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Phytoplasmas: The Evolution of a Molecular Taxonomic Genus*)
Phytoplasmen: Die Evolution einer molekularen taxonomischen Gattung

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It is indeed an honor to attend the 75th anniversary of the BBA research station at Dossenheim. Although scientists at Dossenheim have made numerous contributions to our knowledge of plant diseases that impact tree fruit crops in Germany and around the world, today I would like to discuss some of the contributions that one of your esteemed colleagues, Dr. ERICH SEEMÜLLER and his research group, as well as other researchers have made in advancing our understanding of the elusive group of plant pathogens, the phytoplasmas, which for many years were called mycoplasma-like organisms (MLOs).

These accomplishments are even more noteworthy when one considers that our current detailed taxonomic knowledge of the phytoplasmas has been elucidated only in the past 8 years. I would like to present a historical perspective of the research breakthroughs that led to the proposed formal taxonomic classification of MLOs as phytoplasmas. Although numerous research groups from around the world have contributed to our knowledge about phytoplasmas, I would like to focus on the contributions that have been made here at Dossenheim and in my laboratory at the University or California, Davis.

1 Historical perspectives on the „Yellows Virus“ diseases

At least two major types of graft-transmissible diseases which induced chlorosis in their plant hosts have been recognized for over one hundred years. These diseases were considered to be virus diseases because no bacteria or fungi could be observed or isolated from diseased plants and high temperature often cured plants of these diseases. In addition, all of the diseases could be grafted from diseased to healthy plants and some diseases, such as aster yellows (AY) and X-disease (X), could be transmitted by some phloem-feeding insects such as leafhoppers, in which these plant pathogens multiplied and in some cases caused disease.

One group of the yellows pathogens produced profound changes in normal flower morphology and pigmentation. Phyllody, the production of leaf-like petals and sepal, and virescence, the production of green pigmented petals, were characteristic for the AY and other genetically different phytoplasmas such as the beet leafhopper agent that is found in western North America. Pioneering research conducted by A.O. KUNKEL during the 1920s demonstrated the graft and insect transmissibility of the AY pathogen and he showed that AY could infect a very broad range of herbaceous plant hosts. For this reason, for many years it was thought that any plant showing symptoms of virescence and phyllody was infected with the AY pathogen, a perception we now know is incorrect.

The second group of the yellows pathogens also induced typical foliar chlorosis however they did not induce virescence nor phyllody. In addition, many of these pathogens were associated with severe decline diseases of trees, although in some cases they could also be transmitted to certain herbaceous plants. Examples of this group of phytoplasmas include X-disease of stone fruits in North America, apple proliferation in Europe, coconut lethal decline and yellowing in Africa and the Caribbean, and pear decline which now occurs world-wide.

Although symptomatology, graft and insect transmission, and sensitivity to heat all suggested the yellows diseases were caused by a viral pathogen, all attempts to isolate virions from these infected plants were unsuccessful. In 1967, one of the most profound discoveries in plant pathology was reported by two Japanese scientists, DOI et al. reported numerous wall-less, pleomorphic bodies in the phloem sieve elements infected with several different yellows diseases. They also reported similar organisms in the salivary glands of leafhoppers that could transmit the yellows diseases. ISSHIE et al. reported that application of tetracycline antibiotics to yellows diseased plants caused a temporary remission of symptoms and a concurrent elimination of these organisms in the phloem. Because these organisms morphologically resembled the wall-less prokaryotes that caused pleural pneumonia in laboratory animals, they first called these organisms pleural pneumonia-like organisms or PPLOs. Later the animal PPLOs were taxonomically classified as mycoplasmas so the plant pathogen name was likewise changed to mycoplasma-like organisms or MLOs.

