Rhizobial Microsymbionts of Kamchatka *Oxytropis* Species Possess Genes of the Type III and VI Secretion Systems, Which Can Affect the Development of Symbiosis

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A collection of rhizobial strains isolated from root nodules of the narrowly endemic legume species *Oxytropis erecta*, *O. anadyrensis*, *O. kamtschatica*, and *O. pumilio* originating from the Kamchatka Peninsula (Russian Federation) was obtained. Analysis of the 16S ribosomal RNA gene sequence showed a significant diversity of isolates belonging to families *Rhizobiaceae* (genus *Rhizobium*), *Phyllobacteriaceae* (genera *Mesorhizobium*, *Phyllobacterium*), and *Bradyrhizobiaceae* (genera *Bosea*, *Tardiphaga*). A plant nodulation assay showed that only strains belonging to genus *Mesorhizobium* could form nitrogen-fixing nodules on *Oxytropis* plants. The strains *M. loti* 582 and *M. huakuii* 583, in addition to symbiotic clusters, possessed genes of the type III and type VI secretion systems (T3SS and T6SS, respectively), which can influence the host specificity of strains. These strains formed nodules of two

types (elongated and rounded) on *O. kamtschatica* roots. We suggest this phenomenon may result from Nod factor-dependent and -independent nodulation strategies. The obtained strains are of interest for further study of the T3SS and T6SS gene function and their role in the development of rhizobium-legume symbiosis. The prospects of using rhizobia having both gene systems related to symbiotic and nonsymbiotic nodulation strategies to enhance the efficiency of plant-microbe interactions by expanding the host specificity and increasing nodulation efficiency are discussed.

Keywords: Kamchatka region, *Oxytropis* spp., plant-microbe interaction, symbiosis, T3SS, T6SS

Sequences have been deposited to the NCBI GenBank database under accession numbers CP050292 for *Tardiphaga robiniae* 581, CP050293 to CP050295 for *Mesorhizobium loti* 582, CP050296 to CP050299 for *M. huakuii* 583, CP050300 to CP050303 for *Phyllobacterium* sp. strain 628, CP050304 to CP050308 for *Rhizobium lusitanum* 629, CP050549 to CP050552 for *R. leguminosarum* RCAM0610, CP050553 to CP050557 for *R. leguminosarum* RCAM0626, CP050514 to CP050519 for *R. leguminosarum* RCAM1365, CP050562 to CP050566 for *R. leguminosarum* RCAM2802, CP050558 to CP050561 for *Sinorhizobium meliloti* RCAM1115, and CP050511 to CP050513 for *S. meliloti* RCAM1750. *Rrs* sequences can be found under accession numbers MT192879 to MT225791.

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The legume genus *Oxytropis* does not have much agricultural value but forms natural pastures, successfully growing on poor soils of the northern temperate, subarctic, and arctic regions (Ampomah et al. 2017; Ghobakhloo et al. 2015; Hou et al. 2009; Sprent 2001). The *Mesorhizobium* strains have been identified as the predominant bacteria that nodulate *Oxytropis* plants, whereas the genera *Rhizobium*, *Sinorhizobium*, and *Bradyrhizobium* have been found as their minor symbionts (Ampomah et al. 2017; Ghobakhloo et al. 2015; Hou et al. 2009; Laguerre et al. 1997; Xu et al. 2013). Among *Mesorhizobium*-related strains, representatives of different species were identified as *M. amorphae*, *M. temperatum*, *M. mediterraneum*, *M. loti*, *M. ciceri*, and *M. gobiense* (Han et al. 2008; Hou et al. 2009); however, many isolated strains are still without species definition.

Information about strains isolated from root nodules of Miocene-Pliocene relics *O. triphylla* and *O. popoviana*, which originated from the Baikal Lake region (Siberia), was recently published (Safronova et al. 2017, 2018a and b). It was shown that the rhizobial microsymbionts of these plants belonged to the genera *Bosea*, *Tardiphaga*, *Phyllobacterium*, and *Mesorhizobium*. Although *Bosea* and *Tardiphaga* strains were often found in the root nodules of different legumes, there is no information about their ability to form symbiosis with host plants. It was suggested that they can be present in nodules as carriers of ancestral genes that can contribute to the formation of

symbiosis and affect its effectiveness (Provorov 2013). The ability of most *Phyllobacterium* strains to singly induce nodules was also not clearly demonstrated (Mantelin et al. 2006), although representatives of at least two species (*P. sophorae* and *P. trifolii*) can form effective symbiosis with host plants (Jiao et al. 2015; Valverde et al. 2005). *Mesorhizobium*-related strains were isolated only from *O. popoviana* nodules and were identified as *M. japonicum* and *M. kowhai* species (Safronova et al. 2017, 2018a and b). Previously these rhizobial species were described for *Lotus* and *Sophora* nodulating strains, respectively (De Meyer et al. 2016; Martínez-Hidalgo et al. 2016).

Symbiotic systems of legumes possessing a wide range of microsymbionts are promising models for studying the evolution of host specificity, the impact of natural rhizobial diversity on plant growth, and multipartite interactions between all symbiotic partners. In this regard, the aim of this work was to study rhizobial strains isolated from the root nodules of four narrowly endemic *Oxytropis* species growing in the Kamchatka Peninsula: *O. erecta*, *O. anadyrensis*, *O. kamtschatica*, and

O. pumilio. Particular attention was paid to the phylogeny of the isolated strains and analysis of their symbiotic potential using plant nodulation assays, whole-genome sequencing, and the search for genes (*Sym* genes as well as the type III, IV, and VI secretion systems [T3SS, T4SS, and T6SS, respectively]) that can affect the development of plant-microbe interactions.

RESULTS AND DISCUSSION

Isolation of rhizobial strains from *O. erecta*, *O. anadyrensis*, *O. kamtschatica*, and *O. pumilio* plants and their identification by sequencing the 16S ribosomal RNA (rRNA) gene.

