Immunodominant membrane proteins of phytoplasmas

Alisa Konnerth, Gabriele Krczal and Kajohn Boonrod

Phytoplasmas are plant-pathogenic, phloem-colonizing, cell wall-less microorganisms that are primarily dependent on insect transmission for their spread and survival. The life cycle of phytoplasmas involves replication in insects and host plants. Until recently, phytoplasmas have resisted all attempts at cultivation in cell-free media, making these pathogens poorly characterized on a physiological and biochemical basis. However, host–pathogen relationships can be studied by investigating immunodominant membrane proteins (IDPs), which are located on the exterior surfaces of phytoplasma cells and are the most abundant proteins of the cell membrane. These membrane proteins come in direct contact with both insect and plant hosts and are thought to play a crucial role in phytoplasma spread both within the plant and by insect vectors. Therefore, there is great interest in studying this class of proteins. We summarize and discuss important investigations about these membrane proteins, which have already provided a better understanding of the host–phytoplasma relationship.

Introduction
Phytoplasmas are plant-pathogenic bacteria that cause economically important diseases worldwide. These pathogens are transmitted by insect vectors. While the association between phytoplasmas and their insect vectors can be mutually beneficial (Sugio et al., 2011), phytoplasmas cause numerous diseases in plants, including important food crops, leading to heavy damage to the host plant, considerable yield losses and eventual death of the plant.

In contrast to most other mycoplasmas, phytoplasmas have resisted all attempts at cultivation in cell-free media, indicating that they have a metabolism different from that of other mycoplasmas and/or a greater reliance on their hosts (Kube et al., 2008). Therefore, these pathogens are poorly characterized on a physiological and biochemical basis. Although it was recently shown that specific media support the axenic growth of different phytoplasmas (Contaldo et al., 2012), their application has yet to be reported.

In the past decade many aspects of phytoplasma diseases have been intensively investigated, including their pathology, distribution and vectors, as well as host–phytoplasma interactions. Since phytoplasmas cannot be cultivated, studying their interaction with the host is a major starting point of research. Phytoplasmas are intracellular parasites that lack a cell wall. Thus, their membrane proteins are in direct contact with the cytoplasm of their insect or plant hosts. One group of proteins that comprises a major portion of total cellular membrane proteins is phytoplasma immunodominant membrane proteins (IDPs) (Kakizawa et al., 2004).

IDPs can be classified in three distinct groups: (1) immunodominant membrane protein (Imp), (2) immunodominant membrane protein A (IdpA) and (3) antigenic membrane protein (Amp) (Kakizawa et al., 2006b). However, IDPs with in a single group can be highly variable whereas their adjacent regions share significantly higher similarity. Thus, IDPs are thought to be targets of strong selective pressure (Kakizawa et al., 2006b).

The molecular masses of IDPs range from 15.7 to 23 kDa (Yu et al., 1998). Sequence alignment data indicate that these proteins are not orthologous and that non-homologous proteins function as IDPs in various phytoplasmas.

Surprisingly, some phytoplasmas contain more than one IDP. For example, the Western X disease (WX) phytoplasma contains genes encoding the IDPs Imp and IdpA (Liefing & Kirkpatrick, 2003), whereas Candidatus Phytoplasma (Ca. P.) asteris, at least the strain onion yellows (OY), expresses the IDPs Imp and Amp (Kakizawa et al., 2009).

Due to their variety in amino acid composition in different phytoplasmas, their highly abundant expression and their
direct contact with the host, IDPs are a highly interesting target of phytoplasma research. In this review, we discuss all aspects of many important IDP studies performed to date.

**Genomic organization**

Analysis of the genes surrounding the IDPs shows that the three different types of IDPs described to date are located within different regions of the genome.

To analyse the gene encoding Amp and the surrounding genome area, total DNA was extracted from 14 different phytoplasma strains, and a 3.6 kb DNA fragment harbouring \textit{amp} was amplified using a universal primer pair and sequenced using 11 primers (Kakizawa et al., 2006a). The gene encoding Amp (Fig. 1.3) was localized between two genes encoding molecular chaperonin GroES (\textit{groES}) and GroEL (\textit{groEL}) and a gene encoding NAD synthase (\textit{nadE}).

