

# Nitrogen limitation of chemostat-grown *Rhizobium etli* elicits higher infection-thread formation in *Phaseolus vulgaris*

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**The symbiotic association between rhizobia and legume roots is a complex process involving many steps. An infection thread is a tubular structure of host origin formed during the infection of legume roots by rhizobia. Previous studies with batch cultures have reported that optimal attachment of rhizobia to root hairs coincides with nutrient limitation. In this study, the ability of chemostat-grown, nutrient-limited *Rhizobium etli* cells to form infection threads with its symbiotic partner *Phaseolus vulgaris* was investigated. Rhizobia were grown in a chemostat in synthetic media under C- or N-limiting conditions. Infection-thread formation was examined after inoculation of seedlings with a rhizobial cell suspension from each treatment. The number of infection threads was estimated by light microscopy after staining root sections with o-toluidine. Exopolysaccharide (EPS) production was also measured, and the cellular content and electrophoretic pattern of lipopolysaccharide (LPS) determined semiquantitatively. N-limited cells showed a markedly higher infectivity (measured as infection-thread formation) than C-limited cells. With one of the two bean cultivars used, the number of infection threads produced by N-limited cells was higher than that produced by exponentially growing cells in batch cultures. The higher infectivity of N-limited cells was correlated with higher EPS production. Electrophoretic analysis of LPS showed that C- and N-limited cells shared a common profile but the relative concentration of short LPS forms differed.**

**Keywords:** *Rhizobium etli*, plant–microbe interactions, infection threads, *Phaseolus vulgaris*, nitrogen limitation

## INTRODUCTION

The symbiotic association between rhizobia and legume roots is a multistep process initiated by preinfection events in the rhizosphere. The plant and the bacteria exchange specific signals before infection. Infective rhizobia recognize and attach to the region of the rhizoplane that is most susceptible to infection, the site of emerging root hairs. Root hairs become curled and entrap bacterial cells in a pocket of host cell wall material. Rhizobia enter the roots at sites where the root hair cell walls are

hydrolysed and induce the formation of a tubular structure of host origin called the ‘infection thread’ (van Rhijn & Vanderleyden, 1995). There are several reports on root-hair deforming and curling substances and infection-thread promoting factors (Abe *et al.*, 1982; Dazzo *et al.*, 1991; Higashi & Abe, 1980; Lugtenberg *et al.*, 1991). In the *Rhizobium etli*–*Phaseolus vulgaris* association we previously demonstrated that legume lectin enhances infection-thread formation (Brelles-Mariño *et al.*, 1993, 1996). This effect was also reported by Lodeiro & Favelukes (1995) with soybean lectin and *Bradyrhizobium japonicum*.

Smit *et al.* (1986, 1987, 1989a, b, 1991) and Kijne *et al.* (1988) studied the attachment capacity of *Rhizobium leguminosarum* biovar *viciae* to pea root hairs under various physiological conditions. They concluded that the conditions under which rhizobia are grown strongly influence

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**Abbreviation:** EPS, exopolysaccharide.

their ability to attach to root hairs, and that nutrient limitation always coincides with optimal attachment. C-limited *R. leguminosarum* biovar *viciae* cells did not induce infection-thread formation, while  $Mn^{2+}$ -limited cells yielded the highest number of infection threads (Kijne *et al.*, 1988).

However, the above studies were all carried out with batch cultures and, as demonstrated by Pronk *et al.* (1995), growth in batch cultures can be very different from growth in continuous culture. These authors advised against drawing conclusions on the basis of batch cultures only. The use of a chemostat facilitates study of the response of the organism to its environment. In a batch culture, substrate-limited growth can be obtained only transiently, but in continuous culture it is possible to obtain substrate-limited growth with a constant growth rate.

In the soil and rhizosphere, bacteria are subject to changing environmental conditions including nutritional stresses such as C, N and  $O_2$  limitation (de Bruijn *et al.*, 1995). The use of a chemostat and a synthetic medium allowed us to test the effect of some of these conditions on the *Rhizobium*-legume symbiosis. In the study reported here, we investigated the ability of nutrient-limited cells of *R. etli* to form infection threads within its symbiotic partner, *P. vulgaris*.

## METHODS

**Plant varieties.** Seeds from two *Phaseolus vulgaris* cultivars were used: Alubia (white bean) and Bat 76 (black bean). Both were obtained from INTA (Instituto Nacional de Tecnología Agropecuaria), Cerrillos, Argentina.

**Bacterial strain and culture conditions.** *Rhizobium etli* strain F48 (obtained from INTA, Castelar, Argentina), able to nodulate *P. vulgaris*, was used. Bacteria were maintained on slants consisting of solidified Götzt medium (Götzt *et al.*, 1982). This medium contained the following components ( $g\ l^{-1}$ ): mannitol, 10.0;  $(NH_4)_2SO_4$ , 0.13;  $K_2HPO_4$ , 1.06;  $KH_2PO_4$ , 0.52;  $MgSO_4 \cdot 7H_2O$ , 0.25;  $CaCl_2$ , 0.011; NaCl, 0.006;  $Na_2MoO_4$ , 0.002. Micronutrient components ( $\mu g\ ml^{-1}$ ) were:  $FeSO_4$ , 150; riboflavin, 20; *p*-aminobenzoic acid, 20; pyridoxine.HCl, 20; thiamin.HCl, 20; biotin, 20.

