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Nitrogen-fixing Growth in Continuous Culture of a Strain of *Rhizobium* sp. Isolated from Stem Nodules on *Sesbania rostrata*

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A strain of *Rhizobium* sp. (ORS571), isolated from stem nodules on *Sesbania rostrata*, was grown as a N_2-fixing continuous culture for up to five weeks (>80 culture replacements). Under optimum conditions of O_2 supply [<9 μM dissolved O_2, 100–160 nmol O_2 (mg dry wt)$^{-1}$ min^{-1}], N_2 fixation rates were 300–400 nmol N_2 (mg dry wt)$^{-1}$ h^{-1} and nitrogenase activity in culture samples reached 2000 nmol C_2H_4 (mg dry wt)$^{-1}$ h^{-1}, the highest values yet recorded for any *Rhizobium* species. Other properties of the continuous cultures are described and a comparison is made with previous reports of N_2 fixation by cultures of *Rhizobium* spp. Significant features were the greater tolerance to O_2 of ORS571 and the retention of fixed N in the cells of this strain.

INTRODUCTION

For many years it was believed that the legume root nodule bacteria (*Rhizobium* spp.) fixed atmospheric N_2 only in symbiosis with the respective host plants. Since the simultaneous discovery in 1975 in several laboratories that the slow-growing cowpea strain of *Rhizobium* sp., 32H1, produced nitrogenase activity in axenic culture (for review see Gibson *et al.*, 1977), it has been found that a minority of cultures of *Rhizobium* spp., all of the slow-growing type, are able to produce active nitrogenase in microaerobic culture. For this there are a wide range of nutritional and cultural requirements (e.g. Bergersen & Gibson, 1978; Pankhurst, 1981). Until recently, however, it has not been possible to grow any of these cultures under fully N_2-fixing conditions, that is without addition of a source of combined nitrogen. Indeed, some authors (e.g. Ludwig & de Vries, 1982) consider that the N_2-fixing condition in *Rhizobium* is a function of non-growing cells within the culture. Recently Dreyfus & Dommergues (1981) reported the isolation from stem nodules of the tropical legume *Sesbania rostrata*, of a fast-growing strain of a *Rhizobium* sp. which produced very high rates of nitrogenase activity in culture. This strain (ORS571) was grown in nitrogen-free liquid medium at reduced pO_2 or on agar (Dreyfus *et al.*, 1983). We now report the growth of ORS571 in prolonged continuous culture, confirm that N_2 fixation fully supports growth (except for combined nitrogen in the vitamins provided) and show that this strain tolerates higher concentrations of dissolved O_2 than other strains which produce nitrogenase in culture, but in which N_2 fixation is insufficient to sustain growth on nitrogen-free media.

METHODS

Organism and media. The bacterium used was strain ORS571 isolated from a stem nodule of *Sesbania rostrata* in Sénégal; on arrival in Canberra a single colony was isolated from an agar culture. This isolate fixed N_2 on agar, produced both stem and root nodules on *S. rostrata* in laboratory culture and was used in all subsequent studies. Cultures were maintained on lactate medium with 2% (w/v) agar and 7·6 mM-$(NH_4)_2SO_4$. Lactate medium was prepared from the following solutions to give the final concentrations indicated. (A) sodium lactate,

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0·113 M; $MgSO_4$, 0·4 mM; $CaCl_2$, 0.4 mM (pH 7·1 ± 0·1 adjusted with NaOH). (B) K_2HPO_4, 9·6 mM; KH_2PO_4, 6·4 mM. (C) $FeCl_3$, 25 μM; Na-EDTA, 2·5 μM; Na_2MoO_4, 4 μM; $ZnSO_4$, 0·7 μM; $CoCl_2$, 4 nM; $CuSO_4$, 80 nM; $MnSO_4$, 0·9 μM. (D) Biotin (11·5% N), 0·2 mg l^{-1}; calcium pantothenate (5·9% N), 20 mg l^{-1}; nicotinic acid (11·4% N), 20 mg l^{-1} (unless otherwise stated). Solutions containing A, B and C were separately autoclaved and combined aseptically when cool. The vitamins (D) were filter sterilized and added last, usually with the trace elements (C). The pH of the effluent culture was 7·4–7·8, depending on the rate of growth.

