Identification and Diagnostics of Plant-Symbiotic and Phytopathogenic Bacteria

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Abstract: Rapid and reliable diagnostics and identification of pathogenic and symbiotic bacteria are at the top of the agenda. In the first case, they are important to control and prevent crop damages, and thus reduce economic losses. In the second, it's necessary to design and monitor quality of biofertilizer to raise its effectiveness and crop capacity. Development of accurately, rapidly, technically and commercially accessible methods remains a critical problem for the bacteria with comprehensive phylogenetic structure. In this work, we investigated pathogenic *Xanthomonas* and *Ralstonia* and symbiotic *Sinorhizobium*. The aim of this investigation was to examine the applicability of the novel methods for phylogenetic study, identification and diagnostics of closely related species of these genera. The conventional phenotypic and genotypic (16S rRNA, *gyrB*) methods were applied as referents. Novel polymerase chain reaction (PCR)-based approaches, single-adaptor amplified fragment length polymorphism (saAFLP) and comparative analyses of *hin*-region and *Xcc0006-0007* sequences, were first employed for the investigations. Phenotypic tests, 16S rRNA and *gyrB* analysis distinguished bacteria at the genus level, but failed to identify them to the species robustly. The new methods identified bacteria at the inter-species level more precisely. This identification agreed with the accepted genera's classifications. The only exceptions were *X. fuscans* & *X. citri* and *X. perforance* & *X. euvesicatoria* which clustered together. The further outcome of this study was achieved *hin*-region-based genus-specific PCR primers for the express-diagnostics of the genera. Summary, these new methods can be applied for genome-based phylogeny investigations and as convenient and accurate tools for identification and routine laboratory diagnostics of these comprehensive genera.

Key words: *Hin*-region, saAFLP, *Xanthomonas*, *Rhizobium*, *Sinorhizobium*, *Ralstonia*.

1. Introduction

Plan-associated bacteria share a long history with plants and profound impact on the health and agricultural yield of crop [1]. There are two main groups of bacteria based on their adverse/beneficial effects on the host plants: pathogens and mutualists [2]. Investigation of the plant-bacterial interactions and microorganism's taxonomic classification are the most important and developing directions of the present biology [1]. The received knowledge can be used in agricultural and biotechnological sectors: (1) to diagnose most prevalent and most economically important pathogens and prevent plant diseases; (2) to identify and describe most common groups of

beneficial associates and symbiotic microbes for biofertilizer design; (3) to provide surveillance and control for both pathogenic and beneficial bacteria, and thus prevent a bacterial diseases and increase a crop yield.

For a long time, bacterial classification has been based largely on culture and biochemical tests, DNA-DNA hybridization, and 16S rRNA gene sequencing analysis [3]. Nowadays, classification, identification and diagnostics integrate also new techniques to determine both phenotypic and genotypic characteristics [3-7]. The use of multilocus sequence typing (MLST), multilocus sequence analysis (MLSA) and protein-typing are becoming very important for delineation of plant pathogenic and symbiotic bacteria at species level and lower level [8]. Over the last few years, these methods were improved

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dramatically: increasingly mechanized to raise speed, discrimination power and throughput, and to reduce cost of the laboratory analysis [9-16]. However, every method has its limitations and selection of the integrated approaches significantly depends on the target microorganism, time limits, assay accuracy and geographical scale [3, 17]. These methodical disadvantages has become more complicated due to globalization, climate change, increased human mobility and bacterial evolution. Therefore, there is a strong need to develop more rapid, accurate and universal techniques and methods to identify and detect economically important bacteria.

In this work, we studied two kinds of bacteria characterized by the type of nutrition and agricultural importance: phytopathogenic genera *Xanthomonas* and *Ralstonia* and symbiotic *Sinorhizobium*. *Xanthomonas* and *Ralstonia* affect more than 400 species of agricultural crops, causing bacteriosis and thus leading to major economic losses [18, 19]. Their taxonomic structures are intricate and are being constantly revised [20-22]. These genera had been subjected to numerous studies to clarify the taxonomy and phylogeny, although all applied methods were partially sufficient for their identification and diagnostics [21-27]. The disadvantage of the present diagnostics of these genera is testing of the strain at the one taxonomic level and only a certain known group of bacteria. Therefore, the development of new rapid and efficient methods is still crucial for detection and identification of these phytopathogens.

