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Interactions Between *Azospirillum* and *Arthrobacter* in Diazotrophic Mixed Culture

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Abstract

Azospirillum brasilense and *Arthrobacter giacomelloi* grown in mixed batch culture under diazotrophic conditions, with succinate as carbon source, exhibited improved nitrogenase activity. The interaction between *A. brasilense* and *A. giacomelloi* might be regulated by an interchange of metabolites. In the present work each microorganism was grown in differently diluted cell-free filtrate fluid of the other. To evaluate the effect of metabolites interchange, the bacteria were also grown in a membrane diffusion chamber either in isolated or interacting cultures. From the results it appears that the supply of beneficial metabolites or the removal of some inhibitory products might regulate the interaction between microorganisms.

Keywords: *Azospirillum brasilense*, *Arthrobacter giacomelloi*, microbial interaction, mixed culture, nitrogenase activity

1. Introduction

Examples of microbial interaction with *Azospirillum* have been described, the nature of interactions being various and depending on the interacting microorganisms and environmental conditions (Barea et al., 1983; Jagnow, 1983; Halsall and Goodchild, 1986). In our previous studies (Cacciari et al., 1984,

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1986, 1989a, 1989b) *Azospirillum brasilense* was grown under diazotrophic conditions in both batch and continuous mixed cultures with *Arthrobacter giacomelloi*, a nitrogen-fixing strain isolated from soil (Cacciari et al., 1971). The mixed cultures exhibited an improved nitrogenase activity under partial oxygen pressures ranging from 0.004 to 0.2 atm and also a greater oxygen tolerance, even though a mechanism such as respiratory protection seemed not to be operative (Cacciari et al., 1989a). The aim of the present work was to investigate whether production and diffusion of stimulatory and/or inhibitory substances from the microorganisms might be a main factor affecting growth and nitrogenase activity of the bacteria growing in mixed culture.

2. Materials and Methods

Azospirillum brasilense Cd (ATCC 29710) and *Arthrobacter giacomelloi* (DSM 3681) were grown at 30°C in SuB_7^- medium (Cacciari et al., 1989a). Viable cell number was determined by plate counts using Nutrient Agar medium.

In a first experiment, 48-hour old single cultures (300 ml in 500 ml standing flasks) of *A. brasilense* and *A. giacomelloi* were filtered to take sterile supernatant fluids. Portions of the axenic fluids obtained were then diluted with complete fresh medium at the final concentrations of 50%, 25%, 12%, 6% and 3% by volume. The whole cell-free filtrates from each bacterial culture as well as each diluted axenic fluid (60 ml in 100 ml standing flasks) were then inoculated with 3 ml (5% by volume) of a 3-day old culture of the other microorganism grown in the same medium. The inoculum size was about 7×10^7 c.f.u. ml^{-1} and 25×10^7 c.f.u. ml^{-1} for *A. brasilense* and *A. giacomelloi*, respectively. Control SuB_7^- medium was inoculated and cultured in the same way. Samples were examined after 48 hr of growth.

In a second experiment, interaction between bacteria was studied in the EcoloGen E40 (New Brunswick Scientific Co., Inc., New Brunswick, NJ, USA), (Collins and Tillion, 1977). Two opposite side growth chambers were separated from the central reservoir by membrane filters of 0.2 μm pore diameter which do not permit the passage of cells but allow interchange of diffusing solutes. These side chambers (350 ml), filled with 150 ml medium, were inoculated with *A. brasilense* culture while the central reservoir (550 ml filled with 300 ml medium) was inoculated with *A. giacomelloi* culture. The other two side chambers were isolated by use of stainless steel plates to allow the growth of pure cultures of bacteria at the same conditions. The schematic drawing of EcoloGen and the culture conditions of this experiment are shown in Fig. 1. Cultures were inoculated with about 5% by volume of 3-day old cultures at the

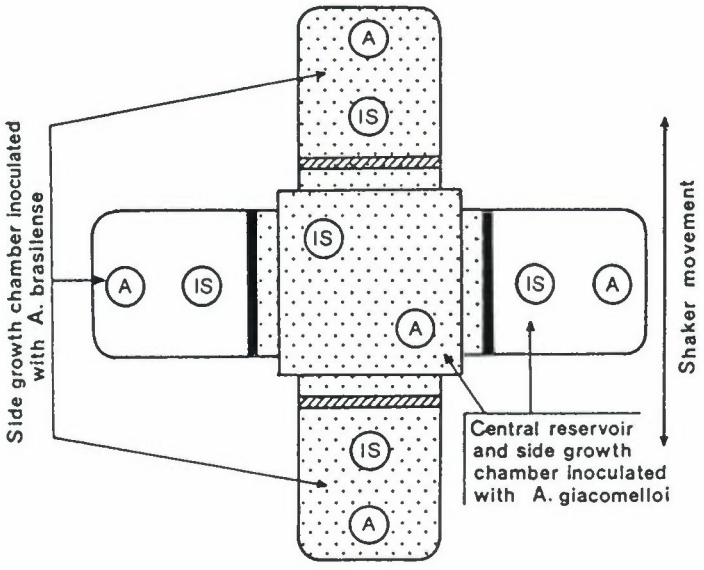

Figure 1. Culture conditions in the EcoloGen E40 experiment. A = aeration port; IS = inoculation and sampling port: ▨ = membrane filter (0.2 μm pore size); ■ = stainless steel plate; □ = isolated cultures; ⊠ = interacting cultures.