Continuous culture. The culture vessel with O_2 electrode, dissolved O_2 (dO_2) feed-back stirrer controller and the analytical methods have been described previously (Bergersen & Turner, 1978, 1980; Ching *et al.*, 1981). The growth temperature was 30 °C and the culture volume 590 ml. The head gas composition was varied by passing measured flows of air and N_2 from rotameters through water, acidified with H_2SO_4, where it was mixed and humidified before being filtered into the culture vessel. Total non-limiting gas flow was approximately 200 or 400 cm^3 min^{-1}. The composition was calibrated by analysis of head-gas samples. Limits of dO_2 in the culture were maintained by small variations in stirrer speed, with a periodicity of 1 to 2 cycles per min (Bergersen & Turner, 1978). These variations were recorded and used with calibrations relating stirrer speed and rates of solution of O_2 together with head gas composition data, to calculate rates of O_2 consumption integrated over periods of 10–15 min. Before inoculation the lactate medium in the culture vessel was equilibrated with 12–15% (v/v) air in N_2. The inoculum was usually 100 ml of a late-exponential-phase culture grown in air in lactate medium containing sodium glutamate (5·3 mM). Nitrogenase activity was detected within 4–5 h and the medium flow started 6–10 h later. Steady states were established with various levels of dO_2 and with calcium pantothenate and nicotinate at 2 or 20 mg l^{-1} and the effects on the composition and activities of the culture determined.

Nitrogenase assays. Preliminary tests showed that, unlike strains 32H1 and CB756 (Bergersen *et al.*, 1976), nitrogenase rates of ORS571 were not stimulated by addition of soybean oxyleghaemoglobin to the assay vessels. Therefore a standard assay was adopted in which 5·0 ml of a sample (collected anaerobically in capped evacuated tubes from the culture vessel) was shaken at 200 r.p.m. and 30 °C in 50 ml rubber-capped Erlenmeyer flasks with a gas phase containing 2% (v/v) O_2, 10% (v/v) C_2H_2 and argon (to 1 atm). These conditions were optimal for moderate nitrogenase activities [400–600 nmol C_2H_4 (mg dry wt)$^{-1}$ h^{-1}] but did not always match conditions in the chemostat. However, it was impractical to optimize assay conditions for every steady state. Samples of gas were analysed for C_2H_4 (Turner & Gibson, 1980) at intervals.

Determination of bacteria. Various standard colorimetric assays often used for determination of cell protein gave variable results due to apparent interference by culture products, as commonly found for cultures of *Rhizobium* spp. (F. J. Bergersen, unpublished observations). Therefore activities were expressed in terms of the dry weight of water-washed cells obtained from a standard curve of optical absorbance of cultures or suspensions at 700 nm.

Nitrogen analyses. Effluent culture was collected in ice-cooled flasks and centrifuged at 0–4 °C, and the bacteria, the culture supernatant and the inflowing medium were analysed for total nitrogen by Kjeldahl digestion, distillation and titration (Bergersen, 1980). The nitrogen was also analysed for natural abundance of ^{15}N with a VG-Micromass 903 isotope ratio mass spectrometer (Bergersen 1980). This was expressed as $\delta^{15}N$ where $\delta^{15}N = 1000[(r_{sample} - r_{reference})/r_{reference}]$; r = mass 29/mass 28, and the reference gas was air N_2.

Plant infection tests. Seeds of *Sesbania rostrata* were surface sterilized with conc. H_2SO_4 (20 min), washed and germinated on agar. Seedlings were planted in cotton-plugged test tubes (16 × 2·5 cm) containing Jensen's seedling agar (Gibson, 1980). Samples from continuous cultures were diluted and plated on lactate agar with $(NH_4)_2SO_4$. Single colonies were sub-cultured on the same medium in McCartney bottles and then tested for ability to nodulate the seedlings. Colonies which failed to nodulate were re-tested in case failure was due to defective plants.

RESULTS AND DISCUSSION

Cultures achieved reasonable rates of growth (up to $D = 0{\cdot}1$ h^{-1} with > 0·2 mg dry wt ml^{-1}) with no combined nitrogen in the medium other than that present in the vitamins and as impurities in the chemicals. They were homogeneous and there was little surface growth in the culture vessel, even after the longest period of growth (37 d; > 80 culture replacements). The yields of cells in these continuous cultures of ORS571 did not appear to be limited by any major constituents of the growth medium although rates of N_2 fixation were affected by vitamin concentration (Fig. 1). At low rates of O_2 consumption (supply) and dO_2, where dilution rate had to be decreased to prevent wash-out (Table 1), the cultures appeared to be O_2-limited since an increase from about 40 to 70 nmol O_2 (mg dry wt)$^{-1}$ min^{-1} usually produced an increase in culture yield from about 6 to 12–14 mg dry wt h^{-1} (data not shown). However, at higher but more uniform dilution rates (0·08–0·10 h^{-1}), and at all concentrations of vitamins tested, further increases in O_2 supply produced no further change in cell yield until both N_2 fixation and yield

