Secretion of immunodominant membrane protein from onion yellows phytoplasma through the Sec protein-translocation system in *Escherichia coli*

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A gene that encodes a putative SecE protein, which is a component of the Sec protein-translocation system, was cloned from the onion yellows phytoplasma (OY). The identification of this gene and the previously reported genes encoding SecA and SecY provides evidence that the Sec system exists in phytoplasma. In addition, a gene encoding an antigenic membrane protein (Amp) (a type of immunodominant membrane protein) of OY was cloned and sequenced. The OY amp gene consisted of 702 nt encoding a protein of 233 aa which was highly similar to Amp of aster yellows phytoplasma (AY). Part of OY Amp was overexpressed in *Escherichia coli*, purified, and used to raise an anti-Amp polyclonal antibody. The anti-Amp antibody reacted specifically with an OY-infected plant extract in Western blot analysis and was therefore useful for the detection of OY as well as Amp. Amp has a conserved protein motif that is known to be exported by the Sec system of *E. coli*. A partial OY Amp protein expressed in *E. coli* was localized in the periplasm as a shorter, putatively processed form of the protein. It had probably been exported from the cytoplasm to the periplasm through the Sec system. Moreover, OY Amp protein expressed in OY and detected in OY-infected plants was apparently also processed. Because phytoplasmas cannot be cultured or transformed, little information is available regarding their protein secretion systems. This study suggests that the Sec system operates in this phytoplasma to export OY Amp.

INTRODUCTION

Phytoplasmas, formerly named mycoplasma-like organisms (MLOs), are endocellular bacteria with a low G+C content genome, and are classified as *Mollicutes*. Phytoplasmas inhabit phloem sieve elements of plants; they cause numerous plant diseases, and are transmitted between plants by phloem-feeding insects (McCoy *et al*., 1989; Kirkpatrick, 1992). Plant-pathogenic phytoplasmas are endocellular and lack cell walls, which allows secreted phytoplasmal proteins to contact host plant and insect cells directly, suggesting possibly important roles for these proteins in host–phytoplasma interactions.

Previous studies have shown that immunodominant membrane protein accounts for a major portion of the total cellular membrane proteins in most phytoplasmas. Genes encoding immunodominant membrane protein have been isolated from eight phytoplasmas: sweet potato witches’broom (SPWB) (Yu *et al*., 1998), aster yellows (AY) (Barbara *et al*., 1999), clover phyllody (CP) (Barbara *et al*., 2002), western X-disease (WX) (Blomquist *et al*., 2001), European stone fruit yellows (ESFY) (Morton *et al*., 2003), pear decline (PD) (Morton *et al*., 2003) and peach yellow leaf roll (PYLR) (Morton *et al*., 2003). These proteins show prodigious

Abbreviations: Amp, antigenic membrane protein; AP, apple proliferation phytoplasma; AY, aster yellows phytoplasma; CP, clover phyllody phytoplasma; ESFY, European stone fruit yellows phytoplasma; E/H/T, export leader, hydrophilic and transmembrane domains, respectively, in the putative Amp; OY, onion yellows phytoplasma; PD, pear decline phytoplasma; PYLR, peach yellow leaf roll phytoplasma; SPWB, sweet potato witches’broom phytoplasma; WX, western X-disease phytoplasma.

The GenBank accession numbers for the secE- and amp-containing DNA fragments are AB110270 and AB110271, respectively.
amino acid and antigenic variations. All of the proteins have a central hydrophilic region, which may be on the outside of the phytoplasmal cell, and one or two transmembrane domains. Thus, immunodominant membrane proteins are probably secreted across the phytoplasmal cell membrane during protein localization. The immunodominant membrane proteins are classified into three types: (i) SPWB, AP, ESFY, PD and PLYR; (ii) WX; and (iii) AY and CP (Barbara et al., 2002). These three types are distinct and are not homologues (Barbara et al., 2002). In this paper, the term immunodominant membrane protein is used as a general name, and the protein and gene names of the third type of immunodominant membrane protein (the antigenic membrane protein) are designated as Amp and amp, respectively.

