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A microarray for screening the variability of 16S–23S rRNA internal transcribed spacer in *Pseudomonas syringae*

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ABSTRACT

The 16S–23S ribosomal internal transcribed spacer (ITS1) is often used as a subspecies or strain-specific molecular marker for various kinds of bacteria. However, the presence of different copies of ITS1 within a single genome has been reported. Such mosaicism may influence correct typing of many bacteria and therefore knowledge about exact configuration of this region in a particular genome is essential. In order to screen the variability of ITS1 among and within *Pseudomonas syringae* genomes, an oligonucleotide microarray targeting different configurations of ITS1 was developed. The microarray revealed seven distinct variants in 13 pathovars tested and detected mosaicism within the genomes of *P. syringae* pv. coronafaciens, pisi, syringae and tabaci. In addition, the findings presented here challenge the using of rRNA analysis for pathovar and strain determination.

1. Introduction

Pseudomonas syringae is a common Gram-negative bacterium that can be found both as a harmless commensal on leaf surfaces or as an important plant pathogen that causes a range of blight, canker, speck and spot diseases in many important agricultural crops (Hirano and Upper, 2000). Approximately 50 different pathovars of this species can be distinguished today (Kůdela et al., 2002) according to their pathogenicity on plants and phenotypic characteristics. To determine the divergence existing at the molecular level, two methodological approaches are routinely employed today: analysis of repetitive elements based on rep-PCR technique (e.g. Peters et al., 2004; Vicente and Roberts, 2007; Gilbert et al., 2009; for a general review see Ishii and Sadowsky, 2009) and analysis of the rRNA operon (*rrn*).

The *rrn* is composed of three rRNA genes and two internal transcribed spacers (ITS) in the order 16S rRNA–ITS1–23S rRNA–ITS2–5S rRNA. At the subspecies level, only the ITS1 region has been reported as a suitable strain and/or pathovar marker (Manceau and Horvais, 1997; Sawada et al., 1997; Peters et al., 2004; Oguiza et al., 2004; Kong et al., 2005; Olczak-Woltman et al., 2007). This region consists of species-conserved domains containing two tRNA genes and hypervariable DNA segments that could allow a strain's characterization (Garcia-Martinez et al., 2002).

There are nevertheless five *rrn* operons within the *P. syringae* genome, and strains possessing different variants of the ITS1 region within the given genome have been described (Milyutina et al., 2004; Stewart and Cavanaugh, 2007). This intragenomic heterogeneity (mosaicism) is brought about by different combinations of the entire hypervariable segments of ITS1, possibly resulting from recombination and horizontal transfer (Milyutina et al., 2004). Similar mosaicism has been found also within other bacteria genomes (Stewart and Cavanaugh, 2007). This may significantly skew the diversity estimates and phylogenetic relationships based on *rrn* analysis.

Although the described variability can be revealed by conventionally used DNA-fingerprinting methods, these have some constraints when probing different versions of hypervariable segments (e.g. the need for several separate enzymes and reactions, sensitivity to point mutations). A competent method is to sequence all the ITS1 copies per genome, but this is too laborious for routine analysis. Therefore, a method that allows simple screening of inter- and intragenomic variability in parallel would be helpful.

DNA microarrays were initially designed for gene expression studies and single nucleotide polymorphism profiling, but since 2000 these have been widely used also for detecting plant pathogens, including viruses (reviewed in Boonham et al., 2008), bacteria (reviewed in Huyghe et al., 2009) or phytoplasmas (Nicolaisen and Bertaccini, 2007). The principle of a microarray is to selectively hybridize the sample sequences labeled fluorescently (targets) to the specific capture probes printed onto a solid surface. The main advantage of the method lies in its ability to detect many different sequences in parallel.

The aims of the study were to a) develop an oligonucleotide microarray for screening the variability of the ITS1 region among *P. syringae* strains, b) test the ability of the microarray to detect mosaicism present within some genomes, and c) assess the method's potential for pathovar differentiation.

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2. Materials and methods

2.1. Bacterial strains and DNA isolation

Samples under study were well characterized strains obtained from several type collections (Table 1). Bacterial cultures were grown overnight on King's B (KB) medium (King et al., 1954) at 25 °C. Three passages in combination with standard streaking method were used to obtain the cell lineage of a single bacterial genome only. Genomic DNA was isolated using Wizard SV Genomic DNA Purification System (Promega, USA) while following the manufacturer's original protocol. Genetic diversity among pathovars and genome homogeneity during the cell subculturing process were proven using the rep-PCR method (Louws et al., 1994) with the following modifications: annealing time was increased to 2 min and the extension step was shortened from 8 to 5 min.

2.2. Target preparation, cloning and sequencing

Purified DNA (1 µl) was amplified using PPP-MasterMix (TopBio, Czech Republic) according to the manufacturer's protocol; non-labeled forward and Cy5-labeled reverse primers (Table 2) were used in the reaction. The PCR conditions were as follow: 95 °C for 5 min, 35 times (94 °C for 1 min, 60 °C for 20 s, 72 °C for 1 min), 72 °C for 10 min and 4 °C for forever. Targets were then purified using GenElute PCR CleanUp Kit (Sigma-Aldrich, USA) into 70 µl of final volume. Targets showing mosaicism under the hybridization experiments were 1000-times diluted, PCR reamplified with non-labeled primers (same conditions as above), and cloned using CloneJET PCR Cloning Kit (Fermentas, Lithuania) and NEB 10-beta competent *E. coli* cells (New England BioLabs, USA) according to the protocols supplied. Colonies were added directly to the PCR mix and targets were prepared as described above. Two microlitres of targets were sequenced (BigDye Terminator Kit, v.3.1, Applied Biosystems, USA) with corresponding non-labeled primers (Table 2). The alignment of the sequences obtained and comparison of targets to all probes was done in ClustalW (Thompson et al., 1994).