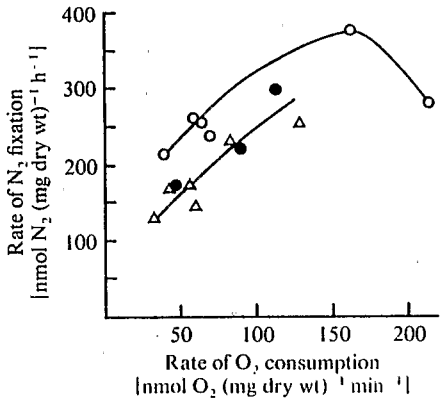

Fig. 1. Relationship between rates of consumption of O_2 and N_2 fixation. Continuous cultures of ORS571 grew at dilution rates of 0·05–0·1 and dO_2 of 0·1–12 μM. ○, 0·2 mg biotin l^{-1}, 20 mg pantothenate l^{-1}, 20 mg nicotinate l^{-1}; ●, 0·02 mg biotin l^{-1}, 2 mg pantothenate l^{-1}, 20 mg nicotinate l^{-1}; △, 0·02 mg biotin l^{-1}, 2 mg pantothenate l^{-1}, 2 mg nicotinate l^{-1}.

declined when O_2 consumption exceeded about 200 nmol O_2 (mg dry wt)$^{-1}$ min^{-1} (Table 1, Fig. 1). The nature of the growth limitation prevailing between O_2 supply rates of 70 and 200 nmol O_2 (mg dry wt)$^{-1}$ min^{-1} was not determined but it was possibly due to intrinsic physiological factors (Hill *et al.*, 1972). In the absence of a clearly-defined limitation, μ_{max} for N_2-fixing growth was not determined.

N_2 fixation increased almost in proportion to rates of consumption of O_2 from about 40 up to 150 nmol O_2 (mg dry wt)$^{-1}$ min^{-1} (Fig. 1). This was generally reflected by increases in nitrogen content of the bacteria. Over all experiments the percentage nitrogen measured in the bacteria ranged from 6 to 10 (mean 8·0, SD 1·7) with yields of 6–15 mg dry wt h^{-1} at dilution rates between 0·05 and 0·1 h^{-1}. The highest rate of nitrogenase activity measured in the standard assay was about 2000 nmol C_2H_4 (mg dry wt)$^{-1}$ h^{-1} when N_2 fixation in the culture was almost 400 nmol N_2 (mg dry wt)$^{-1}$ h^{-1}. This level of nitrogenase activity is equivalent to 60–70 nmol C_2H_4 (mg protein)$^{-1}$ min^{-1} (based on the percentage of N in these bacteria), which is 2–3 times higher than in batch cultures of this strain (Dreyfus *et al.*, 1983) and very much higher than recorded previously for any *Rhizobium* culture. Activities in standard assays were not consistently related to N_2 fixation in the cultures (Table 1) because the conditions in the assay did not always match varying growth conditions.

There seemed to be some loss of symbiotic capacity of the bacteria during continuous culture and this may have been progressive. After 16 d continuous culture, involving several different steady states, 2 of 18 colonies from diluted culture failed to nodulate *S. rostrata* in repeated tests, but they grew on N-free agar and reduced C_2H_2. In another culture, also involving different steady states, after 25 d growth, 5 of 20 single colonies failed to nodulate. In both of these tests all colonies were uniform and appeared to be identical with colonies of the original culture. When 15 colonies from the original stock cultures were tested, all produced nodules on the test plants. The basis for these observations remains to be investigated.

Effects of conditions of O_2 supply

Data from representative steady states with different dO_2 and covering a fivefold range of O_2 consumption rates are presented in Table 1. Higher levels of dO_2 (up to 12 μM) were tolerated by ORS571 than for continuous cultures of 32H1 or CB756 (<1 μM, Bergersen *et al.*, 1976; Bergersen & Turner, 1978, 1980). At dO_2 of up to 12 μM, soybean leghaemoglobin is almost completely oxygenated and thus could not be expected to facilitate the O_2 flux to the bacteria. This explains why leghaemoglobin did not stimulate nitrogenase activity of culture samples as found formerly with CB756 (Bergersen *et al.*, 1976). Above 9 μM dO_2, N_2 fixation was sharply

Table 1. *Effects of supply of O_2 on continuous cultures of ORS571*

The data are from representative steady states of N_2-fixing cultures supplied with pantothenate and nicotinate (20 mg l^{-1}) and biotin (0·2 mg l^{-1}). Values in parentheses are standard deviations of components of the nitrogen analysis.