Forty-five rhizobial strains were isolated from *Oxytropis* species root nodules originating from the Kamchatka Peninsula. The identification of isolates by sequencing the 16S rRNA gene showed that they belonged to different species and genera of the order *Rhizobiales* (Table 1). Only one strain (629) was related to the genus *Rhizobium* and was identified as *R. lusitanum* (*rrs* similarity with the type strain P1-7^T was

Table 1. Identification of strains isolated from *Oxytropis erecta*, *O. anadyrensis*, *O. kamtschatica*, and *O. pumilio* plants by 16S ribosomal RNA gene sequencing

Plant species	Strain ^z	Similarity with the closest species		Identification result
		Type strain	Identity %	
<i>O. erecta</i>	553 a	<i>Bosea vaviloviae</i> Vaf-18	100	<i>B. vaviloviae</i>
	554 a	<i>Tardiphaga robiniae</i> R-45977	99.9	<i>T. robiniae</i>
	558	<i>T. robiniae</i> R-45977	99.9	<i>T. robiniae</i>
	559	<i>Mesorhizobium loti</i> LMG 6125	99.8	<i>M. loti</i>
	561	<i>T. robiniae</i> R-45977	98.6	<i>Tardiphaga</i> sp.
	563 b	<i>Phyllobacterium loti</i> S658	100	<i>P. loti</i>
	565 b	<i>M. loti</i> LMG 6125	99.8	<i>M. loti</i>
	568	<i>T. robiniae</i> R-45977	100	<i>T. robiniae</i>
	569	<i>M. loti</i> LMG 6125	100	<i>M. loti</i>
	571	<i>B. vaviloviae</i> Vaf-18	99.6	<i>B. vaviloviae</i>
<i>O. kamtschatica</i>	572	<i>M. jarvisii</i> ATCC 33669	99.9	<i>M. jarvisii</i>
	576	<i>T. robiniae</i> R-45977	99.9	<i>T. robiniae</i>
	578	<i>M. huakuii</i> NBRC 15243	100	<i>M. huakuii</i>
	581 c	<i>T. robiniae</i> R-45977	99.9	<i>T. robiniae</i>
	582 c	<i>M. loti</i> LMG 6125	99.9	<i>M. loti</i>
	583	<i>M. huakuii</i> NBRC 15243	99.7	<i>M. huakuii</i>
	584	<i>M. jarvisii</i> ATCC 33669	100	<i>M. jarvisii</i>
	585 d	<i>B. vaviloviae</i> Vaf-18	99.9	<i>B. vaviloviae</i>
	586 d	<i>T. robiniae</i> R-45977	99.9	<i>T. robiniae</i>
	589 e	<i>B. vaviloviae</i> Vaf-18	100	<i>B. vaviloviae</i>
<i>O. anadyrensis</i>	590 e	<i>T. robiniae</i> R-45977	99.9	<i>T. robiniae</i>
	592	<i>T. robiniae</i> R-45977	99.9	<i>T. robiniae</i>
	593	<i>T. robiniae</i> R-45977	99.9	<i>T. robiniae</i>
	594 f	<i>B. vaviloviae</i> Vaf-18	100	<i>B. vaviloviae</i>
	595 f	<i>T. robiniae</i> R-45977	99.9	<i>T. robiniae</i>
	596	<i>T. robiniae</i> R-45977	99.9	<i>T. robiniae</i>
	597	<i>T. robiniae</i> R-45977	100	<i>T. robiniae</i>
	608	<i>P. myrsinacearum</i> NBRC 100019	100	<i>P. myrsinacearum</i>
	610	<i>M. jarvisii</i> ATCC 33669	99.9	<i>M. jarvisii</i>
	612	<i>M. jarvisii</i> ATCC 33669	99.9	<i>M. jarvisii</i>
<i>O. pumilio</i>	613	<i>B. vaviloviae</i> Vaf-18	99.4	<i>Bosea</i> sp.
	614	<i>P. loti</i> S658	100	<i>P. loti</i>
	616 g	<i>B. vaviloviae</i> Vaf-18	99.7	<i>B. vaviloviae</i>
	617 g	<i>M. loti</i> LMG 6125	99.8	<i>M. loti</i>
	623 h	<i>B. vaviloviae</i> Vaf-18	99.8	<i>B. vaviloviae</i>
	624 h	<i>M. jarvisii</i> ATCC 33669	99.9	<i>M. jarvisii</i>
	625	<i>B. lathyri</i> R-46060	99.9	<i>B. lathyri</i>
	628 i	<i>P. myrsinacearum</i> NBRC 100019	99.2	<i>Phyllobacterium</i> sp.
	629 i	<i>R. lusitanum</i> P1-7	99.6	<i>R. lusitanum</i>
	631	<i>M. loti</i> LMG 6125	100	<i>M. loti</i>
	638	<i>T. robiniae</i> R-45977	99.6	<i>T. robiniae</i>
	643	<i>T. robiniae</i> R-45977	99.8	<i>T. robiniae</i>
	646	<i>M. jarvisii</i> ATCC 33669	99.9	<i>M. jarvisii</i>
	648	<i>M. jarvisii</i> ATCC 33669	99.9	<i>M. jarvisii</i>
	650	<i>M. qingshengii</i> CCBAU 33460	99.9	<i>M. qingshengii</i>

^z Matching letters indicate pairs of strains isolated from the same nodule.

99.6%). The greatest genetic diversity was observed for *Mesorhizobium*-related isolates belonging to four species: *M. loti*, *M. jarvisii*, *M. huakuii*, and *M. qingshengii* (Table 1; Fig. 1). Strains 559, 565, 569, 582, 617, and 631 belonged to the species *M. loti* (99.8 to 100% *rrs* similarity with the type strain LMG 6125^T). Isolates 572, 584, 610, 612, 624, 646, and

648 were the closest to the type strain *M. jarvisii* ATCC 33669^T, with 99.9 to 100% *rrs* similarity. Strains 578 and 583 were classified as *M. huakuii*, while strain 650 was assigned to the species *M. qingshengii* (Table 1). None of the *Mesorhizobium*-related isolates formed a reliable cluster with statistical support more than 95% (Fig. 1). The species *M. loti*

Fig. 1. Phylogenetic tree generated by the neighbor-joining method, using partial 16S ribosomal RNA gene sequences (1,400 nt) of the *Mesorhizobium*-related isolates from *Oxytropis erecta*, *O. kamschatkana*, and *O. pumilio* (in bold) and closely related species. Bootstrap values more than 30% are given. Type strains are indicated by the letter T.

has been described for a long time and was isolated from nodules of *Lotus corniculatus* (Jarvis et al. 1982) as well as the species *M. jarvisii* (Martínez-Hidalgo et al. 2015). Representatives of the species *M. huakuii* and *M. qingshengii* are known as specific microsymbionts of the Chinese milk vetch (*Astragalus sinicus*) from which they were isolated (Chen et al. 1991; Zheng et al. 2013).

