

Genotyping pathogenic strains of genus *Xanthomonas* causing bacterioses in a number of plants by DDSL technique

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Abstract

In the genus *Xanthomonas*, specialists consider a significant number of species and varieties (pathovars) of phytopathogenic bacteria that infect many agricultural and ornamental plants (about 400 species), which leads to serious economic losses. For the timely detection of these pathogens, accurate diagnosis is necessary, allowing correct and prompt identification. Molecular genetic methods are able to identify populations of *Xanthomonas* strains with a fairly complete characterization of their hereditary material. The proposed method of genotyping — double digest and selective label (DDSL) — is based on the use of two restriction endonucleases for the separation of bacterial genomic DNA. The DNA polymerase (*Taq*) present in the reaction mixture along with biotinylated deoxycytosine triphosphate (Bio-dCTP) allows for the visualization of DNA fragments. The tag only labels DNA fragments that have 3'-recessed ends formed by the first enzyme (*BcuI*). The second restriction endonuclease (*Eco147I*) produces blunt ends that are unable to incorporate the label. As a result, in the DDSL reaction, 20–50 clearly distinguishable DNA fragments are visualized on the filter. The number and distribution of fragments are characteristic for each bacterial strain of the genus *Xanthomonas*. Genotyping these microorganisms makes it possible to identify the specific profile of each strain, i.e., assign it a sort of “bar code” for individual specification. The strains of bacteria of the genus *Xanthomonas*, obtained from different species (tomato, radish, sorghum) are genetically separated from each other, showing a specific pattern in terms of the distribution of DNA fragments, despite the common geographical origin. A comparatively rare case of the identity of strains, despite their geographical and temporal unrelatedness and different cultures, has been recorded.

Keywords: *Xanthomonas*, strain, genotyping, double digest and selective label (DDSL), restriction endonucleases.

Introduction

The genus *Xanthomonas* includes about 40 species and varieties (pathovars) of phytopathogenic bacteria affecting a significant number of agricultural and ornamental plants (more than 400 species), which leads to serious economic losses. Some of its species are characterized by the ability to infect a significant number of plants, others are divided into multiple pathovars, the designation of which is associated with confinement to a particular host. Among them, causative agents of vascular bacteriosis of cabbage (*X. campestris* pv. *campestris*), black bacterial blotch of tomato (pv. *vesicatoria*), leaf streaks and black bacteriosis of cereal seeds (pv. *translucens*), begonia (pv. *begoniae*), yellow hyacinth disease (pv. *hyacinthi*) and others are most known and they are characterized by high severity. So, at

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present, the bacterium *X. campestris* pv. *vesicatoria* is common in more than 70 countries; tomato fruits affected by this pathogen are unsuitable for human consumption, and the proportion of diseased plants in the field can reach 100%. Phytopathogens of the genus *Xanthomonas* cause vascular necrotization and spotting on many terrestrial parts of the plants. The bacteria are short, gram-negative rods with rounded ends, $1.05\text{--}2.4 \times 0.45\text{--}0.9 \mu\text{m}$ in size, mobile (with 1–2 polar flagella), aerobic, oxidative-negative, catalase-positive, and possess amylolytic and pectolytic properties. When developing on nutrient media, colonies of mucous consistency are formed, mainly with yellow pigment (Ignatov et al., 2007; Mansfield et al., 2012).

Currently, genotyping plays an important role not only for identifying interspecific features of pathogens of the genus *Xanthomonas*, but also in differentiating the genetic structure of their individual pathogenic strains at the DNA level (Barak and Gilbertson, 2003; Xiu et al., 2006; Bui et al., 2009; Nabhan et al., 2012; Pritchard et al., 2012). Previous research showed some limitations of individual methods of this testing in recognizing the genetic structure of a population of certain *Xanthomonas* species (Barak and Gilbertson, 2003). They found that amplification of DNA repeats (rep-PCR) and sequencing of the internal transcribed spacer (ITS1) of ribosomal RNA genes does not allow for the specification of genetic heterogeneity in the analyzed phytopathogenic strains. The differences were established using genotyping based on restriction fragment length polymorphism with a labeled probe and analysis of the plasmid profiles of the bacterium. Studies by Spanish authors (Lopez et al., 2006), who managed to divide the groups of phytopathogens into only two clusters, indicate a low level of discrimination between bacterial strains using the methods of rep-PCR and RAPD, although the strains had a significant geographical distance and it was possible to assume more significant genetic differences between them. The results (Bui T.N. et al., 2009) show that despite the presence of a large number of methods for the testing of bacteria there is still the need to develop new methods for specific pathogens (in particular, members of the genus *Xanthomonas*). These authors mention the unequal discriminatory ability of restriction fragment amplification methods (AFLP), tandem repeat variability (MLVA) and PCR-genotyping. Testing microorganisms using the MLVA method is widely used to detect certain differences between strains in bacterial populations. Some researchers show low discrimination between epidemiologically unrelated *Xanthomonas* strains, as determined by AFLP and PCR with random primers (RAPD); the similarity rate in pairwise comparisons of genetic profiles reached 66% and 80%, respectively, indicating low resolution (Ogunjobi et al., 2010). Other authors point out the advantages of MLVA — genotyping in comparison with methods based on sequencing of indi-

