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Plasmids Required for Utilization of Molecular Hydrogen by *Alcaligenes eutrophus*

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Abstract. *Alcaligenes eutrophus* and three other hydrogen bacteria exposed to plasmid-curing agents generated autotrophic-minus mutants at high frequency. These mutants were blocked in the metabolism of H_2 as an energy source and had normal levels of enzymes involved in CO_2 fixation. The loss of hydrogenase activity in *A. eutrophus* was accompanied by the loss or alteration of a plasmid that had molecular weight of approximately 200×10^6. Mobilization of this plasmid from wild-type *A. eutrophus* strains into cured hydrogenase-minus derivatives restored hydrogenase function. It is concluded that *A. eutrophus* contains a large plasmid required for hydrogen metabolism and thereby autotrophic growth.

Key words: *Alcaligenes eutrophus* – H_2 metabolism – H_2 uptake (Hup) – Plasmids – Hydrogenase

Hydrogen bacteria are facultative autotrophs that can obtain energy through the oxidation of hydrogen gas. The physiology and biochemistry of H_2 and CO_2 metabolism in *Alcaligenes eutrophus* has been studied extensively by Schlegel and coworkers (Schlegel 1976) and by McFadden and coworkers (McFadden 1978). This organism has two hydrogenases: one soluble and NAD-reducing, and one membrane-bound and respiratory-chain-linked. Both have been purified and partially characterized (Wittenberger and Repaske 1961; Schneider and Schlegel 1976, 1977; Schink and Schlegel 1979).

Very little is known about the genetic basis of H_2 utilization. The first mutant strains of *A. eutrophus* H16 defective in one or both hydrogenase activities have been described by Pfitzner (1974) and Schink and Schlegel (1978). Preliminary experiments by Reh and Schlegel (1975), Schlegel (1976), and by Pootjes (1977) have led to the interesting notion that plasmids may play a role in hydrogen utilization by some hydrogen bacteria: the best evidence is the loss of hydrogenase activity following treatment with plasmid-curing agents. Furthermore, recent indirect evidence indicates that plasmids may determine H_2 utilization in *Rhizobium leguminosarum* (Brewin et al. 1981). In this communication we describe the isolation, properties, and genetic transfer of plasmids that determine H_2 utilization in *A. eutrophus*.

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Abbreviations: Aut, autotrophic; Hup, hydrogen uptake; NTG, N-methyl-N′-nitro-N-nitrosoguanidine; RuBP, ribulose bisphosphate; RuMP, ribulose monophosphate; Kan, kanamycin; Nal, nalidixic acid; Rif, rifampicin; Tet, tetracycline

Materials and Methods

Organisms and Cultivation

Wild-type strains investigated were *Alcaligenes eutrophus* type strain (ATCC 17697), H1 (ATCC 17698), and H16 (ATCC 17699); *Alcaligenes paradoxus* type strain (ATCC 17713); *Alcaligenes ruhlandii* type strain (ATCC 15749); *Pseudomonas facilis* (ATCC 15376); *Pseudomonas palleronii* type strain (ATCC 17724), and *Pseudomonas saccharophila* type strain (ATCC 15946). See Davis et al. (1969, 1970), Aragno and Schlegel (1977), Pootjes et al. (1966), and Duodoroff (1940) for description of these strains. Mutant strains used are described in Table 1. Conditions for growth were as previously described (Andersen 1979), except for the modifications that follow. The basal mineral medium (Aragno et al. 1977) contained 0.36% Na_2HPO_4, 0.15% KH_2PO_4, 0.10% NH_4Cl, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.001% $CaCl_2 \cdot 2H_2O$, and was supplemented with trace elements (Repaske and Repaske 1976) – the iron source was 5×10^{-5} M ferric ammonium citrate. The medium was supplemented with 0.05% $NaHCO_3$ for autotrophic growth and with 0.1–0.3% organic substrate for heterotrophic growth; pH was adjusted to 6.8 with NaOH. L-broth that contained 1% tryptone, 0.5% yeast extract, and 1% NaCl (Andersen 1979) (supplemented with 0.3% sucrose for *P. saccharophila*) was used as rich medium. Autotrophic growth was under 85% H_2, 5% O_2 and 10% CO_2, and heterotrophic growth was under air; growth temperature was 30° C when not otherwise indicated.

