The role of epiphytic populations in pathogenesis of the genus Xanthomonas bacteria

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Abstract. Global climate warming and involvement of new regions with endemic populations of microorganisms in commercial seed production have led to an increase in the diversity of phytopathogenic bacteria that are affecting major crops, including the fruit trees. As a rule, emergence of new pathogens is associated with importation of infected seeds and planting material, cultivation of new species and varieties of plants, and expansion of agricultural trade with foreign countries. One of the leaders in diversity among phytopathogens is the genus *Xanthomonas* bacteria, affecting more than 400 plant species. Among the characteristic signs of xanthomonads is the high frequency of horizontal gene transfer both within the genus and between phylogenetically removed bacterial taxa – up to 25% of the genes are of this origin. In this paper, we consider another source of increasing the number of phytopathogenic species – by the epiphytic populations. These bacteria are the likely ancestral form of the phytopathogenic bacteria of the genus *Xanthomonas*.

1 Introduction

Representatives of the genus *Xanthomonas* are the widespread phytopathogens that cause diseases of 70 genera of monocotyledonous and 170 genera of dicotyledonous plants. The host range of each *Xanthomonas* group is in most cases limited to several species or related genera of the same plant family [1]. However, this genus includes not only pathogenic strains of bacteria. Isolates of *X. campestris* that do not cause disease symptoms were found in epiphytic populations of various plants [2], avirulent or slightly pathogenic xanthomonads were isolated from cabbage [3], legumes [4], apple trees [2], tomatoes and peppers [5], and rice [6]. In addition, genetic studies of xanthomonads revealed the presence of a group of avirulent strains belonging to the species *X. arboricola* [7]. Most of the isolated non-pathogenic strains belong to *X. arboricola* and cluster separately from typical pathogenic

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bacteria [8-9]. Pathogenic and non-pathogenic strains are likely to exist together. Possibly, epiphytic and pathogenic strains can compete with each other, which, according to some research data, could be used as biocontrol [9].

2 Materials and methods

2.1 Strains

32 strains of *X. arboricola* isolated from tomato, rapeseed, sunflower, barley and cabbage in the Central Chernozemic economic region, the Krasnodar Krai and Stavropol Krai, the Rostov, Saratov and Moscow Oblasts, the republics of Adygea and North Ossetia-Alania in 2009-2018 were used in this work.

2.2 Cultivation of strains and infection of host plants

The strains were cultured on YDC media (glucose – 10.0 g; yeast extract – 10.0 g; CaCO$_3$ – 20.0 g; agar – 18.0 g per 1 l) and SQ media (succinic acid – 10.0 g, quinic acid – 5.0 g, K$_2$HPO$_4$ – 1.5 g, (NH$_4$)$_2$SO$_4$ – 1.0 g, yeast extract – 0.5 g per 1 l). Assessment of the pathogenicity of the strains was carried out by cutting the leaves with scissors that were moistened in a bacterial suspension (10^8 CFU/ml). The suspension was obtained by washing 48-hours long bacteria culture grown on YDC medium at 28 °C. Observation of the disease development was carried out daily, the symptoms were recorded 21 and 30 days after infection.

2.3 Determination of species and genetic traits

The species affiliation of the strains was previously determined by the sequence of 16S rRNA and species-specific PCR amplification [10]. The analysis of the spectrum of genes of the type III secretion system (T3SS) and T3SS effectors (T3E) was performed by PCR amplification with primers to conserved regions of the corresponding genes [11-12]. Fragments of 7 genes were used for multilocus sequence typing (MLST): *gyrB, dnaK, rpoD, purA, nrdB, prpC, fabB*. Amplification was performed according to previously published protocols [13-14]. The genome of the bacterium *X. arboricola* str. 3004 (NCBI AZQY00000000) was sequenced on the Roche GS FLX platform [15].

2.4 Comparative genomics

Comparative genome analysis was carried out using Function based comparison and Sequence based comparison algorithms implemented on the RAST server [16], as well as the Progressive mauve algorithm – the multiple genome alignment with gene gain, loss and rearrangement [17].