A similar method was applied to amplify and analyze a 5.0 kb DNA fragment that was expected to harbor the \textit{imp} gene (Kakizawa et al., 2009). Here, total DNA samples from nine different phytoplasma strains (including strains expressing only Imp and strains expressing Imp and Amp) were used. Gene mapping revealed that \textit{imp} (Fig. 1.1) is surrounded by \textit{rnc} (RNase III), \textit{dnaD} (chromosome replication initiation protein), \textit{pyrG} (CTP synthase), \textit{psKd} (phosphatidylserine decarboxylase), and \textit{pssA} (phosphatidylserine synthase).

In WX phytoplasma, genes encoding IdpA and Imp and the surrounding area were analysed by cosmid cloning and sequencing using DNA extracted from WX-infected celery plants (Liefting & Kirkpatrick, 2003). In PoiBI (Poinsettia branch inducing) phytoplasma, DNA fragments containing the \textit{idpA} and \textit{imp} genes were amplified using information from other phytoplasmas (genomic sequence of OY phytoplasma line M, OY-M) (Liefting & Kirkpatrick, 2003; Oshima et al., 2004) and sequenced (Neriya et al., 2011). In both phytoplasma strains, the following gene order was found (Fig. 1.2): \textit{rnc}, \textit{dnaD}, \textit{imp}, \textit{pyrG}, \textit{psd}, \textit{pssA}, \textit{rpoE} (DNA-directed RNA polymerase \(\delta\) subunit), \textit{dnaX} (DNA polymerase III), \textit{idpA} and \textit{RNA-Ser} (serine transfer RNA).

These data suggest that the localization of genes encoding Amp, Imp or IdpA in the phytoplasma genome is conserved among different phytoplasma strains. Furthermore, in phytoplasmas encoding two IDPs, \textit{imp} is localized in the same area of the genome as that of phytoplasmas expressing only Imp.

**Predicted protein structure**

Like their genomic localization, the protein structures of IDPs are predicted to share the same features within the same sub-type but to differ across sub-types.

Amp of the AY (aster yellows), OY and CP (clover phyllody) phytoplasmas is predicted to contain a single, large, extracellular hydrophilic domain flanked by two hydrophobic domains near the N- and C-termini (Fig. 1.3). While the C-terminal domain forms a transmembrane region that

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**Fig. 1.** Gene organization and transmembrane structure of phytoplasma IDPs Type I (Imp, 1), Type II (IdpA, 2) and Type III (Amp, 3). (a) Localization in phytoplasma genome. (b) Putative translation product. (c) Putative protein structure. (d) Host interaction partners.
anchors the protein to the phytoplasma cell membrane, the N-terminal domain forms a bacterial leader sequence that is probably cleaved during maturation (Barbara et al., 2002; Kakizawa et al., 2004).

Amp, at least that of OY phytoplasmas, appears to be exported via the Sec system (Kakizawa et al., 2004). For several phytoplasmas, e.g. Ca. P. asteris strains OY (Kakizawa et al., 2001, 2004) and AYWB (aster yellows witches broom) (Bai et al., 2006), as well as Ca. P. mali (Kube et al., 2008), three genes (secA, secY and secE) of the Sec protein-translocation pathway have been identified to date. Whether the Sec system is fully functional in phytoplasmas has not yet been reported. Since SecA, SecY and SecE are sufficient to reconstitute protein translocation activity in vitro (Economou, 1999), and the export signal sequence of Amp in OY phytoplasmas is recognized by the E. coli Sec system (Kakizawa et al., 2004), it is very likely that the Sec system is at least functional in OY phytoplasmas and that it functions in a manner similar to that of other bacteria (reviewed in Mori & Ito, 2001).

In four different phytoplasmas of the AP subclade, Imp was predicted to consist of an N-terminal hydrophobic region and a hydrophilic C-terminal region that is probably located outside of the cell (Berg et al., 1999; Morton et al., 2003). Unlike Amp, Imp (Fig. 1.1) does not contain a C-terminal transmembrane region or cleavage sites, leading to the assumption that Imp is anchored to the phytoplasma cell membrane by an N-terminal transmembrane region (Berg et al., 1999; Morton et al., 2003). These findings were later supported by analysis of Imp-encoding genes from a number of other phytoplasmas (Kakizawa et al., 2009; Siampour et al., 2013).