The genus *Sinorhizobium* (*Ensifer*) contains bacteria capable to fixate nitrogen in symbiosis with leguminous plants including agricultural crops, e.g., soybeans [28]. This genus contains closely-related species, and some of them are prospective for soybean biofertilizer production [29]. Identification and characterization of this genus are necessary to reveal and select most efficient symbiotic bacteria, create most productive plant-symbiont combination and thus increase crop capacity of fields. Different phenotypic and genetic

methods were applied before [28]. There is still a need for rapid, accurate, easy and low-cost approaches that can be applied to production for identification (control and monitor) of the industrial strains.

In this work, the new DNA marker *hin*-region and single-adaptor amplified fragment length polymorphism (saAFLP) analysis were first used to identify and describe the closely-related strains of the genera *Xanthomonas*, *Ralstonia* and *Sinorhizobium*. Obtained results were compared with data derived by the traditional pathogenicity and biochemical, comparative analysis of 16S rRNA, *gyrB* and *Xcc0006-0007* nucleotide sequences. The potential of new methods for identification and diagnostics of strains was evaluated for these genera.

2. Materials and Methods

2.1 Bacterial Strains

The original and type strains from weeds and agricultural crops were used in this study. One hundred and fourteen strains of *Xanthomonas* spp. were provided from All-Russian collection of microorganisms, G. K. Skryabin Institute of Biochemistry and Physiology of Microorganisms (VKM IBPM RAS; Pustchino, Russia), and Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine (IMV; Kiev, Ukraine). Twelve *Ralstonia* sp. strains were isolated from wilted tomato and potato in Russia. The known cultures of these genera obtained from the International Collection of Phytopathogenic Bacteria (ICPB; Davis, CA, USA), American Type Culture Collection (ATCC; Manassas, VA, USA) and National Collection of Plant-Pathogenic Bacteria (NCPBP; York, England) were also included for reference. Two referential strains of nitrogen-fixing *Sinorhizobium meliloti* B-117 and B-1009 were received from the collection of VKM IBPM RAS. Nine strains of *Sinorhizobium* spp. were isolated from root nodules of field-grown soybeans in Russia in 2013.

2.2 DNA Isolation

The overall cellular DNA specimens were isolated from strains cultured on agarized yeast extract and tryptone (TY) medium (g/L): 5.0 g/L yeast extract, 10.0 g/L bacto-tryptone, 10.0 g/L NaCl and 20.0 g/L agar. DNA was isolated from cells on days 1-2 of culture by sorption onto magnetic particles (“Silex”, Russia).

2.3 Phenotypic Characterization

The morphological and biochemical characteristics of the pure bacterial cultures were determined based on the general strategy of phenotypic differentiation described in Refs. [30-32].

2.4 Pathogenicity Identification: Plant Nodulation Tests

All strains of genera *Xanthomonas* and *Ralstonia* were tested for their pathogenicity on wide host range using previously described methodology [33]. Four-week-old seedlings (two- to three-leaf-stage) of tomato, cabbage, pepper, geranium and tobacco were inoculated by leaf and steam inoculation methods. The incubated plants were then kept on a greenhouse bench until symptoms development. Each experiment was repeated three times. Results were recorded 7 d and 16 d after inoculation.

2.5 Polymerase Chain Reaction (PCR) Amplification and Sequencing of the 16S rRNA Gene

The PCR analysis and subsequent determination of the 16S rRNA nucleotide sequences were conducted using the universal primers and protocols as described previously [34]. All amplified fragments were separated by an electrophoresis on 1.5% agarose gel. Sequencing was carried out using genetic analyzer 3130xl, “Applied Biosystems” (ABI) automated sequencing machine (USA).

2.6 PCR Amplification and Sequencing of the *gyrB* Gene

Nearly complete DNA sequences of the *gyrB* gene

were amplified and sequenced using previously constructed primers UP1/UP2r and protocols [35].

2.7 PCR Amplification and Sequencing of the *Xcc0006-0007* Region

Nucleotide sequences of the *Xcc0006-0007* region of *Xanthomonas* spp. were amplified and sequenced according previously published protocol [36].

2.8 saAFLP Analysis

We modified amplified fragment length polymorphism (AFLP) method developed before by Vos et al. [37] and named new modification as saAFLP [38, 39]. The phylogenetic relationships between closely related strains *Bacillus cereus* group and *Rhizobium leguminosarum* had been successfully analyzed using this method [38, 39]. The procedure of saAFLP comprises three steps: (1) treatment of the extracted bacterial DNA by one of the genus specific restriction endonucleases and ligation with the single stranded adapter; (2) PCR amplification with the single primer which is complementary to the adapter sequence; (3) electrophoretic separation of the PCR products in agarose gel. The fundamentally new aspects of this methodological procedure include: (1) performing restriction analysis and ligase reaction in the same tube concurrently; (2) selecting restriction endonucleases by using computational analysis (“ReVer”, Remnev and Punina, 2014, unpublished) so that we can obtain 10-30 DNA fragments after the treatment; (3) using the one single-stranded adapter.

