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Naturally Occurring Genetic Transfer of Hydrogen-Oxidizing Ability Between Strains of *Alcaligenes eutrophus*

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Mutants defective in chemolithoautotrophic growth (Aut^-) have been isolated from *Alcaligenes eutrophus* strains H16, N9A, G27, and TF93. Spontaneous Aut^- mutants were obtained only with strain TF93. Mutants of the other strains were selected after conventional mutagenesis or treatment with mitomycin. Most of the mutants, including the spontaneous Aut^- strains, lacked hydrogenase activity (Hox^-) but possessed the ability to fix carbon dioxide (Cfx^+). Agar mating of *A. eutrophus* H16 with Hox^- mutants of the various strains resulted in transconjugants which had recovered the ability to grow autotrophically and to express activity of hydrogenase as examined by enzymatic and immunochemical analysis. Transfer of hydrogen-oxidizing ability occurred in the absence of a mobilizing plasmid such as RP4. The transfer frequency was particularly high (ca. 10^{-2} per donor) when the spontaneous Hox^- mutants of strain TF93 were used as recipients. These strains proved to be plasmid free, whereas donors, transconjugants, and the mutagen-treated Hox^- mutants contained a large plasmid (molecular weight, $270 \pm 10 \times 10^6$) revealed by agarose gel electrophoresis. The results allow the conclusion that *A. eutrophus* H16 harbors a self-transmissible plasmid, designated pHG1, which carries information for hydrogen-oxidizing ability.

Alcaligenes eutrophus is a gram-negative, facultatively chemolithoautotrophic bacterium which is able to grow in minimal salts medium with a gas mixture of hydrogen, oxygen, and carbon dioxide (Aut^+). In strain *A. eutrophus* H16, hydrogen is activated by a soluble NAD^+-reducing hydrogenase (hydrogen:NAD^+ oxidoreductase, EC 1.12.1.2) and a membrane-bound hydrogenase linked to the respiratory chain. The biochemical properties of both enzymes have been thoroughly characterized (16, 21). Although information concerning the genetics of hydrogen-oxidizing bacteria is limited, mutants have been characterized that have lost the ability to utilize either hydrogen as an energy source (Hox^-) or carbon dioxide as a carbon source (Cfx^-) (2, 8, 15). Additionally, in the past few years, evidence has accumulated to indicate a possible role of plasmids in autotrophic metabolism. For instance, mutants of *Nocardia opaca* 1b impaired in autotrophic metabolism have been found relatively frequently. Such mutants regained the ability to grow autotrophically after conjugation with the wild type. Furthermore, the autotrophic character has been transferred via conjugation from *N. opaca* 1b to the nonautotrophic strain *Nocardia erythropolis* (14, 18). *Pseudomonas facilis* also lost its ability to grow autotrophically at an extraordinarily high frequency, indicating that the genes specifying hydrogenase are located extrachromosomally (13). Lim et al. (9) recently obtained evidence for the involvement of a large plasmid in hydrogen metabolism by strains of *A. eutrophus*.

This communication presents evidence for the existence of a self-transmissible plasmid, denoted pHG1, which transfers hydrogen-oxidizing ability from *A. eutrophus* strain H16 to Hox^- mutants of the same strain and other strains of the same species.

MATERIALS AND METHODS

Chemicals. NAD^+, deoxyribonuclease I, and tetracycline hydrochloride were obtained from C. F. Boehringer & Soehne GmbH, Mannheim, Germany. Mitomycin C and agarose type V were purchased from Sigma Chemical Co., St. Louis, Mo. Antibiotic sensitivity disks were from Oxoid Ltd., London, England. All other chemicals were purchased from E. Merck AG, Darmstadt, Germany.

Bacterial strains. *A. eutrophus* strains and plasmid-harboring strains of *Escherichia coli* used in this study are listed in Table 1. Auxotrophic and autotrophic mutants of *A. eutrophus* strain H16 were isolated as previously described (7, 8). Mutants derived from mitomycin treatment were obtained after subjecting the cells to 2 μg of mitomycin per ml for approximately 40 h.

