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Spiralin Polymorphism in Strains of *Spiroplasma citri* Is Not Due to Differences in Posttranslational Palmitoylation

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Spiralin is defined as the major membrane protein of the helical mollicute *Spiroplasma citri*. According to the *S. citri* strain used, spiralin shows polymorphism in its electrophoretic mobility. The spiralin gene sequences of eight *S. citri* strains were determined by direct sequencing of the PCR-amplified genes. All spiralins were found to be 241 amino acids long, except for the spiralin of strain Palmyre, which is 242 amino acids long. The molecular masses calculated from these sequences did not explain the differences observed in the electrophoretic mobilities. In all of the spiralins examined, the first 24 N-terminal amino acids were conserved, including a cysteine at position 24, and had the features of typical signal peptides of procaryotic lipoproteins. When *S. citri* strains were grown in the presence of [^{3}H]palmitic acid, at least 10 proteins, including spiralin, became labeled. In the presence of globomycin, a lipoprotein signal peptidase inhibitor in eubacteria, apparently unprocessed spiralin could be detected. Formic acid hydrolysis of the [^{3}H]palmitic acid-labeled spiralins of four representative *S. citri* strains yielded two peptide fragments for each spiralin, as expected from the gene sequence. One fragment was [^{3}H]palmitic acid labeled, and it had almost the same electrophoretic mobility irrespective of the spiralins used. Samples of the unlabeled peptide fragments from the four representative strains had slightly different electrophoretic mobilities (ΔDa$\cong$800 Da); however, these were much smaller than those of the whole spiralins before formic acid hydrolysis (ΔDa$\cong$8,000 Da). These results suggest that spiralin polymorphism in *S. citri* is not due to differences in posttranslational modification by palmitic acid and is certainly a structural property of the whole protein or could result from an unidentified posttranslational modification of spiralin.

Among the mollicutes (i.e., wall-less eubacteria with low G+C contents and small genomes), spiroplasmas are characterized by their helical morphology and motility. *Spiroplasma citri*, the first spiroplasma to have been cultured, is the causal agent of "Stubborn" disease of citrus (24) and affects many other plant species. *S. citri* also multiplies in various leafhoppers, which act as insect vectors. The spiroplasmas belong to the recently established order *Entomoplasmatales*, which also contains *Entomoplasma* and *Mesoplasma* species (31). All of these mollicutes are associated with arthropods, mainly insects. The spiroplasmas, like the mycoplasmas, ureaplasmas, mesoplasmas, and entomoplasmas, but unlike the acholeplasmas, use the triplet UGA, usually a stop codon, as a tryptophan codon (8, 21).

Spiralin is the most abundant membrane protein in *S. citri* (36). Its amphiphilic and antigenic properties (34, 38) and its membrane location and arrangement (30, 35) have been extensively studied. The spiralin gene has been identified, cloned, and expressed in *Escherichia coli* cells (19). Several forms of spiralin were produced in *E. coli*, including a major form with a slightly larger molecular mass than that of spiralin in *S. citri*. *S. citri* spiralin is free of tryptophan, and its gene has no UGA codon (6). This feature explains why the gene could be efficiently translated in *E. coli* at a time when it was not yet known that in spiroplasmas, UGA is not read as a stop codon but instead is read as a tryptophan codon. However, the function of spiralin in the spiroplasma cell remains undetermined.

Comparison of the amino acid sequence of *S. citri* spiralin with that of *Spiroplasma melliferum* spiralin, as deduced from their spiralin gene sequences, has revealed a putative α-helix, which could anchor the protein in the spiroplasma membrane and subdivide the protein into a large extracellular N-terminal part and a small C-terminal internal portion (7). Dimers and oligomers of spiralin present in the *S. citri* membrane, as determined by cross-linking experiments (35), can be formed by the association of two or more spiralin molecules by their α-helices that are partially hydrophilic. Previous studies with *S. melliferum* spiralin, antigenically related to *S. citri* spiralin (40), have shown that spiralin is a lipoprotein (37). As frequently observed for mollicute membrane proteins (2, 10, 11, 28), *S. melliferum* spiralin becomes preferentially labeled with palmitic acid when radioactive fatty acids are present in the culture medium.

Comparison of the spiralin genes of *S. citri* and *S. melliferum* has shown that the two genes have a conserved N-terminal end. In the latter study, the strain of *S. citri* used was R8A2HP. It is known that other *S. citri* strains have spiralins with electrophoretic mobilities (EMs) different from that of strain R8A2HP (3). We report in this paper the results of further studies of the EM polymorphism of *S. citri* spiralin. This work shows that the spiralin genes of all of the strains studied have a fully conserved signal peptide sequence and that *S. citri* spiralin polymorphism is not due to differences in posttranslational palmitoylation.

