A Study of Nitrogenase Activity in *Mycoplana* Species and Free-living Actinomycetes

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A range of actinomycetes and *Mycoplana* strains were screened for nitrogenase activity. No positive results were obtained with actinomycetes, although some grew on media without added combined nitrogen. In contrast, most *Mycoplana* strains showed nitrogenase activity. Although the strains were unable to grow in anaerobic conditions, sustained nitrogen fixation required microaerobic conditions. The relationship of *Mycoplana* to actinomycetes is discussed.

INTRODUCTION

It has been known for many years that actinomycete-like organisms fix dinitrogen in the nodules of various non-leguminous plants. These organisms have been placed in the genus *Frankia*.

Several successful isolations of *Frankia* endophytes have been reported, for example from *Comptonia peregrina* (Callaham *et al.*, 1978), *Alnus glutinosa* (Quispel & Tak, 1978), *Myrica pensylvanica* (Lechevalier & Lechevalier, 1979), *Elaeagnus umbellata* (Baker *et al.*, 1979; Baker & Torrey, 1979), *Alnus viridis* (Baker *et al.*, 1979) and *A. rubra* (Berry & Torrey, 1979). With the exception of the *Elaeagnus umbellata* isolate, all were shown to re-infect host plant seedlings and produce effective N₂-fixing root nodules. Most current information on dinitrogen fixation by *Frankia* has come from studies with particulate nodule homogenates (Akkermans *et al.*, 1977; Van Straten *et al.*, 1977; Benson & Eveleigh, 1979), or active cell-free extracts of actinorhizal roots (Benson *et al.*, 1979). However, recently, nitrogenase activity has been demonstrated *in vitro* in pure cultures of *Frankia* (Tjepkema *et al.*, 1980; Gauthier *et al.*, 1981).

In contrast, there is no definitive evidence for nitrogenase activity in free-living actinomycetes. Dinitrogen fixation by *Nocardia calcarica* and *N. cellulans* was reported by Metcalfe & Brown (1957) but this was repudiated by Hill & Postgate (1969). Unequivocal evidence for nitrogenase activity in an organism originally called *Mycobacterium flavum* was obtained by Biggins & Postgate (1969). However, subsequent studies showed that *M. flavum* was incorrectly classified as an actinomycete (Biggins & Postgate, 1971) and it was renamed *Xanthobacter flavus*, a new species of the hydrogen bacteria (Malik & Claus, 1979).

In this study we have tested a range of free-living actinomycetes for nitrogenase activity under various experimental conditions. *Mycoplana* species were also included as they have been regarded as members of the Actinomycetales because of their abundant branching (Sukapure *et al.*, 1970).

METHODS

Test organisms and inoculum preparation. The actinomycetes were stored as spores or mycelial macerates in 10% (v/v) glycerol at –20 °C (Wellington & Williams, 1978). Actively growing cultures were produced by inoculation of the thawed glycerol suspensions on to nutrient agar (Oxoid no. 3) or oatmeal agar (Küster, 1959). The *Mycoplana* strains were stored as freeze-dried samples and activated by inoculation on to Bennett's medium (Jones, 1949) solidified with 1% (w/v) agar. Cultures were incubated at 28 °C for 3 to 7 d to provide the inoculum for studies on growth and nitrogenase activity.

Initial screening for nitrogenase activity. The medium designated AM50, contained (g l⁻¹): glucose, 10.0; sodium succinate, 2.0; K₂HPO₄, 1.0; KCl, 0.5; MgSO₄·7H₂O, 0.5; (NH₄)₂SO₄, 0.236 (i.e. 50 mg N l⁻¹); FeSO₄·2H₂O,

0.01; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.005; pH 7.0–7.3. When $(\text{NH}_4)_2\text{SO}_4$ was omitted the medium was termed AM0. Both media were used in liquid form and with agar (1.2% w/v).