Growth conditions. The bacteria were grown in a complex medium consisting of nutrient broth or in minimal salts medium as described by Schlegel et al. (19). The concentration of the heterotrophic carbon

TABLE 1. *Bacterial strains*

Species and strain[a]	Relevant phenotype	Reference or source
A. eutrophus		
H16	Hox^+	Wild type, ATCC 17699, DSM 428
HF54	Hox^+ Trp^- Cys^-	Mutant of H16, this study
HF17	Hox^+ Cfx^-	8
HF41	Hox^+ Cfx^- Met^-	Mutant of HF17, this study
HF18	Hox^-	8
HF09	Hox^-	15
HF60	Hox^-	Mutant of H16, this study
TF93	Hox^+	Wild type, ATCC 17697, DSM 531
TF97	Hox^-	Mutant of TF93, this study
TF100	Hox^-	Mutant of TF93, this study
TF104	Hox^-	Mutant of TF93, this study
N9A	Hox^+	Wild type, DSM 518
N9AF02	Hox^-	Mutant of N9A, this study
G27	Hox^+	Wild type, DSM 516
G27F01	Hox^-	Mutant of G27, this study
E. coli K-12		
402	Pro^- Met^-	J. Beringer, John Innes Institute, United Kingdom
J53(RP4)	Pro^- Met^- Tet^r	H. Meade, Massachusetts Institute of Technology
I5(R1*drd-19*)		11
Rhizobium meliloti		
MVII/I		A. Pühler, University of Bielefeld, Germany
20II		A. Pühler, University of Bielefeld, Germany

[a] Strains carrying the initials HF are derivatives of *A. eutrophus* H16. Strains with the initials TF are derivatives of the parental strain TF93, and strains with the initial N9AF and G27F are derivatives of the parental strains N9A and G27, respectively.

source was 0.4% (wt/vol) if not otherwise stated. Ammonium chloride (0.2% [wt/vol]) served as the nitrogen source. The gas atmosphere for autotrophic growth contained a mixture of hydrogen, oxygen, and carbon dioxide in a ratio of 8:1:1 (vol/vol). Amino acids at a final concentration of 0.01% (wt/vol) were supplemented as indicated. When tetracycline was used, the concentration was 10 μg/ml. Solid media contained 1.5% (wt/vol) agar. The growth temperature was 30°C. Organisms were grown in liquid culture as previously described (6).

Conjugation. Matings were routinely performed on agar as follows. Nutrient both (10 ml) was inoculated with a single colony from an autotrophic plate, and this culture was incubated for about 24 h on a rotary shaker. Recipient and donor cells were mixed at a ratio of 1:1, then 0.3 ml of this mixture was spotted onto fresh nutrient broth plates. Controls of recipient and donor cells were treated similarly. The plates were incubated overnight. Subsequently, the cells were washed off of the agar with 3 ml of minimal salts medium and diluted as indicated. A 0.1-ml portion of the cell suspension was plated onto selective media. Transconjugants appeared after 3 to 5 days of incubation. Viable cell counts were determined on nutrient broth plates.

Enzyme assays. Cells were grown in 100 ml of minimal medium supplemented with fructose and glycerol as described previously (6). They were suspended in 4 ml of 50 mM potassium phosphate buffer (pH 7.0) and subjected to ultrasonic disruption (1-min treatments per 1 ml of cell suspension) in a sonicator (Measuring & Scientific Equipment). The soluble and the particulate fractions were separated by centrifugation (6). The activity of soluble hydrogenase was determined in the soluble fraction by monitoring NADH formation spectrophotometrically by the method of Schneider and Schlegel (21). Membrane-bound hydrogenase activity was assayed with the particulate fraction by spectrophotometric measurement of hydrogen-dependent methylene blue reduction (16). The presence of soluble hydrogenase antigen (cross-reacting material [CRM^+]) in cell extracts was determined by immunodiffusion by the method of Ouchterlony (12). Anti-soluble hydrogenase serum was prepared as previously described (17). Protein was determined by the method of Lowry et al. (10).

