SURFACE PROTEINS OF

SPIROPLASMA CITRI

By

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CHAPTER I

LITERATURE REVIEW

Introduction

This thesis concerns the surface proteins of *Spiroplasma citri*. The introduction will make clear why surface proteins are of interest and why *S. citri* was chosen as the organism in which to study these proteins.

My own research has two parts, one of which is to identify those *S. citri* proteins actually having a surface component, and the other is to clone some of the genes which code for these proteins. Nine surface proteins were identified using protease treatments of intact cells and immunoprecipitation. Only one of these proteins, spiralin, was previously identified as having a surface component. Although several *S. citri* genes were successfully cloned in this project, none of those genes expressed proteins which reacted with antisera generated against several surface proteins that were extracted from SDS polyacrylamide gels.

In order to give the reader a basic understanding of the organism involved, the general characteristics of the class and genus to which it belongs will be related, and then a more in depth description of the species itself will be given. Literature which supports the interest in and use

of this particular organism will then be reviewed, as well as literature which reveals some of the important roles that surface proteins play in other organisms.

Generic names are often used for this and related organisms in the literature, and also in this thesis. Accordingly, the terms mollicute(s), mycoplasma(s), and spiroplasma(s) are often used generically to denote members of the class Mollicutes, and the genera Mycoplasma and Spiroplasma respectively.

Mollicutes

Spiroplasma citri is a member of the family Spiroplasmataceae (5), in the class Mollicutes (25). Mollicutes may be parasites of plants, animals, and arthropods (19). They are the simplest and smallest recognized procaryotes. They differ from other procaryotes in their lack of a cell wall, being bounded only by a plasma membrane, a feature which makes them completely resistant to antibiotics that inhibit cell wall synthesis (e.g. penicillin) (25). Their lack of a cell wall increases the ease with which their membranes may be isolated and manipulated, making them useful for studying membrane structure and function. Having the smallest recorded genome in living cells, their double-stranded circular DNA ranges from about 770 kilobase pairs (kbp) for Mycoplasma and Ureaplasma species to about 1,500 kbp for Acholeplasma, Spiroplasma and Anaeroplasma species (19). Their DNA is relatively poor in G+C content, which

ranges from 25 to 34 mol%.

Mollicutes contain a variety of extrachromosomal DNAs (19). Spiroplasma plasmids appear as open circular, linear, and closed circular DNA, but because viral infections are prevalent in these organisms it may be difficult to distinguish the replicative form of a viral DNA from a plasmid. Most of the viruses which infect mollicutes morphologically resemble classical bacteriophages, having the appendages thought to be necessary for injecting the viral DNA through the cell wall. However, there are two, MLV2 and L172, which have no such appendages, and resemble animal viruses in many respects. Many viruses infecting mollicutes release by budding through the plasma membrane without causing cell lysis.

Two opposing models have been proposed for the phylogeny of mollicutes. One suggests that mollicutes are the descendents of primitive bacteria which existed before the procaryotic-eucaryotic split (19). In this model, the smaller _Mycoplasma_ and _Ureaplasma_ species would have come first, with the larger _Acholeplasma_ and _Spiroplasma_ species evolving through genome doubling. The second model proposes that mollicutes are on the opposite end of the phylogenetic tree, representing the most evolved procaryotes. Some proponents of this model suggest that mollicutes are descendents of low G+C clostridia and that evolution occurred through chromosomal deletions, resulting in part, in the loss of a cell wall. Belief that mollicutes are primitive

organisms seems to be based on their small genome size and their metabolic and structural simplicity. However, rRNA analysis, which is currently the most widely accepted evidence of phylogeny, suggests that mollicutes are a product of degenerative evolution, originating from a branchpoint deep within the Bacillus-Lactobacillus-Streptococcus subgroup of eubacteria (26).

Woese et al. (26) compared 16s rRNA oligonucleotide catalogs of several mollicutes and two clostridia. These catalogs were generated by digesting the 16s rRNA with T1 ribonuclease, which yields G-ending oligonucleotides up to 20 bases in length. Analysis of such data is usually done by comparing the number of nucleotides found in sequences (hexamers and greater) which are common to the catalogs in question. However, the authors state that when dealing with mollicutes, this kind of analysis can be misleading because many of the sequences which are highly conserved in the 16s rRNA catalogs of other eubacteria are absent from their catalogs. Instead, a signature analysis is used, in which sequences occurring in 50% or more of the members of a group, and only rarely, if at all, outside the group, are considered. Signature analysis implies a close phylogenetic relationship of the clostridia, Clostridium ramosum and C. innocuum to the mollicutes and suggests that mollicutes evolved from the Bacillus-Lactobacillus-Streptococcus subgroup and that wall-lessness may have occurred as separate events for different clusters of mollicutes (rather

than the existence of a single wall-less ancestor).

Proponents of either model would probably agree that, because of their size and simplicity, mollicutes may prove very useful in determining the minimal requirements for the survival of free-living organisms.

Spiroplasmas

Spiroplasmas can easily be distinguished from other mollicutes by their helical morphology, which is believed to be linked to their motility (25). In liquid media of low viscosity, spiroplasma cells exhibit vigorous rotation and flexing. When Daniels *et al*. (2) increased medium viscosity by addition of methylcellulose, swimming speed of spiroplasma cells (strain BC3) was proportional to the amount of viscosity. Spiroplasmas (BC3 and *S. citri* strain SP13) also demonstrated viscotactic ability. Ten ul capillary tubes, partially filled with varying concentrations of viscous solutions of either methylcellulose, starch, Ficoll, polyethylene glycol or polyvinylpyrrolidone were suspended in spiroplasma cultures and incubated at 32C. Capillaries were removed after varying lengths of time, wiped with ethanol, and the contents ejected into liquid medium, which was then serially diluted and plated on solid medium. Plate counts revealed an increase in the number of spiroplasmas in capillaries with increasing time and viscosity, reaching a maximum after 30 minutes. There was also an optimum concentration of polymer above which plate counts actually

decreased because capillary contents could not be completely recovered. In similar experiments, spiroplasmas displayed temperature dependent chemotactic abilities with some substances, including certain carbohydrates and amino acids, functioning as attractants, while lactic acid, nicotinamide, uracil and other amino acids were effective repellents (2). Viscotaxis and chemotaxis are most probably involved in the growth and life cycle of spiroplasmas.

Plant spiroplasmas have a complex biological cycle, in which they infect and multiply not only within their plant hosts, but also within insect vectors (24). The ability of spiroplasmas to move rapidly through these viscous environments could aid in their propagation. They have been found in and isolated from the phloem tissue of plants, which are usually adversely affected by spiroplasma infection (3). Effects are often nonlethal however, and transmission from plant to plant occurs via homopterous insects which feed on the phloem of infected plants (25). The spiroplasmas, by an unknown mechanism, pass through the cells of the gut epithelium into the hemolymph, and then pass through salivary acini before being transmitted to another host (24, 25). It is not known whether or not passage through the insect vector has an effect on the plant pathogenicity of the spiroplasma. Accumulation of high concentrations of spiroplasmas in the salivary glands of leafhoppers and in the young shoots of citrus plants may be due to the ability of spiroplasmas to respond to chemical

gradients.