Although the true mycoplasms can be cultured in vitro on complex bacteriological media, all attempts to culture the plant MLOs on a great variety of artificial substrates have failed. Although numerous electron microscopic studies conducted between 1968 to the mid 1980s documented the association of MLOs with more than 200 plant diseases from around the world (McCoy et al., 1989), virtually nothing was known about the genetic diversity of these organisms. The only way that MLOs were classified was on the basis of biological properties such as host range, symptoms and insect-vector relationships.

2 Isolation and serological characterization of phytoplasmas

In 1980 there was only one published report that claimed to isolate MLOs from infected plants (SINHA et al., 1974). Despite numerous attempts by the author and others, no one could repro-
duce these results. In 1982 I developed a procedure which success-
fully isolated western X-disease phytoplasmas (WX-P) from
experimentally infected Collodion montanus leafhoppers.
Leafhoppers, rather than plants, were chosen because previous
EM studies by Nasu et al. (1974) showed high titers of the WX-
P in infected leafhoppers and there would be less chance of pro-
ducing plant antibodies when the WX-P fractions were injected
into rabbits. The procedure consisted of grinding infected
leafhoppers in an osmotically supplemented buffer and removing
many host contaminants by a series of differential centrifuga-
tions. The pathogen enriched fraction was passed through filters
that removed many of the walled bacteria that are found in
leafhoppers but allowed the passage of the wall-less phyto-
plasma. The enriched fraction was finally treated with antibodies
against healthy plant proteins in order to remove these protein
prior to injecting the phytoplasma-enriched fraction into rabbits.
Antibodies produced against the WX-P were used in ELISA to
successfully detect and follow the multiplication of the pathogen
in herbaceous and woody plant hosts, as well as infected leafhopp-
ers. Interestingly, the WX-P preferentially multiplied in celery
plant roots, rather than shoots, following inoculation with insect
vectors. ELISA studies also showed that WX-P first multiplied to
highest titers in cherry fruit peduncles early in the growing sea-
on, and titers were highest in the leaves during late summer and
fall. The ELISA values also correlated very nicely with the abili-
ity of healthy leafhopper vectors to acquire and subsequently
transmit the WX-P to plants. Optimal transmission occurred in
late summer and early fall when titers were highest in the leaves
that leafhoppers fed upon. This information, and further testing
of additional WX-P plant reservoirs, showed that infected sweet
cherry trees were the most important source of WX-P inoculum
in California. Management strategies based upon timely removal
of WX-P-infected cherry trees have significantly reduced the in-
cidence of X-disease in California.

When used in ELISA, antibodies specific for the WX-P did not
react with virescence-inducing agents such as the AY- and beet
leafhopper-transmitted virescence agent (BLTVA). However,
positive ELISA reactions were obtained with other woody de-
cline agents such as peach rosette and walnut witches' broom.
Western blot analyses showed that the WX antiserum recognized
2 major WX-P antigens, results that were similar to those ob-
tained with other phytoplasmas (Clark et al., 1989; Cadwill,
et al., 1988; Lin and Chen, 1985). No reaction was obtained be-
 tween the WX antibodies and any cultivable members of the
class Mollicutes.

Overall, serological detection of phytoplasmas, using either
polyclonal or monoclonal antibodies, has been useful for detect-
ing a specific phytoplasma if it occurs in reasonable titers in a
host. However, because of its relative specificity, serology has
provided only limited information on the relationships between
various phytoplasmas and no information concerning the rela-
tionships between phytoplasmas and other culturable prokary-
ates.

