SECTION II. Bacterial diseases

An Introduction to plant bacterial pathogens

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Short History

The proof that bacteria can cause plant diseases began a new discipline in plant pathology that can be traced back to the 19th century (Goto 1992). It was T.J. Burrill (1839-1916) of the USA who first proposed in 1878 that bacteria caused plant diseases (Schumann and D'Arcy 2006). Burrill demonstrated that fire blight of pear was caused by a bacterium in an inoculation test. He described it ".. under our microscope, the field is seen to be alive with moving atoms known in a general way as bacteria." (cited in Schumann and D'Arcy 2006). He named that bacterium *Micrococcus amylovora* in 1882. The fire blight of pear and other stone fruits have been renamed *Erwinia amylovora*. *Erwinia* was created to honor Erwin F. Smith, also of the USA, who, as a pioneer in plant bacterial diseases, had done a series of descriptions of plant pathogenic bacteria. It may be of interest to students of plant pathology to note that in a heated controversy with S. Fischer from 1897 to 1901 on whether bacteria could cause plant diseases, Smith firmly established the validation and concept of bacterial plant pathogens (Goto 1992). As a specific discipline of plant pathology, bacterial plant diseases were indeed established in 1901 by Smith (Goto 1992). For rice, of course, bacterial blight has been recorded in Japan since 1884. However, it was not established as a "disease" caused by a bacterial pathogen until 1911 when Bokura (Goto 1992) reported isolation of the bacteria and their ability to re-infect the rice plant.

Prospective for Plant Pathogenic Bacteria

The prokaryotes—microscopic single-celled bacterial organisms that have neither a distinct nucleus with a membrane nor other specialized organelles—constitute an essential component of the Earth's biota. No other group of organisms equals the importance of the prokaryotes in supporting and maintaining life on the planet. They possess immense metabolic diversity, dominate Earth's biogeochemistry, and constitute an enormous reservoir of untapped biotechnological potential. Yet to date, less than 5,000 prokaryotic species have been formally described. This relative low number is partly caused by the problems encountered in the isolation of the microorganisms in pure cultures and their characterization (Rosselló-Mora and Amann 2001). Moreover, the advent of molecular methods in recent decades has revealed that a majority of prokaryotes in the biosphere cannot be cultivated by standard methods (Amann et al 1995).

Optimistic estimates suggest that less than 1% of the existing bacterial species are cultivable (Giovannoni and Stingl 2005). The magnitude of prokaryotic diversity, however, still remains to be explored. The pioneering work of Torsvik (Torsvik et al 1990, 2002) has opened our imaginations to the possibility that bacterial species may number in the millions or even billions. Estimates are that a single gram of soil may contain so many different types of prokaryotes that accurate numbers remain unknown, ranging about 4,000-38,000 (Curtis et al 2002). Thus, we only know a fraction of the prokaryotic diversity and their potential in most natural habitats.

Until the discovery of DNA as the biological information containing molecules, prokaryote classification has been traditionally based solely on phenotypic characteristics. An important breakthrough in the late 20th century was the introduction of phylogenetic analyses to examine evolution through the sequencing of the small subunit ribosomal RNA genes (16S rRNA for prokaryotes and 18S rRNA for eukaryotes). Although the term "bacteria" traditionally included all prokaryotes, the scientific classification changed after C.R. Woese had constructed his tree of life showing that prokaryotes consist of two very different evolutionary domains called Bacteria and Archaea (Woese et al 1990). The phylogenetic tree of life reveals that the main diversity of life is not in the eukaryotes, but in the prokaryotes—Bacteria and Archaea (Doolittle 1999). (Archaea were originally seen as extremophiles that live only in harsh environments, such as hot springs and salt lakes. Nowadays they have been found to exist in a broad range of habitats, such as soils and oceans, and are mostly anaerobes. No examples of archaeal plant pathogens are known.)

Bacterial taxonomy

The discipline of taxonomy provides a framework for the scientific community to facilitate understanding and knowledge exchange. Bacterial taxonomy is the practice of three interrelated activities:

- Classification, i.e., the orderly arrangement of organisms into groups on the basis of similarities in morphological, biochemical, physiological, and genetic characteristics. And, since recently, also includes the allocation of organisms into a phylogenetic and evolutionary framework.
- Nomenclature, i.e., the labeling of the units defined by classification with a binomial name according to strict rules.
- Identification, i.e., the process of assigning an unknown organism to one of the units.

The species is the basic unit of bacterial taxonomy and its definition is founded on whole genomic DNA-DNA hybridization values. A bacterial species is essentially considered to be a group of strains that are characterized by at least one diagnostic phenotypic feature and whose DNA molecules show 70% or higher re-association values (Wayne et al 1987).

