Isolation of Acetylene-reducing Spirilla from the Roots of *Potamogeton filiformis* from Loch Leven (Kinross)

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INTRODUCTION

Significant levels of nitrogen-fixation, measured using $^{15}\text{N}_2$, and/or acetylene reduction (Hardy *et al.*, 1968), occur on the roots of non-leguminous aquatic macrophytes (Patriquin & Knowles, 1972; Bristow, 1974), and on the roots of rice seedlings growing in waterlogged soil (Dommergues *et al.*, 1973). This activity is attributed to asymbiotic bacteria. Micro-aerophilic acetylene-reducing spirilla have been isolated from the roots of maize (von Bulow & Dobereiner, 1975) and *Digitaria decumbens* (Day & Dobereiner, 1976) plants in Brazil; these roots also showed high levels of nitrogenase activity by the acetylene-reduction method. The root zone of some aquatic macrophytes is known to be aerated via a channel of tissue (aerenchyma) through which air passes from the shoots to the roots (Armstrong, 1972). This enables the plants to grow in anaerobic sediment. The aerobic or semi-aerobic zone immediately around the roots is likely to be suitable for microaerophilic bacteria. In this study the roots of *Potamogeton filiformis*, one of the major aquatic macrophytes in Loch Leven, Kinross, Scotland (Jupp, Spence & Britton, 1974), were examined for the presence of nitrogen-fixing spirilla, and some characteristics of pure cultures of the organisms isolated from them were investigated.

METHODS

Basic medium. This contained: 0.4 g KH_2PO_4 ; 0.1 g K_2HPO_4 ; 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.1 g NaCl; 0.02 g CaCl_2 ; 0.01 g FeCl_3 ; 0.002 g $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$; 3.5 g (semi-solid) or 15 g (pour plates) Difco Bacto Special Noble Agar; 1 l distilled water. The carbon and nitrogen sources added to this medium in different experiments are described below. All incubations were at 30 °C.

Preliminary enrichment cultures. Samples of *P. filiformis* roots (0.5 to 1.0 cm long) were taken and, on the same day, were washed in lake water and placed in semi-solid medium (1 cm deep in 1 oz vials) containing 0.5 % sodium malate. After incubation for 1 day, the root pieces were removed and acetylene-reduction tests were carried out on the cultures.

Purification. Purification of acetylene-reducing cultures as described by von Bulow & Dobereiner (1975) was not successful. Purification was achieved by mixing samples from preliminary enrichment cultures which reduced the most acetylene with 5 ml sterile distilled water or buffered saline (pH 7.0) for 5 min; and then inoculating 0.1 ml samples into pour plates containing 0.5 % sodium malate and 0.05 % yeast extract, and incubating for 7 days.

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Table 1. *Relative growth and acetylene reduction by five pure spirillum cultures in semi-solid media*

Additions to basic nitrogen-free semi-solid medium*	Gas phase	Visible growth	Acetylene reduction†
1. Sodium malate only	Air	—	0
2. Sodium malate + VfC	Air	+++	80
3. Sodium malate + (NH ₄) ₂ NO ₃	Air	+++	0
4. Glucose + (NH ₄) ₂ NO ₃	Air	—	0
5. Glucose + VfC	Air	+	0
6. VfC only	Air	+	0
1. Sodium malate only	2 % O ₂ in N ₂	+++	60
2. Sodium malate + VfC	2 % O ₂ in N ₂	+++	80

* 0.5 % Sodium malate; 0.5 % glucose; 0.1 % (NH₄)₂NO₃; 0.05 % Difco Bacto vitamin-free casamino acids (VfC).

† Average nmol ethylene produced per culture in 1 h. Variation within experiments was less than 5 %.

Single colonies were selected and inoculated into semi-solid medium containing 0.5 % sodium malate and 0.05 % yeast extract in 1 oz vials. After incubation for 1 day, cultures were assayed for acetylene reduction and those with the highest activity were selected. This dilution, plating and selection procedure was repeated two or three times and the final cultures were streaked on nutrient agar to test for purity.

Growth and acetylene reduction by purified cultures on various media. Washed suspensions of cells of five cultures grown in liquid medium containing 0.5 % sodium malate and 0.1 % (NH₄)₂NO₃ were inoculated in triplicate into semi-solid media (1 cm deep in 7.5 ml vials capped with rubber seals) as shown in Table 1. To obtain 2 % (v/v) O₂ in the gas phase, the bottles were flushed with nitrogen gas and O₂ was added by syringe to 2 %. After incubation for 24 h the rate of acetylene reduction per culture was determined, and a relative estimate of growth was made visually.

Acetylene reduction. Rates of acetylene reduction were determined by adding acetylene to 10 % (v/v), incubating for 1 to 2 h, and analysing the gas phase for ethylene in a Pye Unicam 104 gas chromatograph. Controls without acetylene contained no ethylene. Dilutions of pure ethylene were used to calibrate the gas chromatograph and the ethylene contamination of the acetylene was subtracted. The results were expressed as nmol ethylene produced per culture in 1 h (average), as it was not possible to enumerate cells in semi-solid cultures.