3 Characterization and cloning of phytoplasma DNA

3.1 Isolation and cloning of phytoplasma DNA

It became clear that serological methodologies would not allow
distantly related phytoplasmas to be compared with each other or
with other culturable prokaryotes. Beginning in 1982 many of
the stringent safety protocols which had prevented the routine
use of simple recombinant DNA technologies began to be re-
laxed. As a graduate student, I was fortunate to be in a virology
laboratory at UC Berkeley which quickly adopted recombinant
DNA technologies. We hypothesized that phytoplasmas could be
readily compared using random fragments of the various phyto-
plasmas as hybridization probes. DNA was isolated from the
same type of MLO-enriched fractions that were used to produce
WX-specific antibodies. The DNA was centrifuged in cesium
chloride density gradients and a unique, A+T rich band of DNA
was observed in gradients of infected but not healthy leafhopp-
ers. This band was recovered from the gradient and cloned in E.
coli, using a standard plasmid vector. Twenty- four unique,
cloned fragments of the WX-P chromosome were identified by
screening the recombinants with labelled DNA from healthy and
diseased plants and insects (Kirkpatrick et al., 1987). Similar
strategies were used to clone fragments of the western AY-P
(Kuske et al., 1991a), AP-P (Bonnet et al., 1990), and numerous
other phytoplasmas (Lee et al., 1993a and 1993b).

Another strategy for isolating highly enriched phytoplasma DNA was developed by Kollar et al. (1990) at the BBA Dossen-
hein and used by many others for cloning phytoplasmas. This
method involves the isolation of total nucleic acids from a phyto-
plasma-infected host and repeated centrifugations of the DNA
in cesium chloride gradients containing the DNA intercalating
dye, bisbenzimide. Because bisbenzimide preferentially binds to
A+T-rich DNA, phytoplasma DNA can be readily separated from
host DNA. This strategy greatly increased the efficiency of
cloning low titer phytoplasma DNA such as coconut lethal yel-
lowing (Harrison et al., 1992). In addition, it was possible to es-
timate the G+C content of several phytoplasmas which ranged
from 25 to 30% G+C, a value that is consistent with other cul-
 tural members of the class Mollicutes (Kollar and Seemüll-
ler, 1989).

When radioactively labelled and used as hybridization probes,
cloned fragments of WX-P DNA provided superior detection of
the WX-P in herbaceous and woody plants as well as infected in-
sects. Hybridization analyses greatly facilitated the identification
of plant reservoirs and insect vectors of the WX-P in California.
This information led to the formulation of disease management
procedures that decreased both the incidence and severity of WX
disease in California.

3.2 Genetic relationships among phytoplasmas revealed
by dot blot and RFLP analyses

Hybridization studies using 24 cloned fragments of the WX chro-
omosome showed that the WX-P was closely related to several
tree-infecting phytoplasmas such as Eastern X, peach yellows
and peach rosette, walnut witches' broom, and pecan bunch dis-
cases. Only a few of the WX-P clones hybridized to virescence
phytoplasmas such as AY and BLTVA pathogens, suggesting
these phytoplasmas were not genetically similar to the WX-P.
Numerous other researchers expanded these original observa-
tions with cloned hybridization probes derived from a variety of
phytoplasmas. Thus a picture began to emerge concerning the ge-
etic relatedness between the numerous phytoplasmas. Interest-
ingly, none of the cloned fragments of phytoplasma DNA hy-
bridized with any DNAs from culturable mollicutes, which sug-
gested that the phytoplasmas were either a very different group
of mollicutes or they were completely unrelated to other molli-
cutes.

Cloned phytoplasma probes used in restriction fragment
length polymorphism (RFLP) analyses provided even greater in-
formation concerning the genetic relationship between phyto-
plasmas. For example, early work by Kuske et al. (1991b) showed that considerable genetic variation exists between vari-
ous AY strains, results that were confirmed and greatly expanded by Lee et al. (1992). RFLP studies by Dr. Seemüller’s group
showed that there were at least two distinct genotypes of the AP-
P (Kison et al., 1994), most of the European stone fruit phyto-

plasmas were genetically similar (Ahrens et al., 1993), elm phytoplasmas in Europe and North America were genetically indistinguishable (Maurer et al., 1993), and considerable variation existed in various pear decline strains in Europe (Lorenz et al., 1995).

RFLP analyses of native and PCR-amplified phytoplasma DNAs continue to provide valuable insights into the genetic diversity of a particular phytoplasma species. This type of information is particularly important in evaluating the specificity of a phytoplasma detection assay, and in the development and evaluation of disease management strategies that involve removal of phytoplasma plant reservoirs or management of insect vector populations.

