Mutants of *Pseudomonas facilis* Defective in Lithoautotrophy

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(Received 26 July 1985; revised 2 September 1985)

Mutants of *Pseudomonas facilis* impaired in lithoautotrophic metabolism (Aut⁻) were obtained by Tn5 mutagenesis, mitomycin C treatment or incubation at sublethal temperature. A colony assay provided a fast screening method for hydrogenase-negative mutants. Three classes of mutants were identified: the first had lost the ability to grow autotrophically with H₂ and heterotrophically with NO₃⁻ as nitrogen source; the second lacked both hydrogenase and ribulose-1,5-bisphosphate carboxylase activity but nitrate metabolism was unaffected; the third retained the activities of the key enzymes of lithoautotrophic metabolism but failed to grow with H₂ and CO₂. All of the mutants showed the plasmid pattern of the wild-type.

INTRODUCTION

Hydrogenases are found in many species of bacteria, both aerobic and anaerobic. The molecular properties of these enzymes are rather diverse. According to their physiological function hydrogenases can be subdivided into those that produce H₂ and those that consume it (Cammack *et al.*, 1984). The latter type, the so-called uptake hydrogenase, supplies the cell with energy and/or reducing power from H₂ (Bowien & Schlegel, 1981). Several hydrogenases have been characterized biochemically, as reviewed recently by Cammack *et al.* (1984). Little is known about the genes and the regulation involved in this enzyme system.

Pseudomonas facilis is a Gram-negative, facultatively chemolithoautotrophic bacterium which is able to grow in mineral salts medium under an atmosphere of H₂, O₂ and CO₂. CO₂ is assimilated via the Calvin cycle, the key reaction of which is catalysed by ribulose-1,5-bisphosphate carboxylase (RuBPCase), and H₂ is oxidized by an uptake hydrogenase. *P. facilis* is a member of the major physiological group of hydrogen bacteria which is characterized by the possession of a single, membrane-bound, respiratory-chain-linked hydrogenase (Schneider & Schlegel, 1977).

Indirect evidence has been presented for a plasmid location of hydrogenase genes in *P. facilis* (Pootjes, 1977), which is the host of a relatively large plasmid of about 230 MDa (Gerstenberg *et al.*, 1982). However, plasmid-encoded hydrogenase genes have been conclusively identified only in *Alcaligenes eutrophus* (Andersen *et al.*, 1981; Friedrich *et al.*, 1981a) and *Rhizobium leguminosarum* (Brewin *et al.*, 1980). CO₂ fixation genes are also associated with the indigenous megaplasmid of *A. eutrophus* (Andersen & Wilke-Douglas, 1984; Bowien *et al.*, 1984). Hydrogenase genes in *Escherichia coli* (Krasna, 1984), *R. japonicum* (Haugland *et al.*, 1984) and *Azotobacter chroococcum* (Yates & Robson, 1985) have not been reported to be plasmid encoded.

Genetic investigations of the particulate hydrogenase of *A. eutrophus* were complicated by the fact that mutants impaired in that activity were not altered phenotypically. They grew with H₂ as fast as the wild-type due to the functioning of the soluble, NAD-reducing hydrogenase

Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase; NB, nutrient broth; Km, kanamycin; TTC, 2,3,5-triphenyltetrazolium chloride.

(Schink & Schlegel, 1978; Hogrefe *et al.*, 1984). This prompted us to initiate studies with hydrogen bacteria which contain only the membrane-bound enzyme.

In this communication we report the isolation and characterization of lithoautotrophically defective mutants of *P. facilis* and describe different procedures of mutagenesis and screening.

METHODS

Bacterial strains. *P. facilis* (DSM 550, ATCC 17695) and *A. eutrophus* H16 (DSM 428, ATCC 17699) were obtained from the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, Federal Republic of Germany. *Escherichia coli* strains SM10 and S17-1 carrying pSUP5011 were used for transposon mutagenesis (Simon *et al.*, 1983).

Media and growth conditions. The bacteria were grown either in nutrient broth (NB) or in mineral salts medium as described by Schlegel *et al.* (1961) with NH_4Cl (0.2%) as nitrogen source. Organic carbon substrates were supplied at a concentration of 0.4%. An atmosphere of $\text{H}_2/\text{O}_2/\text{CO}_2$ (8:1:1, by vol.) was used to provide autotrophic growth conditions. Growth experiments were done at 30 °C on a rotary shaker with 30 ml cultures in 300 ml side-arm flasks. Growth was measured with a Klett-Summerson colorimeter equipped with a 520 to 540 nm filter.