vidual DNA segments (for example, multilocus sequencing typing — MLST) (Pruvost et al., 2011, 2014).

Currently, pulse-field gel electrophoresis (PFGE), based on the digestion of the native genomic DNA of a bacterium by restriction endonucleases with a small number of recognition sites (20–30), is considered to be a highly sensitive method for determining genetic features in the DNA structure (the appearance and disappearance of restriction sites, duplication and separation of DNA segments, movement of mobile elements, etc.). It is referred to as a classic in the genotyping of clinically significant isolates of pathogenic microorganisms (for example, *Salmonella* spp., *Pseudomonas aeruginosa*, etc.) (Pappa et al., 2013; Ziebell et al., 2017). Pruvost et al. (2014) studied 100 strains of the genus *Xanthomonas* (isolated in 1985–2012 from different geographical areas of Bulgaria). Despite the strains' geographical separation, researchers identified only two haplotypes of *X. vesicatoria* and one of *X. gardneri*. In a recent paper, researchers used PFGE with *Xba*I and *Spe*I restriction endonucleases to identify the heterogeneity of *Xanthomonas* strains (Kizheva et al., 2018). According to Jacques et al. (2016), at present, the genetic and molecular basis for the practical determination of the host range in the genus *Xanthomonas* has not been sufficiently studied.

The suggested method of genotyping — double digest and selective labeling (DDSL) — of clinical isolates of pathogenic microorganisms *P. aeruginosa* was not inferior in its discriminatory power, and in some cases even slightly exceeded the PFGE method (Terletskiy et al., 2008). It was also tested for the rapid and accurate identification of collection strains of *Bacillus subtilis*, which were similar in a number of biological features, but differed in composition of metabolite complexes (Terletskiy et al., 2016).

The purpose of this work was to evaluate the DDSL method for representatives of the genus *Xanthomonas*, isolated from infected samples of several cultivars obtained from geographically distant regions.

Materials and Methods

The objects of research were strains of phytopathogenic bacteria of the genus *Xanthomonas*, isolated from infected samples of several crops obtained from geographically remote regions (isolated by us, as well as from other institution collections (Table 1). Eleven strains of *Xanthomonas* were used: from cabbage (strains 1–7), tomato (strain 8), radish (strain 9), sorghum (strain 10), and begonia (strain 11). We maintained the collection of phytopathogenic bacteria strains in 2% potato agar in glass tubes by reseeded bacteria every 3–4 months in order to keep their biological properties constant. The studied strains were grown in test tubes with meat-peptone broth for 2 days (25 °C).

Table 1. Characterization of strains of bacteria of the genus *Xanthomonas*, presented for genotyping by the DDSL method

N	Bacterial strain of <i>Xanthomonas campestris</i> (X. c.)	Plant, variety	Place and year of isolation and separation, country, institution
1	<i>X. c. pv. campestris</i> P 1	Cabbage	2006, Belarus, BELNIIZR
2	<i>X. c. pv. campestris</i> 8169	--	1984, Ukraine, IMV
3	<i>X. c. pv. campestris</i> 1305	--	2006, Moscow region, VNIIF
4	<i>X. c. pv. campestris</i> 1306	--	2006, Moscow region, VNIIF
5	<i>X. c. pv. campestris</i> Cab 4	--, Moscow late	1984, Leningrad region, VIZR
6	<i>X. c. pv. campestris</i> Cab 7	--, Moscow late	1984, Leningrad region, VIZR
7	<i>X. c. pv. campestris</i> 7604	--	1984, Ukraine, IMV
8	<i>X. c. pv. vesicatoria</i> 7767	Tomato	1984, Ukraine, IMV
9	<i>X. c. pv. armoraciae</i> 373	Horse radish	1960, UK
10	<i>X. c. pv. holcicola</i> 8954	Sorghum	1984, Ukraine, IMV
11	<i>X. c. spp.</i> 3	Begonia	2018, Leningrad region, VIZR