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MATERIALS AND METHODS

***S. citri* strains.** Morocco strains R8A2HP (high passage) (ATCC 27556), M4, MH2, GII3, and R8A2B; American strains C189 (ATCC 27665) and Hinckley; Israeli strains Israel (NCPPB 2565) and Asp1 (a nonmotile strain) (29); Spanish strain Alcanar 254; Turkish strain 78; Syrian strain "Palmyre;" and French isolate

"Corse" were grown in SP4 culture medium at 32°C (32). The origins of these strains have been recently described (39).

Primers used for amplification and sequencing procedures. The following primers were used: A (5′CTTTTATCGATTTTAGCAG3′), A′ (5′TCAGCTGC TGTTGCTGTTTT3′), B (5′AGGTACATCATTAACAACAA3′), C (5′AGCA GACCCAAAACAAGTTAC3′), D (5′GTATAAAGTAGGGTTAGAAGC3′), D′ (5′CCCTTGTGAATCACCACC3′), E (5′GCAAGTGCTGTTTTAGAGGC 3′), E′ (5′GAGCTGAATAGTCTCCCGC3′), F′ (5′CAATAACCACTTTGTC CT3′), G (5′GCACCAAATGCAGGAGCAAGTG3′), and H (5′TCACCAAA CATTTCTGGTG3′).

DNA isolation. Genomic DNA was obtained by the method of Marmur (18).

Enzymatic amplification of genomic DNA. Genomic DNA (200 ng) was submitted to 40 repeated cycles of amplification (1 min at 92°C, 1 min at 57°C, 2 min at 72°C), followed by an extension step (10 min at 72°C) in a DNA Thermal Cycler (Perkin-Elmer Cetus) with 2 U of *Taq* polymerase (Bethesda Research Laboratories, Inc.) in a reaction mixture described by Saiki et al. (25), except that $MgCl_2$ was at 2 mM and each primer was at 0.1 μM.

Purification of amplified DNA and direct sequencing. After purification of 300 μl of amplified DNA by chloroform-isoamylic alcohol (48:2) extraction, the aqueous phase was precipitated with 1 volume of isopropanol in the presence of 2 M ammonium acetate. After centrifugation (14.000 × *g*), the pellet was washed in 70% ethanol, dried, and dissolved in 300 μl of water. Oligonucleotides were removed by spin dialysis on a Centricon C30 concentrator (Amicon, Inc.) as recommended by the manufacturer. The membrane-retained DNA was washed five times with 500 μl of water before being recovered. The collected DNA was precipitated twice by isopropanol as described above and finally was redissolved in 20 μl of 10 mM Tris-HCl (pH 8)–1 mM EDTA. Sequence determination was carried out with 500 ng of purified DNA, with 1 pmol of the oligonucleotides used as primers according to Casanova's method (5), and with [α-^{35}S]dATP (Amersham Corp.) with the Sequenase version 2.0 sequencing kit (United States Biochemical Corp.). In certain cases, internal primers designed on the basis of the R8A2HP spiralin gene have presented mismatches with the corresponding sequence for one of the strains, preventing us from sequencing regions of the DD′-purified product. To overcome this problem, these oligonucleotides have been used, despite mismatches, for amplification by PCR and for determination of the sequence of a smaller internal fragment (see Fig. 2).

Labeling of *S. citri* cells with [^{3}H]palmitic acid and globomycin treatment. Spiroplasmas were cultured in SP4 medium complemented with 5 μCi of [9, 10(*n*)-^{3}H]palmitic acid (Amersham Corp.) per ml in the presence or absence of globomycin (50 μg/ml). Globomycin was kindly provided by M. Inukai (Sankyo Co., Tokyo, Japan).

PAGE of total proteins, staining, and fluorography. The protein extracts were analyzed by one- or two-dimensional electrophoresis on 15 to 20% polyacrylamide exponential gradient gels as described by Mouches et al. (20). Gels were stained with Coomassie blue or silver nitrate (1). For ^{3}H-labeled proteins, the gels were treated with the fluorographic reagent (Amersham Corp.) according to the manufacturer's instructions and exposed under classical X-ray film for 1 to 3 months.

Production of monoclonal antibody. Monoclonal antibody was obtained by immunization of BALB/c mice with three intraperitoneal injections of extensively phosphate-buffered saline (PBS)-washed total proteins of *S. citri* Israel (70 μg each) and an intravenous booster injection (100 μg). Selection of hybridoma was achieved by enzyme-linked immunosorbent assay (ELISA) of strains R8A2HP, Corse, Israel, and Palmyre. Monoclonal antibody 12G9 was selected because it reacts positively with the spiralins of all *S. citri* strains tested. Immunoglobulin 12G9 was found to belong to isotype immunoglobulin G1.

