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Rhizobium pisi sv. *trifolii* K3.22 harboring *nod* genes of the *Rhizobium leguminosarum* sv. *trifolii* cluster

Monika Marek-Kozaczuk[a], Agnieszka Leszcz[b], Jerzy Wielbo[a], Sylwia Wdowiak-Wróbel[a], Anna Skorupska[a,*]

[a] *Department of Genetics and Microbiology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland*
[b] *Department of Cell Biology, The John Paul II Catholic University of Lublin, Krasnicka Ave 102, 20-718 Lublin, Poland*

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ABSTRACT

The taxonomic status of the *Rhizobium* sp. K3.22 clover nodule isolate was studied by multilocus sequence analysis (MLSA) of 16S rRNA and six housekeeping chromosomal genes, as well as by a subsequent phylogenic analysis. The results revealed full congruence with the *Rhizobium pisi* DSM 30132^T core genes, thus supporting the same taxonomic position for both strains. However, the K3.22 plasmid symbiosis *nod* genes demonstrated high sequence similarity to *Rhizobium leguminosarum* sv. *trifolii*, whereas the *R. pisi* DSM 30132^T *nod* genes were most similar to *R. leguminosarum* sv. *viciae*. The strains differed in the host range nodulation specificity, since strain K3.22 effectively nodulated red and white clover but not vetch, in contrast to *R. pisi* DSM 30132^T, which effectively nodulated vetch but was not able to nodulate clover. Both strains had the ability to form nodules on pea and bean but they differed in bean cultivar specificity. The *R. pisi* K3.22 and DSM 30132^T strains might provide evidence for the transfer of *R. leguminosarum* sv. *trifolii* and sv. *viciae* symbiotic plasmids occurring in natural soil populations.

Introduction

Soil bacteria, commonly referred to as rhizobia, are able to establish root symbioses with legume plants by forming root or stem nodules in which they fix atmospheric nitrogen [21,26]. The *Rhizobium*-legume symbioses vary in both the specificity of the host range and the diversity of bacterial species nodulating a given host plant [26]. Some of them, such as *Ensifer* sp. NGR234, nodulate many plant host species and thus show extreme promiscuity [19,29]. *Rhizobium leguminosarum* is known for its broad host range since it can nodulate *Lathyrus* spp., *Pisum sativum*, *Melilotus indicus*, *Robinia pseudoacacia*, *Securigera varia* and, more frequently, *Trifolium* and *Vicia* plants, forming several symbiovars (biovars) [1,6,31,33,44]. However, *R. leguminosarum* sv. *trifolii* has a very narrow host range and only nodulates clover plants (*Trifolium* spp.), and forms symbiovar *trifolii* [33].

Rhizobial genomes are complex, dynamic structures composed, in most cases, of the chromosome (core genome) and plasmids (accessory genome) that may represent up to 50% of the genome [15,23,48]. In most rhizobial species, symbiotic functions are encoded by independent replicons, known as symbiotic plasmids (pSym) [16,26]. Comparative whole-genome analyses have revealed several events of horizontal gene transfer (HGT), particularly in the symbiosis-related genes that, to some extent, explain the evolution of certain symbioses [3,15,41]. Also, the ability of different species of rhizobia to undergo HGT was demonstrated, which explains the commonly observed exchange of plasmid- or island-located symbiosis genes [7,11,36,38].

In rhizobial taxonomy, species identification is performed on the basis of relatively stable chromosomal (core) genes but the nodulation capability, designated as the species symbiovar [33], is encoded by genes of plasmids or genomic islands that are unstable and usually have their own phylogenic histories. The *R. leguminosarum* species comprises three symbiovars: *viciae* (nodulating pea and vetch), *trifolii* (nodulating clover), and *phaseoli* (nodulating *Phaseolus vulgaris* beans), which show a common chromosomal background and different host range specificities [18,33]. *R. phaseoli* and *Rhizobium etli*, which also nodulate bean (*P. vulgaris*), belong to the same *R. leguminosarum* group [35]. This group has recently been revised and species *novum Rhizobium pisi* DSM 30132^T has been described [30,33]. It was isolated from pea nodules (*P. sativum*) and was also described as being able to nodulate *P. vulgaris* and *Trifolium repens* [30].

Recently, *R. leguminosarum* sv. *trifolii* strain K3.22 was found to be genetically distinct from other closely related clover nodule isolates [23]. Therefore, in this study, we investigated the taxonomic position of the K3.22 isolate on the basis of multilocus sequence analysis (MLSA) of 16S rRNA and six housekeeping chromosomal

* Corresponding author. Tel.: +48 81 5375972; fax: +48 81 5375959.
E-mail address: anna.skorupska@poczta.umcs.lublin.pl (A. Skorupska).

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genes, which showed its close relatedness to *R. pisi* DSM 30132^T, the only strain described to date. The common *nod* gene clusters of both strains were partially sequenced and the symbiotic capabilities of both strains were assessed.

Materials and methods

Rhizobium strains

The K3.22 isolate was obtained from nodules of red clover (*Trifolium pratense* L. cv. Dajana) [47]. *Rhizobium pisi* DSM 30132^T was kindly supplied by Dr. Encarna Velazquez [30]. *Rhizobium* strains were grown and maintained on 79CA [43], tryptone-yeast (TY) complex media, or M1 medium [34] at 28 °C for 2–3 days.

Plasmid profiles

Plasmid contents of K3.22 and DSM 30132^T were analyzed using the Eckhardt method [9] and pulsed-field gel electrophoresis (PFGE), as described previously [23]. Plasmid size estimation was performed using Bio-Profile V11.01 (Vilber-Lourmat, France) by comparison with *R. leguminosarum* sv. *viciae* 3841 standard plasmids [48].