Unlike plant pathogenic mycoplasmas, many spiroplasmas can be cultivated in artificial medium (20). This, of course, increases the ease and precision with which they can be isolated and manipulated, thereby increasing their usefulness in the study of plant diseases caused by mollicutes. Early culture media were patterned after the composition of plant sap, but later media resembling insect hemolymph aided in cultivating the more fastidious spiroplasmas (25). In general, a medium containing 10-20% animal serum, peptone, tryptone, carbohydrates, amino acids, yeast extract and yeast hydrolysate is used. The precise ingredients and optimal concentrations vary for different spiroplasmas. A pH indicator is usually added to detect changes in medium acidity which occur during growth of the organism. Because spiroplasmas are resistant to penicillin, it is often added to culture medium to retard bacterial contamination.

It is unknown how spiroplasmas maintain their helical morphology. However, it has been proposed that fibrils, which can be released from spiroplasma membranes by treatment with detergent, may be part of the cytoskeletal system (22). In order to investigate the nature of these fibrils, Townsend, _et al_. (22) grew honeybee spiroplasmas (strain BC3) in liquid medium, harvested via centrifugation, and solubilized in detergent. When triton X-100-insoluble material was subjected to isopycnic centrifugation, two proteins of approximately 26,000 and 55,000 daltons were

found in the same bands. However, treatment of this material with KCl and EDTA prior to centrifugation caused a significant dissociation of these proteins. Two-dimensional electrophoretic separation of SDS-treated purified fibrils revealed one protein of approximately 55KD. Fibrils were 3.5nm in diameter and had an axial repeat of 8.5nm. Those subjected to high-salt treatment and EDTA formed highly ordered aggregates with a striated appearance, while those not subjected to high-salt conditions tended to associate in pairs. The fibrils exhibited pronase sensitivity, but were resistant to trypsin.

The obvious association of fibrils with the 26KD protein lends to interesting speculation, because the major membrane protein of Spiroplasma citri, spiralin, has a similar molecular weight (22). Perhaps the fibrils are linked to spiroplasma membranes by an ionic bond with spiralin (or, in the case of BC3, a spiralin-like protein) because the high-salt-EDTA treatment disassociated the fibrils from the 26KD protein. The fact that these fibrils associate in such a highly ordered manner in vitro, suggests that their interaction might also have biological function. They are thought to be involved in the maintenance of cell shape and motility. However, it is unlikely that they have sole responsibility for these features, since no evidence of contraction and relaxation of purified fibrils was found. It is more likely that motility and helicity involve other proteins which are associated with partially purified fibrils.

One of these proteins, extracted from *S. citri* cells, has similar molecular weight and solubility properties to actin, a contractile protein found in eucaryotic cells. Antiserum directed against invertebrate actin was shown to specifically bind to *S. citri* cells (25). Another fibril-associated protein (MW 40,000) is thought to be identical to that of a missing protein from *S. citri* (strain ASP-1). This variant is both nonhelical and nonmotile, indicating that these two functions are probably related.

Spiroplasma citri

Growth in Artificial Medium

Spiroplasma citri, the causative agent of citrus stubborn disease (21) and horseradish brittle root disease (3) has been the most widely studied spiroplasmal pathogen. Garnier *et al*. (4) examined *S. citri* during different phases of its growth in liquid medium. Samples of broth cultures were fixed with glutaraldehyde and deposited onto agar. The agar was then flooded with procelloidine, and the resultant dried film (with adsorbed cells) was transferred onto collodion membranes and examined with an electron microscope; a technique which preserves the cellular helicity of the spiroplasma cells. In a mid-logarithmic phase culture grown at 32C, helices with one to more than ten turns were observed, the shortest organisms having the widest helices. Two-turn helices were the most prevalent, and were one of three distinct morphological types. The most common were

cells with one blunt end and one tapered end, while cells with two tapered ends or two blunt ends were seen less frequently. Long helices possessed one or more constrictions, which led to the formation of tapered ends. These observations strongly suggested that cells with two tapered ends result from multiple division of a long helix containing more than one constriction, while cells with one blunt end and one tapered end are the product of division of a four-turn parent helix. Accordingly, a broth culture of S. citri exhibiting good growth can be recognized by high frequency of two-turn and four-turn helices.

Aging cultures of S. citri exhibit the appearance of round bodies and long, unconstricted filaments. While exhaustion of medium nutrients may be partially responsible, it is also known that the pH of older cultures is lower, which increases the binding of serum proteins to the membranes, causing morphological alterations (4). The use of buffered media, however, can help prevent these changes from occurring.

The optimum temperature for cell growth is 32C. When cells were grown at 32C and then shifted to 37C, the number of short helices (associated with good growth) decreases, and longer helices are more often seen (4). These longer helices are observed to have very few constrictions, indicating their decreased ability to divide. These cells are still viable, however, and division is resumed when optimal temperature is restored.

Growth in Insects

The biological cycle of S. citri includes growth and multiplication within its insect vectors (24). Three different leafhoppers are known to be involved in the natural transmission process (18). Among these, S. citri has most frequently been isolated from Circulifer tenellus, which occurs widely in North America. Scaphytopius nitridus and Scaphytopius acutus delongi, which can both be found in California, have also been recognized as natural vectors of S. citri. It is likely that there are many more as yet unidentified homopterous insects which harbor this pathogen, as indicated by evidence that seven species of leafhoppers in Morocco demonstrated S. citri antigens when subjected to an enzyme-linked immunosorbent assay (ELISA) (25).

Although S. citri cells can be located in the intestines, salivary glands and somatic muscles (20) of its insect vectors, the highest concentration of cells is found in the hemolymph, where the organisms most probably multiply (25). When Russo et al. (20) fed S. nitridus on a sucrose solution containing S. citri, they found that most of the organisms within the leafhoppers appeared spherical instead of helical when viewed by electron microscopy. Townsend et al. (23) obtained similar results a few days after injecting Euscelis plebejus with S. citri. When salivary glands were excised and observed under the electron microscope, non-helical S. citri cells in densely packed membrane bound pockets were observed. Although S. citri has been con-

sistently found to lose its helicity in the hemolymph of leafhoppers, the Drosophila, corn stunt and honey bee spiroplasmas have each been found to retain their helicity within the hemolymph of their respective hosts (4, 23). Corn stunt spiroplasmas, however, do lose helicity within the cells of their vectors, suggesting that the medium and environment which spiroplasmas are exposed to may play a role in determining their morphology.

Townsend et al. (23) demonstrated conclusively that S. citri did multiply within the leafhopper E. plebejus after a lag period of four days, reaching a maximum titer of over 10 million colony forming units per insect after 10-12 days. They also found that survival rate of E. plebejus was good even when high concentrations of S. citri were attained, in contrast with similar experiments with the leafhopper Macrosteles fascifrons, in which S. citri significantly decreased longevity. It would seem reasonable that the relationship between S. citri and its natural vectors would be one of tolerance rather than lethality.

Effect on Plant Hosts

S. citri infects a wide variety of plant hosts, including members of the families Brassicaceae, Fabaceae, Plantaginaceae, Apocynaceae, Crassulaceae, Asteraceae and Violaceae, with varying symptoms in different species (25). In citrus, small malformed fruit, shortened internodes, axillary proliferation, stunting and chlorosis are among

those often expressed at high temperatures (5, 25). Although _S. citri_ is rarely lethal to citrus trees, it can produce sudden wilting and death in periwinkle, bean, and pea plants. The mechanism(s) of pathogenesis are unclear, however, the number of spiroplasmas present within the sieve tubes of infected plants has been correlated with symptom severity (5). It is feasible that damage to the plant host may be caused by physical blockage of the phloem and/or competition for nutrients. It has also been suggested that _S. citri_ may produce a translocatable toxin within the phloem. A small, very stable toxin was isolated from culture fluids, but its role, if any, in plant pathogenesis is unknown.

