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Are classification and phytopathological diversity compatible in *Xanthomonas?*

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The genus *Xanthomonas* is characterized by its phytopathogenic diversity and the host specificity of its members. In the past, the classification of the members of this genus has been based primarily on the criterion of host specificity. This has led to a classification system which focused only on naming phytopathogenic variants on different hosts. Extensive taxonomic examination of *Xanthomonas* has shown that the phytopathogenic specialization of the bacteria is not correlated with the actual relationships within the genus. Based upon total genomic DNA homology, the genus has been reclassified into 20 species. At present, non-pathogenic xanthomonads are frequently isolated from plant material. As these strains often cannot be classified to existing species, it becomes clear that the diversity of the genus is much greater than expected from the phytopathogenic subpopulation, which has been the primary subject in the past. The example of *Xanthomonas* also illustrates that attempts to divide bacterial populations into discrete taxa conflict with the actual continuous nature of biodiversity.

Keywords: *Xanthomonas*; diversity; classification; pathogenicity

Xanthomonas is a typical genus of plant pathogenic bacteria. Its representatives occur in many climatic regions and especially subtropical and tropical areas all over the world. The pathogens cause a variety of diseases including wilt, necrosis, gummosis and vascular or parenchymatous diseases on leaves, fruits or stems on diverse monocotyledonous and dicotyledonous plant families [2]. According to the most thorough study in this domain [18], the host range of *Xanthomonas* includes at least 268 dicotyl and 124 monocotyl plant species. However, since most known plant pathogens are associated with crops and other cultivated plants, the real number of plant species that is susceptible to xanthomonad pathogens might be far greater.

Members of the genus *Xanthomonas* infect many economically important crops. Among the most devastating of them are those pathogens affecting primary food crops in third world regions, such as *X. oryzae* on rice [22] and *X. axonopodis* (*X. campestris*) pv *manihotis* on cassava [21]. Other important pathogens include *X. axonopodis* pv *phaseoli* causing bacterial blight of bean, *X. axonopodis* (*X. campestris*) pvs *glycines* causing bacterial pustule of soybean, *citri*, responsible for citrus canker, *X. vesicatoria* (*X. campestris* pv *vesicatoria*), the causal agent of bacterial spot of pepper and tomato, *X. campestris* pv *campestris* which causes black rot of crucifers, *X. translucens* (*X. campestris* pv *translucens*), causing leaf streak and black chaff of small grains. This list is not complete as many other *Xanthomonas* species and pathovars are highly specialized pathogens for various crops, trees and ornamental plants. A recent comprehensive survey is given by Hayward *et al* [12].

In spite of the importance of *Xanthomonas* as plant pathogens, the genus has its beneficial aspects as well. Most xanthomonads produce an extracellular polysaccharide called xanthan. This characteristic polymer of pentasaccharides, which is responsible for the typical mucous appearance of colonies and cultures of xanthomonads, has a number of attractive physico-chemical features (for a recent review, see [31]). It renders solutions a high degree of viscosity and is resistant to high temperatures and salt concentrations, as well as to acid pH. Xanthan gum is produced industrially on a large scale as a stabilizing, emulsifying, and gelling agent in numerous commercial products, particularly in the food industry [31].

A classification based on phytopathogenicity

One of the most remarkable characteristics of *Xanthomonas* is the phytopathogenic diversity and the apparent host specificity of the members. Originally, each variant showing a different host range or producing different disease symptoms was classified as a separate species. This practice, denounced as the 'new host – new species' concept [29], led to a complex genus, finally containing more than 100 species. In contrast to the phytopathogenic diversity of *Xanthomonas*, the general phenotypic characteristics of the bacteria are remarkably uniform, at least as determined by available tests. Several comprehensive phenotypic studies have been performed in attempts to differentiate the phytopathological groups by means other than the host from which isolated [3,6,34], but these have only illustrated the phenotypic homogeneity of the genus. This knowledge, and also the fact that insufficient information was available about the actual phytopathogenic specialization of the taxa, was the major motive for merging almost all *Xanthomonas* species into the single species *X. campestris* by Dye and Lelliott [7]. Later, Young *et al* [47] have proposed to reclassify the former *nomenspecies* (ie, species only distinguished by their name) into *pathovars*. A pathovar is an

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infraspecific group which is defined only by the fact that it is, or is believed to be, characterized by a unique host range or disease. Pathovar names are usually derived from the name of the host plant. The pathovar subdivision is a special-purpose classification which is designed to meet the practical needs of plant pathologists to name important plant pathogens. It was adopted as a provisional solution until a classification would be established based on more generally accepted principles. Thus far, more than 140 pathovars have been defined within *X. campestris* [2,12].