Plasmid Curing and Mutant Isolation

Cells for inoculum were taken from single autotrophic colonies. Log-phase cultures growing in 33% L-broth, 66% minimal medium containing fructose (*A. eutrophus*, *A. paradoxus*, *P. facilis*) glucose (*A. ruhlandii*), sucrose (*P. saccharophila*), or sodium malate (*P. palleronii*) were diluted to 10^7 cells/ml. Curing agents were added at a concentration that caused severe but incomplete inhibition of growth; the concentration varied from strain to strain. Incubation continued until the culture had reached a density of at least 10^9 cells/ml (24–48 h). The cells were collected by centrifugation, washed, resuspended in fresh medium without curing agents, and incubated until rapid growth resumed. Dilutions were plated on L-broth plates. After incubation under 5% O_2 and 95% N_2 at 30° C, the resulting colonies were replica-plated onto autotrophic medium and scored for autotrophic growth. Autotrophic-minus clones were tested for heterotrophic growth on minimal media with the carbon sources indicated above. Autotrophic-minus clones were purified and studied further.

Spontaneous nalidixic-acid (Nal)-resistant strains were isolated from cultures growing in L-broth after exposure to Nal concentrations that increased by steps (20, 50, 100, 200, and 400 μg/ml). Spontaneous rifampicin (Rif)-resistant strains were isolated after being plated on L-broth with 100 μg/ml Rif. A tetracycline (Tet)-sensitive derivative of the

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Table 1. Bacterial strains

Strain	Genotype[a]	Relevant phenotype	Derivation/source
Escherichia coli K-12			
C600	*thr leu thi*	$Thr^-Leu^-Thi^-$	JR Mielenz
C600/R68.45		$Amp^RKan^RTet^R$	JR Mielenz
C600/RP4		$Amp^RKan^RTet^R$	JR Mielenz
Alcaligenes eutrophus (type strain)			
AE1	wild-type (/pAE1)	Hup^+	ATCC17697
AE1/RP4		$Hup^+Kan^RTet^R$	C600/RP4 × AE1
AE101	*met-1*/pAE1	Hup^+Met^-	NTG mutagenesis AE1
AE102	*met-1*/R68.45 pAE1	$Hup^+Met^-Kan^RTet^R$	C600/R68.45 × AE101
AE103	*met-1*/RP4 pAE1	$Hup^+Met^-Kan^RTet^R$	C600/RP4 × AE101
AE113	*glc-1*/pAE1	Glc util.$^-$	NTG mutagenesis AE1
AE122	*glc-1*/R68.45 pAE1	$Hup^+Glc^-Kan^RTet^R$	AE102 × AE113
AE126	*glc-1*/R68.45 *tet*^{s} pAE1	$Hup^+Glc^-Kan^RTet^S$	NTG mutagenesis AE122
AE131	/ΔpAE1	Hup^-	Mitomycin C treatment of AE1
AE156	*glc-1*/R68.45 *tet*^{s} ΔpAE1	$Hup^-Glc^-Kan^RTet^S$	Spont. from AE126
AE157	*glc-1 nal-1*/R68.45 *tet*^{s} ΔpAE1	$Hup^-Glc^-Nal^RKan^R$	Spont. from AE156
AE159	*nal-2 rif-1*/ΔpAE1	$Hup^-Nal^RRif^R$	Spont. from AE131
AE160	*nal-2 rif-1*/R68.45 *tet*^{s} ΔpAE1	$Hup^-Nal^RRif^RKan^R$	AE126 × AE159
AE165	*nal-2 rif-1*/RP4 pAE1	$Hup^+Nal^RRif^RTet^R$	AE103 × AE160
AE166	*nal-2 rif-1*/RP4 pAEH1	$Hup^+Nal^RRif^RTet^R$	H1/RP4 × AE160
AE167	*nal-2 rif-1*/RP4 pAEH16	$Hup^+Nal^RRif^RTet^R$	H16/RP4 × AE160
A. eutrophus H1	wild-type (/pAEH1)	Hup^+	
H1/RP4		$Hup^+Kan^RTet^R$	AE103 × H1
A. eutrophus H16	wild-type (/pAEH16)	Hup^+	
H16/RP4		$Hup^+Kan^RTet^R$	AE103 × H16

[a] glc = glycollate; Δ = plasmid deleted

R-factor R68.45 was isolated following mutagenesis of the R68.45-containing *A. eutrophus* strain AE122 with nitrosoguanidine (NTG) under conditions described previously (Andersen 1979). The mutagenized cell suspension was incubated in L-broth for 3 h and dilutions were then plated on fructose minimal medium plates that contained 100 µg/ml kanamycin (Kan). Tet-sensitive colonies were identified by replica-plating them onto fructose minimal plates containing 400 µg/ml Kan and 40 µg/ml Tet.