Previously, we identified the genes of onion yellows phytoplasma (OY) that encode the SecA and SecY proteins, which are essential components of the Sec protein translocation system. Based on those findings, we suggested that the Sec system exists in phytoplasma (Kakizawa et al., 2001). However, the details of the protein secretion mechanism or secreted proteins in phytoplasmas have not been analysed. Proteins secreted via the Sec system are likely to be important (Kakizawa et al., 2001). The identification of the secreted proteins in phytoplasma may elucidate the phytoplasmal pathogenesis. Our goal is to identify the components of the secretion pathway and the consensus signal sequence of secreted proteins in phytoplasmas.

SecA, SecY and SecE are required for protein translocation and cell viability in *Escherichia coli* (Economou, 1999), and protein translocation activity can be reconstituted in *vitro* with only these three proteins (Akimaru et al., 1991). In this study, a gene encoding the SecE protein, one of the essential components of the Sec protein translocation system, was cloned from OY and sequenced. Together with the previously cloned SecA and SecY genes from OY, the components of a complete Sec system have now been identified, strongly suggesting that the Sec system exists in phytoplasma. This study also reports the cloning and expression analysis of the amp homologue encoding a putative immunodominant membrane protein of OY, and provides additional information about the variability of immunodominant membrane proteins. The relationship between the phytoplasmal Sec system and Amp is discussed, and a common signal sequence recognition mechanism is proposed for the Sec systems of phytoplasma and *E. coli*.

**METHODS**

**Phytoplasma and DNA extraction from phytoplasma-infected plants.** OY used in this study was isolated in Saga Prefecture, Japan (Shiomi et al., 1996). The preparation of a phytoplasma-enriched fraction from OY-infected plants (*Chrysanthemum coronarium*) and DNA extraction were performed as described previously (Oshima et al., 2002; Kakizawa et al., 2001).

**Cloning and sequencing analysis of phytoplasma DNA.** To construct a plasmid library containing the OY genome, DNA that was enriched with OY genomic DNA was completely digested with the restriction endonucleases *XbaI* or *HindIII*, and fragments were ligated into pUC18. *E. coli* JM109 was transformed with the resultant recombinant plasmids. Cloned DNA inserts were sequenced using *Taq* FS DNA polymerase and fluorescent deoxy terminators, and the results were analysed using an automated DNA sequencer (model 3100, Applied Biosystems). Similarities between the ORF sequences identified in the clones and known gene sequences were analysed using sequence interpretation tools (Institute of Medical Science, University of Tokyo, Japan), the *BLAST* algorithm, and the PSI-BLAST algorithm (Altschul et al., 1990, 1997; http://www.ncbi.nlm.nih.gov/BLAST/). Deduced amino acid sequences were aligned using CLUSTAL W, version 1.7 (Thompson et al., 1994); alignments were checked manually. The putative transmembrane domain in the predicted protein sequence was identified by the SOSUI program (Hinokawa et al., 1998; http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html). The coiled-coil protein structure was predicted by the Lupas algorithm and the Paircoil program (Lupas et al., 1991; Bonnie et al., 1995; http://www.york.ac.uk/depts/biol/units/coils/coilcoi.html).

**Preparation of antiserum against Amp protein.** A *pET* system (Novagen) was used to express the histidine-tagged OY Amp in *E. coli*. The OY amp gene contained an N-terminal export signal sequence (amp-E), a central hydrophilic domain sequence (amp-H), and a C-terminal transmembrane sequence with a short hydrophilic domain (amp-T) (see Fig. 3). The entire gene was amplified by PCR using the primers Amp-N1 (5′-AAG AAT TCC ATA TGG AAA ATG AAA AAA CTC A-3′) and Amp-C1 (5′-AAG AGC TGG AGT TTA TGG TTT TTT TTT TAC A3′). A truncated amp gene without amp-T DNA (i.e. consisting of only amp-E/H) was amplified by PCR using the primers Amp-N1 and Amp-C2 (5′-AAG AGC TGG AGT TGG TAC CAA GGT GTT TTA TIA GC-3′). A truncated amp gene without amp-E/H DNA segment alone was amplified by PCR using the primers Amp-N2 (5′-AAG AAT CCA ATA TGG AAG AAC TAG ATT TAA GCA CTT-3′) and Amp-C2. These three PCR products were doubly digested with both *NdeI* and *XhoI* and inserted into pET30a (+). Each construct was introduced into *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene).

