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Regulation of Hydrogenase Formation Is Temperature Sensitive and Plasmid Coded in *Alcaligenes eutrophus*

CORNELIUS G. FRIEDRICH* AND BÄRBEL FRIEDRICH

Institut für Mikrobiologie der Universität Göttingen, D-3400 Göttingen, Federal Republic of Germany

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Alcaligenes eutrophus grew well autotrophically with molecular hydrogen at 30°C, but failed to grow at 37°C (Hox Ts). At this temperature the strain grew well heterotrophically with a variety of organic compounds and with formate as an autotrophic substrate, restricting the thermolabile character to hydrogen metabolism. The soluble hydrogenase activity was stable at 37°C. The catalytic properties of the wild-type enzyme were identical to those of a mutant able to grow lithoautotrophically at 37°C (Hox Tr). Soluble hydrogenase was not rapidly degraded at elevated temperatures since the preformed enzyme remained stable for at least 5 h in resting cells or was diluted by growth, as shown in temperature shift experiments. Immunochemical studies revealed that the formation of the hydrogenase proteins was temperature sensitive. No cross-reactivity was detected above temperatures of 34°C. The genetic information of Hox resides on a self-transmissible plasmid in *A. eutrophus*. Using Hox Tr mutants as donors of hydrogen-oxidizing ability resulted in Hox^+ transconjugants which not only had recovered plasmid pHG1 and both hydrogenase activities but also were temperature resistant. This is evidence that the Hox Tr phenotype is coded by plasmid pHG1.

Alcaligenes eutrophus is a facultative chemolithoautotrophic bacterium which is able to grow with hydrogen or formate as energy source and carbon dioxide as the sole source of carbon (5). The initial reactions of autotrophic carbon dioxide fixation are catalyzed by phosphoribulokinase (EC 2.7.1.19) and ribulosebisphosphate carboxylase (RuBPCase; EC 4.1.1.39). The formation of these key enzymes of the Calvin cycle is derepressed during carbon starvation irrespective of whether the cells are cultivated autotrophically or heterotrophically (4).

Molecular hydrogen is activated by *A. eutrophus* by a soluble NAD^+ reducing hydrogenase (hydrogen: NAD^+ oxidoreductase; EC 1.12.1.2) and a membrane-bound hydrogenase linked to the respiratory chain (10, 11). The synthesis of the hydrogenases is derepressed during limitation by the electron donor, whether it is inorganic (molecular hydrogen) or organic (e.g., succinate). Evidence has been obtained that the redox level of the cell is involved in the transcriptional control of these enzymes (4). Previously, we have demonstrated that the ability to oxidize molecular hydrogen (Hox) is coded by a large self-transmissible plasmid designated pHG1 (2). It is unknown whether plasmid pHG1 harbors the structural genes or regulatory components or both of the hydrogenases.

In this communication we report that lithoautotrophic growth of *A. eutrophus* is temperature sensitive and that this is due to a failure of hydrogenase formation at 37°C. At this temperature the bacterium grows well with organic substrates. By means of temperature-resistant (Hox Tr) mutants we demonstrate that the temperature-sensitive control of hydrogenase formation is determined by plasmid pHG1.

MATERIALS AND METHODS

Chemicals. NAD^+, DNase I, and tetracycline hydrochloride were obtained from C. F. Boehringer & Soehne GmbH, Mannheim, West Germany. All other chemicals were of analytical grade and were purchased from E. Merck AG, Darmstadt, West Germany.

Bacterial strains. Strains of *A. eutrophus* used in this study and mutants derived therefrom are listed in Table 1.

Growth conditions. The bacteria were grown in a mineral salts medium as described by Schlegel et al. (12). The concentration of the organic carbon source was 0.4% (wt/vol) and that of ammonium chloride was 0.2% (wt/vol) if not otherwise stated. The gas atmosphere for chemolithoautotrophic growth contained hydrogen, oxygen, and carbon dioxide in a ratio of 8:1:1 (vol/vol/vol). Hydrogenase-derepressing conditions were the same as described previously (1). The growth temperature of the precultures was 30°C, and that of the main cultures was as stated in the text. Solid media contained 1.5% (wt/vol) agar.