Culture parameter	pO_2 in culture head space (%): 3·5	5·2	8·7	16·6
O_2 supply				
Dissolved O_2 (μM)	1·6	4·3	5·9	12·5
O_2 consumption [nmol (mg dry wt)$^{-1}$ min^{-1}]	39	58	161	213
Culture characteristics				
Dilution rate (h^{-1})	0·055	0·090	0·096	0·097
Bacteria (μg dry wt ml^{-1})	193	259	224	168
(mg dry wt h^{-1})	6·3	13·8	12·7	9·6
(μg N ml^{-1})	20·8 (1·0)	20·7 (1·0)	23·7 (0·5)	13·4 (1·2)
Culture supernatant (μg N ml^{-1})	2·7 (0·4)	3·0 (0·4)	3·6 (0·6)	2·9 (0·4)
Nitrogen fixation				
μg N_2 h^{-1}*	678	1118	1398	777
nmol N_2 (mg dry wt)$^{-1}$ h^{-1}	213	261	378	279
Standard nitrogenase assay [nmol C_2H_4 (mg dry wt)$^{-1}$ h^{-1}]	700	839	1816	610

* The inflowing medium contained 2·65 (0·35) μg N ml^{-1} by analysis. N_2 fixed (μg N_2 h^{-1}) = (bacterial N ml^{-1} + supernatant N ml^{-1} − 2·65) × flow (ml h^{-1}).

curtailed. Generally it was found that N_2 fixation could not sustain growth when O_2 consumption was < 30–40 nmol O_2 (mg dry wt)$^{-1}$ min^{-1}. However, the rates of O_2 consumption associated with maximum N_2 fixation by ORS571 [100–160 nmol O_2 (mg dry wt)$^{-1}$ min^{-1}; Table 1, Fig. 1] were similar to those in CB756. The reasons for the apparently greater respiratory efficiency, leading to greater cell yields and higher N_2 fixation rates of ORS571, are not known but appear to be a feature of this faster-growing strain.

Effects of vitamin concentration

Dreyfus & Elmerich (1981) first reported that N_2 fixation by ORS571 in batch cultures was greatest when the vitamins were provided at 20 mg l^{-1}. Later (Dreyfus *et al.*, 1983), this was changed to 10 mg l^{-1} for biotin and nicotinate and 20 mg l^{-1} for pantothenate. These high vitamin levels may be required for conditions in batch culture; they represent a small but significant source of combined nitrogen because of the nitrogen content of pantothenate and nicotinate. Figure 1 presents data from a series of steady states in continuous cultures supplied with biotin (0·02 or 0·2 mg l^{-1}) and pantothenate and nicotinate at 2 or 20 mg l^{-1}. The higher concentration of combined vitamins increased N_2 fixation but there were no consistent effects of high concentrations of any one of them. Moreover, at constant values of D, variations in vitamin concentrations did not affect yield. Therefore the reasons for the differences between the two curves of Fig. 1 remain unresolved.

Features of N_2-fixing cultures

Fixation of N_2 by other strains of *Rhizobium* spp. in culture has been characterized by rapid equilibration of newly-fixed N with the medium (e.g. Bergersen & Turner, 1978), sometimes called 'export' of fixed nitrogen (O'Gara & Shanmugam, 1976; Ludwig, 1980), which results in most of the fixed nitrogen being found in the medium as NH_4^+. This rapid dilution of fixed nitrogen by passage into the medium may be a major reason for failure of these strains to grow without a supplementary source of combined nitrogen. In contrast, in the continuous cultures of ORS571 about 90% of the fixed nitrogen was found in the bacteria (Table 1); supernatants contained only 10% of the N_2 fixed and there was no detectable NH_4^+ (< 5 nmol NH_4^+ ml^{-1}). However, $\delta^{15}N$ and total nitrogen data (Table 2) indicate that there was some exchange of fixed

Table 2. *Influence of N-containing vitamins on nitrogen balance and natural abundance of ^{15}N for the medium, bacteria and culture supernatants from continuous cultures of ORS571*

Apart from the effects of rates of O_2 supply on rates of nitrogen fixation, which are reflected in the relatively large standard deviations for cell nitrogen values, there were no discernible effects of culture parameters and the data are pooled. Means and standard deviations (in parentheses) are given for the nitrogen analyses of samples taken from 6, 3 or 6 steady states respectively for each concentration of N-containing vitamins. Biotin concentrations were too small to influence the data.

