‘Candidatus Phytoplasma mali’, ‘Candidatus Phytoplasma pyri’ and ‘Candidatus Phytoplasma prunorum’, the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively

Erich Seemüller and Bernd Schneider

Apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY) are among the most economically important plant diseases that are caused by phytoplasmas. Phylogenetic analyses revealed that the 16S rDNA sequences of strains of each of these pathogens were identical or nearly identical. Differences between the three phytoplasmas ranged from 1.0 to 1.5% of nucleotide positions and were thus below the recommended threshold of 2.5% for assigning species rank to phytoplasmas under the provisional status ‘Candidatus’. However, supporting data for distinguishing the AP, PD and ESFY agents at the species level were obtained by examining other molecular markers, including the 16S–23S rDNA spacer region, protein-encoding genes and randomly cloned DNA fragments. The three phytoplasmas also differed in serological comparisons and showed clear differences in vector transmission and host-range specificity. From these results, it can be concluded that the AP, PD and ESFY phytoplasmas are coherent but discrete taxa that can be distinguished at the putative species level, for which the names ‘Candidatus Phytoplasma mali’, ‘Candidatus Phytoplasma pyri’ and ‘Candidatus Phytoplasma prunorum’, respectively, are proposed. Strains AP15 R, PD1 R and ESFY-G1 R were selected as reference strains. Examination of available data on the peach yellow leaf roll (PYLR) phytoplasma, which clusters with the AP, PD and ESFY agents, confirmed previous results showing that it is related most closely to the PD pathogen. The two phytoplasmas share 99.6% 16S rDNA sequence similarity. Significant differences were only observed in the sequence of a gene that encodes an immunodominant membrane protein. Until more information on this phytoplasma is available, it is proposed that the PYLR phytoplasma should be regarded as a subtype of ‘Candidatus Phytoplasma pyri’.

INTRODUCTION

Phytoplasmas are plant-pathogenic, yet uncultured, mollicutes. In plants, they usually inhabit phloem sieve tubes and are transmitted from plant to plant by phloem-feeding insects, primarily leafhoppers. They are associated with diseases in about 1000 plant species (Seemüller et al., 2002). These diseases include three economically important disorders of temperate fruit trees: apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY). AP is only known in Europe and was first described in Italy (Rui et al., 1950). ESFY is mainly known in Europe, but has also been reported in Turkey (Jarauhs et al., 2000a) and was first described as a decline of Japanese plum (Prunus salicina) in Italy (Goidanich, 1933). Until recently, the disease was known by the names plum leptonecrosis, apricot chlorotic leaf roll and others, according to the crop affected and the symptoms induced (Lorenz et al., 1994). PD was first reported in North America (McLarty, 1948; Woodbridge et al., 1957) and seems to have been introduced...
from Europe (Shalla et al., 1961; Seemüller, 1992). These data indicate that AP, PD and ESFY are of European origin and were present in Europe long before the first reports were published. In contrast, peach yellow leaf roll (PYLR), which is caused by a phytoplasma that is related closely to the AP, PD and ESFY agents, has only been reported in western North America (Seemüller, 1999).

Recent investigations, particularly sequence analysis of 16S rDNA, have revealed that phytoplasmas constitute a coherent, genus-level taxon. In the monophyletic phytoplasma clade, groups and subgroups have been delineated, many of which are being considered as putative species under the provisional status ‘Candidatus’ (Seemüller et al., 2002) for incompletely described prokaryotes, according to Murray & Stackebrandt (1995). Several provisional species have been described to date and rules for future putative species delineation have been defined (IRPCM Phytoplasma/ Spiroplasma Working Team – Phytoplasma taxonomy group, 2004). According to these recommendations, ‘a phytoplasma can be described as a novel ‘Candidatus Phytoplasma’ species if its 16S rDNA sequence has < 97.5% similarity to that of any previously described ‘Candidatus Phytoplasma’ species’. In cases where phytoplasmas share ≥ 97.5% 16S rDNA sequence similarity, description as different ‘Candidatus’ species is only recommended if there is an indication that these phytoplasmas clearly represent separate populations, as evidenced by significant differences based on molecular markers other than 16S rDNA, antibody specificity, host range and vector transmission specificity.