Citrus Stubborn Disease. Citrus stubborn disease was first observed in naval orange trees in California around 1915, and derived its name from the poor growth characteristics these trees exhibited (5). It was later found to be widely distributed in Arizona and many countries of the Mediterranean and Mideast including Algeria, Iran, Syria, Egypt, Morocco, Lebanon, Jordan, Cyprus, Sicily and Tunisia. The disease was thought to be caused by a virus until 1970, when mycoplasma-like bodies were discovered within the sieve tubes of infected plants. It was not until 1973 that the causative agent of citrus stubborn disease was identified, characterized, and given the status of a new genus and species (21).

Saglio _et al_. (21), in order to obtain pure cultures of

the "stubborn" agent, homogenized leaves of Madame Vinous sweet orange seedlings and sour orange rootstocks at the time these leaves started showing symptoms of the disease. Each homogenate was then passed through a Ø.45um filter under sterile conditions and grown in an appropriate liquid culture medium, after which, cultures were transferred to agar medium. Single fried-egg colonies were then subjected to serial passages in broth, subcultured on agar, and passed through Ø.22um filters before being used for biochemical and biological analysis. Assurance that the organisms isolated in this manner were not due to contamination is based on the fact that they were repeatedly obtained from "stubborn" affected leaves but never from healthy trees growing next to them. Growth was best in 95% nitrogen-5% carbon dioxide, and organisms displayed helical structure and absence of cell wall. Gram staining revealed gram-positive pleomorphic filaments. Growth was serum and sterol dependent, and 32C was found to be the optimum growth temperature. Esculin fermentation and arginine and urea hydrolysis were negative. Penicillin resistance and high tetracycline sensitivity were exhibited, and G+C content was found to be about 26 mol%. Based on these results and others Saglio _et al_. (21), suggested a close relationship between this organism and recognized _Mycoplasma_ and _Acholeplasma_ species, and offered a new genus and species name, _Spiroplasma citri_.

Horseradish Brittle Root Disease. Brittle root (BR), named for the loss of root flexibility, is an extremely

destructive, often fatal disease of horseradish in Illinois (3). Other symptoms of brittle root include stunting, foliar chlorosis, and discoloration of the phloem tissue, appearing as a darkened ring. Diseased plants are found each year to some degree, but brittle root has also been known to occur in epidemics. When the disease was first described in 1929, it was attributed to curly top (CT) virus because it resembled CT disease of sugar beet and was reported as being experimentally transmissible from sugar beet to horseradish and back by Circulifer tenellus, the beet leafhopper. This belief was supported in 1954 when C. tenellus, bearing CT virus, was found in Illinois horseradish fields during the 1953 BR epidemic. It was not until 1980 that mycoplasmalike organisms were found in electron micrographs of tissue from a horseradish plant with BR. Shortly following this, a spiroplasma was cultured from this same plant. In 1981, Fletcher et al. (3) reported evidence that S. citri was the causative agent of BR disease.

Horseradish plants, collected from fields in Illinois, were designated as brittle root (BR), nonbrittle root (NBR), or questionable brittle root (QBR) based on foliar and root symptoms, sealed in plastic bags, and stored at 4C until used (3). Root tissue containing the phloem was removed, minced, and passed through 0.45um filters in sterile medium, and used for primary cultures. Isolates were cloned by passing broth cultures through 0.2um filters, plating on solid medium and then inoculating sterile broth

with single colonies. Spiroplasmas were cultured from each of 31 BR and never from 32 NBR horseradish plants. Isolates exhibited helical cellular morphology in both dark field and electron microscopy, and, as is typical for spiroplasmas, their growth appeared to be sterol dependent. Growth inhibition tests were performed by soaking filter paper disks in antisera to different strains of spiroplasmas and placing them onto solid medium which had been seeded two hours previously with horseradish isolates. After 12 days incubation at 31C, zones of inhibition were measured, and the spiroplasmas were determined to be closely related to S. citri and AS576, the honeybee strain, both of which belong to Serogroup 1. The protein patterns of spiroplasmas isolated from horseradish with BR and from beet leafhoppers fed on BR plants were indistinguishable from S. citri (Maroc R8A2) by polyacrylamide gel electrophoresis. When deformation tests (13) were performed, in which spiroplasmas were exposed to specific antibody and then monitored via dark field microscopy for loss of spiral shape, it was clearly shown that the isolates tested were strains of S. citri (3).

Interest in S. citri

Studies on S. citri would certainly be of interest to farmers growing horseradish crops because of devastating monetary losses due to brittle root epidemics. Citrus growers are also affected by this mollicute because, although S. citri is rarely lethal to citrus trees, there is a

great reduction in the quantity and quality of fruit production. The diseases caused by S. citri provide enough reason for an in depth study of this organism, although many other points of interest exist. The study of surface membrane proteins, which have been shown to play key roles in such events as attachment (7) and invasiveness to host cells (6) in other organisms, is facilitated because of the lack of cell wall. Characteristics of S. citri pose several interesting questions, such as: How is entry into insect cells and movement between plant cells accomplished? Are different proteins (or alternate levels of the same proteins) present on the surface of S. citri in different environments (i.e. plant, insect and artificial media)? If so, how is expression of these proteins modulated? Since these organisms lack flagella and axial filaments, what is responsible for their motility? How is helicity within plants and artificial media maintained without the presence of a cell wall, and why is this property lost within the hemolymph and cells of homopterous insects?

Proteins on the membrane outer surface are available for interaction with host or artificial media, and therefore, knowledge about these proteins will aid in answering these questions.

Membrane Composition and Properties

Mudd, et al. (17) proposed that S. citri membrane cholesterol and phospholipid, and the ratio of these to

membrane protein, may be relevant to cellular shape and motility. Their analysis of the membrane fraction of a two liter culture of S. citri (strain 189) revealed 35.0 mg protein, 14.3 umol phospholipid, 9.55 umol cholesterol, 5.96 mg fatty acid, 28.9% by weight lipid, and 25.6% by weight cholesterol in membrane lipid. Palmitic acid was the dominant fatty acid, with roughly equal amounts of stearic and oleic acids and a small amount of linoleic acid. Enzymatic activities of S. citri membranes were also investigated. Adenosine triphosphatase (ATPase) and p-nitrophenyl phosphatase (PNPPase) were found to be closely associated with the membranes, while reduced nicotinamide adenine dinucleotide (NADH) oxidase activity was found in early washes of the membranes and was inhibited at temperatures above 35C.

Kahane et al. (10), to provide more information about the carbohydrate moieties of the S.citri membrane, incubated membranes of strain ATCC 27556 with radioactively labeled lectins, washed, and incubated them with different sugars. Specific displacement of lectins by the sugars was monitored, and results indicated that some of the mannose, glucose, and galactose residues that are present in the membrane are exposed to the surface. Although N-acetylglucosamine is present in S. citri membranes, it is not available for interaction with its specific lectin unless membranes are treated with proteolytic enzymes. However, overall, the digestion of membrane proteins proportionally

decreased their lectin-binding activity, which would suggest the presence of glycoproteins, but none were detected by SDS polyacrylamide electrophoresed gels stained with periodic acid-Schiff reagent. Results of enzymatic activity assays were similar to those of Mudd *et al*. (17), identifying ATPase and PNPPase as integral membrane proteins, but, in addition, *S. citri* membranes were found to have both ribonuclease and deoxyribonuclease activity (10).

