EVIDENCE FOR PLASMID CODING OF THE ABILITY TO UTILIZE HYDROGEN GAS BY PSEUDOMONAS FACILIS

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Summary

Growth of Pseudomonas facilis in the presence of sublethal concentrations of rifampin, ethidium bromide, and mitomycin C and at a sublethal temperature, 37 C, results in a high percentage of cells unable to utilize hydrogen gas as an energy source. This high rate of curing indicates an extrachromosomal location for the hydrogenase. This is further substantiated by lack of reversion of this characteristic upon repeated transfer or back mutation.

Introduction

Pseudomonas facilis, formerly known as Hydrogenomonas facilis, is characterized by its ability to grow either heterotrophically on an organic medium or autotrophically on a mineral salts medium with a gas mixture containing hydrogen, carbon dioxide and air. In the latter medium hydrogen serves as the energy source and carbon dioxide as the carbon source (5).

The ability of many pseudomonas strains to utilize a wide variety of substances has been found to be due to the presence of extrachromosomal DNA encoding the degradative enzymes. Chakrabarty has provided genetic evidence for the presence of these degradative plasmids coding for enzymes involved in the utilization of salicylate, octane, and camphor. Recently, by use of special methods, physical evidence for these plasmids in P. putida was found (3). Plasmids which code for drug resistance in pseudomonas have been extensively studied by Datta et al. (2).

These reports led us to investigate whether the genes for hydrogenase in P. facilis are located on a plasmid. P. facilis utilizes

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hydrogen by means of a hydrogenase bound to membranes which oxidize hydrogen by a complete respiratory chain in which oxygen or nitrate can serve as terminal acceptors. The primary hydrogen acceptor is unknown but the enzymatic action provides hydrogen for respiration and coupled ATP synthesis. The hydrogenase is induced by hydrogen and induction is repressed by the presence of organic compounds. Hydrogenase activity is assayed by measuring the rate of methylene blue reduction by the intact cells in the presence of hydrogen gas (6). To examine the possibility that hydrogenase is plasmid coded, we grew cells in the presence of agents which are reported to "cure" bacteria of plasmids. The isolation of a large number of strains which lost the ability to utilize hydrogen gas as an energy source and were unable to reduce methylene blue in the presence of hydrogen gas suggests that the information for this enzyme is plasmid coded.

Materials and Methods

Bacterial strain and bacteriophage. The original strain of P. facilis ATCC 15376 was obtained as Hydrogenomonas facilis from R. Burris, Department of Biochemistry, University of Wisconsin. Phage strain HF was isolated in this laboratory.

Growth conditions. For autotrophic growth, a defined basal medium (Ks) was used which contains KH_2PO_4, 3.0 g; K_2HPO_4, 7.0 g; $(NH_4)_2SO_4$, 1.0 g; NaCl, 1.0 g and distilled water, 1 l, supplemented with 1 mM $MgSO_4$ and 0.1 mM $CaCl_2$ (autoclaved separately) and 15 g agar. The cells were grown at room temperature in a gas mixture containing 10% CO_2, 60% H_2, and 30% air.

For heterotrophic growth, the gas mixture was replaced with sodium acetate, 1.2 mg/ml (1).

Hydrogenase assay. The cells were grown on Ks medium supplemented with sodium acetate in the gas mixture on solid medium. The cells were suspended in 0.07 M Sorensen phosphate buffer (pH 7.4) centrifuged and the pellet resuspended in phosphate buffer to give a Klett reading (red filter) of 150. Hydrogenase activity was measured by adding 1 ml of the cell suspension to the side arm of a Thunberg tube. Four ml of phosphate buffer (pH 7.4) and 1 ml of 0.12 mM methylene blue were added to the main tube. The tube was evacuated, and the air was replaced with hydrogen. The contents were mixed, and the decrease in adsorption was measured using a Klett colorimeter (red filter) (1).

Curing. The cells were grown in peptone broth, (4 g/l) on a shaker at 29 C in the presence of the curing agent at the highest concentration

just permitting growth. The final concentration of agents used were ethidium bromide (Calbiochem) 50 µg/ml; mitomycin C (Schwarz-Mann) 4 µg/ml; and rifampin (Schwarz-Mann) 20 µg/ml. After 24 hr incubation the culture was streaked on peptone agar, the colonies which arose were then picked and spotted on Ks medium with acetate and Ks medium supplemented with H_2, CO_2 and air gas mixture. Strains unable to grow autotrophically were assayed for hydrogenase activity and tested for phage sensitivity.