Mutagenesis. *P. facilis* was grown in NB medium in the presence of the mutagen mitomycin C (0.05 to 0.5 $\mu\text{g ml}^{-1}$) for 24 to 72 h. The cells were washed with NB medium and plated onto NB agar.

For transposon mutagenesis the method of Srivastava *et al.* (1982) was modified as follows: Tn5-mob was transferred from *E. coli* SM10 or S17-1 to *P. facilis* by the vector plasmid pSUP5011 containing a P-type-specific recognition site for mobilization in Tn5. This plasmid is unable to replicate in hosts other than *E. coli* (Simon *et al.*, 1983). Kanamycin (Km)-resistant colonies were selected on mineral medium containing fructose and 80 $\mu\text{g Km ml}^{-1}$.

Mutant detection. The simplest, although laborious, procedure for mutant identification was the transfer to mineral agar of single colonies obtained after mutagenesis. The plates were incubated under $\text{H}_2/\text{O}_2/\text{CO}_2$. Growth was examined after 4 to 5 d incubation.

Hydrogenase-negative mutants of *P. facilis* could be identified by the specific colony staining technique of Schlegel & Meyer (1985). The assay was modified as follows. After mutagenesis, cells were plated on mineral agar containing 0.1% glycerol and incubated for 5 to 6 d. The plates were sprayed with 2 to 3 ml of a dye solution consisting of 2 vols 4% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) and 1 vol. 0.2 M-monoiodoacetate, both dissolved in 35 mM-phosphate buffer. The two solutions were mixed before use, and the pH was adjusted to 7.0. The plates were incubated for 1 h under an atmosphere of H_2 . Hydrogenase-containing colonies turned purple-red due to H_2 -dependent TTC reduction whereas hydrogenase-free cells remained unstained. Kanamycin-containing plates were incubated under H_2 with complete exclusion of O_2 , achieved by the addition of a gas pack (Anaerocult; Merck).

Enzyme assays. Whole cells were used to determine the activity of key enzymes of lithoautotrophic metabolism. The growth conditions are specified in Results and Discussion. Membrane-bound hydrogenase activity was measured by H_2 -dependent methylene blue reduction (Schink & Schlegel, 1979), modified as follows. Cells were washed and resuspended in 50 mM-phosphate buffer, pH 7.0. The OD_{436} , measured with a Zeiss PL4 spectrophotometer, was adjusted to 30. The assay mixture contained 2.9 ml 0.17 mM-methylene blue in 50 mM-potassium phosphate buffer, pH 7.0, saturated with H_2 . The reaction was initiated by the addition of cells. The cuvettes were incubated anaerobically at 30 °C, and the decrease in A_{570} was followed. Controls were run routinely by substituting N_2 for H_2 gas.

RuBPCase (EC 4.1.1.39) was determined according to Leadbeater *et al.* (1982). One unit (U) of enzyme activity is defined as 1 μmol substrate converted or product formed min^{-1} . Protein was determined by the Lowry method.

Immunodiffusion experiments. The presence of hydrogenase antigen in the membrane fraction was determined by double immunodiffusion in Ouchterlony plates (Friedrich *et al.*, 1981a). Cells were subjected to ultrasonic disruption (0.5 min treatments per ml of cell suspension, amplitude 10) in an MSE sonicator. The soluble and particulate fractions of the extract were separated by centrifugation. Membrane extracts were obtained by solubilizing the particulate fraction as described by Schink & Schlegel (1980). Purified antiserum raised against the particulate hydrogenase of *A. eutrophus* H16 was provided by H. G. Podzuweit and K. Schneider (Institut für Mikrobiologie, Göttingen, FRG).

Analysis of plasmid DNA. Crude lysates were prepared by the method of Kado & Liu (1981), modified as described by Hogrefe & Friedrich (1984). Lysates were analysed by agarose gel electrophoresis as described by Friedrich *et al.* (1981a). Purification of plasmid DNA and endonuclease digestion were done as reported by Hogrefe & Friedrich (1984).

Chemicals. Mitomycin C, kanamycin sulphate and agarose type V were obtained from Sigma. Ribulose 1,5-bisphosphate was a gift from G. Whitesides (Harvard University, Cambridge, Mass., USA). $\text{NaH}^{14}\text{CO}_3$ was from Amersham. Other chemicals were purchased from Merck.