Spiralin. The membrane proteins of *S. citri* have been resolved into approximately 29 bands by SDS-PAGE (16). These proteins range in size from 12,000 to 170,000, with one dominating in quantity at about 22% of the total membrane protein (28). This protein, spiralin, has a molecular weight of 26-28,000 daltons (16). Wroblewski *et al*. (28) reported that by extracting *S. citri* (strain C189) membranes first with Tween 20 and then with sodium deoxycholate, a supernatant enriched for spiralin could be obtained.

The amino acid composition of purified spiralin and unfractionated membranes were determined with an amino acid analyzer (28). No tryptophan or methionine was detected in spiralin, and arginine and histidine values were too low to be accurate, though all four of these amino acids were present in the membranes. When comparing the mol% of different amino acids in spiralin with those of the *S. citri* membrane, spiralin has a considerably higher content of threonine, alanine, and valine, and a lower content of glycine, leucine, and phenylalanine. Although small amounts

of amino sugars were detected in the membrane, spiralin contained none, indicating that it is not a glycoprotein. The fact that spiralin can not be extracted from the membrane in Tween 20 or in aqueous solutions indicates that it is an intrinsic membrane protein.

In a later paper, Wroblewski et al. (27) compared the amino acid compositions of spiralins from S. citri (strains C189, R8A2, and Scaph) and a spiralin (or spiralin-like protein) from the honey-bee spiroplasma (strain B88). They were all similar, but lack of Cys, less Asx and Ile, more Thr and Lys and the presence of His distinguish B88 spiralin from that of S. citri. In crossed immunoelectrophoresis experiments, the spiralins isolated from each of the S. citri strains demonstrated antigenic cross-reactivity with each other, while that of B88 had no shared epitopes with S. citri spiralins. Although spiralin is the most abundant S. citri membrane protein, its function remains unknown.

Cloning the Spiralin Gene. The S. citri spiralin gene has been successfully cloned and expressed in Escherchia coli (15, 16). Mouches et al. (16) isolated the genomic DNA from S. citri (strain R8A2) and digested it with the restriction endonuclease HindIII. The purified fragments were ligated with alkaline phosphatase-treated, HindIII-cut pBR328 (a deletion derivative of an E. coli plasmid), and the products were used to transform E. coli (strain HB101). Only one clone, Tsp, out of 565, elicited a strong positive reaction in an enzyme-linked immunosorbent assay using

rabbit anti-*S. citri* IgG.

Proteins from Tsp, *E. coli* carrying pBR328, and *S. citri* were separated by two-dimensional electrophoresis and transferred to nitrocellulose (16). Filters were probed with either rabbit antibodies against whole *S. citri* cells or with a mouse monoclonal antibody directed against spiralin. Several Tsp proteins reacted with the anti-*S. citri* and also with the anti-spiralin antibodies, while *E. coli* carrying pBR328 did not react with either. These results suggested that more than one form of spiralin was being produced by Tsp.

To characterize the *S. citri* DNA fragment responsible for the expressed spiralin in Tsp, the recombinant plasmid (pES1) was isolated (16). The plasmid was found to be 11.4kbp in size, and restriction mapping revealed that the *S. citri* DNA inserted into the *Hin*dIII site of pBR328 was 6.5kbp in size. Since only about 0.8kbp is required to encode for spiralin, it was unclear whether the additional *S. citri* DNA contained a promoter from which the spiralin was being expressed, or whether expression was being effected from the nearby Tet resistance promoter in pBR328. Another recombinant plasmid (pES2), which harbored the same 6.5kbp *S. citri* DNA fragment, was created and inserted into the same site within pBR328, but in the opposite orientation. Spiralin was found to be expressed in pES2 to the same degree as in pES1, indicating that a spiroplasmal DNA sequence was functioning as a promoter in *E. coli*.

Mouches et al. (15) investigated more deeply the apparent size discrepancy between the spiralin of S. citri and that produced by the E. coli transformant Tsp. Immunoaffinity chromatography of solubilized, carbon 14-labeled proteins from S. citri (R8A2) and Tsp was performed using monospecific antibodies to spiralin. When proteins retained by the column were analyzed by SDS-PAGE, most of the spiralin produced by Tsp was found to be 30.5kd, but a small amount of it had the same molecular weight as the S. citri spiralin, 28.0kd. A very small quantity of the S. citri spiralin was found to have a molecular weight of 30.5kd. These results suggest that spiralin produced in S. citri is expressed as a preprotein that is posttranslationally modified into the mature form, but that this processing is inefficient in E. coli.

Surface Proteins

The focus of the original research presented in this thesis is on surface proteins. Surface proteins, because of their location, are available for interaction with their host and environment. Therefore, it is likely that they contribute to the preservation and propagation of various organisms. Surface proteins have been found to play some important roles in other organisms.

The protein II family of outer membrane proteins of Neisseria gonorrhoeae play important roles in adherence and in susceptibility to killing by serum (9). Conformational

changes of these proteins may be responsible for confusing the host with new immunogenic sites during the course of illness.

Manning *et al*. (12) cloned the *Vibrio cholerae* gene for a 22kd outer membrane protein into *E. coli* K-12 in a variety of recombinant plasmids. The strains harboring one of these plasmids were capable of spontaneous agglutination, suggesting that this outer membrane protein has an adhesive function. This is important because antibodies that are capable of preventing adherence of *V. cholerae* are also capable of preventing disease.

Isberg and Falkow (6) reported converting *E. coli* K-12 into an invasive organism by addition of a single genetic locus from *Yersinia pseudotuberculosis* (an organism capable of invading epithelial cells). A 3.2kbp region of *Y. pseudotuberculosis*, coding for a protein targeted to the surface of *E. coli* K-12, was responsible for invasiveness of K-12 into animal tissue culture cells.

Investigations into the identification and function of surface proteins have also been undertaken with mycoplasmas. Many diseases caused by mycoplasmas have been found to require physical association with the host cell surface (1, 7, 11, 14), implicating mycoplasma surface components. The initiation of disease by *Mycoplasma pneumoniae* is brought about by attachment to the host cell (7). Attachment is accomplished by a surface protein located on the tip structure of *M. pneumoniae*, and monoclonal antibodies

directed against this adhesin inhibit binding (8). Clyde and Hu (1) found that monoclonal and monospecific antibodies directed against the attachment protein of *M. pneumoniae* reacted with a *Mycoplasma gallisepticum* protein of similar molecular weight and with a smaller protein of *Mycoplasma genitalium*. Since these mycoplasmas similarly parasitize host cells, shared epitopes suggest the possibility that these proteins may have similar functions.

Minion *et al.* (14) have shown that the ability of *Mycoplasma pulmonis* to agglutinate erythrocytes was removed by proteolytic treatment of the mycoplasma, suggesting that a membrane protein(s) was responsible for this property. Since *M. pulmonis* infections require intimate association with the membranes of host mucosal surfaces, it is likely that surface proteins, such as the one responsible for hemagglutination, may be involved.

As previously stated, *Spiroplasma citri* cells bind to and enter the gut epithelial and salivary cells of their homopterous insect vectors. The mechanism(s) are unknown, but may be similar to those of mycoplasmas. Investigation into the identification and function of surface proteins should shed some light on the subject.