Isolation of plasmids. The bacteria were grown either in 20 ml of nutrient broth or in minimal salts medium with fructose and glycerol as carbon sources (0.2% [wt/vol]) for 34 to 40 h. The cells were collected by centrifugation and washed once with 36 mM potassium phosphate buffer (pH 7.0) and then with TE buffer (50 mM Tris-hydrochloride and 20 mM EDTA; pH 8.0). The washed cells (50 mg, wet weight) were resuspended in 0.5 ml of TE buffer. Crude lysates were prepared by treatment of the cells with an alkaline solution of sodium dodecyl sulfate by the method of

Casse et al. (4) with the following modification. The precipitated DNA was recovered by centrifugation at 5,000 × *g* at −4°C for 35 min and subsequently dissolved in 200 μl of TES buffer (pH 8.0) (50 mM Tris-hydrochloride, 50 mM EDTA, and 5 mM NaCl).

Agarose gel electrophoresis. The method of Meyers et al. (11) was used, but it was slightly modified. DNA samples were subjected to electrophoresis in 0.7% (wt/vol) agarose in Tris-borate buffer (pH 8.5) (89 mM Tris-hydrochloride, 2.5 mM Na_2-EDTA, 89 mM boric acid). Electrophoresis was carried out in a vertical slab gel (120 by 130 by 3 mm) at 40 mA and 130 V for 3.5 h. DNA bands were stained with ethidium bromide and visualized on a UV transilluminator.

RESULTS

Characterization of mutants defective in autotrophic metabolism. The stability of the autotrophic character was examined by using selected strains of the hydrogen-oxidizing bacterium *Alcaligenes*. Cells of *A. eutrophus* strain H16, G27, N9A, and TF93 (Table 1) were cultivated under heterotrophic conditions in complex medium for at least eight generations and plated on nutrient agar. Approximately a thousand colonies of each strain were examined to determine their ability to grow with hydrogen and carbon dioxide. For strains H16, G27, and N9A of *A. eutrophus*, no mutants defective in autotrophic metabolism could be detected. However, spontaneous mutants were derived from strain TF93, and their frequency varied from 80% to less than 1% for different experiments. Treatment with mitomycin C yielded mutants defective in autotrophic metabolism of all strains examined. Most of the *A. eutrophus* H16 mutants were obtained by using conventional mutagenesis with nitrous acid or ethyl methane sulfonate after enrichment with cycloserine (8). Mutants used in the subsequent experiments were characterized with respect to reversion and biochemical properties. All spontaneous and mitomycin-induced mutants were found to belong to the same phenotype, as they were unable to oxidize hydrogen but were capable of growth on formate (Table 2), which was evidence that they can fix carbon dioxide (8). This phenotype was denoted Hox^-. Among the various mutants examined, those which originated spontaneously and a few mitomycin-induced mutants did not exhibit any reversion. The majority of mutants did revert, and the frequency of reversion ranged from 10^{-7} to 10^{-9}.

Enzyme analyses revealed that all of the Hox^- mutants tested had lost both the soluble and the particulate hydrogenase activities (Table 2). Mutants lacking activity of either the soluble or the particulate hydrogenase have been isolated from *A. eutrophus* H16 which is the most intensively studied strain of the species (15). However, in contrast to the Hox^- mutants listed in Table 2, the mutants lacking only one of the two hydrogenase activities were still able to grow with hydrogen (data are not shown).

A comparison of enzyme activities among parental strains led to the interesting observation that the membrane fraction of *A. eutrophus* TF93 contained a very low, barely detectable activity of particulate hydrogenase (Table 2). The activity of the NAD-reducing hydrogenase in the soluble extract was as high as that found in the other wild-type strains.