Continuous cultures were performed in a chemostat,

TABLE 1. Bacterial strains of *A. eutrophus*

Strain	Relevant phenotype[a]	Reference or source
H16	Hox Ts	Wild type: ATCC 17699, DSM 428
HF116	Hox Tr	Mutant of H16; this study
HF94	Hox Tr	Mutant of H16; this study
HF123	Hox$^-$ Sm^r Tc^r	Mutant of H16; this study
HF30	Hox Ts Sm^r Tc^r	Transconjugant of HF123; this study
N9A	Hox Ts	Wild type; DSM 518
N9AF17	Hox$^-$ Sm^r Tc^r	Mutant of N9A; this study
N9AF34	Hox Ts Sm^r Tc^r	Transconjugant of N9AF17
TF93	Hox Ts	Wild type; ATCC 17697, DSM 531
TF121	Hox$^-$ Sm^r Tc^r	Mutant of TF93; this study
TF132	Hox Tr Sm^r Tc^r	Transconjugant of TF121; this study

[a] Phenotypes are designated as follows: Hox, ability to oxidize hydrogen; Hox Ts and Hox Tr, inability or ability, respectively, to grow with H_2 and CO_2 at 37°C; Sm^r, resistant to 600 μg of streptomycin per ml; Tc^r, resistant to 7 μg of tetracycline per ml.

using a 2-liter Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) of 910-ml working volume and a pH control unit, model TTT2 (Radiometer, Copenhagen, Denmark). The experimental conditions of succinate-limited growth were identical to those described before (4) with the exception of the growth temperature, which is as specified in the text. The steady state was reached when the optical density of the culture and the specific activity of hydrogenase remained constant with time.

Enzyme assays. Cells of about 1 g (wet weight) were suspended in 4 ml of 50 mM potassium phosphate buffer, pH 7.0, and subjected to ultrasonic disruption (1-min treatments per milliliter of cell suspension) in a sonicator (Measuring and Scientific Equipment, Crawley, U.K.). The soluble and particulate fractions were separated by centrifugation (1). The activity of the soluble hydrogenase was assayed from the soluble fraction by monitoring NADH formation spectrophotometrically (13). The activity of the membrane-bound hydrogenase was assayed from the particulate fraction by spectrophotometric determination of hydrogen-dependent methylene blue reduction (10). Soluble hydrogenase and RuBPCase were also determined with whole cells according to Friedrich et al. (6) and Leadbeater et al. (7a), respectively.

The presence of soluble and particulate hydrogenase antigen (cross-reacting material) in cell extracts was determined by double immunodiffusion as reported previously (2). Antisera were prepared as described previously (11). One unit of enzyme activity was defined as the amount converting or producing 1 μmol of substrate or product, respectively, per min at 30°C. Protein of whole cells and cell extracts was determined by the method of Lowry et al. (8).

Isolation of mutants and transconjugants. Mutants able to grow autotrophically with hydrogen at 37°C were isolated by two methods. (i) Cells were either treated with nitrous acid (3) followed by phenotypic expresssion of the mutation in fructose-minimal medium, or (ii) cells were directly plated on minimal medium and incubated lithoautotrophically at 37°C. Temperature-resistant colonies appeared after 5 to 10 days of incubation. Spontaneous drug-resistant mutants were isolated by plating approximately 10^8 cells per plate on fructose-minimal agar supplemented with the antibiotic specified. Mutants which had lost the ability to oxidize hydrogen (Hox$^-$) were isolated as described previously (2, 3).

Transconjugants of the Hox$^-$ mutants were derived from matings with Hox$^+$ donors on nutrient broth followed by selection under lithoautotrophic conditions at 30°C, according to Friedrich et al. (2). Tetracycline was present in the agar to counterselect the donor cells. After purification of the transconjugants, they were screened for ability to grow with hydrogen at 37°C.

RESULTS

Temperature-dependent autotrophic and heterotrophic growth. Under heterotrophic growth conditions, *A. eutrophus* grew at 37°C with a variety of carbon sources such as fructose, glutamate, pyruvate, proline (data not shown), succinate, isoleucine, or glycerol (Table 2). The growth rate on substrates which support only slow growth was even enhanced at 37°C compared with 30°C. No growth occurred above 42°C. Under autotrophic growth conditions with hydrogen, oxygen, and carbon dioxide, the wild type was unable to grow at 37°C. We questioned whether the cells failed to oxidize hydrogen or to fix carbon dioxide.