Nomenclature. The purpose of classifications is that they should be useful in first instance for identification. The purpose of nomenclature is to create a common language to ensure accurate communication among scientists, regulators, growers and the public, and thus avoid confusion. Appropriate scientific names are important as they carry information about the basic biology of the organisms, including statutory or quarantine actions in the case of pathogens. Nomenclature is practiced according to strictly defined rules (actually it is the only element of taxonomy that is controlled by strictly regulated processes). The naming of bacteria is regulated by the rules set forth in the International Code of Nomenclature of Prokaryotes (ICNP). However, these rules only apply to ranks at the level of subspecies and above, whereas infrasubspecific ranks are not covered. The pathovar is one infrasubspecific rank that is widely used in the classification and nomenclature of plant pathogenic bacteria (Bull et al 2008). Therefore, a special purpose nomenclature was developed for the naming of plant pathogens as pathovars at the infrasubspecific rank codified in the International Standards for Naming Pathovars (Dye et al 1980). Thus, the ICNP regulates the application of all prokaryote names, and the International Standards for Naming Pathovars give regulation to the names of plant pathogenic bacteria that are not regulated by the ICNP (Young 2008).

For many years, the common characteristic of plant pathogenic bacteria was their perceived host plant specificity; they were often named after the host from which they

were first isolated. However, the host from which the pathogen was first isolated may only reflect part of the host range as extensive host studies were often lacking. Thus, a given pathovar found on a host other than the 'normal' one might as well have been identified as a new pathovar. The formal prokaryote classification was exclusively based on classical phenotypic analyses and had not reliably produced clearly differentiated species. Classification systems were often influenced by the aims of the taxonomist and based on a few convenient shared characters, irrespective of any relationship to other bacteria. Many species had been named without comprehensive descriptions or lacked authenticating reference strains, making revision impossible. It was only when adequate culture collections were made available, allowing the comparison of strains, that it became clear that many previously published bacterial names were synonyms.

Recognition of the enormity of nomenclatural confusion and the lack of regulation of nomenclature led the International Committee on Systematics of Prokaryotes (ICSP) to make a new start in bacterial nomenclature. Central to the revision of bacterial nomenclature was the publishing in 1980 of the approved lists (Skerman et al 1980). When the approved lists were adopted, names of many important bacterial plant pathogenic species, especially those in *Pseudomonas* and *Xanthomonas*, were not included, thus leaving them without standing in nomenclature. The loss of names of important plant pathogens with implications for quarantine procedures caused considerable confusion in communication among plant pathologists, administrators, and agriculture advisers.

Therefore, to ensure that the plant pathogenic species published prior to 1980 remained recognizable, the International Society of Plant Pathology (ISPP) Committee on the Taxonomy of Plant Pathogenic Bacteria (CTPPB) adopted the infrasubspecific category pathovar in a special purpose nomenclature regulated by the International Standards for Naming Pathovars (Dye et al 1980). The primary intentions of the International Standards for Naming Pathovars were to maintain nomenclatural continuity for established plant pathogen species published before the implementation of the approved lists in 1980. The pathovar nomenclature allows plant bacteriologists to name pathogens for practical purposes. The International Standards for Naming Pathovars define a pathovar as "... a strain or set of strains with the same or similar characteristics, differentiated at infrasubspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts" (Young 2008). The concept 'distinctive pathogenicity' was introduced specifically to permit the formal differentiation of pathogens based on symptoms, e.g., *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. Within a pathovar, subpopulations pathogenic to different cultivars of a same host are differentiated as races. The principal requirements from the ICNP for species names are essentially the same for pathovars. Pathotype strains have the same function as type strains for species; they are name-bearers.

Classification is an outcome of ongoing research and therefore undergoes constant reinterpretation and change. It is obvious that the processes of classification determine nomenclature, and as classification schemes may change to better represent relationships among bacteria, the result may be a change in name for an organism from one genus to another. In order to keep track of these changes, a complete list of all plant pathogenic bacteria is maintained online on the website of the International Society of Plant Pathology (ISSP) Committee on Taxonomy of Plant Pathogenic Bacteria (CTPPB), which is regularly updated.

Thus, the ICNP and the International Standards for Naming Pathovars are complementary systems to set out the rules for the nomenclature of plant pathogenic bacteria. The International Standards for Naming Pathovars deal specifically with the naming of pathovars of plant pathogens. ICNP determines the overall rules for the naming of prokary-

otes. It is solely concerned with the correct formation and change of names according to a natural hierarchy (class, order, family, genus, species and subspecies). The correct name of a species is an italicized binary combination consisting of the genus name followed by a species epithet. Names of taxa above the rank of species are single words. For a species or subspecies the type is a designated strain. A type must be designated when a new genus, species or subspecies is proposed. The type strain is the name-bearer of the species or subspecies. It is also a requirement that pure cultures of type strains are deposited in at least two recognized culture collections in two different countries in order to be available to the scientific community for information and comparison. The preservation and availability of type strains are essential to taxonomic research (Tindall and Garrity 2008).