TABLE 2. *Properties of mutants defective in autotrophic metabolism*

Strain	Origin	Growth on		Relevant phenotype	Hydrogenase activity[a]		Presence of plasmid pHG1[b]
		Formate	$CO_2 + H_2$		SH	PH	
H16	Wild type	+	+	Hox^+	4.800	0.920	+
HF18	Nitrous acid	+	−	Hox^-	0	0	+
HF09	EMS[c]	+	−	Hox^-	0.032	0.004	+
HF41	Nitrous acid	−	−	Hox^+ Cfx^- Met^-	3.560	0.920	+
HF60	Mitomycin	+	−	Hox^-	0	0	+
TF93	Wild type	+	+	Hox^+	2.880	0.050	+
TF97	Spontaneous	+	−	Hox^-	0	0	−
TF100	Spontaneous	+	−	Hox^-	0	0	−
TF104	Mitomycin	+	−	Hox^-	0	0	+
N9A	Wild type	+	+	Hox^+	1.970	0.200	+
N9AF02	Mitomycin	+	−	Hox^-	0	0	+
G27	Wild type	+	+	Hox^+	3.600	0.310	+
G27F01	Mitomycin	+	−	Hox^-	0	0	+

[a] Cells were grown in fructose-glycerol-minimal medium, and extracts were prepared as described (6). Soluble (SH) and particulate (PH) hydrogenase activities are expressed as units per milligram of protein.

[b] The presence of plasmids was examined by agarose gel electrophoresis of a crude lysate as described in the text.

[c] EMS, ethyl methane sulfonate.

Plasmid analysis. The fact that Hox^- mutants of *A. eutrophus* appeared spontaneously and after treatment with mitomycin C, which is known as a curing agent, indicated a possible involvement of plasmid DNA in hydrogen metabolism. Advantage was taken of a method developed specifically for the identification of large plasmids (4), and this led to the discovery of a plasmid in all of the hydrogen-oxidizing wild-type strains of *A. eutrophus* included in this study (examples are shown in Fig. 1, wells a and b). The molecular weight of plasmid pHG1 was estimated to be approximately $270 \pm 10 \times 10^6$ (Fig. 2, wells c and e). The following molecular weight standards were used: (i) plasmids of *Rhizobium meliloti* (Fig. 2, wells b and d), whose size is known from their contour lengths measured with the electron microscope and (ii) plasmids of *E. coli* (Fig. 2, wells a and f). The plasmid pattern of Hox^- strains revealed two types; the majority of the mutants derived from *A. eutrophus* H16, N9A, G27, and TF93 still contained the plasmid typical of the corresponding wild types (Fig. 1, wells e, h, and b).

Only a few derivatives of *A. eutrophus* TF93, namely the spontaneous mutants TF97 and TF100 (Table 2), did not contain any plasmid, as judged by agarose gel electrophoresis (Fig. 1, wells c and f).

Conjugational transfer of plasmids conferring antibiotic resistances. By using the broad host range plasmid RP4 (5), it was our initial intention to develop a conjugation system for *A. eutrophus*, and, specifically, we wanted to be able to transfer those genes concerned with autotrophic metabolism. To accomplish this, RP4 was transferred from *E. coli* to various strains of *A. eutrophus* by using the spot-agar mating technique. Transconjugants were selected on minimal medium supplemented with tetracycline. Tetracycline-resistant colonies appeared at a frequency of approximately 5×10^{-6} per donor cell. In intraspecific crosses, this frequency was considerably higher, up to 10^{-3}. Transconjugants showed a multiple resistance against tetracycline, ampicillin, and kanamycin. Occasionally, it was observed that the kanamycin-resistant phenotype was not expressed; however, despite this fact, transconjugants were stable, and RP4 could be transferred back to *E. coli* at a frequency of approximately 10^{-1}. Apparently, *A. eutrophus* is a good donor but a bad recipient of the RP4 plasmid. Mating in liquid medium was possible, but resulted in a much lower frequency of transconjugants; concentrating the cells on filters did not improve the transconjugant frequency as compared with the spot-agar mating technique. Thus, the latter method was routinely used. A temperature of 30°C and a 1/1 ratio of donor and recipient cells provided optimum conditions for conjugation.

Transfer of hydrogen-oxidizing ability between strains of *A. eutrophus*. Attempts to mobilize chromosomal genes of *A. eutrophus* by using the RP4 plasmid resulted in a low frequency of recombinants of approximately 10^{-6} for markers such as Ile or His; however, it was difficult to differentiate between true recombinants and revertants in these experiments. Surprisingly, if Hox^+ transconjugants were selected, the frequency of transfer was at least 100-fold higher. Additionally, a control experiment revealed that Hox^+ transconjugants even appeared in the absence of RP4, and the transfer frequency was the same with RP4-free strains as with RP4-harboring donors (Table 3). Thus, transfer of hydrogen oxidizing ability appeared to be a self-transmissible character.