Conjugation Experiments

Donor and recipient strains were grown in L-broth to densities of 5×10^8 to 10^9 cells/ml, cultures were mixed to a ratio of 5 donor cells per one recipient cell, and a total of 5×10^9 cells were collected on membrane filters 25 mm in diameter with 0.2 µm sized pores. The filters were incubated on L-broth plates overnight, the cells were resuspended, and dilutions were plated on the appropriate media. R-factor transfer was scored as growth on fructose minimal medium containing Kan and/or Tet when auxotrophic donors were used. Transfer of the Hup^+ phenotype was scored as autotrophic growth on plates containing Kan and Tet. Nal^R recipients were also used, and the Nal^S donors were then selected against using Nal-containing media. Donors and recipients were plated separately in control experiments. Antibiotic concentrations used were 400–1000 µg/ml Kan, 500 µg/ml Nal, 25 µg/ml Tet, and 100 µg/ml Rif.

Enzyme Assays

Ribulose bisphosphate (RuBP) carboxylase activity was determined as RuBP-dependent fixation of $^{14}CO_2$ using toluene-treated cells (Andersen 1979). RuMP kinase activity was determined as RuBP-dependent fixation of $^{14}CO_2$ in toluene-treated cells, which coupled ribulose 1,5-bisphosphate formation to CO_2 fixation by endogenous RuBP carboxylase (Tabita et al. 1978). Both activities were determined at 25°C.

Hydrogenase activity was determined a) at 30°C by measuring incorporation of radioactivity from tritium gas (predominantly exchange between tritium gas and water) by a suspension of washed whole cells in minimal medium buffer to 0.01 to 0.2 mg cell protein/ml and incubated under N_2 with 1% $^3H_2$ (35.9 Ci/mol) (Lim 1978); b) at 30°C by measuring H_2 uptake in whole cells or cell-free extracts in 0,05 M potassium-phosphate buffer pH 7.0 with 5 mM methylene blue as acceptor under N_2 atmosphere with 1% H_2; c) at 25°C by following NAD reduction spectrophotometrically using freshly prepared cell-free extract in 0.05 M potassium-phosphate buffer pH 8.0 and 0.8 mM NAD as acceptor under 100% H_2 (Schneider and Schlegel 1977). Hydrogen uptake at 30°C was determined in whole cells respiring with O_2 under air containing 1% H_2. H_2 was in all cases determined by gas chromatography (Lim 1978). NADH oxidase activity in cell-free extracts in 0.05 M potassium phosphate buffer pH 7.5 was determined spectrophotometrically by following the NADH concentration at 365 nm. Cell-free extracts were prepared by passing washed cell suspensions (15–20 mg protein/ml) in 0.05 M potassium-phosphate buffer pH 7.5 through a French pressure cell (Aminco, USA) at 0–4°C and 140 MPa. Unbroken cells and cell debris were removed by centrifugation at 10,000 × *g* for 20 min. Protein was determined according to the method of Drews (1965); bovine serum albumin was used as a standard.

Plasmid Isolation

Ten-milliliter cultures grown in L-broth were concentrated by centrifugation. Cell pellets were frozen and then resuspended in 200 µl 10% sucrose containing 24 mM Tris-HCl pH 8.0, 5 mM EDTA and 10 µl lysozyme. After samples spent 10 min on ice, 0.5 mg nuclease-free pronase was added. Cells were lysed by adding 10 µl 20% sodium dodecyl sulphate (SDS). After 10 min on ice, each lysate received 50 µl of 1 mg/ml pancreatic RNase and was incubated 15 min on ice. Three ml of phenol equilibrated with 50 mM Tris-HCl pH 7.5 plus 0.1 mM EDTA was added to each lysate; addition of 200 µl of H_2O followed. After 5 min of gentle mixing, the phenol extract was centrifuged for 10 min in an Eppendorf 5412 centrifuge. The upper aqueous phase contained a viscous "pellet" and 50–100 µl of clear, slightly viscous supernatant. The clear super-

natant was withdrawn and an additional 200 µl of H_2O was added to the viscous "pellet". After the "pellet" was mixed gently and centrifuged, the clear supernatant was again withdrawn. The supernatants were combined and 70 µl was examined for the presence of plasmid DNA by electrophoresis in a 0.8% agarose gel (Bolivar et al. 1977).

Results

Curing of the Autotrophic Phenotype

As summarized in Table 2, growth of *Alcaligenes eutrophus* (type strain) in the presence of the plasmid-curing agents ethidium bromide and mitomycin C, or near the maximum temperature that allows growth, led to substantial rates of loss of the autotrophic$^+$ (Aut$^+$) phenotype. Spontaneous Aut$^-$ clones also were found occasionally after long periods of heterotrophic growth at 30° C.