Thesis organization

Chapters two and three of this thesis describe research on the surface proteins of *Spiroplasma citri*. Hopefully, the literature review has made clear the interesting features of

this organism, and surface proteins in general. Chapter two is a transcript of an article that is awaiting approval for publication (Fletcher, J., J. W. Wills, and S. E. Denman. 1988. Newly identified surface proteins of *Spiroplasma citri*. Submitted to the Journal of Bacteriology). It describes the identification and partial characterization of several *S. citri* surface proteins. The research was performed by the authors and by Amy James (Department of Plant Pathology). Chapter three describes the expression of *S. citri* genes cloned in *Escherichia coli*. This research is principally my own.

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CHAPTER II

NEWLY IDENTIFIED SURFACE PROTEINS OF *SPIROPLASMA CITRI*

Introduction

Spiroplasma citri is the causative agent of a lethal stunting disease in a number of host plants including citrus and several Brassicaceous species. The pathogen replicates in the phloem and is transmitted by several species of leafhoppers. Spiroplasmas are wall-less members of the class Mollicutes, but unlike mycoplasmas they are helical in morphology. Spiroplasmas are currently the only phyto-pathogenic Mollicutes to be cultivated, although a number of mycoplasmas have been cultured from animal hosts and many mycoplasma-like organisms have been associated with plant diseases.

Surface proteins of Mollicutes have been implicated in a variety of activities related to interactions with their host cells. Several mycoplasmas, including *Mycoplasma pulmonis*, *M. genitalium*, and *M. gallisepticum* bind to their host animal cell using a specific adherence structure containing adhesin proteins without which attachment is inhibited (1, 3, 14, 20). It is not known whether *S. citri* binds to its plant host during the infection process.

However, spiroplasma cells will bind to several tissue cultured lines of insect cells, including Drosophila cell line Dm-1 (10, 18), indicating that surface proteins may be involved in such attachments.

Surface proteins may also function as enzymes in the infection process. M. pulmonis seems to contain two such surface enzymes; a nuclease and an enzyme with hemolytic activity (19). Several enzymes including ribonuclease, deoxyribonuclease, adenosine triphosphatase, and p-nitrophenyl phosphatase have been associated with the membranes of S. citri; whether any of these are surface proteins is unknown (14, 21).

The attachment of viruses to Mollicutes has been associated with surface proteins in the case of Acholeplasma laidlawii (11). However, in other virus-Mollicute interactions attachment may occur to a specific lipid molecule (22). S. citri serves as a host for viruses of several kinds, but the nature of the attachment sites is unknown (4, 5, 6, 7).

Little is known about the proteins on the surface of plant Mollicutes including spiroplasmas. The most studied spiroplasma protein is spiralin, which is 29,000 daltons and comprises 22% of the total S. citri membrane protein (30). Spiralin has been found to be amphiphilic and to have a surface component (28, 30). The importance of surface proteins in the pathogenicity of plant Mollicutes is unknown. As an initial step to investigate the molecular

interactions between S. citri and its plant and insect host, we chose to look for proteins on the surface of this pathogen. In this paper we describe the identification of several surface proteins of S. citri by protease treatments and by surface immunoprecipitation.

Materials and Methods

Culture Maintenance

S. citri strains BR3, BR6, and BR18 were originally cultivated from Illinois horseradish affected by brittle root disease and had been subcultured fewer than twenty-seven, seventeen and six times, respectively. Strains R8A2 and ASP-1 were kindly provided by R. E. Davis, USDA-ARS, Beltsville, MD. Strain Ex Calavan Citrus and Iran were gifts from B. Kirkpatrick, University of California, Davis, and R. Whitcomb, USDA-ARS, Beltsville, MD, respectively. S. floricola 23-6, S. melliferum AS576, and S. kunkelii CSS were also from R. E. Davis. All spiroplasmas except S. kunkelii were maintained aerobically in LD8 broth (17) at 31C. S. kunkelii was grown under anaerobic conditions (Gas-Pak Anaerobic Jar; BBL Microbiology Systems, Cockeysville, MD) at 31C in C3-G broth (2).

Isolation of S. citri BR3 Membranes

Membranes were prepared using a modification of the method reported by Razin (23). Briefly, cells (1 L) were pelleted at 13,700 xg, 15 min, and washed twice in 1/2

volume of 0.07M HEPES-S (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5, with 10% sucrose). The final pellet was resuspended in 2 ml of 0.5 M NaCl in 25mM Tris, pH 7.0 and homogenized in a glass tissuemizer. The homogenate was diluted 100-fold in 20 mM $MgCl_2$, 25mM Tris, pH 7.0; DNase 1 was then added to 10 ug/ml. The solution was agitated for 15 min at 37C, and then centrifuged at 30,000 xg for 30 min at 4C. The pellet was washed once with water and once with 0.150 M NaCl in 25 mM Tris, pH 7.0 before final resuspension in 200 ul water. Small aliquots were frozen at -80C.

Antiserum Preparation

Antiserum against *S. citri* BR3 membrane preparation was produced in a New Zealand white rabbit in a manner similar to that described previously (8). Approximately 250 ug membrane protein (determined by Bio-Rad protein assay, Bio-Rad Laboratories, Richmond, CA) per injection was thawed and 50% of the volume was denatured by adding SDS to 1% and boiling for 5 min. Ice-cold absolute ethanol (10 volumes) was added to precipitate the proteins. After micro-centrifuging for 5 min the supernatant was removed and the pellet resuspended in a small volume of phosphate buffered saline (PBS). This preparation was mixed with the remaining, undenatured membrane sample. Four injections were given over a period of 8 wk and the final antiserum had a titer of 1:25,600 as determined by the serological deformation test

(27).

Protease Treatments of Intact Spiroplasmas

All protease treatments were performed using *S. citri* strain BR3. Trypsin, chymotrypsin, proteinase K, and papain (all from Sigma Chemical Co., St. Louis, Mo.) were dissolved in water at 10 ug/ul. For each test sample, 10 ml of log phase cells of *S. citri* were centrifuged at 12,100 xg for 20 min, washed twice in HEPES-S, and then resuspended in 1.0 ml HEPES-S. Aliquots of these preparations either were incubated with enzyme or served as controls with no enzyme. Enzymes were tested at a range of concentrations, from 0-100 ug/ml of sample. Optimum concentrations were 25 ug/ml for trypsin, chymotrypsin and proteinase K and 0.025 ug/ml for papain. Because the pH optimum of papain is 6.7, HEPES-S for papain treatments was adjusted to pH 6.7 rather than 7.5. After 10-60 min incubation (optimum 60 min) at 37C the contents of tubes were microfuged at 9550 xg for 5 min and washed in HEPES-S. Final pellets were dissolved in 60-100 ul sample buffer (0.5 M Tris-HCl, pH 6.8, containing 10% glycerol, 0.1% bromphenol blue, 2.0% SDS, and 2.0% B-mercaptoethanol), boiled for 2 min, and loaded onto polyacrylamide gels (described below). In some experiments protease inhibitors were added at different steps to inactivate the enzyme. Enzyme inhibitors were soybean trypsin inhibitor (0.1 ug/ul), N-p-tosyl-L lysine

chloromethyl ketone (TLCK; 0.021 ug/ml), N-tosyl-1-phenylalanine chloromethyl ketone (TPCK; 0.33 ug/ml), and phenylmethylsulfonyl fluoride (PMSF; 1.0 mM). With the exception of proteinase K,inhibitors did not affect results. The addition of PMSF at 1.0 mM following proteinase K incubation and further addition of PMSF at 1.0 mM in sample buffer was necessary to prevent complete degradation of cellular proteins. In some experiments, proteins from enzyme treated cells were transferred to nitrocellulose and Western blots were performed (described below) using our anti-*S. citri* membrane serum, diluted 1:300 in antibody buffer (20 mM Tris, 500 mM NaCl, pH 7.5 (TBS), containing 0.05% Tween 20 and 1% BSA).