Transformation can be excluded since (i) attempts to transform *A. eutrophus* with isolated

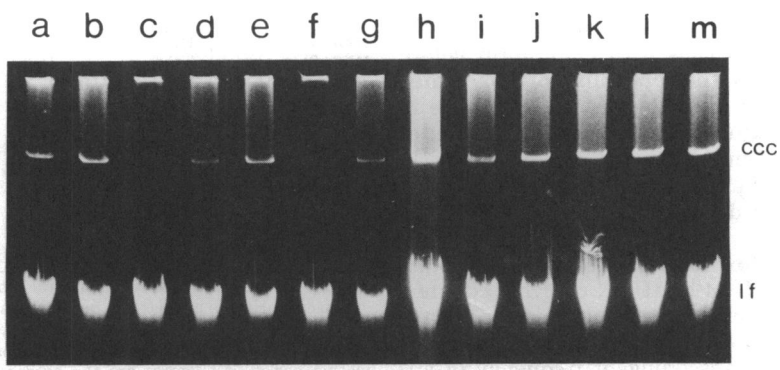

FIG. 1. *Agarose gel electrophoresis of ethanol-precipitated DNA from crude lysates of A. eutrophus strains TF93 (a), HF54 (b), TF97 (c), TF111 (d), HF09 (e), TF100 (f), TF110 (g), HF18 (h), HF67 (i), HF41 (j), N9A (k), N9AF02 (l), and N9AF04 (m). The lower bands in the photograph are linear DNA fragments (lf), and the upper bands represent plasmid DNA (ccc).*

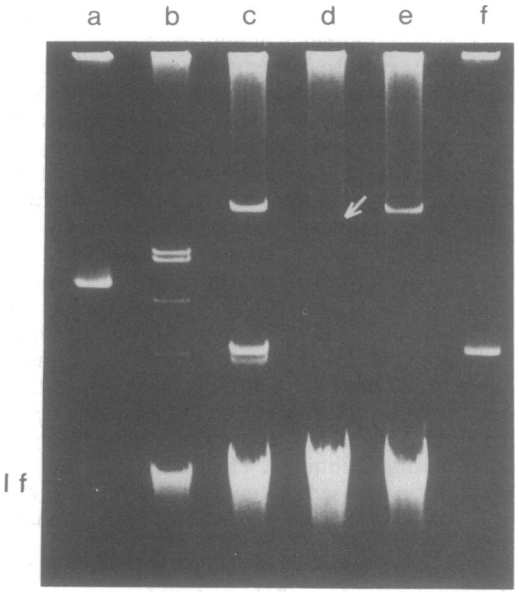

FIG. 2. *Agarose gel electrophoresis of ethanol-precipitated DNA from crude lysates. Lysates were run in wells as follows. (a) E. coli I5 showing plasmid R1drd19 with a molecular weight of 62 × 10⁶ (11). (b) R. meliloti MVII/1 showing four different plasmids. The sequence of bands is listed from the top to the bottom: plasmid pRmMVII/1-a with a molecular weight of 140 ± 8 × 10⁶; pRmMVII/1-b with a molecular weight of 90 ± 2 × 10⁶; pRmMVII/1-c with a molecular weight of 54 ± 1 × 10⁶; pRmMVII/1-d with a molecular weight of 37 × 10⁶ (A. Pühler, unpublished data). (c and e) A. eutrophus HF54 showing, in addition, to plasmid pHG1 (upper band) plasmid RP4 (lower band). (d) R. meliloti 2011 showing a faint band (indicated by arrow) of plasmid pRm2011-a with a molecular weight of 260 × 10⁶ (22). (f) E. coli J53 showing plasmid RP4 with a molecular weight of 36 × 10⁶. lf, Linear DNA fragments.*

plasmid DNA consisting of RP4 and pHG1 under mating conditions were unsuccessful, (ii) killing of the donor cells with chloroform before mating prevented the appearance of Hox⁺ transconjugants, and (iii) the presence of DNase in the mating mixture did not affect the transfer of hydrogen-oxidizing ability. The results clearly indicate that the transfer of hydrogen-oxidizing ability requires direct cell contact and is due to conjugation.