Western immunoblotting. Spiroplasma cells from a 100-ml culture were collected by centrifugation (20,000 × *g* for 30 min) and washed three times in PBS buffer (4 mM $NaHPO_4$, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl [pH 7.2]). Proteins were solubilized in 1 ml of solubilization buffer and separated through a 15 to 20% polyacrylamide exponential gradient gel. Colored molecular weight markers were purchased from Amersham Corp. Proteins were electroblotted at 12 V for 1 h to nitrocellulose by using a semidry blot system with cathodic transfer buffer containing 25 mM Tris base, 190 mM glycine, and 3.5 mM sodium dodecyl sulfate (SDS) and anodic transfer buffer containing 25 mM Tris base, 190 mM glycine, and 20% methanol. After saturation in TBS buffer (50 mM Tris-HCl [pH 7.4], 200 mM NaCl) containing 3% bovine serum albumin for 30 min, the nitrocellulose filter was incubated overnight at room temperature with the monoclonal antibody 12G9. After three washes in TBS, polypeptides reacting with the primary antibody 12G9 were visualized by using a sheep anti-mouse immunoglobulin horseradish peroxidase conjugate with 4-chloro-1-naphtol as a chromogenic substrate.

Formic acid hydrolysis. The spiralin band was cut out from gels weakly stained with Coomassie blue. Spiralin within the gel was cleaved by formic acid as described by Sonderegger et al. (27), except that the resulting peptides were eluted from the gel into charge buffer by an overnight incubation at 20°C. Electrophoretic analysis of hydrolysis products was performed with 20% polyacrylamide gels (bisacrylamide/acrylamide ratio, 0.5%) in the case of fluorography or high-resolution gels for small peptides (26), followed by silver staining.

Nucleotide sequence accession number. The NUCALN alignment program of Wilbur and Lipman (33) was used for nucleotide sequence analysis. Predicted amino acid sequences were obtained with the NUMSEQ translational program of Fritensky et al. (13). The nucleotide sequences reported in this paper have been submitted to the GenBank databases under accession numbers U13996 (strains Israel and Asp1), U13994 (strain Alcanar 254), U13993 (strain 78), U13995 (strain Corse), U13998 (strain R8A2B), and U13997 (strain Palmyre).

TABLE 1. Apparent and calculated molecular masses of various *S. citri* spiralins

Spiralin from strain	Mol mass (kDa)	
	Apparent[a]	Calculated[b]
Group 1		
Israel, Asp1, Alcanar 254	24.0	25.257
78	24.0	25.285
MH2, M4, GII3	24.0	ND[c]
Group 2; Corse	24.5	25.186
Group 3		
R8A2HP, C189	28.0	25.300
Hinckley	28.0	ND
Group 4; R8A2B	28.5	25.372
Group 5; Palmyre	32.0	25.563

[a] Apparent molecular masses are deduced from the EM of spiralin.
[b] Calculated molecular masses are deduced from the primary amino acid sequence of the corresponding spiralin gene.
[c] ND, not done.

RESULTS

Polymorphism of spiralin. Total proteins of 13 *S. citri* strains have been submitted to SDS-PAGE. Five groups of *S. citri* strains could be distinguished on the basis of the EM of their major protein (Table 1). Results from the SDS-PAGE analysis of the total proteins of a representative strain of each group are presented in Fig. 1A. Western blot analysis with spiralin-specific monoclonal antibody 12G9 shows that the major protein of all *S. citri* strains tested is spiralin (Fig. 1B). Strain Israel spiralin (lane 4) and the slightly different Corse spiralin (lane 3) have the highest EM. The spiralins of R8A2HP (lane 1) and R8A2B (lane 2) have an intermediate EM, whereas Palmyre spiralin (lane 5) has the smallest EM.

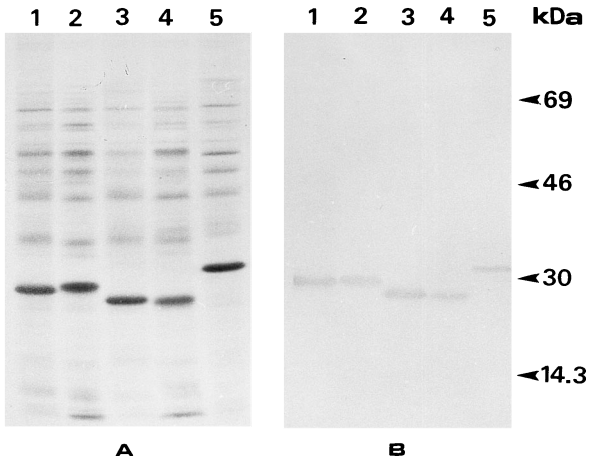

FIG. 1. Coomassie blue-stained SDS-PAGE pattern (A) and Western blot analysis with monoclonal antibody 12G9 (B) of *S. citri* R8A2HP (lane 1), R8A2B (lane 2), Corse (lane 3), Israel (lane 4), and Palmyre (lane 5).

