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The epidemicity of facultative microsymbionts in faba bean rhizosphere soils

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ABSTRACT

The epidemicity of bacteria facultatively associated with eukaryotes, involves not only housekeeping genes but also genes linked with the pathogenesis and symbiosis. Here, by characterizing both housekeeping (*rpoB* and 16S rRNA gene) and nodulation (*nodD*) genes, we explore processes shaping epidemic patterns of facultative microsymbionts from rhizosphere soils of faba bean in three ecoregions. Although total bacterial communities in rhizosphere were not significantly differentiated across ecoregions, *rpoB* amplicon sequencing uncovered that *Rhizobium laguerreae* and *Rhizobium anhuiense* were predominant in different samples with contrasting pH or salt content. However, *R. anhuiense* can outcompete *R. laguerreae* in certain sterilized soils where *R. laguerreae* originally dominated, and *viceversa*. Contrasting bacterial taxa associated with either *R. laguerreae* or *R. anhuiense* in soils. The biogeographical pattern of *nodD* was more clear than that of rhizobial species in both rhizosphere soils and nodules. Competitive nodulation experiments demonstrated a hierarchical selection on *nodD* genotypes and their genomic backgrounds by faba bean cultivars. Taken together, abiotic and biotic factors in soils and the selection by legume hosts are either indirectly or directly involved in shaping rhizobial species-level taxonomic biogeography, which however cannot be used to infer spatial patterns of nodulation gene.

1. Introduction

Understanding the epidemicity of mutualistic and pathogenic bacteria associated with eukaryotes is crucial for the further practical management of their positive or negative impacts on host fitness (Hussa and Goodrich-Blair, 2013; Martínez and Baquero, 2002; Nemergut et al., 2013). Specific interactions between these bacteria and their hosts are usually at the species or even intraspecific level as demonstrated using pure cultures isolated from infected host tissues. However our knowledge on the diversity of these bacteria in the environment is limited due to both the lacking of selective medium and the low resolution (above the genus level) of the 16S rRNA gene survey, which is widely used in the culture-independent studies (Nemergut et al., 2013). Alternative housekeeping genes such as *rpoB* encoding the beta subunit of RNA polymerase has been proposed as a good candidate due to its intraspecific resolution and its robustness as a taxonomic and phylogenetic marker (Adekambi et al., 2008; Drancourt et al., 2009; Vos et al., 2012).

The rhizobium-legume symbiosis has been widely studied as one of the model mutualistic systems (more than 7000 papers in the past 5 years) and is an essential component of sustainable agriculture due to its high efficiency in nitrogen fixation. The symbiotic interaction is initiated through the specific sensing of host symbiotic signal by rhizobial NodD. The activated form of NodD further induces the transcription of nodulation genes involved in the biosynthesis of Nod factors that are specifically recognized by legume receptors before the intracellular infection of nodules by rhizobia (Remigi et al., 2016). These key nodulation genes including *nodD* are organized in a genomic island in either the symbiosis plasmid or the chromosome of different rhizobial

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species and usually exhibit a distinct evolutionary history compared to the chromosomal background due to their horizontal transfer potential (Guo et al., 2014; Ling et al., 2016; Tian et al., 2010; Zhang et al., 2014).

As typical facultative microsymbionts, rhizobia live saprophytically in soils and can enter the nitrogen-fixing symbiosis with legumes under appropriate conditions. It is hypothesized that rhizobia in soils experience a transient bottleneck mediated by the host selection of the most compatible strain during the symbiotic interactions. Indeed, by examining the nodule occupancy of signature-tagged mutants in an inoculant mixture, it has been demonstrated that most nodules are usually occupied by a single mutant clone (Pobigaylo et al., 2008; Wang et al., 2016). Competition by indigenous strains in rhizosphere soils can affect symbiotic interaction between an inoculant strain and the host plant, and this has been the major obstacle for improving the inoculation efficiency of rhizobia (Brockwell and Bottomley, 1995). However, rhizobial relative abundance in rhizosphere soils and factors affecting its variations have not been directly addressed.

Faba bean (*Vicia faba* L.) was domesticated at least 7000 years ago in southwestern Asia (Tanno and Willcox, 2006; Zeder, 2009). It is now being cultivated either in the season of winter or spring around the world, with China and Europe as the main producers (Duc et al., 2010). Recent studies have revealed a considerable genetic differentiation in faba bean germplasms, generally corresponding to the geographical origin (Bao et al., 2006; Duc et al., 2010; Wang et al., 2012; Zong et al., 2009). *Rhizobium leguminosarum* bv. *viciae* (*Rlv*), *R. fabae*, *R. laguerreae* and *R. anhuiense* have been isolated from faba bean nodules in the field (Mutch and Young, 2004; Saïdi et al., 2014; Tian et al., 2008; Zhang et al., 2015), and these isolates can be collectively grouped into the symbiovar of *viciae* reflecting bacterial adaptation to legumes (Kumar et al., 2015; Remigi et al., 2016; Rogel et al., 2011).

