The effect of biotic and physical factors on the competitive ability of *Rhizobium leguminosarum*

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Abstract: Background: *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) is a soil bacterium which can form nitrogen-fixing symbiotic relationships with leguminous plants. Numerous rhizobial strains found in soils compete with each other. Competition can occur both during the saprophytic growth phase in the rhizosphere and inside plant tissues, during the symbiotic phase. Competition is important as it may affect the composition of rhizobial populations present in the soil and in the root nodules of plants.

Methodology: We examined the link between physiological traits and bacterial competitive ability in eighteen *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) isolates during root nodule colonization using laboratory and field experiments. The competitive ability of *Rlv* strains was measured as the percentage of root nodules colonized by *gusA*-tagged rhizobia in two types of host plants, peas and vetch.

Results: The competitiveness of *Rlv* strains was significantly affected by soil type and the identity of the host plant. Of the eighteen bacterial traits examined in this study, the metabolic potential (number of utilized carbon and energy sources) and the responsiveness of nod genes to flavonoid activation were most important in affecting the competitive ability of *Rlv* strains. The amount of acylated homoserine lactones (AHL) produced by the strains was less important in influencing competitiveness. Finally, the preactivation of strains with flavonoids or the addition of AHL to *gus*-*tagged* *Rlv* strains did not significantly enhance competitiveness: of the *gus*-tagged inoculants in comparison to indigenous soil populations of vetch microsymbionts.

Conclusions: The competitiveness of *Rlv* strains is dependent upon numerous physiological traits. However, environmental factors such as soil type and the type of host plant may be even more important in affecting rhizobial competitiveness.

Keywords: *Rhizobium leguminosarum* • *Rhizobium-legume symbiosis* • Strain competitiveness

1. Introduction

*Rhizobium leguminosarum* bv. *viciae* (*Rlv*) is a soil bacterium, which is able to establish nitrogen-fixing symbiotic relationships with leguminous plants of the tribe Vicieae (*Pisum Vicia, Lens* and *Lathyrus*). Symbiosis is a multi-stage process which involves co-recognition of the symbiotic partners via an exchange of plant and bacterial molecular signals. Flavonoids secreted by plant roots activate a rhizobial regulatory Nod protein resulting in the induction of nodulation genes that encode enzymes for the biosynthesis of lipochitin oligosaccharides. These oligosaccharides are referred to as the nodulation factors (Nod factors), which trigger early symbiotic responses in roots and initiate the developmental program of nodule formation. Infection threads filled with growing bacterial cells are then formed and release rhizobia into plant cells in the nodule primordia. Rhizobia colonize the developing nodules and differentiate into dinitrogen fixing bacteroids inside them. Bacteroids provide the host plant with ammonium from nitrogen fixation. In return, the plant supplies the bacteria with carbohydrates derived from photosynthesis [1-3]. Vegetative forms of bacteria multiply in the infection threads and saprophytic zones of indeterminate type nodules, and are released into the soil during the decay of nodules [4,5]. Amongst the heterogeneous microbial communities in the soil, rhizobia constitute numerous and very differentiated populations.
Several factors can influence the number and diversity of indigenous rhizobia, including nutrient availability, soil type, acidity and agricultural management regimes [6,11,12]. However, despite the numerous availability, soil type, acidity and agricultural management diversity of indigenous rhizobia, including nutrient [6-10] world [6,14,15].

Numerous biological traits affecting rhizobial nodulation competitiveness have been described in the literature [16]. They include the ability of rhizobia to utilize specific carbon and energy sources [17,18], the overall metabolic potential of bacteria [19], bacteriocin production, resistance to bacteriocin [20], the susceptibility to plant molecular signals [21,22] and bacterial motility [23]. In addition, the legume hosts themselves [24-26] and the distribution of rhizobia in the soil [27] may also influence the outcome of competitive interactions between rhizobia. Furthermore, it is possible that compounds other than plant-derived flavonoids and rhizobial Nod factors may influence strain competitiveness for nodule occupancy in Rhizobium-legume interactions [28,29]. As a result of competition between rhizobia during nodule colonization, strain composition inside root nodules may be diverse [30]. Therefore, nitrogen fixation efficiencies of these strains may also vary, which is important in the context of the development of commercial rhizobial inoculants [13].