Apart from the fact that pathovars are defined by one single feature and thus have no place in a modern taxonomic environment [39], this might have represented a useful convenience if there were not three major practical problems with the system: (i) In most cases our knowledge of the host range of strains of a particular pathovar is limited as no extensive host range study including numerous cross-inoculations has ever been performed or at least published; (ii) In an early DNA hybridization study, Murata and Starr [23] have reported that there is significant heterogeneity within a number of pathovars, at that time nomenspecies, at the genomic level. Later, this finding was confirmed and extended in numerous taxonomic studies on *Xanthomonas* [14,25,33,39,40,42,43]; (iii) Non-pathogenic xanthomonads, which are isolated from healthy as well as diseased plants, cannot be classified in a pathovar system. Schroth and Hildebrand [27] were among the first to discuss the shortcomings of a pathovar system in the light of general taxonomy, and suggested that a taxonomic scheme for plant pathogenic bacteria should be based on DNA hybridization matrices.

A basis for a new classification by a polyphasic approach

To sort out the relationships between the many pathovars and species, a series of studies on the taxonomy of *Xanthomonas* has been undertaken. These studies have mainly addressed the species delineation within the genus. Rather than extending classical phenotypic comparisons by testing individual biochemical and physiological features [34], analytical fingerprinting techniques such as electrophoresis of whole-cell proteins [38] and gas-chromatographic analysis of cellular fatty acids [32] have been applied. The idea of this approach was to analyze a large number of isolates (more than 1000) from diverse origins using these fast yet sensitive fingerprint techniques, and then select a more restricted number of representative strains for further genomic study by DNA hybridization. This approach combines the benefits of: (i) analyzing large numbers of strains, which is necessary to obtain a representative picture of the biological diversity of the organisms; (ii) overcoming restrictions and errors inherent in a single typing method, by comparing more than one fingerprint technique; and (iii) establishing genomic relationships between the obtained groupings by hybridizing DNA between selected strains.

Partial results on protein electrophoresis applied on 307 *Xanthomonas* strains [37] have shown that the *X. campestris* pathovars are much more heterogeneous than expected. Based upon cluster analysis of similarities between scanned and digitized protein patterns, 19 clusters could be delineated, of which *S. maltophilia* (formerly *X. maltophilia*) was the most aberrant. In some cases pathovars from related hosts such as members of the plant families *Fabaceae*, *Poaceae*, and *Brassicaceae* seemed to be related to each other [36]. Another striking result of this study was the demonstration of the heterogeneity of many pathovars eg *X. campestris* pv *vesicatoria*, *X. campestris* pv *poinsettiicola*, and *X campestris* pv *dieffenbachiae*.

Similarly, quantitative comparison of cellular fatty acid contents of more than 1000 strains in total demonstrated an unexpected high heterogeneity within *Xanthomonas*, especially between a number of *X. campestris* pathovars [30,46]. Many of the groupings recovered were the same as those revealed by protein analysis, although significant discrepancies between the results were also found.

The decisive information came from DNA homology measurements determined by DNA hybridization among 183 xanthomonad strains, selected from both the protein and fatty acid groupings [35] and additional strains hybridized in other studies [14,25]. In complex genera like *Xanthomonas*, where phenotypic features either yield no discrimination, or are in part contradictory, we have to rely on total genomic DNA hybridization as the standard criterion for the delineation of species [27,39].

Thus, the largest DNA homology matrix presently published allowed the distinction of 20 genomic groups [35]. Four groups contained respectively the existing species *X. albilineans*, *X. fragariae*, *X. populi*, and *X. oryzae*, whereas 16 DNA homology groups were new and not consistent with the existing pathovar classification. The latter 16 genomic groups were consequently described as new species [35]. The complex rearrangements resulting from the DNA homology relationships within *Xanthomonas* are schematically represented in Figure 1. In general, DNA homology values between the different genomic groups were below 40%, whereas internal DNA homology values typically were higher than 80% [35]. This discontinuity is a strong argument in favour of the existence of discrete taxa within *Xanthomonas*, and justifies the proposed reclassification into species. Below, we use the new species nomenclature proposed by Vauterin *et al* [35].

