Native bradyrhizobia isolated from *Lupinus mariae-josephae* possess an essential T3SS for symbiosis.

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ABSTRACT

Analysis of the genome sequence of bradyrhizobia strains isolated from root nodules of *Lupinus mariae-josephae* revealed the presence of a type III secretion system (T3SS). Mutagenesis of *ttsI* gene that codes for the transcriptional activator (TtsI) resulted in the formation of white, non-fixing nodules in *L. mariae-josephae*. The T3SS cluster includes a gene coding for a NopE-like protein with an autocleavage motif. The NopE protein is an effector in the *Bradyrhizobium*-soybean symbiosis (Wenzel *et al*., 2010). The autocatalytic properties of the purified NopE-like protein have been studied.

INTRODUCTION

Lupinus mariae-josephae is an endemic lupine found in basic soils from a small area in Eastern Spain. Endosymbiotic bacteria that form nodules with *L. mariae-josephae* have been isolated from this area and characterized by multilocus phylogenetic analysis as belonging to *Bradyrhizobium* genus. These bradyrhizobia gather in six different operational taxonomic units (OTUs) unrelated to any other isolated from lupines (Duran *et al*. 2013). Draft genomic sequences from representative strains corresponding to the different OTUs have been obtained and analyzed in this work.

MATERIAL AND METHODS

Genome draft.

Genome sequencing was performed with a HiSeq2000 instrument (500bp library, PE100, 100 x coverage) at BGI (Beijing Genomics Institute, Shenzhen, China), and the reads were assembled into several contigs and scaffolds using the Short Oligonucleotides Alignment Program (SOAPdenovo).

Construction of a ttsI mutant.

For mutant construction, a DNA fragment containing an internal deletion of *ttsI* gene was cloned into plasmid pK10mobSac and then conjugated to the wild-type strain.

NopE expression and purification.

Plasmid pT7.7 was used for the expression of NopE-Strep in *E. coli*. Cloning of LmjC *nopE* gene, modified to code for a C-terminal Strep-tag, was performed by PCR using specific primers. NopE was purified by StrepTactin affinity chromatography.

RESULTS AND DISCUSSION

Bradyrhizobia isolated from Lupinus mariae-josephae contains a T3SS.

Analysis of the genome drafts of several isolates from *L. mariae-josephae* (Lmj strains) revealed the presence of a type III secretion system (T3SS) in a cluster of about 30 genes. Genes belonging to this cluster encode the transcriptional activator TtsI, structural components of the secretion apparatus, secreted proteins including a NopE-like found among a few uncharacterized proteins of plant-associated bacteria (Schirrmeister *et al*. 2011), as well as hypothetical and unknown proteins. The highest

gene conservation was observed with the genes encoding T3SS structural components of *B. diazoefficiens* USDA110, *B. japonicum* USDA6 and *B. elkanii* USDA61. However, the genes of non-structural proteins from Lmj strains are not conserved in other bacterial species.

T3SS of Bradyrhizobium sp. LmjC is essential for symbiosis with L. mariae-josephae.

TtsI has been described as the positive regulator of the T3SS. In order to ascertain the relevance of T3SS in Lmj strains, a *ttsI* mutant was generated in strain LmjC. Symbiosis of *L. mariae-josephae* with the mutant appeared to be severely impaired, since plants were significantly smaller than those inoculated with the wild type strain and since nodules produced by the mutant were white and unable to reduce acetylene. This suggests that the T3SS from strain LmjC is required for effective symbiosis with *L. mariae-josephae.*

Autocleavage of purified LmjC NopE protein in the presence of different cations.

NopE-like protein from LmjC strain presents just one autocatalytic motif (DUF1521) unlike NopE1 and NopE2 proteins secreted by the T3SS of *B. diazoefficiens* that contain two cleavage sites that play a role in the symbiotic host-range definition (Schirrmeister *et al*. 2011). Consistent with this trait, LmjC NopE protein, purified from *E. coli*, was autocleaved in two fragments of the predicted size in the presence of Ca^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and Mn^{2+}. In contrast, autocleavage did not take place in the presence of Ni^{2+}, Co^{2+} or Mg^{2+}. Site-directed mutagenesis of the DUF1521 motif of LmjC NopE in *E. coli* abolished the in vitro self-cleavage. The role of NopE in LmjC strain is unknown, and the behavior of the NopE-DUF1521 mutant in LmjC strain is required.

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