Presently, extensive taxonomic information on the prokaryotes is available online in web-based databases: the Approved Lists of Bacterial Names, Bacterial Nomenclature up-to-date, rRNA gene and genome sequences (GenBank/NCBI–EMBL–DDBJ), and microbial reference material in microbial resource centers. Amendments of the ICNP by the International Committee on Systematics of Prokaryotes (ICSP) are published in the International Journal of Systematic and Evolutionary Microbiology (IJSEM). Central to the regulation of bacterial nomenclature is the principle that all legitimate names must be validated by publication (either as an original manuscript or through listing on the Validation Lists) in the IJSEM, the official journal of the ICSP, before it can be considered to have a standing in nomenclature. In contrast, no journal is specified for valid publication of pathovar names but authors are requested to send a copy of the publication to the ISPP-CTPPB to facilitate inclusion of published names in the lists. More detailed information on the nomenclatural rules can be found in the reviews by Bull et al (2008) and Young (2008).

Aside from the taxonomic categories species, genus, and family, a number of infrasubspecific groups, distinguished in terms of some independent special characteristic, have been introduced. For example, strains of any species can be grouped in terms of biochemical properties (biovar or biotype), pathogenic reactions (pathovar), or antigenic characteristics (serovar). The term genomovar refers to a group of strains that are phenotypically similar but genotypically distinct. Genomovars are often seen as potential species but cannot be named until they can be differentiated from other genospecies by a phenotypic feature. Such infrasubspecific categories are not covered by the ICNP and papers on such taxa are outside the scope of IJSEM.

The prerequisite of the current prokaryotic species concept is the isolation of the microorganisms in pure cultures. In recent years, it became clear that the majority of bacteria are unculturable and therefore cannot be investigated in ways that provide the descriptions and type strains of pure cultures. As they cannot be cultured, there is no method by which type strains can be isolated or maintained. Sequence-based methods, such as the rRNA gene approach for exploring uncultured prokaryotes in natural samples, allow the retrieval of some information on uncultured organisms and permit the recognition of their uniqueness. As a temporal solution, the International Committee on Systematics of Prokaryotes (ICSP) implemented the category of *Candidatus*, which is not a rank but a provisional status. The reference material for *Candidatus* is either a DNA sample or a specific rRNA gene sequence. Most plant pathogenic *Candidatus* are members of the genus *Candidatus* Phytoplasma. Phytoplasmas are phloem-restricted bacteria lacking a true cell wall and belong to the class of *Mollicutes*. PCR-amplified 16S rRNA gene sequences, the plant host(s), the insect vector(s), and other molecular data are usually included in the descriptions of *Candidatus* Phytoplasma species.

Classification and identification. Every classification should be an attempt to arrange the natural diversity among bacteria into a hierarchical system. Classifications should in the first place be useful for identification and to produce as much relevant

information about an organism as possible. The appropriateness of classification systems and the methods used to develop them are evaluated through peer review (Tindall 1999). There are no strict rules for classification. Acceptance of a classification system and its associated nomenclature occurs as researchers adopt it as the preferred system and use it in the literature.

Chronological development: Originally, prokaryotic classification schemes were dominated by a phenotypic approach, based mainly on morphological and physiological characters. Classification systems were influenced by the aims of the taxonomist and, hence, based on subjective interpretations of phenotypic characters. In many cases, this led to artificial or special-purpose classifications with taxa based on a few convenient shared characters, irrespective of any relationship to other bacteria. For example, phytobacteriologists placed significantly more importance on pathogenicity rather than other properties and used host range as the primary standard for distinguishing organisms. However, knowledge of the host range is limited as, in most cases, no extensive host range studies have been performed. As such, this practice often leads to nomenclatural confusions, where a plant pathogenic species could be simultaneously classified under several different names (synonyms).

Since the 1960s, a number of breakthroughs were made that have resulted in our present nomenclature and classification of the Bacteria and Archaea. Computer-assisted numerical comparison of phenotypic data came up in parallel with the development of computers (Sneath and Sokal 1973), and allowed to interpret large numbers of phenotypic characters in an unbiased way, by giving equal weight to each character tested. The period of computer-assisted classification coincided with the rise of chemotaxonomy, which is considered another breakthrough in the development of modern bacterial classification. In the late 1970s, the cataloging of ribosomal ribonucleic acids (rRNA) introduced a phylogenetic dimension into prokaryotic taxonomy. The discovery of genetic information supported the idea that bacteria might best be classified by comparing their genomes, and classification based on the inference of phylogenetic relationships became popular.

DNA-DNA similarity: In 1987, DNA-DNA hybridization, an indirect parameter of the sequence similarity between two entire genomes, became the reference standard for species delineation. As early as 1970, this strategy had already been applied to plant pathogens (De Ley et al 1970). The rationale for using this standard for species delineation is based on numerous studies, in which a high degree of correlation was found between genomic DNA similarity and phenotypic similarity. Members of the same bacterial species have at least 70% DNA-DNA hybridization values and less than 5°C difference in melting temperature (**ΔTm**) between the homoduplex and heteroduplex (Wayne et al 1987, Stackebrandt et al 2002). For practical identification purposes, phenotypic consistency within the species and differences between species are required.