The AP, PD and ESFY agents are among the phytoplasmas that have been studied most intensively, using both molecular and biological methods. 16S rDNA sequence data indicated that each of them represents a relatively uniform organism (Lorenz et al., 1995; Seemüller et al., 1998b; Kison & Seemüller, 2001). The results also revealed that the three pathogens are closely related phylogenetically and form, together with the PYLR phytoplasma, a cluster designated the ‘AP phytoplasma group’ (Seemüller et al., 1998b) or 16SrX group (Lee et al., 2000) within the AP subclade, which is one of the major branches of the phytoplasma clade. The four phytoplasmas differ from each other in < 2.5% of 16S rDNA nucleotide sequence positions. Thus, the objective of this work was to examine whether the above-mentioned requirements for differentiation of closely related phytoplasmas are fulfilled. The data examined include sequence and/or RFLP analyses of the 16S–23S rDNA spacer region, protein-encoding genes and randomly cloned DNA fragments, serological comparisons and differences in insect vector and host-plant specificity. Further data were generated in this work. In particular, rDNA sequences of more strains were examined and previously sequenced strains were re-examined in order to obtain unambiguous sequences. Also, RFLP analyses of macrorestriction fragments and Western blot studies were performed to demonstrate inter- and intraspecific differences of the phytoplasmas examined.

**METHODS**

Most data on which these taxonomic descriptions are based were generated previously. Additional work was carried out as follows. Sequence and RFLP analyses of ribosomal fragments were performed by using rDNA that was amplified with the primer pair P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995) and extended from the 5’ end of the 16S rRNA gene to the 5’ region of the 23S rRNA gene, thus including the 16S–23S rDNA spacer region. RFLP analysis was carried out as described previously (Lorenz et al., 1995; Kison & Seemüller, 2001). For sequencing of strains AP1/93, PD1, ESFY-G1R, and ESFY-G2 and resequencing of strains AT and AP15B, P1/P7 amplions were cloned by using the pGEM-T vector system (Promega). Sequencing of both strands was done by a commercial service (SEQLAB, Göttingen, Germany) by using M13 forward and reverse primers and six primers that were complementary to conserved 16S rDNA regions (Seemüller et al., 1994). Sequencing data obtained with these primers usually provided threefold coverage of the sequences. To obtain unambiguous sequences, three or four clones were sequenced from each of strains AP15B, AT, PD1R and ESFY-G1R, which represent the three taxa treated in this description. One clone was sequenced from each of strains AP1/93 and ESFY-G2. Sequence evaluation, including manual alignment, was done by using the software package HUSAR (Bionocomputing Service Group, German Cancer Research Center, Heidelberg, Germany). Gaps and ambiguities were removed from the final dataset. Phylogenetic and molecular evolutionary analyses were conducted by using the neighbour-joining program of the genetic analysis software MEGA, version 2.1 (Kumar et al., 2001). The data were resampled 500 times and bootstrap percentage values are given at the nodes of the tree. Phylogenetic distances were calculated by pairwise comparison. Numbering of nucleotide positions corresponds to that of the 16S rRNA gene of aster yellows phytoplasma strain OAY (Lim & Sears, 1989).

Full-length chromosomes of the AP, PD and ESFY phytoplasmas were isolated from diseased tobacco plants and RFLP analysis was performed as described previously (Lauer & Seemüller, 2000). ELISA and Western blot experiments were carried out by using standard procedures (Ausubel et al., 1987). The strains examined in this work are listed in Table 1.

**TAXONOMIC EVIDENCE**

**Phylogenetic relationships based on 16S rDNA sequences.** Four AP phytoplasma strains (AP15B, AT, AP1/93 and APS), three PD phytoplasma strains (PD1R, PD2 and PD3), five ESFY phytoplasma strains (ESFY-G1R, ESFY-G2, ESFY3, ESFY4 and ESFY5) and one strain from the PYLR agent (PYLR1) were used for 16S rDNA sequence analysis (Fig. 1). Of these phytoplasmas, which were collected in several European countries and California and, in the case of the ESFY agent, from several stone fruit species (Table 1), strains AP15B, PD1R and ESFY-G1R were selected as reference strains. AP15B is known to occur in several European countries and is probably the most common type of AP agent (Kison et al., 1994; Jarausch et al., 2000).