A. eutrophus grows on formate. Formate is oxidized by formate dehydrogenases to carbon dioxide, which is then assimilated autotrophically via the Calvin cycle (5). Thus, it was necessary to examine whether growth on formate was

TABLE 2. Autotrophic and heterotrophic growth of *A. eutrophus* at 30 and 37°C

Substrate	Doubling time (h) of strains[a]			
	H16		HF116	
	30°C	37°C	30°C	37°C
H_2, CO_2	3.4	NG	3.3	3.1
Formate	4.6	4.1	4.8	4.2
Isoleucine	10.6	4.7	8.8	5.2
Glycerol	38.5	18.7	39.5	20.5
Succinate	1.5	1.3	1.5	1.4

[a] Strain H16 is the wild type (Hox Ts) and strain HF116 is a temperature-resistant mutant derived therefrom. NG, No growth.

TABLE 3. Activities of the key enzymes of autotrophic metabolism during growth of *A. eutrophus* at 30 and 37°C

Strain	Substrate	Sp act (U/mg of protein)			
		Soluble hydrogenase		RuBPCase	
		30°C	37°C	30°C	37°C
H16	H_2, CO_2	0.312	NG[a]	0.083	NG
	Formate	0.153	0	0.060	0.069
	Isoleucine	1.227	0	0.022	0.038
	Glycerol	2.704	0.010	0.028	0.035
	Succinate	0.010	0	<0.001	<0.001
HF116	H_2, CO_2	0.340	0.252	0.074	0.081
	Formate	0.164	0.152	0.044	0.059
	Isoleucine	1.153	0.559	0.028	0.058
	Glycerol	2.896	2.043	0.031	0.051
	Succinate	0.095	0.026	<0.001	<0.001

[a] NG, No growth.

possible at elevated temperatures. The bacterium grew well at 37°C on formate, indicating that the thermosensitivity of lithoautotrophic growth was due to hydrogen oxidation (Table 2).

Autotrophic enzyme activities of cells grown at 30 and 37°C. *A. eutrophus* forms the key enzymes of autotrophic metabolism not only during autotrophic growth, but also under various conditions of heterotrophic growth (4, 6). Thus, it was possible to grow the cells at different temperatures and to examine, independently of autotrophic growth, the activities of the hydrogenases and of RuBPCase. In cells grown at 30°C with hydrogen or formate, the soluble hydrogenase was found at intermediate activities. Cells cultivated heterotrophically with a poor carbon source such as isoleucine or glycerol (Table 3), 2-oxoglutarate, or proline (data not shown) exhibited high hydrogenase activities. Only traces of hydrogenase activities were present in cells grown on a carbon source such as succinate (Table 3). No hydrogenase activities were detected in cells grown at 37°C no matter what the organic substrate was. On the other hand, at 37°C the activity of RuBPCase was not significantly altered; however, it was slightly enhanced compared with that at 30°C (Table 3). Mutant HF116 had gained the ability to grow with hydrogen at 37°C. The growth rates on organic substrates were identical to those of the parent strain (Table 2). The mutant was not altered in RuBPCase activity (Table 3). However, in contrast to the wild type, the temperature-resistant (Hox Tr) mutant HF116 exhibited hydrogenase activities at 37°C. The levels of soluble hydrogenase activity were similar in cells grown autotrophically or heterotrophically at 30 and 37°C (Table 3). Therefore, the question arose as to whether the lack of catalytically active hydrogenases in the wild type at 37°C was due to a thermolability of the hydrogenase proteins, a temperature-sensitive regulation of hydrogenase, or a rapid degradation of the hydrogenases at elevated temperatures.

Temperature-dependent hydrogenase activity. The temperature optimum of the homogeneous soluble hydrogenase (33°C [13]) differed from that of the membrane-bound enzyme (45°C [10]). Mutants lacking either soluble hydrogenase (Hos^-) or particulate hydrogenase (Hop^-) activity were still able to grow autotrophically at 30°C but failed to grow at 37°C (data not shown). This indicated that both hydrogenases were affected by elevated temperatures. Thus, the relatively low temperature optimum of the soluble hydrogenase in vitro could not account for the Hox Ts phenotype. Therefore, the catalytic activity of the soluble hydrogenase from the wild type was determined in vivo at various incubation temperatures and compared with the activity of the Hox Tr mutant HF116. However, no difference in catalytic activity was observed between the two strains. At an assay temperature at 37°C, both strains revealed about 90% of the activity determined at 30°C. The reaction rate decreased substantially above 40°C and ceased above 47°C (Fig. 1). This result suggested that the temperature sensitivity of chemolithotrophic growth was not due to a thermolabile catalytic function of hydrogenases.