DNA analyses

Genomic DNA was extracted from 5 mL of a 2-day bacterial culture in liquid TY using the method of Pitcher et al. [28]. The primers and protocols used for amplification and sequencing the genes encoding 16S rRNA, *recA*, *glnA*, *gyrB*, *rpoB*, *dnaK*, and *atpD* are described in Table 1. PCR reactions were carried out using the Ready Mix Taq PCR Reaction (Sigma). DNA probes for Southern hybridizations were obtained by PCR using K3.22 and DSM 30132^T genomic DNA as the templates with appropriate primers (Table 2). The hybridization probes were labeled with the DIG DNA Labeling and Detection Kit (Roche). The primers used for *nod* gene amplification are shown in Table 2. Sequencing was performed using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 3730 XL Genetic Analyzer (Applied Biosystems).

The sequences were aligned with those from GenBank using the MEGA5.05 software package [39]. The distances were calculated according to Kimura's two-parameter model [17]. Phylogenetic trees were inferred using the neighbor-joining (NJ) method. Bootstrap analyses were calculated based on 1000 replications [10]. All gene sequences (chromosomal and *nod*) determined in this study have been deposited in the EMBL database, and the GenBank accession numbers are listed in Tables 1 and 2.

Physiological tests

The utilization of different carbon and energy sources by strains K3.22 and DSM 30132^T was assayed using API 20NE test kits (bioMérieux) and the Biolog GN2 MicroPlate panel (BIOLOG Hayward, CA), as described previously [47]. To assay salt tolerance, *Rhizobium* cultures were grown in liquid 79CA with sodium DL-lactate as the sole carbon source and different concentrations of NaCl ranging from 0 to 4% (w/v) [14]. The pH tolerance of *Rhizobium* in the range 4–10 was tested in 79CA with mannitol as the sole carbon source. Antibiotic resistance was determined on 79CA agar plates by the disc diffusion method. To study motility, a 10 μL water culture suspension (OD_{550} 0.1) was inoculated at the center of a swarm plate (0.3% 79CA agar medium) and incubated at 22 °C for 7 days.

Plant tests

For nodulation tests, seeds of two cultivars of red clover (*T. pratense* L. cv. Rozeta and cv. Nike), white clover (*T. repens* cv. Grasslands Huia), vetch (*Vicia villosa* cv. Wista), pea (*P. sativum* cv. Łówiecki), a Polish cultivar (*P. vulgaris* var. Majorka), two Spanish cultivars of bean (*P. vulgaris* cv. Slenderette and cv. Borlotto Lengua de Fuego Enana) and fava bean (*Vicia faba* cv. Muchamiel sel. Goliath) were used.

Seeds were surface sterilized and cultivated on agar slants (clover, vetch) with nitrogen-free Fåhraeus medium [43] or in pots (pea, bean, fava bean) with sterilized sand. The plants were grown for five weeks in a greenhouse under natural light supplemented with artificial light (14 h day/10 h night, at 24/19 °C).

Results and discussion

Phylogenetic analysis of the K3.22 isolate

To examine the phylogenetic position of the K3.22 clover nodule isolate, the entire 16S rRNA gene (1319 bp) was sequenced for genus identification (Fig. 1). This sequence was identical (100%) to *R. pisi* DSM 30132^T and nearly identical (99%) to *Rhizobium fabae* CCBAU 23163, *R. fabae* CCBAU 33202, and *R. phaseoli* ATCC 14482. Subsequently, for further species identification, the *atpD* (460 bp), *dnaK* (704 ± 6 bp), *glnA* (935 ± 3 bp), *gyrB* (660 ± 18 bp), *recA* (533 bp), and *rpoB* (925 bp) housekeeping genes of K3.22 and DSM 30132^T were sequenced. The phylograms constructed for the individual core genes by the neighbor-joining (NJ) method with high bootstrap replication yielded similar grouping when compared to the 16S rRNA tree (Fig. S1 A–F). Next, the concatenated sequences of the *recA* and *atpD* core genes available for all

Table 1
Oligonucleotide primers and PCR cycling conditions used for chromosomal gene amplification.

Gene name	Primer	Sequence 5′–3′	Anneal. temp. (°C)	Accession number (length bp)		Reference
				K3.22	DSM 30132T	
dnaK	dnaK1466F	AAGGARCANCAGATCCGCATCCA	65	JQ771759	JQ795193	
	dnaK1777R	TASATSGCCTSRCCRAGCTTCAT		(697)	(710)	[20]
atpD	atpD294F	ATCGGCGAGCCGGTCGACGA	58	JQ771756		
	atpD771R	GCCGACACTTCCGAACCNGCCTG		(460)		[13]
16S rDNA	fD1	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG	55	JQ795192		
	rD1	CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC		(1319)		[45]
glnA	glnA144F	GTCATGTTCGACGGYTCYTCG	61	JQ771757	JQ771758	
	glnA1142R	TGGAKCTTGTTCTTGATGCCG		(931)	(938)	[20]
gyrB	gyrB343F	TTCGACCAGAAYTCCTAYAAGG	58	JQ795182	JQ795183	
	gyrB1043R	AGCTTGTCCTTSGTCTGCG		(641)	(678)	[20]
rpoB	rpoB83F	CCTSATCGAGGTTCACAGAAGGC	56	JQ795189	JQ795190	
	rpoB1061R	AGCGTGTTGCGGATATAGGCG		(925)	(925)	[20]
recA	recA6f	CGKCTSGTAGAGGAYAAATCGGTGGA	56	JQ795188		
	recA640r	ACATSACRCCGATCTTCATGC		(533)		[2]