The 16S rRNA gene of AP-group phytoplasmas consists of 1521 nt, of which 97·0–99·6% was analysed. Alignment revealed that the sequences of strains of each phytoplasma type were identical or nearly identical, with similarity...
values between 99·9 and 100 %. A difference of 0·2 % was determined between strains ESFY3 and ESFY5. In sequences of the AP phytoplasma strains, only one polymorphism occurred (at position 1442 where, in the AP15 R sequence, the C was replaced by a T residue). This substitution, the only polymorphism that was detected in the PD sequences, is shared by strain PD2. Three polymorphisms were observed in the ESFY strains, with the following substitutions: a G at position 30, present in all other AP-group members, that is replaced by a T residue in strain ESFY5; a G at position 645 that is replaced by an A in strains ESFY-G1R and ESFY3; and an A at position 1251, present in other ESFY strains, that is replaced by a G in strain ESFY3. There was no indication of sequence heterogeneity between the two rRNA operons that are present in all phytoplasmas (Schneider & Seemuëller, 1994a).

The AP, PD and ESFY phytoplasmas are closely related phylogenetically. In interspecific comparisons of the AP/PD, AP/ESFY and PD/ESFY agents, differences in 16S rDNA sequences were 1·0–1·1, 1·3–1·5 and 1·2–1·3 %, respectively. Strain PYLR1 is related most closely to the PD strains, with which it shares 99·6 % sequence similarity, whereas similarity values between the AP and ESFY strains were 98·4–98·6 %. Sequence similarity values within taxa and divergence between taxa largely confirm the results of previous work (Seemuëller et al., 1994, 1998b).

Phylogenetic relatedness of the phytoplasmas examined is depicted in Fig. 1. Strains of each of the three pathogens cluster tightly together and form three distinct branches. The PYLR agent clusters with the PD strains. The closest relatives of the AP-group phytoplasmas are the spartium witches'-broom (SpaWB; Marcone et al., 1999a) and buckthorn witches'-broom (BWB; Mäurer & Seemuëller, 1996) agents, which compose, together with the fruit tree agents, the AP subclade. Their 16S rDNA sequence similarity with the fruit tree phytoplasmas is slightly less than 97·5 % and is thus below the defined 2·5 % threshold. Other selected phytoplasmas that represent other major

Table 1. Designations and other details of the AP phytoplasma group strains examined