The IdpAs (Fig. 1.2) of WX and PoiBI phytoplasmas were predicted to contain two hydrophobic regions near the C- and N-termini of the protein and a large central hydrophilic region without cleavage sites leading to an extracellular domain, which is anchored to the membrane by two transmembrane regions (Blomquist et al., 2001; Neriya et al., 2011).

**Nomenclature**

Although IDPs are categorized into three groups, the nomenclature of each group is not explicitly organized in a standardized way and cannot fully be equated with the characteristics of their members. Since all IDPs are both membrane-localized and immunodominant, the name immunodominant membrane protein (IDP) should apply to all groups. IDPs should be divided into defined types, such as IDP type I, II and III, according to their genomic organization, the protein structure of their prototype, their transmembrane domains or their protein topology. This nomenclature would help prevent misunderstandings and would allow for expansion if more IDPs that do not fit into the existing groups are identified.

Based on the existing classification of IDPs (Kakizawa et al., 2006b), we suggest the following nomenclature:

**IDP Type I: Imp-type proteins (Fig. 1.1)***

- Genomic organization: *rnc, dnaD*, *imp*, *pyrG*, *psd*, *psA*
- Protein structure: one N-terminal hydrophobic region, large hydrophilic C-terminal region, no cleavage site
- Protein topology: main C-terminal portion is located outside of the cell and anchored to the membrane by an N-terminal transmembrane domain

**IDP Type II: IdpA-type proteins (Fig. 1.2)***

- Genomic organization: *psA*, *rpoE*, *dnaX*, *idpA*, tRNA-Ser
- Protein structure: large central hydrophilic region flanked by two hydrophobic regions near the C- and N-termini, no cleavage sites
- Protein topology: main extracellular domain is anchored to the membrane by two transmembrane domains

**IDP Type III: Amp-type proteins (Fig. 1.3)***

- Genomic organization: *groES*, *groEL*, *amp*, *nadE*
- Protein structure: large central hydrophilic region flanked by two hydrophobic regions near the C- and N-termini, N-terminal domain forms a bacterial leader sequence, cleavage site between the N-terminal and central domain
- Protein topology: main extracellular domain is anchored to the membrane by a single C-terminal transmembrane domain, N-terminal region is cleaved during protein maturation and translocation

**Positive selection as the driving force of IDP evolution**

In general, IDPs belonging to the three currently designated groups (Amp, Imp and IdpA) do not share any similarity in amino acid sequence. Furthermore, despite their similarities in protein structure, the amino acid sequences of IDPs within a single group can also vary considerably. For example, the amino acid sequences of different groups of phytoplasmas within the Imp group show little or no sequence identity (Kakizawa et al., 2009). Therefore, gene localization and predicted protein structure were used to compare IDP genes from different phytoplasma strains.

Sequence similarities among IDPs from three related phytoplasmas were also compared to the inter-relationships among the phytoplasmas, as measured by *rRNA* gene sequence similarity. This comparison showed that the degree of similarity of IDPs is not reflected in the degree of similarity deduced from the comparison of *rRNA* sequences (Morton et al., 2003). This finding suggests that the high variability reflects other parameters in addition to evolutionary time.
One factor that might lead to high variability in a gene is positive selection, indicating the importance of the gene for the evolution of an organism. Positive selection is the process by which new, advantageous genetic variants sweep a population. Although advantageous mutations are of great interest, they are difficult to detect and analyse because neutral and deleterious mutations are much more predominant in terms of frequency. Different programmes for calculating the probability of positive selection acting on the genes of interest are available. The ratio of non-synonymous substitutions (amino acid altering mutations) to synonymous substitutions (silent mutations), also referred to as $K_a/K_s$, $d_N/d_S$ or $\omega$, is basically determined by comparing sequences of the same gene from different strains or different groups of the organism of interest.