Culture parameters:					Nitrogen analysis:					
Vitamin concn (mg l^{-1})		O_2 supply			Medium		Cells		Supernatant	
Nicotinate	Pantothenate	dO_2 (μM)	Rate (nmol O_2 mg^{-1} min^{-1})	Dilution rate (h^{-1})	μg N ml^{-1}	$\delta^{15}N$	μg N ml^{-1}	$\delta^{15}N$	μg N ml^{-1}	$\delta^{15}N$
20	20	0·5–12·5	40–213	0·06–0·10	2·40 (0·23)	−1·54* –	19·60 (3·6)	−0·27 (1·06)	2·90 (0·36)	4·24 (1·43)
20	2	0 3·5	46 111	0·054 0·089	1·93 (0·98)	−2·45 (0·47)	20·96 (4·08)	0·71 (0·80)	1·29 (0·36)	4·97 (0·54)
2	2	0·7–10·5	31–128	0·056–0·097	2·19 (0·80)	1·43 (0·38)	13·48 (1·59)	0·61 (0·86)	1·84 (0·32)	4·33 (1·39)

* One estimate only.

Table 3. *A comparison between N_2-fixing continuous cultures of ORS571 and of the cowpea Rhizobium sp. strain CB756*

Data for ORS571 are from the present paper and for CB756 from the references cited.

Parameter	ORS571	CB756	Reference*
Max. dissolved O_2 tolerated (μM)	⩽12	⩽1	(1)
Max. O_2 solution rates tolerated (μmol ml^{-1} h^{-1})	1–2·4	0·9–2·4	(1)
O_2 consumption [nmol min^{-1} (mg dry wt)$^{-1}$]	30–200	80–180	(4)
Yield for 600 ml culture (mg dry wt h^{-1})	⩽14	⩽2·5	(3)
Nitrogen fixation [nmol N_2 (mg dry wt)$^{-1}$ h^{-1}]	200–300	32–55	(2)
Distribution of fixed N (%)			
Cells	88–92	1–9	(2)
Medium	8–12	91–99	(2)
Nitrogenase assays [nmol C_2H_4 (mg dry wt)$^{-1}$ h^{-1}]	600–2000	140–776	(3)

* (1) Bergersen & Turner (1976); (2) Bergersen & Turner (1978); (3) Bergersen & Turner (1980); (4) Ching *et al.* (1981).

nitrogen with the medium. The medium and supernatants from culture effluent had similar low nitrogen contents but the $\delta^{15}N$ values were quite different. Further, spectrophotometrical examination of the culture supernatants showed that no detectable nicotinate remained in the cultures, so nicotinate nitrogen had been replaced by nitrogen with a higher $\delta^{15}N$ (presumably arising from isotopic fractionation occurring during exchange between the medium and the bacteria). As expected, $\delta^{15}N$ values for the bacteria were closer to zero (i.e. to air N_2) than the medium or supernatant nitrogen, consistent with an overall average of 92% of culture N from the atmosphere. The data also show that traces of nitrogen were present in the media in addition to that in the vitamins.

Table 3 makes a comparison between continuous cultures of the previously-studied strain CB756 and ORS571. The main differences are the greater tolerance of ORS571 for dissolved O_2, the distribution of fixed nitrogen and the faster growth and N_2 fixation of ORS571. Further, CB756 required that the medium be supplemented with combined N (glutamine or NH_4^+) for growth, whilst ORS571 needed only the vitamins. For additional comparison, recently reported nitrogenase activities for cultured *Rhizobium* spp. have been 10·120 nmol C_2H_4 (mg

protein)$^{-1}$ h^{-1} for agar cultures of two cowpea strains (Pankhurst, 1981), and for *R. japonicum* strain 311b110 supplied with CO_2 under microaerobic conditions in liquid cultures the values were 40–100 nmol C_2H_4 (mg protein)$^{-1}$ h^{-1} (Aguilar & Favelukes, 1982). These seem to be typical values for agar and small batch cultures in liquid media.

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