Practical Benefits of Knowing the Enemy: Modern Molecular Tools for Diagnosing the Etiology of Bacterial Diseases and Understanding the Taxonomy and Diversity of Plant-Pathogenic Bacteria

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Abstract

Knowing the identity of bacterial plant pathogens is essential to strategic and sustainable disease management in agricultural systems. This knowledge is critical for growers, diagnosticians, extension agents, and others dealing with crops. However, such identifications are linked to bacterial taxonomy, a complicated and changing discipline that depends on methods and information that are often not used by those who are diagnosing field problems. Modern molecular tools for fingerprinting and sequencing allow for pathogen identification in the absence of distinguishing or conveniently tested phenotypic characteristics. These methods are also useful in studying the etiology and epidemiology of phytopathogenic bacteria from epidemics, as was done in numerous studies conducted in California's Salinas Valley. Multilocus and whole-genome sequence analyses are becoming the cornerstones of studies of microbial diversity and bacterial taxonomy. Whole-genome sequence analysis needs to become adequately accessible, automated, and affordable in order to be used routinely for identification and epidemiology. The power of molecular tools in accurately identifying bacterial pathogenesis is therefore of value to the farmer, diagnostician, phytobacteriologist, and taxonomist.

INTRODUCTION

If you know your enemies and know yourself, you will not be imperiled in a hundred battles . . . if you do not know your enemies nor yourself, you will be imperiled in every single battle.

From: The Art of War
By General Sun Tzu (544–496 BCE)

This admonition, from a sixth century BCE military strategist and philosopher, provides words of wisdom still applicable today. Plant-pathogenic bacteria are enemies that confront farmers and agriculturalists worldwide and threaten to reduce the quality and quantity of food, fiber, and ornamental commodities. When outbreaks occur, growers need to know the source of the pathogen and the likelihood of disease spread as well as other epidemiological information. Diagnosticians, extension professionals, and plant pathologists likely want to know the precise identity of the pathogen. Bacterial pathogens need to be understood so that appropriate measures can be implemented on the farm.

Plant-pathogenic bacteria are also enemies that have defied attempts to thoroughly understand them. We cannot easily differentiate between species because of their microscopic size and lack of distinguishing morphological traits. Thus, indirect methods are required for detection, classification, and identification. These methods make it possible to answer questions that farmers have on the epidemiology and management of diseases they face. To more fully know these adversaries is therefore to be less imperiled by them.

In this article, we describe the need for a strong theoretical understanding of the taxonomy of plant-pathogenic bacteria, despite its complexities and previous attempts to demystify phytobacterial taxonomy (14, 140, 141, 144). Examples are provided from our research to demonstrate the use of modern molecular tools for meeting the current taxonomic challenges in determining the etiology and epidemiology of bacterial plant pathogens. The usefulness of a coherent genomic species concept to phytobacteriology is explained. We then present California's Salinas Valley as a case study that illustrates the dynamics between plant host, bacterial pathogen, diagnostic needs, and search for clarity regarding etiology. Lastly, we describe the needs of end users for training and automated processes in order to press forward with the application of molecular tools.

WHAT'S TAXONOMY GOT TO DO WITH IT?

Taxonomy is the starting point for all biological scientific study. It allows us to precisely define an organism in relation to all other life on the planet. Within bacteriology, "the ultimate goal of taxonomy is a clear framework that allows researchers to understand relationships among bacteria and communicate effectively" (133, p. 42). Taxonomy is made up of three distinct subdisciplines (identification, classification, and nomenclature) that when appropriately integrated form a coherent and useful system (14). Bacterial unknowns are identified and assigned to previously named taxa. If the unknowns do not fit the description of previously described taxa, modern methods are used to classify organisms into new taxa. These groups are then named using prescribed rules of nomenclature. Thus, as we describe novel diseases and attempt to identify or classify the pathogens causing them, we employ the principles of taxonomy to aid our work.

As currently practiced, taxonomy is inherently unstable (45, 61, 91, 123, 133). Classifications are essentially hypotheses about how organisms are related to each other. Hypotheses change as new methods or concepts are applied to the diversity of organisms studied. As taxonomy is currently practiced, nomenclature reflects classifications; thus, as hypotheses change, so do names. The instability is augmented by the Linnaean binomial system of nomenclature in which all

species names consist of a genus name and a specific epithet. When species are proposed, they are hypothesized to belong to a particular genus. Upon further study, the species may be hypothesized to belong to a different genus, and this new species is proposed with a binomial that includes the new genus name as part of the species name (*Pseudomonas solanacearum* was hypothesized to belong to the genus *Burkholderia* as *Burkholderia solanacearum* and then to the genus *Ralstonia* as *R. solanacearum*). For more than 50 years, phytobacteriologists and others have understood that bacterial taxonomy is inherently unstable and many have questioned whether the binomial system is a suitable system (28, 47, 91, 120, 121). A further complication of the current taxonomic system is that once a name is valid, it remains valid even when new names are proposed (14, 125, 135). Scientists decide which valid name to use based on their estimation of the appropriateness of the classification proposal from which it was generated. Thus, classification and nomenclature complicate identification.

Type strain: strain of a species or subspecies with which the name is permanently associated

Nomenspecies: a species defined by the characteristics of a type strain

As described elsewhere, bacterial taxonomy was rife with poorly described species having multiple synonyms as a result of poor classification based on relatively few phenotypic characteristics and a lack of comparisons with previously named taxa (100, 133). In order to rectify this and other problems, the International Code of Nomenclature of Prokaryotes (80) restructured bacterial nomenclature in part by establishing the Approved List of Bacterial Names (116). Upon its publication on January 1, 1980, only names of species that had type strains that matched modern published descriptions distinguishing them from other species were considered valid and were included on this list. This publication created major complications in phytobacterial taxonomy because many plant-pathogenic species did not meet these criteria and were not included; thus, their names were no longer valid and the organisms had no place in the new structure. Nonetheless, plant pathologists still needed to communicate about these distinct pathogens. To provide continuity between historical scientific literature and current and future research, plant pathologists adopted an infra-subspecific taxon, the pathovar, which allowed threatened nomenspecies to be preserved as pathovar epithets originally within the species *Corynebacterium flaccumfaciens*, *Erwinia chrysanthemi*, *Pseudomonas syringae*, and *Xanthomonas campestris* (40). There are currently validly named pathovars in 26 species (13, 15, 16).

One significant result of this solution was the creation of a trinomial, which further exacerbated the issue of apparent nomenclatural instability. A pathovar name (e.g., *P. syringae* pv. *alisalensis*) consists of a genus name (*Pseudomonas*), a specific epithet (*syringae*), and a pathovar epithet (*alisalensis*). When a proposal transfers a pathovar from one species to another, the pathovar name changes even though the pathovar epithet usually remains the same (40, 142). For example, *P. syringae* pv. *alisalensis* was transferred to *P. cannabina* as *P. cannabina* pv. *alisalensis* (24). Determining the host range of the pathogen is essential to determining its pathovar status (40, 142). Below we describe complications related to identifying a pathovar within a species and provide molecular strategies to help ease these burdens. However, prior to determining the pathovar status of an organism, hypotheses first about the genus and then about the species should be made (**Figure 1**). For plant pathologists and others, this is problematic because methods and species concepts and/or definitions continue to change, so defining or assigning an organism to a species is a moving target (7, 110, 133, 148).

