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Alkalinity of Lanzarote soils is a factor shaping rhizobial populations with *Sinorhizobium meliloti* being the predominant microsymbiont of *Lotus lancerottensis*

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ABSTRACT

Lotus lancerottensis is an endemic species that grows widely throughout Lanzarote Island (Canary Is.). Characterization of 48 strains isolated from root nodules of plants growing in soils from eleven locations on the island showed that 38 isolates (79.1%) belonged to the species *Sinorhizobium meliloti*, whereas only six belonged to *Mesorhizobium* sp., the more common microsymbionts for the *Lotus*. Other genotypes containing only one isolate were classified as *Pararhizobium* sp., *Sinorhizobium* sp., *Phyllobacterium* sp. and *Bradyrhizobium*-like. Strains of *S. meliloti* were distributed along the island and, in most of the localities they were exclusive or major microsymbionts of *L. lancerottensis*. Phylogeny of the nodulation *nodC* gene placed the *S. meliloti* strains within symbiovar lancerottense and the mesorhizobial strains with the symbiovar loti. Although strains from both symbiovars produced effective N_2-fixing nodules, *S. meliloti* symbiovar lancerottense was clearly the predominant microsymbiont of *L. lancerottensis*. This fact correlated with the better adaptation of strains of this species to the alkaline soils of Lanzarote, as *in vitro* characterization showed that while the mesorhizobial strains were inhibited by alkaline pH, *S. meliloti* strains grew well at pH 9.

Introduction

The great diversity of the rhizobia nodulating *Lotus* spp. has been revealed in recent years, with descriptions of novel species [7,27] and the detection of many other new rhizobial genotypes that are also potentially not-yet-described species. Moreover, several species originally described from other legume hosts have also been recovered as microsymbionts of *Lotus* spp. [4,13,15–17,34]. In consequence, the idea has been abandoned that the symbiosis between the rhizobia and *Lotus* was highly specific with only two divergent groups of rhizobia, with *Mesorhizobium loti* and *Bradyrhizobium* sp. strains being considered as the typical microsymbionts. Currently, *Lotus* rhizobia include strains belonging to many species within the genera *Mesorhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Rhizobium* [2,4,15–17] and *Aminobacter* [4]. Nevertheless, species of the genus *Mesorhizobium* are the most common microsymbionts of the *Lotus* spp. worldwide. Surprisingly, in recent years it has been shown that *M. loti*, previously considered the most typical microsymbiont of *Lotus*, is a rather infrequent symbiont. The other genera of rhizobia are more sporadically isolated from the *Lotus* spp. or nodulate only certain species of *Lotus*. Strains of *Bradyrhizobium* sp. are typical symbionts of *L. uliginosus* (*pedunculatus*) or *L. angustissimus*. With a few exceptions [5,8], it is debatable whether most of the species of the genus *Rhizobium* isolated from root nodules of diverse *Lotus* spp. are true microsymbionts of *Lotus*, as re-infection has neither been proved nor has it failed. Some strains of the genus *Sinorhizobium* are true predominant symbionts of endemic *Lotus* species growing in arid soils, where salinity and alkalinity are also other stress factors [13,28,22]. In these stressed soils, *Sinorhizobium* strains appear to be better adapted than the mesorhizobia.

Since the taxonomy of the rhizobia based on core genes does not reflect their symbiotic traits, usually the characterization of new isolates includes a parallel phylogeny of symbiotic genes (usually *nodC* and *nifH* genes) besides the symbiotic phenotypes. Thus, *S. meliloti* strains, apart from symbiovar meliloti (that nodulates *Medicago* and *Melilotus*), have been found associated in other

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legume hosts with other symbiovars (sv.): sv. medicaginis [41], sv. mediterranense [23], sv. rigiduloides [6] or sv. lancerottense [13]. *S. meliloti* symbiovar lancerottense were the first strains of this species described as nodulating *Lotus*. In the case of the *Lotus* mesorhizobia, despite the great number of species described as microsymbionts, most of them harbour symbiotic genes belonging to symbiovar loti or closely related symbiovars [4,7,16,17], and the *Bradyrhizobium* nodulating *Lotus* belong to symbiovar genistearum [17]. *Rhizobium* sp. strains isolated from *Lotus* spp. have usually failed to reinfect them [2,4,16,17], although there are some exceptions [5].

Lotus lancerottensis is an endemic species limited to the easternmost islands of the Canary Islands, Lanzarote and Fuerteventura. It is also found in Madeira, but is very rare. Lanzarote is an arid island with alkaline soils, as well as saline soils in some locations. A previous study showed that seven strains isolated from the root nodules of *L. lancerottensis* belonged to *Sinorhizobium meliloti* [13]. Therefore, the aim of this work was to study the genetic diversity in a wider sample of bacteria nodulating *L. lancerottensis* on Lanzarote and determine the importance of *S. meliloti* as a *Lotus* symbiont. For this purpose, soil samples were collected at eleven points along the longitudinal axis of the island, and a large collection of root nodule bacteria were isolated by using *L. lancerottensis* as a trap-legume.