The first step in genotyping is DNA isolation, which was performed using standard phenol/chloroform protocol. DNA was precipitated by isopropanol, washed in 70° ethanol, and dissolved in water. The DDSL method of genotyping is based on the use of two restriction endonucleases for digestion of the bacterial genomic DNA: the first enzyme cuts DNA, forming 3'-recessed fragment ends, and the second one forming 3'-protruding or blunt ends. Present in the reaction mixture, *Taq* DNA polymerase provides for labeling DNA fragments with biotinylated deoxycytosine triphosphate (Bio-dCTP). The label is incorporated in a fill-in reaction only in DNA fragments that have 3'-recessed ends formed by the first rare cutting enzyme. The second frequently cutting restriction endonuclease generates blunt or 3'-protruding ends of the fragments that are not capable of tagging. This enzyme makes DNA fragments shorter to be suitable for separation in a standard agarose gel. As a result, 20–50 clearly distinguishable DNA fragments, the number and distribution of which is characteristic for each bacterial strain, are visualized on the filter. An *in silico* search (<http://insilico.ehu.eus/digest/>) allowed us to propose a combination of genotyping enzymes. As the first enzyme, the restriction endonuclease *BcuI*, which has several dozen cleavage sites in the genomes of the representatives of the genus *Xanthomonas* and forms 3'-recessed fragment ends, is the most promising and was used in the first stage of the development of the technique. The second enzyme that digests the DNA of *Xanthomonas* in more than 1000 sites was the restriction endonuclease *Eco147I*. The latter generated ends of DNA fragments that are not able to be labeled with biotin. An important criterion for the selection of enzymes is their compatibility in one buffer and appropri-

ate number of cleavage sites as mentioned above. In particular, in buffer B (Thermo Fischer Scientific), *BcuI* is active at 50–100%; the activity of the *Eco147I* enzyme in this manufacturer-recommended buffer is 100%. Other enzyme combinations can also be used but in our hands the best data are generated with *BcuI/Eco147I* combination. *In silico* simulation of number of cleavage sites in all sequenced bacterial genomes is available on the website <http://insilico.ehu.es/digest/>.

Technically, the DDSL reaction is performed in a single microtube containing 15 µl H₂O, 2 µl buffer B (Thermo Fischer Scientific), 2 µl bacterial DNA, and 1 µl enzymatic mix. The latter includes 0.5 µl of *BcuI*, and *Eco147I* each, 1 µl of *Taq* polymerase (diluted by water in proportion 1:10), and 0.5 µl of Bio-dCTP per one reaction. Normally, the number of reaction microtubes is more than one, and the amount of preformulated mix is calculated depending on the number of bacterial samples in a given experiment. The reaction is complete within 1 hour at 37 °C. Gel electrophoresis is performed in 0.8% agarose gel at 60V for 16 hours (alternatively, 3 hours is enough at 150V), transfer to nylon filter is carried out in distilled water for 30 minutes immediately after electrophoresis. Detection of biotin-tagged fragments on the filter is based on streptavidin-alkaline phosphatase conjugate chemistry using NBT and BCIP color forming substrates (Thermo Fischer Scientific).

Results

After theoretical selection (*in silico*) of enzymes, experiments were carried out with several strains of the genus *Xanthomonas*. It was found that the selected enzymes form the optimal number of labeled DNA fragments