3 Results and discussion

3.1 Results

3.1.1 Infection of plants

All 32 studied strains were virulent for at least one tested host plant. The oblong watery spots of light green color appeared on the cereal leaves 14-21 days after inoculation, then they acquired a yellow-brown color. The leaves died after severe damage. Pathogens caused leaf blight, leaf and stem spots in sunflower. Oilseed rape had the following symptoms: wilting, the V-shaped yellowing and necrosis of the tissues of the affected leaves between the leaf veins, as well as the darkening of the stem vessels. Walnut seedlings had the following symptoms: leaf blight, wilting of shoots and death of plants within a month after infection.

3.1.2 Genetic analysis

PCR analysis has not revealed T3SS genes and associated T3E genes among 32 studied strains of the tested phytopathogenic bacteria. MLST confirmed the identification of all bacteria as *X. arboricola*, but did not allow to refer the studied strains to any of the known pathovariants of this species. At least one or more of the studied genes in the MLST were closer to *X. campestris*, *X. oryzae*, or *X. vesicatoria* than to *X. arboricola*. Diversity of these polymorphic genes was explained more by the process of parallel adaptive evolution, than by interspecies horizontal transfer of the household genes that were used for MLST.

3.1.3 Genome annotation and analysis

The genome of strain 3004, typical of the entire group, was annotated on the RAST server. A total of 4230 coding sequences were identified. GC composition of the genome is 66%, which is within the standard range for bacteria of the species *X. arboricola*. When annotating the genome, phage sequences of Xantho Xp10 NC 004902 were detected. Phage sequences flank the VirE protein gene (COG5545). Gene sequences were allegedly obtained from *Xylella fastidiosa* 9a5c. Therefore, the fact of horizontal transfer of the virulence factor is assumed. A detailed study of the genes of the membrane transport system confirmed the absence of T3SS genes and associated effector protein genes. The presence of a fully functional type IV secretion system was revealed. The type IV secretion system of strain 3004 is represented by 10 *virB* genes, the *virD4* gene, and 23 genes responsible for the synthesis and assembly of pili for the secretion of Vir proteins. The presence of the *virD4* gene is the key for protein secretion via T4SS, and the presence of *virB8, virB9, virB10,* and *virB11* is essential for T4SS *Xanthomonas* as a whole [18].

3.1.4 Comparative genomics

Reconstruction of metabolic models and comparison of nucleotide sequences of the genome of strain *X. arboricola* 3004 with sequences of the reference genome (*X. arboricola* pv. *Juglandis* 417) revealed 17 genes and 249 unique nucleotide sequences that were present only in strain 3004. Most of the unique genes related to phage sequences and genes type IV secretion systems. The functional annotation of unique genes revealed the fact of their high homology (98%, 93%, and 93%) with the genes of the species *X. campestris, X. hortorum, X. gardneri*. According to the comparison results, only phage sequences are truly unique (present exclusively in the genome of strain 3004). With functional annotation of 249 unique

nucleotide sequences, the fact of the high homology of most of them with species *X. campestris, X. hortorum* and *X. gardneri* was also revealed. Truly unique are a number of genes belonging to the classes of tyrosine hydroxylases, M4 peptidases, MarR family transcription regulators, histidine kinases, NAD-malic enzymes, MutT proteins, esterases, thioridoxins, MFS transporters, and genes with unknown function. Multiple alignment using the Progressive mauve algorithm also revealed the proximity of many DNA fragments to other species of xanthomonads, and not to previously published genomes of *X. arboricola* strains. The abovementioned facts may be a sign of mosaic genome. Mosaic genome, in turn, may indicate that *X. arboricola* str. 3004 is closer to the last common ancestor of the genus *Xanthomonas* bacteria than representatives of the specialized pathovariants of *X. arboricola*.