Stability of hydrogenase. The soluble hydrogenase was stable in resting cells of *A. eutrophus* at 37°C for at least 5 h. Moreover, hydrogenase activities of both the parent and the Hox Tr strain HF116 were even enhanced by 9 to 17% under this condition (data not shown). To investigate the possibility of a growth-dependent temperature-induced inactivation or degradation of the hydrogenases, wild-type cells were grown in a chemostat with succinate as the limiting nutrient. Under this condition the hydrogenases are highly derepressed (Fig. 3; 4). At a dilution rate

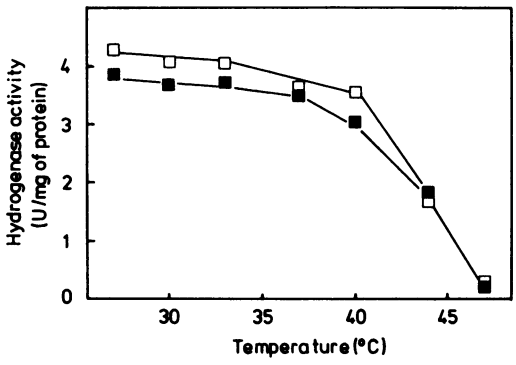

FIG. 1. Temperature-dependent activity of soluble hydrogenase of *A. eutrophus* in situ. Cells were grown in 100 ml of fructose-glycerol mineral medium (1) for 48 h at 30°C. Soluble hydrogenase activities of the wild-type H16 (■) and the Hox Tr mutant HF116 (□) were determined with whole cells.

of 0.055/h, the growing culture was subjected to a temperature shift from 32 to 34°C and the activity of the soluble hydrogenase was followed. At 32°C *A. eutrophus* formed high activities of the soluble hydrogenase. However, after the temperature shift the activity decreased in first-order kinetics with time at a rate of 0.056/h (Fig. 2). This rate was consistent with the dilution rate and evidence that the hydrogenase

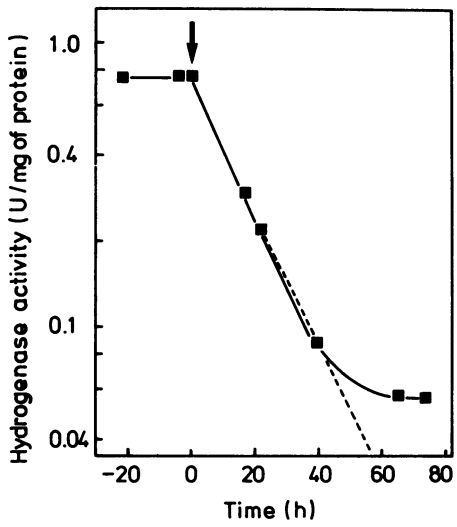

FIG. 2. Decrease of soluble hydrogenase activity of *A. eutrophus* after a temperature shift in a chemostat. *A. eutrophus* was grown in a succinate-limited chemostat at 32°C at a dilution rate of 0.055/h as described in the text. The activity of the soluble hydrogenase was followed by using the whole-cell assay. Arrow indicates the temperature shift to 34°C. Dashed line indicates the theoretical decrease in activity caused by washout.

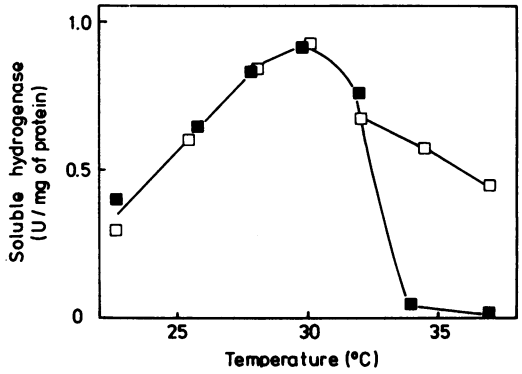

FIG. 3. Temperature-dependent hydrogenase formation during growth of *A. eutrophus* in a chemostat. The wild-type strain H16 (■) and the Hox Tr mutant strain HF116 (□) were grown at various temperatures in a succinate-limited chemostat as described in the text. The dilution rate of strain H16 was 0.055/h and that of HF116 was 0.058/h. From both strains the soluble hydrogenase activities were determined from whole cells.

protein was not rapidly degraded at this temperature.