The history of bacterial classification and species concepts has been reviewed (34, 45, 100, 108, 110, 114, 133, 148). In summary, the science has its origin in an earlier subjective era of taxonomy that was based on using phenotypic characteristics for both classification and identification, with identification being the priority in the selection of methods. With our knowledge of evolution, the elucidation of the relevance and structure of DNA, and the development of powerful computers, a more objective era of taxonomy emerged in which defining natural relationships among organisms took precedence over being able to identify them once these relationships were defined. A

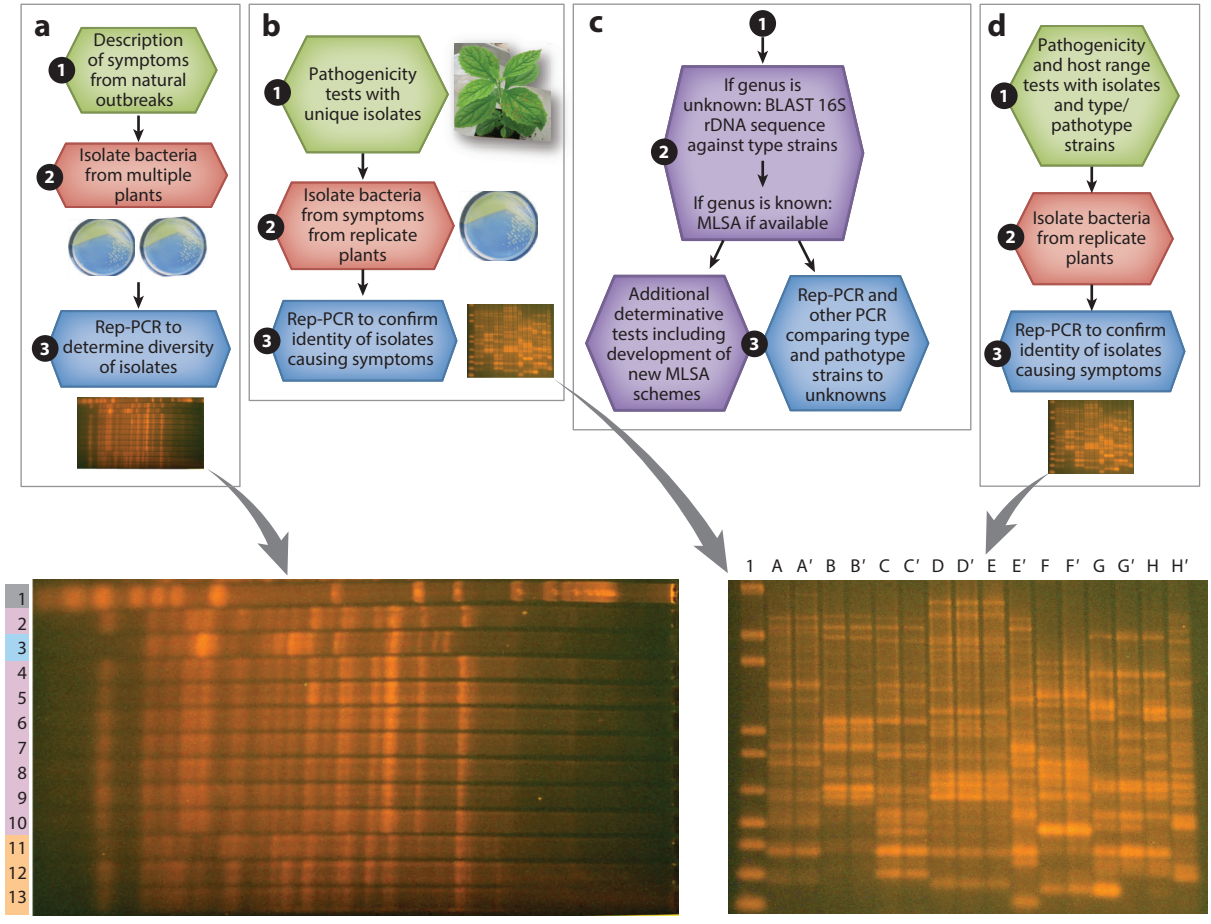


Figure 1

Framework for determining the etiology of bacterial diseases of plants. (*a*) Isolation of presumptive pathogens. 1. Note symptoms possibly caused by a bacterial pathogen and document species and cultivar of host. 2. Isolate bacteria from multiple disinfested lesions, plants; often see one predominant colony type. Note colony color, fluorescence (on King's medium B), and other features. 3. Compare isolates from different plants and different outbreaks to select strains representing different genotypes for pathogenicity testing. *Inset*: Rep-PCR (repetitive extragenic sequence palindromic chain reaction) gel from individual isolates from a disease outbreak. Lane 1 (*gray*) is the DNA size standard; lanes 2, 4–10 (*purple*) have identical banding patterns (Pattern 1); lanes 11–13 (*orange*) have identical banding patterns (Pattern 2); lane 3 (*blue*) has a unique banding pattern (Pattern 3). Two isolates from Patterns 1 and 2 and the single isolate of Pattern 3 are tested for pathogenicity to determine which isolates might be phytopathogenic. (*b*) Completion of Koch's postulates. 1. Pathogenicity tests on host of origin, ideally using the same cultivar. 2. Describe symptoms and reisolate pathogen from symptomatic tissue. 3. rep-PCR and other tests to confirm identity or reisolates with original isolates. *Inset*: Rep-PCR gel with original isolates from a disease outbreak (e.g., A) and corresponding reisolates (e.g., A′) from pathogenicity tests. Koch's postulates have been confirmed. Isolates represented by fingerprints in lanes A, B, C, D, and F. The experiment should be repeated for publication. Lane 1 is the DNA size standard. (*c*) Identification of bacterial pathogens. 1. Record characters of colonies on diagnostic media such as SX (semiselective for *Xanthomonas*) and KBBC (semiselective for *Pseudomonas*). 2. Make a hypothesis about the genus and species of the organism BLAST (Basic Local Alignment Search Tool) 16S rDNA sequences. The Ribosomal Database Project (81) (**http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp**) has a search tool specific for type strains. 3. MLSA (multilocus sequence analysis)/rep-PCR in comparison with type and pathotype strains to confirm identity or make a hypothesis about isolates. (*d*) Determine pathovar status by conducting pathogenicity tests to evaluate host range. 1. Replicated and repeated pathogenicity tests on hosts of related pathotypes. Related pathotypes included as replicate control treatments. 2. Describe symptoms and isolate bacteria from symptomatic tissue. 3. Use rep-PCR and other tests to confirm identity of the isolates in comparison with original isolates. Repeat for publication.

polyphasic approach incorporating multiple phenotypic, biochemical, and genetic characteristics became the standard practice for classification (34, 57, 100, 110, 112, 129). However, by the 1980s significant weight was given to one method above others for defining bacterial species (45, 108, 110, 133).

Polyphasic classification: classification that integrates all phenotypic and genotypic data available

Species definition: a species is a group of strains having DNA-DNA hybridization values of at least 70% and a ΔTm of less than 5°C, and that is phenotypically distinct from previously described species

Genomospecies: a species as defined by the species definition for which no diagnostic phenotypes have been discovered

sensu lato: derived from Latin to mean in the largest sense

DNA-DNA Hybridization

DNA-DNA hybridization (DDH) "has mainly driven construction of the current prokaryotic taxonomy" (108, p. 19,126) and has become the gold standard for determining relationships among bacteria (110, 118, 133). In addition to conforming to the genetic criteria of the species definition, species are phenotypically distinct from previously described species (110, 119, 136). This species definition harmonized criteria for all species regardless of the taxa being studied and did a great deal to organize the known microbial diversity, leading to a less subjective classification system. However, in addition to being difficult to perform, no database of pairwise comparisons can be made because DDH has large experimental error, is intransitive, and is not reciprocal (106, 126, 133). Therefore, data are not cumulative and all pairwise comparisons with type strains from related species need to be repeated in each laboratory for every experiment. In practice, only specialized laboratories associated with public strain collections perform DDH regularly because of the difficulty of the method and access to type strains (45, 100, 108, 110, 133).