Correlation between taxonomic groups and phytopathogenicity groups

The apparent contradiction between phenotype and real genomic diversity in *Xanthomonas* becomes particularly true when the genomic groups (now species) are examined more closely (Figure 1). In some obvious cases, former single *X. campestris* pathovars have become one new species. Examples are *X. hyacinthi*, *X. theicola*, *X. cassavae*, *X. cucurbitae*, and *X. melonis*. However, even more examples can be found of former pathovars that fall unexpectedly in two or more species. The pathovar '*vesicatoria*', a pathogen of tomato, pepper and a few other solanaceous hosts, which has always been described as a homogeneous group, causing one consistent disease, appears to be composed of two completely unrelated genomic types which now constitute *X. vesicatoria* and a subgroup of *X. axonopodis*, respectively. The two types were discovered previously by DNA

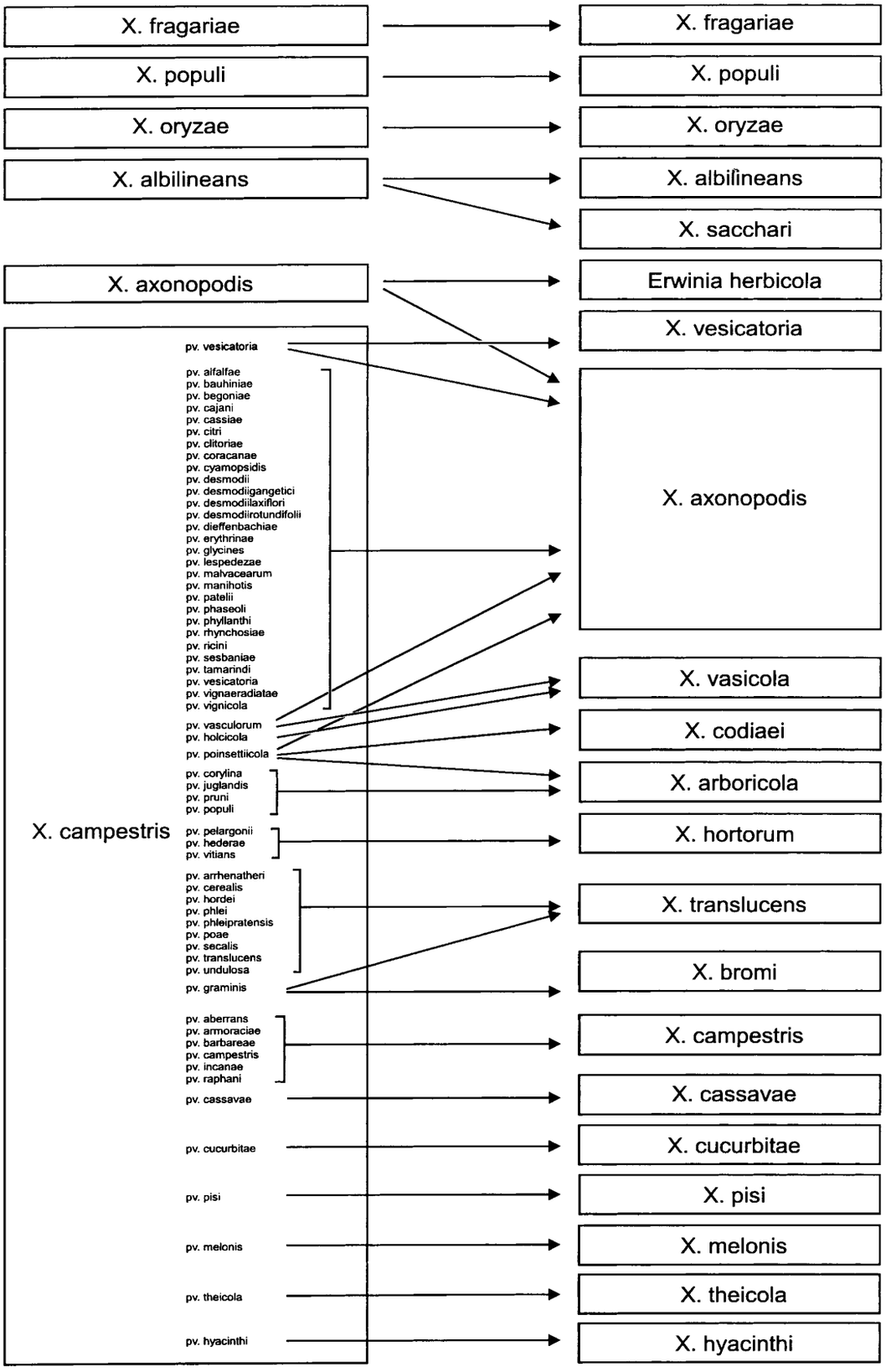

Figure 1 Schematic representation of the rearrangements proposed within the genus *Xanthomonas*, resulting from a global taxonomic study of more than 1000 strains and DNA hybridization experiments between 183 selected strains [35].

