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BEIJERINCKIA DERXII STIMULATES THE VIABILITY OF NON-N_2-FIXING BACTERIA IN NITROGEN-FREE MEDIA

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ABSTRACT

The interactions between the nitrogen–fixing microorganism *Beijerinckia derxii* with two non-diazotrophic bacteria, either *Escherichia coli* or a facultative sulphur-oxidizing chemolitotroph, were studied in mixed cultures. Direct and indirect contact between *B. derxii* and *E. coli* were tested. *B. derxii* increased CFU numbers and/or maintained the viability of the non-diazotrophic bacteria, but neither growth nor nitrogenase activity of the nitrogen-fixing bacterium were affected by either partner.

Key words: *Beijerinckia derxii*, coculture, viability.

INTRODUCTION

Mixed cultures of microorganisms are suitable systems for studying the interactions between organisms and their impact on the environment and may open up new perspectives that could lead to new discoveries. Some mixed cultures containing N_2- fixing bacteria provide conditions more suitable for N_2 fixation than pure cultures. For example, Halsall (10) showed that the nitrogenase activity of *Beijerinckia indica* B15 was stimulated by coinoculation with *Cyathus stercoreus* both in axenic culture and in native soil. An extremely unlikely association is the mixed culture of *Azospirillum brasilense* Cd with the non-N_2-fixing marine mangrove rhizosphere bacterium *Staplylococcus sp,* which increased the N_2 fixation of the former (11). Benefits to both partners were observed in a coculture of *Bacillus* and *Azospirillum,* where pectin degradation by *Bacillus* and N_2 fixation by *Azospirillum* were enhanced (13). Studies on mixed cultures generally emphasize the effects of the non-diazothophic on N_2-fixing bacteria, mainly with respect to nitrogenase activity. However, little is known about the effects of diazotrophs on the growth and maintenance of viability of compatible partners. Tsuchiya *et al* (22) and Trivedi and Tsuchiya (21) concluded that the association of a nitrogen-fixing organism from the genus *Beijerinckia* with the leaching bacterium *Thiobacillus ferrooxidans* enhanced the rate and extent of copper, nickel and ore leaching, indicating that nitrogen fixation can be important in bioextractive metallurgy.

In this study the N_2-fixing microorganism *Beijerinckia derxii,* frequently found in Brazilian acid soils (8) was cocultured with two non-N_2-fixing bacteria, *Escherichia coli* or a facultative sulphur-oxidizing chemolitotroph, in order to understand some of the ecological roles of free-living N_2-fixing bacteria.

MATERIALS AND METHODS

Microorganisms

The bacterial strains used were: (a) *Beijerinckia derxii* ICB10, a free-living N_2-fixing bacterium isolated from soil under cerrado (1) and identified by biochemical and morphological tests (4); (b*) Escherichia coli* ICB19, isolated from human feces; (c) a facultative chemolitotrophic sulphur-oxidizing bacterium isolated from garden soil in the city of São Paulo and named Bacterium C. Although *E. coli* is an unlikely natural partner of *B. derxii*, it was chosen because it is unable to fix N_2 and is a non-fastidious bacterium. *B. derxii* grows in a simple medium composed of glucose and mineral salts. Bacterium C, unable to fix N_2, was chosen because it is a soil microorganism with a metabolism quite different from that of the other two bacteria studied

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Culture media

The following media were used: Nutrient Broth (NB), Nutrient Agar (NA) and mineral media which compositions are specified in Table 1.

Isolation of the facultative sulphur-oxidizing chemolitotroph bacterium

Medium S6 (12), containing $Na_2S_2O_3$ as energy source and CO_2 as carbon source, was used to isolate the facultative sulphur-oxidizing chemolitotroph bacterium. One gram of soil was suspended in 10 ml of sterile distilled water and shaken at 300 rpm for 30 minutes. 2ml of this suspension were inoculated in 20 ml of S6 liquid medium to which 0.0018 $g.l^{-1}$ phenol red was added and incubated at 30°C with shaking (200 rpm). When the color of the medium changed to yellow, indicating a drop in the pH of the medium, 2 ml of the culture were transferred to fresh liquid medium. After 5 consecutive transferences, a sample was inoculated onto S6 (12) solid medium for isolation of colonies. The neutrophilic, autotrophic facultative sulphur bacterium recovered was not properly classified because ordinary tests were not sufficient to meet its complex identification requirements.