Cells were heat cured by transferring the culture to peptone agar slants every 48 hrs for eight transfers and incubating the slants at 37 C.

Results

Growth of P. facilis cells at the upper temperature limit, 37 C, resulted in the selection of cells which were unable to utilize hydrogen gas as an energy source. To insure that the repeated transfers had not resulted in the isolation of a contaminant, the hydrogen negative strains were tested for sensitivity to a phage which is specific for P. facilis. All isolated strains were phage sensitive.

Isolation of a hydrogen negative strain by repeated transfer at 37 C may be the result of selection of a chromosomal mutant able to grow better at higher temperature. Therefore, the cells were grown in sublethal concentrations of various agents previously shown to cure bacterial cells of carried plasmids. The agents used were ethidium bromide, mitomycin C, and rifampin, added to peptone broth. The resultant growth was streaked on peptone agar and the colonies tested for the ability to grow autotrophically on Ks medium with H_2, CO_2, and air or heterotrophically on Ks medium supplemented with sodium acetate. The results are given in Table 1.

Colonies unable to grow autotrophically but able to grow heterotrophically were then streaked on Ks + acetate and incubated in a H_2, CO_2 and air mixture and tested for presence of the hydrogenase by the ability to reduce methylene blue in a hydrogen atmosphere. The results of the ability to reduce methylene blue by representative strains is given in figure 1. All strains unable to grow autotrophically in the presence of H_2, CO_2 and air were also unable to reduce methylene blue.

Table 1. Curing of Plasmid in P. facilis

Agent	No. of colonies K_s + acetate	No. of colonies K_s + H_2, CO_2 + air	% Cured
Rifampin 20 µg/ml	196	104	46
Ethidium Bromide 50 µg/ml	122	52	57
Mitomycin C 4µg/ml	146	22	85
Control	228	228	0

Discussion

Several colonies isolated after growth of P. facilis following exposure to the agents rifampin, ethidium bromide, and mitomycin C were no longer able to utilize hydrogen gas as an energy source. The high percentage of cured cells in the population indicates that the ability to utilize hydrogen gas is coded for on a plasmid rather than on the chromosome. The heat cured strain has been transferred frequently over the period of a year and no back mutations have been found. Back mutation would be expected if the loss of hydrogen utilization were due to a chromosomal point mutation. All strains are still sensitive to a phage strain which is specific for P. facilis which precludes the possibility of the isolation of a contaminant.

The ability to utilize hydrogen gas depends upon the enzyme, hydrogenase, which in P. facilis is bound to the membrane. The membrane bound hydrogenase oxidizes hydrogen by a complete respiratory chain in which oxygen can serve as a terminal acceptor and is coupled to ATP synthesis (6). Since the hydrogenase is induced by the presence of

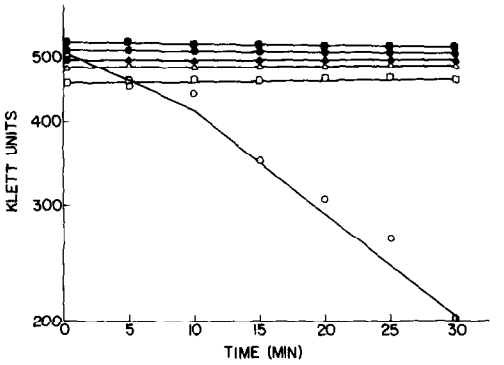

Figure 1. Hydrogen Utilization by P. facilis Strains.

Time course of methylene blue reduction in the presence of hydrogen gas by P. facilis ATCC 15376 O; heat cured strain Δ; rifampin cured strain Δ; mitomycin C cured strain O; ethidium bromide cured strain ; control, no cells .

hydrogen and repressed by organic compounds, the cured strains were tested after growth on a synthetic medium in which sodium acetate was the energy source and where hydrogen gas was present in the atmosphere.

The presence on a plasmid of the genetic code for hydrogen utilization is not surprising. Plasmids found in other pseudomonas strains are related to fertility, antibiotic resistance, a mercury resistance and some code for catabolic enzymes (7). The presence of plasmids which can be transferred from strain to strain undoubtedly contributes to the metabolic diversity of these soil organisms.

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