Dependence on DDH for classification significantly diminished phytopathologists' abilities to identify pathogens and describe new species. The few heroic efforts made to analyze relationships among pathovars in *P. syringae* and *X. campestris* (44, 132) were incomplete and soon out of date. For example, of the 153 pathovars of *X. campestris*, Vauterin et al. (132) and others (15) conducted pairwise DDH comparisons of 83 validly named pathovars. Seventy-six of the pathovars were transferred to species other than *X. campestris*, and seven pathovars were verified to be members of *X. campestris*. Because of the daunting task of completing all pairwise comparisons, 70 pathovars were not evaluated and largely remain orphaned in *X. campestris*, although it is not clear whether they are members of this species (15, 132). Prior to the application of new methods, such as those described below, to these and other orphans, phytobacterial taxonomy was essentially in limbo (12, 143, 145).

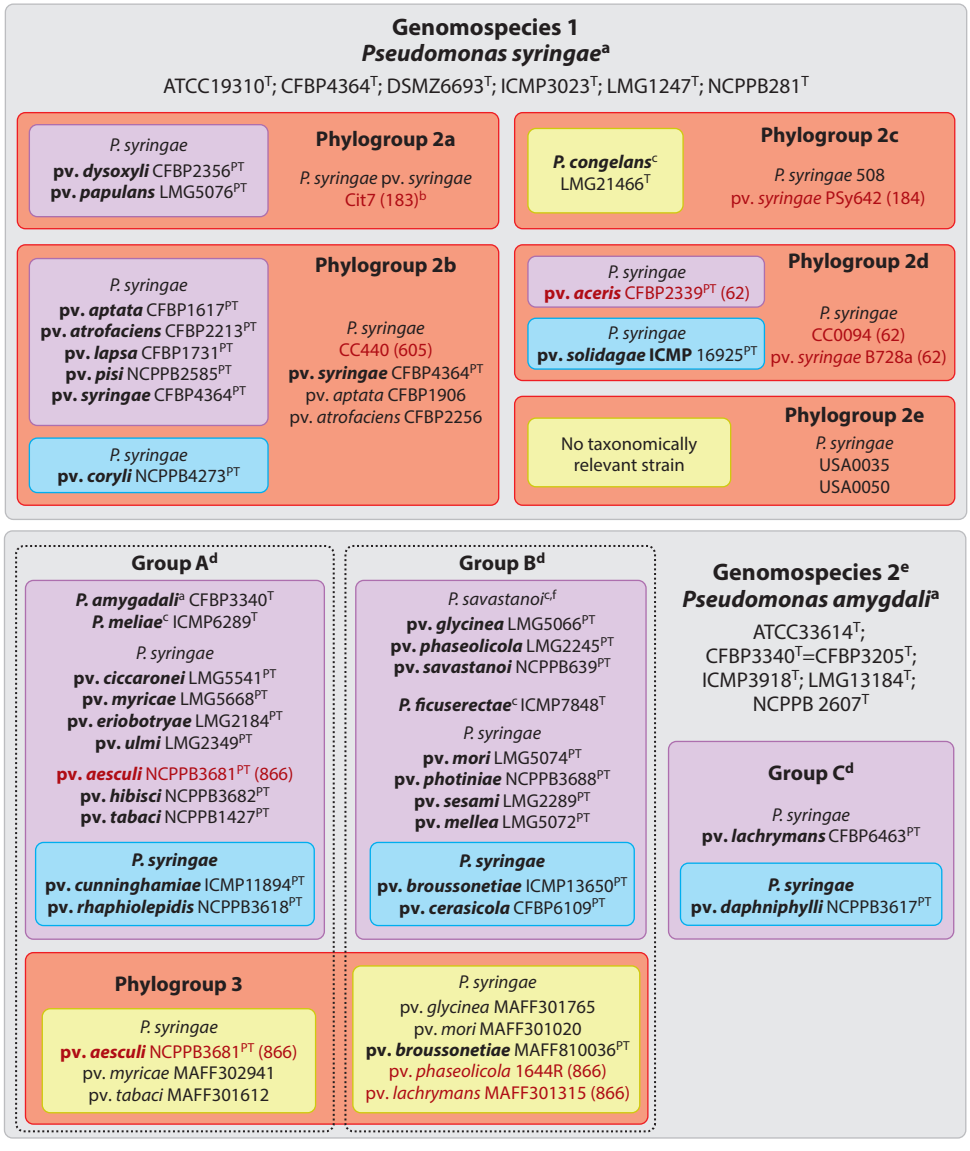
For historical reasons and because most laboratories were not prepared to use DDH for species identification, Wayne et al. (136) recommended that genomospecies (having DDH values of at least 70% and ΔT_m of less than 5°C) remain unnamed if they could not be distinguished from other species by phenotypic characteristics. This recommendation significantly influenced phytobacterial taxonomy. For example, *P. syringae* sensu lato [*P. syringae*–related species and pathovars (44, 98)] was shown by Gardan et al. (44) to consist of nine genomospecies (**Figure 2**). Strains from genomospecies 9 (*P. syringae* pv. *cannabina*) were elevated to species status and named *P. cannabina* because phenotypic characteristics were discovered that allowed *P. cannabina* to be distinguished from previously named species, including *P. syringae*. Distinguishing characteristics were not identified for the remaining genomospecies and they have yet to be named (12) (**Figure 2**).

The most unfortunate consequence of the commitment to the species definition was that DDH was used for classification, but entirely different methods were needed for identification. Additionally, DDH-based classification was problematic because plant pathologists need to define organisms at taxonomic levels below the species level. The gel- and sequenced-based methods described below are used for both classification and identification at and below the species level. We highlight these techniques because we employ them to investigate etiology and epidemiology of bacterial diseases of Salinas Valley crops.

rep–Polymerase Chain Reaction Fingerprinting

Biotype: a group of organisms having the same biological characteristics

The use of various fine-typing strategies important to studying the taxonomy and epidemiology of phytobacteria has been reviewed (84, 87, 102, 107, 115, 128). The methods are not interchangeable, but many of the same questions are answered by these various methods. Gel-based methods for observing genetic polymorphisms allow researchers to type organisms and characterize diversity without having to first identify them (48, 87, 105, 128, 130). We have chosen to use rep-PCR (polymerase chain reaction) to investigate the etiology and epidemiology of bacterial diseases of Salinas Valley crops because the technique is universal for all bacteria, fingerprints differentiate between important biotypes within pathovars (12, 24), it requires little special equipment, and tools are available for analysis of relationships.