Materials and methods

Soil sampling

This study was carried out on Lanzarote, the north-easternmost island of the Canary Island chain (Spain). It is 862 km^2 in size and formed largely by basaltic rock of volcanic origin. This arid/semi-arid island is situated in the Atlantic Ocean, approximately 115 km off the west coast of Africa, between parallels 29°17 and 28°02 latitude north, and meridians 13°25 and 14°30 longitude west. Annual precipitation on most of the island is below 150 mm, with no part receiving more than 300 mm $year^{-1}$. Rainfall is seasonal (October–March) and presents high inter-annual variability. The combination of solar radiation (annual average of 7.8 h sunshine per day), high temperatures (annual average of 18 °C) and moderate–strong winds leads to extremely high evaporation rates (≈1800 mm $year^{-1}$ in evaporation pans). Most sampled soils had basaltic pyroclasts on the surface, which act as a mineral mulch improving the soil properties, such as infiltration capacity, humidity, evaporation and salinity [3,36–38].

Soil samples were taken from the first 5–15 cm depth at different locations on the island (Table 1).

Analysis of the soils

All the samples were air-dried and passed through a 2 mm sieve prior to analysis. The following parameters were then analysed: pH (H_2O), electrical conductivity (ECe), exchangeable cations (Ca^{2+}, Mg^{2+}, Na^+, and K^+), calcium carbonate equivalent ($CaCO_3$), organic matter (OM), total nitrogen (TN) and P-Olsen. All the soil analyses followed standard methods [33]. When electric conductivity was above 2 dS m^{-1}, soluble cations in saturated paste extract were determined (data not shown) and this value was subtracted in order to calculate exchangeable cations.

Isolation of bacteria and growth conditions

Sterilized *L. lancerottensis* seeds were germinated and grown in soil samples from Lanzarote collected at several locations, from south to north, where natural populations were growing (Table S1). The bacteria were recovered from root nodules 8–10 weeks after sowing. The isolates were grown and maintained in the laboratory following standard protocols, as previously described [13]. The isolates were grown at 28 °C on yeast-extract mannitol agar (YMA). Purified strains were stored at −80 °C in YM broth with 20% v/v glycerol.

Physiological tests: salt and pH tolerance

Tolerance to acid and alkaline pH was tested by growing the bacterial strains on YMA plates adjusted to pH 5.5 (20 mM MES buffer) or pH 9.0 (50 mM TRIS). YMA plates with 20 mM MES or 50 mM TRIS at pH 7 were also inoculated in order to verify that the buffer did not influence bacterial growth. Salt tolerance to sodium chloride (NaCl), 0.5%, 1%, 1.5%, 2% and 2.5%, was assessed on YMA medium supplemented with the required concentration of salt. Plates were inoculated by adding 5 μL drops of a diluted bacterial suspension containing approximately 10^5–10^6 cells mL^{-1}. Growth was recorded after 5 and 7 days incubation at 28 °C, respectively, for fast-growing (*Sinorhizobium* sp., *Rhizobium* sp.) and moderate-growing rhizobia (*Mesorhizobium* sp. LLAN60 and *Phyllobacterium* sp.). Slow-growing strains *Mesorhizobium alhagi*-type LLAN46, LLAN63, LLAN64 and LLAN67 required 14 days. All assays were performed in triplicate.

Restriction fragment length polymorphism of the 16S rRNA *and* nodC *genes*

The near full-length 16S rRNA gene was amplified, restricted (*HinfI*, *MspI* and *RsaI*) and analysed as previously described [4], together with a fragment of approximately 900 nt from the *nodC* gene that was also amplified and restricted [13].

Sequencing of rss, gyrB, nodC *and* nifH *genes and phylogenetic analyses*

The 16S rRNA gene was sequenced as above. The *gyrB* (subunit B protein of DNA gyrase) gene [20], as well as the *nodC* and *nifH* genes [11] were amplified as previously described. The PCR-amplified products were purified (Qiaquick extraction kit, Qiagen) and sequenced in an ABI3730XL (Macrogen, Inc.) or in a Genetic Analizer 3500 (Applied Biosystems; Servicio de Genómica, Universidad de La Laguna).

Sequence alignments (ClustalW) and phylogenetic analyses were conducted using the MEGA version 6 software package [35]. The phylogenetic trees were inferred by the neighbor-joining method (NJ) [31] using Kimura's-2-parameter model [10] and maximum likelihood (ML) with nucleotide substitution model parameters as implemented in MEGA v6 [35]. Confidence of the tree branches was estimated with 1000 and 500 bootstrap (BS) replications for NJ and ML, respectively.

Infectivity tests

A selection of representative strains was used in order to check re-infection on the original host, *L. lancerottensis*, and *L. corniculatus* was additionally evaluated as a potential host. Seeds were surface scarified in concentrated H_2SO_4 for 3–4 min, washed with sterile distilled water, then suspended for 5 min in 50% diluted commercial sodium hypochlorite (NaClO) and thoroughly washed. The sterilized seeds were germinated on 1% agar-water. Seedlings were inoculated by submerging them in a dense suspension (5U McFarland turbidity standard) of the corresponding strain. Uninoculated plants served as controls. *L. lancerottensis* plants were grown in tubes with vermiculite and *L. corniculatus* in tubes with the plant nutritive solution plus 0.8% agar. Three replicates *per* strain were used. The N-free nutrient medium of Rigaud and Puppo [29] was