hybridization [39] and by protein electrophoresis [37]. By subjecting this pathovar to more extensive studies, other workers have shown that these two groups can be distinguished by a number of features [28]. Even more notable is the case of the former pathovar '*poinsettiicola*', pathogenic for various members of the family *Euphorbiaceae*. Part of the strains within this pathovar, ie the strains isolated from *Codiaeum variegatum*, a houseplant known as croton, comprise the actual species *X. codiaei*, whereas the other strains, all isolated from *Euphorbia pulcherrima*, are found in two other separate species: *X. axonopodis* and *X. arboricola* (Figure 1). Strains of the former pathovar '*dieffenbachiae*' remain within the same species (*X. axonopodis*) but Brazilian strains isolated from *Anthurium* and strains isolated from *Dieffenbachia* in the United States share DNA homology levels as low as 66%. The pathogens from *Anthurium* and *Dieffenbachia* could also be differentiated on the basis of fatty acid analysis [4].

The reverse case, where apparently unrelated pathogens together form one genomic group is also found in several examples. The most striking example is the relatedness between the pathovars *pelargonii* (from *Pelargonium* and *Geranium*), *vitians* (from *Lactuca* spp) and *hederae* (from *Hedera helix*), associated with different hosts and diseases but together forming the new species *X. hortorum*. The close relationship between the pathovars *vitians* and *hederae* was predicted by protein electrophoresis [37] whereas the relationship between *pelargonii* and *hederae* was revealed by fatty acid profiles [46].

Another group of highly related pathogens is composed of the pathovars *corylina*, *juglandis* and *pruni*, now classified in the species *X. arboricola*. It has been suggested that these pathogens, infecting hazelnut, walnut and prune, respectively, could have originated from a common xanthomonad, that was able to infect and colonize trees in the temperate regions [36]. Lee *et al* [16] found the members of this species to be distinguishable from other xanthomonads by their ability to metabolize quinate.

The largest and most problematic group within *Xanthomonas* is now the new species *X. axonopodis*. It is the largest group because it contains, besides the emended species *X. axonopodis*, at least 32 former *X. campestris* pathovars or subgroups of pathovars from the most diverse origin and hosts. It is the most problematic group for the following reasons: (i) there is no known phenotypic method or combination of methods that can define this complex species as a whole; (ii) phenotypic relationships between some of the strains within *X. axonopodis* are sometimes lower than those with other species; and (iii) internal DNA homology values are variable, ranging between 50 and 100%. It would be helpful to further split this loose group into more species, if there were clear subgroups. But this is neither the case by DNA homology nor by phenotypic relationships. Rather, a continuous range of DNA homology between 50 and 100% is observed, and phenotypic methods such as protein electrophoresis and fatty acid profiling are not always consistent. Hildebrand *et al* [13] determined nutritional characteristics of 88 *Xanthomonas* strains using 143 carbon sources. Similarly, they found that most *Xanthomonas* DNA groups could be differentiated from each other, except members of the largest group, ie the species *X. axonopodis*, which showed substantially different patterns among the pathovars that comprise the group.

Is the taxonomic diversity of Xanthomonas a continuum?

Up to now, an odd 80 pathovars have been allocated in the new classification. Although this classification is completely based on genetic grounds, there is growing evidence that the genomic groups can be differentiated by phenotypic features. Using Biolog and other metabolic tests, it was possible to discriminate among a number of genomic groups [13,35]. The database of fatty acid fingerprints of *Xanthomonas* by Yang *et al* [46] was revised in the light of the new *Xanthomonas* species, and most new species could be discriminated on the basis of quantitative fatty acid composition [44]. There are, however, more than 140 former *X. campestris* pathovars in total, of which at least 60 have never been analyzed taxonomically. Although most of the unstudied pathovars concern single isolations of a xanthomonad from endemic hosts, it implies that the real diversity of the genus is probably even greater than observed up to now. Some strains will obviously fit within known species, but others may form new entities, or may further confuse existing groups. The situation is becoming even more complex as so-called opportunistic xanthomonads are frequently isolated from plant material. These are xanthomonad populations, living in close association with plants but causing no apparent disease symptoms on the host and missing the *hrp* genes typical of pathogenic members of the genus [17]. In the past, this group of non-pathogenic xanthomonads has been largely overlooked as they were unimportant from an economic point of view. With the increasing interest in bacterial ecology and biodiversity however, their existence should not be neglected. A recent study of 70 presumptive non-pathogenic xanthomonads by protein electrophoresis, fatty acid analysis, and monoclonal antibody testing [41] revealed that the population was very heterogeneous. When the strains were identified with the databases of protein patterns and fatty acid profiles established by the authors, only forty-two strains were identified as belonging to the same species, whereas five strains were identified as a different species. Eight strains remained unidentified by both methods, whereas in 15 cases the identification was ambiguous. Interestingly, the identification at pathovar level was always ambiguous and not concordant, and none of the non-pathogenic xanthomonads was identified as belonging to the pathovars of the plant from which they were isolated.