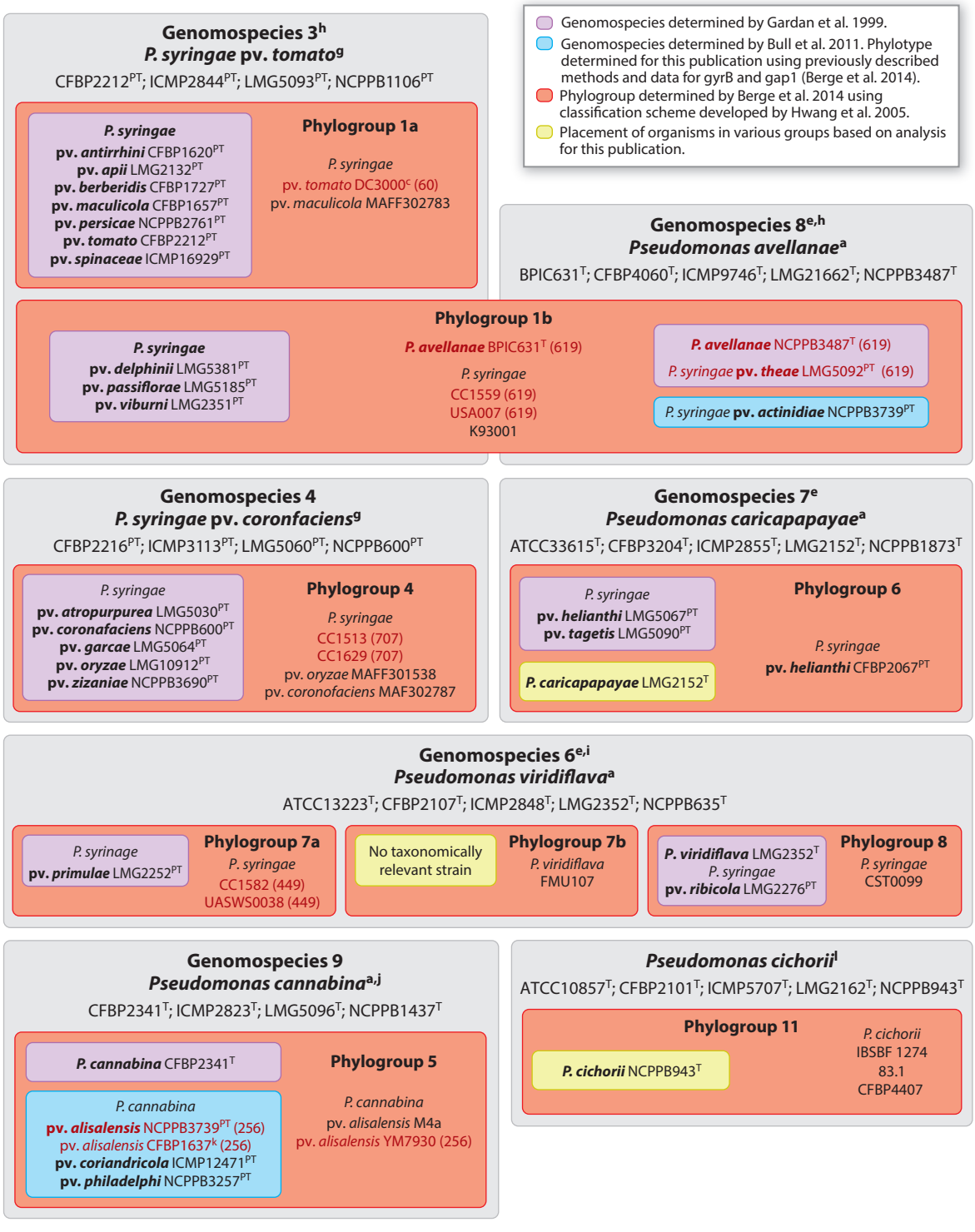


Figure 2

(*Continued*)

LOPAT: five phenotypic tests (levan production, oxidase, potato soft rot, arginine dihydrolase, tobacco hypersensitivity) that can differentiate among broad groups of fluorescent pseudomonads

Pathotype strain: the strain on which the description of the pathovar and the pathovar name is based and with which the name is permanently associated

PCR methods using universal primers that bind to dispersed repetitive palindromic (rep) sequences located throughout the genome allow amplification of genomic fragments regardless of their gene content (87, 105). The sizes of the resulting fragments are dependent on the distance between rep sequences. Gel electrophoresis of amplicons results in a fingerprint of genomic DNA (**Figure 1**). Because the target sequences are present in all bacteria, this technique can used to fingerprint any isolate. Several different rep targets (BOX, REP, ERIC) are available (87, 105). This technique is particularly powerful because only a small amount of DNA is needed and results in a specific, sensitive, and rapid method for fingerprinting bacteria. We have been successful in typing bacteria directly from lesions using this technique. As we describe below, we use rep-PCR as an important tool in confirming Koch's postulates and experimental host range results (**Figure 1*b*,*d***).

Whereas phenotypes distinguish major groups of organisms, they usually are not able to distinguish pathovars. For example, LOPAT (levan production, oxidase, potato soft rot, arginine dihydrolase, tobacco hypersensitivity) characteristics (83) can help to identify pathogens such as *P. syringae* sensu lato but are not useful to identify genomospecies or pathovars within *P. syringae* (12). rep-PCR and other gel-based methods accurately identify strains to the correct species and pathovar when relevant type and pathotype strains are included in comparisons. rep-PCR has been adapted to classification (130), but like DDH, rep-PCR data are not accumulative and cannot be used directly by other laboratories. Additionally, these methods cannot be used in species descriptions but are used to support hypotheses about the taxonomy of the organisms (126).

Figure 2 (*Continued*)

Species and pathovars within *Pseudomonas syringae* sensu lato. *Pseudomonas syringae* sensu lato currently consists of nomenspecies and genomospecies (not formally named) that may be further divided into pathogenic varieties called pathovars. In addition, populations within *Pseudomonas syringae* sensu lato have been classified by MLSA (multilocus sequence analysis) into phylogroup (8, 52) that frequently correspond to species or genomospecies. More recently, whole-genome sequences have been used to classify species within this group into cliques and clique groups (131). The relationship between cliques, phylogroup designation, nomenspecies, and genomospecies is presented. Relationships were determined for this manuscript using previously published methods, published sequences of gene fragments from *gyr*B and *gap*1 (8, 12), or unpublished sequences from a broader group of fluorescent pseudomonads (C. Bull & O.M. Martins, unpublished results). Superscripts T and PT designate the type and pathotype strains (*in bold*) for each taxon.
[a]Nomenspecies and their type strains with priority within genomospecies are in bold beneath the genomospecies designation.
[b]Strains listed in red are followed by clique numbers as classified by Varghese et al. (131).
[c]Other synonymous nomenspecies within a genomospecies are given.
[d]Within genomospecies 2, there are three phylogroups. Three groups designated A, B, and C are shown with the species and pathovars that belong to each group.
[e]Pathovars within genomospecies 2, 6, 7, and 8 could be, but have not yet been, formally transferred to *Pseudomonas amygdali*, *Pseudomonas caricapapayae*, *Pseudomonas viridiflava*, and *Pseudomonas avellanae*, respectively.
[f]In addition to the pathovars listed *Pseudomonas savastanoi* pv. *fraxini*, *P. savastanoi* pv. *nerii*, *P. savastanoi* pv. *retracarpa* are also likely to be members of genomospecies 2 group B.
[g]Pathovar names with priority and recommended by Gardan et al. (44) to serve as the type strain if a named species is proposed for genomospecies 3 and 4.
[h]Genomospecies 3 and 8 are closely related (12) and were designated as two subgroups within one phylogroup by Berge et al. (8). These subgroups do not correspond exactly to genomospecies as described by Gardan et al. (44).
[i]Phylogroups 9, 10, 12, and 13 do not correspond to any named species. However, phylogroup 9 is closely related to strains previously identified as *P. viridiflava*.
[j]*P. syringae* pv. *coriandricola*, *P. syringae* pv. *philadelphi*, and a pathogen of broad bean are in the process of being formally proposed as members of *Pseudomonas cannabina*.
[k]*P. cannabina* pv. *alisalensis* strain CFBP1637 was previously known as *P. syringae* pv. *maculicola* and is strain B-70 isolated by Williams & Keen (137).
[l]*Pseudomonas cichorii* was not examined by Garden et al. (44).