*E. coli* cells harbouring each construct were cultured in 2 x YT medium (Sambrook et al., 1989), and protein expression was induced with IPTG at a final concentration of 1 mM. Cells were collected after induction, resuspended in TBS (20 mM Tris/HCl, pH 7-9, 500 mM NaCl), sonicated, and centrifuged at 10 000 g for 15 min. The supernatant (TBS-soluble fraction) and the precipitate (TBS-insoluble fraction), which was resuspended in TBS containing 6 M urea, were applied separately to a nickel NTA column (Novagen), and the fusion protein was purified from each fraction. The protein purity was evaluated by SDS-PAGE. An antibody against the OY Amp purified from the TBS-soluble fraction was generated following the method described previously (Kakizawa et al., 2001).

**Periplasm and cytoplasm fractionation.** Both periplasmic and cytoplasmic fractions from *E. coli* were prepared using previously described methods (Ueguchi & Ito, 1990) with some modifications. *E. coli* cells expressing OY Amp were collected, washed with 10 mM Tris/HCl buffer (pH 7-5), centrifuged, and resuspended in a suspension buffer (30 mM Tris/HCl, pH 7-5, 0-2 g sucrose ml−1) at a volume equal to that of the culture medium. One-tenth volume of freshly prepared lysozyme solution (1 mg lysozyme ml−1 in 0-1 M EDTA, pH 8-0) was added, and the cells were incubated for 30 min at 0 °C followed by centrifugation at 10 000 g for 10 min. The supernatant was used as the periplasmic fraction. The precipitate was resuspended in suspension buffer and centrifuged at 10 000 g for 10 min. The precipitate was resuspended in 10 mM Tris/HCl buffer (pH 7-5) and used as the cytoplasmic fraction. Each of these two fractions was treated with 4 vols cold acetone, stored at −20 °C.
overnight, centrifuged at 10 000 g for 15 min, and precipitated to give protein preparations. These two protein preparations were subjected to SDS-PAGE and Western blot analysis.

**SDS-PAGE and Western blotting.** These were performed using previously described procedures (Kakizawa et al., 2001), with two exceptions: the polyacrylamide concentration for SDS-PAGE was 15%; the detection reagent for Western blots was 0.2 M Tris/HCl, pH 8.2, 3 mg Fast Red TR salt ml⁻¹ and 0.5 mg naphthol AS-MX phosphate ml⁻¹ and was mixed immediately before use.

**RESULTS**

**Cloning and sequence analysis of the secE gene**

A plasmid clone with a 2374 bp insert containing a putative secE gene was identified from a randomly sequenced HindIII plasmid library of the OY genome. The insert contained four ORFs and one incomplete ORF (Fig. 1, OY). The first, second and fourth ORFs (360, 186 and 615 bp, respectively) encoded deduced proteins that were similar to a highly conserved hypothetical protein (yacO), 50S ribosomal protein L33 (rpmG) and transcription anti-termination factor (nusG) from *Bacillus subtilis*, respectively (20–1 %, 41–0 % and 38–2 % identity, respectively). The third ORF (411 bp) encoded a protein of 136 aa. No homologous proteins were found with a normal BLAST search; however, a PSI-BLAST search revealed weak homology with the putative SecE proteins of *Neisseria meningitidis*, *Staphylococcus carnosus* and *Haemophilus influenzae*. The incomplete ORF (339 bp) encoded a protein that was homologous to the 50S ribosomal protein L11 (rplK) which is conserved in several bacteria (e.g. 69–9 % identity with rplK of *B. subtilis*). Regions of the OY sequence that were homologous to these five genes were located on the OY chromosome in the order 5'-yacO-rpmG-secE-nusG-rplK-3' (Fig. 1, OY); this organization was compared with those of the secE-containing regions from *Mycoplasma genitalium* (nt 61407–64911), *E. coli* (nt 4173523–4176453) and *B. subtilis* (115266–119013) (Fig. 1). The gene organization around secE is well conserved in several bacterial genomes. In *M. genitalium*, the order of three of the five genes is identical to that in OY, rpmG-secE-nusG. In *E. coli*, the order of three genes, secE-nusG-rplK, is identical to that in OY. In *B. subtilis*, two genes, encoding a conserved hypothetical protein (yacP) and RNA polymerase sigma factor (sigH), are located between yacO and rpmG, but the organization of the other genes is identical to that in OY. In OY, *M. genitalium*, *Mycoplasma pneumoniae*, *B. subtilis*, *Helicobacter pylori*, *E. coli*, and several other bacteria, the secE gene is upstream from the nusG gene (data not shown).