Seven other strains of hydrogen bacteria were treated with mitomycin C. The frequencies of loss of the Aut$^+$ phenotype after this treatment were as follows (the numbers in parentheses refer to the percentage of curing observed): *A. eutrophus* type strain (4.5%); *A. eutrophus* Hl (43%); *A. eutrophus* H16 (0.1%); *A. paradoxus* (0.3%); *A. ruhlandii* (< 0.1%); *Pseudomonas facilis* (28%); *P. palleronii* (1.3%); and *P. saccharophila* (< 0.2%).

All the Aut$^-$ derivatives of these bacteria grew at the same rate as the wild-type on minimal media under heterotrophic conditions. No auxotrophs were detected after the mitomycin C treatment, as would have been expected if mitomycin C acted as a general mutagen. The proportion of auxotrophs of *A. eutrophus* type strain after mitomycin C treatment was $< 10^{-3}$ of the number of Aut$^-$ clones generated.

Several of the Aut$^-$ clones derived from the various hydrogen bacteria were characterized further. Two classes of Aut$^-$ mutant strains were generated by the mitomycin C treatment: a) completely Aut$^-$ (all mutants derived from *A. eutrophus* type strain, *A. paradoxus*, and *P. palleronii*), or b) leaky (all mutants derived from *P. facilis*). *A. eutrophus* H1 and H16 gave rise to both classes. These two classes corresponded to complete or partial loss of H_2 utilization activity.

Table 2. Loss of the autotrophic phenotype in *A. eutrophus* (type strain) after exposure to plasmid curing agents

Treatment	Colonies examined		
	Total	Autotrophic$^-$	Autotrophic$^-$, %
Control[a]	2500	0	< 0.04
Sodium dodecyl sulphate; 300 µg/ml	490	0	< 0.20
Rifampicin; 100 µg/ml	1200	0	< 0.10
Acridine orange; 200 µg/ml	221	0	< 0.50
Ethidium bromide; 50 µg/ml	123	28	22.8
Mitomycin C; 2 µg/ml	447	20	4.5
High temperature; 42° C	160	4	2.5

[a] Cells grown in fructose medium for 7 doublings at 30° C

Loss of Hydrogenase Activity in Cured Derivatives

The cured Aut$^-$ derivatives were grown heterotrophically in isoleucine minimal medium to induce measurable levels of enzymes required for autotrophic growth (Andersen 1979). The *A. eutrophus* mutant strains AE131, H1 – 3, and H16-1 had no hydrogenase activity (< 0.1% of the activity for wild type cells, measured as $^3H_2$ uptake, Table 3). Several independently isolated Aut$^-$ derivatives of *A. eutrophus* (type strain) that resulted from mitomycin C treatment, exposure to high temperature (42° C) or long periods of heterotrophic growth at 30° C, were characterized, and all were completely blocked in H_2 metabolism (data shown only for AE131, isolated after mitomycin C treatment). The partially Hup$^-$ *A. eutrophus* H1 and H16 mutants (Table 3) will be discussed later. All the Hup$^-$ derivatives of the three *A. eutrophus* strains had both RuBP carboxylase and RuMP kinase activities (Table 3). The cured derivatives PF-2 of *P. facilis* and PP-1 of *P. palleronii* also had RuBP carboxylase and RuMP kinase activities similar to their parental strains, whereas the hydrogenase activities ($^3H_2$ uptake) were greatly

Table 3. Characterization of *A. eutrophus* Hup$^-$ mutants isolated after curing with mitomycin C[a]

Characteristic	*A. eutrophus* (type strain)		*A. eutrophus* H1				*A. eutrophus* H16		
	Wild-type AE1	AE131	Wild-type H1	H1-1	H1-2	H1-3	Wild-type H16	H16-1	H16-2
Enzyme activities[b]									
RuBP carboxylase	0.18	0.21	0.30	0.36	0.41	0.20	0.26	0.18	0.30
RuMP kinase	0.15	0.13	0.22	0.27	0.34	0.13	0.15	0.15	0.14
Hydrogenase, electron acceptor:									
O_2	18	< 0.2	16	24	20	< 0.2	16	< 0.2	16
methylene blue	38	< 0.2	24	25	35	< 0.2	26	< 0.2	19
NAD	131	< 0.5	155	16	< 0.5	< 0.5	118	< 0.5	< 0.5
Plasmid content (molecular weight · 10^{-6})	~200	absent	~200	~180	~200	absent	~200	~200	~220