2.9 PCR Amplification and Sequencing of *Hin*-Region

Hin-region amplification was carried out using primers designed specifically for the genera *Sinorhizobium*, *Ralstonia* and *Xanthomonas*. The primers and PCR amplification protocols were patented for the genera *Rhizobium* and *Xanthomonas* [40].

2.10 Analysis of the Nucleotide Sequences

The primary comparative analysis of the DNA

sequences determined in this study and represented in the Gen Bank database was carried out using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (NCBI BLAST) [41]. Sequence alignment was performed using the Clustal W 1.75 v software [42]. Based on nucleotide data, the phylogenetic trees were concatenated and constructed with the neighbor joining (NJ) [43] and minimum evolution (ME) [44] methods of MEGA 3.1 software [45].

3. Results and Discussion

3.1 Genus *Xanthomonas*

All bacterial cultures with different biochemical characteristics were tested on the corresponded test plants and produced either systemic or leaf spot lesions under glasshouse conditions. According to the 16S rRNA analysis, all strains were reliably (> 97% similarity with type strain ATCC 33913T) identified as *Xanthomonas* spp.. After confirmation that investigated strains were affiliated to the same genus, DNA sequences of the *gyrB* gene [35] were sequenced (2,395 bp, 97.96% of whole its length) for all of them. Through cluster analysis (Fig. 1a), eight clades were branched with high bootstrap values (> 70%). Seven clades robustly comprised seven species (*X. campestris*, *X. gardneri*, *X. translucens*, *X. oryzae*, *X. arboricola*, *X. vesicatoria* and *X. albilineans*). Seven strains were poorly discriminated. They formed the one clade together with other *X. euvesicatoria*, *X. perforance*, *X. citri*, *X. fuscans* and *X. axonopodis* reference strains, and probably were just synonyms. These data were supported by recent genomic comparative analysis [26] and MLST analysis [21]. For example, it had been hypothesized that *X. citri* and *X. fuscans* could belong to a single species [26]. Comparison of the *gyrB* DNA sequences revealed high variability for intra- (similarity values were greater than 79.3%) and inter-species (> 97.5%) levels for *Xanthomonas* spp..

Despite the fact that this analysis is considered as a

convenient and accurate tool to discriminate the majority of established species in the genus *Xanthomonas* [46], four close-related species were not supported with high branching level.

As it was shown before, comparative analysis of the DNA sequences of the *Xcc0006-0007* operon (length 1,971 bp, 86.30% per whole length) is sufficient to

Fig. 1 NJ tree of the nucleotide sequences for the *gyrB* gene (a) and *hin*-region (b), based on 115 strains of *Xanthomonas*.

The scale corresponds to the number of substitutions per 100 base pairs (genetic distances). The numerals show statistical reliability of the branching order (%) defined by bootstrap analysis (1,000 replicas). Bootstrap values less 70% are not shown. The numbers in parentheses shows the amount of the investigated strains.

Fig. 2 Schematic structures of the *hin*-regions of *X. campestris*, genotype *hin* I corresponding to the type strain ATCC 33913T.

distinguish close-related *Xanthomonas* spp. strains below species level [36]. Based on the nucleotide polymorphism and pathovar-specific deletions in intergenic region (IR) (Fig. 2), robust species and inter-specific identification had been provided for *X. campestris* pv. *campestris* (referent strain, the length of the IR—140 bp), *X. campestris* pv. *raphani* (2 bp deletion, type I), *X. vesicatoria* (25 bp deletion, type IIa), *X. gardneri* (27 bp deletion, type IIb), *X. oryzae* pv. *oryzae* (35 bp, type IIIa), *X. oryzae* pv. *oryzicola* (36 bp, type IIIb), and *X. arboricola* (7 bp, type IV). The cluster analysis of the DNA sequences (data not shown) had revealed six major groups, authentically. Five of them corresponded to the previously revealed species. As it was shown by 16S rRNA, *gyrB* and whole-genome sequence analysis, *X. axonopodis* (9 bp deletion, type V), *X. citri* (type V) and *X. fuscans* (V) formed the single clade based on the 100% homologous IR sequences. *X. albilineans* and *X. translucens* did not contain *Xcc0006* gene and IR. The results obtained by saAFLP correlated with the previously defined topology. We robustly revealed eight species (as for *gyrB*), three subgroups for *X. campestris*, according to the pathovars pv. *campestris* and pv. *raphani*; two subgroups for *X. oryzae* (pv. *oryzae*, pv. *oryzicola*); two subgroups for *X. arboricola*.