<table>
<thead>
<tr>
<th>Designation*</th>
<th>Origin</th>
<th>Original host</th>
<th>Reference/collector</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP15†‡</td>
<td>Udine, Italy</td>
<td>Malus domestica</td>
<td>Carraro et al. (1988)</td>
</tr>
<tr>
<td>AT‡</td>
<td>Heidelberg, Germany</td>
<td>Malus domestica</td>
<td>Marwitz et al. (1974)</td>
</tr>
<tr>
<td>APS</td>
<td>Spain</td>
<td>Malus domestica</td>
<td>L. Avinent</td>
</tr>
<tr>
<td>AP2</td>
<td>Dossenheim, Germany</td>
<td>Malus domestica</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>AP1/93‡</td>
<td>Hohenstaufen, Germany</td>
<td>Malus prunifolia</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>AP5/93</td>
<td>Dossenheim, Germany</td>
<td>Malus domestica</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>AP-Bx</td>
<td>Bordeaux, France</td>
<td>Malus domestica</td>
<td>F. Dosba</td>
</tr>
<tr>
<td>AP-Ro</td>
<td>Pitesti, Romania</td>
<td>Malus domestica</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>PD1†‡</td>
<td>Dossenheim, Germany</td>
<td>Pyrus communis</td>
<td>Marcone et al. (1999a)</td>
</tr>
<tr>
<td>PD2</td>
<td>Dossenheim, Germany</td>
<td>Pyrus communis</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>PD3</td>
<td>Basilegica, Italy</td>
<td>Pyrus communis</td>
<td>C. Marcone</td>
</tr>
<tr>
<td>PD5</td>
<td>Dossenheim, Germany</td>
<td>Pyrus communis</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>PD471</td>
<td>Dossenheim, Germany</td>
<td>Pyrus communis</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>PD477</td>
<td>Dossenheim, Germany</td>
<td>Pyrus communis</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>PD484</td>
<td>Dossenheim, Germany</td>
<td>Pyrus communis</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>PD489</td>
<td>Dossenheim, Germany</td>
<td>Pyrus communis</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>ESFY-G1†‡</td>
<td>Dossenheim, Germany</td>
<td>Prunus persica</td>
<td>Marcone et al. (1999a)</td>
</tr>
<tr>
<td>ESFY-G2‡</td>
<td>Dossenheim, Germany</td>
<td>Prunus armeniaca</td>
<td>Marcone et al. (1999a)</td>
</tr>
<tr>
<td>ESFY1†‡</td>
<td>Udine, Italy</td>
<td>Prunus salicina</td>
<td>Loi et al. (1995)</td>
</tr>
<tr>
<td>ESFY2</td>
<td>Udine, Italy</td>
<td>Prunus salicina</td>
<td>R. Osler</td>
</tr>
<tr>
<td>ESFY3</td>
<td>Dossenheim, Germany</td>
<td>Prunus persica</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>ESFY4</td>
<td>Czech Republic</td>
<td>Prunus armeniaca</td>
<td>Z. Vorackova</td>
</tr>
<tr>
<td>ESFY5</td>
<td>Austria</td>
<td>Prunus armeniaca</td>
<td>M. Laimer</td>
</tr>
<tr>
<td>ESFY6</td>
<td>Dossenheim, Germany</td>
<td>Prunus persica</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>ESFY176</td>
<td>Kallstadt, Germany</td>
<td>Prunus dulcis</td>
<td>K.-H. Lorenz</td>
</tr>
<tr>
<td>ESFY163</td>
<td>Weinsberg, Germany</td>
<td>Prunus salicina</td>
<td>E. Seemuëller</td>
</tr>
<tr>
<td>PYLR1</td>
<td>California, USA</td>
<td>Prunus persica</td>
<td>B. C. Kirkpatrick</td>
</tr>
<tr>
<td>PYLR2</td>
<td>California, USA</td>
<td>Prunus persica</td>
<td>B. C. Kirkpatrick</td>
</tr>
</tbody>
</table>

*For rDNA sequence GenBank accession numbers, see Fig. 1 and text.
†Reference strain.
‡Maintained in periwinkle.
specific sequence was 5'-329–351, which was specific for PYLR. A second ESFY-5' could be distinguished from each other by the sequence TATGGTCT-3'.

The 16S–23S rDNA spacer of AP-group phytoplasmas was 5'-9-ATACGGCCCAAACTCATACGGA-3'.

The 16S rRNA gene subclades differ from the AP-group agents in >7% of 16S rDNA nucleotide positions.

16S rDNA signature sequences. The 16S rRNA gene sequences from the AP, PD and ESFY agents were aligned with those of phytoplasmas that represent most phylogenetic groups. Several oligonucleotides that are unique for the AP-group phytoplasmas were selected and used as query sequences in a BLAST 2.0 search (Altschul et al., 1997). This analysis resulted in the determination of signature sequences that are characteristic for the AP-group phytoplasmas and are absent in other organisms or prokaryotes. Sequences identified as unique for the AP group were at positions 1393–1409, where 5'-AATACCGGAACCAGTA-3' and 5'-AATACC CGGAACCAGTA-3' were specific for the AP and ESFY phytoplasmas, respectively, and 5'-AATACTCAAACCAGTA-3' was specific for the PD and PYLR agents. Another sequence that was only shared by PD and PYLR strains was 5'-AATACTCAAAACCAGTA-3'.