The value of 70% DNA-DNA hybridization proposed by Wayne et al (1987) as a recommended standard for delineating species is, however, not that strict. Sometimes strains of the same plant pathogenic species can show DNA–DNA hybridization values below 70% (e.g., within *Pseudomonas syringae*) (Konstantinidis and Tiedje 2005). In such cases, more relaxed boundaries of 50-70% DNA-DNA hybridization values are recommended for the species delineation. The use of DNA–DNA hybridization in bacterial taxonomy has recently been reviewed in detail by Rossello´**-Mora (**2006). DNA–DNA hybridization is a time-consuming, complex and labor-intensive method, and therefore, usually carried out with a relatively small set of strains. Moreover, the method is not applicable to non-culturable organisms, which constitute the largest proportion of prokaryotes (Amann et al 1995).

16S rRNA gene sequence: The advent in the mid-1980's of rRNA gene sequencing (5S, 16S, 23S) introduced a phylogenetic dimension into prokaryotic taxonomy. Especially

16S rRNA gene sequencing has been widely used as a primary technique to determine bacterial classification and phylogenetic relationships between distantly related bacteria at the family or genus level. Nowadays, almost complete sequencing of 16S rRNA genes is routine and it is recommended, as part of a polyphasic approach, to include a 16S rRNA gene sequence for new descriptions of bacterial species (Stackebrandt et al 2002).

The rRNA sequencing approach has important advantages (Staley 2006): the presence of universal bacterial sequences in the conserved portions of the rRNA molecule allows, by using PCR, performing a global classification of all bacteria including the unculturable ones present in environmental samples. It has allowed for directly positioning any uncharacterized organism, whether cultured or not, onto the tree of life by its 16S rRNA gene sequence. And most importantly, unlike the DNA–DNA hybridization technique, the 16S rRNA gene sequence information is archival. Once it has been determined, it is a definitive comparative feature of the strain of interest. And comprehensive databases of aligned rRNA sequences are publicly accessible online, such as: GenBank at the National Center for Biotechnology Information (NCBI), the European Molecular Biology Laboratory (EMBL), and the DNA Data Bank of Japan (DDBJ).

At the beginning of the 1990s, it was found that organisms with DNA–DNA hybridization values above 70% usually share more than 97% 16S rRNA sequence similarity. A recent study increased the recommended 16S rRNA gene sequence similarity for inclusion in the same species from 97% to 98.7–99% (Stackebrandt and Ebers 2006). If a strain shows less than 97% 16S rRNA sequence similarity to all known taxa, then it can be considered a new species. However, the bacterial species definition can never be based solely on sequence similarity of rRNAs because of the low resolving power of 16S rRNA at the species level. The comparative analysis of 16S rRNA gene sequences has been especially useful for measuring phylogenetic relationships at the genus level and higher taxonomic levels.

Phylogenetic reconstruction based on 16S rRNA gene sequence analyses has been responsible for many revisions of bacterial classification, especially for taxa above species. It has allowed the recognition of badly circumscribed taxa and their further reclassification. One remarkable example is the genus *Pseudomonas* that harbored many species that have been reclassified into different (often new) genera in the alpha subclass (e.g., *Aminobacter, Brevundimonas, Devosia, Oligotropha, Sphingomonas, Zavarzinia*), beta subclass (e.g., *Acidovorax, Burkholderia, Comamonas, Hydrogenophaga, Ralstonia, Telluria*), gamma subclass (e.g., *Chryseomonas, Flavimonas, Pseudomonas* sensu stricto), or between the beta- and gamma-subclasses (e.g., *Stenotrophomonas*) of the *Proteobacteria* (reviewed by Kersters et al 1996).

An example for which revisions were mainly based on extensive DNA-DNA hybridizations is the reclassification of the genus *Xanthomonas*, particularly the rearrangement of former *X. campestris* pathovars (Vauterin et al 1995). Indeed, revisions of nomenclature also concern plant pathogenic bacteria and sometimes cause confusion in the diagnostic and regulatory fields of plant pathology. For example, *Erwinia herbicola* was first redefined as *Enterobacter agglomerans* (Beji et al 1988) and then transferred to the new genus *Pantoea* as *Pantoea agglomerans* (Gavini et al 1989). Other named *Erwinia* plant pathogens were also reclassified in the new genera *Brenneria, Dickeya*, and *Pectobacterium* based on comparative analyses of 16S rRNA gene sequences (Hauben et al 1998, Samson et al 2005). Rice pathogens that were previously named *Pseudomonas* species and reclassified in other genera include *Burkholderia glumae, B. gladioli* and *B. plantarii* (Urakami et al 1994), and *Acidovorax avenae* subsp. *avenae* (Willems et al 1992) for which recently the new name *Acidovorax oryzae* has been proposed based on a polyphasic study by Schaad et al (2008).

Polyphasic classification: Prokaryotic species are currently characterized using a polyphasic approach that aims at the integration of many different properties including

genotypic, phylogenetic, and phenotypic information (Vandamme et al 1996). Diversity among bacteria is expressed in numerous different molecules, which explains why classifying bacteria by a single approach are bound to fail. Any character that reveals part of this diversity is therefore useful and can be considered. The number of different molecules that have been applied in taxonomic studies is large and their applications as markers are manifold. These include nucleic acids, protein- and carbohydrate-containing macromolecules, and lipids. Each of these molecules has advantages over the others in specific uses and resolution level.