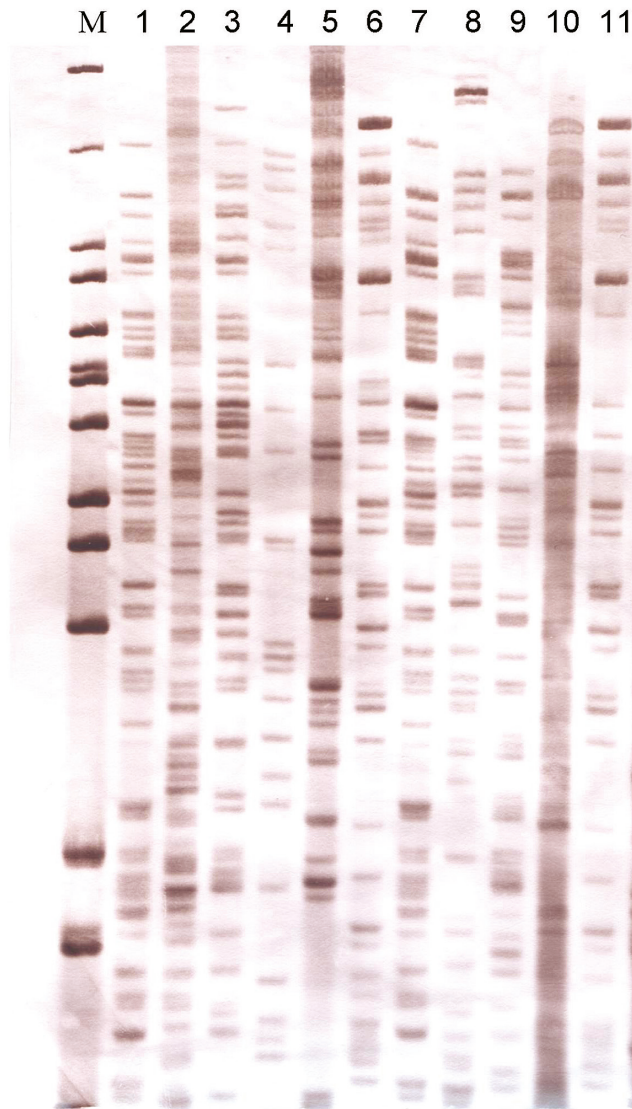


Fig. 1. Genotyping of 11 strains of the genus *Xanthomonas* by the DDSL method with restriction endonucleases of *BcuI/Eco147I*

(from 22 to 60), which are well separated in a 0.8% agarose gel. At the next stage of the study, 11 phytopathogenic strains were examined (Figs. 1, 2). Molecular weight marker was lambda DNA digest (*HindIII* and *BstEI* enzymes, tagging of Bio-dCTP in fill-in reaction), the fragment size varied from 23130 to 500 bp. The number of common (and different) bands between strains was up to 40, which made it possible to construct a phylogenetic tree using software package Statistica 6.0. (module Cluster analysis, Euclidian distance). In general, high genetic diversity of the strains was established, and they differed both in the number of detected DNA fragments and in their distribution. This fact emphasizes the high resolution of this method, which was previously demonstrated on several types of pathogenic clinically significant pathogens, as well as phytopathogen antagonistic bacteria (Terletskiy et al., 2016).

Analysis of the distribution of DNA fragments shows that strains 1 and 7 are genetically identical. Both strains were isolated from cabbage grown in different places (Belarus and Ukraine) and their genetic proximity may indicate a common host of these crops or the use of the original planting material from a specific place where the pathogen was further spread. The data on the genotyping of strains 3 and 4 are also interesting (Table 1). Although they were isolated in 2006 from the Vologda leaf cabbage variety plants in the Moscow region, their characteristics differ substantially in almost all DNA fragments. In addition, strain 4 was genetically distant from all other analyzed strains, since it has a relatively small number of DNA fragments. A similar pattern was noted for strains 2 and 7, 5 and 6, which have a common origin (Ukraine, IMV, and Russia, Leningrad region, VIZR, respectively), but are genetically distant from each other.

Analysis of differences in the position of DNA fragments in strains derived from different crops shows that these variants are no more distant from each other than variants from the same plant species. Moreover, it was found that strain 6 (cabbage, Leningrad region, VIZR, 1984) is genetically close to strain 11, obtained from the begonia greenhouse plant (Leningrad region, 2018); the position of most DNA fragments is the same with almost the same number of all fragments in both genomes.

As was expected, various subspecies (for example, strains 8, 9 and 10), despite the geographical proximity of origin, are genetically distant from each other. Strains isolated from other host plants (not cabbage strains) show a specific pattern in terms of the distribution of DNA fragments. In this case, we cannot talk about the transmission of the pathogen between different hosts.

Discussion

Counting the number of common and differing DNA fragments revealed no association between geographic location and genetic distance between the strains. Analysis of differences in the position of DNA fragments in strains derived from different crops shows that these variants are no more distant from each other than variants from the same culture. The fact that strain 6 (cabbage, Leningrad region, VIZR, 1984) is genetically close to strain 11, obtained from the begonia greenhouse culture (Leningrad region, 2018) can be explained by the probability of transmission through soil, despite a significant time gap in bacteria isolation; the position of most DNA fragments is the same with almost the same number of all fragments in both genomes. This is a relatively rare case when the identity of the strains is noted, despite their geographical and temporal distance and their different host plants. This observation drawn on the base of DDSL data indicates the absence of any relation be-