Surface Immunoprecipitation

Thirty ml of log phase cultures of intact spiroplasmas were centrifuged at 9750 xg for 15 min; pellets were then resuspended in 3 ml of HEPES-S. Samples of 300 ul were incubated for 2 hr at 4C with varying amounts of undiluted anti-*S. citri* membrane serum to allow binding of the antibody to proteins on the surface, then microcentrifuged 2 min and washed twice with HEPES-S to remove unbound antibodies. Pellets were resuspended in 500 ul of a hypotonic solution (10 mM Tris, pH 7.6, 10 mM NaCl, and 2 mM $MgCl_2$) and incubated at room temperature for 20 min. The membranes were solubilized by the addition of 100 ul of 5X lysis buffer (5% Triton-X 100, 5% Na deoxycholate, 0.5% SDS, 0.75 M NaCl and

125 mM Tris-HCL, pH 7.5); antigen-antibody complexes were then collected by the addition of 100 ul Staph A protein (15) for 1 hr at room temperature. After microcentrifugation the Staph A pellets were resuspended, boiled in 25 ul sample buffer and loaded onto SDS polyacrylamide gels. Proteins were transferred to nitrocellulose and Western blots were performed using our anti-S. citri membrane serum, diluted 1:300 in antibody buffer.

Separation and Visualization of Spiroplasma Proteins

All samples were loaded and electrophoresed by the method of Laemmli (16) in 1.5 mm SDS slab gels containing 10% acrylamide. To visualize the proteins, gels were either stained with Coomasie blue or blotted onto nitrocellulose. For Western blotting (24), the separated proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA) using a Transphor apparatus (Hoefer Scientific Instruments, San Francisco, CA) for 1.25 hr at 100 volts in transfer buffer (0.192 M glycine, 0.025 M Tris pH 8.3, and 20% v/v methanol). The molecular weights of proteins were determined by the co-electrophoresis and transfer of prestained molecular weight standards (Diversified Biotech, Newton Centre, MA) which appear as blue bands on the nitrocellulose. To block unbound sites on the nitrocellulose membranes, filters were incubated overnight at 4C in a 7.5% solution of Carnation

powdered skim milk (12). After three washes in TBS they were incubated for 3 hr at room temperature in the anti-membrane serum, diluted 1:300 in antibody buffer. After washing as above, the nitrocellulose was incubated for 1 hr in protein-A-horseradish peroxidase (Sigma Chemical Co., St. Louis, MO), diluted to 2 ug/ml in antibody buffer. A final wash was followed by incubation in the substrate color developing reagent (5 parts TBS, 1 part 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO) at 3 mg/ml in methanol, and 0.018 part of 3% H_2O_2). Maximal color developed in 10-15 min; blots were then washed in water and air-dried.

Results

Protease Treatments

After intact S. citri BR3 cells were treated with various proteases, washed, and boiled in sample buffer, the remaining proteins were viewed on Coomasie blue stained polyacrylamide gels. Examples from typical experiments are shown in Figure 1 (Appendix B). Bands which decreased in intensity when treated with a given protease were interpreted as corresponding to BR3 surface proteins. Only proteinase K degraded spiralin, a 29 KDa protein (Figure 1A, lanes a and b), while chymotrypsin was the only enzyme to degrade proteins of 37 and 54 KDa (Figure 1A, lanes e and f; Figure 1B). Both trypsin and chymotrypsin have protease activity on a 45 KDa protein (Figure 1A, lanes c-f), and all three proteases cleave a 58 KDa protein (Figure 1A, lanes a-

f). Proteinase K and trypsin both had enzymatic activity on a 61 KDa protein (Figure 1A, lanes a-d), while only proteinase K degraded 86 and 89 KDa proteins (Figure 1A, lanes a and b). The effects of these enzymes are further substantiated by the successive decrease in the intensity of the indicated protein bands with increasing amounts of enzyme, as shown for chymotrypsin in Figure 1B. These results are summarized in Table 1 (Appendix A). Papain treatment did not result in the visible loss of bands in the gels. However when these gels were blotted, immunologically reactive degradation products were detected, indicating that digestion of proteins had occurred (data not shown). We were unable to identify the intact papain-sensitive surface proteins from which these peptides were derived.

It is possible that larger surface proteins were not detected by this treatment. The bands near the top of the gels were unclear or faded, possibly due to residual activity of proteases in the samples. Inclusion of protease inhibitors did not improve our results for proteins in this region (data not shown). Question marks on Figure 1A indicate bands which seem to disappear with enzyme treatment; however, the visual clarity of the gel precludes a definite conclusion.

Surface Immunoprecipitation

An immunoprecipitation procedure (Figure 2, Appendix B)

was designed to allow identification of membrane proteins having antigenic determinants exposed to the cell surface. Proteins on the surface of intact S. citri BR3 cells were directly collected by incubating the cells with anti-BR3 membrane serum. After washing to remove unbound antibodies, the cells were lysed and solubilized and cell lysates were incubated with formalinized staphylococcal cells (Staph A). The precipitate was collected by centrifugation, washed to remove unbound antigen and boiled in sample buffer. Spiroplasma proteins recovered by this method were examined by SDS-PAGE using both Coomasie blue staining (Figure 3, panel A, Appendix B) and Western blot analysis (Figure 3, panel B, Appendix B). In this scheme, bands which appear on these gels presumably represent cell surface proteins as opposed to the protease treatments in which we looked for disappearing bands. When stained with Coomasie blue, the gels exhibited faint bands corresponding to BR3 surface proteins with molecular weights of 29, 58, 86 and 89 KDa. The intensity of these bands increased with increasing amounts of antiserum (Figure 3A, lanes a-c and lanes d-g) and/or BR3 cells (Figure 3A, lanes c, f and g). Bands representing Staph A proteins are seen in Figure 3A, lane k. The antibody heavy chain is clearly visible between the 45 and 58 KDa standards in lanes c, f, and g, while the light chain is a faint smear below the 31 KDa standard in the same lanes. In Figure 3B, lane i, precipitation of the spiralin protein (29 KDa) has occurred in the absence of antibody or

Staph A. However, the quantity of "self-precipitating" spiralin is not great enough to visualize on the stained gel in Figure 3A. Treatments with Staph A in the absence of antibody (Figure 3A, lane h) gave the same bands as similar treatments without Staph A (Figure 3A, lane i), indicating that the S. citri proteins do not stick nonspecifically to Staph A. The Western blot using anti-BR3 membrane serum revealed the same surface protein bands, but an additional 77 KDa surface protein was also seen (Figure 3B; Table 1).