The alignment of the deduced amino acid sequence of OY SecE with the deduced SecE amino acid sequences of several other bacteria is presented in Fig. 2. The putative OY SecE protein was predicted by the SOSUI program to contain three transmembrane regions (Fig. 2). The amino acid identity scores between the full-length OY SecE and the SecE proteins of *M. genitalium*, *E. coli* and *B. subtilis* were 13–8 %, 13–0 % and 11–6 %, respectively. The amino acid identity scores for only the third transmembrane regions were 18–8 %, 18–8 % and 23–2 %, respectively. Most of the phylogenetically conserved residues of SecE (Medigue et al., 2002) were also conserved in the putative OY SecE (data not shown).

**Cloning and sequence analysis of the amp gene**

A plasmid clone with a 2364 bp insert containing a putative amp gene was identified in a randomly sequenced *XbaI* plasmid library of the OY genome. The insert contained one complete ORF (702 bp; 233 aa; ~24.7 kDa) and two incomplete ORFs (Fig. 3A). The 24.7 kDa protein showed significant homology with Amps from AY and CP (Barbara et al., 2002), and thus was designated as OY Amp.

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**Fig. 1.** Physical map of the 2374 bp HindIII fragment containing the secE gene from onion yellows phytoplasma (OY) compared with secE-containing genome regions from *Mycoplasma genitalium* (Mg, 61407–64911 bp), *Escherichia coli* (Ec, 4173523–4176453 bp) and *Bacillus subtilis* (Bs, 115266–119013 bp). ORFs are indicated as arrow boxes; secE ORFs are indicated as black arrow boxes. yabC, conserved hypothetical protein; mamB, phosphomannomutase; tufB, elongation factor Tu; the designations of the other genes appear in the text.
As observed for AY and CP Amps, three structural domains, Amp-E, -H and -T, were also identified in OY Amp (Fig. 3B). OY Amp showed the highest identity (96·6%) with AY Amp; AY Amp is the same length as OY Amp, but has eight amino acid substitutions. The amino acid sequence identities between the Amp-E and -T regions of OY Amp and CP Amp were 84·4% and 70·0%, respectively. The Amp-H regions were variable not only in sequence (only 16·4% identical), but also in length (171 and 104 aa residues for OY and CP, respectively). The N-terminal and C-terminal transmembrane regions of OY Amp were predicted with the Sosui program (Fig. 3B). Three repeated coiled-coil sequences in the H-domain (aa residues 47–74) were barely detectable using the Lupas algorithm and the Paircoil program (Fig. 3B). The OY Amp-E domain matched closely with the consensus sequence of Sec system signal peptides (Fig. 3C). Namely, the signal sequence consists of a positively charged N-terminal domain with at least one Arg or Lys residue (two Lys residues in OY, AY and CP), a hydrophobic core domain, and an A-X-A consensus sequence of cleavage site, in which two Ala residues can be replaced by other, preferably small and uncharged residues (V-F-A in OY, AY and CP) (Tjalsma et al., 2000).

The upstream incomplete ORF (888 bp) encoded a protein that was homologous to the molecular chaperonin GroEL (groEL) of AY (~99·7% identity within the sequenced regions) and several other bacteria. The downstream incomplete ORF (209 bp) encoded a protein that was homologous to the NAD synthase (nadE) of several bacteria and to a putative small ORF from AY (98·1% identity within the sequenced region of 163 bp), which is also located downstream from the amp gene in the AY genome. The organization of these three ORFs in OY is identical to the gene organization in AY and CP.

Expression of OY Amp in E. coli

We attempted to express three combinations of the OY Amp protein domains in E. coli: the entire OY Amp (OY Amp-E/H/T), the export signal sequence followed by the hydrophobic domain (OY Amp-E/H), and the hydrophobic domain alone (OY Amp-H). Six histidine residues were fused onto the C-terminal end of each of the three proteins as a tag. The OY amp-E/H/T plasmid could not be introduced into E. coli; the OY amp-H plasmid was introduced into E. coli, but it significantly retarded the growth of the transformants (data not shown). The growth of E. coli harbouring the OY amp-E/H plasmid was the same as that of E. coli containing vector

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**Fig. 2.** Protein sequence alignment of SecE. Black boxes indicate similar residues that are conserved in three or four bacterial SecE. Three transmembrane regions (TM) predicted by the Sosui program are underlined. Abbreviations are as in Fig. 1.