All test strains were initially inoculated into liquid AM50 medium in 100 ml Erlenmeyer flasks and incubated at 28 °C with shaking. After 7 to 10 d growth, these cultures were used as inocula in screening experiments for nitrogenase activity as follows. Inocula of all the test strains were streaked on to slopes of AM50 and AM0 media contained in McCartney vials fitted with serum caps and grown under $p\text{O}_2$ of 0, 0.025, 0.05, 0.10 and 0.20 atm (1 atm = 101.32 kPa) in N_2 . The gas phase in each vial was renewed every day. In addition, the test strains were also inoculated into AM0 and AM50 liquid media and incubated aerobically as static and shake cultures. After 2, 5, 10 and 15 d incubation, the presence or absence of growth was noted and nitrogenase activity determined.

A modified acetylene reduction technique was used to estimate nitrogenase activity (Stewart *et al.*, 1971). Samples (3 ml) from liquid cultures were transferred (with minimum disturbance), to McCartney vials fitted with serum caps. Cultures already growing in vials were assayed *in situ*. Acetylene was injected into the vials to 10% (v/v). They were then incubated at 28 °C on an orbital shaker (80–120 rev. min^{-1} , 2–3 cm amplitude) except for samples of the static-grown liquid cultures, which were gently swirled immediately after the addition of acetylene and then left to stand. Vials containing either samples of the test organisms or pure media, to which no acetylene had been added, were also incubated under the various experimental conditions. These controls were to check for any extraneous ethylene production. After 1, 6 and 24 h, 1 ml samples of the gas phase in each of the vials were removed and analysed for ethylene and acetylene using a Perkin-Elmer F11 gas chromatograph (oven temperature 60 °C) fitted with a 0.5 m 80–100 mesh Porapak T column.

¹⁵N tracer studies. One of the strains which gave a positive reaction with the acetylene reduction test (*Mycoplana dimorpha* LL-C4) was selected for confirmatory testing using the ¹⁵N tracer technique. The methods of Rai (1980) were used for the exposure of 15 ml samples of aerobic static-grown liquid cultures (in AM0 medium) to ¹⁵N gas in McCartney vials and for their subsequent digestion and analysis. The samples were incubated for 2 h at 26 °C under a gas phase comprising $\text{N}_2/\text{A}/\text{CO}_2$ (30:69.96:0.04, by vol.) with a 60 atom % enrichment of the nitrogen with ¹⁵N. Controls comprised sterile AM0 medium exposed to the ¹⁵N enriched gas phase and samples of *Mycoplana* incubated under the gas phase without ¹⁵N enrichment. A VG Micromass 601 mass spectrometer was used to analyse for ¹⁵N enrichment.

Effect of carbon sources on nitrogenase activity. A range of carbon sources (see Results) were substituted for the glucose/succinate combination in media AM50 and AM0. *Mycoplana dimorpha* LL-C4 was inoculated into 15 ml of the variously substituted liquid media contained in McCartney vials and incubated aerobically at a $p\text{O}_2$ of 0.05 atm at 28 °C with shaking. Growth was estimated after 4 and 7 d by measuring the increase in turbidity of samples removed from the cultures at 540 nm using a Pye Unicam SP600 spectrophotometer. Nitrogenase activity was determined in the substituted AM0 series and after 2 and 4 d. The samples were incubated with acetylene for 1 and 6 h.

Effect of different oxygen concentrations on nitrogenase activity. The influence of a range of sub-atmospheric oxygen concentrations on nitrogenase activity by *Mycoplana dimorpha* LL-C4 was studied. Samples of a dilute N_2 -fixing liquid culture grown at a $p\text{O}_2$ of 0.05 atm were dispensed into McCartney vials fitted with serum caps. These were exposed with shaking at 28 °C to $p\text{O}_2$ in the range 0 to 0.1 atm. Nitrogenase activity in replicate vials was followed during a 4 h exposure to the oxygen regimes. Longer-term effects were studied by exposing cells to $p\text{O}_2$ in the range 0 to 0.1 atm for periods of 20 and 40 h.

The influence of changing oxygen regimes on nitrogenase activity was also investigated. Cultures grown under a $p\text{O}_2$ of 0.05 atm were either: (i) incubated under pure nitrogen for 40 h and then flushed with a gas mixture with a $p\text{O}_2$ of 0.025 atm, or (ii) incubated under a $p\text{O}_2$ of 0.025 atm for 40 h and then reflushed with the same gas mixture or with pure nitrogen, or (iii) incubated under a $p\text{O}_2$ of 0.05 atm for 40 h and then reflushed with the same gas mixture or with pure nitrogen.