In practice, a polyphasic approach for the characterization of a collection of isolates starts with making a choice of appropriate screening methods that allow the more closely related isolates to be clustered and to be distinguished from unrelated isolates. Screening methods must be appropriate for the group of bacteria under investigation and be relatively quick to compare a large number of isolates. Often used screening methods include whole-cell fatty acid analysis and a variety of DNA fingerprinting methods such as restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphism (AFLP), and several PCR-based methods using random primers (e.g., Random Amplified Polymorphic DNA) or repetitive sequences (rep-PCR). Subsequently, representative isolates are chosen from the different clusters and 16S rRNA gene sequencing and comparison of their sequences to those of known species in public databases, is the primary method for determining their phylogenetic affiliation. Based on the results of this analysis, few isolates are selected for DNA–DNA hybridization experiments to classify them at the species level using the 70% cut-off criterion. For describing new species, genomic information and phenotype need to be investigated. And one needs to find a diagnostic phenotype that discriminates a given species from its closest relatives to facilitate identification in routine microbiology laboratories.

The polyphasic approach to classification has widespread support because it is accepted that a species can only be classified through the analysis of a large set of features, combining phenotypic data (e.g., biochemical tests), chemotaxonomic data (e.g., fatty acid composition), genotypic data (e.g., DNA fingerprints) and phylogenetic information (e.g., rRNA gene sequences) in order to achieve a consensus type of classification (Vandamme et al 1996).

Methods for classifying bacterial diversity. A wide range of phenotypic and genotypic methods has been used for prokaryote classification. The rise of molecular and sequence-based techniques has caused a significant shift towards genotypic approaches. The different methods can be ordered according to their resolving power and their technical complexity. But it should be emphasized that limitations exist in the applicability of each technique depending on the group of organisms under study. Therefore, it is of primary importance to understand at which level a particular method provides information, and to select the most appropriate method according to the organisms under investigation. One has to keep in mind that a single, ideal identification technique does not exist. An extensive review on the different methods and their resolving power for prokaryote classification has been published by Vandamme et al (1996).

Phenotypic methods: Phenotypic and chemotaxonomic methods comprise all methods that are not directed toward DNA or RNA. They can roughly be divided into classical phenotypic tests, chemotaxonomic typing methods, and miniaturized identification systems.

The formal prokaryote classification was exclusively based on classical phenotypic analyses. The classical phenotypic characteristics of bacteria comprise morphological, physiological and biochemical features. The morphology of a bacterium includes both cellular (shape, endospore, flagella, fimbriae or pili, inclusion bodies, gram staining) and

colonial (color, dimensions, form). The cell size and shape of bacteria are described as follows.

Bacteria are small, single-celled prokaryotic organisms that reproduce by binary fission and evolve mainly through mutation. Most plant pathogenic bacteria are rod-shaped and have cell walls. The size of the cells varies depending on several factors such as temperatures for incubation, types of culture medium, and age of the lesions from which cells are isolated. When cells age due to either continuous culture or old lesions, their size tends to be smaller. In general, the size of most plant pathogenic bacteria is between 1.0-5.0 x 0.5-1.0 µm.

Phytoplasmas lack cell walls and are pleomorphic. There are two rice diseases caused by phytoplasma organisms, which are discussed in the section of on rice virus diseases. The phytoplasma-like organisms are usually spherical or ovoid in shape with a size of 0.3 to 0.2 µm in diameter. (For more details on phytoplasma-like organisms, refer to the section on Virus and Phytoplasma Diseases of Rice in this online resource.)

The physiological and biochemical features include data on growth at various environmental factors such as temperature, pH, salinity or oxygen, growth on various carbon sources, growth in the presence of various substances such as antibiotics, and data on the presence or activity of various enzymes. These phenotypic tests are still commonly used for identification and diagnostic purposes in routine microbiology laboratories. Hence, there is a requirement for each new species description to include discriminative phenotypic features.

In phytopathology, phenotypic features commonly tested for plant pathogens include production of plant cell wall-degrading enzymes, the ability to elicit a nonhost hypersensitive response (HR), and the ability to cause disease on certain host plants.

The hypersensitive response (HR) is a plant defense mechanism elicited by the presence of a pathogen in a non-host tissue. Most plant pathogens can induce a HR in non-hosts plant species (Klement et al 1964). Tobacco (*Nicotiana tabacum*) is frequently used in HR tests because its large leaf panels can be easily infiltrated with a bacterial suspension. The rapid and localized cell death at the site of infection limits the spread of the invading bacteria and results in the collapse of the infiltrated leaf tissue. A positive HR is an indirect indication of the presence of molecular determinants for pathogenicity in the tested bacterium.