[a] Cells were grown to mid log-phase in isoleucine minimal medium under air, and enzyme activities were then determined using whole cells or cell-free extracts as described in Materials and Methods

[b] µmol $^{14}CO_2$, H_2 or NAD/h · mg cell protein

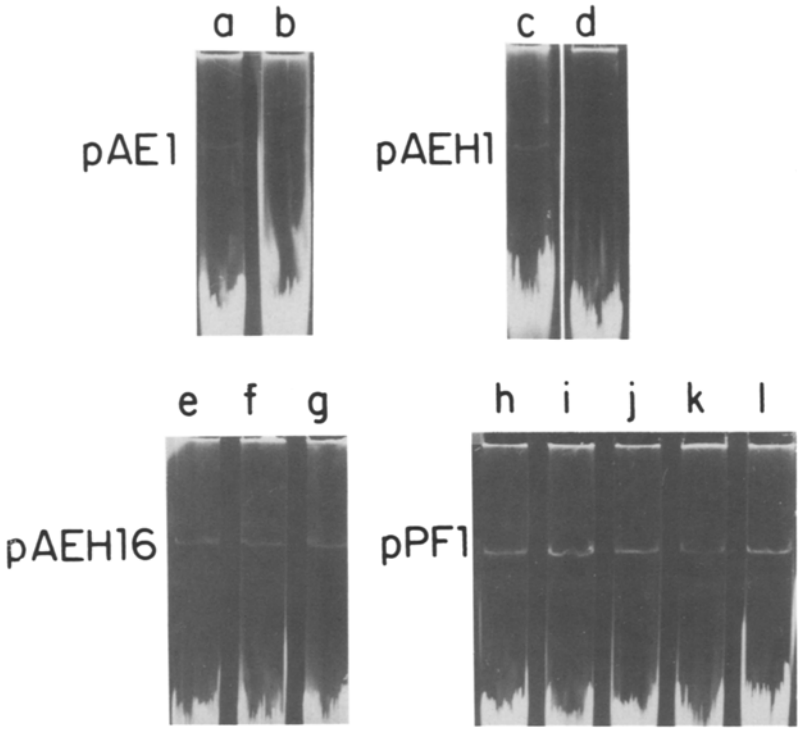

Fig. 1
Plasmid DNA from hydrogen bacteria examined by electrophoresis on a 0.8% agarose gel. The migration positions of the plasmids designated pAE1, pAEH1, pAEH16, and pPF1 are indicated. The lanes contain DNA isolated from **a**) *Alcaligenes eutrophus* type strain (Hup^+); **b**) AE131 (Hup^-); **c**) *A. eutrophus* H1 (Hup^+); **d**) H1-3 (Hup^-); **e**) *A. eutrophus* H16 (Hup^+); **f**) H16-1 (Hup^-); **g**) H16-2 ($Hup^\pm$); **h**) *Pseudomonas facilis* (Hup^+); **i–l**) four cured (Hup^+ derivatives of *P. facilis*. $Hup^\pm$ designates partial activity

reduced (PF-2) or not detected (PP-1) (after growth in Ile minimal medium, data not shown).

None of the completely Hup^- strains isolated reverted back to Hup^+ phenotype (frequency $< 10^{-10}$) when incubated under autotrophic conditions. The non-reverting nature of the Hup^- phenotype is consistent with the theory that the loss of this trait frequently involves deletions that affect genes essential to H_2 metabolism.

Loss of a Large Plasmid in Cured Strains

Various strains were examined for plasmid content by agarose gel electrophoresis of DNA extracted by a procedure modified to allow for the detection of very large plasmids. Figure 1 a, c, e, and h show that the wild-type *A. eutrophus* strains and *P. facilis* each contained a plasmid of high molecular weight. By co-electrophoresis of these plasmids with plasmids of known molecular weight, a minimum molecular weight estimate of 200×10^6 was obtained for the plasmid present in *A. eutrophus* (type strain) (data not shown). These plasmids have been designated pAE1, pAEH1, pAEH16, and pPF1, as indicated in Fig. 1. In repeated attempts, these plasmids could not be detected in the completely Hup^- derivatives of *A. eutrophus* type strain (data shown only for AE131), or in H1-3 (Fig. 1 b, d). These results suggest that the loss of the plasmids pAE1 or pAEH1 is accompanied by the loss of both the NAD-dependent and the membrane-bound hydrogenase activities, which generated a cell incapable of growth with hydrogen gas.