3.3 Many phytoplasmas possess extrachromosomal (plasmid) DNA

Hybridization analyses using the first cloned fragments of phytoplasma DNA revealed that many of the cloned fragments of phytoplasma DNA did not hybridize to chromosomal DNA fractions but rather to extrachromosomal elements (plasmid) of the phytoplasma (Davis et al., 1988; Kuske et al., 1991; Schneider et al., 1992). Plasmid DNA was particularly abundant in those phytoplasmas that caused virescence and phyllody symptoms in their plant hosts. Although a few plasmid DNAs have been reported in some of the culturable mollicutes, the overall abundance of these elements is much greater in the phytoplasmas. In many of the culturable mollicutes, especially the spiroplasmas, these extrachromosomal elements are actually replicative forms of DNA phages that infect the mollicute hosts. It is not known if most of the phytoplasma extrachromosomal DNAs are of viral origin, however virus particles have not been observed in most phytoplasmas that possess extrachromosomal DNAs. Hybridization analyses with cloned phytoplasma plasmid DNA showed that there are a number of genetically distinct groups of plasmids that contain similar DNA sequences. In general, phytoplasmas which were found to be related on the basis of cross hybridization with cloned fragments of chromosomal DNA usually possessed genetically related extrachromosomal DNAs, although the number and sizes of the plasmids varied between the strains. Collaborative work between Doessheim and UC Davis showed that cloned fragment of the western AY-P plasmids hybridized with a number of aster yellows type phytoplasmas from around the world (Kuske et al., 1991). The Doessheim group also expanded these original studies and found that some of the woody plant-infecting phytoplasmas, such as vaccinium witches' broom, also possessed a number of genetically distinct extrachromosomal DNAs (Schneider et al., 1992).

Although plasmid DNAs in other plant pathogenic prokaryotes such as Agrobacterium tumefaciens and several Pseudomonas species contain genes that greatly influence plant pathogenesis, it is not known if phytoplasma plasmids contain similar pathogenicity determinants. However, Northern blot hybridization analyses with a cloned 11-kb plasmid from the BLTVA-P showed this plasmid contained several genes that were differentially expressed in either plant or leafhopper hosts (Shaw et al., 1993). Further characterization of these plasmid-encoded genes is currently in progress.

The discovery of plasmid-free strains of the maize bushy stunt (MBS) phytoplasma, which caused symptoms that were indistinguishable from plasmid-containing MBS-P strains, suggests these elements may be dispensible for this pathogen. The widespread occurrence of these self-replicating elements offers the possibility that phytoplasma plasmids could be used as transformation vectors to introduce and express foreign DNA in heterologous phytoplasmas or perhaps other culturable mollicutes. Considerable additional research will be necessary to further characterize plasmid genes and origins of replication before these elements can be genetically engineered and used as phytoplasma cloning vectors.

3.4 Isolation and size determination of phytoplasma chromosomes

In addition to lacking a cell wall, one of the most important taxonomic characteristics of the culturable mollicutes is their very small genome size. In general, mollicute genomes are from one-third to one-sixth the size of most bacterial genomes. Thus determination of the size of a phytoplasma chromosome would provide key additional insights into the proper classification of these nonculturable prokaryotes.