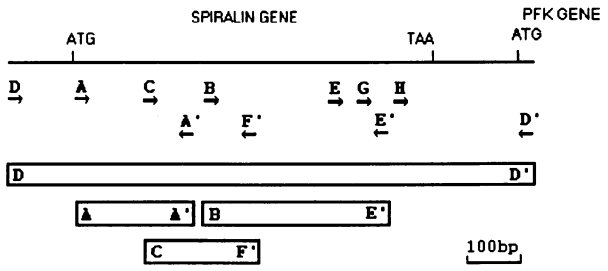

FIG. 2. Schematic representation of direct sequencing strategy with PCR-amplified spiralin genes. Oligonucleotides are represented by arrows. Open boxes DD′, AA′, BE′, and CF′ symbolize the PCR-amplified fragments.

Direct sequencing of PCR-amplified spiralin genes of eight *S. citri* strains. To determine the molecular bases of the spiralin polymorphism, we have sequenced the spiralin genes of eight *S. citri* strains. Since the nucleotide sequence of the spiralin gene of *S. citri* R8A2HP was already known (6), PCR was used to obtain the sequences of the other spiralin genes. For this purpose, two primers were chosen. Primer D is located about 108 bp upstream of the spiralin gene start codon, and primer D′ is located at the beginning of the gene coding for the phosphofructokinase (Fig. 2).

As expected from the gene sequence, PCR of genomic DNA of strain R8A2HP with the primer pair DD′ leads to a 1,053-bp amplified product. Similar amplifications have been obtained for all *S. citri* strains tested (data not shown).

Direct sequencing of the DD′-amplified product has been carried out as shown in Fig. 2 with primers D and D′ and internal oligonucleotides based on the R8A2HP spiralin gene. The strains selected were grouped as follows: group 1, Israel, Asp1, Alcanar 254, and 78; group 2, Corse; group 3, C189; group 4 R8A2B; and group 5, Palmyre.

The nucleotide sequences of all DD′ fragments determined show an open reading frame that has more than 96% homology to the spiralin gene of *S. citri* R8A2HP (taken as the reference strain). The predicted amino acid sequences are shown in Fig. 3. Seven spiralins have 241 amino acids; the spiralin of strain Palmyre has 242 amino acids. The strains C189 (group 3) and R8A2HP (group 3) have identical spiralin gene sequences. Compared with the R8A2HP spiralin sequence, one and three nucleotide changes, respectively, are found in R8A2B (group 4) and Corse (group 2) spiralin genes, and the corresponding spiralins have one and three amino acid changes. Asp1, Israel, and Alcanar 254 spiralins (group 1) are identical and differ from R8A2HP spiralin by 12 amino acids. This difference is the result of 13 point mutations, including the same 3 mutations observed in strain Corse, except for strain Alcanar 254, in which an additional 14th nucleotidic mutation occurs without an amino acid change. Spiralin of strain 78 (group 1) differs from those of Asp1, Israel, and Alcanar 254 by one amino acid change at position 39. Palmyre spiralin (group 5) is the most different spiralin, with 21 amino acid differences. Compared with the R8A2HP spiralin gene, 33 mutations affect 21 triplets, including a 3-bp insertion that occurs nine nucleotides upstream of the end of the gene.

The spiralin gene polymorphism can be useful to distinguish *S. citri* strains from group 1, group 2, groups 3 and 4, and group 5, because they have a different spiralin gene restriction map. From an *S. citri* culture, the spiralin gene can be amplified by PCR, followed by restriction of the amplified product by three restriction enzymes: *Rsa*I, *Dra*I, and *Mbo*I (data not shown). This *S. citri* spiralin gene typing is currently used in our laboratory and has been applied throughout our work to verify the absence of strain mistakes or contamination of one strain by another.