Plasmid Deletions or Insertions in Strains Defective in NAD-Dependent Hydrogenase

The partially Hup^- *A. eutrophus* mutants H1-1, H1-2 (derived from H1), and H16-2 (derived from H16) had generation times of 7.5, 8.5, and 12 h, respectively, during autotrophic growth, compared with 3.0 – 3.1 h for the wild-types. These strains had high total hydrogenase activity (measured as methylene-blue reduction or $^3H_2$ uptake), and high H_2 uptake activity in respiring cells (Table 3). However, NAD-dependent hydrogenase activity was greatly reduced (H1-1) or completely absent (H1-2 and H16-2). This activity is therefore not obligatory for H_2-dependent growth, which agrees with previous reports for *A. eutrophus* H16 (Pfitzner 1974; Schink and Schlegel 1978). These derivatives had plasmids of molecular weight similar to those of the wild-type strains (Table 3; see also Fig. 1 g for H16-2). In the case of H1-1, co-electrophoresis with the plasmid pAEH1 revealed that the plasmid present in H1-1 had a molecular weight approximately 10% lower than that of pAEH1, (Table 3), which suggests that a deletion event may be responsible for the loss of the NAD-dependent hydrogenase activity. Similar examination of the plasmid present in H16-2 indicated that this plasmid was of slightly greater size than pAEH1 (Table 3), which suggests that loss of the NAD-dependent hydrogenase activity in this strain may have been caused by the insertion of a small DNA fragment into pAEH16. Isolation and analysis of such altered plasmids should allow for the identification the regions of the plasmids necessary for H_2 utilization. Because of the difficulty in resolving large, supercoiled plasmids of similar molecular weights, it is possible that the plasmids present in other Hup^- derivatives, such as H1-2, H16-1, and PF1-PF4, Fig. 1, may contain small, undetected deletions or insertions as well.

Genetic Transfer of Hup Plasmids

A conjugation system based on R-factors was developed that allowed the Hup plasmids in *A. eutrophus* to be mobilized. The R-factors RP4, R68.45, and RK2 were transferred into *A. eutrophus* (type strain) from *Escherichia coli* at frequencies similar to those reported for several other bacterial species

Table 4. Genetic transfer of hydrogen uptake (Hup) plasmids in *A. eutrophus*

Donor	Recipient	Frequency of R factor transfer (Tet^R per recipient)	Frequency of Hup^+ transfer[a] (Hup^+ per R factor transfer)
E. coli C600/RP4	AE101	8×10^{-6}	
E. coli C600/R68.45	AE101	5×10^{-6}	
AE102	*E. coli* C600	3×10^{-1}	
AE103	*E. coli* C600	4×10^{-1}	
AE103	AE131	6×10^{-4}	$< 10^{-6}$
AE103	AE157	1×10^{-2}	2×10^{-4}
AE1/RP4	AE160	2×10^{-2}	4×10^{-4}
AE103	AE160	2×10^{-2}	3×10^{-4}
H1/RP4	AE160	1×10^{-2}	8×10^{-5}
H16/RP4	AE160	2×10^{-2}	7×10^{-4}

[a] Scored as Aut^+ on plates containing 25 μg/ml Tet and 400 μg/ml Kan when AE131 was the recipient; when AE157 or AE160 were the recipients 500 μg/ml Nal was also included to select against the Nal^S donors (Table 1). Controls where donors and recipients were plated separately gave no colonies

(Holloway 1979) (Table 4). The drug-resistance markers conferred high levels of resistance to Tet and Kan. Transfer between *A. eutrophus* strains occurred at a frequency of about 10^{-4}. Transfer to *E. coli* C600 occurred at high frequency, which indicated that *A. eutrophus* behaved as an efficient donor but was a poor recipient of these R-factors. Strains that contained the Tet-sensitive derivative of R68.45 were better recipients: they increased the frequency of R-factor transfer to 1–2% (Table 4).

The use of such improved recipients allowed for the R-factor-mediated transfer of the Hup^+ phenotype from Hup^+ *A. eutrophus* strains to Hup^- derivatives at frequencies of about 10^{-4} (per R-factor transfer, Table 4). No transfer was detected ($< 10^{-6}$ per R-factor transfer) of markers believed to reside on the chromosome (data not shown). Also, no transfer of the Hup^+ phenotype was observed when donors lacking R-factors were used. Donors and recipients plated separately gave no colonies.