Pathogenicity, i.e., the ability of an organism to cause disease on host plants, is determined by performing a pathogenicity test. Fulfillment of Koch's postulates is required to establish a causal relationship between a causative organism and a disease. During the nineteenth century, Robert Koch proved the 'germ theory' by demonstrating the role of bacteria as pathogens causing disease. Koch's postulates are still used today, and set out criteria to test if an organism causes a disease. Confirmation that an organism causes disease symptoms (fulfilling Koch's postulates) requires a host on which a pathogenicity test is performed. A pure culture of bacteria recovered from the diseased tissue is artificially inoculated into the host species. The inoculated bacteria must reproduce the same disease symptoms, and must be re-isolated and confirmed to be identical with the inoculated culture.

Chemotaxonomic methods provide information on various chemical constituents of the cell, notably amino acids, lipids, proteins and sugars in order to classify bacteria. Several cellular compounds that belong to the bacterial phenotype have been used in typing systems to characterize the variability within species. Serotyping is based on the variability in the antigenic constituents of the cells (cell envelopes, flagella, fimbria). In multilocus enzyme electrophoresis (MLEE), native enzymes are electrophoretically separated and stained for enzyme profile and their mobilities are compared. Sophisticated analytical

techniques, such as pyrolysis mass spectrometry and UV resonance Raman spectroscopy, which examine the total chemical composition of bacterial cells, have only been used on particular groups of bacteria.

Chemotaxonomic methods that have been extensively used for comparing and grouping large numbers of isolates are whole-cell fatty acid analysis and whole-cell protein analysis. Whole-cell protein profiles are obtained by extraction of proteins and separation on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and they provide discriminative information at or below the species level. Whole-cell fatty acid analysis is a chemotaxonomic method in which the lipids that are present in bacterial cells are analyzed by gas chromatography and used to delineate clusters at the genus level. The method comes with a database, is cheap, rapid, and has been widely applied and proven useful for classification and identification of bacterial taxa (Welch 1991).

Miniaturized identification systems (e.g., Biolog, Biolog Inc., Hayward, CA; Vitek, Biomérieux Inc., Hazelwood, MO) come as commercially available kits and are mostly based on the classical phenotypic tests. These methods are highly automated and have computer-generated identification databases. The major problem of such methods, however, is the dependence of the identification results on the quality of the database. The current databases used in automated identification systems are heavily dominated by clinical isolates. Therefore, these systems have been less successful in the identification of environmental isolates and should be applied very cautiously to samples from natural environments.

Genotypic methods: The methods of genomic information retrieval dominate modern taxonomic studies because of the present view that classification should reflect genotypic relationships as encoded in the DNA. The taxonomic information about a bacterium is incorporated in the complete nucleotide sequence of its genome (Stackebrandt et al 2002). One of the most fundamental direct genomic analysis methods is the total DNA-DNA hybridization technique, an indirect parameter of the sequence similarity between two entire genomes. In fact, the 70% value of DNA–DNA hybridization between two genomes has been accepted as the standard for the operational species definition (Wayne et al 1987) and is still being widely used (Stackebrandt et al 2002).

Another direct genomic analysis method based on the digestion of total genomic DNA with restriction enzymes and analysis of the resulting banding patterns after hybridization with specific genomic probes is restriction fragment length polymorphism (RFLP). A typical example of such approach is the ribotyping method, which uses rRNA gene-specific oligonucleotide probes.

Comparative analysis of rRNA sequences has become the standard for studying phylogenetic relationships and is today indispensable in polyphasic taxonomy. The universal bacterial sequences in the rRNA molecule can be used for PCR techniques and have made it possible to detect uncultivated microorganisms and to understand them in a phylogenetic context. Also, techniques applying phylogenetic markers such as 16S rDNA sequences and novel separation techniques to the PCR amplified gene are widely used in microbial ecology studies to rapidly screen microbial communities. Such methods as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and single-strand-conformation polymorphism (SSCP) involve the separation of PCR-amplified DNA products of the same length but of different primary sequence composition based on electrophoretic mobility. Alternatively, length polymorphisms can be assayed directly, as in ribosomal intergenic spacer analysis (RISA) and terminal restriction fragment length polymorphism (T-RFLP). More information on methodologies used for microbial community analyses can be found in reviews by Tiedje et al (1999) and van Elsas et al (1998).

The introduction of the PCR methodology has led to the development of a vast array of PCR-based DNA-typing methods, which have attracted much interest because of their simplicity and general applicability. PCR-based genomic fingerprinting methods include arbitrarily primed (AP-PCR) or randomly amplified polymorphic DNA (RAPDs), repetitive sequence-based rep-PCR, and amplified fragment length polymorphism (AFLP) analysis. The AFLP-method is a combination of restriction enzyme analysis and PCR-based typing that screens for amplified fragment length polymorphisms by selective amplification of restriction fragments. Rep-PCR genomic fingerprinting refers to the protocols known as REP-, ERIC-, and BOX-PCR, which respectively use primers binding to conserved repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences, and BOX elements, distributed in the genomes of diverse bacteria. Each of these protocols generates a collection of genomic fragments via PCR, which are resolved as banding patterns that allow the detection of infraspecific diversity, e.g., the differentiation of strains within a named species. The principle of enhanced specificity using restriction enzyme analysis is applicable for any amplified product and generically may be referred to as PCR-RFLP. PCR-RFLP applied to ribosomal gene sequences is known as amplified ribosomal DNA restriction analysis (ARDRA), in which universal primers are used to amplify bacterial rDNA sequences (with or without spacer regions), followed by digestion with frequently cutting restriction endonucleases and gel electrophoresis, to characterize bacterial isolates at the genus and species level.