Cocultures assays

Bacterial interactions were tested in mixed cultures as specified in Table 2.

Preparation of cocultures

Coculture I was prepared as follows: - pure cultures of *B. derxii* (in LGb medium) and *E. coli* (in NB medium) were grown for 74h at 30°C in a rotary shaker (200 rpm). *E. coli* cells were centrifuged to discard the medium; the pellet was washed 3 times and ressuspended in LGb medium at a concentration of 1.8 x 10^8 $CFU.ml^{-1}$. 15 ml of the *E. coli* suspension was mixed with 150 ml of a *B. derxii* culture (contained in a 500 ml Erlenmeyer flask). In **coculture II**, pure cultures of *B. derxii* and *E. coli* were prepared as in coculture I. The suspension of *E. coli* was inoculated in a 500 ml Erlenmeyer flask containing 100 ml of LGb medium, so as to reach about 2.4 x 10^4 or 1.8 x 10^7 $CFU.ml^{-1}$. A sterile dialysis

Table 1. Composition (in mM) of the culture media used in the experiments

Nutrient (*)	LGa (17)	LGb	LGc	S6
K_2HPO_4	0.860	40.750	______	______
KH_2PO_4	2.200	9.250	13.240	13.240
$NaHPO_4.12H_2O$	______	______	8.960	8.960
$CaCl_2.2H_2O$	0.140	0.140	0.280	0.280
$MgSO_4.\ 7H_2O$	0.810	0.810	0.810	0.405
$Na_2MoO_4.2H_2O$	0.008	0.008	0.008	______
$FeCl_3$	0.060	0.06	0.120	0.120
$CoCl_2.2H_2O$	0.006	0.006	0.006	______
$MnSO_4.H_2O$	______	______	0.086	0.088
$(NH4)_2SO_4$	______	______		0.100
Glucose	58.800	58.800	22.220	______
$Na_2S_2O_3.5H_2O$	______	______	40.320	40.320
pH	5.7	6.2	5.7	6.6

(*) For solid medium, 12 $g.L^{-1}$ agar were added.
All chemicals were of analytical grade.

Table 2. Bacterial interactions studied conditions.

Coculture	Bacteria involved	Contact	Media
I	*B. derxii* X *E. coli*	Direct	LGb -medium was used for co-culture I and II and respective controls.
II	*B. derxii* X *E. coli*	Indirect*	The same media used for coculture I were used for coculture II
III	*B. derxii* X Bacterium C	Direct	LGc- medium used for co-culture III and respective controls

* The different bacterial species were separated by a membrane with a cut-off MW 50 kDa or greater (VWR Scientific Prod. CO).

tubing containing 8 ml of the *B. derxii* pure culture was introduced into the *E. coli* suspension. This system was set according to Reporter (18). In **coculture III**, the *B. derxii* pure culture was prepared as in coculture I. The Bacterium C pure culture, grown in S6 liquid (12) medium for 120h at 30°C, with shaking (200 rpm) was centrifuged; the pellet was washed 3 times and ressuspended in LGc medium. About 5 x 10^8 $CFU.ml^{-1}$ (0.1 ml) of this suspension was mixed to 100 ml of *B. derxii* pure culture grown for 74 hours. Coculture III was incubated at 30°C with shaking (200 rpm). Still pure cultures of *B. derxii* and *E. coli* in LGb were used as controls of cocultures I and II; shaken pure cultures of *B. derxii* and Bacterium C in LGc were used as controls of coculture III**.**

Analytical Procedures

Samples of the three mixed cultures and controls were taken periodically for enumeration of Colony Forming Units (CFU) by the drop method (2). Diluted suspensions of pure *B. derxii, E. coli* and Bacterium C cultures were plated on solid LGa (16), NA and S6 medium, respectively. The dilutions of cocultures I and II were plated on both LGa and NA medium and those of coculture III on both LGa and S6 media. Six replicates of each dilution were plated.

The concentration of ammonia the supernatants of coculture I was determined according to Chaney and Marbach (5).