**Fig. 3.** (A) Physical map of the 2364 bp Xbal fragment. It contains a complete gene encoding antigenic membrane protein (amp) and two incomplete genes encoding molecular chaperonin GroEL (groEL) and NAD synthase (nadE). (B) Schematic representation of the putative Amp translation product. Hatched boxes, TM, transmembrane regions predicted by the Sosui program; black box, CC, predicted coiled-coil sequence; E, predicted export leader signal sequence; H, central hydrophobic domain; T, C-terminal transmembrane plus short hydrophilic intracellular sequence. Definitions of E, H, and T are the same as those reported previously (Barbara et al., 2002). (C) Amino acid alignment of the leader signal sequence of Amps from onion yellows phytoplasma (OY), aster yellows phytoplasma (AY), and clover phyllody phytoplasma (CP). Black boxes are as Fig. 2. Three domains of the Sec system signal sequence, a positive charged N-terminal domain, a hydrophobic transmembrane domain and a cleavage motif are indicated. Two asterisks and an arrowhead indicate the positively charged residues and the cleavage point, respectively.
without an insert (data not shown); this strain was used to express OY Amp. A crude extract of *E. coli* that expressed OY Amp-E/H was separated into TBS-soluble and TBS-insoluble fractions, and each fraction was applied to a nickel column to purify the histidine-tagged proteins. SDS-PAGE analysis of these two fractions showed protein bands of different sizes; the major protein (~25 kDa) in the TBS-soluble fraction (Fig. 4, lane 1; open arrowhead) was approximately 3 kDa smaller than the major protein (~28 kDa) in the TBS-insoluble fraction (Fig. 4, lane 2; filled arrowhead). The protein purified from the TBS-soluble fraction (the smaller protein) was collected and used to immunize a rabbit.

**Periplasm and cytoplasm fractionation**

*E. coli* cells expressing OY Amp E/H were subjected to periplasmic and cytoplasmic fractionation using a lysozyme treatment procedure. Western blot analysis of the two fractions with the anti-OY Amp antibody revealed that the major protein (~28 kDa) in the cytoplasmic fraction (Fig. 5, lane 2, filled arrowhead) was approximately 3 kDa larger than the major protein (~25 kDa) in the periplasmic fraction (Fig. 5, lane 4, open arrowhead).

**Western blot analysis**

To confirm the expression of OY Amp in the phytoplasma OY, a crude protein extract was prepared from a phytoplasma-enriched fraction of OY-infected garland chrysanthemum (OY extract) and from uninfected, healthy control plants (healthy extract). These two protein fractions were subjected to Western blot analysis using an antibody against OY Amp. A distinct protein band (~28 kDa) and a minor protein band with smaller size (~18 kDa) were detected in the OY extract (Fig. 6, lane 2, open and filled arrowheads, respectively), but not in the healthy extract (Fig. 6, lane 1).
DISCUSSION

Our results identified a putative SecE (136 aa residues) in OY; however, the identity scores between the OY secE gene and secE genes in other bacteria were extremely low. A comparison of SecE proteins from several bacteria demonstrated variability in the sizes of the proteins, the amino acid sequence homologies between the proteins, and the estimated number of transmembrane domains. Compared with OY SecE, SecE proteins from B. subtilis and H. pylori were much shorter (59 and 60 aa residues, respectively) and SecE from Ureaplasma urealyticum was much longer (166 aa residues). Three transmembrane domains were predicted in the SecE proteins of OY and E. coli, but only one was predicted to occur in the SecE proteins of M. genitalium, M. pneumoniae, H. pylori, B. subtilis and U. urealyticum (data not shown). It was reported that the identification of the secE gene of H. pylori was difficult using normal homology search methods (Medigue et al., 2002). Although the entire genomes of M. genitalium and M. pneumoniae have been sequenced, the only secretory protein genes identified in some databases, including that of the Institute for Genomic Research (TIGR) (http://www.tigr.org/db/mdb/mdbcomplete.html), are secA and secY (Fraser et al., 1995). However, the secE gene is recognized in other databases, such as the COG database (http://www.ncbi.nlm.nih.gov/COG/). Clearly, in general, identification and annotation of the secE gene are quite difficult based only on sequence homology information. In the amino acid alignment of the putative OY SecE protein with several other bacterial SecE proteins, only a weak homology, which was restricted to the third transmembrane region, was seen (Fig. 2). It has been reported that an N-terminal domain of E. coli SecE encompassing the first two transmembrane regions is unnecessary for protein translocation, but a SecE derivative lacking this domain is very unstable (Nishiyama et al., 2000). The difficulties in annotating the secE genes are probably due to the variability in all regions except the third transmembrane domain. The gene organization around secE is well conserved in several bacteria (Fig. 1); the secE gene is located upstream from the nusG gene in most bacteria, including OY (Fig. 1; other data not shown). Therefore, despite dissimilarities in length, sequence homology, and the estimated number of transmembrane structures, the annotation of OY secE is reasonable based on its conserved sequence homology within and around the third transmembrane region and its similar gene organization.