Then, we sequenced and provided cluster analysis for the *hin*-region that, possibly, played the role into host-plant specificity and virulence capacity [39] (Fig. 1b). The schematic structure of the *hin*-region is shown in Fig. 2.

At the intra-species level, *hin*-region (R1) sequences were unique, but at the inter-species level, the sequences identities were 30%-98%. On average, six substitutions were found for the strains belonging to the same pathovar. Based on the *hin*-region structure, all strains were reliably delineated into eight distinct *hin* genotypes (I-VII). These groups correlated with the species and pathovar origins, in other words, reflected the practical taxonomic classification. The

data exposed *hin*-region as a potential marker for the diagnostics of comprehensive genus *Xanthomonas* at the inter-species level.

3.2 Genus *Ralstonia*

After inoculation of tomato and geranium test plants and analysis of complete DNA sequences of the 16S rRNA gene (Gene Bank numbers are KJ606332-KJ606343), it had been shown that all studied strains belonged to the *R. solanacearum* (*Rs*) species complex (> 99.98% similarity with type strain *Rs* K60), race 3, biovar 2 (R3B2). Through the analysis of the *gyrB* gene, we authentically identified all strains as *Rs*, phylotype IIB [22]. They were grouped together with strains isolated from temperate region all over the world [47-49]. The *gyrB* nucleotide sequences were 100% identical to the DNA sequences of the strain UW551, introduced long time ago to the cold regions of USA and Europe from Kenya [49]. The saAFLP analysis of the strains revealed the same pattern with UW551 and was specific for phylotype IIB. The saAFLP pattern was comprised of 32 DNA fragments. To design specific primers for PCR amplification and sequencing of the phylotype II *hin*-regions, we used whole-genome sequences of *Ralstonia* sp. (Table 1) and related bacteria *Cupriavidus* spp. retrieved from Gene Bank. The primers (5'-CTTCGCAGCGTAGACGGAAAC-3' (*Rs*F79) and 5'-CTTTGCAATGCGCTTCTGATG-3' (*Rs*R715)) were tested for the specificity on the wide-range sampling of *Ralstonia* spp. strains. The 566 bp PCR product (R1 located between the 1st and 2nd tRNA (Glu) genes) was revealed only for the strains of the phylotype II, R3B2. The determined *hin*-region sequences were compared to the corresponding sequences of the other phylotypes retrieved from Gene Bank. The amount of specific substitutions for phylotype I was three, for phylotype IIA-6, IIB-13, IV-4. The average sequence variability (15.8%, including indels) exceeded the 16s rRNA (3.7%) and *gyrB* (8.2%) ones. These values could be

understated partially due to inclusion of relatively small sample size. Using saAFLP and *hin*-region, better separation of closely related *Rs* strains was obtained

compared to *mutS* gene [22, 50], indicating the potential of these two approaches for diagnostics of four phylotypes (Table 2).

Table 1 Presence of tRNA (Glu) copies and structure of *hin*-region in the whole sequenced genomes of the strains of genera *Ralstonia* and *Sinorhizobium*.

Strain	CTC*	TTC*	tRNA structure	Begin**	End**	Role
<i>S. fredii</i> NGS234	1	3	(Glu) 4	1451200	1451275	s
				1451466	1451540	
				1455542	1455616	
				1455835	1455909	
<i>S. medicae</i> WSM419	1	3	(Glu) 4	1531855	1531930	s
				1532147	1532222	
				1532279	1532353	
				1532539	1532614	
<i>S. meliloti</i> 1021	1	3	(Glu) 4	1862108	1862183	s
				1862401	1862476	
				1862534	1862608	
				1862790	1862865	
<i>R. eutropha</i> JMP134	0	2	(Ala-Glu-Asp) 2	2339565	2339640	n/p
				2340159	2340234	
<i>R. solanacearum</i> UW551	0	2	(Ala-Glu-Asp) 2	5012672	5012747	p/p
				5013162	5013237	
<i>R. solanacearum</i> GMI1000	0	2	(Ala-Glu-Asp) 2	1241767	1241842	p/p
				1242236	1242311	
<i>R. solanacearum</i> CFBR2957	0	2	(Ala-Glu-Asp) 2	2236101	2236026	p/p
				2236591	2236516	
<i>R. solanacearum</i> PCI07	0	2	(Ala-Glu-Asp) 2	2308786	2308711	p/p
				2309250	2309175	
<i>R. syzygii</i> R24	0	2	(Glu) 2	343062	343137	p/p
				343526	343601	

*Anticodon; **tRNA (Glu) positions; s—symbiont; p/p—plant pathogen; n/p—non pathogen.