Using these methods, various studies strongly suggest that positive selection is acting on all three types of IDPs, but not on the genes adjacent to the IDPs (Kakizawa et al., 2006a, 2009; Neriya et al., 2011; Siampour et al., 2013). For Imp and Amp proteins, most amino acid sites that are targets of positive selection are located within the hydrophilic regions of the proteins, suggesting that the selective pressure is acting mainly on the predicted extracellular region of each protein (Kakizawa et al., 2006a, 2009).

Since other membrane proteins of phytoplasma that are not targets of positive selection have been identified (Mounsey et al., 2006), this feature appears to be specific to membrane proteins belonging to the IDP group. Thus, it is likely that the high variability of IDPs is important for their function. The variability of bacterial membrane proteins can be promoted by host–bacterium interactions (Tummler & Cornelis, 2005), leading to the suggestion that the variability of IDPs could be triggered in a similar manner (Kakizawa et al., 2009). For example, selective pressure can occur via adaptation of the pathogen to avoid the immune system of the insect vector or by attachment to host proteins; the latter is often an important step that occurs when pathogens invade the host and establish infection (Kakizawa et al., 2009). Therefore, an important step in understanding the function of IDPs is to identify host proteins that interact with the IDPs.

According to current knowledge, phytoplasma genomes usually contain one gene encoding an IDP. If two IDP genes are present, one always encodes Imp. Furthermore, it appears that Imp can have a much more variable amino acid sequence with constant protein structure (Kakizawa et al., 2009) than Amp (Kakizawa et al., 2006a) or IdpA (Neriya et al., 2011). These observations suggest that Imp could represent an ancestral type of IDP from which Amp and IdpA have somehow emerged (Kakizawa et al., 2006b, 2009). This notion is supported by the finding that in OY phytoplasmas, Amp is tenfold more highly expressed than Imp (Kakizawa et al., 2009). Thus, Amp, not Imp, appears to be the main IDP of OY. In contrast to OY phytoplasmas, in PoiBI phytoplasma, which carries imp and idpa, Imp is expressed at a higher level than IdpA, suggesting that Imp is the main IDP of PoiBI (Neriya et al., 2011). Taking this point into account and due to the high variability of the amino acid sequences of all three types of IDPs, conclusive evidence that Imp is the ancestor of Amp and/or IdpA is still lacking.

### Protein isolation and recombinant expression

Expressing recombinant membrane proteins often results in protein aggregation and misfolding due to the hydrophobic nature of their transmembrane segments. Moreover, overexpressing transmembrane proteins in *E. coli* could lead to the saturation of the membrane protein biogenesis pathway (Sec translocon), which causes cell death (due to inefficient ATP synthesis) and/or inclusion body formation (Wagner et al., 2008).

Due to their high abundance, IDPs can be purified by phase partitioning and centrifugation (Berg et al., 1999). There are several reports of the expression of recombinant IDPs in *E. coli* and plants. Expressing the full-length Imp from different phytoplasmas in *E. coli* led to either retardation of cell growth, protein aggregation or very low soluble protein yields, while the expression of truncated Imp without the transmembrane region yielded a highly soluble protein (Boonrod et al., 2012; Kakizawa et al., 2009; Siampour et al., 2013). Like Imp, only the hydrophilic region of Amp was successfully expressed in *E. coli* in soluble form (Arashida et al., 2008; Barbara et al., 2002). Nevertheless, only the export leader and hydrophilic domain of Amp from OY were expressed and processed by *E. coli* protease in the cytoplasm before being exported to the periplasmic space, probably by the Sec secretion system (Kakizawa et al., 2004). IdpAs were expressed in *E. coli*; however, the yield of full-length, expressed protein was much lower than that of the truncated protein (Blomquist et al., 2001).

Taken together, these studies demonstrate that IDPs can be expressed in *E. coli* and purified via histidine fusion tagging (for a detailed protocol see Galetto et al., 2013).