These observations suggest that the pool of xanthomonads present in the environment is even more diverse and complex than what has been previously obvious as determined from mainly pathogenic populations. Especially in *Xanthomonas*, but also in other genera such as *Stenotrophomonas* (Vauterin and Swings, unpublished data), it is becoming clear that the biodiversity is much greater than expected. When numerous strains are analyzed and grouped by various methods, as in *Xanthomonas*, it appears that this genus constitutes a continuum of geno- and phenotypes with cloudy condensed nodes representing ecologically more successful types. Thus, any attempt to divide biologi-

cal populations into discrete taxa, as is done in the current classification systems, will always be more or less artificial because of its inconsistency with the real continuous nature of the biodiversity. Obviously, this situation will be more pronounced in one genus than in another. *Xanthomonas*, with more than 140 phytopathogenic, and probably many more opportunistic variants, is an excellent example of bacterial biodiversity.

A view on current developments

Assessing the taxonomic diversity and relationships of bacteria requires that large numbers of strains are investigated by revealing their genomic resemblance. DNA hybridization is considered as a reliable technique for this purpose, as this technique measures levels of homology between complete genomes. However, a restriction of DNA hybridization methods is that they are not sensitive enough to detect close relationships between strains and populations. Many genomic fingerprinting techniques are described that are based on specific cleavage of DNA by restriction endonucleases. A common feature of such techniques is that they are suitable for detecting clonal relationships, but cannot replace DNA hybridization in measuring more distant relatedness. Whole genomic fingerprints were successfully used to differentiate among *X. axonopodis* groups causing diseases on citrus [9]. The same group of bacteria as well as other pathovars were studied by RFLP analysis [10,11] and pulsed field electrophoresis [8]. Berthier *et al* [1] studied a large number of *Xanthomonas* species and pathovars by ribotyping. These authors concluded that pathovars with a broad host range are often more heterogeneous than pathovars with a narrow host range. They showed that for the pathovars they studied, ribotyping can be correlated with the pathogenicity on the host plants.

A second restriction of DNA hybridization techniques is that they provide pairwise similarities between strains, but no descriptive information about individual strains. Thus, based on DNA hybridization, it is impossible to generate databases with the purpose of identification, as can be done with fingerprints. In other words, this implies that placing an unknown strain into a DNA homology matrix of *n* strains requires up to *n* experiments to be carried out. In the example of *Xanthomonas*, consisting of 20 species at present, identification of an unknown strain would be very laborious based on the DNA homology matrix. A fingerprint in contrast, once available, can be readily compared with hundreds or thousands of other fingerprints stored in a database, by using appropriate computer software. It is believed that the correspondence between restriction fragment fingerprints can be used to estimate genetic distance or relatedness [24], provided that the obtained fingerprints offer a sufficient number of bands to exceed the statistical and experimental error due to mismatches, wrong alignments etc. Janssen *et al* [15] conducted a restricted study on *Xanthomonas* species and pathovars using AFLP, a PCR-based fingerprinting technique originally developed in plant breeding [45]. AFLP yielded some 30–50 PCR products per banding pattern, which is far superior to most other fingerprinting techniques. The groupings of *Xanthomonas* revealed after clustering of AFLP patterns could be nicely correlated with DNA hybridization data obtained previously [35]. Likewise, Louws *et al* [19,20] and Schneider and de Bruijn [26] demonstrated that the combined use of PCR fingerprints generated with REP, ERIC and BOX primers [5] can be used to detect phylogenetic relationships among strains. These, and perhaps other similar fingerprint techniques that reveal information about the total genome, are likely to become a valuable substitute for DNA hybridization in the future, provided that the fingerprints can be sufficiently standardized to allow databases for identification to be generated.

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