whereas the parent strain contained a membrane-bound enzyme. The activity present in MCD-124 also differed from the parental enzyme with respect to O₂ sensitivity, pH optimum and behaviour with electron acceptors. Preliminary results (M. G. Yates, unpublished) indicate that the partly purified and solubilized hydrogenase from *A. chroococcum* MCA-1 behaves differently from the MCD-124 enzyme with electron acceptors. However, whether this difference reflects the mutation which prevents the enzyme, once synthesized, from binding to the membrane is not known. Alternative possibilities which could cause this phenotype include mutations in other membrane components or processing enzymes.

The wide variation of hydrogenase activity in MCD-124 cannot be explained by O₂ sensitivity. Maximum O₂-dependent H³H uptake occurred at <2% (v/v) O₂ but some 30% of activity was still left at 50% (v/v) O₂; moreover, exposure of crude extracts to air only slowly decreased the activity by 30% in 45 min.

The secondary phenotypes (fast and slow growth, high or low acid production and resistance to methylene blue) may be due to secondary mutations, induced by MNNG, which are unrelated to the *hup* genes, since these differences were confined to few of the Hup⁻ mutants. However, MCD-118 (defective for methylene blue uptake) and MCD-124 (soluble hydrogenase and acid⁻) belong to the third group of mutant types, those with discernible H₂-uptake and H₂-evolving activity, and these mutations might be pleiotropic. This possibility could be resolved by isolating Hup⁺ revertants.

High hydrogenase activity at pH 8.0 compared to that at pH 5.5 indicated that the four plasmid pHU1 transconjugants of MCD-105, 115, 117 and 122 contained particulate hydrogenase. This suggests that *R. japonicum* hydrogenase, which also has a pH optimum at pH 8.0, is so similar to *A. chroococcum* hydrogenase that it is incorporated into *Azotobacter* membranes. However, by its failure to complement MCD-124, plasmid pHU1 either does not contain genes for processing hydrogenase into the membranes or, if present, they will not operate in *A. chroococcum*. On the other hand, the fact that pHU1 did not increase the soluble hydrogenase activity in MCD-124 suggests that either *R. japonicum* hydrogenase was very unstable when soluble *in vivo* or, alternatively, that it was not synthesized by pHU1 in MCD-124. This leads to the alternative possibility that pHU1 carries *R. japonicum hup* regulatory genes whose products activate *A. chroococcum hup* promoters. The *hup* genes apparently span at least 15.5 kb in this plasmid (Haugland *et al.*, 1984) but it seems not to contain all the genes necessary for *hup* expression (Cantrell *et al.*, 1983). MCD-104, 115, 117 and 122 could be regulatory Hup⁻ mutants of *A. chroococcum*. This possibility must await further characterization of plasmid pHU1 or *hup* clones from *A. chroococcum*.

Some organisms contain more than one hydrogenase, e.g. *Alcaligenes eutrophus* (Schink & Schlegel, 1978), *Clostridium pasteurianum* (Chen & Blanchard, 1984; Adams & Mortenson, 1984) and *E. coli* (Ballantine & Boxer, 1985). Ackrell *et al.* (1966) found that *Azotobacter vinelandii* contained several hydrogenase fractions, but these may not have contained different hydrogenase proteins. Zero hydrogenase activity in several Hup⁻ mutants of *A. chroococcum* suggests that either only one hydrogenase exists in this organism or if more than one is present they may have common genetic determinants.

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