The absolute molecular masses of the different spiralins, as deduced from the amino acid sequences (Table 1), are 25,563 (strain Palmyre), 25,372 (strain R8A2B), 25,300 (strain R8A2HP), 25,186 (strain Corse), 25,257 (strains Israel, Asp1, and Alcanar 254), and 25,285 (strain 78) Da. The difference in molecular mass between the smallest and largest spiralins is only 377 Da. The difference in the apparent mass of spiralins as observed by PAGE is 8 kDa, more than 20 times higher.

```
                1       10        20        30        40        50        60        70        80
R8A2HP/C189:    MKKLLSILAVFGVSAVGTTSVVACNKTESNNLSIVKTIAVPATVATANPKQVTNAEIKTALEANVLKAVQGVVKTATAADFQ
R8A2B      :    --------------------------------------------------------------------------------
CORSE      :    ----------------------------------------A---------------------------------------
ISR/ASP/ALC:    ----------------------------------------A---------------------------------------
78         :    ---------------------------------------VA---------------------------------------
PALMYRE    :    ----------L-----------------------N---A-------------K------------Q-----------D---

                                                                                          ↓
                      90        100       110       120       130       140       150       160
R8A2HP/C189:    FDVYQDNKGTSLTTINLEEGNVEVYVQITPAKDKTVVIGETGYIKVTLPKIKVDISGVVIDQQIVEIKAADPKQVTKDELNA
R8A2B      :    --------------------------------------------------------------------------------
CORSE      :    ------------------G-------------------------------------------------------------
ISR/ASP/ALC:    -------E---------QG--------------------K----------------S---N--------------------
78         :    -------E---------QG--------------------K----------------S---N--------------------
PALMYRE    :    ---------E--------G-------------------------------------------------------------

                   170       180       190       200       210       220       230       240
R8A2HP/C189:    VNTYATLASAVLEAIKNKAPNAGASDFEITNNCDAGDYSAQKDVKVTVKAKDESPNISGEFKVNAKVKATLAPPK*AG
R8A2B      :    ------------------------------------E-------------------------------------*--
CORSE      :    ------------------------------------------------------------------------N*--
ISR/ASP/ALC:    ------------------------------------N--E----------------------------I---AN*--
78         :    ------------------------------------N--E----------------------------I---AN*--
PALMYRE    :    --------------------------------D-NE----T--N---------K---------F------INKKVTP--
```

FIG. 3. Amino acid sequences of spiralin from various *S. citri* strains. The spiralin amino acid sequence of strain R8A2HP (6) is taken as a reference. Dashes correspond to amino acid identity, and stars indicate the absence of the corresponding residue. The putative signal sequence is underlined. The arrow indicates the conserved formic acid cleavage site.

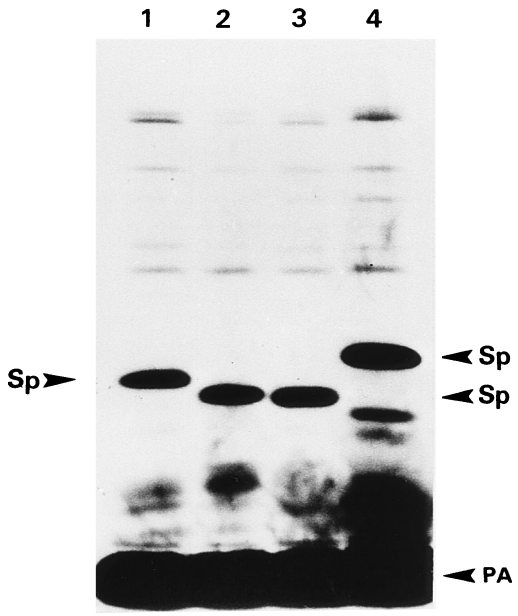

FIG. 4. Fluorography of SDS-PAGE of [^{3}H]palmitic acid metabolically labeled proteins of four representative *S. citri* strains. Lanes: 1, R8A2HP; 2, Corse; 3, Israel; 4, Palmyre. PA, nonincorporated palmitic acid; Sp, spiralin.

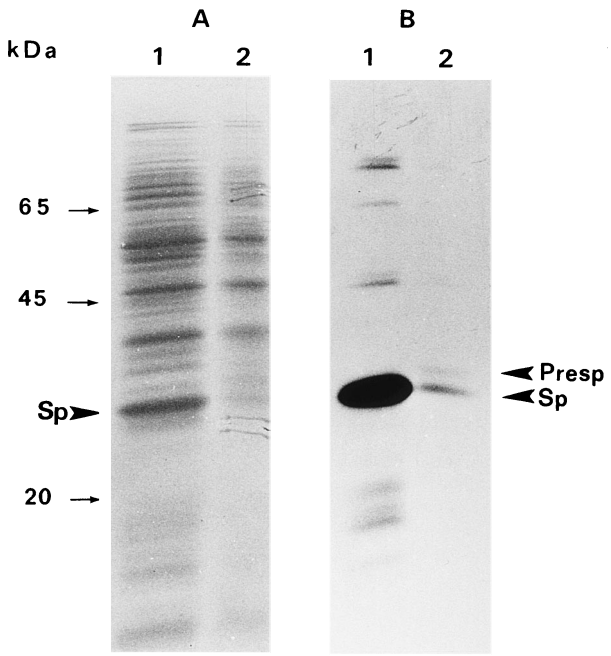

FIG. 5. SDS-PAGE pattern of [^{3}H]palmitic acid metabolically labeled proteins of *S. citri* R8A2HP, after Coomassie blue staining (A) or fluorography (B), in the absence (lane 1) or presence (lane 2) of globomycin at 50 μg/ml. Sp, spiralin; presp, prespiralin.