A modern sequence-based approach, multilocus sequence typing (MLST), uses the allelic mismatches of seven housekeeping genes for the genotypic characterization of prokaryotes at the infraspecific level. MLST has emerged as the method of choice for studying intraspecies genetic diversity (Maiden 2006). The term MLSA (multilocus sequence analysis) is used when, instead of the allelic mismatches, the concatenated sequences of multiple protein-coding genes are used for analysis. MLSA is better suited for the genotypic characterization at different levels ranging from infraspecific to genus (Gevers et al 2005). The selection of suitable housekeeping genes for MLSA can represent the overall genotypic characteristics of bacterial taxa, including plant pathogens. In addition to its use in typing bacteria, MLSA can be used to estimate recombination rates between bacteria (Maiden 2006). Multilocus sequence analysis has been proposed as an alternative method that can replace the cumbersome DNA-DNA hybridization experiments (Gevers et al 2005).

The most complete genomic source of information is, of course, the entire bacterial genome. Dozens of prokaryote genomes have currently been sequenced and many more will be sequenced in the near future (completed bacterial genomes can be found online at the website of NCBI). Recent studies with strains for which complete genome sequences are available have shown that DNA-DNA hybridization values correlate well with the genome sequence-derived parameters ANI (average nucleotide identity) and the percentage of conserved DNA (Goris et al 2007, Konstantinidis and Tiedje 2005). Both genome sequence-derived parameters are limited in their application to sequenced genomes. The percentage of conserved DNA between two strains is calculated as the sum of the lengths of the alignable regions divided by the total length of the genome (Goris et al 2007). The average nucleotide identity (ANI) is calculated from pair-wise comparison of all genes shared between two strains. The classical cut-off point of 70% DNA-DNA hybridization similarity for species delineation corresponds to 94% ANI and 69% conserved DNA. Thus, it was concluded that using the 94% ANI criterion can accurately replace the 70% DNA-DNA hybridization value for strain assignment to species (Goris et al 2007).

Prokaryotic taxonomy is being influenced by the ease with which sequence data can be obtained. Today, the genomes of important plant pathogens have been or will be

sequenced, and the molecular basis of pathogenicity is beginning to be deciphered. It is noted that bacterial pathogenicity genes commonly are found in specialized areas termed pathogenicity islands, or on one or more extra-chromosomal elements (plasmids).

Additionally, it has been observed that substantial parts of the chromosomes of many bacterial species have been acquired from unknown sources by horizontal gene transfer (Ochman et al 2000). For example, homologous *hrp* genes, implicated in the interactions between bacterial pathogens and their plant hosts, are found in separate subclasses of the *Proteobacteria*, which is likely to be explained by horizontal gene transfer. The acquisition of genes by horizontal gene transfer can lead to new properties such as virulence or resistance to antibiotics. Processes such as gene loss, horizontal gene transfer, homologous recombination and chromosomal rearrangements shape the prokaryotic genome and are far more widespread than previously thought.

Species definition challenged

The advent of genomics has increased our understanding of the prokaryotic genetic diversity including the diversity of bacterial plant pathogens. Consequently, it has been noticed that many named species are diverse in their genomic content and ecological properties, and appear to consist of multiple ecotypes. The species concept is continuously being challenged by the growing amount of genomic information, and opinions differ widely on what constitutes a microbial species. The ways that microbiologists view the species concept for prokaryotes have been reviewed recently in papers by Cohan (2006), Gevers et al (2005), Staley (2006). and Ward et al (2008).

The present species definition, being used for the delineation of bacterial species including plant pathogens, is based on

- a certain degree of phenotypic consistency,
- 70% DNA–DNA hybridization between the total genomic DNA of two species,
- 97-98% similarity in the 16S rRNA gene sequence, and
- about 94% average nucleotide identity (ANI) of genomes.

This operational species definition, while pragmatic and universally applicable within the prokaryotic world, has been criticized for being too broadly defined to provide a clear picture of bacterial diversity since the genetic differences revealed among strains of a named species are often large enough to justify the description of the strains as different species (Cohan 2006). Research on plant pathogens consists of describing the genetic diversity within plant pathogen populations and to correlate the revealed genetic diversity to ecological and functional diversity within those populations (Vinatzer and Bull 2009). In the present situation, however, modern genomic approaches uncover genetic diversity at ranks below the level of the described bacterial species and, these subpopulations may represent ecologically relevant units, for which there are no codified taxa or nomenclature available. Therefore, a classification and nomenclature that matches our understanding of the genetic and ecological diversity of a bacterial plant pathogen is most wanted, as the current species definition is not up to this task.