Four strains, 563, 608, 614, and 628, were closely related to genus *Phyllobacterium* (Table 1; Supplementary Fig. S1). Isolate 608 formed the highest supported cluster with the type strain *P. myrsinacearum* NBRC 100019^T (100% bootstrap support, 100% *rrs* similarity), while isolate 628 had only 99.2% *rrs* similarity with this type strain and was left without species definition. Strains 563 and 614 had 100% *rrs* similarity with type strain *P. loti* S658^T. It is known that *P. myrsinacearum* strains, originally isolated from leaf nodules of *Ardisia* plants (Knosel 1984), are also found on the phylloplane and rhizoplane of other plants from the families *Myrsinaceae* and *Rubiaceae* (Mergaert et al. 2002). The type strain *P. loti* S658^T was isolated from a *Lotus corniculatus* nodule in Uruguay (Sánchez et al. 2014).

Strains 553, 571, 585, 589, 594, 616, and 623 had 99.6 to 100% *rrs* similarity with the type strain *Bosea vaviloviae* Vaf18^T, while isolate 625 was identified as *B. lathyri* (Table 1; Supplementary Fig. S2). Previously, *B. lathyri* (De Meyer and Willems 2012) strains were isolated from root nodules of *Lathyrus latifolius* plants in Belgium, and the species *B. vaviloviae* (Safronova et al. 2015) was described for isolates from root nodules of *Vavilovia formosa* growing in the North Caucasus. Most isolates (14) were identified as *Tardiphaga robiniae* (99.6 to 100% *rrs* similarity with the type strain R-45977^T) and the species affiliation of one *Tardiphaga*-related strain 561 was not determined (Table 1; Supplementary Fig. S3). The species *T. robiniae* were isolated from *Robinia pseudoacacia* root nodules in Belgium and it is the only member of this genus belonging to the family *Bradyrhizobiaceae* (De Meyer et al. 2012).

It was noted that pairs of phenotypically and taxonomically different strains were isolated from some nodules of *O. erecta*, *O. anadyrensis*, *O. kamtschatica*, and *O. pumilio* plants (Table 1). The combination of strains in pairs was diverse: *Bosea* + *Tardiphaga*, *Phyllobacterium* + *Mesorhizobium*, *Tardiphaga* + *Mesorhizobium*, *Bosea* + *Mesorhizobium*, *Phyllobacterium* + *Rhizobium*. The ratio of different strains in the same nodule did not exceed 1:10. Previously, several pairs of rhizobial strains (*Bosea* + *Phyllobacterium* and *Mesorhizobium* + *Bradyrhizobium*) were isolated from the root nodules of the Miocene-Pliocene relict legumes *O. triphylla* and *O. popoviana* originating from the Baikal Lake region (Safronova et al. 2017; 2018a). Moreover, coinoculation with paired strains significantly accelerated nodule formation and it was suggested that taxonomically different strains of the archaic symbiotic system can be co-microsymbionts infecting the same nodule and resulting in promotion of nodulation process due to complementary sets of symbiotic genes (Safronova et al. 2018a).

Thus, the analysis of the 16S rRNA gene sequence showed a significant species diversity of *Oxytropis* microsymbionts belonging to three families of the order *Rhizobiales*: *Rhizobiaceae* (genus *Rhizobium*), *Phyllobacteriaceae* (genera *Mesorhizobium* and *Phyllobacterium*), and *Bradyrhizobiaceae* (genera *Bosea* and *Tardiphaga*). It was noted that the spectrum of microsymbionts depended on the host plant. The greatest genetic diversity was observed among rhizobial isolates of the species *O. pumilio*, while only *Bosea* and *Tardiphaga* strains were isolated from *O. anadyrensis* nodules. Among the isolated strains, both typical microsymbionts of legumes belonging to genera *Mesorhizobium* and *Rhizobium* were present. Among atypical

strains, only certain species possess nodulating ability (genus *Phyllobacterium*) or may be present in nodules but do not form them (genera *Tardiphaga* and *Bosea*). Thus, a number of individual strains and pairs of taxonomically different bacteria in various combinations were isolated from nodules of the studied *Oxytropis* plants, demonstrating high variability of microsymbionts and low symbiosis specificity of these host plants.

Plant nodulation assays.

The plant nodulation tests were performed on *O. erecta*, *O. kamtschatica*, and *O. pumilio* plants with the participation of some isolated strains representing different rhizobia genera (*Bosea*, *Tardiphaga*, *Mesorhizobium*, *Phyllobacterium*, and *Rhizobium* [Table 1]). Four different variants of joint inoculation with pairs of strains isolated from the same nodule were also studied (*B. vaviloviae* 553 + *T. robiniae* 554, *P. loti* 563 + *M. loti* 565, *T. robiniae* 581 + *M. loti* 582, *Phyllobacterium* sp. strain 628 + *R. lusitanum* 629). The results of preliminary and representative tests are presented in Supplementary Table S1 and Table 2, respectively. No nodules were formed on plants inoculated with isolates of the genera *Bosea*, *Tardiphaga*, *Phyllobacterium*, and *Rhizobium*. At the same time, most strains of the genus *Mesorhizobium* (*M. jarvisii*, *M. loti*, and *M. huakuii*) formed nitrogen-fixing symbiosis. The nitrogen-fixing activity of the strains was more associated with the host plant than with the bacterial species. The maximum parameter, nanomoles C₂H₄ per nodule per hour (nmol C₂H₄ nodule⁻¹ h⁻¹), was possessed by the strains *M. jarvisii* 572 and *M. huakuii* 583 in symbiosis with *O. kamtschatica* plants. Inoculation led to a significant increase in plant biomass only in these two variants (Table 2). The symbiosis with *O. pumilio* plants was less active. In the coinoculations, the pair of strains *B. vaviloviae* 553 + *T. robiniae* 554 did not lead to the appearance of nodules, while the combination of strains *P. loti* 563 + *M. loti* 565 did not change the symbiotic parameters (number of nodules, plant biomass, and nitrogen fixation) compared with strain *M. loti* 565. After coinoculation with strains *T. robiniae* 581 + *M. loti* 582, a significant decrease in the parameter nmol C₂H₄ plant⁻¹ h⁻¹ was observed as compared with the mono-inoculation with the strain *M. loti* 582. However, a decrease in this parameter occurred as there tended to be fewer nodules (Table 2). The most interesting results were that single-strain inoculations of *Phyllobacterium* sp. strain 628 and *R. lusitanum* 629 did not nodulate *O. pumilio* plants but their coinoculation formed inactive nodules.