3.2 Discussion

Ability to cause the disease is associated with the presence of pathogenicity genes. A typical bacterial genome of the genus *Xanthomonas* encodes about 4,000 genes. Pathogenicity genes are usually found in clusters or operons: xps – encoding a type II secretion system (T2SS); rpf – regulatory synthesis of pathogenicity factors; *hrp* – encoding T3SS; *gum* – responsible for the synthesis of xanthan polysaccharide; T4SS genes [19-20]. Differences between *Xanthomonas* spp. in the composition of genes that encode enzymes for degradation of the plant cell wall, may be associated with a difference in the cell wall composition among the corresponding host plants, as well as with differences in the symptoms. But the presence of these genes in both pathogenic and non-pathogenic strains does not indicate their key role in pathogenesis. T3SS genes are found in most species of *Xanthomonas*. The sets of T3E genes are very diverse [21], and are associated with the process of adaptation to the host response to infection. The secretion system of type IV in *Xanthomonas* bacteria is not fully understood. T4SS carries effectors with bacteriolytic activity and the ability to degrade peptidoglycan in target cells [18]. The *virB + virD4* gene complex is found in many species of xanthomonads, regardless of their pathogenic activity [19-20].

Most authors believe that the presence of a type III secretion system determines the strain's pathogenicity. When studying the set of T3SS and T3E genes in the species *X. arboricola*, it was shown that they are necessary for virulence [22]. Acquisition of T3SS allows previously avirulent strains to suppress the hypersensitive response-like necrosis [23]. Non-pathogenic strains either do not contain T3SS genes or contain only a limited number of them [24-29]. Epiphytic bacteria *X. campestris* contain only the *hrpG* and *hrpX* genes. It is possible that these genes are induced to regulate plant cell wall degradation genes, which allows the epiphyte to receive nutrients [9].

Results of our studies show that it is not necessary to acquire the T3SS and T3E genes for the appearance of pathogenicity. According to genomic analysis, *X. arboricola* str. 3004 should belong to epiphytic strains, since it does not have T3SS genes and associated T3E genes. However, *X. arboricola* str. 3004-like strains isolated in the Russian Federation are pathogenic in a wide range of plants. As a factor contributing to the acquisition of pathogenic properties is the probable horizontal gene transfer of the VirE protein (COG5545) from *Xylella fastidiosa* using the Xantho Xp10 phage.

4 Conclusion

It is still not completely clear what role epiphytic strains play in the evolution and pathogenesis of *Xanthomonas* spp. All theories about evolution of the genus *Xanthomonas* bacteria can be divided into two groups: (1) pathogenic strains evolved from non-pathogenic ones by acquiring virulence genes; (2) non-pathogenic strains evolved from pathogens by the loss of virulence genes.

The fact that the loss of a cluster of pathogenicity genes could be the initial event of evolution is considered by the example of *Pseudomonas* spp. in a study of Mohr et al. [29]. Meline et al. [23] also noted that acquiring T3SS genes may reduce the ability of *X. campestris* strains to survive on a plant [23]. This fact may explain the loss of virulence genes assumed by Merda et al. [24].

We support the hypothesis that it is epiphytic strains that are the ancestral form, and horizontal virulence gene transfer is the main driving factor of evolution. This hypothesis is supported by the results obtained in the analysis of the genome of *X. arboricola* str. 3004. It affects a wide range of host plants: barley, wheat, rapeseed, cabbage, tomato, walnut and sunflower. Genome *X. arboricola* str. 3004 has a mosaic structure, i.e. has many genes that are closer to other species of the genus *Xanthomonas*, which may indicate the relative proximity of this group of bacteria to the last mutual ancestor of xanthomonads. We suggest that the strain could be an epiphyte until the *virE* gene was obtained as a result of the horizontal gene transfer. In addition, we believe that acquisition of T3SS, described by Meline et al. [23], is not the only prerequisite for pathogenicity. Close attention should be paid to studying the role of T4SS in the pathogenesis of *Xanthomonas*. It was shown by using the symbiotic species *Mesorhizobium loti* as an example that type IV and type III secretion systems can be used interchangeably [30]. It is possible that T3SS plays a role only in the specialization of strains that is acquired by the mechanism described by Monteil et al. for bacteria of the genus *Pseudomonas* [31].

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