In contrast to the C-terminal parts of the spiralins, which are relatively variable, the putative N-terminal signal peptide, including the cysteine at position 24, and the putative transmembrane α-helix are highly conserved (Fig. 3). The only change occurs in strain Palmyre, in which leucine replaces phenylalanine at position 11 (Fig. 3), but both amino acids are hydrophobic. In view of the highly conserved N-terminal sequence, it is very likely that if a signal peptide cleavage occurs, the same peptide would be removed from all spiralins. This removal would yield a mature spiralin with a size of 218 amino acids, except for Palmyre spiralin, in which 219 amino acids would be present. Therefore, the observed differences in spiralin electrophoretic mobilities cannot be due to differences in size or modification of the N terminus.

It must be noted that amino acid changes affect serine or threonine, proposed as a candidate for acylation (7, 37). Compared with the spiralin of strain R8A2HP, the spiralins of strains Asp1, Alcanar 254, 78, and Israel have an additional serine residue in place of a glycine at position 139. Strain Palmyre spiralin has the same number of threonine residues as strain R8A2HP spiralin; it has lost threonines at positions 92 and 234, which were replaced by a glutamic acid residue and an isoleucine residue, respectively, but it has gained two threonine residues at positions 204 and 239 in replacement of alanine and lysine residues.

[^{3}H]palmitic acid labeling of spiralin. We attempted to directly demonstrate the fatty acid modification of *S. citri* spiralin by metabolic labeling of intact cells with [^{3}H]palmitic acid. Ten-fold-diluted cultures of four representative strains of *S. citri* (R8A2HP, Corse, Israel, and Palmyre) were incubated for 24 h in SP4 medium in the presence of [^{3}H]palmitic acid. Total spiroplasmal proteins were examined by SDS-PAGE, and the [^{3}H]palmitic acid labeling was visualized by fluorography (Fig. 4). About 10 labeled protein bands were detected. Spiralin was the most intensively labeled band in each strain.

Effect of globomycin on spiralin maturation. A 10-fold-diluted culture of *S. citri* R8A2HP was incubated for 24 h in SP4 medium containing [^{3}H]palmitic acid, in the presence (50 μg/ml) or absence of globomycin, a signal peptidase II inhibitor in *E. coli* (12). Total proteins were analyzed by SDS-PAGE. The Coomassie blue-stained proteins (Fig. 5A) revealed an important modification of the protein pattern for strain R8A2HP grown in the presence of globomycin (lane 2) in comparison with the same strain grown in the absence of globomycin (lane 1). The quantity of spiralin was strongly reduced in the presence of globomycin. Fluorography was then performed with the same gel to visualize the [^{3}H]palmitic acid-labeled proteins (Fig. 5B). In the absence of globomycin, spiralin was the most labeled protein. When globomycin was added to the culture medium (lane 2), the labeled proteins, including spiralin, were present in smaller quantities, even though the total amounts of proteins detected in lanes 1 and 2 were similar, as shown by the Coomassie-stained gel (Fig. 5A). An additional band was detected above the spiralin band. This additional acylated protein is very likely prespiralin that has not been induced to mature by the lipoprotein signal peptidase, because it is inhibited in the presence of globomycin.

Formic acid hydrolysis of spiralin. It would be important to know which part of spiralin is involved in polymorphism. Interestingly, a cleavage site for formic acid (16), an aspartylprolyl bond, is conserved in all of the spiralins studied (Fig. 3, arrow). Theoretically, two peptide fragments must be obtained upon formic acid hydrolysis of spiralin: a large fragment representing the N-terminal part of the spiralin and a small fragment corresponding to the C-terminal part and carrying the putative transmembrane α-helix. In the case of strain R8A 2HP, the molecular masses of these two peptide fragments, as deduced from the gene sequence, are 9,165 Da (88 amino acids) for the C-terminal part and 14,597 Da (130 amino acids) for the N-terminal part when the signal peptide is removed. These calculated molecular masses include a classical dipalmi-