Where rates of acetylene reduction are expressed on a dry weight basis, samples of the cultures were filtered on to 2.5 cm diameter GS type membranes (Millipore), pore size 0.22 μm , and dried to constant weight at 90 °C.

Effect of different ammonium and nitrate concentrations on growth and activity. One ml quantities of freshly prepared filter-sterilized stock solutions of $(\text{NH}_4)_2\text{SO}_4$ or KNO_3 were added to sterile AM0 liquid medium contained in Erlenmeyer flasks to give known final concentrations in the range 0 to 150 mg N l^{-1} . The flasks were inoculated with a uniform suspension of *Mycoplana dimorpha* LL-C4 and incubated at 28 °C for up to 10 d, either aerobically in static and shake culture, or at a $p\text{O}_2$ of 0.05 atm. Growth was estimated spectrophotometrically and nitrogenase activity by the acetylene reduction technique.

RESULTS

Screening of actinomycetes and Mycoplana strains for nitrogenase activity

The defined medium containing ammonium (AM50) supported growth of all strains, but few strains were able to grow on the medium lacking combined nitrogen (AM0) (Table 1). Single

Table 1. Screening of actinomycetes and *Mycoplana* strains for growth in the absence of combined nitrogen, and for nitrogenase activity

Strain*	Growth in medium lacking combined nitrogen (AM0)†	Nitrogenase activity (in AM50 or AM0)‡
<i>Nocardia asteroides</i> ATCC 19247	—	—
<i>N. asteroides</i> NCTC 8595	—	—
<i>N. asteroides</i> N671	—	—
<i>N. autotrophica</i> NCIB 9574	—	—
<i>N. brasiliensis</i> ATCC 19296	+	—
<i>N. calcarica</i> NCIB 8863	—	—
<i>N. opaca</i> CBS 330	—	—
<i>N. opaca</i> NCIB 9409	+	—
<i>N. otitidis-caviarum</i> Mariat 751	—	—
<i>N. pellegrino</i> ATCC 15998	—	—
<i>N. saturnea</i> ATCC 15778	—	—
<i>Nocardioides albus</i> IMET 7801	—	—
<i>Oerskovia turbata</i> IMET 7153	+	—
<i>O. xanthineolytica</i> LL-Y13-3	—	—
<i>Rhodococcus coprophilus</i> CUB 628	—	—
<i>R. coprophilus</i> CUB 118	—	—
<i>R. corallinus</i> ATCC 60004	—	—
<i>R. erythropolis</i> NCIB 9158	—	—
<i>R. ruber</i> N361	—	—
<i>Streptomyces aureofaciens</i> NRRL B-1286	—	—
<i>S. michiganensis</i> ATCC 14970	—	—
<i>S. violaceoruber</i> ATCC 14980	—	—
<i>S. viridochromogenes</i> CBS	—	—
<i>Streptomyces</i> sp. soil isolate M1	+	—
<i>Streptomyces</i> sp. soil isolate F1	—	—
<i>Streptomyces</i> sp. soil isolate C1	+	—
<i>Streptomyces</i> sp. soil isolate T1	—	—
<i>Mycoplana dimorpha</i> LL-C4	+	+
<i>M. dimorpha</i> LL-C3	+	+
<i>M. dimorpha</i> LL-4-9	+	+
<i>M. dimorpha</i> LL-5-20	+	+
<i>M. dimorpha</i> LL-8-32	+	+
<i>M. dimorpha</i> LL-100-32	+	+
<i>M. dimorpha</i> NCIB 9439	+	+
<i>M. dimorpha</i> ATCC 4279	—	—
<i>M. bullata</i> NCIB 9440	+	+
<i>M. bullata</i> ATCC 4278	—	—

* Sources: ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; CBS, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; CUB, Dr T. Cross, School of Biological Sciences, University of Bradford, Bradford, W. Yorks., U.K.; IMET, Institute for Microbiology and Experimental Therapy, Jena, G.D.R.; LL, Dr M. P. Lechevalier, Waksman Institute of Microbiology, Rutgers University, New Jersey, U.S.A.; M. Mariat, Institut Pasteur, Paris, France; N, Dr M. Goodfellow, Microbiology Department, University of Newcastle, U.K.; NCIB, National Collection of Industrial Bacteria, Aberdeen, U.K.; NCTC, National Collection of Type Cultures, London, U.K.; NRRL, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill., U.S.A.

