Nitrogen Fixation by *Thiobacillus ferrooxidans*

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Cultures of *Thiobacillus ferrooxidans* grown in the absence of a source of fixed nitrogen reduced acetylene to ethylene suggesting the presence of nitrogenase. This presumptive evidence has now been substantiated by the demonstration of the enrichment of ^{15}N in the protein of the organism when grown under $^{15}\text{N}_2$. The possibility that *T. ferrooxidans* was being grown in association with other nitrogen-fixing organisms was eliminated by the use of cultures derived from colonies whose development had been observed from single organisms.

INTRODUCTION

The acetylene reduction test is widely used to demonstrate the presence of nitrogen-fixing enzyme systems in bacteria. It is based on the observation of Dilworth (1966) and Schöllhorn & Burris (1967) that preparations of nitrogenase reduce acetylene specifically to ethylene. The iron-oxidizing bacterium *Thiobacillus ferrooxidans* reduces acetylene to ethylene (Mackintosh, 1971); this activity is repressed by growth in the presence of NH_4^+ , which is good presumptive evidence for the presence of nitrogenase. Nitrogen fixation has not previously been demonstrated in this group of bacteria and therefore it is necessary to show that this organism does not just reduce acetylene *per se* but incorporates dinitrogen into cell material.

When *T. ferrooxidans*, whether isolated in a liquid culture or received from a culture collection, is plated out by the technique described in this paper at least two types of colonies are seen. One produces a brown precipitate of iron but the other does not; the latter may be *T. acidophilus* (Guay & Silver, 1975). It has often been assumed that a liquid salts medium of acid pH containing FeSO_4 as the source of energy is selective for *T. ferrooxidans* and that by subculture in such a medium a pure culture will be obtained. This is not so. Mixed cultures are still present after numerous subcultures in FeSO_4 medium. Unless a strain is specifically purified and maintained under strict aseptic conditions it is highly probable that work is being carried out with a mixed culture. For this reason care was taken in the purification of the strain of *T. ferrooxidans* used for the nitrogen-fixation experiments.

METHODS

Bacterial strain. The strain of *T. ferrooxidans* was supplied by M. G. Yates (Unit of Nitrogen Fixation, University of Sussex). To eliminate the possibility of having a mixed or contaminated culture (Guay & Silver, 1975; Tsuchiya, Trivedi & Schuler, 1974), the strain used in this work was purified by a modification of the slide chamber technique (Postgate, Crumpton & Hunter, 1961) which allowed the development of a bacterial colony from a single organism to be followed microscopically. A 5 cm Petri dish containing 2.5 ml solid medium was placed in a metal holder which could be used on an ordinary microscope stage. Lugs on the holder were aligned with reference marks on the plate to ensure that the plate could be relocated precisely if movement occurred during incubation. The plates were inoculated with a dilute suspension of *T. ferrooxidans* and the positions of single organisms were identified by vernier scale readings. The plates, in their

holders, were put inside larger Petri dishes containing damp filter paper to prevent desiccation of the medium. These were placed in a glass anaerobic jar under air/N₂/CO₂ (1:94:5, by vol.) and incubated at 30 °C for 36 h. Colony growth of *T. ferrooxidans* was favoured by conditions of reduced oxygen tension (Mackintosh, unpublished observation). After incubation the plates were observed microscopically and the positions of microcolonies were noted. The plates were then re-incubated under the same conditions for 7 d during which time the microcolonies grew into visible colonies. A deep brown precipitate of ferric sulphate within and around the colony identified the organism as *T. ferrooxidans*. Organisms from one of these colonies were picked and inoculated into a flask of liquid medium and the culture was incubated at 30 °C for 5 d. The whole procedure was repeated and the final strain was considered to be uncontaminated *T. ferrooxidans*.