The possibility exists that a protein band appearing in the surface immunoprecipitation tests may not be a surface protein. A cytoplasmic protein stably associated with a trans-membrane protein, for example, might be pelleted as a Staph A-antibody-surface protein-internal protein complex, and react with the anti-membrane serum in the blots. To address this possibility, the surface immunoprecipitation experiments were repeated using additional antisera prepared against enriched (i.e. partially purified) individual surface proteins. Antigens for the immunizations were prepared by excising four bands (the 86/89 KDa doublet, the 77 KDa band, the 58 KDa band and the 29 KDa band) from SDS polyacrylamide gels and grinding them in PBS. Antisera were prepared as described (Materials and Methods) and shown to react with the appropriate proteins in Western blots of whole cell protein preparations. When these antisera against individual proteins were used in surface immunoprecipitations, all presumptive surface proteins, except the 58 KDa

protein were consistently collected with the corresponding antiserum (data not shown), suggesting that they are indeed located on the surface. In the case of the 58 KDa protein, the SDS treatment of the preparative gel may have eliminated native antigenic determinants, so that the resulting antibody does not recognize the protein in its native state. This would explain why the anti-membrane serum (prepared against non-detergent treated antigens) reacted with the 58 KDa protein. This interpretation seems likely because the protease treatments clearly removed a 58 KDa protein, indicating its surface location.

Strain Comparisons

We compared different spiroplasma strains to determine whether the presence of these proteins on the surface is universal among spiroplasmas. Using the surface immunoprecipitation technique, several different strains of *S. citri* and three other species of spiroplasmas were examined by Western blot analysis (Figure 4, Appendix B). Although the panels in this photograph are from different gels, resulting in bands which do not line up from one panel to another, the proper alignment is indicated by the letters (a-d) to designate bands of proteins that are probably related antigenically. All of the tested strains that are known to be antigenic for plants (BR3, BR6, and BR18) contained an 89 (a_1) and/or 86 KDa (a_2) surface protein(s) which reacted with our antiserum. Because the relative intensity of these

two proteins (86 and 89 KDa) routinely differed from experiment to experiment, and even with different treatments within a single experiment for a given strain, we predict that these are related forms of the same protein. One may be a degradation product of the other. For these reasons, we have designated the two bands a_1 and a_2. The resolution of this issue awaits further experimentation. Strain R8A2, presumed to be non-pathogenic for plants, does not contain these proteins. Pathogenicity of the other strains is uncertain. Bands corresponding to the 77 KDa (b_1) and 58 KDa (c_1) surface proteins can be seen in all strains except 23-6. Again, sometimes two bands can be seen with each of these (b+c) and it is uncertain if they represent more than one protein each. Spiralin or a spiralin-like protein (d) can be seen in all strains except 23-6.

Discussion

This is the first report of the identification of proteins on the surface of the membrane of _S_. _citri_ other than the major membrane protein, spiralin. We have identified eight other proteins having a surface component, and explored their presence in multiple strains of _S_. _citri_ and in several other spiroplasma species.

Interpretations of the protease treatments were made by comparing gels from a number of experiments and observing the relative decrease in intensity of the protease-treated preparations. All proteins degraded by proteases in these

treatments were presumed to have surface components. The success of this method relies on the integrity of the S. citri membrane during treatment. Cells were pelleted and washed before enzyme treatment to eliminate any endogenous proteases released from S. citri. The fact that no protein was attacked by all four proteases suggests that the exposure of the cells to the buffer alone did not induce protease of spiroplasma origin to become activated causing the disappearance of particular bands. In addition, cells treated with each protease were still helical when examined with 1000x dark field optics, and were still viable when transferred into liquid medium (data not shown), indicating that most of the cells were intact following treatment.

It is possible that certain surface proteins may have gone undetected by this method. For example, the 77 KDa protein identified by surface immunoprecipitation was not detected by proteases in our experiments, and other such proteins could be present in levels below detection by Coomasie blue staining. If present in detectable levels, their disappearance could also be masked by the presence of a predominant band at the same position on the gel. Alternatively, there may be surface proteins that are not susceptible to degradation by any of the four proteases used in these experiments.

The specificity of action of the four proteases used in these experiments may provide some clues regarding the nature of the proteins attacked. Trypsin and chymotrypsin

cleave specific amino acid linkages. Trypsin cleaves after lysine and arginine, and chymotrypsin cleaves after the large hydrophobic or aromatic residues phenylalanine, tyrosine, and tryptophan (9). Proteinase K and papain are rather nonspecific, and are capable of completely hydrolyzing proteins, although papain is especially active at phenylalanine, lysine, glutamine, histidine, glycine and tyrosine (9). Wroblewski et al. (31) reported that spiralin lacks methionine, histidine and tryptophan, and has a low content of glycine, leucine, tyrosine and phenylalanine, but a high content of threonine, alanine and valine. Thus it is not surprising that chymotrypsin does not attack spiralin, and although trypsin is specific for two residues present in the molecule, they may be located in sites unavailable for attack due to folding or masking by other molecules. Proteinase K would be expected to digest spiralin, and it does. The reason for non-degradation of other surface proteins by proteinase K is unknown, but may relate to the positioning of these proteins in the membrane. Positioning may also explain the inefficiency of papain in cleaving the surface proteins.

Spiralin is the most studied protein of S. citri, probably due to its strong antigenicity and its prevalence as a membrane component (25, 30). Our work with proteases has shown that, of the four proteases used, only proteinase K attacked spiralin. There was no evidence of substantial breakdown products of this protein, even when 15% gels were

used (data not shown), suggesting that there are no major protein residues of spiralin on the interior of the membrane, extending into the cytoplasm. This is not inconsistent however, with the concept of an intrinsic protein.

The surface immunoprecipitation method was undertaken to answer questions raised by the protease treatments and to confirm the presence of surface proteins observed with the latter treatment. The requirement of surface immunoprecipitation that cells be intact and not contaminated with intracellular antigens at the time of antibody treatment was satisfied by washing steps in the protocol. The antibody incubation is a mild treatment, especially when compared to protease treatment, and would not be expected to lyse cells. Although spiroplasmas are deformed by antibody binding, any intracellular (non-membrane bound) proteins released by such a reaction would be washed away whether bound to antibody or not.

It would be possible to miss a surface protein by this method if the protein were of low antigenicity or were inaccessible to the antibody. For example, we found that a 45 KDA protein was degraded by trypsin and chymotrypsin treatment, but was not detected by surface immunoprecipitation. Surface proteins present at low concentrations might also be missed. In conclusion, we have identified at least nine membrane proteins of S. citri which have surface components. These proteins are approximately 29, 37, 45, 54, 58, 61, 77, 86 and 89 KDa (summarized in Table 1, Appendix

A); only the 29 KDa protein has been previously reported as a surface protein (28). It is noteworthy that Wroblewski (29) identified seven major and several minor proteins in the membranes of S. citri, suggesting that they were intrinsic. These proteins are approximately 39, 51, 69, 76, 89 and 95 KDa. Several of these proteins may be identical to the surface proteins described in this paper.

Surface immunoprecipitation experiments using several pathogenic and nonpathogenic strains of S. citri and other spiroplasmas revealed the presence of the 77, 58 and 29 KDa protein bands in all tested spiroplasmas except 23-6. The latter spiroplasma is the only one in the test group not in Serogroup I (26); therefore it is not surprising that 23-6 would have different surface antigens. It is interesting that the 86 and 89 KDa protein bands were seen in the immunoprecipitation blots only in S. citri strains BR3, BR6, and BR18 (all early passage strains from brittle root diseased horseradish, believed to be pathogenic), and Ex Calavan citrus and Iran (both from stubborn diseased citrus, pathogenicity uncertain). These bands were not seen in S. citri strains R8A2 (believed nonpathogenic) and ASP-1 (a nonhelical derivative of R8A2) or other species of spiroplasmas (S. kunkelii, a pathogen of corn, and S. melliferum and S. floricola, both nonpathogenic to plants). Whether the 86 and 89 KDa proteins are related to pathogenicity or transmissibility is uncertain at this time.