Genes encoding immunodominant membrane proteins were isolated and sequenced from seven phytoplasmas as described above. These immunodominant membrane proteins were divided into three distinct types, all of which possess a central hydrophilic region, possibly external to the phytoplasma cell. The organization of the hydrophobic transmembrane anchor is a distinguishing feature of the different types. The first type is anchored by only N-terminal transmembrane regions; the second type has N-terminal and C-terminal transmembrane regions, and neither of them is cleaved; the third type also has two transmembrane regions, but the N-terminal one is cleaved and only the C-terminal one serves as an anchor (Barbara et al., 2002). The structural properties of OY Amp (Fig. 3) are consistent with the third type of immunodominant membrane protein. On the basis of a phylogenetic analysis using the 16S rRNA genes from the seven phytoplasmas (Jung et al., 2002), OY was found to be most related phylogenetically to AY. The structural resemblance between OY and AY Amps is therefore in agreement with the phylogenetic relationship between these two phytoplasmas.

The amino acid identity between the GroEL proteins of OY and AY was 99.7%; only one of the 292 residues was different. The amino acid identity between OY Amp and AY Amp was 96.6%; eight of the 232 residues were different. These comparisons suggest that Amp is more variable than GroEL. The variability of Amp has been discussed previously (Barbara et al., 2002), and our study provides additional information. Among the eight amino acid residues of Amp that differed between OY and AY, six were similar, and the two that were dissimilar were both localized in the central domain (data not shown). The central hydrophilic domains of OY Amp and CP Amp were variable in both sequence and length, even though the N-terminal signal sequences and C-terminal transmembrane regions resembled each other. These data indicate the variability of the central domain of Amp.

Three repeated coiled-coil sequences were barely detectable in the central hydrophilic domain of OY Amp (Fig. 3B, black box). Four coiled-coil sequences have been predicted in WX Amp (Blomquist et al., 2001). Since the coiled-coil structure is often responsible for protein–protein binding (Voet & Voet, 1995), it has been suggested that OY Amp and WX Amp may function by binding to another protein. AY Amp has been predicted to contain a ligand-binding sequence, and a ligand-binding function has been proposed for this protein (Barbara et al., 2002). These potential interactions indicate that the phytoplasma immunodominant membrane proteins may be important in the host–phytoplasma interaction.

The calculated molecular masses of the histidine-tagged Amp-E/H and -H regions of OY Amp were 22.3 kDa and 18.9 kDa, respectively. However, protein bands of 28 kDa and 25 kDa, each of which is ~6 kDa larger than the calculated weights, were detected by SDS-PAGE (Fig. 2). This phenomenon was also observed for AY Amp (Barbara et al., 2002). Western blot analysis to detect OY Amp in an OY-infected plant extract revealed a major band of 28 kDa (Fig. 6, lane 2, open arrowhead). Considering the difference between the size estimated by electrophoresis and that calculated from the sequence, the molecular mass of this major band is thought to be ~3 kDa smaller than the full-length OY Amp (E/H/T). The size of the OY Amp band detected in the plant extract corresponded to OY Amp without the 32 aa leader peptide. This strongly suggested that the signal sequence of OY Amp was cleaved in infected plants. These results were consistent with previous reports,
i.e. an N-terminal, 32 aa signal peptide of AY Amp was cleaved upon protein expression (Barbara et al., 2002).