RESULTS AND DISCUSSION

All the initial enrichment cultures from the root pieces showed thin undulating pellicles of microbial growth 1 to 2 mm below the surface of the agar, and reduced acetylene after the root had been removed. Phase-microscopic examination revealed motile spiral-shaped organisms (2 to 3 × 5 to 10 μm) containing many refractile bodies. From these enrichment cultures five pure cultures of bacteria which consistently reduced acetylene were obtained. These cultures originated from samples of *P. filiformis* collected from five different sites in the loch. The bacteria were smaller than the organisms in the original enrichment cultures (0.5 to 1.0 × 3.0 μm), more motile, and did not contain refractile bodies. They had half to one spiral turn per cell and sometimes joined together to form spiral chains. They formed white opaque colonies in pour plates containing malate and yeast extract, and buff-coloured colonies on nutrient agar. They were Gram-negative. Of the *Spirillum* species described in

Bergey's Manual of Determinative Bacteriology (1957), they were most closely related to *Spirillum lipoferum*.

Some characteristics of the spirilla in pure culture are shown in Table 1. Under air at 30 °C they did not grow in completely nitrogen-free malate medium (medium 1); but grew in a sub-surface pellicle and reduced acetylene vigorously in semi-solid malate medium containing 0.05 % vitamin-free casamino acids (VfC) (medium 2). They did not grow in liquid medium 2. In semi-solid malate medium containing 0.1 % $(\text{NH}_4)_2\text{NO}_3$ but no VfC (medium 3), they grew nearer the surface and did not reduce acetylene. They grew well in liquid medium 3 (although in wet mounts a microaerotactic response, as described by Wells & Krieg (1965) for *S. volutans*, was observed). This apparent decrease in oxygen sensitivity and concomitant lack of acetylene reduction when grown in medium containing $(\text{NH}_4)_2\text{NO}_3$ was as expected for aerobic nitrogen-fixing bacteria: in other species, nitrogenase synthesis is inhibited by fixed nitrogen, and continuous cultures are very sensitive to oxygen when fixing nitrogen (Hill, Drozd & Postgate, 1972).

Other organisms have been reported to require yeast extract or VfC in the growth medium in order to fix nitrogen from air. In *Mycobacterium flavum* 301 the need for yeast extract was eliminated by growing cultures at low oxygen tensions (Biggins & Postgate, 1969). Hill (1975) found that with VfC at 2 mg ml⁻¹ all colonies of *Klebsiella pneumoniae* grown under air reduced acetylene, and suggested that VfC contains enough combined nitrogen to support growth until respiratory activity lowers the oxygen tension within the colony sufficiently to allow nitrogenase activity. Table 1 indicates a similar effect on the spirilla. When the oxygen tension over the semi-solid medium was reduced to 2 %, they grew and reduced acetylene in nitrogen-free medium almost as well as they did in medium containing VfC. Thus, apparently, VfC is required to enable the spirilla grown under air to overcome oxygen toxicity, even when growing in a subsurface pellicle in semi-solid medium. This may be because nitrogen gas is less soluble than oxygen in water, and becomes limiting just below the surface of the agar; under air the bacteria would be forced to grow at super-optimal P_{O_2} to obtain sufficient nitrogen.

The *S. lipoferum* isolated by Day & Dobereiner (1976) grows under air on completely nitrogen-free semi-solid media, will grow on glucose, although not in nitrogen-free media, and forms a pink pigment in older cultures. The isolates described here do not grow on glucose with $(\text{NH}_4)_2\text{NO}_3$ under air (medium 4), and do not grow better on glucose plus VfC (medium 5) under air than can be explained by their growth on VfC alone (medium 6) (Table 1). No pigment has been observed. Thus, although they are somewhat similar to the maize and *Digitaria* isolates, they differ in these properties and in the difficulty in isolating them by the method described by von Bulow & Dobereiner (1975) and Day & Dobereiner (1976). The nitrogen-fixing spirilla described by Becking (1963) did not grow in the absence of yeast extract and may have been the same as the isolates described here. These preliminary observations support the suggestion that there is more than one form of *S. lipoferum*.

Experiments described elsewhere (Sylvester-Bradley, 1976) have shown that washed *P. filiformis* roots from Loch Leven can reduce acetylene at an optimum oxygen concentration between 0 and 5 % (v/v) in the dark [average rates observed at lake temperature were up to 90 nmol C₂H₂ (g dry roots)⁻¹ h⁻¹]. Some of the acetylene-reducing activity described by Patriquin & Knowles (1972) was also higher under aerobic than under anaerobic conditions. Thus it is possible that this activity is due to micro-aerophilic organisms. The absence of reports of nitrogen-fixing spirilla in aquatic systems and temperate climates may be due to the lack of methods for isolating them. Further investigations might reveal that they are of considerable ecological and economic significance.

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