DNA Sequencing

The availability of rapid and cheap DNA sequencing and searchable sequence databases has allowed a sea change in bacterial taxonomy, resulting in the replacement of the untenable DDH method for classification and identification. Sequences from any gene or from whole genomes can be easily compared and analyzed using phenetic or phylogenetic methods for a multitude of bacteria.

Phenetic: classification based on overall similarity

Phylogenetic: classification based on hypothesized evolutionary history

The era of sequence-based classification and identification was ushered in by the use of 16S rDNA sequences to build a phylogenetic framework for understanding how all organisms are related (42, 43, 138). 16S rDNA is the most-used gene for classification and identification because of its universality, ease of use (sequences are easily obtained from DNA that is amplified using universal primers), and reproducibility as well as the large number of retrievable sequences from curated searchable databases (55, 81). Nearly full-length 16S rDNA sequences became a standard part of classification and species description during the 1990s, and because they are archived, sequences of type strains are available for comparisons to researchers worldwide. Therefore, after confirming that an isolate is pathogenic, a first step in identification of an unknown organism is a search for 16S rDNA sequence similarity using the Basic Local Alignment Search Tool (BLAST) (3, 96) (**Figure 1**). For 16S rDNA sequences, strains with 98.65% similarity or less are likely to be different species (59).

Because 16S rDNA sequence analysis does not discriminate below the species level, additional methods are needed for plant pathogens. There is ample evidence to demonstrate that analysis of multiple loci (which may include 16S rDNA sequences) provides greater confidence in the phylogenetic conclusions than analysis of single genes (45, 126, 133). Additionally, 16S rDNA sequence analysis does not discriminate at the taxonomic levels needed by plant pathologists, whereas multilocus sequence analysis (MLSA) or typing (MLST) does.

MLST was first developed to identify clones of species of bacteria by indexing sequence variation at housekeeping loci (89). MLST is especially useful for identification if sequenced gene fragments are identical to those of type or pathotype strains. MLSA is a modification that allows the study of phylogeny of organisms within a given group (45). In practice, fragments of four to eight genes are sequenced individually and small sequence fragments are joined in silico prior to analysis (88, 90). Analysis of these concatenated gene fragments provides a powerful phylogenetic tool useful in classification, identification, epidemiology, and evolutionary biology (27, 41, 55, 90, 133).

MLSA has greatly increased our ability to clarify relationships among plant-pathogenic bacteria. MLSA allows pathovars to be grouped in their correct genomospecies and helps establish hypotheses about the pathovar status of unknown isolates (12, 143). One drawback of MLSA/T is that different schemes (primers and gene fragments) are used for different genera and for individual genera by different laboratories (2, 8, 12, 52, 98, 133). The development of MLSA/T schemes requires knowledge of the diversity of the group being studied and the whole-genome sequence (WGS) for a number of strains (55, 102, 133). Most plant pathologists and clinicians use strategies that have already been developed for the genera with which they are working. The Plant Associated and Environmental Microbes Database (PAMDB) provides a significant resource of methods for MLSA/T schemes developed for phytobacteria and includes the sequences for many type and pathotype strains (2, 12, 143).

Ultimately, whole genomes will be used to understand phylogeny and identify bacteria. WGS analysis is the basis of a developing genomic species concept that will allow us to organize diversity at all levels (30, 123, 131, 133, 148). Next-generation sequencing technologies, the aforementioned drop in sequencing prices, single-cell genomics technology, and better tools for interpreting sequencing data have led to a boom in the number of WGSs archived in retrievable databases. The

targets for sequencing are no longer just model strains; the type strains of more than 15% of validly named species have been sequenced (30). There is an international movement to sequence all type strains (60, 79, 114, 139). There are efforts underway to sequence type and pathotype strains of plant pathogens. For example, all type and pathotype strains within *Pseudomonas* are being sequenced (92; D. Guttman & J. Loper, personal communication). Thus, the building blocks for a taxonomic framework based on WGSs will be available for classification and identification. Likewise an elegant approach (**https://ani.jgi-psf.org**) for delineating species using whole-genome sequences has been developed (131). WGS of type strains will likely be the standard for species descriptions in the near future (5, 78), although the criteria to be used for a genetic species definition are still being debated (6, 38, 76, 91, 106, 108, 124, 148).

Regardless of the target of sequencing, data from database searches are often misinterpreted because comparisons are made to organisms of unreliable taxonomy. Sequence data often have no quality assurance, and the databases are full of incorrectly labeled and poor quality sequences (126). Also, researchers differ in the rigor they use to identify the organisms for which they deposit sequences. Thus, unless sequences are compared with high quality sequences of the type or pathotype strains, the data have little taxonomic relevance and identifications may be misleading and wrong (14, 126, 133).

Why Haven't We Clarified the Taxonomy of Plant-Pathogenic Bacteria Once and For All?

WGS may be thought of as a solution to taxonomic instability because it provides a nonsubjective and complete data set from which to derive phylogeny. However, as previously stated, opinions continue to differ about how to analyze the data. Additionally, our understanding of microbial diversity is on the verge of a significant conceptual adjustment due to the initiation of large microbiome projects that will reveal the extent of microbial diversity in the majority of uncultured bacteria (78, 100, 109, 122). Therefore, taxonomy will continue to be dynamic and names of organisms will change as classifications are developed.

Already, scientific understanding of microbial diversity has surpassed the ability of the current taxonomic system to name classified taxa (34, 45, 91, 99, 123). There are numerous unnamed genomospecies because no phenotypic characteristics have been identified to distinguish them from previously described species (100, 109, 114). Prohibition on naming phenotypically indistinguishable genomospecies is supported by researchers who argue that phenotypes provide insight into unique biology that we are not yet able to decipher from the DNA sequences (115, 129). However, WGS has been used to predict virulence levels of pathogens as well as identify phenotypic differences (5, 56).

Although phenotypic data are always included in descriptions of new species, they are not as definitive as sequence data and often are not determinative (100, 122). Moreover, phenotypically distinct named species may not remain distinct. Phenotypic characteristics were found to distinguish the three known strains of *P. syringae* pv. *cannabina* (genomospecies 9) from *P. syringae*, thus allowing genomospecies 9 to be elevated to *P. cannabina* (44). However, as emended, *P. cannabina* is no longer phenotypically distinct because newly added pathovars (based on MLSA, DDH, and rep-PCR) varied for these phenotypes (12, 24). Nonetheless, the named species helped confirm the distinction between *P. cannabina* and the other species and between genomospecies within *P. syringae* sensu lato. The lack of distinctive phenotypes for this species is more than compensated for by the ability to rapidly identify pathovars of *P. cannabina* by MLSA and rep-PCR. Likewise, researchers would have greater understanding of the genetic relatedness of pathovars

within the other genomospecies of *P. syringae* if they were named. Finally, because we have been unwilling to name genomospecies, parallel systems of nomenclature are being suggested (91) for even clearly delineated genomospecies that were named previously (8, 52) (**Figure 2**).

MLSA and other sequencing schemes, including WGS, are perfectly suited for identification of organisms to species and below; several different criteria are being suggested for a genomic species definition (6, 38, 76, 91, 106, 108, 124, 131, 148). Using a genomic species definition helps speed the naming of microorganisms as they are discovered (78, 109). Although phenotypes of bacteria tell us a great deal about organisms and should be reported and used when helpful, the requirement for determinative phenotypes should be removed from species prescriptions when adequate (usable by all, clearly determinative) genotyping methods are available and the hypothesis of distinct species is clearly supported.