Sequence analysis of the 16S–23S rDNA spacer region. The 16S–23S rDNA spacer of AP-group phytoplasmas consists of about 210 bp. The gene that encodes tRNA^{ile} was present and identical in all strains examined, which comprised AP15\textsuperscript{R}, AT, AP1/93, APS (GenBank accession no. X76426), PD1\textsuperscript{R}, PD3, PD5 (accession no. U54989), ESFY-G1\textsuperscript{R}, ESFY-G2, ESFY3, ESFY4, ESFY6 (accession no. U54988), PYLR1 and PYLR2 (accession no. U54990) (see Table 1 for strain identities and Fig. 1 for other accession numbers). Flanking sequences within these phytoplasmas were also highly conserved; those of strains of each the AP and ESFY phytoplasmas were identical, whereas one polymorphism in the PD spacer (in the form of a deletion of an A residue in PD1\textsuperscript{R} and PD5, just downstream of the tRNA^{ile} gene) was identified. Divergence between taxa was slightly higher in the 16S–23S rDNA spacer region than in the 16S rDNA sequence, being 1-9, 3-0 and 1-5% for the comparisons AP/PD, AP/ESFY and PD/ESFY, respectively. The sequence of PYLR strains PYLR1 and PYLR2 was identical to that of strain PD3. Relationships between the phytoplasmas examined here agree with results of previous comparisons that were made with fewer strains (Kirkpatrick et al., 1994; Schneider et al., 1995).

Sequence analyses of non-ribosomal DNA. Examination of putative translation products of immunodominant membrane protein (IMP) genes of the AP, PD, ESFY and PYLR phytoplasmas revealed a similar size for all. The IMPS consisted of a transmembrane region towards the N-terminus, with only a short N-terminal intracellular sequence (together, about 35 aa), and a large hydrophilic C-terminal domain (about 130 aa), probably held on the outside of the cell membrane. The extracellular hydrophilic domain, which may play a role in pathogen–host interactions, is not conserved. Similarities of nucleotide and deduced amino acid sequences were 47–76 and 31–57%, respectively. Sequence similarity values for the combined N-terminal and transmembrane domains were higher, ranging from 82 to 98% and 65 to 100%, respectively. Whilst the PD and PYLR phytoplasmas differed significantly in the nucleotide and amino acid sequences of their large hydrophilic domain (54 and 31% similarity, respectively), sequences of their combined N-terminal and transmembrane domains were nearly identical or identical. However, sequence similarity of the entire gene was highest between the PD and AP phytoplasmas (Berg et al., 1999; Barbara et al., 2001; Morton et al., 2003). There is no sequence similarity in antigenic membrane proteins between AP-group and aster yellows-group phytoplasmas (Barbara et al., 2002).

The DNA sequence of randomly cloned DNA fragment AT67 from strain AT of the AP phytoplasma (Schneider & Seemüller, 1994b), which contains three ORFs that encode a putative ATP-binding protein and two putative permease proteins, was compared with analogous, PCR-amplified sequences of Californian, German and Italian PD phytoplasma isolates and a Californian PYLR phytoplasma isolate. This analysis revealed that the Californian and German PD isolates and the Californian PYLR isolate had identical sequences. DNA sequence similarity of these...
isolates to the Italian PD isolate was 98 % and to AP strain AT was 92 % (Guerra, 1997).