Mating of Hox⁺ derivatives of *A. eutrophus* H16, such as HF54 or HF41, carrying counterselectable markers with the plasmid- free Hox⁻ mutants TF97 or TF100 resulted in an unexpectedly high transconjugant frequency of approximately 10^{-2} per donor (Table 3). Plasmid analyses revealed that transconjugants from these crosses, such as TF110 and TF111, had recovered the plasmid pHG1 (Fig. 1, wells g and d). The frequency of transconjugants was lower, (ca. 10^{-4} per donor) when plasmid-harboring Hox⁻ mutants of *A. eutrophus*, such as HF18, N9AF02, or TF104, were used as recipients (Table 3).

The fact that HF41, defective in carbon dioxide fixation and methionine biosynthesis, was as efficient a donor strain as the double auxotrophic mutant HF54 indicated that the self-transmissible property was restricted only to energy metabolism and not to carbon metabolism. Of 12 Hox⁻ mutants examined, only HF09 was able to function as a donor of hydrogen-oxidizing ability in crosses with TF97 or TF100 (Table 3). However, in contrast to the other Hox⁻ mutants, HF09 appeared not to be a recipient of hydrogen-oxidizing ability, since no transconjugants of HF09 could be detected after crosses with HF41 or HF54.

Biochemical studies showed that all of the transconjugants examined had recovered hydrogenase activity (data are not shown). For instance, transconjugants such as HF66 or HF67 (Table 3), derived from mutant HF18 which was defective in soluble and particulate hydrogenase (Table 2), had acquired wild-type activity of both enzymes. The same was observed with transconjugants derived from N9A or G27. Transconjugants obtained from crosses by using derivatives of *A. eutrophus* H16 such as HF41 or HF54 as a donor and Hox⁻ mutants of *A. eutrophus* TF93 such as TF97 or TF100 as the recipient showed the phenotype of the parental

TABLE 3. *Conjugational transfer of hydrogen-oxidizing ability*

Donor	Recipient[a]	Transfer frequency[b]	Transconjugant designation
HF54(RP4) Trp⁻ Cys⁻	HF18(pHG1)	1.1×10^{-4}	NR[d]
HF54 Trp⁻ Cys⁻	HF18(pHG1)	1.8×10^{-4}	NR
HF54 Trp⁻ Cys⁻	TF97	6.3×10^{-2}	TF111
HF41 Cfx⁻ Met⁻	HF18(pHG1)	1.8×10^{-4}	HF66
HF41 Cfx⁻ Met⁻	TF100	2.5×10^{-2}	NR
HF18 Hox⁻	TF97	NT[c]	
HF09 Hox⁻	TF100	6.5×10^{-2}	TF110
HF09 Hox⁻	HF18(pHG1)	1.1×10^{-4}	HF67
HF41 Cfx⁻ Met⁻	HF09(pHG1)	NT	
HF41 Cfx⁻ Met⁻	N9AF02(pHG1)	1.7×10^{-4}	N9AF04
HF41 Cfx⁻ Met⁻	TF104(pHG1)	8.9×10^{-5}	NR
TF104 Hox⁻	TF97	NT	

[a] The phenotype of the recipients was Hox⁻. The presence of plasmid pHG1 is indicated in parentheses.

[b] Transfer frequency results are expressed as the number of Hox⁺ transconjugants obtained per donor in an overnight mating period. All figures are the average of at least two separate determinations. Variation of transfer frequency between different experiments was generally less than fivefold.

[c] NT, No transconjugant, obtained in at least three attempts.