Although the sizes of culturable mollicute genomes have been estimated by a number of methods, including renaturation kinetics and electron microscopy, it was the development of pulsed-field gel electrophoresis (PFGE) that greatly simplified the isolation and size determination of prokaryotic chromosomes. Early PFGE size determinations of prokaryote chromosomes usually involved embedding the cells in an agarose block, digesting away the cell wall and proteins on the chromosome with several enzymes, digesting the chromosome with rare-cutting endonucleases and resolving the fragments on a PFGE gel. The total size of the bacterial genome was then estimated by adding up the size of the resulting fragments. Neimark and Lange (1990) developed a novel way to more rapidly and accurately determine the size of full-length mollicute chromosomes. Cells were embedded and treated as previously described except that the embedded chromosomes were gamma irradiated to introduce a single break in the circular chromosome. Once linearized, the full-length chromosomes were subjected to PFGE and the size of the chromosome was directly determined by comparison with yeast chromosome size markers. We used these same basic methods to determine the size of several phytoplasma chromosomes (Neimark and Kirkpatrick, 1993). Phytoplasma chromosome sizes varied from approximately 600 kb for the WX-P, BLTVA-P, and peach rosette (PR-P) to approximately 1150 kb for the AYP. Interestingly, the AYP also contained a second „mini-chromosome” of approximately 550 kb which lacked ribosomal RNA genes. These genome sizes were clearly within the general size range of other culturable members of the mollicutes (630 to 2,000 kb). Thus this important taxonomic character also suggested that the phytoplasmas were similar to other mollicutes.

In addition to accurately determining the size of phytoplasma chromosomes, the PFGE method also allows the isolation of the complete phytoplasma genome free from contaminating host DNA for the first time. This should greatly facilitate the construction and characterization of complete genomic libraries of the phytoplasma DNA. Recently, Firrao et al. (1996) further demonstrated the utility of this approach by using these PFGE methodologies to construct a physical map of the WX-P genome.

4 Phylogenetic characterization of phytoplasmas

Although serological and DNA hybridization studies provided important tools for detecting phytoplasmas and provided considerable insight into the relationships between various phytoplasma strains, critical information concerning the exact relationship between phytoplasmas and other culturable mollicutes remained uncertain.

In 1987 Woese published a landmark paper that revolutionized prokaryote systematics and taxonomy. He proposed that the detailed, comparative sequence analysis of evolutionarily conserved genes such as the 16S ribosomal RNA (rRNA) would pro-
vide a phylogenetic basis for classifying and better understanding the relationships between major groups of prokaryotes. This approach was especially applicable to the classification of nonculturable prokaryotes because 16S rRNA genes could be isolated, cloned and sequenced without having to culture the organisms.

In 1989 Weisburg et al. published an extensive study on the phylogenetic relationships of numerous genera and species within the class Mollicutes. Given the availability of this large data of mollicute and other prokaryote 16S rRNA sequences it seemed justified to pursue the phylogenetic characterization of the phytoplasmas.

In 1988 our laboratory began cloning and sequencing the 16S rRNA genes of the western AY-P and the X-P. This was a comparatively laborious task that used a cloned 16S rRNA gene from E. coli as a heterologous probe to identify cloned fragments which contained the phytoplasma 16S rRNA gene. Lim and Sears (1989) were the first to completely clone and sequence a 16S rRNA gene from a midwestern strain of the AY-P. Subsequent sequence analysis of the western and midwestern AY-Ps and the WX-P showed that all three phytoplasmas were more closely related to each other than to any other prokaryote, which suggests the phytoplasmas were all derived from a common ancestor. Additional analyses revealed that the phytoplasmas were more closely related to Acholeplasma species than the true mycoplasmas. Thus, it appeared from this initial work that the phytoplasmas were indeed members of the class Mollicutes however they seemed to form a unique clade that was unrelated to any other cultured mollicute. However, since there were over 200 plant diseases associated with phytoplasmas it would be necessary to clone and sequence the 16S rRNA genes of several more phytoplasmas before this group of plant pathogens could be unambiguously classified as members of the mollicutes.