Table 1. *Hydrogenase and RuBPCase activities in P. facilis*

Growth substrate	Doubling time (h)	Specific activity [mU (mg protein) ⁻¹]*					
		Hydrogenase			RuBPCase		
H ₂ /O ₂ /CO ₂	24.0	731	1765	1995	89	105	106
Pyruvate	4.2	125	105	59	0	0	0
Succinate	3.3	76	74	32	0	0	0
Fructose	3.5	99	192	153	0	0	0
Gluconate	5.5	458	476	444	0	0	0
Glycerol	8.5	504	471	218	0	0	0

* The first value represents the activity of cells taken from the mid-exponential phase, followed by values from the early and the late stationary phase of growth. Each number is the mean value of three independently conducted assays.

RESULTS AND DISCUSSION

Formation of key lithoautotrophic enzymes

To characterize lithoautotrophically deficient (Aut⁻) mutants, conditions had to be defined which allowed the expression of the key enzymes independent of growth with H₂ and CO₂. Hence, *P. facilis* wild-type was cultivated heterotrophically on organic substrates such as succinate and fructose, which supported fast growth, or glycerol and gluconate, which supported slow growth (Table 1). The activity of hydrogenase was determined at different stages of growth and was found to be present under both autotrophic and heterotrophic conditions. Slowly growing cells had relatively high hydrogenase activity whereas the enzyme activity was low in rapidly growing cells. On organic substrates a decrease in hydrogenase activity was observed when the cells entered the late stationary phase of growth. RuBPCase activity was only detected in cells cultivated autotrophically with H₂, and remained constant over a wide period of time (Table 1).

The increased hydrogenase activity in slowly growing cells of *P. facilis* is consistent with data obtained with *A. eutrophus*, in which high hydrogenase activities are achieved under conditions of limited energy supply (Friedrich *et al.*, 1981*b*), starvation for electron donors being the regulatory signal of hydrogenase derepression (Friedrich, 1982).

Lithoautotrophically defective mutants

The fact that little is known about hydrogenase genes and their regulation is mainly due to the absence of a suitable procedure for the isolation of mutants. Strains of *P. facilis* which failed to grow with H₂ and CO₂ were detected at a frequency of 0.2% among cells which had been grown at a sublethal temperature of 39 °C or treated with mitomycin C. Transposon Tn5 provided an efficient tool to generate Aut⁻ mutants (Table 2). Km-resistant colonies of *P. facilis* occurred at a frequency of 2×10^{-6} per donor cell in matings with *E. coli* S17-1/pSUP5011 or Sml10/pSUP5011; 0.4–0.5% of the Km-resistant colonies were Aut⁻ mutants.

Mutants specifically impaired in H₂ metabolism were identified by a colony staining assay with TTC as electron acceptor in a H₂-dependent reduction reaction. This assay was particularly useful for the detection of Tn5-induced mutants. Km-resistant colonies were selected under hydrogenase-derepressing conditions on glycerol-containing agar and subsequently screened for hydrogenase activity. TTC eliminated the need to test every presumptive mutant biochemically for enzyme activity. Although the procedure is relatively simple it is important to note that consistent dye reduction was only observed under conditions of hydrogenase derepression. Care must also be taken to avoid non-specific reduction of TTC which was observed with cells grown at a substrate concentration exceeding 0.1%. This was easily achieved by growing the cells under glycerol-limitation (0.1%) that allowed derepressed hydrogenase synthesis and simultaneously decreased non-specific reduction of TTC. The method was further improved by inhibiting intermediary metabolism by monoiodoacetate as recently described by Schlegel & Meyer (1985), and by excluding oxygen which is a competitive electron acceptor of TTC.

Table 2. *Lithoautotrophically defective mutants of P. facilis*

Strain	Mutagen/ mutagenic treatment	Growth on:*		Specific activity [mU (mg protein) ⁻¹]†	
		H ₂ /CO ₂ /O ₂	Nitrate	Hydrogenase	RuBPCase
Wild-type	None	+	+	760	90
JF19	Tn5	—	—	0	0
JF46		—	—	0	0
JF42		—	+	0	0
JF43		—	+	0	0
JF78		—	+	0	0
JF26		—	+	920	77
JF9	Mitomycin C	—	+	1030	94
JF11		—	+	990	102
JF4	Growth at 39 °C	—	+	920	120
JF6		—	+	840	117

* +, growth; —, no growth. Nitrate served as the nitrogen source in the presence of fructose as the carbon source.

