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Short Communications

N_2-Fixation by Chemoautotrophic Hydrogen Bacteria

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Abstract. The Gram-positive coryneform bacteria strains *14g* and *7C* were found to be able to grow with N_2 as sole nitrogen source when incubated under microaerobic conditions. Nitrogenase activity in whole cells was assayed by acetylene reduction. High rates of ethylene production (50—120 nmole/h× mg cell protein) were observed in N_2 or glutamate grown cell suspensions shaken in an atmosphere of 2.5% O_2, 10% acetylene and 87.5% argon.

Key words: Hydrogen-Bacteria — Nitrogen Fixation — Acetylene Reduction — Coryneform Bacteria Strains *14g* and *7C*.

None of the characterized chemolithoautotrophic bacteria has been previously reported to be able to fix nitrogen. Some evidence has been obtained for the utilization of molecular nitrogen as the sole nitrogen source by a bacterium growing under an atmosphere of nitrogen, hydrogen, oxygen and carbon dioxide (Ooyama, 1971). But the bacterium, which was isolated from an oily soil in northern Japan, was not identified.

This report describes a study of acetylene reduction in the following strains of hydrogen oxidizing bacteria: *Hydrogenomonas eutropha H 16* (Wilde, 1962), *Nocardia opaca 1b* (Aggag and Schlegel, 1973) and the coryneform bacteria strain *7C* (Siebert, 1969; Tunail and Schlegel, 1972; Tunail, 1973) and strain *14g* (Schneider *et al.*, 1973). Only the coryneform bacteria were capable of nitrogen fixation.

Cells were grown in the mineral medium of Schlegel *et al.* (1961) modified by the omission of NH_4Cl and containing trace elements (1 ml/l; Ormerod *et al.*, 1961) in which the ferrous-ammonium citrate was replaced by ferrous citrate. The pH was adjusted to 7.0—7.2. The mineral medium was supplemented with the following sources of nitrogen, oxygen, hydrogen and carbon (the associated figures are % v/v, gas phase, for gases and % w/v, liquid phase, for solids): A) NH_4Cl,0.1, $+O_2$,5—10, $+H_2$,85—80, $+CO_2$,10; B) N_2,60, $+O_2$,10, $+H_2$,20, $+CO_2$,10; C) NH_4Cl,0.1, $+N_2$,85, $+O_2$,10, $+CO_2$,5, +succinate 0.3; D) Gluta-

mate, .05, $+N_2$,85, $+O_2$,10, $+CO_2$,5, +succinate, 0.3; E) N_2,85, $+O_2$,10, $+CO_2$,5, +succinate, 0.3; F) N_2,85, $+O_2$,10, $+CO_2$,5, +fructose, 0.3.

Cultures (1 or 3 l) were grown in round, flat-bottomed flasks (2 or 6 l respectively) at 30° C and gassed by a magnetically driven, plastic coated stirring bar (7 cm, 400 rpm; Schlegel *et al.*, 1961). The appropriate gas mixture was supplied from containers (10 l) connected to the growth flask, and the gas mixture over the culture was flushed and renewed every 6—10 h. Growth was measured turbidimetrically at 436 nm (*Hydrogenomonas eutropha H16* and *Nocardia opaca 1b*) or 546 nm (strains *14g* and *7C*).

The rate of acetylene reduction by resting cells was used as a measure of nitrogenase activity (Dilworth, 1966). Assays (2 ml final volume) were done in Warburg vessels (18 ml) at 30° C using conventional Warburg apparatus (Type P 166, Braun, Melsungen, BRD) with reciprocal shaking (120 strokes/min, amplitude 4 cm). Cell suspension (0.5 ml, 9 mg cell protein) was added to the main vessel, which also contained 0.067 M phosphate buffer pH 7.2; substrate (20 μmole) was added to the side arm. The Warburg vessels were then evacuated and filled with a gas mixture containing O_2 (2.5—10%), acetylene (10%) and argon (87.5—80%); when hydrogen was present, the argon concentration was correspondingly reduced. At intervals after tipping the Warburg vessels, gas samples (0.2 ml) were removed by syringe for analysis by gas chromatography and replaced by argon (0.2 ml). The gas samples were injected into a Perkin Elmer F 11 gas chromatograph filled with a 45 cm Porapak R column and coupled to a hydrogen flame ionization detector.

Linear rates of ethylene production were obtained when rates did not exceed 700 nmole ethylene/h × mg cell protein. The protein content of intact cells was measured using the modified Biuret method of Schmidt *et al.* (1963).

When *Hydrogenomonas eutropha H 16*, which had been grown in media containing combined nitrogen, was inoculated into the supplemented mineral media without a source of combined nitrogen (supplements B or D), a limited increase in turbidity was observed (two- to threefold). No further turbidity increase was observed and further subcultures in media without combined nitrogen did not grow. Under no condition was acetylene reduction detected. This absence of nitrogenase was also observed in *Nocardia opaca 1b*.

In contrast, both strains of coryneform hydrogen bacteria grew rather fast under the microaerobic conditions used. With succinate as a substrate, the turbidity increased 30-fold with media supplements D and E.

Both strains *14g* and *7C* have been subcultured several times in media free from combined nitrogen. The cells exhibited high nitrogenase activities.

Using strains *14g* and *7C* the gas phase for optimal acetylene reduction was studied. The rate of ethylene production increased with increasing acetylene concentrations; 10% acetylene was saturating. Oxygen, the hydrogen acceptor for aerobic respiration and energy generation, inhibited acetylene reduction. With the relatively high cell density used, the maximum rates of acetylene reduction were measured at oxygen concentrations of 10% (strain *7C*) or 2.5% (strain *14g*); 40% and 20% O_2, respectively, gave approximately 95% inhibition.

The highest rates of acetylene reduction (120 nmole ethylene/h × mg cell protein) were measured with cells grown in the presence of glutamate or with N_2 as the sole nitrogen source (media supplements D, E, F). Nitrogenase activity was not detected in cells grown in the media containing ammonia (supplements A and C). Pyruvate, succinate or malate served as substrates for acetylene reduction in the presence of oxygen. Only pyruvate gave rise to a modest rate of acetylene reduction in the absence of oxygen (about 5% of the rate observed under aerobic conditions). Hydrogen supported acetylene reduction under microaerobic conditions, but only at low partial pressures (2.5% for *7C* and 5% H_2 for *14g*).

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