[d] NR, Not relevant.

strain TF93. Transconjugants examined thus far expressed high activity only of soluble hydrogenase, whereas the membrane fraction contained very low particulate hydrogenase activity. The biochemical data were supported by immunochemical analysis. Extracts of Hox^- mutants including HF09 were antigenically inactive (CRM^-) against antiserum of purified soluble hydrogenase from *A. eutrophus* H16. The corresponding transconjugants, however, were CRM^+, as shown by immunodiffusion (Fig. 3).

DISCUSSION

The data presented in this report clearly establish the existence of a large plasmid (molecular weight, $270 \pm 10 \times 10^6$) in hydrogen-oxidizing strains of *A. eutrophus*. This result is consistent with recent observations of Lim et al. (9) who reported the involvement of a large plasmid in the hydrogen metabolism of *Alcaligenes* strains. In the present communication, we have shown the transfer of hydrogen-oxidizing ability between various strains of *A. eutrophus* via natural conjugation. Cell contact, but no additional mobilizing plasmid such as RP4, was required to promote this transfer. The results presented here contrast with the data of K. Andersen, (personal communication) who achieved only an R-factor mediated transfer of hydrogen utilization at a fairly low frequency. From our experiments, it appears that the frequency of hydrogen-oxidizing ability transfer is dependent upon the type of recipient employed because the most efficient transfer was observed when plasmid-free Hox^- mutants, such as TF97, served as recipients. In these experiments, Hox^+ transconjugants occurred at a frequency of approximately 10^{-2} per donor. A 10- to 100-fold-reduced transfer frequency was obtained with Hox^- recipients which still harbored the plasmid. Presumably, the different yield of transconjugants reflects incompatibility.

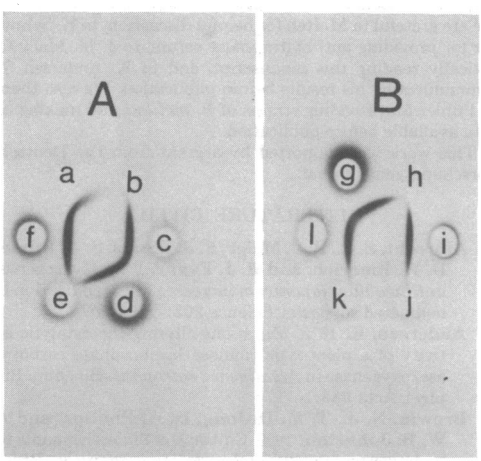

FIG. 3. *Agar gel immunodiffusion (Ouchterlony) of antisoluble hydrogenase serum with crude extrtacts of wild-type strains, Hox^- mutants, and Hox^+ transconjugants of A. eutrophus. The center wells of gel (A) and (B) contained 4 μl of a 1:1 dilution of purified antisoluble hydrogenase serum. To the outer wells were added 7 to 10 μl (ca. 40 μg of protein) of soluble extracts derived from fructose-glycerol-grown cells. The extracts were as follows. Gel A: HF41 (a), HF18 (b), HF66 (c), N9A (d), N9AF02 (e), N9AF04 (f). Gel B: TF93 (g), TF97 (h), TF111 (i), HF09 (j), TF100 (k), TF110 (l). Gels were developed in a moist, sealed atmosphere at room temperature, stained, and photographed.*

A natural conjugation system permitting the transfer of large chromosomal segments has been found in the nitrogen-fixing, hydrogen-oxidizing bacterium *Xanthobacter autotrophicus* GZ29. Linkage analysis of recombinants gives rise to the assumption that the genetic determinants of autotrophic metabolism reside on the chromosome (23). The ability to fix carbon dioxide could be cotransferred with the ability to oxidize hydrogen between strains of *Nocardia*. Direct cell contact is required to allow this transfer (14, 18). Attempts to correlate the autotrophic character with a specific extrachromosomal element have been unsuccessful thus far (M. Reh, personal communication). Recently, Brewin et al. (3) reported that determinants for hydrogenase activity in a strain of *Rhizobium leguminosarum* are genetically linked to determinants for nodulating ability and are probably carried on a large plasmid of molecular weight 190×10^6. Although this plasmid was not self-transmissible, it could be transferred to other rhizobia after recombination with a self-transmissible plasmid.