DOWN INTO THE VALLEY: FROM THEORY TO PRACTICE

The battles against bacteria take place in diverse fields, locales, and settings. For us, California's coastal Salinas Valley is a productive outdoor agricultural laboratory that we have used to know our phytobacterial foes. A number of Salinas Valley features have facilitated this research. The temperate coastal climate and the long growing season (February through November) provide excellent conditions for growing a wide range of crops. The more than 70 vegetable and fruit crops (82) provide an ample host base for both well-established and newly introduced bacterial pathogens. As participants in a dynamic and innovative industry, farmers are constantly adding new species and cultivars of crops, which further increases opportunities for pathogens to be introduced. The cycle of crops is both intensive and rapid. Direct seeded lettuce, for example, can be harvested 60 to 65 days after planting; spinach crops can be harvested after 22 to 28 days. This means that there is a constant turnover of annual vegetables, with any one acre of land being cropped two to four times a year. These features translate into a complex of crops being grown back-to-back and side-by-side for most of the calendar year, providing a context for phytobacterial interactions with host plants.

The Salinas Valley system also provides researchers with ample opportunities to study families of plant hosts. These botanical arrays of hosts create scenarios in which bacterial pathogens may or may not cross-infect closely related species. The pathogen that causes bacterial leaf spot of celery, for instance, can be studied within a field context of side-by-side plantings and rotations with other members of the Apiaceae, namely cilantro, fennel, and parsley. Bacteria known to be pathogenic on crucifers can be researched within the Salinas Valley context that includes thousands of acres of arugula, broccoli, rapini, cabbage, cauliflower, kale, and other brassicas.

Partnering our respective bacteriological research and extension programs has allowed us to keep one foot in the laboratory and one in the farm furrows of the Salinas Valley. In doing so, we have sampled slices of the diverse etiology of bacterial diseases found on these crops and developed a valuable strain collection representing the diversity of bacterial pathogens from the valley. The collection currently includes approximately 900 well-characterized fluorescent bacteria from more than 120 disease outbreaks and large numbers of strains from the genus *Xanthomonas* and the Sphingomonadacae (17, 19, 21, 75, 146). This research resulted in numerous first reports (**Table 1**) and allowed us to apply our knowledge of the enemy to develop management strategies (17, 33, 51).

To best manage bacterial plant diseases, agricultural professionals need to know one fundamental piece of information about their microbial opponents: What is the enemy's name? Armed with the proper name of the agent, one has access to the following: (*a*) the means to clearly discuss

Table 1 New diseases and identifications of the causal bacterial pathogens for crops from the coastal region of California from 1990 to 2014

Crop	Pathogen	Reference
Vegetable crops		
Arugula	*Pseudomonas syringae*	62
Arugula	*Pseudomonas cannabina* pv. *alisalensis*	20
Basil	*Pseudomonas viridiflava*	85
Broccoli	*P. cannabina* pv. *alisalensis*	69
Broccolini	*P. syringae* pv. *maculicola*	31
Brussels sprout	*P. cannabina* pv. *alisalensis*	25
Cabbage	*P. cannabina* pv. *alisalensis*	94
Catnip	*Xanthomonas campestris*	64
Cauliflower	*P. cannabina* pv. *alisalensis*	73
Celery	*P. syringae* pv. *apii*	86
Cilantro	*P. syringae* pv. *coriandricola*	35
Fennel	*P. syringae* pv. *apii*	54
Italian dandelion	*P. syringae*	68
Kale	*P. syringae* pv. *maculicola*	18
Leek	*P. syringae* pv. *porri*	66
Parsley	*P. syringae* pv. *apii*	12
Parsley	*P. syringae* pv. *coriandricola*	12
Radicchio	*Xanthomonas hortorum*	146
Rapini	*P. cannabina* pv. *alisalensis*	71
Rutabaga	*P. cannabina* pv. *alisalensis*	74
Spinach	*P. syringae* pv. *spinaciae*	65
Swiss chard	*P. syringae* pv. *aptata*	72
Water spinach	*P. syringae* pv. *syringae*	29
Fruit crops		
Raspberry	*P. syringae*	67
Strawberry	*Pseudomonas marginalis*	22
Ornamentals		
Impatiens	*P. syringae*	36
Lavender	*X. campestris*	75
Four o'clock	*X. campestris*	63

the disease and pathogen with researchers, regulators, and other industry personnel; (*b*) the known host range of the pathogen; (*c*) the likely source(s) of inoculum; (*d*) epidemiological aspects such as the ability to survive in the soil and the ability to persist epiphytically or saprophytically; and (*e*) the collective biological information upon which to base management decisions. A grower may choose to apply bactericides, change irrigation and other production practices, find resistant cultivars to plant, implement a modified crop rotation strategy, insert crop-free periods, or purchase only pathogen-tested and/or treated seed. Therefore, real-world, economically critical decisions may ultimately depend on the name of the bacterium. The creation of a workable, accurate, scientifically acceptable taxonomy based on research and modern molecular tools is therefore not purely an academic or theoretical exercise.

Risks in Shortcutting the Diagnosis

Diagnosing plant diseases is accomplished at various levels and with varying degrees of thoroughness and analysis. An intensive and extensive investigation of every plant disease situation is neither possible nor even desirable. The level of rigor in diagnosis and pathogen identification often depends on the available management strategies. A visual diagnosis of a commonly seen bacterial disease for which treatment options are well known may be sufficient to make management decisions; however, there are occasions when a deeper analysis may uncover surprises. Even for commonly seen diseases, diagnoses made primarily on the basis of symptoms and knowledge of previous host-pathogen relationships and foregoing formal pathogen identification may lead to a missed opportunity to discover new pathogens or observe changing pathogen populations. Our own experience in identifying a novel pathogen that many others missed and still confounds acts as a cautionary tale in plant disease diagnostics.

The Salinas Valley is home to wide expanses of crucifer crops, including more than 36,000 ha of broccoli, cauliflower, cabbage, bok choy, rapini, Chinese cabbage, and kale as well as a host of other specialty crucifers, such as arugula, mizuna mustard, red mustard, and tatsoi (82). Like other diagnostic laboratories, the University of California Cooperative Extension (UCCE) in Salinas is accustomed to receiving crucifer samples having angular, water-soaked leaf spots; technicians regularly recover the cream-colored, fluorescent colonies of *P. syringae* pv. *maculicola* (*Psm*), causal agent of bacterial leaf spot of crucifers [first described as pepper spot in 1911 by Lucia McCulloch (95)]. In 1995, the lab began receiving severely blighted rapini (*Brassica rapa* subsp. *rapa*; also called broccoli raab) that exhibited similar symptoms and from which the expected cream-colored, fluorescent bacteria were recovered. Although the symptoms differed by being more severe than usual, the lab initially diagnosed this rapini problem as bacterial leaf spot on the basis of previous experience with pseudomonads recovered from crucifers. Phenotypic tests (LOPAT, fluorescence, gram character, and ice nucleation ability) indicated that the pathogen belonged to *P. syringae* sensu lato (83, 113); however, additional research showed that the rapini pathogen had a novel host range (it could also infect monocots!) (32, 53) and phenotypes distinct from the pathotype strain of *Psm* (32, 71). Differences in pathogenicity were sufficient to justify a new pathovar (40, 142). Moreover, rep-PCR patterns demonstrated that the rapini isolates were genetically distinct from *Psm* and other pathovars of *P. syringae* and supported the designation of a novel pathovar *P. syringae* pv. *alisalensis* (32). Although phenotypically similar to most *P. syringae* pathovars, it was later shown to be a member of genomospecies 9, *P. cannabina*, and was renamed *P. cannabina* pv. *alisalensis* (*Pca*) (24).