Pictures of nodules obtained in the representative plant nodulation test are shown in Figure 2. Strains of the genus *Mesorhizobium* formed single, slightly pinkish or pink elongated nodules on plants of all *Oxytropis* species. Nodules formed by the strains *M. jarvisii* 572 and *M. huakuii* 583 on *O. kamtschatica* plants had an intense pink color, which correlated with their high nitrogen fixation (Fig. 2C1 and C2). In mono-inoculations with the strains *M. loti* 582 and *M. huakuii* 583, in addition to elongated nodules, the formation of atypical rounded nodules was noted (Fig. 2C2 to C5). These nodules retained their shape during the experiment and were bright yellow or orange. A representative of the rounded nodules that were observed on *O. pumilio* plants in the coinoculation with strains *Phyllobacterium* sp. strain 628 + *R. lusitanum* 629 is shown in Figure 2B3.

Nodules obtained in some mono- and coinoculations were studied for the presence of bacteria. Strains *M. loti* 582 and *M. huakuii* 583 were found in both elongated and rounded nodules formed in mono-inoculations. The ratio of bacteria in nodules obtained in the coinoculations was approximately 1 to 10 for strains *P. loti* 563 and *M. loti* 565 and 10 to 1 for strains *T. robiniae* 581 and *M. loti* 582, respectively. Nodules formed in

the variant *Phyllobacterium* sp. strain 628 + *R. lusitanum* 629 contained none of the introduced bacteria.

Whole-genome sequencing of the selected isolates.

To obtain whole-genome sequences, five strains were selected: the non-nodulating strain *T. robiniae* 581, strains *M. loti* 582 and *M. huakuii* 583, forming elongated and rounded nodules on *O. kamschatatica* plants, and the pair *Phyllobacterium* sp. strain 628 and *R. lusitanum* 629, which formed only rounded nodules and only in the coinoculation (Table 2; Fig. 2). The search results for the symbiotic *fix*, *nif*, *nod*, *noe*, and *nol* genes are presented in Table 3. It was shown that strains *M. loti* 582 and *M. huakuii* 583 possessed a set of symbiotic genes necessary for the formation of effective symbiosis and were grouped into clusters. In contrast, strain *T. robiniae* 581 possessed only a reduced number of *fix* genes, while *Phyllobacterium* sp. strain 628 and *R. lusitanum* 629 had only individual symbiotic genes, which obviously did not participate in the formation of nodules.

The presence of two clusters of *fix* genes in strains *T. robiniae* 581 and *M. loti* 582 was noteworthy. Strain *M. loti* 582, in addition to cluster 1 containing all the necessary nitrogen fixation genes (*fixXCBANOQPGHIS*), had a cluster 2 with fewer genes (*fixKNOQPGHIS*). The strain *T. robiniae* 581 did not possess *fixABCX* genes, which play a central role in nitrogenase functioning, but had a “long” cluster 2 (*fixKNOQPGHS*) and a “short” cluster 3 (*fixNOQPI*). Since the strains *T. robiniae* 581 and *M. loti* 582 were isolated from the same nodule, we studied the intra- and interstrain similarity between *fix* gene copies of all these clusters. It turned out that the interstrain homology between the nucleotide sequences of the corresponding gene copies was below 70% (data not shown), which indicates the absence of horizontal transfer of these genes between two strains localized in the same nodule. Percentages of the intra-strain similarity between the gene copies present in different

Fix clusters of strains *T. robiniae* 581 and *M. loti* 582 are shown in Supplementary Table S2. It was demonstrated that the level of similarity between copies of most *fix* genes was quite high (more than 80% nucleotide and protein identity) in strain *M. loti* 582. At the same time, the gene copies of Fix clusters 2 and 3 had a similarity level of less than 70% (Supplementary Table S2) in strain *T. robiniae* 581. The genes within Fix cluster 2 had the highest percentage of similarity with representatives of genus *Tardiphaga*, whereas the genes of Fix cluster 3 were closest to the strain *Oligotropha carboxidovorans* (data not shown), which is not a known nitrogen fixator but is closely related to *Nitrobacter hamburgensis* (Paul et al. 2011). Therefore, it can be concluded that, in strain *T. robiniae* 581, Fix gene clusters 2 and 3 were obtained from different microbial sources.