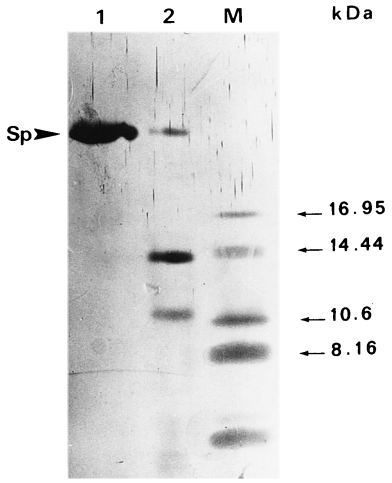

FIG. 6. Analysis of the peptides released by formic acid hydrolysis of spiralin (Sp) from *S. citri* R8A2HP. Lanes: 1, untreated spiralin; 2, formic acid-treated spiralin. M, molecular mass markers.

toyl diglyceride modification of the N-terminal cysteine and a palmitic acid molecule linked to the N terminus (14).

The spiralin band was excised from two-dimensional polyacrylamide gels of *S. citri* R8A2HP total proteins. Spiralin was submitted to formic acid treatment, and the hydrolysis products were analyzed with high-density polyacrylamide gels (Fig. 6) as described by Schägger and von Jagow (26). Two peptides were obtained, with apparent molecular masses of 14.3 and 11.1 kDa (Fig. 6, lane 2). To see which part of the protein is responsible for the polymorphism and where acylation is located, formic acid hydrolysis was carried out with [^{3}H]palmitic acid-labeled spiralin of the four representative strains (R8A 2HP, Corse, Israel, and Palmyre). The peptides released by formic acid hydrolysis were analyzed with a high-density polyacrylamide gel and detected by silver staining (Fig. 7A) or fluorography (Fig. 7B). For all four *S. citri* strains, two peptides with molecular masses close to those of molecular mass markers with sizes of 14,440 and 10,600 Da were detected by silver staining, whereas only the 14.3-kDa peptide was revealed by fluorography (Fig. 7B). The labeled peptides had an electrophoretic mobility close to 14.3 kDa (Table 2) for each strain analyzed. This peptide is very probably the N-terminal part of spiralin after acylation and signal peptide cleavage. The unlabeled peptides present a more variable electrophoretic mobility, even though the greatest difference in apparent molecular mass is only 0.8 kDa (Table 2), between strain Israel (group 1) and strain Palmyre (group 5). These peptides represent the C-terminal part of the spiralin, known to be more variable, according to the gene sequence analysis, than the N-terminal part of the protein (Fig. 3). Moreover, the apparent molecular mass of the C-terminal peptides of the four spiralins analyzed is 1.5 to 2 kDa higher than the molecular mass calculated from the gene sequence (Table 2).

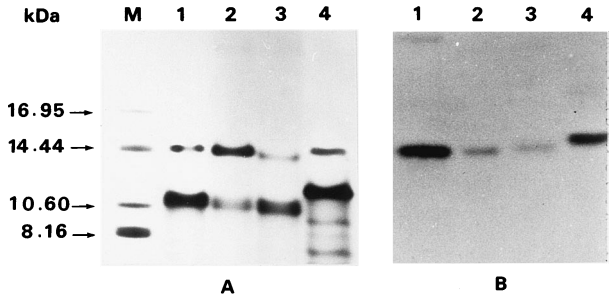

FIG. 7. Formic acid hydrolysis of [^{3}H]palmitic acid metabolically labeled spiralin of four representative *S. citri* strains. Hydrolysis products were analyzed with a high-density polyacrylamide gel and silver stained (A) or analyzed with a 20% polyacrylamide gel and fluorographed (B). Lanes: 1, R8A2HP; 2, Corse; 3, Israel; 4, Palmyre; M, molecular mass markers.

TABLE 2. Expected and apparent molecular masses of the two peptides released by formic acid hydrolysis of four representative *S. citri* strains

Strain	Mol mass (kDa) of peptides			
	N-terminal part		C-terminal part	
	Calculated[a]	Observed[b]	Calculated[a]	Observed[b]
R8A2HP	14.597	14.3	9.165	11.1
Corse	14.497	14.2	9.151	11.0
Israel	14.525	14.0	9.194	10.7
Palmyre	14.529	14.3	9.530	11.5

[a] Calculated molecular masses of the N-terminal part on the basis of signal peptide cleavage and maturation of the cysteine 24 by a dipalmitoyl diglyceride and fixation of a myristic acid on the N terminus.

[b] Apparent molecular masses deduced from the EM of the peptides on a high-density polyacrylamide gel.