**RFLP analysis of ribosomal and non-ribosomal DNA.**

rDNA, PCR-amplified with universal phytoplasma primer pairs fU5/rU3 or P1/P7 or with primers fO1/rO1, fPD/rO1 or P1/PYL Rint, which specifically amplify some or all phytoplasmas of the AP group, was predominantly used in RFLP analysis (Seemüller et al., 1998a). Following AluI digestion, the AP, PD and ESFY phytoplasmas showed the same restriction pattern, which was also shared by the PYLR agent (Kison et al., 1997). The AP, PD and ESFY phytoplasmas can be distinguished from each other by separate restriction of 16S rDNA with Rsal and Sspl. Following Rsal digestion, the ESFY agent differs from the AP and PD phytoplasmas by one additional restriction site at position 420, whereas Sspl digestion cleaves the AP phytoplasma at position 413, a site that is absent in the PD and ESFY agents. Differentiation of AP and PD from ESFY phytoplasmas is also possible with BsaAl and SfcI. The ESFY agent shows a BsaAl site at position 422 that is absent in the two other phytoplasmas, whilst both the PD and ESFY phytoplasmas have an SfcI site at position 624 that is absent in AP. In addition, the PD and ESFY agents share a unique Sspl site in the 16S–23S rDNA spacer region that does not occur in the AP phytoplasma (Fig. 2). A supplementary figure showing restriction maps of rDNA from the AP, PD and ESFY agents is available in IJSEM Online. In all comparisons, the PYLR agent resembled the PD phytoplasma (Lorenz et al., 1995; Marcone et al., 1996a; Guerra, 1997; Kison et al., 1997; Del Serrone et al., 1998; Seemüller et al., 1998a; Topchiiska et al., 2000; Kison & Seemüller, 2001). Hundreds of AP-group phytoplasma isolates from various locations in European countries and California, including the ESFY agent taken from several Prunus species and hybrids (Bonnet et al., 1990), also proved to be useful for AP-group phytoplasma characterization and differentiation. Work by Jarausch et al. (1994, 2000a, 2000b) showed that this fragment contains three ORFs, including a putative nitroreductase gene. Restriction enzyme digestion with Rsal and HincII of a product obtained with primers AP9/AP10 enabled the differentiation of AP from PD and ESFY agents. All three phytoplasmas could be distinguished from each other by digestion of an AP3/AP10 amplicon with these enzymes. One hundred and seventy-five ESFY phytoplasma isolates, which were collected from many Prunus species and hybrids in four Mediterranean countries, responded identically in this work. However, by examining a 1·5 kbp fragment that was obtained from AP phytoplasma isolates with primers AP8/AP10, three RFLP types were identified by restriction digestion with RcaI and HincII. Nucleotide sequence analysis revealed that the three RFLP types are nearly identical and differ only by a point mutation in each of the two restriction sites that are responsible for the polymorphisms.

Primers that were derived from the non-ribosomal DNA fragment AT67 (see above) of strain AT were used to amplify the analogous sequence of the PD phytoplasma. The primers designed from the sequence obtained were PD-specific and were used to examine 11 Californian PD isolates and a German reference strain. All showed the same RFLP profile following digestion with AluI and Rsal (Guerra, 1997). In another experiment, AP and PD phytoplasmas were distinguished by digestion of an 835 bp tuf gene fragment with both HinfI and HpaII (Berg, 1998). The PYLR agent also showed the same profiles as the PD phytoplasma in the present study.

**Southern blot hybridizations.** Randomly cloned chromosomal DNA fragments from strain AT were employed in Southern blot analysis to characterize and differentiate AP-group phytoplasmas. HindIII fragments IH184 and IH196 (Bonnet et al., 1990) and EcoRI fragments AT17, AT27, AT67 and AT72 (Schneider & Seemüller, 1994b) were used as probes (see above for inserts IH196 and AT67). With both sets of fragments, which were employed mostly as
cocktail probes, distinctly different RFLP patterns were obtained with *HindIII* - and *EcoRI*-digested DNA from plants that were infected with the AP or ESFY agents (Ahrens et al., 1993; Lorenz et al., 1994; Kison et al., 1997). The *EcoRI* probes also enabled clear differentiation of the PYLR agent from the AP and ESFY phytoplasmas. In contrast, following hybridization with probes *HI*184 and *HI*196, *HindIII*-digested PYLR phytoplasma DNA showed the same RFLP profile as strain AP15<sup>R</sup>, which was different from that of strain AT (Kison et al., 1997). Hybridization experiments using *HindIII* - or *EcoRI*-digested DNA from PD-diseased pear trees with both sets of probes gave inconclusive results; this is probably due to too low phytoplasma DNA concentrations in extracts from PD-diseased trees. The probes used were specific for AP-group phytoplasmas and did not hybridize or hybridized only weakly to DNA of phytoplasmas from other phylogenetic groups. When Southern blots of *EcoRI*-digested DNA were probed with IMP gene 318B from strain AT, hybridization signals were also obtained from the PD and ESFY phytoplasmas. However, the fragment size was different for each phytoplasma (Berg et al., 1999).