Nitrogenase activity of cocultures I and III was evaluated by the acetylene reduction method (23).

E. coli cell protein content in coculture II was measured by the method of Lowry *et al*. (15). Protein concentration was performed in samples collected periodically from the *E. coli* culture grown outside from the dialysis bag.

The presence of extracellular proteins in supernatants of cocultures I and II as well as in *B. derxii* and *E. coli* pure cultures was tested running the samples in a SDS polyacrylamide electrophoresis gel (PAGE) (14); the gels were silver stained (Merck AG. Darmstadt).

RESULTS

The growth curves in N-free medium (coculture I, Fig. 1b) show that *E. coli* in pure culture at 1.8x10^7 $CFU.ml^{-1}$ was practically unable to multiply during the first 24 hours. Following this period, a constant drop in CFU values was observed, with a 91.2% reduction to 340h. Conversely the association with *B. derxii* enabled *E. coli* to gradually increase in numbers (CFU values corresponding to 2 generations of growth) but mainly prevented loss of cell viability.

When *E. coli* cells were cocultured with *B. derxii* separated by a dialysis membrane (coculture II) two different initial concentrations of *E. coli* were used . When the initial CFU value was 1.7 x 10^7 ml^{-1}, i.e. similar to coculture I, this number reached, after 24 hours, 2.7 x 10^7 ml^{-1} in pure culture and 3.1 x 10^7 ml^{-1} in mixed culture; after 72 hours, the corresponding concentrations were 2.3 x 10^7 ml^{-1} and 3.0 x 10^7 ml^{-1}. Following this time, the pure population entered in decline whereas cells in coculture kept their viability (data not shown). By using a lower initial CFU value (2.8 x 10^4 ml^{-1}), it was possible to see that both mixed and pure cultures increased in number (Fig. 2) but the rate of *E. coli* growth was slightly enhanced in the mixed culture. This multiplication probably occurred because of contamination of the medium with traces of combined nitrogen, which could have been assimilated by the bacteria. After this period, while *E. coli* cells viability in pure culture began to decline, in the mixed culture it remained stable, indicating, again, that the presence of *B. derxii* enhanced *E. coli* survival. The data shown in Fig. 2 indicated that in coculture II the higher influence of *B. derxii* was in the maintenance of viability after exponential growth phase.

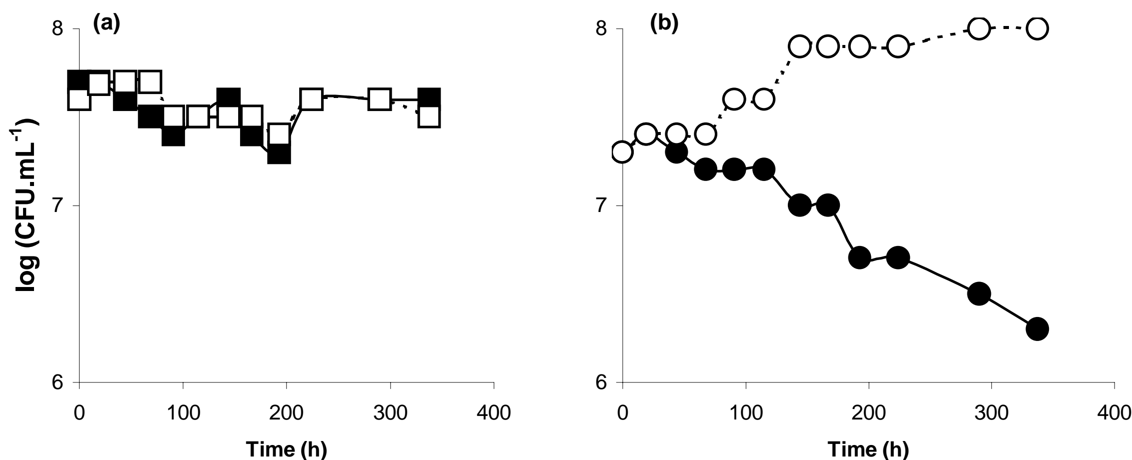

Figure 1- Growth curves of *B. derxii* (a) and *E. coli* (b) in pure and mixed cultures in direct contact. (■ *B. derxii* in pure culture; —□— *B. derxii* in mixed culture; ● *E. coli* in pure culture; —○— *E. coli* in mixed culture)