Media. For the growth of the inoculum, a 'low phosphate' salts medium was used containing: (NH₄)₂SO₄, 1.0 mM; KH₂PO₄, 0.2 mM; MgCl₂, 125 µM; CaCl₂, 1.0 mM; MnCl₂, 0.5 µM; ZnCl₂, 0.5 µM; CoCl₂, 0.5 µM; H₃BO₃, 0.5 µM; Na₂MoO₄, 0.05 µM; CuCl₂, 0.5 µM; H₂SO₄, 9.6 mM; FeSO₄, 180 mM; pH 1.8 (D. Herbert, personal communication). This medium considerably reduced the amount of precipitate of basic iron sulphates formed during growth and also decreased the loss of bacteria which adsorb to this precipitate. The ¹⁵N₂ incorporation experiments were carried out in a modified version of the medium devised by Leathen, Kinsel & Braley (1956) (F₂ medium) which contained: KCl, 0.7 mM; KH₂PO₄, 0.3 mM; MgSO₄, 2.0 mM; CaCl₂, 0.06 mM; Na₂MoO₄, 0.04 mM; FeSO₄, 180 mM; pH 2.2. All media were sterilized by autoclaving at 103 kPa for 15 min except for the ferrous sulphate which was filtered through a 0.45 µm membrane filter (Millipore) and added aseptically. *Thiobacillus ferrooxidans* was maintained on F₂ medium with added 1 mM-(NH₄)₂SO₄ and purified on the same medium solidified with 0.8 % (w/v) agarose (BDH).

Gases. The ¹⁵N₂ isotope was 99.4 % pure (BOC). It was transferred from its container, which had been sealed with a Suba-seal closure (William Freeman & Co, Staincross, Barnsley), to the culture vessel using a hypodermic syringe. The gas removed was replaced with an equal volume of sterile distilled water. Acetylene was made from calcium carbide and water (Postgate, 1972). Ethylene (99.9 % pure) was obtained from BOC.

Preparation of the inoculum. A 1 litre culture of *T. ferrooxidans* was grown in F₂ medium plus (NH₄)₂SO₄ at 30 °C for 3 d. This was used as the seed culture for a bottle containing 20 l 'low-phosphate' medium which was incubated at 30 °C with forced aeration through a sintered glass sparger at 2 l min⁻¹ for 48 h. The culture was harvested by continuous flow centrifugation at 47000 g at 100 ml min⁻¹. The final yield of cells (about 0.5 g) was resuspended in 20 ml 5 mM-H₂SO₄.

¹⁵N₂ incorporation and acetylene reduction experiments. Experiments were carried out at room temperature (22 °C) in Bellco jars (A. R. Horwell, London), working volume 250 ml; these had a hanging bar magnet stirrer and two side-arms which could be loosely covered with screw caps, to prevent contamination but allow free passage of air, or hermetically sealed with Suba-seals. Jars and seals were sterilized by autoclaving at 103 kPa for 15 min. A series of seven jars were prepared each containing 210 ml F₂ medium; subsequent treatments are detailed in Results.

Isotopic nitrogen ¹⁵N₂ analysis. Analysis of the ¹⁵N in the concentrated samples was carried out by conventional Kjeldahl & Markham distillation to recover NH₄⁺. N₂ was liberated from the samples by hypobromite oxidation and analysed in an AEI MS10 mass spectrometer.

Acetylene reduction test. Acetylene and its reduction product ethylene were determined by gas-liquid chromatography on a Pye model 104 chromatograph using a hydrogen flame ionization detector and a 152 cm glass column packed with Porapak R held at 65 °C. The carrier gas was nitrogen at a flow rate of 45 ml min⁻¹. Gas samples (1 ml), taken from the cultures which had received acetylene, were injected directly into the chromatograph through a gas sampling valve. Ethylene production was determined by comparing the areas of the peaks with those obtained with authentic ethylene diluted in N₂.

RESULTS AND DISCUSSION

Acetylene reduction tests (Mackintosh, 1971) indicated that the presumptive N₂-fixing system in *T. ferrooxidans* was oxygen sensitive as discussed by Drozd & Postgate (1970), and that highest activities were obtained with organisms in the exponential phase of growth. *Thiobacillus ferrooxidans* would be expected to require some oxygen for N₂ fixation since the necessary metabolic energy must be generated from the oxidation of ferrous to ferric iron. It was therefore necessary to supply sufficient oxygen to allow enough iron oxidation to support measurable ¹⁵N₂-fixation but insufficient to inhibit the expression of the nitrogenase system.