In this study, we aimed to investigate the epidemicity of rhizobia in rhizosphere soils of faba bean across three ecoregions in China and the potential factors shaping the epidemic patterns. To this end, the diversity of rhizobial genospecies and nodulation gene in rhizosphere soils was studied by using the high-throughput sequencing analyses of *rpoB* and *nodD*. Given the horizontal transfer ability of symbiotic functions across bacterial species, theoretically it is challenging to distinguish all rhizobial species compatible with faba bean from the huge pool of bacteria in soils. To determine a conservative list of faba bean microsymbionts in rhizosphere soils, we also studied the diversity of nodule isolates from the same faba bean plants, from the rhizosphere of which soil samples were collected. The geographical patterns of *rpoB* genospecies and *nodD* genotypes in rhizosphere soils and nodule isolates were analyzed, and abiotic or biotic factors potentially shaping these patterns were investigated. The significance of our findings was further discussed in the context of the ecology and evolution of bacteria, which form either pathogenic or mutualistic associations with eukaryotic hosts.

2. Material and methods

2.1. Sampling, DNA extraction and soil characterization

Rhizosphere soils were collected from 44 sites in the northwest (Gansu and Qinghai provinces), east (Jiangxi, Anhui, Henan and Zhejiang), and southwest (Yunnan) growing regions of faba bean in China (Table S1 in Supporting Information). The distance between the sites ranges from 3.3 km to 2264.4 km. During the blooming stage of faba bean, soil attached to roots of 5–10 random plants within 100 m^2 were pooled together after gentle shaking by hand, and defined as one “thick” rhizosphere soil sample (~500 g) as described earlier (Sørensen et al., 2009; Thrane et al., 2000). DNA was extracted from 0.5 g samples using the Fast DNAtm SPIN Kit for soil (MP Biomedicals, Cleveland, OH, USA). Root nodules were collected from the same faba bean plants. Isolation of rhizobia and DNA extraction were done as described earlier (Tian et al., 2007). Soil chemical properties (Table S1) were analyzed, using the standard procedures described earlier (Lu, 2000), at the Plant Nutrient and Resource Research Institute, Beijing Academy of Agriculture and Forestry Sciences, China. Briefly, pH was determined for a suspension of 1:5 soil:water (w/v) using a pH meter; total N was measured by the Kjeldhl method; available phosphorus (P) was extracted by sodium bicarbonate (0.5 M, pH 8.5) and determined by Mo-Sb colorimetric method; available potassium (K) was extracted with ammonium acetate (1 M, pH 7.0) and determined using a flame photometer; organic carbon was measured by potassium dichromate oxidation and subsequent titration with ammonium ferrous sulfate (0.2 M), and organic matter (OM) was determined by multiplying the organic carbon content by 1.724 assuming that 58% of OM is carbon; concentrations of soil K^+, Ca^{2+}, Na^+, Mg^{2+}, and SO_4^{2-} extracted using deionized water (1:5 w/v) were determined by inductively coupled plasma atomic emission spectrometry; the level of HCO_3^- in a suspension of 1:5 soil:water was measured using an automatic potentiometer and hydrochloric acid (20 mM); Cl^- content in the soil:water suspension (1:5 w/v) was titrated using silver nitrate (20 mM); electrical conductivity (EC) was measured in deionized water (1:5 w/v). To determine the total salt content, the aqueous extract from a suspension of 1:5 soil:water was evaporated in a water bath; organic matter within the resultant product was then removed by adding 15% H_2O_2 and by heating in a water bath; and the salt content was finally determined by gravimetry of the residue evaporated in a drying oven.

2.2. DNA fingerprinting and analyses of rpoB and nodD for nodule isolates

Total DNA from 1456 purified nodule isolates was used as the template for repetitive extragenic palindromic PCR (REP PCR) with the BOX-A1R primer as described earlier (Koeuth et al., 1995; Tian et al., 2007). Three hundred ninety-seven representative strains of 85 BOX fingerprint types were further characterized by amplification and sequencing of *rpoB* using primers rpoB-F/rpoB-R (TCGCAGTTCATGGACCAGG/GTAGCCGTTCCAGGGCATG) as described previously (Zhang et al., 2017). Since all published genomes and *nod* genes of rhizobia belonging to the symbiovar *viciae* have a conserved single copy of *nodD* (Kumar et al., 2015; Marek-Kozaczuk et al., 2013; Tian et al., 2010; Young et al., 2006), the primers nodDviciaeF88/Y6 (TGCAGAGACGGGAGCTARTTC/CGCA WCCANATRTTYCCNGGRTC) were used to amplify *nodD* gene of all nodule isolates as described earlier (Macdonald et al., 2011; Zézé et al., 2001). The *nodD* PCR products were analyzed by restriction fragment length polymorphism (RFLP) analysis using endonucleases *Hae*III, *Mse*I and *Bst*NI. The restriction fragments were separated by electrophoresis and visualized under UV light as previously described (Tian et al., 2007). The *nodD* PCR products of 366 representative strains were purified and sequenced on an ABI 3730xl sequencer.

2.3. High-throughput sequencing of rpoB, 16S rRNA and nodD from rhizosphere soils

The *rpoB*, 16S rRNA gene and *nodD* were amplified using the primers rpoB1479-F/rpoB1831-R (*rpoB*) (Zhang et al., 2017), 515f/806r (the V4/V5 region of the 16S rRNA gene) (Caporaso et al., 2012) and nodDviciaeF88/nodDviciaeR443 (*nodD*) (Macdonald et al., 2011), respectively. The *nodD* fragment obtained using this pair of