The earlier inability to distinguish *Pca* from *Psm* was due, in part, to incomplete pathogen identification and to the prevailing concept of host specificity described as the "new host–new species cliché" (120, 121, 133), which prejudiced researchers against the idea that a fluorescent pseudomonad other than *Psm* could cause leaf spots on crucifers. For example, in 1965, Williams & Keen (137) isolated a fluorescent pseudomonad from radish in Wisconsin and identified it as *P. maculicola* B-70 (also called ES4326, M4, CFBP1637, and ICMP4326 and later employed as the pathogen in a well-studied model for molecular host-pathogen interactions). Although they documented significant differences in host range and virulence of B-70 as compared with both *P. maculicola* (now *Psm*) and *P. cichorii* on crucifers, they considered this pathogen different from *P. cichorii* but not *P. maculicola*. Strain B-70 has since been shown to be *Pca* (12, 24). Given this context, all strains identified as *Psm* prior to 2002 could actually be either *Psm* or *Pca*. We have documented additional *Pca* strains previously identified as *Psm* (26, 103) and misidentification continues (37) despite the availability of rapid molecular methods such as MLSA and rep-PCR (24). Misidentification and delayed discovery of *Pca* were largely due to a lack of comparison of new isolates with the pathotype strain of *Psm* coupled to a misunderstanding of the taxonomic

significance of differences in host range and rep-PCR patterns (37, 58, 103, 147). Therefore, crucifer diseases caused by *Psm* and *Pca* cannot be differentiated on the basis of symptoms, host, or recovery of fluorescent pseudomonads; differentiation requires the use of other diagnostic tools. Knowing whether the agent is *Psm* or *Pca* is important if crop rotation is the main management method employed, because of the broad host range of *Pca* versus the restricted host range of *Psm*.

A Host of Problems or the Problem with Hosts

The crux of many of our taxonomy and etiology questions still turns on host range of the pathogen. Prior to the 1980s, species definitions for plant-pathogenic bacteria included host range information (9, 39). The changes in the Bacteriological Code urged against "special purpose" classifications such as pathogenic specificity (14, 80). Because pathovars are defined by "distinctive pathogenicity to one or more plant hosts" (40, p. 154), host range information is no longer just useful in classification but is instead a codified criterion of formal nomenclature.

For purposes of disease management, epidemiology, and taxonomy, it is important to conceptualize host range in two different ways (11). The natural host range of a pathogen consists of plants growing in environmental settings, developing a disease from which the organism was isolated and to which the organism is subsequently confirmed to be pathogenic by completion of Koch's postulates. The experimental host range of a pathogen is determined by artificial inoculation of additional potential host plants under controlled conditions and subsequent reisolation of the causal agent. Whereas pathogenicity tests corroborate the association of a pathogen with the original and naturally occurring host, for experimental hosts the only association occurs under the ideal but artificial conditions of an experiment. Experimental host range lists can be much broader than natural host range lists. Therefore, the natural host range tells us what has happened in the field, and the experimental host range tells us what might happen in the future. Consequently, the natural host range of the pathogen may be the most important criterion for determining pathovar status.

A good case study of the use of molecular tools to help define the natural and experimental host range of pathogens involves Apiaceae crops in the Salinas Valley, which are planted on extensive acreage (7,829 ha) (82). Initially, bacterial leaf spot caused by *P. syringae* pv. *apii* was the only bacterial disease of celery (*Apium graveolens*) reported in the Salinas Valley (86). Likewise, the bacterial leaf spot pathogen of cilantro (also known as coriander; *Coriandrum sativum*), *P. syringae* pv. *coriandricola*, was the only known bacterial pathogen of cilantro (35, 70). Worldwide reports of these pathogens indicated they were host specific and only caused disease on celery or cilantro, respectively (12, 127). Other Apiaceae crops such as parsley (*Petroselinum crispum*) and fennel (*Foeniculum vulgare*) suffered from no bacterial diseases in our region. However, starting around 2002, a new and severe bacterial leaf spot disease of parsley was detected in the Salinas Valley. Interestingly, through field surveys, molecular analysis of isolates (using rep-PCR and MLSA), and pathogenicity experiments, we found that bacterial leaf spot of parsley was caused by three pathogens: *P. syringae* pv. *apii*, *P. syringae* pv. *coriandricola*, and *P. viridiflava* (12, 93). On parsley, the symptoms caused by these three pathogens were indistinguishable from each other. When we explored the experimental host ranges of these organisms, we found that each of the three pathogens caused indistinguishable symptoms on all three Apiaceae hosts (celery, cilantro, parsley).

Differences reported between experimental and natural host ranges may be due to lack of scrutiny of isolates from the reciprocal hosts (12). In order to determine the natural host ranges of *P. syringae* pv. *apii* and *P. syringae* pv. *coriandricola*, we conducted epidemiological studies in which we isolated 205 bacteria from individual lesions from 43 different regional outbreaks on all three Apiaceae crops. Identities of the isolates were confirmed by rep-PCR and in some cases by MLSA.

Parsley was clearly a natural host for *P. syringae* pv. *apii* and *P. syringae* pv. *coriandricola*. *P. syringae* pv. *apii* (38 isolates) was isolated from 4 of the 13 outbreaks on parsley, whereas *P. syringae* pv. *coriandricola* was isolated 68 times from 8 outbreaks. *P. viridiflava* has a broad host range but is a transient pathogen on parsley. Eight isolates of *P. viridiflava* were obtained from one outbreak of bacterial leaf spot on parsley during this study. Although plants were collected from disparate locations in fields, isolates recovered from each outbreak consisted of a single pathogen. On celery and cilantro, only the anticipated pathogens were isolated, only *P. syringae* pv. *apii* (85 isolates from 16 outbreaks) was isolated from diseased celery, and only *P. syringae* pv. *coriandricola* (141 isolates from 14 outbreaks) was isolated from cilantro. Subsequently, these methods were used to enlarge the natural host ranges for *P. syringae* pv. *apii* and *P. syringae* pv. *coriandricola* to include fennel (54), and parsnip and carrot (104), respectively.

Fingerprinting and sequencing tools can be used to help define the natural host range of bacterial plant pathogens. This level of detailed analysis of multiple strains from many outbreaks indicates that for some pathovars, although the experimental host range is large, the natural host range is more restrictive and clearly suggests differences in pathovars. Thus, experimental host range data should be carefully considered when contemplating biological and taxonomic significance. This may be especially true for environmental isolates for which no natural host has been identified (8). In order to further probe the relevance of natural and experimental host ranges, we are evaluating strains from our diverse collection of *P. syringae* sensu lato isolated from diseased plants in the Salinas Valley.

Revisiting Koch's Postulates

According to Alvarez (4, p. 339) ". . .diagnosis of truly unknown pathogens requires field observation, examination of plant tissues, isolation of the pathogen, characterization, and proof of Koch's postulates." However, application of Koch's postulates varies, and they are not specifically required by all journals for descriptions of new pathogen-host combinations (the natural host range of the pathogen). Also the methods for completing Koch's postulates vary widely. Experimental host range studies are likely to vary even more given that they are generally not treated as rigorously as natural host range studies; rarely are reisolates carefully compared with the original strains, if reisolations are made at all. Ideally, all four tenants of Koch's postulates (1, 39) should be required for any manuscript evaluating the natural or experimental host range of the pathogen.

Rapid genotyping methods have facilitated the implementation of Koch's postulates for bacteria because they allow for the fingerprinting of any strains without knowing their identity. This is helpful because isolations from single lesions, multiple plants, several different outbreaks, or different fields may yield a variety of colony types (4, 23). Thus, a systematic method of distinguishing genotypes among isolated strains is useful and allows for selection of a subset of isolates for use in preliminary pathogenicity tests (**Figure 1*a***). The greatest advantage is that the reisolates from all inoculated, symptomatic plants can be compared with the original strains used to inoculate the plants, thus confirming Koch's postulates (**Figure 1*b***). As stated, we use rep-PCR because it distinguishes the pathovars and, sometimes, interesting biotypes. Identification of the verified pathogens then proceeds using additional methods. Thus, we only invest resources in identification or classification of organisms that we first determine are pathogenic.

