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Investigations into the Growth of a Nitrogen Fixing Bacterium in Gradients relevant to the Rhizosphere of Rice*

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Abstract

Experiments with a nitrogen fixing bacterium grown in C, N, and O_2 gradients established in a gradostat (multistage chemostat) are described which model the rhizosphere of rice. In a C gradient the carbon source was fully used and gave rise to a biomass gradient in its place. Addition of an ammonium gradient increased the overall biomass, but did not change the location of maximum growth. Superimposing an oxygen gradient on top of the glucose gradient shifted the growth position due to a partial inhibition of the diazotrophic microorganism at higher oxygen concentrations (16 μM). Such inhibition was overcome by further superimposing an ammonium gradient.

Introduction

The exudates of roots are carbon sources for a wide variety of rhizosphere organisms. These substrates, as well as other substances, form spatial gradients through diffusion and uptake by plants or microorganisms. For paddy rice (*Oryza sativa* L.) growing in anaerobic soil, oxygen reaches the rhizosphere through the aerenchyma of roots also forming a gradient. As carbon, ammonium, and oxygen are important factors for the growth of heterotrophic microaerobic nitrogen fixing organisms, a diazotrophic isolate was investigated in various gradients of these compounds. These experiments were performed in a bidirectional compound chemostat or gradostat. Continuous culture systems are more appropriate models for rhizosphere research than batch cultures since the substrates need not be given in excess.

Material and Methods

The gradostat

A gradostat is a multistage continuous culture system with bidirectional pumping

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of media (Lovitt et al., 1981). The media from the two reservoirs mix in each vessel in different proportions. If a soluble component is present in different amounts in the two media it will form a gradient in the system depending only on pumping rates and the concentration in the two reservoirs. Allowing some time for the gradients to be become established, time-independent steady-state gradients are obtained. If a soluble compound is the carbon source for a heterotrophic microorganism it will be metabolized and replaced by a gradient of biomass instead, since the organisms are transfered at the same rate as other medium constituents. We built a gradostat with five vessels (V_1–V_5) each containing 700 ml of culture. The pH and pO_2 were separately controlled in each vessel making it possible to maintain pH as well as pO_2 gradients. For a detailed description of the system, see Fritzsche et al., (in preparation).

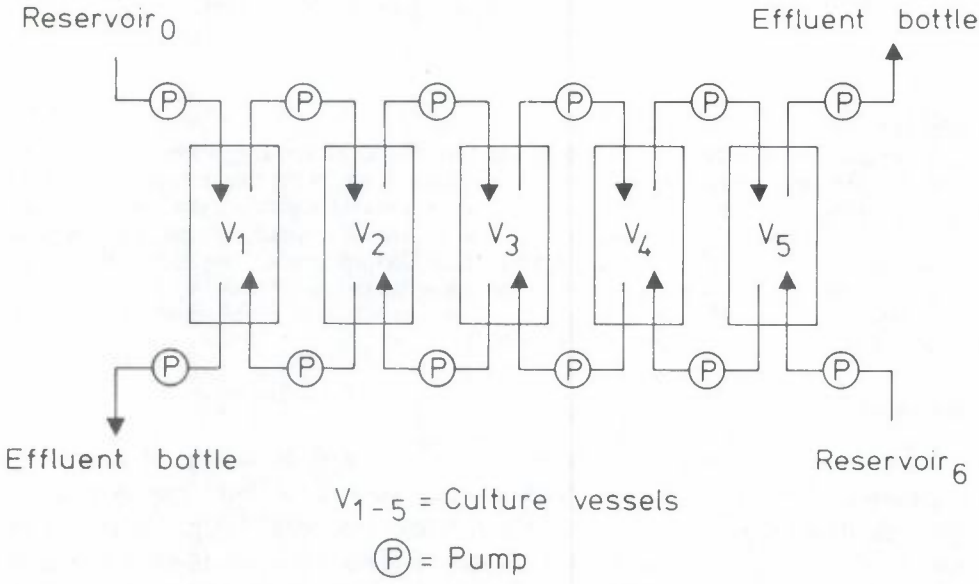

Figure 1. Pumping scheme for the gradostat described in this paper.

Media and growth conditions

Basal medium: (g l^{-1} of distilled water) K_2HPO_4, 0.2; KH_2PO_4, 0.3; $MgSO_4 \cdot 7\ H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2\ H_2O$, 0.026; $MnSO_4 \cdot H_2O$, 0.01; $Na_2MoO_4 \cdot 2\ H_2O$, 0.002; $FeSO_4 \cdot 7\ H_2O$, 0.01; pH 6.4.

Using the properties of the gradostat, gradients were set up to model the rhizosphere of rice with V_1 simulating a point near to the root and V_5 a point further away in the soil. Both reservoirs contained the basal medium and all pumps were set to the same pumping rate (50 ml $\cdot h^{-1}$) which resulted in a linear carbon gradient maintained by supplying the medium in reservoir 0 (R_0) with 1 g glucose l^{-1} while no glucose was

added to reservoir 6 (R_6). In R_0 50 $\mu g \cdot l^{-1}$ of biotin, 50 $\mu g \cdot l^{-1}$ of thiamine, and 25 $\mu m \cdot l^{-1}$ of panthotenic acid were also added. Ammonium gradients were maintained by adding 45 mg ammonium chloride l^{-1} into R_0 or R_6. Where O_2 gradients were required concentrations were 16, 12, 8, 4, 2 μM in V_1–V_5, respectively. For all other experiments the concentration was 6 μM O_2 in all vessels. Temperature was maintained at 30°C, stirring rate at 400 rpm and all vessels were operated at pH 6.4.