† Strains are listed positive if growth on AM0 occurred under any of the incubation conditions described in Methods.

‡ Strains are listed as positive if nitrogenase activity was measurable under any of the incubation conditions described in Methods.

strains of *Nocardia brasiliensis*, *N. opaca*, *Oerskovia turbata*, and two *Streptomyces* isolates, were able to grow on solidified AM0 medium. However, none of these strains showed nitrogenase activity even when grown and assayed under sub-atmospheric oxygen concentrations. They, presumably, were able to utilize traces of combined nitrogen carried over at inoculation, or present in the agar or gas phase. In contrast, most strains of *Mycoplana* grew on AM0 and showed nitrogenase activity under aerobic conditions and at pO_2 values below 0.2 atm as indicated by the acetylene reduction technique.

Fig. 1. Growth of *Mycoplasma dimorpha* LL-C4 in aerobic shake culture in liquid media containing different concentrations of $(\text{NH}_4)_2\text{SO}_4$ (●) and KNO_3 (○).

Dinitrogen fixation by *Mycoplasma*

One of the strains showing nitrogenase activity, *Mycoplasma dimorpha* LL-C4, was selected for more detailed study.

Nitrogen fixation by *M. dimorpha* LL-C4 was confirmed by the ^{15}N tracer technique. Samples of an aerobically-grown static liquid culture exposed to $^{15}\text{N}_2$ under anaerobic conditions for 2 h showed a mean atom % ^{15}N enrichment of 0.430, compared with a natural enrichment of 0.366 in control samples of *Mycoplasma* which were not exposed to the ^{15}N gas phase.

The influence of various factors on nitrogenase activity in *M. dimorpha* LL-C4 was also studied. Growth and nitrogenase activity were optimal at 30°C ($\pm 2^\circ\text{C}$) and between pH 6.5 and 7.5. Good growth and nitrogenase activity occurred when L-arabinose, ethanol, D-glucose, sodium lactate or sodium succinate were provided as sole carbon sources. In contrast, D-fructose, maltose and sucrose gave poor growth and nitrogenase activity. The cell yield of *M. dimorpha* (LL-C4) increased exponentially with increasing concentrations of ammonium up to 50 mg N l^{-1} (Fig. 1) and growth was always better on ammonium than in combined-nitrogen-free medium, regardless of the oxygen regime. An ammonium concentration of 15 mg N l^{-1} totally suppressed nitrogenase activity. In contrast, concentrations of nitrate up to 150 mg N l^{-1} did not support substantially greater growth than that obtained in combined-nitrogen-free cultures. Preliminary screening studies suggested that nitrogenase activity in *Mycoplasma* cultures might be stimulated by sub-atmospheric concentrations of molecular oxygen, a view also supported by the observation that cultures with good levels of nitrogenase activity could be more reliably produced by growing them at an initial pO_2 of 0.05 atm rather than in air. The influence of oxygen concentration on nitrogenase activity was therefore investigated further. Nitrogenase activity in *M. dimorpha* LL-C4 previously grown at a pO_2 of 0.05 atm was progressively stimulated by decreasing oxygen concentrations over a 4 h period (Fig. 2). Little activity was detected at oxygen concentrations above 0.06 atm.

However, *Mycoplasma dimorpha* LL-C4 was not able to grow in slope or stab cultures incubated under anaerobic conditions. Therefore, cultures were incubated under a range of oxygen concentrations for 20 and 40 h and then assayed for nitrogenase activity. There was no activity in the absence of oxygen and the highest rates occurred at 0.025 atm oxygen (Fig. 3). Loss of nitrogenase activity with prolonged incubation under anaerobic conditions was reversible. Samples from a culture grown under a pO_2 of 0.05 atm were transferred to anaerobic conditions for 40 h; nitrogenase activity was re-established after a short lag by transfer to 0.025 atm oxygen (Fig. 4a). When samples of the culture grown under 0.05 atm oxygen were transferred to 0.025 atm, nitrogenase activity was initially high. The decline in activity after 40 h was temporarily

Fig. 2

Fig. 3

Fig. 2. Nitrogenase activity by *Mycoplana dimorpha* LL-C4 grown at a pO₂ of 0.05 atm and then incubated at pO₂ of 0.00 (○); 0.02 (●); 0.04 (□); 0.06 (■) and 0.08 (△) atm. Values are the means of at least triplicate determinations.