Bacteria from slants were grown at 30 °C in 250 ml Erlenmeyer flasks containing 50 ml Götzt medium with vigorous aeration (200 r.p.m. incubator shaker, New Brunswick Scientific).

The synthetic medium for the C-limited chemostat cultures contained ( $g\ l^{-1}$ ): mannitol, 2.00;  $(NH_4)_2SO_4$ , 2.60;  $KH_2PO_4$ , 0.71;  $MgSO_4 \cdot 7H_2O$ , 0.25. The other components of Götzt medium were added in the same quantities given above, except that no  $K_2HPO_4$  was used. The synthetic medium for the N-limited chemostat cultures contained ( $g\ l^{-1}$ ): mannitol, 10.0;  $(NH_4)_2SO_4$ , 0.52 and the other components of Götzt medium as indicated above. In both nutrient-limited chemostat cultures  $MnSO_4 \cdot 4H_2O$  was also added to obtain a final concentration of 1.0  $\mu M$ . This concentration was chosen because it has been demonstrated that rhizobial growth is maximal with 1.0  $\mu M$   $Mn^{2+}$  (Wilson & Reisenauer, 1970).

Media were autoclaved in 81 batches for 45 min. The stock solution of micronutrients was sterilized by filtration through a 0.2  $\mu m$  pore size membrane and aliquots were stored frozen.

Synthetic media were supplemented with 1 ml micronutrient solution  $l^{-1}$  before inoculation.

**Chemostat culture experiments.** These were carried out in laboratory chemostats (LKB Bromma 1001 Ultroferm or New Brunswick BioFlo C30; 2.5 or 0.4 l volume, respectively) at 30 °C. The aeration rates were 8–10 and 11.5–16.5  $l\ h^{-1}$ , respectively. The medium flow was regulated to obtain a dilution rate (*D*) of  $0.03 \pm 10\% h^{-1}$ . Foam formation was prevented by the automatic addition of an antifoaming agent and pH was maintained at a value of  $6.95 \pm 0.15$  by the automatic addition of NaOH or  $H_2SO_4$ .

**Analytical methods.** These were performed once the culture reached steady state conditions (after at least 10–15 retention times). Samples (10 ml) were centrifuged at 6000 *g* for 10 min. Pellets were washed twice with distilled water and dried at 105 °C until constant weight was attained. Bacterial concentration was measured as dry wt ( $l\ culture$ ) $^{-1}$ . Supernatants were separated and stored at –20 °C to determine exopolysaccharide (EPS), mannitol and  $NH_4^+$ . EPS was determined colorimetrically with anthrone (Herbert *et al.*, 1971). Mannitol and  $NH_4^+$  were determined colorimetrically according to Lambert & Neisch (1950) and Chaney & Marbach (1962), respectively. Culture samples containing approximately the same biomass concentration were centrifuged and pellets used to compare lipopolysaccharide (LPS) content of cells grown under C or N limitation. LPS were extracted by using 100 mM EDTA, pH 7.0 (C. Valverde, D. Hozbor & A. Lagares, unpublished). Samples were separated in SDS-PAGE gels and silver-stained as described by Tsai & Frash (1982).

**Infection thread assay.** Infection-thread formation was studied after inoculation of *P. vulgaris* seedlings with suspensions of  $1 \times 10^7$  cells  $ml^{-1}$ . The infection-thread assay was performed with secondary roots of bean plants growing in plastic growth pouches. The number of infection threads was estimated by microscopy after staining the root sections with *o*-toluidine (Pueppke, 1983). Uninoculated seedlings were used as controls. All assays with seedlings were performed under micro-biologically controlled conditions. Samples were taken 8 and 14 d after inoculation.

## RESULTS AND DISCUSSION

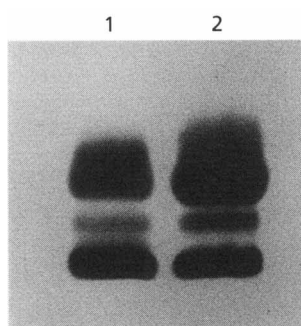
The number of infection threads per root produced by *R. etli* grown in continuous culture under different substrate limitations was determined. Plants were scored 8 and 14 d after inoculation, but no significant differences occurred between the two sampling dates (data not shown). We evaluated infection threads mainly in pre-existing root hairs but, because of the fibrous morphology of bean roots (Graham & Halliday, 1977), some roots developed after inoculation could also be excised. The anatomical nature of the bean root did not allow us to use the method of Bhuvaneshwari *et al.* (1981) in which soybean and alfalfa roots formed before and after inoculation were clearly distinguished by marking the root tip at the time of inoculation.