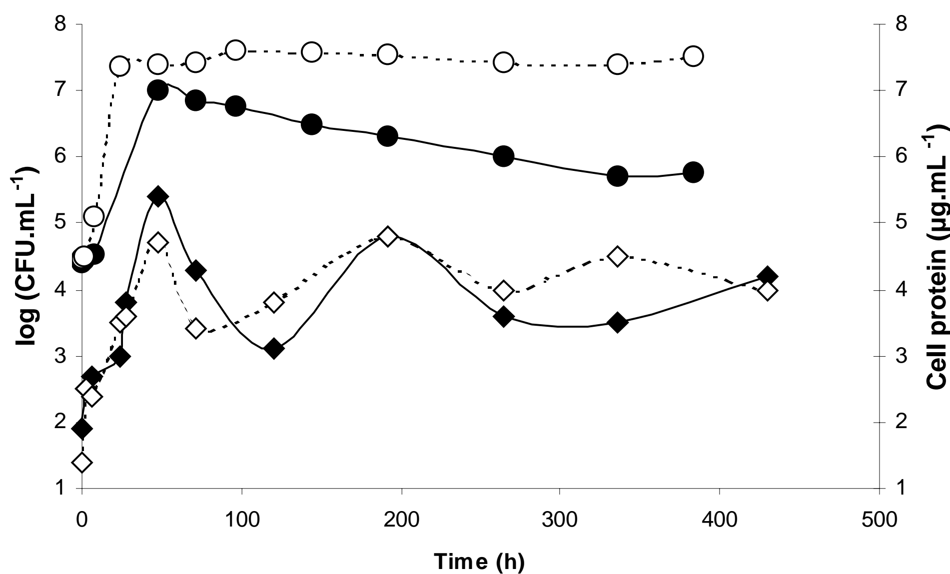

Figure 2- Growth curves of *E. coli* in pure and mixed cultures in indirect contact. (● CFU *E. coli* in pure culture; —○— CFU *E. coli* in mixed culture; ◆ *E. coli* cell protein in pure culture; —□—-*E. coli* cell protein in mixed culture)

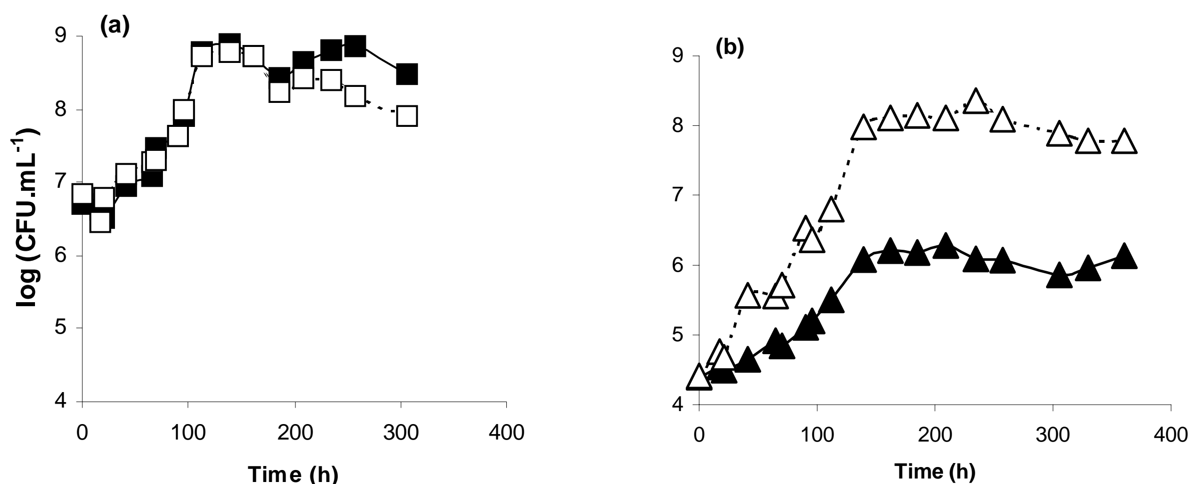

Figure 3 – Growth curves of *B. derxii* (a) and Bacterium C (b) in pure and mixed cultures in direct contact (■ *B. derxii* in pure culture; —□— *B. derxii* in mixed culture; ▲ Bacterium C in pure culture; —Δ— Bacterium C in mixed culture).