Different donors and recipients gave similar transfer frequencies for the Hup^+ phenotype (Table 4). The R-factor R68.45 mobilized pAE1 at the same frequency as RP4. The identity of the exoconjugants was ensured by using multiply marked strains. AE165, AE166, and AE167, which were selected on medium without Rif, were thus Rif^R (Table 1). As shown in Table 4, *A. eutrophus* (type strain), strains H1 and H16 could serve equally well as donors of the Hup^+ phenotype to AE160. The Hup^+ exconjugants AE165-AE167 grew at rates similar to the wild-types under autotrophic conditions (generation time 2.8–3.1 h) and had levels of hydrogenase activity similar to those in the wild-types (Table 5). A specific assay exists only for the soluble (NAD-reducing) hydrogenase in *A. eutrophus*. Both the soluble and the membrane-bound hydrogenase can reduce methylene blue (Schneider and Schlegel 1976; Schink and Schlegel 1979). Centrifugation of cell-free extracts of AE165-AE167 at 140,000 × *g* for 1 h sedimented < 5% of the NAD-reducing hydrogenase activity, 30–40% of methylene-blue-reducing hydrogenase activity and 80–90% of the NADH oxidase activity (membrane bound). It is therefore concluded that the exconjugants contained both soluble and membrane-bound hydrogenases.

As shown in Fig. 2f–h, the exconjugants contained not only RP4 (and possibly R68.45 Tet^S, which is of similar size as RP4), but also pAE1, pAEH1, or pAEH16, respectively. The fact that the genetic complementation of the Hup^- phenotype coincided with the reappearance of the large plasmid lost during the generation of the Hup^- phenotype strengthens the theory that genes necessary for the metabolism of H_2 are present in these large plasmids.

Table 5. Characterization of *A. eutrophus* Hup^+ exconjugants[a]

Strain	Hydrogenase activity with electron acceptor		
	O_2[b]	methylene blue[b]	NAD[c]
Donors			
AE1/RP4	23	38	83
H1/RP4	26	21	146
H16/RP4	29	24	113
Recipient			
AE160	< 0.2	< 0.2	< 0.5
Exconjugants			
AE165[d]	31	31	112
AE166	28	24	93
AE167	27	20	90

[a] Experimental details were as described for Table 3
[b] μmol H_2/h per mg cell protein
[c] μmol NAD/h per mg cell protein
[d] AE165 was derived from AE103 × AE160 (Table 1)

Discussion

Evidence for Hup Plasmids: Curing, Transfer, Gel Analysis

Derivatives that had lost their H_2-utilization capability were isolated after six strains of hydrogen bacteria were exposed to agents that interfere with plasmid maintenance. The Hup^- derivatives characterized were found to be defective only in H_2 metabolism and had no discernible defects in heterotrophic metabolism, such as auxotrophic requirements. The non-reverting nature the Hup^- phenotype suggested that this phenotype was the result of the deletion of genes necessary for H_2 metabolism. Generation of the Hup^- phenotype was found to be accompanied in *Alcaligenes eutrophus* type strain, and strains H1 and H16 by the loss of alteration or a plasmid