same conditions described above. The EcoloGen was mounted on a reciprocating shaker (170×35 mm strokes/min, 90° to membrane) at 30°C. Samples were taken after 1, 3, 7 and 10 days of growth.

Culture protein content and bacterial dry weight were determined as previously described (Cacciari et al., 1989a). The nitrogenase activity was determined by the acetylene reduction assay. Five ml culture samples were collected by a sterile syringe and directly injected into vials in the presence of argon and acetylene (10%) at the partial oxygen pressure of 0.01 atm. Duplicate samples were incubated at 30°C and the ethylene produced was determined with a gas chromatograph (Perkin-Elmer 900, Porapak N-column, f.i.d.).

Statistical analysis of results was performed by the one-way analysis of variance and significance was calculated on the basis of four replicates.

3. Results and Discussion

A. brasilense and *A. giacomelloi* were able to grow in the whole or diluted cell-free filtrate fluids of the other (Fig. 2). Diluted supernatants from *A. gia-*

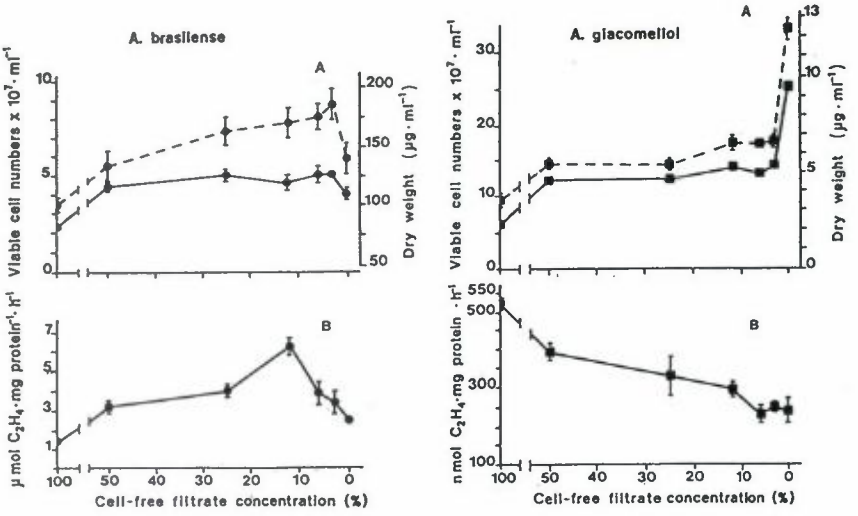

Figure 2. Viable cell number (A; unbroken line), dry weight (A; broken line) and nitrogenase activity (B) of *Azospirillum brasilense* Cd and *Arthrobacter giacomelloi* grown for 48 hr in differently diluted cell-free filtrates, as described in Materials and Methods section. At 100% and 0% concentrations are reported values obtained by growing bacteria in the whole cell-free filtrates of the other strain culture or in the control medium, respectively. Error bars indicate standard error of the mean and in some cases they are within the dimensions of the symbols.

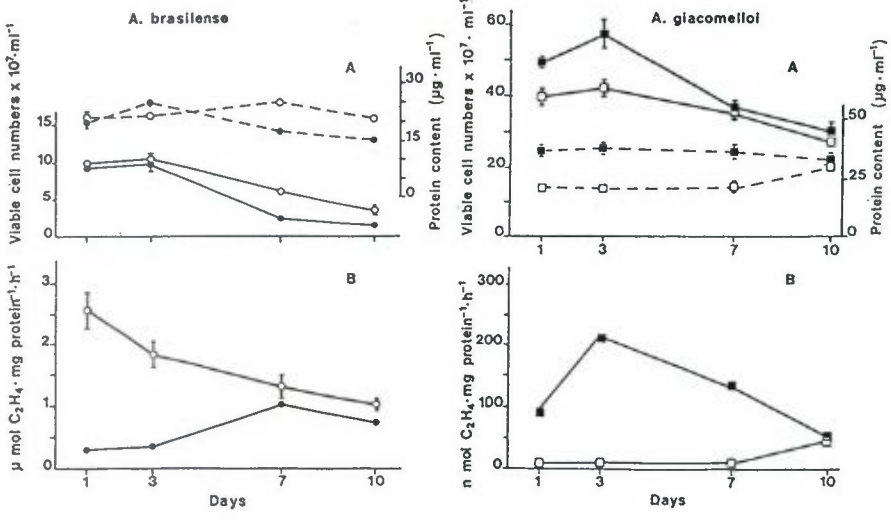

Figure 3. Viable cell number (A; unbroken line), protein content (A; broken line) and nitrogenase activity (B) of *Azospirillum brasilense* Cd and *Arthrobacter giacomelloi* grown in the EcoloGen, as described in Materials and Methods section. Open symbols: cultures grown in isolated side chambers; closed symbols: cultures grown in chambers with membrane filters which allow interchange of diffusing solutes. (See Fig. 1). Statistical details as for Fig. 2.