Table 2 The resolutions of the methods to study the biodiversity of the genera *Sinorhizobium*, *Ralstonia* and *Xanthomonas*.

Methods	Genus	Species	Phylotype/Pathovar	Strain/Sequevar
Phenotypic methods				
Biochemical analysis	+	+/-	-	+/-
Inoculation/nodulation	+	+/-	+/-	-
Genotypic methods				
16S rRNA	+	+/-	-	-
16S-23S rRNA	+	+	-	-
<i>gyrB</i>	+	+	-	-
<i>Xcc0006-0007</i> *	+	+	+	-
<i>mutS</i> **	+	+/-	N/A	-
<i>nodD</i> ***	+	+/-	+/-	-
<i>egl</i> **	+	+	N/A	+/-
saAFLP	-	+	+	+/-
<i>hin</i> -region PCR	+	+	+	-

*only for genera *Xanthomonas*; **only for *R. solanacearum*; ***only for genus *Sinorhizobium*; N/A—not applicable.

3.3 Genus *Sinorhizobium*/*Ensifer* Group

Nine nitrogen-fixing fast-growing strains *Sinorhizobium* spp., prospective for commercialization as a microbial seed inoculant to soybeans seeds prior to planting, were studied in this work. These strains were isolated from nodules of the different cultivars of soybeans (“Ustya”, “Svetlaya”, *Glycine soja*) in Russia in 2013. On the basis of the 16S rRNA sequences, biochemical characteristics and host specificity, nine investigated strains were attributed to the genus *Ensifer*. Based on the 16S rRNA sequence analysis, all strains were clustered together with *S. americanum*, *S. fredii* and *S. xinjiangense*, the last is considered as heterotypic synonym of *S. fredii* [51]. Thus, 16S rRNA gene sequence analysis lacked resolving power at species (or genomovars) level and below (genus variability < 0.5%) [52]. The *gyrB* nucleotide sequences of the studied strains were 97%-100% identical to the type strain *S. fredii* ATCC 35423T. The “Ustya” strains (U1, U2 and U3) were 100% identical to the ATCC 35423T. The strains isolated from *G. soja* (Gs1, Gs2 and Gs3) formed separate branch and were closely related to the broad-host-range strains USDA257 (enter symbiosis with wild soybean cultivars and some agronomical soybean cultivars) and NGR234 (unable to enter symbiosis with any soybean). The strains from “Svetlaya” (S1, S2, S3) formed a tight cluster with HH103 (forms nodules on agronomically advanced soybean cultivars) [53]. The sequences were highly similar between all *Ensifer* species, and their variability for the genus saAFLP analysis, ranging from 0% to 2.1%, revealed three types of saAFLP patterns correlated with host plant of studied strains. Based on the conservative regions of the tRNA (Glu) genes, the specific primers for the genus were developed. Nearly 300 bp PCR fragments (R1) were sequenced and compared to the corresponding sequences from Gene Bank. The R1 sequences were unique for the *S. fredii* group and did not reveal any

homology with whole-genome sequences of other species or genera. The topology of NJ tree was similar to the data obtained before [54, 55]. The strains U1-3 and S1-3 were robustly clustered together with the strains HH103 (97%-99% identity) and ATCC 35423T, which are able to nodulate soybeans. These data were supported by inoculation test, which showed the occurrence of wide-host range for these strains. Beside *G. soja* and *G. max*, they were able to effectively nodulate *Phaseolus vulgaris* and *Vigna* sp. The strains Gs1-3 formed separate clusters closely related to USDA257. The sequence variability of the *hin*-regions for species *S. fredii* was ranging from 0% to 10.5%, which was reflected in the high separability values.

4. Conclusions

Summarizing all data below, we concluded that only integrated approach should be applied for identification and diagnostics of genera with intricate taxonomic structure. This can be based on the combination of phenotypic and genotypic tools. Genotypic approach should include the analysis of more than one phylogenetic marker gene, or methods with different resolution. Thus, we suggested: (1) 16S rRNA or *gyrB* for analysis of bacteria at the genera-species level; (2) *hin*-region and saAFLP for determination of the inter-species variability. Furthermore, *hin*-region PCR may be applied singly as a rapid, efficient and low-cost method for direct monitoring and diagnostics of the species in the investigated genera.

Acknowledgments

This work was supported by the programme “support of young scientists” by Presidium Russian Academy of Science. The authors are grateful to Vasily Zotov and Sofia Khapchaeva for their technical assistance.

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