Fig. 2 indicates that *E. coli*'s cell protein content increased at the exponential phase, in parallel with increasing CFU values; it also shows that there was no difference in protein concentration between cells grown in pure culture and those in mixed culture. The similarity between results may be explained because the protein measurement does not discriminate between alive and dead cells present in the pure culture. The advantage of coculture II was that it enabled the determination of a single partner's cell protein content in a mixed population. In coculture III (Fig. 3) the presence of *B. derxii* enhanced Bacterium C to multiply and reach a CFU value that corresponds to 12 generations of growth. In pure culture, the growth of Bacterium C by 6 generations, was, probably for the same reason that did *E. coli* in coculture II. The specific growth rate of Bacterium C in mixed culture (0.05514) was twice as high as that in pure culture (0.02559). The effect of *B. derxii* on Bacterium C multiplication was higher than that observed on *E. coli* multiplication (Fig. 1 and 3).

The results were negative regarding the presence of ammonia in culture supernatants, at least for concentrations above 0.5 $mg.ml^{-1}$. SDS gel electrophoresis of supernatants from pure cultures and cocultures I and II revealed that no proteins were present. Neither *E. coli* nor Bacterium C did affect *B. derxii* growth (Fig. 1 and 3) or nitrogenase activity (Fig. 4).

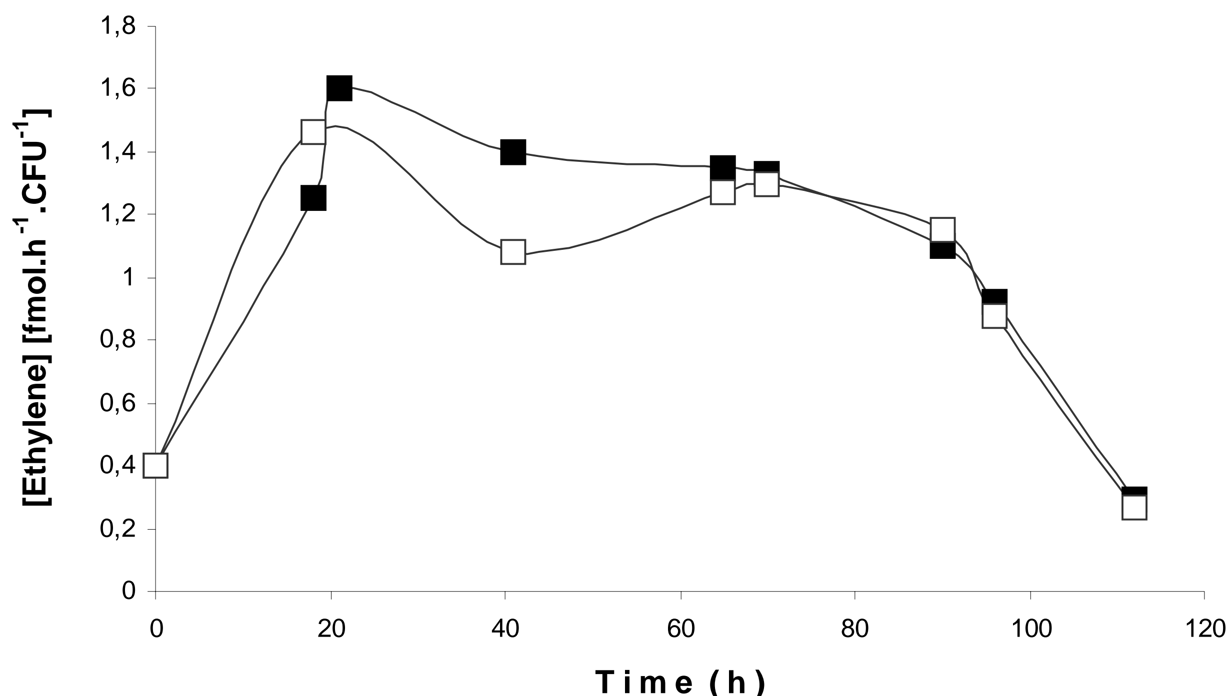

Figure 4 – Specific nitrogenase activity curves of *B. derxii* in pure (■) and in mixed culture (□) with Bacterium C (coculture III), evaluated by acetylene reduction method.