Tree Diagram for 11 *Xanthomonas* strains
Single Linkage
Euclidean distances

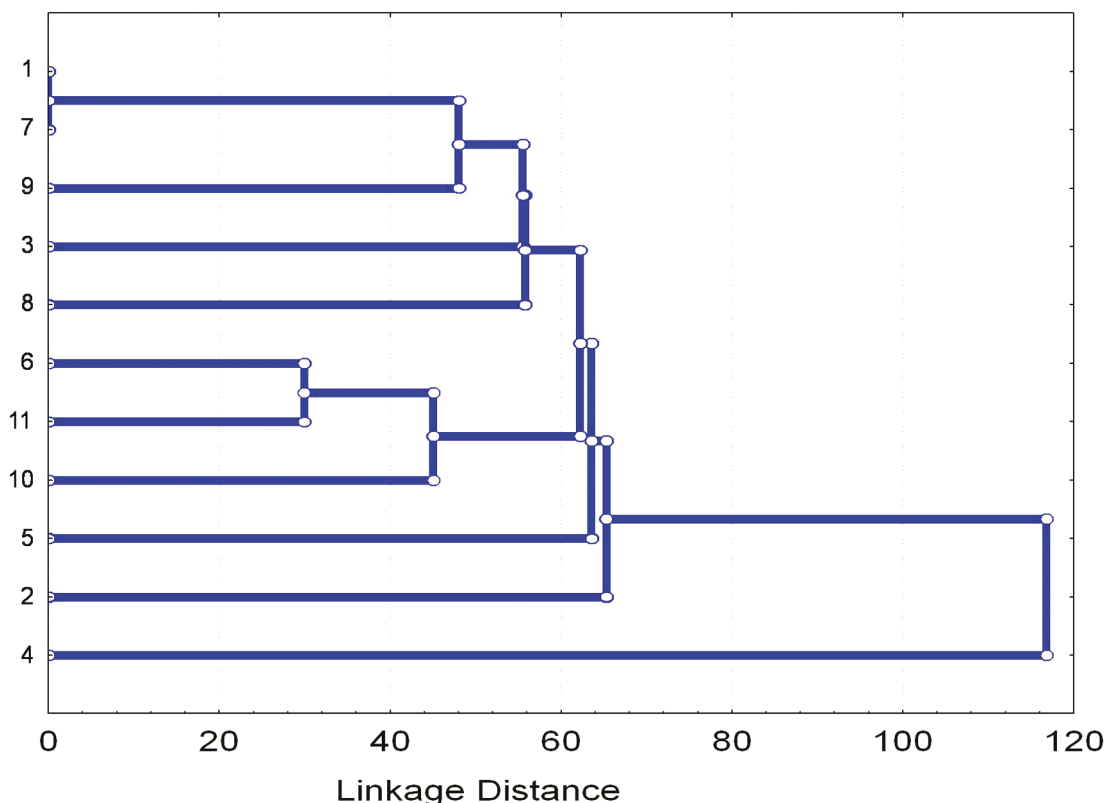


Fig. 2. Dendrogram of genetic relationships between bacterial strains of the genus *Xanthomonas*

tween the pathogen genotype and the host plant species, and underlines that pathogens of this kind are able to infect a wide range of different plants. Prolonged storage of bacterial culture in collection did not affect the genetic structure, as can be seen in strains 6 and 11 collected in 1984 and 2018 from the same source. So, we can assume a slow rate of accumulation of mutations during reseed-ing. The same slow rate we observed in *Salmonella* spp. when we used LT2 reference strains kept in a Spanish hospital collection for more than 50 years. Only a few differences in genetic profiles were detected.

Thus, it has been shown that the DDSL method, tested on a number of strains of the genus *Xanthomonas*, isolated from different crops, allows us to prove the transmission of the pathogen between hosts and show the specific prevalence of individual strains of phytopathogens among crops. It shows the possibility of identifying and unambiguously identifying strains of phytopathogenic bacteria of this kind in the process of phytosanitary monitoring during the development of epiphytities of potato bacteriosis. The fundamentals of the developed technique for genotyping pathogens in pure culture of the *Xanthomonas* genus bacteria will fur-

ther help to identify them in samples of plant material with characteristic symptoms of the disease and in the form of latent infection, which will make it possible to effectively plan phytosanitary measures for growing and storing agricultural plant products.

As DDSL technique is based on a simple restriction digestion of genomic DNA and simultaneous frag-ment labeling, the generated genetic profiles are highly reproducible. *Taq* polymerase is still able to incorporate a single Bio-dCTP molecule into 3'-recessed fragment ends at 37°C, the observation that makes it possible to perform digestion and labeling in a single reaction mic-totube at this temperature.

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