Southern blot analysis also revealed sequence heterogeneity between isolates of the AP phytoplasma. After hybridization of *HindIII*-digested DNA from 34 isolates of the AP agent with *HindIII* fragments *HI*184 and *HI*196, five different RFLP patterns were observed. Two of them were represented by strains AT and AP15<sup>R</sup> (Bonnet et al., 1990; Kison et al., 1994). These two genotypes correspond to PCR-RFLP subtypes AT-1 and AP of Jarausch et al. (2000b). Sequence heterogeneity within the AP agent was also observed when *HindIII*-digested DNA from three AP phytoplasma isolates was hybridized with a 5 kbp probe from an aster yellows phytoplasma, which comprised part of an rDNA operon and a 2-8 kbp upstream region. In this case, each strain showed a different RFLP pattern (Schneider & Seemüller, 1994a). However, no polymorphisms were observed following hybridization of *EcoRI*-digested AP DNA with *EcoRI* fragments AT17, AT27 and AT67 (Kison et al., 1997). By hybridization of *HindIII* - or *EcoRI*-digested DNA from over 50 ESFY phytoplasma isolates with cocktail probes *HI*184 and *HI*196 or AT17, AT27 and AT67, polymorphisms were only observed in two samples following hybridization with the *HindIII* fragments as probes. No heterogeneity was observed in the PYLR phytoplasma following hybridization with both sets of probes (Ahrens et al., 1993; Lorenz et al., 1994; Kison et al., 1997).

**Genome sizes, chromosome restriction maps and G+C content.** PFGE was used to estimate genome sizes of the AP, PD and ESFY phytoplasmas by resolving full-length chromosomes and/or chromosomal macrorestriction fragments that were generated by single or double digestions with rare-cutting enzymes (Marcone et al., 1999b; Lauer & Seemüller, 2000). PFGE analysis showed that the chromosome sizes of the three fruit-tree pathogens are in the lower range for phytoplasmas. Estimated sizes of the chromosomes of strains AT and AP15<sup>R</sup> of the AP agent were 645 and 690 kbp, respectively, whereas the chromosome length of ESFY phytoplasma strains ESFY1, ESFY-G1<sup>R</sup> and ESFY-G2 was uniformly 630 kbp and that of strain PD1<sup>R</sup> was 660 kbp. Physical maps were constructed from the chromosomes of strains AT and ESFY-G1<sup>R</sup> (Lauer & Seemüller, 2000; Marcone & Seemüller, 2001). Although there were differences in the restriction enzymes that were suitable for map construction and in the frequency and location of restriction sites, the overall arrangement of the chromosomes was similar, as evidenced by gene positions on the maps. Interspecific variations were detected by digestion of full-length chromosomes from strains AT, AP15<sup>R</sup>, PD1<sup>R</sup> and ESFY-G2 with BssHII. Also, polymorphisms were observed between strains AT and AP15<sup>R</sup> (Fig. 3). The G+C content of the chromosome of strain AT was estimated to be 23.7 mol% (Kollar & Seemüller, 1989).

**Antibody specificity.** Two mAbs that were derived from antigens of strain AP15<sup>R</sup> proved to be highly specific for the AP agent. In ELISAs, they detected both the homologous strain and strain AT, but did not react with antigen preparations from the PD or ESFY phytoplasmas, nor with extracts from plants infected with phytoplasmas from other phylogenetic groups (Loi et al., 2002). In our Western blot experiments, in which five different strains of the AP agent were included, these mAbs reacted with only three strains (AP15<sup>R</sup>, AP1/93 and AP12/93) and poorly with IMP 318B from strain AT (see above). A
polyclonal antiserum that was raised to IMP 318B of strain AT (Berg et al., 1999) gave different results when the same samples were analysed in Western blot experiments. This antiserum reacted with the recombinant protein and antigens of strains AT and AP5/93, but not with antigens of strains AP15R, AP1/93 or AP12/93 (Fig. 4). In another experiment, polyclonal antibodies purified from antisera that were raised to antigens from the AP, PD and ESFY phytoplasmas recognized, in Western blot assays, only the homologous antigens and did not cross-react (Davies & Adams, 2001).