DISCUSSION

In the present study the effects of *B. derxii* on its partners can not be ascribed to the excretion of both proteins and ammonia once the results were negative for these analysis. These results are in contrast to Narula *et al*. (17) who demonstrated the presence of ammonia in the culture media of several species of the free-living *Azotobacter* isolated from soils of India. Few papers in the literature deal with the increase of nitrogenated substances in co-cultures of different microoganisms. Cohjo *et al*. (6) showed that the total protein accumulation of *Acetobacter diazotrophicus*, an endophytic N_2-fixing bacterium, was 25% higher when in mixed culture with *Lipomyces kononenkoae,* as compared with pure cultures. However, the excretion of nitrogenated substances by this N_2-fixing microorganism was not directly demonstrated.

Enhanced cell multiplication of the non-diazotrophic microorganism was observed in cocultures I and III (Fig. 1b and 3b) but in coculture II, the *E.coli* population was less stimulated (Fig. 2). This fact suggests that the dialysis tube retained substance(s) with MW higher than 50 KDa that could contribute to the non-diazotrophic multiplication. The similar *E. coli* cell protein concentration in both coculture II and pure culture indicates that no nitrogenated material, liberated by *B. derxii* was available to be assimilated by *E. coli,* increasing its biomass.

The observed maintenance in CFU numbers of non-diazotrophs in the cocultures may be explained by the diazotrophic bacterium secreting hormone-like substances into the medium. These may be nitrogenated substances, necessary in a very low concentration (7) so that this nitrogen does not influence biomass. A preliminary result showed that *B. derxii* is able to excrete indoleacetic acid (IAA) in the culture media supplemented with 0.5 $g.l^{-1}$ tryptophan (20). In the present work this IAA precursor was always absent.

Substances like cytokinin (19) and indole-acetic acid (24) have been detected in the culture media of different diazotrophic bacteria. These substances are known to promote plant growth (9). The influence of phytohormone-like substances on microorganisms was studied by Barea *et al.* (3), who proposed a method by which the concentrations of auxins, kinetin and gibberelic acid could be determined using yeast cells. However, there is little information about their effects on bacteria.

In the present study, the effect of *B. derxii* on multiplication and maintenance of viability of both *E. coli* and Bacterium C co-cultures, indicated that the former produces substance(s) that promote proliferation and/or increased viability of bacteria. The extension of this effects may vary depending on the partners. While Bacterium C had its growth enhanced, *E. coli* had both its multiplication stimulated and its viability preserved in mixed cultures. A less marked effect on cell growth was that of *Beijerinckia lacticogenes*, which caused an increase of only 2 generations in *Thiobacillus ferrooxidans,* a strict sulphur-oxidizing chemolitotroph cocultured as reported by Tsuchiya (22).

In the present paper, it was observed that *B. derxii* in coculture with *E. coli* or a facultative sulphur-oxidizing chemolitotroph bacterium was not affected by the partners yet had a positive influence on these non-N_2-fixing microorganisms. Even taking into account that the results were obtained under laboratory conditions, it can still be considered that *B. derxii* may also influence different soil microorganisms in the natural environment.

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RESUMO

Beijerinckia derxii estimula a viabilidade de bactérias não fixadoras de N_2 em meio sem nitrogênio

Co-culturas com a bactéria fixadora de nitrogênio *Beijerinckia derxii* e duas bactérias não diazotróficas (*Escherichia coli* e uma bactéria quimiolitotrófica facultativa oxidante de enxofre) foram empregadas para o estudo de interações bacterianas. *B. derxii* foi colocada em contato direto e indireto com *E. coli*. Um aumento no número de UFC e a manutenção da viabilidade das não diazotróficas foi promovido por *B. derxii,* porém seu crescimento e atividade da nitrogenase não foram afetadas por nenhuma das parceiras.

Palavras-chave: *Beijerinckia derxii*, co-cultura, viabilidade.

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