Fortunately the development of polymerase chain reaction (PCR) technologies greatly facilitated this task. Two general approaches were taken to characterize the 16S rRNA genes of approximately 40 additional phytoplasma strains. Both approaches relied on PCR primers which allowed the specific amplification of phytoplasma 16S rRNA genes from infected plants (Deng and Hikuki, 1991; Ahrens and Seemüller, 1992). In one strategy the amplified fragments were digested with a number of restriction endonucleases and the resulting fragments were analyzed by polyacrylamide gel electrophoresis (PAGE). Approximately 10 phytoplasma groups were identified on the basis of similarities and differences in their RFLP patterns (Ahrens and Seemüller, 1992; Schneider et al., 1993; Lee et al., 1993b). Although this method was useful for rapidly classifying what group an unknown phytoplasma belonged to, this method could not precisely determine the phylogenetic relationships of phytoplasmas with other prokaryotic genera; this could only be determined by sequencing the full-length 16S rRNA genes.

Fortunately, two research groups were willing to undertake this enormous challenge. Seemüller et al. (1994) and Gunderson et al. (1994) PCR-amplified, cloned and sequenced the full-length 16S rRNA genes from approximately 40 phytoplasma strains. Approximately 12 distinct phylogenetic clades were identified by this sequencing effort and these clades probably represent the equivalent of individual phytoplasma species. Their combined efforts showed that all of the phytoplasmas examined were more closely related to each other than to any other prokaryote, indicating that the phytoplasmas represent one monophyletic clade of organisms that were derived from a common gram-positive, clostridial, walled ancestor. In addition, all of the phytoplasmas were more closely related to acholeplasmas rather than the true mycoplasmas, thus, the trivial name mycoplasma-like organism was clearly inaccurate and needed to be changed.

The validity of these 12 major phytoplasma taxa was reinforced by analyzing another phylogenetically conserved region—the intergenic (spacer) region that separates the 16S from the 23S rRNA genes. Because this region is not transcribed into functional rRNA it is less highly conserved than the structural rRNAs. In addition, this region is only about 350 bp long so it can be readily sequenced in both directions using only two sequencing reactions. Thus a larger number of strains can be rapidly examined using detailed sequence analysis rather than the less precise RFLP analysis of the PCR-amplified 16S rDNA. Kirkpatrick et al. (1994) PCR-amplified and directly sequenced the spacer regions from more than 60 phytoplasma strains and the phylogenetic tree constructed from these sequences agreed completely with the major phylogenetic clades that were determined by full-length 16S rRNA sequences. Thus it is now comparatively easy to determine the relative phylogenetic position of an unknown phytoplasma by PCR-amplifying a 1800-bp region that contains the entire 16S rRNA and the 16/23S spacer region and either rapidly sequencing the 16/23S spacer region or digesting the fragments with several restriction enzymes and determining its RFLP group.

Recently, the editors of the two leading journals on microbial taxonomy proposed that nonculturable prokaryotes, which have been extensively characterized by molecular techniques, could be formally classified using a Candidatus prefix (Murray and Schleifer, 1994). Thus the IOM Working Team on MLOs (Phytoplasmas) has proposed that the trivial term MLO is replaced by Candidatus Phytoplasma species. The Working Group is now preparing a series of formal taxonomic descriptions of the major phytoplasma clades which will be assigned tentative species level designations, e.g. Candidatus Phytoplasma pruni, for members of the X-disease clade.

Summary and conclusions

The recent application of molecular biological techniques to studying the phytoplasmas has resulted in a tremendous advancement of our knowledge about this recalcitrant group of plant pathogens. Their DNA composition, genome size and most importantly their phylogenetic characterization have all supported their proper classification as an entirely unique group of organisms within the class Mollicutes. Given the fact that these pathogens have been associated with more than 200 plant diseases around the world the detailed characterization of these organisms has required the hard work of many research groups, especially the extensive phylogenetic research conducted in Dossenheim by Dr. Seemüller’s group and USDA scientists in Beltsville (Gunderson et al., 1994). By better understanding the genetic diversity and interrelationships of these important plant pathogens and developing both broadrange and specific PCR detection techniques (Smart et al., 1996) it is now possible to identify important plant reservoirs and insect vectors of these pathogens. The development of better phytoplasma detection capabilities will also benefit the production of phytoplasma-free germplasm and expedite international importation and plant quarantine programs.

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