The natural abundance of ¹⁵N in dinitrogen and combined nitrogen is 0.366 atom %. If incubation of cultures with ¹⁵N₂ leads to an enrichment under the conditions of the exper-

Table 1. *Incorporation of $^{15}\text{N}_2$ into cell material by *T. ferrooxidans* under different conditions*

The various components (10 ml unless otherwise stated) were added to the jars at the times indicated. Each jar also received 150 mM- FeSO_4 at 18 h and 100 mM- FeSO_4 at 42 h. Jars 1 and 2 were sealed at the start of the experiment, the other jars were sealed after 18 h. After 96 h, the extent of $^{15}\text{N}_2$ incorporated was determined as described in Methods.

		Jar number						
		1	2	3	4	5	6	7
Times of addition	0 h	$^{15}\text{N}_2$	$^{15}\text{N}_2$	—	—	—	—	—
	18 h	$^{15}\text{N}_2$	$^{15}\text{N}_2$	$^{15}\text{N}_2$	$^{15}\text{N}_2$	$^{14}\text{N}_2$	$^{14}\text{N}_2$	$^{14}\text{N}_2$
		air					C_2H_2 (2 ml)	C_2H_2 (2 ml) (NH_4) $_2\text{SO}_4$ (1.0 mM)
	42 h	air	—	$^{15}\text{N}_2$	$^{15}\text{N}_2$	$^{14}\text{N}_2$	$^{14}\text{N}_2$	$^{14}\text{N}_2$
^{15}N (atom %) incorporated into cell material		0.518 ± 0.015	0.369	0.479	0.421 ± 0.007	0.360	—	—
Excess ^{15}N (atom %)		0.152	0.003	0.113	0.055	—		

Table 2. *Acetylene reduction by *T. ferrooxidans**

Jars were prepared and incubated as described in Table 1.

Time (h)	Ethylene produced (nmol ml ⁻¹)	
	Jar 6	Jar 7 (with NH_4^+)
0	0.024	0.031
24	6.4	0.028
48	80	0.030
96	112	0.031

iment then fixation of dinitrogen must have occurred. Seven culture jars were prepared, four being used to test for $^{15}\text{N}_2$ fixation (Table 1). The vessels were about 550 ml total volume with 210 ml medium and received a 1.5 ml inoculum (3.0 mg dry wt). They were supplemented with 150 mM- FeSO_4 after 18 h incubation and 100 mM- FeSO_4 after 42 h. Two jars were sealed immediately and injected with 10 ml $^{15}\text{N}_2$ (Table 1, jars 1 and 2). Jar 1 received an additional 10 ml of air after 18 and 42 h; jar 2 did not. Significant $^{15}\text{N}_2$ fixation occurred in jar 1 only, indicating that fixation probably occurred only under the micro-aerophilic conditions prevailing after the 18 and 42 h injections of additional air. Failure to observe fixation in jar 2 indicated that the oxygen concentration in air was inhibitory to N_2 fixation in the initial stages of growth, as also shown by the failure to observe acetylene reduction during the early stages of growth (Table 2). Jars 3 and 4 (which represent separate experiments performed on two occasions) were sealed after 18 h and received 10 ml $^{15}\text{N}_2$ at 18 and 42 h. Both showed positive $^{15}\text{N}_2$ -fixation, consistent with microaerophilic conditions developing in the cultures because of rapid iron oxidation following sealing of the jars during exponential growth. The other three jars were also sealed after 18 h. Jar 5 received no additions and was a control for the $^{15}\text{N}_2$ analysis. Jars 6 and 7 were supplemented with acetylene for a standard acetylene reduction test (Table 2). Ethylene production was highly significant over the 96 h incubation (Table 2, jar 6) and was completely inhibited by NH_4^+ (jar 7).

The data presented here show that a pure culture of *T. ferrooxidans* grown on a nitrogen-free medium is able to reduce acetylene to ethylene and incorporate $^{15}\text{N}_2$ into its cell protein. It should, therefore, be considered a true nitrogen fixer.

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