Temperature optimum of hydrogenase formation. The temperature-dependent formation of hydrogenase activities was determined with the wild-type strain H16 and the Hox Tr mutant HF116. Cells were grown in a succinate-limited chemostat at a low dilution rate of about 0.06/h. The soluble hydrogenase activity of the wild type was at a maximum at a growth temperature of 30°C. A drastic decrease in the steady-state activity was observed between 32 and 34°C, and no activity could be determined during growth at 37°C (Fig. 3). The Hox Tr mutant HF116 also exhibited maximum soluble hydrogenase activity at 30°C. In contrast to the parent, however, the activity decreased gradually with increasing growth temperature. At 37°C about 50% of the maximum activity at 30°C was present (Fig. 3). In the experiments shown in Fig. 3 the activities of the membrane-bound hydrogenase also were determined from the particulate fraction of cell extracts of both strains. These activities followed the kinetics characteristic for the soluble hydrogenase (data not shown).

Furthermore, immunological analysis with antisoluble and antiparticulate hydrogenase sera revealed that no cross-reacting material was formed by the wild type at the nonpermissive temperature at 37°C (Fig. 4A and B, well f). However, at this temperature the Hox Tr mutant HF116 had gained the ability to form cross-reacting material (Fig. 4C and D, well f). This result supported the notion that the synthesis of the hydrogenases was a thermolabile character.

Genetic transfer of the temperature-resistant phenotype. Mutants able to grow at 37°C (Hox

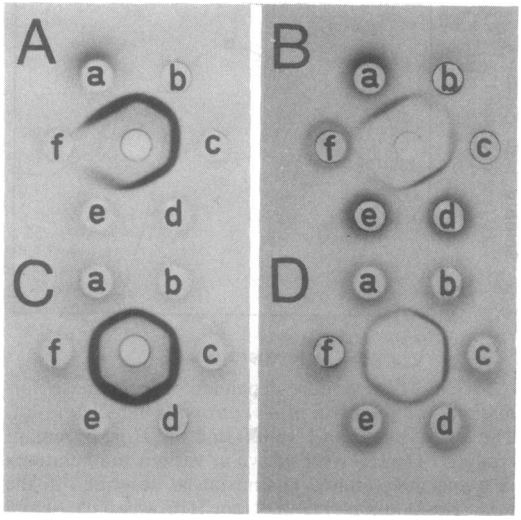

FIG. 4. Ouchterlony double-diffusion analysis of hydrogenase formation at various temperatures. Cells of the wild type (A and B) and the Hox Tr mutant HF116 (C and D) were grown in a succinate-limited chemostat as described in the legend to Fig. 3. Samples were taken at the following temperatures: 26°C (a), 28°C (b), 30°C (c), 32°C (d), 34°C (e), 37°C (f); extracts derived therefrom were analyzed for the presence of hydrogenase protein (2, 11). The inner wells of (A) and (C) contained purified antisoluble hydrogenase serum; those of (B) and (D) contained purified antiparticulate hydrogenase serum. Soluble (A and C) and particulate (B and C) extracts were added to the outer wells as indicated above.

Tr) with hydrogen as the sole energy source occurred spontaneously at a frequency of approxiately 1.4×10^{-7}. After treatment of the cells with a mutagen such as nitrous acid the frequency was enhanced to 2×10^{-5}. To examine whether the mutation that leads to the temperature-resistant phenotype is located on plasmid pHG1 or on the chromosome, mating experiments were performed. Two Hox Tr mutants were used as donors: the spontaneous mutant HF94 and mutant HF116, which had been isolated after mutagen treatment. Hox^- mutants, which lacked catalytically and serologically active hydrogenases were used as recipients (Table 4). Mutant TF121 was plasmid-free, whereas the other Hox^- mutants presumably harbored plasmid mutations (2). All of the recipients were tetracycline resistant. Hox^+ transconjugants were isolated at 30°C under autotrophic conditions in the presence of tetracycline to counterselect the donor cells. Although the drug decreased the transfer frequency significantly, Hox^+ transconjugants could still be isolated at a frequency of 10^{-3} to 10^{-5}. After purification of the transconjugants, they were examined for their ability to grow autotrophically at 37°C in the presence of tetracycline. Of several hundred colonies tested so far, all were temperature resistant, indicating that this character is self-transmissible and plasmid coded. Biochemical analysis revealed that the Hox Tr transconjugants had recovered both the soluble and the particulate hydrogenase activities irrespective of whether the cells were cultivated at 30 or 37°C (Table 4).