Fig. 3. Nitrogenase activity by *Mycoplana dimorpha* LL-C4 grown at a pO₂ of 0.05 atm and then incubated at different pO₂ for 20 h (●) and 40 h (○). Values are the means of at least triplicate determinations.

Fig. 4. Nitrogenase activity by *Mycoplana dimorpha* LL-C4 grown under a pO₂ of 0.05 atm and then: (a) transferred to and, after 40 h, either retained under pure nitrogen (●) or refushed with a gas mixture with a pO₂ of 0.025 atm (○); (b) transferred to a pO₂ of 0.025 atm and then, after 40 h, either refushed with a gas mixture with a pO₂ of 0.025 atm oxygen (○), or refushed with pure nitrogen (●); (c) retained under a pO₂ of 0.05 atm and, after 40 h, either refushed with the same gas mixture (○) or refushed with pure nitrogen (●). The arrows show the point at which the gas phases were changed. Values are the means of at least triplicate determinations.

reversed by refushing with a gas mixture with a pO_2 of 0.025 atm oxygen, but not by refushing with pure nitrogen (Fig. 4*b*). If samples from the culture grown under 0.05 atm oxygen were maintained at this oxygen tension and refushed with the same gas mixture after 40 h, the steady increase in nitrogenase activity was resumed after a short initial drop in the rate immediately after refushing (Fig. 4*c*). However, when samples were refushed with nitrogen instead of 0.05 atm oxygen, there was a short-term stimulation of nitrogenase activity, but the rate then dropped below that in the samples maintained under 0.05 atm oxygen.

These results suggest that although nitrogenase activity is optimum under anaerobic conditions in the short-term, it is dependent on reductant and/or ATP generated by aerobic respiration. Under the experimental conditions used here, an oxygen concentration of approximately 0.025 atm appears best to drive aerobic respiration at a rate sufficient to meet the energy requirements of nitrogen fixation, while not disturbing the oxygen protection mechanism of the nitrogenase complex. The nitrogenase activity detected in *Mycoplana* strains grown on solid medium or in static liquid culture under aerobic conditions could be explained by decreased oxygen tensions within the mass of the individual colonies or in the liquid medium as a result of respiratory activity. Nitrogenase activity in samples of aerobic static liquid cultures of *Mycoplana dimorpha* LL-C4 was lost by vigorous shaking in air.

DISCUSSION

It has been shown for the first time that species of the genus *Mycoplana* are capable of dinitrogen fixation. The rates of acetylene reduction (Fig. 3) are low compared with those for other free-living bacteria, but higher than values reported for liquid cultures of free-living *Rhizobium* sp. 'cowpea' strain 32 H1 (Tjepkema & Evans, 1975). Although *Mycoplana* species are apparently obligate aerobes, sustained nitrogenase activity is dependent on the maintenance of microaerobic conditions. Little is known about the ecology of this genus, but most strains originate from soil, where they probably represent another component of the free-living, nitrogen-fixing population.

Nitrogenase activity was demonstrated in eight *Mycoplana* strains, including type cultures of *M. dimorpha* (NCIB 9439) and *M. bullata* (NCIB 9440). The genus was erected by Gray & Thornton (1928) and these cultures were deposited by the senior author. However, the two cultures deposited in the American collection (*M. dimorpha* ATCC 4279, *M. bullata* ATCC 4278) failed to show nitrogenase activity. Therefore, the strains from NCIB and ATCC are either different, or the latter have lost the facility to express nitrogenase activity.