Table 1
Isolates in the study with configurations observed. BCCM/LMG = Belgian Co-ordinated Collections of Microorganisms, Ghent University, Belgium; RICP = Research Institute of Crop Production (since 2007 renamed to Crop Research Institute), Prague, Czech Republic; NCPPB = National Collection of Plant Pathogenic Bacteria, Harpenden, UK; CCM = Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic. If multiple accession numbers are given for one strain, their order corresponds to the order of ITS1 versions found.

Collection number	Pathovar	Host	Locality	Version of ITS1 found	Accession number
BCCM/LMG 5060	Coronafaciens	Oat	UK	a + e	HM032069, HM032070
RICP 7001	Glycinea	Soybean	Czech Republic	e	HM032071
BCCM/LMG 5066	Glycinea	Soybean	New Zealand	e	HM032072
BCCM/LMG 5067	Helianthi	Sunflower	Mexico	f	HM032073
BCCM/LMG 5070	Lachrymans	Cucumber	USA	d	HM032074
BCCM/LMG 2245	Phaseolicola	Bean	Canada	a	HM032075
BCCM/LMG 5079	Pisi	Pea	New Zealand	a	HM032076
NCPPB 3496	Pisi	Pea	USA	a + e	HM032077, HM032078
BCCM/LMG 2330	Striafaciens	Oat	Unknown (Elliot)	a	HM032079
CCM 4073	Syringae	Cummin	Czech Republic	b + e	HM032081, HM032082
BCCM/LMG 1247	Syringae	Lilac	UK	a + e + g	HM032080, HM032083, HM032084
BCCM/LMG 5393	Tabaci	Tobacco	Hungary	a + e	HM032085, HM032086
BCCM/LMG 5090	Tagetis	Marigold	Zimbabwe	f	HM032087
BCCM/LMG 5092	Theae	Tea plant	Japan	c	HM032088
RICP 9921	Tomato	Tomato	Czech Republic	d	HM032089
RICP 9932	Tomato	Tomato	Czech Republic	d	HM032090
RICP 100	Tomato	Tomato	Czech Republic	d	HM032091
RICP 101	Tomato	Tomato	Czech Republic	d	HM032092
BCCM/LMG 5093	Tomato	Tomato	Guernsey - UK	d	HM032093
BCCM/LMG 2349	Ulmi	Elm tree	Yugoslavia	e	HM032094

2.3. Probes design and microarray fabrication

The ITS1 sequences of *P. syringae* were retrieved from GenBank, aligned using the POA alignment programme (Lee et al., 2002), and then the alignment was justified manually. Required probe-to-target similarity was >= 75% homology and >= 15 nt of identity, and each probe designed was allowed to fulfill these criteria for targeted sequences only. The lengths of the probes were 35 to 40 nt, and the shorter specific probes were extended by random sequences on both ends (Table 2). The similarity between the probes themselves was checked by ClustalW in an all-against-all comparison. The probes were synthesized by Sigma-Aldrich and spotted (Lambda, Austria) onto aldehyde-coated plastic slides (HTA™ Slide12, Greiner Bio-One, Germany).

2.4. Hybridization, scanning and data analysis

The 15 µl of each target were mixed with 15 µl of preheated (42 °C) hybridization buffer (500 µl 100% formamide, 500 µl 20 × SSC, 20 µl 10% SDS) and denatured at 95 °C for 3 min. After cooling to room temperature, targets were applied onto the microarray slide and hybridized at 42 °C for 45 min. The arrays were washed with pre-warmed (42 °C) buffers: 2× SSC and 0.1% SDS for 1 min, 1× SSC for 1 min and 0.1 × SSC for 5 min. Air-dried slides were scanned using a Typhoon 9410 scanner and processed with ImageQuant TL v2003.02 software (Amersham Bioscience, USA). Signal-to-noise ratio (SNR) values were computed as the background subtracted signal of the particular spot divided by the standard deviation of the background. If a SNR >= 3 was obtained for both spots in the double-dot, the signal was considered to be positive.

3. Results

3.1. Probes and microarray design

Alignment of 78 sequences of *P. syringae* revealed three variable blocks of ITS1, denoted here as A, B and C (Fig. 1). Outside of these blocks, the alignment was highly conserved except for sequence AF209771, which differed from the consensus sequence onwards from block B. A BLAST-search found significant similarity only to *Xanthomonas* species, and this part of the sequence was therefore excluded from the alignment. Three different versions of each hypervariable block were found, and each was represented by a group of sequences with more than 70% nucleotide similarity. Nine probes were designed in order to have only one probe targeting each of the block versions found (Fig. 1, Table 2). The similarity between C2 and C3 versions did not allow the design of a unique probe, and 16 of 29 sequences targeted by probe C3 showed 75% homology also to probe C2. Similarity between probes did not exceed 55% homology in all-to-all comparison and maximal length of identity