In addition to symbiotic genes, a search was made for genes of T3SS, T4SS, and T6SS involved in bacterial virulence due to secretion of broad classes of effector proteins, cytotoxins, lysozymes, lipoproteins, and factors of adherence to epithelial cells and the penetrating system, such as pilus formation, conjugation, protein translocation (Cascales and Christie 2003; Notti and Stebbins 2016; Okazaki et al. 2016; Russell et al. 2014). It is known that these secretion systems in rhizobia can affect the host specificity of strains and determine the number of nodules formed (Nelson and Sadowsky 2015). The T3SS and T4SS have an important role in suppressing the plant immune response along with rhizobial surface polysaccharides (Masson-Boivin et al. 2009). It was shown that deletions of the T3SS or the T4SS had both positive and negative effect on symbiosis (Sugawara et al. 2013; Tampakaki 2014). For example, the partial deletion of *vir* genes (T4SS) in *M. loti* R7A led to a decrease in the number of nodules on *Lotus corniculatus* plants (Hubber et al. 2004). However, deletion of the T4SS allowed the R7A strain to nodulate *Leucaena leucocephala*, in contrast to the wild-type strain that formed only uninfected tumor-like structures on this plant species. The T6SS is

Table 2. Effects of mono- and coinoculation of *Oxytropis erecta*, *O. kamschatatica* and *O. pumilio* plants with the isolated strains representing different rhizobia genera in the gnotobiotic plant nodulation assay^y

Host plant	Treatment		Nitrogen fixation ^z			
	Bacterial species	Strain	NN	PB	Plant	Nodule
<i>O. erecta</i>	<i>Bosea vaviloviae</i>	553	0	92.4 ± 10 a	0	0
	<i>Tardiphaga robiniae</i>	554	0	87.4 ± 9 a	0	0
	<i>B. vaviloviae</i> + <i>T. robiniae</i>	553+554	0	80.4 ± 8 a	0	0
	<i>Mesorhizobium loti</i>	559	6.6 ± 2.0 a	97.0 ± 10 a	770 ± 240 a	117 ± 35 a
	<i>Phyllobacterium loti</i>	563	0	77.5 ± 20 a	0	0
	<i>M. loti</i>	565	8.0 ± 2.0 a	81.9 ± 16 a	1228 ± 153 a	154 ± 14 a
	<i>P. loti</i> + <i>M. loti</i>	563+565	4.3 ± 0.7 a	82.4 ± 8 a	928 ± 211 a	216 ± 43 a
	Control without inoculation		0	109.7 ± 14 a	0	0
<i>O. kamschatatica</i>	<i>M. jarvisii</i>	572	2.3 ± 0.6 a	95.0 ± 8 b	1408 ± 29 c	612 ± 11 c
	<i>T. robiniae</i>	581	0	70.8 ± 10 ab	0	0
	<i>M. loti</i>	582	6.0 ± 2.3 bc	74.0 ± 19 ab	927 ± 98 b	155 ± 15 b
	<i>T. robiniae</i> + <i>M. loti</i>	581+582	3.4 ± 1.5 ab	54.2 ± 13 a	443 ± 90 a	130 ± 21 b
	<i>M. huakuii</i>	583	1.0 ± 0.5 a	76.5 ± 2 ab	970 ± 10 b	970 ± 10 d
	<i>M. jarvisii</i>	584	8.0 ± 2.1 c	103.3 ± 6 b	273 ± 85 a	34 ± 10 a
	Control without inoculation		0	74.4 ± 9 ab	0	0
<i>O. pumilio</i>	<i>M. jarvisii</i>	610	5.0 ± 0.1 b	45.0 ± 10 a	450 ± 10 c	90 ± 2 b
	<i>M. jarvisii</i>	612	1.5 ± 0.3 a	36.3 ± 8 a	37 ± 12 a	25 ± 7 a
	<i>B. vaviloviae</i>	616	0	36.7 ± 6 a	0	0
	<i>M. loti</i>	617	4.0 ± 1.6 ab	40.3 ± 5 a	115 ± 32 a	29 ± 6 a
	<i>Phyllobacterium</i> sp.	628	0	45.3 ± 7 a	0	0
	<i>Rhizobium lusitanum</i>	629	0	40.3 ± 5 a	0	0
	<i>Phyllobacterium</i> sp. strain 628 + <i>R. lusitanum</i>	628+629	1.5 ± 0.4 a	42.7 ± 5 a	0	0
	<i>M. loti</i>	631	1 ± 0.3 a	26.0 ± 9 a	29 ± 6 b	29 ± 6 a
	Control without inoculation		0	34.8 ± 4 a	0	0

^y The data is shown as mean ± standard error of one representative experiment (*n* = 5). Different letters show significant differences between treatments (least significant difference test, *P* < 0.05). NN = number of nodules (per plant), PB = plant biomass (milligrams of fresh weight per plant).

^z Shown as nanomoles C₂H₄ per plant or nodule per hour.

among the least-studied secretion system involved in protein translocation. The sequence for the T6SS has been found in five different species of rhizobia (*R. leguminosarum*, *Bradyrhizobium japonicum*, *M. loti*, *Sinorhizobium saheli*, and *S. fredii*), but their role in symbiosis is still not well-understood (Bingle et al. 2008; Sugawara et al. 2013). It was previously shown that strains *M. kowhaii* Ach-343 and *M. japonicum* Opo-235, isolated from nodules of relict legumes *Astragalus chorinensis* and *Oxytropis popoviana*, respectively, in addition to *Sym* genes had a representative set of T6SS genes (*icmF*, *tssABCEGJKL*, *tagFH*, *VgrG*, *vasA*, *Hcp* and *clpV*). These strains demonstrated a wide range of host specificity, including species of different

legume genera from tribes Galegeae and Trifolieae (genera *Astragalus*, *Oxytropis*, *Glycyrrhiza*, *Medicago* and *Trifolium*) and the presence of both strains in the same nodules and plant cells of *Glycyrrhiza uralensis* was accompanied by a significant increase in the number of nodules and plant biomass (Safronova et al. 2019).

Search results for genes of the T3, T4, and T6 secretion systems in strains *T. robiniae* 581, *M. loti* 582, *M. huakuii* 583, *Phyllobacterium* sp. strain 628, and *R. lusitanum* 629 are presented in Table 4. The whole genomes of six commercial rhizobia strains used for inoculation of vetches, clovers, and alfalfa were studied as reference sequences: *R. leguminosarum*

Fig. 2. Root nodules formed in the gnotobiotic plant nodulation assay by the strains isolated from *Oxytropis erecta*, *O. kamtschatica*, and *O. pumilio* in the variants of mono- and coinoculation. Plant species: A to C, *O. erecta*, *O. pumilio*, and *O. kamtschatica*, respectively. Inoculation treatment: *Mesorhizobium loti* 559 (A1), *M. loti* 565 (A2), *Phyllobacterium loti* 563 + *M. loti* 565 (A3), *M. jarvisii* 610 (B1), *M. loti* 617 (B2), *Phyllobacterium* sp. strain 628 + *Rhizobium lusitanum* 629 (B3), *M. jarvisii* 572 (C1), *M. huakuii* 583 (C2 and C3, elongated and rounded nodules, respectively), *M. loti* 582 (C4 and C5, elongated and rounded nodules, respectively), and *Tardiphaga robiniae* 581 + *M. loti* 582 (C6). Scale bar = 500 μm.