Mycoplana strains were originally included in this study because some workers have included the genus in the Actinomycetales (Gray & Thornton, 1928; Sukapure *et al.*, 1970; Cross & Goodfellow, 1973). However, the relationship of these Gram-negative, motile, branching bacteria to actinomycetes is questionable. In the 7th edition of *Bergey's Manual of Determinative Bacteriology*, *Mycoplana* was placed in the *Pseudomonadaceae* (Breed & Smith, 1957); it was omitted from the 8th edition (Buchanan & Gibbons, 1974). Results from their DNA-RNA hybridization studies on *M. dimorpha* NCIB 9439 and *M. bullata* NCIB 9440 led De Smedt & De Ley (1977) to conclude that these organisms were remote relatives of the family *Rhizobiaceae*, which contains many nitrogen-fixing species. However, the taxonomic implications of nitrogenase activity should not be over-estimated as it occurs in free-living, aerobic bacteria which belong to different rRNA superfamilies (De Smedt *et al.*, 1980).

Nitrogenase activity was not detected in any of the strains which are unequivocally actinomycetes, even when growth was initially stimulated by the addition of ammonia and incubation carried out under sub-atmospheric pO_2 s. Most common soil genera were represented, including *Nocardia calcaria* NCIB 8863 in which Hill & Postgate (1969) were also unable to detect nitrogenase activity, despite an earlier claim that it fixed dinitrogen (Metcalf & Brown, 1957). A few strains grew on agar media without added combined nitrogen but further testing of these at low pO_2 with the various carbon sources which supported growth and nitrogenase in *Mycoplana dimorpha* LL-C4 still failed to stimulate nitrogenase activity. Thus, although *Frankia*

species fix nitrogen when growing as root endophytes and *in vitro*, there is still no clear evidence that free-living actinomycetes have this ability.

It is hoped that the demonstration of nitrogenase activity in *Mycoplana* will stimulate further studies on the physiology and ecology of this neglected genus.