Is It a New or Existing Pathovar?

One significant problem for plant pathologists is determining the pathovar status of isolates after species identification. Within a species, the unknowns could be members of a previously described

pathovar or a new pathovar. The ideal scientific approach for determining pathovar status would be to compare the new isolates with the pathotypes of all previously described pathovars within the species in reciprocal pathogenicity tests (all pathotypes and new isolates on all original hosts of the pathogens). Because this is a practical impossibility, researchers may arbitrarily choose handy isolates or hosts, leaving host range studies, and thus pathovar determination, incomplete. If the pathotypes are available, rep-PCR and other fingerprinting techniques help reduce the number of hosts that need to be tested. As discussed below, the use of MLSA is even more effective at eliminating the need to compare unknowns with the majority of pathovars within a species and can simultaneously identify organisms to species within a known genus. MLSA allows the researcher to strategically concentrate on the pathogenic profile (unknown and pathotype strains on reciprocal hosts) of the pathovars most similar to the unknowns.

Phylogroup: group of organisms determined phylogenetically

sensu stricto: derived from Latin to mean in the strictest or smallest sense

As discussed earlier, *P. syringae* sensu lato (*P. syringae*–related species) (98) consists of nine species or genomospecies (**Figure 2**). Because these can be differentiated by MLSA, unknowns can be identified to their respective species or genomospecies and occasionally to phylogroups (8, 12). Pathovars from all other species and genomospecies are eliminated from comparisons, and the most important pathotypes to be compared are those from the same phylogroup. For *P. syringae* strains isolated from melon and squash rep-PCR, patterns were different from the pathotype strains of the known cucurbit pathogens: *P. syringae* pv. *aptata* (genomospecies 1) and *P. syringae* pv. *lachrymans* (genomospecies 2). An MLSA with *P. syringae* pathotypes demonstrated that these strains belong to genomospecies 1 (*P. syringae* sensu stricto) (111). This allowed us to concentrate our research effort (host range studies and other taxonomic comparisons) on the 11 members of genomospecies 1 and in particular to the most closely related pathovars. Because the sequences of the pathotypes and type strains within *P. syringae* sensu lato are available [PAMDB (2) and from C.T. Bull] researchers worldwide can use this approach to strategically identify which pathotypes and hosts are critical in their comparisons.

Additionally, plant pathologists and breeders are interested in identification of levels of diversity below the pathovar level. Using a published MLSA typing scheme (143), we identified 6 genotypes from 120 strains of *X. campestris* pv. *vitians* that cause bacterial leaf spot of lettuce and belong to *Xanthomonas hortorum* (132; C.T. Bull & R. Hayes, unpublished data). Strains from three closely related MLSA genotypes appear to represent a single race of the pathogen commonly found in California (17; C.T. Bull & R. Hayes unpublished results). We identified germplasm that differentiates an additional race that is distinguishable by MLSA and has not yet been found in California. We are currently working out the race structure for strains in the remaining MLSA genotype. In other epidemiological studies, alternative fine-typing strategies, especially WGS, are more useful for predicting races because of the difference between phylogeny of the genome and pathogenicity-related genes (46, 49, 50). Recently, WGS was used to identify genetic elements linked to genes involved in host interactions in *P. syringae* pv. *tomato* and allowed development of primers and PCR reactions to distinguish this pathogen from other *P. syringae* strains (56). MLSA and WGS therefore are powerful tools for predicting potential races and pathovars.

CONCLUSIONS: TRANSFERRING WHAT IS KNOWN TO THE PROBLEM SOLVERS

Louws et al. (87) and Alvarez (4) list obstacles to bringing modern molecular tools to extension and diagnostic laboratories: (*a*) the belief that exact identifications are not essential for disease management; (*b*) lack of equipment and training; (*c*) lack of confidence with the protocols; and (*d*) reliance on culturing techniques. A brief survey of 26 diagnostic laboratories (see **Supplemental Table 1**; follow the **Supplemental Material link** from the Annual Reviews home page at

Supplemental Material

http://www.annualreviews.org) indicated that half of the laboratories use pathogen-specific PCR, indicating that some of the technical and equipment issues have been overcome. However, only one and three laboratories use rep-PCR and MLSA, respectively, for identification. Thus, diagnosticians are using modern molecular tools developed by others (including commercial laboratories) for specific named plant pathogens (4, 97, 134) (**Supplemental Table 1**) but not for pathogen discovery.

Supplemental Material

Given the lack of training and funding for taxonomic research available nationally and internationally, there is a general lack of understanding of bacterial taxonomy by plant pathologists and other microbiologists (10, 101, 117). This leads to poor selection of taxonomically relevant controls, inappropriate data analysis, inadequate editorial review, and lack of placement of a broad array of findings in a taxonomic framework. Considering the high volume of samples analyzed by extension and diagnostic laboratories, it is understandable why these frontline scientists find it difficult to invest in new methodologies and apply these tools to pathogen discovery.

In contrast, many extension plant pathologists and diagnosticians may be more familiar with identifying fungi because of the abundance of observable morphological features of sporulating fungi and an understanding of basic fungal taxonomy because of those features. The UCCE lab in Salinas handles fungal diagnoses internally, but like approximately half of the laboratories surveyed, they rely on others to lead the bacterial identification and classification efforts when greater detail is needed (134) (**Supplemental Table 1**). In lieu of each diagnostic lab having access to a bacterial taxonomist, how do we ensure that the needed tools (technical and theoretical) are available for recognizing and characterizing novel bacterial pathogens?

Workshops combining bacterial taxonomy with hands-on coaching in the application of techniques have helped researchers in developing countries (in workshops funded by the USDA Foreign Agricultural Service) apply these methods appropriately within a taxonomic framework (104). It is the combination of theory and application together that takes researchers beyond being just consumers of techniques for identification of specific pathogens to pathogen discovery. Extension researchers and diagnostic laboratory leaders have expressed interest in having similar training provided through the National or Regional Plant Diagnostic Networks. This organization could also play a vital role in ensuring that researchers have access to DNA from the approximately 450 type and pathotype strains of plant pathogens. A central laboratory could obtain the appropriate permits and purchase the strains (for as little as $50 per strain) and then distribute DNA as needed for rep-PCR and other comparisons.

Experts predict that in the future researchers will use WGS for the identification of pathogens (5, 30, 41, 77). Because type and pathotype strains are being sequenced, curated sequences of these strains will be available to all researchers, eventually eliminating the need to have these strains or their DNA on hand for initial identification. Despite being available, widespread use of WGS data for routine identification will not occur until plant pathologists can send bacterial cultures to commercial laboratories and receive back WGS sequences. Even then, expertise would be required to assemble, process, and analyze sequence data, although some predict that post-DNA sequence analysis can be fully automated and delivered (30, 41).

Thus, we are headed into a future that will be less dependent on cultural phenotypes and will rely on genomic species concepts for bacterial classification and identification. Significant automation and affordability will be needed to allow plant disease clinicians and others to use MLSA, WGS, or other sequence-based analyses to make pathogen identifications that might advance the science of plant pathology as well as benefit the farmer in the field. Nomenclatural recommendations requiring diagnostic phenotypes (119, 136) must change to allow genomospecies to be named via a genomic species definition, or else the Bacteriological Code and the binomial system of nomenclature will become obsolete without that being our thoughtful intent.

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