Because the data presented here are as yet preliminary, it is not possible to draw a firm conclusion concerning the genetic origin of mutations that have led to the Hox^- phenotype. Nevertheless, three classes of mutants can be distinguished as follows. (i) The plasmid-free mutants of *A. eutrophus* TF93 that do not revert; their existance supports the notion that the plasmid contributes an essential function to hydrogen metabolism. (ii) The Hox^- mutants that harbor the plasmid; most of these mutants reverted to the Hox^+ phenotype, although at a relatively low frequency. In mating experiments, they functioned exclusively as recipients and were not donors in crosses with plasmid-free Hox^- strains. These results suggest that the mutation is localized on the plasmid and not on the chromosome. We were unable to detect any differences in the plasmid pattern of these mutants, as compared with the wild type, by agarose gel electrophoresis. However, in view of the

large size of the plasmid, small deletions or rearrangements within the molecule cannot be detected by using this relatively insensitive method. It is anticipated that these differences will be made clear as a result of current restriction analyses. Since Hox^- mutants of *A. eutrophus* H16 have lost the soluble and the particulate hydrogenase activities, it is likely that the plasmid is involved in the expression of both enzymes. This is supported by the fact that transconjugants originating from this class of mutants after crosses with Hox^+ derivatives of *A. eutrophus* H16 have recovered the soluble and particulate hydrogenase activities. Finally, immunochemical studies revealed that the mutants were free of soluble hydrogenase protein, whereas cross-reacting material was present in the corresponding transconjugants. (iii) The third class of mutants is represented by a single mutant HF09. It belongs to the same plasmid-harboring Hox^- phenotype as does the second group. HF09 is unable to grow autotrophically with hydrogen and has almost no activity of soluble and particulate hydrogenase; this mutant is also able to revert. However, HF09 differs from the second group of mutants in that it was capable of transferring hydrogen-oxidizing ability but was unable to function as a recipient. We suspect that the mutation in this strain is the result of a chromosomal lesion occurring while the plasmid is intact. If this is the case, hydrogen-oxidizing ability is not only genetically coded by a plasmid, but requires chromosomal determinants as well. Biochemical studies have shown that both hydrogenases consist of different subunits (16, 20), and it is possible that the enzymes share a common polypeptide. As yet, it is too early to speculate as to whether the plasmid carries all or only a portion of the structural genes, with the chromosome harboring certain regulatory components, or vice versa. In view of increasing interest in hydrogenase systems, for instance, in their energy-recycling function during nitrogen fixation (1), it is certainly important to gain more knowledge concerning the genetic regulation of hydrogen metabolism.

Our results eliminate the possibility that the plasmid of *A. eutrophus* is involved in both the hydrogen and carbon metabolism of autotrophy, as appears to be the case for *N. opaca* (14). The fact that mutants unable to fix carbon dioxide (Cfx^-), such as HF41, transferred hydrogen-oxidizing ability as efficiently as Cfx^+ strains makes it unlikely that carbon dioxide fixation is determined by plasmid genes. Finally, no Cfx^+ transconjugants were observed after conjugation.

The results presented here provide evidence that the strains of *Alcaligenes* investigated in this study are closely related. However, *A. eutrophus* TF93 appears to differ with regard to the following properties. (i) It was the sole strain which could be cured of the plasmid. Although numerous attempts were made, we failed to isolate plasmid-free derivatives of other *Alcaligenes* strains which were characterized by an extremely high stability of the autotrophic marker. (ii) Undoubtedly, the particulate hydrogenase of *A. eutrophus* TF93 is different from the enzyme of the other strains investigated in this study. Under our assay conditions, we could merely detect traces of hydrogenase activity in the membrane fraction. An earlier communication reported a very low methylene blue reducing activity in particles of *A. eutrophus* TF93 (24). Current biochemical investigations indicate that the particulate hydrogenase is present in the strain but differs in some properties from the enzyme of *A. eutrophus* H16 (K. Schneider, personal communication).

We conclude that hydrogen metabolism by *A. eutrophus* is genetically linked to a self-transmissible plasmid, pHG1, which may offer a useful tool for future genetic studies on hydrogenases.

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