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REFERENCES

- AKKERMANS, A. D. L., VAN STRATEN, J. & ROELOFSEN, W. (1977). Nitrogenase activity of nodule homogenates of *Alnus glutinosa*: a comparison with the *Rhizobium*-pea system. In *Recent Developments in Nitrogen Fixation*, pp. 591–603. Edited by W. E. Newton, J. R. Postgate & C. Rodriguez-Barrucce. London: Academic Press.
- BAKER, D. & TORREY, J. G. (1979). In *Symbiotic Nitrogen Fixation in the Management of Temperate Forests*, pp. 38–56. Edited by J. C. Gordon, C. T. Wheeler & D. A. Perry. Corvallis: Oregon State University Press.
- BAKER, D., TORREY, J. G. & KIDD, G. H. (1979). Isolation by sucrose-density fractionation and cultivation *in vitro* of actinomycetes from nitrogen fixing root nodules. *Nature, London* **281**, 76–77.
- BENSON, D. R. & EVELEIGH, D. E. (1979). Nitrogen-fixing homogenates of *Myrica pensylvanica* (Bayberry) non-legume root nodules. *Soil Biology and Biochemistry* **11**, 331–334.
- BENSON, D., ARP, D. J. & BURRIS, R. H. (1979). Cell-free nitrogenase and hydrogenase from actinorhizal root nodules. *Science* **205**, 688–689.
- BERRY, A. & TORREY, J. G. (1979). In *Symbiotic Nitrogen Fixation in the Management of Temperate Forests*, pp. 69–83. Edited by J. C. Gordon, C. T. Wheeler & D. A. Perry. Corvallis: Oregon State University Press.
- BIGGINS, D. R. & POSTGATE, J. R. (1969). Nitrogen fixation by cultures and cell-free extracts of *Mycobacterium flavum* 301. *Journal of General Microbiology* **56**, 181–193.
- BIGGINS, D. R. & POSTGATE, J. R. (1971). Confusion in the taxonomy of a nitrogen-fixing bacterium currently classified as *Mycobacterium flavum* 301. *Journal of General Microbiology* **65**, 119–123.
- BREED, R. S. & SMITH, N. R. (1957). *Bergey's Manual of Determinative Bacteriology*, 7th edn. Baltimore: Williams & Wilkins.
- BUCHANAN, R. E. & GIBBONS, N. E. (1974). *Bergey's Manual of Determinative Bacteriology*, 8th edn. Baltimore: Williams & Wilkins.
- CALLAHAM, D., TREDICI, P. D. & TORREY, J. G. (1978). Isolation and cultivation *in vitro* of the actinomycete causing nodulation in *Comptonia*. *Science* **199**, 899–902.
- CROSS, T. & GOODFELLOW, M. (1973). Taxonomy and classification of the actinomycetes. In *Actinomycetales, Characteristics and Practical Importance*, pp. 11–112. Edited by G. Sykes & F. A. Skinner. London & New York: Academic Press.
- DE SMEDT, J. & DE LEY, J. (1977). Intra- and inter-generic similarities of *Agrobacterium* ribosomal ribonucleic acid cistrons. *International Journal of Systematic Bacteriology* **27**, 222–240.
- DE SMEDT, J., BAUWENS, M., TYTGAT, R. & DE LEY, J. (1980). Intra- and inter-generic similarities of ribosomal ribonucleic acid cistrons of free-living, nitrogen-fixing bacteria. *International Journal of Systematic Bacteriology* **30**, 106–122.
- GAUTHIER, D., DIEM, H. G. & DOMMERGUES, Y. (1981). *In vitro* nitrogen fixation by two actinomycete strains isolated from *Casuarina* nodules. *Applied and Environmental Microbiology* **14**, 306–308.
- GRAY, P. H. H. & THORNTON, H. G. (1928). Soil bacteria that decompose certain aromatic compounds. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung II)* **73**, 82–84.
- HILL, S. & POSTGATE, J. R. (1969). Failure of putative nitrogen-fixing bacteria to fix nitrogen. *Journal of General Microbiology* **56**, 277–285.
- JONES, K. L. (1949). Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic. *Journal of Bacteriology* **57**, 141–145.
- KÜSTER, E. (1959). Outline of a comparative study of criteria used in characterisation of the actinomycetes. *International Bulletin of Bacterial Nomenclature and Taxonomy* **9**, 97–104.
- LECHEVALIER, M. P. & LECHEVALIER, H. A. (1979). In *Symbiotic Nitrogen Fixation in the Management of Temperate Forests*, pp. 111–122. Edited by J. C. Gordon, C. T. Wheeler & D. A. Perry. Corvallis: Oregon State University Press.
- MALIK, K. A. & CLAUS, D. (1979). *Xanthobacter flavus*, a new species of nitrogen-fixing hydrogen bacteria. *International Journal of Systematic Bacteriology* **29**, 283–287.
- METCALFE, G. & BROWN, M. E. (1957). Nitrogen fixation by new species of *Nocardia*. *Journal of General Microbiology* **17**, 567–572.
- QUISPEL, A. & TAK, T. (1978). Studies on the growth of the endophyte of *Alnus glutinosa* (L.) Vill. in nutrient solutions. *New Phytologist* **81**, 587–600.
- RAI, A. N. (1980). *Studies on the nitrogen-fixing lichen Peltigera aphosa* Willd. Ph.D. thesis, University of Dundee, U.K.
- STEWART, W. D. P., MAGUE, T., FITZGERALD, G. P. & BURRIS, R. H. (1971). Nitrogenase activity in Wisconsin lakes of differing degrees of eutrophication. *New Phytologist* **70**, 497–509.

- SUKAPURE, R. S., LECHEVALIER, M. P., REBER, H., HIGGINS, M. L., LECHEVALIER, H. A. & PRAUSER, H. (1970). Motile nocardial Actinomycetales. *Applied Microbiology* **19**, 527-533.
- TJEPKEMA, J. D. & EVANS, H. J. (1975). Nitrogen fixation by free-living *Rhizobium* in a defined liquid medium. *Biochemical and Biophysical Research Communications* **65**, 625-628.
- TJEPKEMA, J. D., ORMEROD, W. & TORREY, J. G. (1980). Vesicle formation and acetylene reduction activity in *Frankia* sp. CP11 cultured in defined nutrient media. *Nature, London* **287**, 633-635.
- VAN STRATEN, J., AKKERMANS, A. D. L. & ROELOFSEN, W. (1977). Nitrogenase activity of endophyte suspensions derived from root nodules of *Alnus*, *Hippophae*, *Shepherdia* and *Myrica* spp. *Nature, London* **266**, 257-258.
- WELLINGTON, E. M. H. & WILLIAMS, S. T. (1978). Preservation of actinomycete inoculum in frozen glycerol. *Microbios Letters* **6**, 151-157.