† Hydrogenase activity was determined in cells cultivated in glycerol–mineral medium for 4 d. RuBPCase activity was determined in cells pregrown in fructose–mineral medium for 1 d under air and shifted to an atmosphere of H₂/O₂/CO₂ for 4 d. The numbers are mean values of three independently conducted assays.

Three phenotypically different classes of Aut[−] mutants were discovered (Table 2). Among the Tn5-induced mutants we detected Aut[−] isolates which also failed to use nitrate as a nitrogen source (Nit[−]) under heterotrophic conditions. These pleiotropic mutants are represented by strains JF19 and JF46 (Table 2). Aut⁺ revertants simultaneously recovered the ability to grow with nitrate; likewise, reversion to Nit⁺ led to the reconstitution of the Aut⁺ phenotype. All of the revertants were Km-sensitive, indicating the excision of the transposon. The data are consistent with the notion that the pleiotropic strains resulted from a single mutation. The occurrence of similar pleiotropic, hydrogenase-deficient mutants has been described for *A. eutrophus*. The possibility has been discussed that these mutants suffer from a lesion in a regulatory gene whose product is necessary for the expression of genes involved in hydrogen and nitrate metabolism (Hogrefe *et al.*, 1984). Moreover, mutants of *E. coli* K12 have been isolated which were simultaneously affected in nitrate reductase, nitrite reductase, fumarate reductase, hydrogenase activity and in cytochrome C-552 production (Chippaux *et al.*, 1978).

The pleiotropic Aut[−] mutants had lost both hydrogenase and RuBPCase activities. Since there is immunological cross-reactivity between the hydrogenase of *P. facilis* and antiserum raised against the particulate hydrogenase from *A. eutrophus* (Schink & Schlegel, 1980; Fig. 1*a*), we were able to use immunological techniques to further characterize the Aut[−] mutants of *P. facilis*. The pleiotropic mutants were immunologically active (Fig. 1*b*, wells 1 and 5). However, the cross-reaction was not completely identical with that of the parental strain (Fig. 1*b*). Thus we assume that the hydrogenase protein of the pleiotropic mutants is at least structurally different from the wild-type enzyme.

A second class of Aut[−] mutants, represented by strains JF42, JF43 and JF78, was exclusively impaired in lithoautotrophic metabolism (Table 2). Neither hydrogenase nor RuBPCase activities could be detected. Immunological studies of the membrane fraction showed two precipitin bands one of which was identical with that of the wild-type (Fig. 1*b*, wells 2 and 4).

The absence of RuBPCase activity in cells of all hydrogenase-negative mutants tested so far may reflect a regulatory problem of expressing the CO₂ fixing enzymes under conditions where hydrogen is not metabolized. We have shown that the wild-type had hydrogenase activity when cultivated heterotrophically. However, RuBPCase activity was only measurable in cells grown either lithoautotrophically or mixotrophically.

A third class of Aut[−] mutants, including the Tn5-induced strain JF26, had hydrogenase and RuBPCase activities (Table 2). The biochemical lesion of these mutants is still unknown.

Fig. 1. Double-diffusion immunoprecipitation. The centre well contained 4 μ l (a) or 8 μ l (b) of antiserum raised against the particulate hydrogenase of *A. eutrophus*. The outer wells of (a) contained 14 μ l of the membrane fraction of *P. facilis* (1) and *A. eutrophus* H16 (2); the outer wells of (b) contained 20 μ l of the membrane fraction of *P. facilis* wild-type (3, 6) and of the hydrogenase mutants JF19 (1), JF42 (2), JF43 (4) and JF46 (5).

Analysis of plasmid DNA from lysates of the Aut⁻ mutants indicated neither a loss nor an alteration in the restriction map of the plasmids (data not shown). Moreover, preliminary hybridization studies revealed a transposon location in the chromosomal DNA and not in the plasmid (Warrelmann, unpublished result). Pootjes (1977) concluded that the high genetic instability of the hydrogen-oxidizing ability of *P. facilis* may result from the curing of a hydrogenase-encoding plasmid. Our results do not support this conclusion. The final identification and cloning of hydrogenase genes from *P. facilis* will be facilitated in the future by the availability of Tn5-labelled hydrogenase mutants. This will answer the controversial question as to whether extrachromosomal DNA is involved in the lithoautotrophic metabolism of *P. facilis*.

We thank H. G. Podzuweit and K. Schneider for the supply of hydrogenase antiserum and Ute Warnecke for the isolation of some of the mutants. We also thank Waltraud Kues for typing the manuscript.

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