RCAM0610, RCAM0626, RCAM1365, and RCAM2802 and *S. meliloti* RCAM1115 and RCAM1750. It was shown that, among isolates from genus *Oxytropis*, strain *T. robiniae* 581 possessed only three T4SS genes (*virB4*, *virB11*, *virB10*), strain *Phyllobacterium* sp. strain 628, in addition to ten *virB* genes, had gene *clpV1* of T6SS, although it was split into two parts. Strain *R. lusitanum* 629, in addition to the T4SS, had T3SS genes (region *y4yB-y4xM*), while strains *M. loti* 582 and *M. huakuii* 583 possessed a significant set of genes for all three secretion systems (Table 4). At the same time, only *virB* genes of the T4SS were detected in the four commercial strains (RCAM0610, RCAM1365, RCAM1115, and RCAM1750), and no T3SS, T4SS, or T6SS genes were detected in the two remaining strains, RCAM0626 and RCAM2802.

Thus, it can be stated that three of five strains isolated from the Kamchatka *Oxytropis* species have a significantly larger set of secretion system genes compared with the six commercial

rhizobial strains used as reference. We propose that these genes can affect the host specificity and the formation of symbiosis with the studied *Oxytropis* species. The question remains whether the ability of strains *M. loti* 582 and *M. huakuii* 583 to form nodules of two types on *O. kamtschatica* plants (typical elongated and atypical rounded) is associated with the presence of the T3SS or T6SS genes. It is known that the T3SS secretion system allows some *Bradyrhizobium* strains with no common *nodABC* genes (for example strains STM3809, ORS285, ORS375, STM3847, STM4509, STM4523, ORS278, and BTAi1) nodulating the leguminous plant *Aeschynomene indica* independently of Nod factors (NF) by *crack* entry (Bonaldi et al. 2011; Mornico et al. 2012; Okazaki et al. 2016). Therefore, it can be assumed that the development of symbiosis by strains simultaneously possessing gene systems for NF-dependent and NF-independent nodulation can occur in two ways: i) by the infection thread formation and ii) by the direct

Table 3. Presence of symbiotic genes in the strains isolated from *Oxytropis kamtschatica* and *O. pumilio* plants and representing different rhizobia genera^z

Symbiotic genes	Strain				
	<i>T. robiniae</i> 581	<i>M. loti</i> 582	<i>M. huakuii</i> 583	<i>Phyllobacterium</i> sp. strain 628	<i>R. lusitanum</i> 629
Fix cluster 1	–	<i>XCBA NOQPGHIS</i>	<i>XCBA SIHQGPON</i>	–	–
Fix cluster 2	<i>KNOQPGHS</i>	<i>KNOQPGHIS</i>	–	–	–
Fix cluster 3	<i>NOQPI</i>	–	–	–	–
<i>fixBA</i>	+	+	+	+	+
<i>fixR</i>	+	–	–	–	–
Nif cluster	–	<i>A TZBA SVW Q HDKENX</i>	<i>HDKENX A TZBA SVW Q</i>	–	–
<i>nifL</i>	+	–	–	–	–
<i>nifR</i>	+	–	–	+	+
<i>nifS</i>	+	–	–	–	+
<i>nifU</i>	–	–	–	–	+
Nod cluster	–	<i>D AFEGBCIJHPQ TM</i>	<i>QPHJICBGEFA D MT</i>	–	–
<i>nodB</i>	+	–	–	–	–
<i>nodG</i>	+	–	–	+	+
<i>nodM</i>	+	–	–	+	+
<i>nodN</i>	–	–	+	+	–
<i>nodP</i>	–	+	–	–	+
<i>nodQ</i>	+	–	+	+	+
<i>nodT</i>	–	–	–	–	+
<i>noeK</i>	–	–	–	–	+
Nol cluster	–	<i>VUTBW X</i>	<i>XWBTUV</i>	–	–
<i>nolL</i> like	+	+	+	+	+

^z Strains *Tardiphaga robiniae* 581, *Mesorhizobium loti* 582 and *M. huakuii* 583 were isolated from *O. kamtschatica* and *Phyllobacterium* sp. strain 628 and *Rhizobium lusitanum* 629 from *O. pumilio*.

Table 4. Presence of type III, IV, and VI secretion system (T3SS, T4SS, and T6SS, respectively) genes in the strains isolated from *Oxytropis kamtschatica* and *O. pumilio* plants^y

Bacterial species	Strain	Secretion system		
		T3SS	T4SS	T6SS
<i>R. leguminosarum</i>	RCAM0610	–	<i>virB1,2,3,4,5,6,8,9,10,11</i>	–
	RCAM0626	–	–	–
	RCAM1365	–	<i>virB1,2,3,4,5,6,8,9,10,11</i>	–
	RCAM2802	–	–	–
<i>S. meliloti</i>	RCAM1115	–	<i>virB1,2,3,4,5,6,7,8,9,10,11</i>	–
	RCAM1750	–	<i>virB1,2,3,4,5,6,8,9,10,11</i>	–
<i>T. robiniae</i>	581	–	<i>virB4,11; 10</i>	–
<i>M. loti</i>	582	<i>rhcV; rhcUTSRQNL, nolUTW, nopX, rhcC, ttsI</i>	<i>virB1,2,3,4,5,6,8,9,10,11</i>	<i>clpV1, tssABC, hcp1, tssEFG, tagH, tssJKL, icmF, tagF, vgrG, FHA domain, dotU family, icmF fragment</i>
<i>M. huakuii</i>	583	<i>ttsI, rhcC, nopX, nolWTU, rhcLNQRSTU; rhcV</i>	<i>virB1,2,3,4,5,6,8,9,10,11</i>	<i>clpV1, tssABC, hcp1, tssEFG, tagH, tssJKL, icmF, vgrG1, tagH, tssL, icmF fragment</i>
<i>Phyllobacterium</i> sp.	628	–	<i>virB1,2,3,4,5,6,8,9,10,11</i>	<i>clpV1^z</i>
<i>R. lusitanum</i>	629	region <i>y4yB-y4xM</i>	<i>virB1,2,3,4,5,6,8,9,10,11</i>	–