DISCUSSION

In this communication we have shown that autotrophic growth of *A. eutrophus* with molecular hydrogen is a temperature-sensitive character. That the bacterium grew well at 37°C with a

TABLE 4. Hydrogenase activities of Hox mutants and Hox Tr transconjugants

Donor	Recipient	Transconjugant	Temp of growth (°C)[a]	Hydrogenase activity[b]			
				Catalytic (U/mg of protein)		Serological	
				SH	PH	SH	PH
HF116			30	3.84	2.56	+	+
			37	3.82	0.30	+	+
HF94			30	3.10	2.83	+	+
			37	4.70	0.72	+	+
	HF123		30	0	0	−	−
	N9AF17		30	0	0	−	−
	TF121		30	0	0	−	−
HF116	HF123	HF30	30	3.03	1.56	+	+
			37	3.27	0.99	+	+
HF116	N9AF17	N9AF34	30	2.56	2.15	+	+
			37	2.84	0.70	+	+
HF94	TF121	TF132	30	0.95	<0.01	+	+
			37	0.69	<0.01	+	+

[a] Cells were grown in fructose-glycerol medium as described previously (1).

[b] SH, Soluble hydrogenase; PH, particulate hydrogenase. The serological activity was determined by double immunodiffusion with antisoluble and antiparticulate hydrogenase serum: +, cross-reacting material present; −, cross-reacting material absent.

variety of organic substrates clearly restricts the temperature sensitivity to the lithoautotrophic metabolism. Furthermore, cells grew well at 37°C with formate and revealed active RuBPCase at this temperature. *A. eutrophus* oxidizes formate to CO_2, which is then assimilated via the Calvin cycle (5). These results exclude the possibility that elevated temperatures affect autotrophic carbon dioxide fixation and rather suggest that hydrogen oxidation is thermolabile.

We took advantage of the observation that derepression of hydrogenase formation does not depend on lithoautotrophic growth conditions (6). Cells grown in batch cultures with fructose-glycerol-mineral medium (1) or in a succinate-limited chemostat (4) at 30°C contain extremely high activities of soluble and particulate hydrogenases. However, at the restrictive temperature the cells were free of catalytically and serologically active hydrogenases. By shifting the temperature from 32 to 34°C we could demonstrate that hydrogenase was diluted by growth and not rapidly degraded or inactivated. Furthermore, preformed hydrogenase of resting cells remained active for several hours upon exposure to elevated temperatures. These results are in good agreement with the properties described for homogeneous soluble and particulate hydrogenase (10, 13). Finally, mutants which had gained the ability to grow lithoautotrophically at 37°C did not exhibit any alteration in the catalytic properties of hydrogenases or in the regulation pattern at 30°C. However, in contrast to the wild type, which failed to derepress hydrogenase formation at 37°C, the temperature-resistant mutants escaped this repression.

A temperature control has also been reported for nitrogen fixation of *Klebsiella pneumoniae* (7). It was shown recently that mutations in the regulatory gene *nifL* altered *nif* regulation such that nitrogenase synthesis was no longer repressed by high temperature (9).

Hydrogenase genes of *A. eutrophus* or other hydrogen-oxidizing bacteria have not been precisely mapped so far. We recently reported on a large self-transmissible plasmid of *A. eutrophus* which harbors essential information of hydrogen-oxidizing ability (2). By using the temperature-resistant (Hox Tr) mutants as donors for Hox in matings with hydrogenase-deficient (Hox^-) mutants, we were able to show that the Hox Tr phenotype was transferable by conjugation. Since we never observed the mobilization of chromosomal markers at such a high frequency (2), we conclude that the mutation which rendered the mutants temperature resistant is plasmid-borne. These results led to our current model that the expression of the hydrogenase structural genes requires a gene product whose formation or structure is temperature sensitive.

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