There are very few studies relating rhizobial nutrient limitation with preinfection and infection processes in legumes. As far as we know, only Smit *et al.* (1986, 1987, 1989a, b, 1991) and Kijne *et al.* (1988) have reported data on infection-thread formation in *R. leguminosarum* biovar *viciae*. Many of these results were obtained with batch cultures which, as indicated above, are not an appropriate

**Table 1.** Infectivity of *R. etli* F48 grown under different nutrient limitations

*P. vulgaris* seeds were pregerminated in petri dishes and transferred aseptically to plastic pouches. Each root was inoculated with 1 ml cell suspension containing  $1 \times 10^7$  cells  $\text{ml}^{-1}$ . Infection threads were counted 8 d after inoculation. Data are the average of at least 5 roots and were analysed by the Student's *t*-test. Values obtained from C- and N-limited chemostat cultures are highly significantly different ( $P < 0.01$ ). Results are the average of four independent experiments.

Growth conditions	No. infection threads per <i>P. vulgaris</i> root	
	Cv. Alubia	Cv. Bat 76
Chemostat culture, C-limited	2.5	2.2
Chemostat culture, N-limited	16.0	7.5
Batch culture, exponential phase	7.8	4.5



**Fig. 1.** LPS profile of N-limited (lane 1) and C-limited (lane 2) *R. etli* F48 cells after SDS-PAGE (15%, w/v, acrylamide) visualized by silver-staining. LPS was extracted and determined as given in Methods.

system for this type of physiological experiment. Lower infectivity of cells from C-limited cultures was reported for *R. leguminosarum* biovar *viciae* (Smit *et al.*, 1986, 1987). In this case, the authors used cells that had been grown in a complex medium in which they reported C as the growth-limiting nutrient. To verify that the substrate was actually limiting growth, we measured the concentration of the limiting nutrient under steady-state conditions. Mannitol and  $\text{NH}_4^+$  were undetectable in the supernatants from C- or N-limited cultures, respectively. Moreover, we measured bacterial growth (cell count and cell dry wt) after pulses of each nutrient-limiting substrate. C-limited cultures responded to the addition of mannitol and N-limited cultures to the addition of ammonium sulfate by increasing biomass concentration immediately after nutrient addition (data not shown).

There was a marked difference in infectivity (measured as number of infection threads) between C-limited and N-limited cells (Table 1). N-limited cells were much more infective than cells under C limitation. In cultivar Alubia, the number of infection threads was even higher than that obtained with exponentially growing cells in batch cultures. It has been reported that cells obtained from the exponential growth phase in batch culture are the most infective population (Boiardi & Galar, 1988). The higher infectivity of N-limited cells relative to C-limited ones was

correlated with differences in EPS production. EPS yield under N- and C-limited conditions was 115 and 6.4 mg EPS (g biomass) $^{-1}$ , respectively. However, nutrient limitation appeared to alter the relative intensity (as judged by silver-staining) of short LPS forms without a noticeable band profile modification (Fig. 1).

Some of the initial stages of nodulation are believed to involve EPS and LPS but the actual role of these polysaccharides in the nodulation process is not clear. Although EPS is not essential for attachment (Bauer, 1981), it stimulates the process and might also facilitate the infection of legumes by rhizobia. Yang *et al.* (1992) found that *R. meliloti* *exo* mutants (EPS $^-$ ) were not able to form complete infection threads. Kijne *et al.* (1988) proposed that C-limited rhizobia are not infective at the time of inoculation because of the lack of lectin receptor molecules and/or the lack of EPS on the rhizobial surface. The correlation between EPS production and infection-thread formation found in our study is consistent with the above hypothesis. It can be speculated that the enhanced infectivity obtained with N-limited rhizobia could be explained by an appropriate concentration of EPS on the bacterial surfaces. Carlson *et al.* (1987) tentatively suggested that the ability to synthesize LPS molecules containing the O-antigen is essential for initial stages of infection. Cava *et al.* (1989) demonstrated that complete LPS structures, in normal amounts, are necessary for infection-thread development in bean plants. Changes in the relative concentration of LPS forms were correlated with nutrient limitations (Fig. 1), therefore enhanced infectivity of N-limited cells might also be attributed to differences in the composition of this polysaccharide. However, further research is required to determine whether EPS and/or LPS (or neither) are responsible for the enhanced infectivity of N-limited rhizobia.

Our study correlated rhizobial infectivity with different growing conditions and showed the potential of chemostat culture as a tool for studying the effects of different limiting substrates on the symbiotic competence of rhizobia. The rhizosphere environment is quite different from the conditions under which rhizobia are classically grown in the laboratory and the chemostat allowed us to approximate some of the conditions under which the

micro-organisms proliferate and infect the host plant in the soil.

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