The antibody produced against OY Amp in this study reacted specifically with OY Amp from an OY-infected plant extract in Western blot analysis, and no signal was observed in a healthy plant extract (Fig. 6). The additional smaller band observed in the infected extract (Fig. 6, lane 2, filled arrowhead, ~18 kDa) could be a degradation product of OY Amp because a corresponding band was not observed in the healthy plant extract (Fig. 6, lane 1). This specificity could be due to the fact that the antigen used was a bacterially expressed, pure protein, as opposed to the crude protein preparation purified from phytoplasma-infected plants. The advantages of antibodies against bacterially expressed phytoplasmal protein were discussed earlier (Berg et al., 1999; Barbara et al., 2002).

In the fractionation analysis of OY Amp-E/H overexpressed in E. coli, the molecular mass of the major protein observed in the periplasmic fraction was less than that of the band observed in the cytoplasmic fraction (Fig. 5). This suggests that OY Amp-E/H was exported from the cytoplasm to the periplasm in E. coli through an export system that involved post-translational processing. The predicted export signal sequence of OY Amp matched closely with the consensus sequence of Sec system signal peptides. Specifically, the signal sequence consists of a positively charged N-terminal domain with at least one Arg or Lys residue (two Lys residues in OY, AY and CP), a hydrophobic core domain which forms an $\alpha$-helix and penetrates the inner membrane, and an A-X-A consensus sequence serving as the signal peptidase I (SPaseI) cleavage site, in which two Ala residues can be replaced by other, preferably small and uncharged residues (V-F-A in OY, AY and CP) (Tjalsma et al., 2000). Therefore, it is presumed that the predicted export signal sequence of OY Amp is recognized by the E. coli Sec system and that OY Amp-E/H is exported through the Sec system in E. coli. This presumption is also supported by the fact that the size difference estimated by SDS-PAGE (~3 kDa) between small and large Amp-E/H proteins (Fig. 5) is consistent with the predicted molecular mass of the leader signal sequence (32 residues).

The possible toxicity of phytoplasmal Amp in E. coli cells was previously documented (Barbara et al., 2002). In this study, when the entire OY amp-E/H/T construct was introduced into E. coli, no transformants were obtained, suggesting that full-length OY Amp is toxic to E. coli. When the OY amp-H construct was introduced, the growth of transformed E. coli cells was very slow (data not shown). Thus the OY Amp-H protein might also be detrimental to E. coli. On the other hand, E. coli that were transformed with the OY amp-E/H construct and induced to overexpress had the same growth rate as did E. coli transformed with the identical vector lacking an insert (data not shown). If OY Amp-E/H is exported through the Sec system of E. coli, the signal peptide would be cleaved after the translocation, because the active site of SPaseI is localized on the periplasmic side of the cell (Wolfe et al., 1983). Accordingly, the central domain of OY Amp-E/H is found in the periplasmic space because it has no C-terminal transmembrane region to serve as an anchor. The lack of toxicity of the OY amp-E/H construct might be explained by its specific localization.

In OY, the genes encoding the SecA and SecY proteins, essential components of the Sec system (Economou, 1999), were reported previously (Kakizawa et al., 2001). In this study, another essential component protein, SecE (Economou, 1999), was identified. Therefore, the complete set of components of a Sec system have been identified in OY, strongly suggesting that the Sec system exists in this phytoplasma. Moreover, OY Amp seemed to be exported to the periplasm through the Sec system of E. coli. OY Amp that was observed in OY-infected plant extracts also appeared to be processed. These results strongly suggest that OY Amp may be transported through a Sec system in phytoplasma. If the OY Amp is exported through the Sec systems of both phytoplasma and E. coli, the mechanisms of signal sequence recognition in phytoplasma and E. coli share some commonality. This would mean that programs such as SignalP (Nielsen et al., 1997) or PSORT (Nakai & Kanehisa, 1991) could be used to recognize signal sequences in phytoplasmal proteins and thereby to identify exported phytoplasmal proteins. The secreted proteins of phytoplasmas, both those that remain membrane-embedded and those that are secreted into the host cytoplasm, are expected to interact directly with host plant and insect cells and to play critical roles in host–phytoplasma interactions. Our future work will focus on the search for proteins that are exported by phytoplasmas and the investigation of the functions of exported phytoplasmal proteins, including Amp, with respect to host–phytoplasma interactions.

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