DISCUSSION

Spiralin polymorphism in *S. citri* strains. We have shown that spiralins of different *S. citri* strains do not have the same EMs in SDS-PAGE. *S. citri* strains can be grouped according to the EMs of their spiralins. In addition to spiralin polymorphism, restriction fragment length polymorphism studies (3) with ribosomal or viral probes made it possible to define similar groupings of *S. citri* strains. It must be stressed that no variation in the EM of spiralin has been observed for any *S. citri* strain over many years of culture passaging (unpublished data). No evidence of phenotypic switching has been found for *S. citri* spiralin (21a), in contrast to many animal mycoplasmal lipoproteins involved in high-frequency phase variation and high-frequency size variation (4, 22, 23). Moreover, the spiralin gene is present as a single copy in the chromosomes of all *S. citri* strains examined (7, 39). Sequence determination of spiralin genes of eight different *S. citri* strains by direct sequencing of the PCR-amplified DNA has revealed differences according to the spiralin EMs. These differences are almost exclusively point mutations, except for a 3-bp insertion at the end of the spiralin gene of strain Palmyre. All *S. citri* spiralin genes encode 241- or 242-amino-acid-long proteins. The differences in spiralin molecular masses, deduced from the gene sequences, cannot explain the EM polymorphism. In the same way, the number of these changes does not seem to be the only reason for spiralin polymorphism, since the EM of spiralin of strain Corse with 3 amino acid changes is very similar to that of strain Israel with 12 changes.

As shown by [^{3}H]palmitic acid metabolic labeling, *S. citri* spiralin is an acylated protein, like *S. melliferum* spiralin (37). Unusual internal acylation on serine or threonine residues has been proposed for *S. melliferum* (37) and *S. citri* spiralin (7). Variations in the number and/or in the location of serine and threonine residues occur in the different *S. citri* spiralins studied and could result in variation in the number of these hypothetical posttranslational sites of acylation. Such acylation could also be affected by modifications of the spatial arrange-

ment of spiralin as a result of amino acid changes other than those affecting serine and threonine. As a consequence, variation in the number of such posttranslational acylations could explain the spiralin polymorphism. However, as demonstrated by formic acid hydrolysis of [^{3}H]palmitic acid-labeled spiralin, the acylated region of spiralin is certainly the N-terminal part of the protein and shows only a very small polymorphism. According to these data, we conclude that spiralin polymorphism in *S. citri* is not due to differences in posttranslational palmitoylation from one strain to another. The polymorphism observed for the EM of the C-terminal peptide can be estimated to be about 0.8 kDa (Table 2). This value must be compared with the difference in EM observed for the whole spiralin (i.e., about 8 kDa). We cannot exclude that polymorphism of the whole spiralin could be the effect of a hypothetical posttranslational modification sensitive to the acidic conditions occurring during the formic acid treatment. Determination of the molecular masses of whole spiralins and peptides by Electrospray ionization-mass spectrometry will allow investigation of this hypothesis.

Spiralin signal peptide maturation. When the lipoproteins of *S. citri* cells were labeled with [^{3}H]palmitate in the presence of globomycin, a new labeled peptide appeared in addition to spiralin and had a molecular mass similar to that expected for prespiralin (Fig. 5). However, control cells labeled in the absence of the antibiotic had larger amounts of spiralin, suggesting that the presence of globomycin results in a loss of spiralin and probably prespiralin. The globomycin sensitivity of *S. citri* cells suggests that spiralin is processed as a classical bacterial lipoprotein (14) by lipid modification of the N terminus and removal of the signal peptide by a globomycin-sensitive signal peptidase II (12, 14). The following two results are in favor of this proposal. (i) The *S. citri* spiralin gene has been recently cloned in *Acholeplasma laidlawii*. The expressed protein contained the signal peptide and had a molecular mass 5 to 6 kDa larger than that of the mature signal peptide-free *S. citri* spiralin (15). (ii) Spiralin purified from *S. citri* has an amino acid composition very similar to that corresponding to the signal peptide-free spiralin (17). According to these data and if our own *S. citri* spiralin matures as a classical bacterial lipoprotein, this processing would yield a mature protein with a size of 218 amino acids in all strains examined, except in strain Palmyre, which has a spiralin with a size of 219 amino acids. Also, the N-terminal cysteine of spiralin would carry three fatty acid molecules: two as a diglyceride on the SH group and one as free acid on the NH_2-terminal group. However, in the case of *S. melliferum* spiralin, the signal peptide sequence of which is identical to that of *S. citri* spiralin (7), only one fatty acid molecule was reported (37), not three. The value of only one fatty acid molecule for mature *S. melliferum* spiralin is probably an underestimate, because of technical difficulties, as indicated by the authors themselves (17), and should not weaken the proposal that *S. citri* spiralin is processed as a classical bacterial lipoprotein.

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