Host-plant and vector transmission specificity. By using PCR, DNA hybridization and serological techniques, the AP, PD and ESFY phytoplasmas were almost exclusively detected in the genera Malus (apple), Pyrus (pear) and Prunus (stone fruits), respectively. Within these genera, several species are known to be natural or experimental hosts of the respective pathogen and develop disease following infection (Kartte & Seemüller, 1991; Seemüller, 1992; Jarausch et al., 2001; Kison & Seemüller, 2002; Carraro et al., 2002). Using universal phytoplasma primers or AP group-specific primers, phytoplasmas showing rDNA RFLP profiles that were specific for the three fruit-tree agents were occasionally identified in plants other than the typical host. On the basis of these patterns, additional AP phytoplasma hosts include stone fruits and both European and Asian pear (Davies & Adams, 2001).

Phylogenetic analyses revealed that the AP, PD and ESFY phytoplasmas are closely related, differing by only 16–19 nucleotide positions in their 16S rDNA, which corresponds to 98–99·1% sequence similarity. An even closer relationship occurs in other mollicute species. For example, Mycoplasma clemmensen and Mycoplasma yeatsii share 99·7% sequence similarity (Heldtander et al., 1998) and Mycoplasma gallisepticum and Mycoplasma imitans differ by only 2 nt in their 16S rDNA sequences. However, the latter two taxa have other characteristics that show that they can be regarded as different species (Bradbury et al., 1993). Similarly, the AP, PD and ESFY phytoplasmas differed clearly in all comparisons made, in addition to 16S rDNA analyses. From this, it can be concluded that the requirements for defining phylogenetically closely related phytoplasmas as putative species are fulfilled and that the AP, PD and ESFY phytoplasmas represent discrete, coherent taxa.

In contrast to the AP, PD and ESFY agents, there is not sufficient evidence to justify a formal taxonomic classification of the PYLR phytoplasma at present. This pathogen is
phylogenetically closely related to, but not identical to, the PD agent. In most molecular analyses in which ribosomal and non-ribosomal DNA sequences were included, this pathogen was indistinguishable from the PD agent. Only recent comparisons of structurally similar IMP genes showed distinct differences between the two phytoplasmas (Morton et al., 2003). This supports geographical and pathological evidence that indicates that PD and PYLR are caused by different organisms. Based on these results, it appears to be most appropriate to regard the PYLR agent as a subtype of the PD phytoplasma.

**Taxonomic descriptions**

To facilitate reference to unique phytoplasma lineages and to have names by which distinct phytoplasmas can be known, we propose to designate the AP, PD and ESFY phytoplasmas as novel *Candidatus* species: ‘Candidatus Phytoplasma prunorum’, ‘Candidatus Phytoplasma mali’, ‘Candidatus Phytoplasma pyri’ and ‘Candidatus Phytoplasma prunorum rosea’, respectively, with the following descriptions.

‘Candidatus Phytoplasma prunorum’ (mali, epithet referring to the plant host) [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. AJ542541); oligonucleotide sequence complementary to unique region of the 16S rRNA gene is 5'-ATATCGGAAAACCAGTA-3'; P (Malus, phloem); M].

‘Candidatus Phytoplasma mali’ (mali, epithet referring to the plant host) [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. AJ542543); oligonucleotide sequence complementary to regions of the 16S rRNA gene, which are only shared by the PYLR agent, are 5'-ATATCTCAAAACCAGTA-3' and 5'-ATACGGGCCCACAATCATAACGTA-3'; P (Pyrus, phloem); M]. We propose to regard the PYLR agent as a subtype of ‘Candidatus Phytoplasma pyri’.

‘Candidatus Phytoplasma prunorum’ (prunorum, epithet referring to the plant host) [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. AJ542544); oligonucleotide sequences complementary to unique regions of the 16S rRNA gene 5'-ATATCCGAAAACCAGTA-3' and 5'-TGAGTTTGGAGCATCTCAGAA-3'; P (Prunus, phloem); M]. Reference strains AP15R, PD1R and ESFY-G1R and other strains mentioned herein that are maintained in *Catharanthus roseus* are available from the authors.

**ACKNOWLEDGEMENTS**

We thank J. Thompson and C. Marcone for critical reading of the manuscript and helpful suggestions.

**REFERENCES**


Roma