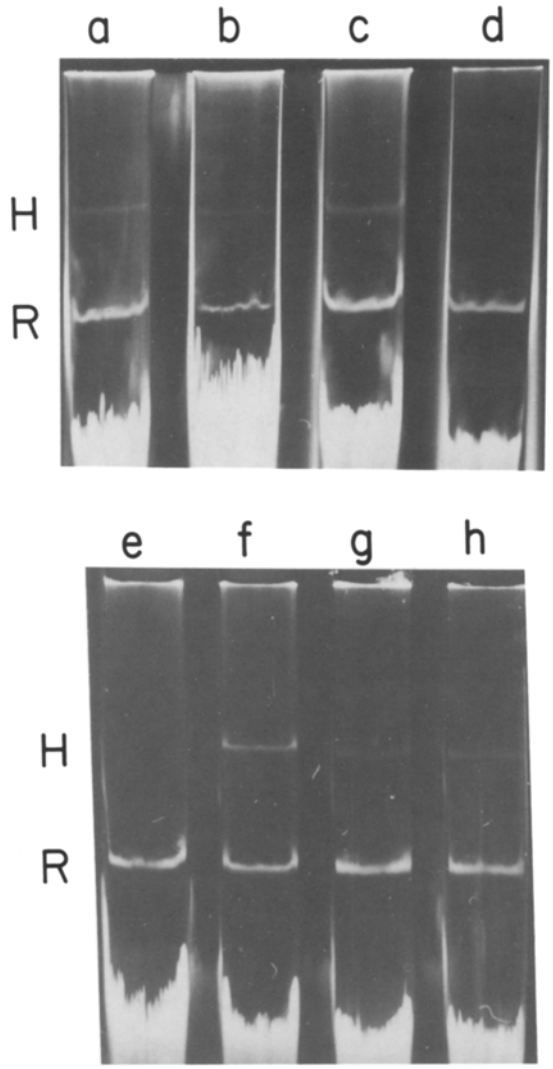

Fig. 2. Genetic transfer of Hup plasmids in *A. eutrophus*. DNA isolated from the Hup^+ donor bacteria, the Hup^- recipient, and the Hup^+ exconjugants was examined by electrophoresis on a 0.8% agarose gel. The lanes contain DNA isolated from **a**) AE103 (Hup^+); **b**) H1/RP4 (Hup^+); **c**) H16/RP4 (Hup^+); **d** and **e**) AE160 (Hup^-); **f–h**) the Hup^+ exconjugants AE165–167, respectively. The migration position of the R-factors RP4 and R68.45 Tet^S is indicated by R. The migration position of the Hup plasmid is indicated by H

with molecular weight of approximately 200×10^6. RP4-mediated transfer of the Hup^+ phenotype was accompanied by transfer of these large plasmids. These results indicate that the plasmid pAE1 and the similar, if not identical, plasmid pAEH1 are required for autotrophic growth of *A. eutrophus* type strain and strain H1, respectively, where H_2 is the sole source of energy. The genetic-transfer data indicates that plasmid pAEH16 plays a similar role in strain H16, but this plasmid was not observed to be cured completely. The present curing data for *Pseudomonas facilis*, *A. paradoxus* and *P. palleronii*, and the previous reports on *P. facilis* (Pootjes 1977), *Nocardia opaca* (Reh and Schlegel 1975), and *Rhizobium loguminosarum* (Brewin et al. 1981) indicate that similar plasmids may exist in other H_2-utilizing bacteria as well. However, several independently cured derivatives of *P. facilis* isolated according to the procedure of Pootjes (1977) all had levels of hydrogenase activity ($^3H_2$ uptake) that were 3–6% of wild-type levels. Loss of plasmid or plasmid deletions or insertions were not detected in these strains (Fig. 1h–l).

What Genes Are Coded for by the Hup Plasmid?

Autotrophic growth of *A. eutrophus* involves the use of H_2 as an energy source and CO_2 as the sole carbon source, and it might have been anticipated that if an "autotrophy plasmid" exists in this organism, it would code for enzymes necessary for the metabolism of H_2 and for the fixation of CO_2. The Hup^- strains AE131 and H1-3 were cured of the plasmid pAE1 and pAEH1, respectively, and contained less than 0.1% of the wild-type levels of hydrogenase, which suggested that the plasmids determine hydrogen metabolism. However, both strains had normal levels of RuBP carboxylase and RuMP kinase activities, which are enzymes involved in the fixation of CO_2. Furthermore, AE131 and H1-3 both grew on formate as sole source of carbon and energy. Formate is assimilated via CO_2 both in *Alcaligenes eutrophus* H16 (Friedrich et al. 1979) and the type strain (Andersen, unpublished observation). Thus, although pAE1 and pAEH1 determine H_2 metabolism, they apparently do not encode the enzymes necessary for CO_2 fixation. Whether the structural genes for the two hydrogenases in *A. eutrophus* (Schneider and Schlegel 1976, 1977; Schink and Schlegel 1979) or for necessary electron transport components are located on the plasmid, or whether the plasmid only regulates H_2 metabolism is not known. A plasmid of this size may carry much genetic information. We have, however, been unable to identify any other phenotypes determined by it.

Finally, the possible involvement of Hup plasmids in biological nitrogen fixation is of considerable interest. As much as one-third of the energy consumed in the nitrogenase reaction may be lost in the form of H_2 gas (Andersen and Shanmugam 1977). Some of this energy may be recovered by recycling the H_2 through H_2 uptake (Hup) systems. Evans and coworkers have recently reported that some free-living *Rhizobium* strains behave as "hydrogen" bacteria: they are capable of using H_2 gas as the sole source of energy for growth (Hanus et al. 1979). However, many agronomically important *Rhizobium* spp. do not have an active H_2 utilization system, which leads to less efficient symbiotic N_2 fixation (Schubert and Evans 1976; Lim et al. 1980; Albrecht et al. 1979). Thus, the identification and characterization of naturally occurring plasmids that determine utilization of H_2 may facilitate the understanding and genetic manipulation of H_2 metabolism in these economically important symbiotic bacteria.

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