Recombinant IDP proteins have been used to generate antibodies specific to IDPs (Berg et al., 1999; Blomquist et al., 2001; Galetto et al., 2008; Hong et al., 2001; Mergenthaler et al., 2001). These antibodies can serve as specific tools for investigating the expression patterns of IDPs within the infected host plant. In addition, these antibodies have been used to localize phytoplasma via *in situ* immunofluorescence, immunosorbent electron microscopy and gold-labelled antibody decoration (Narayanasamy, 2011). The recombinant IDPs are also important tools for studying protein–protein interactions by pull-down assays and far-western blot analysis (Galetto et al., 2011; Suzuki et al., 2006). However, for diagnostic purposes, the sensitivity of the respective anti-IDP antibodies is far below the detection limit of nucleic acid-based techniques (Nejat & Vadamalai, 2010).
Protein interactions

Recombinant IDPs have been successfully expressed as full-length and truncated proteins in *E. coli*, which opens up the possibility of studying the host interactions of these proteins *in vitro*. Many microbial pathogens bind to carbohydrate moieties of glycoproteins and glycolipids covering the eukaryotic cell surface, which function as attachment sites (Kato & Ishiwa, 2015). In contrast to spiralin (Killiny *et al.*, 2005), a major membrane protein of the helical mollicute *Spiroplasma citri*, Imp of lime witches’ broom (LWB) phytoplasma does not bind to sugar residues of ovalbumin and therefore does not behave like a lectin. This finding suggests that Imp does not bind to the glyco-proteome of the insect vector (Siampour *et al.*, 2013). However, since glycoconjugates are not involved in the adherence of *S. citri* to insect cells (Yu *et al.*, 2000), it is possible that Imp may be involved in mediating phytoplasma adherence to the epithelial cells of the vector insect’s gut or salivary glands.

Recently, Marzachi’s group found that pre-feeding of two leafhopper vectors with anti-Amp antibody resulted in a significant decrease in the acquisition efficiency for both vectors (Rashidi *et al.*, 2015). Moreover, the inoculation efficiency of Chrysanthemum yellows phytoplasma (CYP) was significantly reduced when it was co-injected with anti-Amp antibody into *Eriphus variegatus*, a leafhopper vector of CYP phytoplasma (Rashidi *et al.*, 2015). Although it is not yet clear whether Amp binds to the glyco-proteome to mediate phytoplasma adherence to epithelial cells of the insect vector’s gut or salivary glands, a recent study provided the first *in vivo* evidence that Amp is somehow involved in the specific crossing of the gut epithelium, as well as salivary gland colonization, during the early phases of vector infection, at least for CYP (Pacifico *et al.*, 2015).

Suzuki *et al.* showed that in a pull-down assay, Amp of *Candidatus Phytoplasma asteris*, onion yellows strain, line W (OY-W) co-precipitated with actin and myosin (heavy and light chains) of the host insects (Suzuki *et al.*, 2006). Interestingly, Amp-microfilament complexes were only detected in OY-transmitting leafhoppers, suggesting that the capacity of leafhoppers to transmit a phytoplasma is correlated to Amp-microfilament complex formation. The reduced acquisition and inoculation efficiencies observed during *in vivo* Amp-inhibiting assays in which anti-Amp antibody was used to pre-feed or micro-inject insect vectors support this finding (Pacifico *et al.*, 2015). This finding raises the question of why Amp cannot form complexes with the microfilaments of non-transmitting vectors. One possible explanation is that the microfilamentous proteins of transmitting and non-transmitting insect vectors are somewhat different, even though their amino acid sequences coding for actin and myosin are highly homologous (Suzuki *et al.*, 2006). Alternatively, the formation of Amp-microfilament complexes may require some other unknown factors, which are probably absent in non-transmitting vectors. In order to understand the impact of IDP-microfilament complexes on vector transmission, further investigations are needed.