^y In comparison with six commercial strains, namely, *Rhizobium leguminosarum* (RCAM0610, RCAM0626, RCAM1365, RCAM2802) and *Sinorhizobium meliloti* (RCAM1115 and RCAM1750).

^z The gene *clpV1* split into two parts. Strains *Tardiphaga robiniae* 581, *Mesorhizobium loti* 582, and *M. huakuii* 583 were isolated from *O. kamtschatica* and *Phyllobacterium* sp. strain 628 and *R. lusitanum* 629 from *O. pumilio*.

penetration of bacteria through the host cell wall. This possibility was previously shown for *Bradyrhizobium* sp. strain ORS285, which used NF-dependent strategy for nodulation of *A. afraspera* plants and NF-independent strategy for nodulation of *A. indica* (Bonaldi et al. 2011). In that case, no nodule formation differing in morphology was detected on *A. indica*. However, development of outgrowths at the top of nodules was observed on *A. afraspera* plants inoculated with strain ORS285. These outgrowths were probably derived from outer cortical cells and were filled with undifferentiated bacteria that did not release into the host cytoplasm. A possible reason for the appearance of various structures in the course of nodule organogenesis on the *A. afraspera* roots is the availability of different nodulation strategies in *Bradyrhizobium* sp. strain ORS285.

In *R. lusitanum* 629, which has no common *nodABC* genes needed for nodule formation, a *y4yB-y4xM* region similar to that of strain *S. fredii* NGR234 was found (Table 4). *S. fredii* NGR234 has an exceptionally broad host range and is able to nodulate more than 112 genera of legumes (Pueppke and Broughton 1999; Streit et al. 2004). It was shown that, in *S. fredii* strains, the *rhcI* (*Rhizobium*-conserved) gene family of the T3SS contains a group of genes *y4yB*, *y4yA*, *y4xP*, *y4xO*, *y4xN*, and *y4xM* that may be related to the secretion of nodulation outer proteins called Nops (Krishnan et al. 2003; Weidner et al. 2012). Although it was shown that expression of *y4yA*, *y4yB*, and *y4xP* is flavonoid-dependent (Jiang and Krishnan 2000; Streit et al. 2004), the role of this group of genes in rhizobial symbiosis remains unknown. Our results showed that *R. lusitanum* 629 was not able to form nodules or nodule-like structures on *O. pumilio* plants. However, in the coinoculation with *Phyllobacterium* sp. strain 628, which was isolated from the same nodule, the formation of nonfixing, rounded nodules on the roots of the host plant was observed (Table 2; Supplementary Table S1; Fig. 2). The reason for this phenomenon is difficult to explain, since *Phyllobacterium* sp. strain 628 has neither common *sym* genes nor T3SS or T6SS genes, except for the *clpVI* gene (Tables 3 and 4). Perhaps other genes present in *Phyllobacterium* sp. strain 628 and *R. lusitanum* 629 and performing complementary functions allow the formation of atypical nodules in the coinoculation. Such factors may be, for example, surface polysaccharides involved in symbiosis specificity, namely, lipopolysaccharides, extracellular polysaccharides and capsular polysaccharides (Nelson and Sadowsky 2015; Streit et al. 2004).

Thus, the obtained results showed very high variability among the strains isolated from the narrow endemic species of *Oxytropis* plants originating from Kamchatka. These strains may be useful for the study of functions of the T3SS, T4SS, and T6SS genes and their role in the development of rhizobia-legume symbiosis. The rhizobial strains having gene systems for both symbiotic and nonsymbiotic nodulation may be also applied for enhancing the efficiency of plant-microbe interactions by expanding host specificity and increasing the efficiency of nodulation of agricultural legumes.

MATERIALS AND METHODS

Isolation of rhizobial strains from the root nodules.

Rhizobial strains were isolated from root nodules of four narrowly endemic species of the legume genus *Oxytropis* (*O. erecta*, *O. anadyrensis*, *O. kamtschatica* and *O. pumilio*) originating from the Kamchatka Peninsula (Russian Federation). The standard method was used for isolation of bacteria from surface-sterilized single nodules (Novikova and Safronova 1992). The isolates were grown at 28°C on the modified yeast extract mannitol agar (Vincent 1970) supplemented with 0.5% succinate (Safronova et al. 2015). One or two taxonomically

different strains were isolated from the same nodule. All isolates are deposited in the Russian Collection of Agricultural Microorganisms (RCAM) and are stored at –80°C in the automated Tube Store (Liconic Instruments).

Identification of isolates by the sequencing of the 16S rRNA gene.

For identification of all isolates, the following PCR primers were used: fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-CTTAAGGAGGTGATCCAGCC-3') for an approximately 1,400-bp segment of the 16S rRNA (*rrs*) gene (Weisburg et al. 1991). PCR was performed in 25-μl reaction mixtures containing 150 μM dNTPs (Promega), 5 pmol of each primer, 1 U of *Taq* polymerase (Helicon) and 50 to 100 ng of purified template DNA. PCR conditions for amplification of the 16S rRNA gene were as follows: initial denaturation at 95°C for 3 min 30 s; 35 cycles of denaturation at 94°C for 1 min 10 s, annealing at 56°C for 40 s, and extension at 72°C for 2 min 10 s; final extension at 72°C for 6 min 10 s. Electrophoresis was carried out with 1% agarose gel (Invitrogen) in Tris-acetate-EDTA. A 100-bp GeneRuler and Lambda DNA/*Hind*III markers (Fermentas) were used for sizing and approximate quantification of DNA fragments. Purification of the PCR products was usually performed by using PureLink Quick kit (Invitrogen) according to manufacturer guidance. The direct sequencing of PCR products was performed by an ABI PRISM 3500xl genetic analyzer (Applied Biosystems). The sequences were compared with related sequences of the type strains available in the GenBank database, using BLAST analysis (basic logical alignment search tool) at the National Center for Biotechnology Information (NCBI). Rrs dendrograms were constructed using the neighbor-joining method in MEGA 6.0 software package (Tamura et al. 2011). The evolutionary distances were computed using the maximum composite likelihood method. Bootstrap analysis with 1,000 replicates was performed to estimate the support of clusters.