Furthermore, the competition and proliferation of vegetative forms of bacteria inside the nodules may affect the total yield of rhizobia, which return to the soil after a vegetative period in the host plants [31-33]. Each of these factors affects the potential success of individual strains to persist in local populations. In the case of rhizobial inoculants, introduced strains may quickly disperse in the native population in rhizobia-rich soils [34] or remain as a significant proportion of a population for years in rhizobia-depleted soils [35].

Here, we investigated the contribution of important bacterial physiological and metabolic traits to the competitiveness of Rlv strains used to inoculate vetch and pea plants grown in different types of soil under noncompetitive and competitive conditions. We specifically examined the role of the rhizobial metabolic potential, the responsiveness of nod genes to flavonoid treatment, AHL production, and chemotaxis in influencing the competitive ability of Rlv strains.

2. Experimental Procedures

2.1 Rhizobial strains

Eighteen Rhizobium leguminosarum bv. viciae (Rlv) strains used in this study were isolated from root nodules of pea (Pisum sativum cv. Ramrod) grown on arable sandy loam soil in Lublin, Poland [36].

2.2 Chemotaxis assay

Rhizobia were cultured in TY liquid medium [37] to an optical density of 0.3-0.4 at l=550 nm (OD 550 ) and centrifuged at 1000×g for 20 min at room temperature. Pellets were suspended in chemotaxis buffer (10 mM potassium phosphate, 0.1 mM sodium EDTA, pH 7.0) [38] to ~5x10^7 CFU/ml. A chemotaxis assay was conducted in capillary tubes placed into chemotaxis chambers [39,40]. Capillary tubes filled with chemotaxis buffer (control) or flavonoid extract (10µM) and the bacterial suspension (~5x10^7 CFU/ml) were placed inside the chambers for 4 h at 28°C. After incubation, the content of capillary tubes was suspended in 100 µl of chemotaxis buffer, plated in dilutions onto 79CA medium and incubated for 72 h at 28°C. The chemotaxis ratio was calculated by dividing the number of bacteria recovered from capillary tubes filled with flavonoid extract by the number of bacteria recovered from capillary tubes containing chemotaxis buffer. The assay was replicated four times for each strain.

2.3 Assay of P nodA-lacZ promoter fusion activity

The derivatives of the Rlv strains harbouring pMPA221 plasmid with P nodA-lacZ fusion [22] were used to study the effect of flavonoid induction of rhizobial nod genes. Overnight cultures of rhizobia in TY medium supplemented with tetracycline (10 µg/ml) were centrifuged, washed twice with M1 medium supplemented with 1 µg/ml thiamine, 0.5 µg/ml biotin and 1 µg/ml panthotenate [22], and resuspended in M1 to OD 550 of 0.05. Then, flavonoid extract was added to the culture at a final concentration of 10 µM. The cultures were grown for an additional 24 h at 28°C. The level of nod gene expression was determined in Miller units by assaying β-galactosidase activity with the o-nitrophenyl-β-D-galactoside (ONPG) cleavage assay [41].

2.4 Isolation and detection of rhizobial AHLs

AHLs were isolated by ethyl acetate extraction from supernatants of 5 ml overnight cultures of Rlv grown in TY medium [42]. The samples were separated by thin-layer chromatography on C8 reversed-phase TLC plates (Merck), and overlaid with Chromobacterium violaceum CV026 [43]. Synthetic AHLs (Sigma-Aldrich) N-butyryl-dl-homoserine lactone (C4-AHL), N-hexanoyl-dl-homoserine lactone (C6-AHL), N-octanoyl-dl-homoserine lactone (C8-AHL) and N-decanoyl-dl-homoserine lactone (C10-AHL) (solutions of 1 mg/ml in ethyl acetate) were used as standards.