Galetto *et al.* showed that Amp of CYP binds to the α and β subunits of ATP synthases of insect vectors (Galetto *et al.*, 2011). Although ATPases primarily localize to the inner membranes of mitochondria, there are several reports showing that ATPases can also localize to the outer surface of the plasma membrane (Martinez *et al.*, 2003; Moser *et al.*, 1999) or to the cell surface (Lin *et al.*, 2009; Paingankar *et al.*, 2010; Zalewska *et al.*, 2009). In an immunofluorescence assay, the ATP synthase β subunit was shown to be localized to the cell surfaces of the midgut and salivary glands of *E. variegatus* (Galetto *et al.*, 2011). A comparison of the β subunits of ATP synthase from a non-vector aphid and the leafhopper vector revealed that they are different despite their high sequence similarity. The specific binding of Amp to ATP synthase, especially the localization in the midgut and salivary glands of the insect vector, suggests that extracellular ATP is required as an exogenous energy source for more efficient colonization, resulting in high phytoplasma multiplication and transmission (Bosco *et al.*, 2007; Galetto *et al.*, 2011). Although it was shown that Amp binds specifically to ATP synthase from leafhopper vectors and differs from that of a non-vector aphid, no experimental testing of the potential binding of ATP synthase from a non-vector leafhopper with Amp has been performed. Therefore, whether this interaction is the decisive factor in vector specificity is an open question.

Boonrod *et al.* showed that Amp of *Candidatus Phytoplasma mali* binds to plant actin (Boonrod *et al.*, 2012). Transgenic plants expressing Imp did not show phytoplasma-like symptoms, indicating that the binding of Imp to plant actin is probably crucial for phytoplasma mobility and survival. Interestingly, recent studies using transmission electron microscopy combined with immunogold labelling revealed co-localization of sieve-element actin and *Candidatus Phytoplasma solani* in infected tomato plants (Buxa *et al.*, 2015). The results of this study indicate that phytoplasma infection triggers a re-organization of sieve-element sub-structures, leading to the anchoring of sieve-element actin to the phytoplasma surface.

Blocking Amp of Stolbur Phytoplasma via *in planta* expression of a single chain variable fragment (scFv) specific to Amp resulted in reduced phytoplasma infection in a grafting assay (Le Gall *et al.*, 1998), indicating that Amp plays an important role in infectivity. Although the exact inhibition mechanism is not known, it is possible that the scFv coats the phytoplasma cells, preventing bacterial attachment to the host cell (opsonization) through binding to Amp. Alternatively, scFv-Amp complexes could reduce the mobility of the phytoplasma due to the increase in its overall size. Revealing the precise mechanism will help us better understand the functions of IDPs in planta.
IDPs as a target for engineering phytoplasma resistance

Several strategies are used to protect crops from pathogens. Engineering RNA-mediated pathogen resistance using transgenic approaches is one of the most successful methods for controlling viral infection (Duan et al., 2012). A protein-mediated strategy can also be utilized: genetically engineered plants expressing peptides or fragments of antibodies (scFv) targeting different pathogens were resistant to viral or bacterial infection (Boonrod et al., 2004; Peschen et al., 2004). Blocking of IDPs using a specific scFv (Le Gall et al., 1998) or antibody (Pacifico et al., 2015) in plants or an insect vector was highly effective at reducing phytoplasma infection in both hosts. Therefore, IDPs are highly suitable targets for developing resistance strategies to combat phytoplasma infections. However, due to the high variability of IDPs, it is difficult to generate an antibody specific to all IDPs. Ideally, the IDP inhibitor should confer a broad-spectrum resistance and be easily applicable to plants without the need for using a transgenic approach, considering the current political situation in Europe.

Conclusion

Since phytoplasmas cannot be cultivated, studying interactions with their insect and plant hosts has helped uncover the biology of phytoplasmas. IDPs appear to be important proteins that help phytoplasmas interact with their hosts. The IDP studies discussed in this review have provided important knowledge about the biology of phytoplasmas, their virulence and their mode of action.

On one hand, the variable amino acid composition of IDPs could reflect the adaption of these pathogens to various aspects of pathology (transmission, vector selection and the severity of infection). Like viral coat proteins, it is possible that phytoplasmas use the variability of IDPs to enhance their virulence, to survive in the host and/or for motility. Further studies of IDPs will provide more insights into the biology of phytoplasmas. Furthermore, the variability of IDPs is currently used as a criterion to distinguish between different phytoplasmas.

On the other hand, further elucidating the interactions of phytoplasmas with their hosts could facilitate the development of tools to protect plants from phytoplasma infection. IDPs have actin-binding activity, which does not disturb the viability of the host but appears to facilitate phytoplasma mobility and survival. Interrupting this binding could prevent phytoplasma infection, or at least reduce symptoms.

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