Plant nodulation assays.

Seeds of *O. erecta*, *O. kamtschatica*, and *O. pumilio* plants were surface-sterilized by H₂SO₄ for 15 min, were washed with sterile tap water, and were germinated on filter paper in Petri dishes at 25°C in the dark for 4 days. The *O. anadyrensis* species was not used, due to lack of seeds. Germinated seedlings were transferred to 50-ml glass test tubes (two seedlings per test tube) that contained 3 g of sterile vermiculite. In each tube, 6 ml of the nutrient solution [grams per liter: 1.0 K₂HPO₄, 0.25 KH₂PO₄, 1.0 MgSO₄, Ca₃(PO₄)₂ 0.2, 0.02 FeSO₄, 0.005 H₃BO₃, 0.005 (NH₄)₂MoO₄, 0.005 ZnSO₄ · 7 H₂O, 0.002 MnSO₄] was added (Novikova and Safronova 1992). Seedlings were inoculated with individual strains or with a pair of strains isolated from the same nodule in the amount of 10⁶ cells of each strain per test tube. The uninoculated plants were used as negative control. Plants were cultivated for 5 weeks in the growth chamber with 50% relative humidity and four-level illumination/temperatures mode: night (dark, 18°C, 8 h), morning (200 μmol m⁻² s⁻¹, 20°C, 2 h), day (400 μmol m⁻² s⁻¹, 23°C, 12 h), evening (200 μmol m⁻² s⁻¹, 20°C, 2 h). Illumination was performed by L 36W/77 Fluora lamps (Osram). The nodulation assay experiment was performed twice (preliminary and representative tests). The number of replicates in each treatment was two in the preliminary test and five in the representative test. At the end of the experiment, nodules were counted and the fresh biomass of plants (shoots plus roots) was determined. The nitrogen fixation of nodules was measured by the acetylene-reduction method (Turner and Gibson 1980), using a gas chromatograph GC-2014 (Shimadzu). Strains were re-isolated from the obtained nodules and were identified by

16S rDNA sequencing as described above. Pictures of nodules were taken by the stereo microscope Stemi 508 (Carl Zeiss). The data were processed by the standard method of variance analysis using the Statistica version 10 software (StatSoft Inc.). Fisher's least significant difference test was used to evaluate differences between means.

Whole-genome sequencing of the selected isolates.

For whole-genome sequencing, strains *T. robiniae* 581, *M. loti* 582, *M. huakuii* 583, *Phyllobacterium* sp. strain 628, and *R. lusitanum* 629 were selected. The whole genomes of six commercial rhizobia strains deposited in the RCAM collection and used as reference (*R. leguminosarum* RCAM0610, RCAM0626, RCAM1365, and RCAM2802 and *S. meliloti* RCAM1115 and RCAM1750) were obtained previously (*data not published*). Genomic DNA was extracted using a Genomic DNA purification kit (Thermo Fisher Scientific) according to manufacturer recommendations. Long-read whole-genome sequencing was performed using a Minlon sequencer (Oxford Nanopore) of the Core Centrum "Genomic Technologies, Proteomics and Cell Biology" at the All-Russia Research Institute for Agricultural Microbiology. The SQK-LSK109 ligation sequencing kit with the EXP-NBD104 native barcoding expansion 1-12 kit was used to prepare the library according to manufacturer instructions. The reads were base-called and demultiplexed using the guppy_basecaller (v. 3.3.0). The Flye pipeline (v 2.6-release) was used to assemble the Nanopore reads (Kolmogorov et al. 2019). The resulting assembly was corrected four times, using Racon (v1.3.2) (available online) with the following modifiers (-m 8 -x -6 -g -8 -w 500), following with a single polish using the medaka (v 0.10.0) program with default parameters available online. A search for genes in the assembled contigs was performed using the RAST annotation service (Overbeek et al. 2014). All the genomes were assembled into circular replicons. Search for homologs of the symbiotic *fix*, *nif*, *nod*, *noe*, and *nol* genes as well as genes of the T3SS, T4SS, and T6SS secretion systems in annotated genomes was performed using CLC Genomics Workbench 7.5.1 software using local BLASTn and tBLASTx (available online).

Accession numbers.

All sequences have been deposited to the NCBI GenBank database. The whole-genome sequences are under accession numbers CP050292 for *Tardiphaga robiniae* 581, CP050293 to CP050295 for *Mesorhizobium loti* 582, CP050296 to CP050299 for *M. huakuii* 583, CP050300 to CP050303 for *Phyllobacterium* sp. strain 628, CP050304 to CP050308 for *Rhizobium lusitanum* 629, CP050549 to CP050552 for *R. leguminosarum* RCAM0610, CP050553 to CP050557 for *R. leguminosarum* RCAM0626, CP050514 to CP050519 for *R. leguminosarum* RCAM1365, CP050562 to CP050566 for *R. leguminosarum* RCAM2802, CP050558 to CP050561 for *Sinorhizobium meliloti* RCAM1115, and CP050511 to CP050513 for *S. meliloti* RCAM1750. *Rrs* sequences can be found under accession numbers MT192879 to MT225791.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

BLAST software: <https://blast.ncbi.nlm.nih.gov>
Medaka (v 0.10.0): <https://github.com/nanoporetech/medaka>
Racon (v1.3.2): <https://github.com/lbcb-sci/racon>

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