Bacterial strain

Isolate MB 35 is a Gram negative rod which belongs to the β subclass of proteobacteria. It fixes nitrogen under microaerobic conditions and was isolated from rice rhizosphere using continuous culture enrichment (Fritzsche, C. et al., in preparation).

Sampling and analysis

Each experiment was continued until a steady-state was obtained after approximately 6 days. 5 ml samples were taken then and after another 1 or 2 days. Optical density (OD_{578}) was measured, protein content obtained by the micro-Goa method (Bergersen 1980). An enzymatic assay was used for determinations of remaining carbon source in the sterile filtered supernatant (glucose assay, Sigma, Deisenhofen, FRG). Each analysis was repeated twice leading to four replicates for each steady-state. The growth rates were calculated for each vessel under steady-state conditions.

Results and Discussion

Table 1 shows the data obtained for growth in a glucose gradient. Isolate MB 35 has a high substrate affinity ($K_s < 1$ μM glucose) and growth was only observed in V_1. All glucose was consumed for growth and a biomass gradient was established. Negative growth rates are probably due to loss of biomass through bacterial maintenance. The results of this experiment are in good agreement with the mathematical model described by Newmann et al., (1977) which predicted that growth occurs near the root surface, a steep gradient of biomass develops, and that exudates are present in only very small concentrations.

Table 1. Analysis of growth of the diazotrophic isolate MB 35 in a glucose gradient.

vessel		V_1	V_2	V_3	V_4	V_5
OD_{578}		0.445	0.287	0.204	0.133	0.067
protein	[$mg \cdot l^{-1}$]	95	62	44	30	15
glucose	[$mg \cdot l^{-1}$]	0	0	0	0	0
growth rate	[h^{-1}]	0.10	−0.02	0.00	0.00	0.00

In opposing gradients of ammonium (added to R_6) and glucose no differences could be detected from results in the glucose gradient alone. Presumably only small amounts of ammonium was able to reach V_1 where all the growth occurred and this had little influence on growth.

A glucose as well as a parallel ammonium gradient, modelling exudates containing N at a C:N ratio of 40:1, increased the measured protein content of the culture (152, 96, 73, 46, and 22 $mg \cdot l^{-1}$ in V_1–V_5, respectively), but did not change the growth position (Table 2). No glucose could be detected in the supernatants of any vessel. The protein content measured in this experiment could not be explained by growth on the ammonium source alone since under comparable conditions in a chemostat a protein:N ratio of 7.5:1 was determined. Additional experiments performed in single stage continuous culture demonstrated that Isolate MB 35, *Azospirillum brasilense* Sp 7, and another nitrogen fixing isolate (isolate R7) were able to grow simultaneously on ammonium and N_2 as N sources (Fritzsche et al., 1990). It is most likely that this ability also occurred in the aforementioned gradostat experiment. When an oxygen gradient was superimposed on the glucose gradient, the growth rate in V_1 decreased (Table 2) and 0.26 g glucose l^{-1} could be detected in the supernatant of this vessel. This was due to inhibition of isolate MB 35 by the high oxygen concentration. The residual glucose was able to reach V_2 where growth occured (Table 2). A partial inhibition of MB 35 at 16 μM O_2 was also demonstrated in a separate chemostat experiment.

Table 2. Growth rates [h^{-1}] of the diazotrophic isolate MB 35 in the gradostat in parallel gradients of glucose (C), NH_4Cl (N), and O_2 as described in the text.

vessel	V_1	V_2	V_3	V_4	V_5
C gradient	0.10	−0.02	0.00	0.00	0.00
C+N gradients	0.10	−0.02	−0.01	−0.01	−0.01
C+O_2 gradients	0.06	0.05	−0.03	−0.01	0.00
C+O_2+N gradients	0.09	−0.02	−0.01	−0.01	0.01

Oxygen inhibition could not be seen when parallel glucose, oxygen, and ammonium gradients were investigated together. Growth occured in V_1 and only a low concentration of glucose was detected in this vessel (0.07 $g \cdot l^{-1}$). No growth and no glucose were measured in V_2–V_5. This experiment demonstrates that ammonium and oxygen might have opposing effects on the growth of diazotrophic microorganisms in gradients. In this experiment the protein contents (93, 56, 33, 24, and 10 $mg\,l^{-1}$ in V_1–V_5, respectively) indicated simultaneous growth on ammonium and N_2 though the gain of N by nitrogen fixation seems to be smaller compared to the experiment with glucose and parallel NH_4Cl gradients.

In spite of its technical complexity the gradostat is a valuable tool to model spatial

gradients relevant to the rhizosphere and investigate the physiological abilities of diazotrophic and non diazotrophic microorganisms in these gradients.

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