comelloi culture produced a significant stimulation on *A. brasilense* growth and nitrogenase activity, as compared with those obtained in the control medium, whereas the whole supernatant inhibited both growth and nitrogenase activity. The growth of *A. giacomelloi* in *A. brasilense* cell-free filtrates was strongly inhibited at any dilution level, though a significant increase of nitrogenase activity was observed in cells grown in the more concentrated filtrates. Changes in pH of the cell-free filtrates or depletion in carbon source seem not to be responsible for the fall in cell number observed in both *Azospirillum* and *Arthrobacter* when grown in the whole supernatant of the other. In fact pH of cultures was always near neutral and addition of succinate to cell-free filtrates did not remove the inhibition.

No inhibition but rather a stimulation of *A. giacomelloi* growth occurred when the bacteria were grown in a membrane diffusion chamber, in the second experiment (Fig. 3). During the first days of growth, cell number and protein content of the interacting culture were significantly higher than those of the isolated culture. Nitrogenase activity by the isolated culture was very low, while the interacting culture showed an increase in activity with a maximum of 210 nmol/mg protein/hr after 3 days of growth. The number of cells and the protein content of *A. brasilense* did not change greatly during the first 3 days. Then cell number decreased with time and appeared significantly lower after 7 days, mainly in the interacting culture. Nitrogenase activity of the isolated culture was high after 24 hr of growth but decreased with time. By contrast, a strong inhibition occurred during the first period of growth in the interacting culture. After 7 days the nitrogenase activity of both cultures reached very similar values.

The suitability of the diffusion chamber for studying microbial interactions was extensively examined and discussed by Crump and Richardson (1985) who demonstrated that membrane porosity, thickness and area, as well as fluid velocity can affect metabolites' diffusion rate. In the experiment described here, *A. giacomelloi* was inoculated in the central reservoir and *A. brasilense* in two side chambers, but the most favourable membrane area/medium volume ratio seemed not to account for increased cell number of *A. giacomelloi* culture. In fact, significantly similar c.f.u. ml^{-1} values have been obtained also when *A. giacomelloi* was inoculated in the side chambers, the central reservoir functioning only as a diffusion chamber. Moreover, the geometry of the vessels and the shaking conditions of EcoloGen were not responsible for these contradictory results. When the membranes were substituted by stainless steel discs and the bacteria were allowed to grow in mixed cultures, the cell number of

A. giacomelloi was 2–4 times lower than that of isolated culture, thus confirming the results previously obtained in mixed batch culture (Cacciari et al., 1984, 1989a).

At this stage of the research no attempts have been made to determine the nature of metabolites but it seems that the enhancement of *A. giacomelloi* growth under dialysis conditions might be due to a removal of an inhibitory product that did not pass through the membranes utilized in this experiment. On the other hand, the significant increase in nitrogenase activity of *A. brasilense* grown in diluted cell-free filtrates of *A. giacomelloi* seemed to indicate that some metabolite may be also involved in the nitrogenase stimulation during growth in mixed batch culture and that the response of *A. brasilense* is strictly dependent on their concentration. Dose depending modified nitrogenase activity by some amino acid or herbicide have been reported in *Azospirillum* (Gadkari, 1988; Haahtela et al., 1988; Hartmann et al., 1985). Moreover different concentrations of phytohormone-like substances that are produced by both microorganisms (Cacciari et al., 1980, 1989b; Tien et al., 1979) can differently influence the nitrogen fixing activity of *Azospirillum* (Christiansen-Weniger, 1988).

Positive interactions, resulting in increased nitrogenase activity, were observed in experiments on *Azospirillum* grown in mixed cultures with other nitrogen-fixer (Jagnow, 1983) or non-nitrogen-fixer bacteria (Gracioli and Ruschel, 1978; Halsall and Goodchild, 1986). In addition, Whitehead et al. (1978) reported that in a rhizosphere community a non-nitrogen-fixer *Arthrobacter* sp. greatly stimulated the nitrogenase activity of an *Azospirillum* sp. and of another diazotrophic *Arthrobacter* sp. These reports suggest the importance of bacterial associations in promoting nitrogen fixation, irrespective of the mechanisms involved.

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