Population geneticists have used a high-resolution method, multilocus sequence typing (MLST), to show that the subpopulations within named species can resemble ecologically adapted species populations (ecotypes). MLST is a method for recognizing distinct strains within named species, using the allelic mismatches of seven housekeeping genes (Gevers et al 2005). Population geneticists advocate an evolutionary-ecologically based species concept that envisions ecotypes to be fundamental species-like units that occupy unique niches within microbial communities. Ecotypes are defined as populations

that are genetically cohesive and ecologically distinct. It also recognizes that novel properties (for example, the acquisition of pathogenicity islands through horizontal gene transfer) may influence ecotype.

The species definition, whatever it may be, should be pragmatic and useful, as the ultimate function of the definition is to serve as a tool for the identification of individual isolates and to allow communication. Bacterial taxonomy is constantly evolving together with the rapid technical advances in molecular biology that provide new insights into prokaryotic diversity. DNA-DNA hybridization is currently the accepted standard but is likely to be replaced by sequence-based techniques in the not-too-far future.

Rice Bacterial Diseases

There are more than 10 rice diseases caused by bacterial pathogens in a straight sense. If we include those caused by phytoplasma-like organisms, the number will be increased to a dozen or more in total. However, in this online reference, we intentionally place rice diseases caused by phytoplasma-like organisms together with those caused by viruses because of the similarity in disease symptoms, and mode of transmission.

Over the past decades, the importance of bacterial diseases of rice comes from two stage of their occurrence, which has caught the attention of rice scientists. This is due primarily to the response of the bacterial pathogens to changes of rice cultivation: one relates to the change of new plant types and the other to new methods of planting. The response to the change of new plant types was a series of epidemics of bacterial blight occurring in the early seventies and has continued to occur periodically until this very day. These epidemics on then newly released or introduced semidwarf, nitrogen responsive, high-yielding varieties such as TNI, IR8, and Jaya in South and Southeast Asia have alarmed rice scientists and policymakers alike.

The outcome is to realize that not only bacterial blight, specifically, but bacterial diseases, in general, could be a threat to rice production if there is no built-in resistance. Because of mechanization taken place first in Japan then followed by Korea, Taiwan, and mainland China, a new method of raising rice seedlings has been developed—the indoor seedbox method that has replaced the traditional outdoor nursery bed. As a result of this change of method of raising seedlings, seedling blight or seedling rot caused by *Burkholderia glumae* and others was an outbreak in seedling boxes. (For more information, see Chapter 1 in this Section on seedling diseases.)

With this change, we suddenly experienced a group of new bacterial diseases that appear to be closely associated with the seedling stage and all appear to be carried by rice seeds to become a nuisance in rice crop production. Although rice seeds are known to harbor a large number of bacteria (Cottyn et al 1996a,b), their importance as rice diseases affecting rice crop production is only evident when the traditional seedbed nursery has changed to indoor seedbox due to mechanization in response to labor shortage. Obviously, there is always a cost associated with the change but some of the changes are necessary as inevitable technological advancement. There is always a challenge in our ability to predict or project the potential problems associated with these changes to make new approaches in rice production more sustainable.

In some areas, climate change can also have an effect on the outbreak of rice bacterial diseases that might have just escaped our attention. While many of the bacterial diseases are common in rice in various regions of the world, grain rot in Asia (Uematsu et al 1976) or bacterial panicle blight in the southern United States appears to arise when night temperature increases during flowering time (Nandakumar et al 2009). High night temperature causes rice yield decline in tropical environments (Peng et al 2004). The high night temperature likely promotes the growth of a specific bacterial pathogen. *Burkholde-*

ria glumae is frequently detected from rice seeds (Cottyn et al. 2001, 2009), and may have been involved, together with other seedborne bacteria and fungi, in causing grain discoloration in tropical environments (Cottyn et al 1996a,b). The sudden occurrence of grain rot in the southern United States (Nandakumar et al 2009) and in southern Korea (Jeong et al 2003) appeared to be related to the increase in night temperature.

The types of symptoms caused by bacterial pathogens are not drastically different from those caused by fungal pathogens. For the types of symptoms on host plants infected by fungal pathogens, see **Section 1** on fungal diseases. As a diagnostic procedure, symptoms associated with bacterial diseases are often water-soaked in appearance and bacterial ooze exuding from lesions. When an infected plant tissue such as leaf is cut and placed in a drop of water on a glass slide, bacterial stream can be observed oozing out. Uniquely on rice, many bacterial diseases are caused by *Burkholderia* and *Pseudomonas* spp., but those bacterial diseases, which have posed serious production threats in the past on a global scale, are those caused by *Xanthomonas* spp.

The chapters on rice bacterial diseases that follow are organized according to target plant organs for attack such as seedlings, leaves, grains, and culms. Thus far, there is no known rice root disease that is caused by a bacterial pathogen.

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