Surface proteins of animal mycoplasmas have been

implicated in a variety of activities related to interactions with their host cells, including adsorption to the host cells, enzyme activity, and attachment of mycoplasma viruses (1, 3, 11, 14, 19, 20, 22). These and other possible roles of surface proteins in the pathogenicity of Mollicutes to hosts or insect vectors are important reasons to continue the investigation of the nature and activity of the surface proteins of the phytopathogen _S. citri_.

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CHAPTER III

CLONING OF *SPIROPLASMA CITRI* GENES IN *E. COLI*

Introduction

Very little is known about the structure or function of those *S. citri* proteins that are exposed to the cell surface. The previous chapter described the identification of several of these proteins. The cloning of *S. citri* surface protein genes would be a step toward understanding the nature of these proteins and therefore the nature of some of the interactions between *S. citri* cells and their environment. This chapter contains data on the construction of an *S. citri* BR3 genomic library in *E. coli* and the search for clones expressing *S. citri* surface antigens.

Methods and Materials

DNA Isolation, Fragmentation and Fractionation

Total DNA of *S. citri* strain BR3 (origin and maintenance related in Chapter 2) was isolated by a modified method (8) of Maniatis *et al.* (3). Spiroplasma cells were pelleted and resuspended in resuspension buffer (100 mM

NaCl, 50 mM EDTA, 100 mM Tris, pH 8.0). Proteinase K (Sigma Chemical Co., St. Louis, MO.) was added to a final concentration of 100 ug/ml and SDS was added to a final concentration of 1% followed by incubation at 50C for 15 min. The DNA was then extracted with phenol/chloroform (1:1), followed by chloroform, ethanol precipitated, resuspended in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0), treated with RNase and again precipitated with ethanol. The isolated DNA was dissolved in resuspension buffer and mechanically sheared by passage through a French pressure cell at 2,000, 3,000 and 4,500 pounds of force. This method of fragmentation was favored over the use of restriction endonucleases because of the very low G+C content of spiroplasmas (3), which makes them less susceptible to most of the commonly used restriction enzymes, yielding fragments too large for efficient insertion into plasmid cloning vectors. The fragmented BR3 DNA was extracted with phenol/chloroform, followed by chloroform, ethanol precipitated, centrifuged at 9,000 xg for 20 min at 4C, and resuspended in TE buffer. Fragments were separated on the basis of size by ultracentrifugation on a 5-20% continuous sucrose gradient and fractions were collected. Fractions containing DNA fragments of 2,000 base pairs (bp) and smaller were pooled, purified by phenol and chloroform extractions, ethanol precipitated and dissolved in TE buffer.

Library Constructions

Sheared, fractionated BR3 genomic DNA was incubated for 1 hr at room temperature with the Klenow fragment of DNA polymerase I and all four deoxyribonucleotides in to cause the fragments to become blunt ended. The bacterial plasmid pUC19 (Bethesda Research Laboratories (BRL) Gaithersburg, Maryland) was used as a cloning vector. It is a derivative of an E. coli plasmid, pBR322, and contains a polylinker located near the 5' end of the genetically engineered alpha complementing portion of the E. coli lac z gene, between the promoter and the coding sequence. Foreign DNA inserted into the polylinker region may be expressed by the lac promoter as a truncated B-galactosidase fusion protein if it is in the correct reading frame and orientation, contains a number of base pairs which is a multiple of three and has no stop codons (Figure 5, Appendix B). The portion of B-galactosidase coded for by pUC19, together with the complementary portion expressed by the bacterium used, yields the functional protein. This system was chosen for several reasons which will be explained in the discussion section of this chapter. The plasmid was digested with Sma I, for which there is a unique site located within the polylinker, producing blunt ends. The Sma I-cut pUC19 was then treated with alkaline phosphatase (4 units/ 30 ug DNA), incubated 15 min at 37C, then 15 min at 55C. This procedure was repeated with a second volume of alkaline phosphatase (AP) and then the reaction mixture was made 0.1 M NaCl, 10 mM EDTA and 1%

SDS before adding 1 ul of 25 ug/ul proteinase K. This mixture was incubated for 30 min at 55C, after which, phenol/chloroform and chloroform extractions, followed by ethanol precipitation, were performed. The precipitate was collected by centrifugation and dissolved in TE buffer.

One ug of sheared, Klenow treated BR3 DNA and 0.3 ug of Sma I-cut, AP treated pUC19 were ligated with T4 DNA ligase (total volume 55 ul) at 15C for either 45 or 90 min. The ligation products were used to transform *E. coli* strain JM107 (BRL, Gaithersburg, Maryland). Frozen, transformation competent JM107 cells (200 ul) were thawed slowly and incubated for 30 min on ice with 10 ul of a 1:100,000 dilution of the reaction products of either the 45 min ligation or the 90 min ligation in Falcon 2059 tubes. Cells were then heat shocked at 42C for 90 seconds and placed back on ice for 2 min. The cells were diluted with 800 ul of SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose), followed by a 1 hr incubation with agitation at 37C. A portion of the transformed JM107 cells was frozen at $^-$80C in 50% SOC, 25% glycerol and 25% LB (10% w/v tryptone, 10% w/v NaCl, 5% w/v yeast extract) for later use.

An additional library of BR3 DNA was constructed (by Dan Obermark) using larger fragments. In the event that only part of a gene was cloned in the previously described library, DNA/DNA hybridizations would allow the entire gene to be isolated from this second library. To this end, total

BR3 genomic DNA was partially digested with *Sau* 3A and the fragments size fractionated as before. The restriction enzyme *Bam* HI (which produces ends that are compatible with *Sau* 3A ends) was used to digest pUC19, followed by treatment with AP. Fragments of *Sau* 3A cut BR3 with average sizes around 10,000 bp were ligated with the *Bam* HI cut, AP-treated pUC 19. The recombinant plasmids were used to transform *E. coli* strain TB1 (BRL, Gaithersburg, Maryland).

Immunological Screening

Transformants from both libraries were spread onto LB agar plates (1.5% agar) containing 50 ug/ml ampicillin (Amp) and incubated at room temperature. When colonies were clearly visible (about 2 days), autoclaved nitrocellulose filters were placed on top of the growing colonies and allowed to remain there until wet. Filters were marked for orientation and suspended in a sealed chamber partially filled with chloroform (to allow the vapor to lyse the cells) for 20-30 min. These were then transferred to petri dishes (2 filters, back to back/dish) containing 20 ml blocking/lysing solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 5% w/v Carnation nonfat dry milk, 0.01% antifoam A and 1 ug/ml DNase and 40 ug/ml lysozyme, both added immediately before use) and incubated at 4C overnight. Filters were washed three times with TTBS (20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% v/v Tween 20) and incubated at room temperature for 9 hr in a 1:350 dilution of anti-*S. citri*

(whole cell) antiserum (1) in antibody buffer (1% w/v bovine serum albumin Fraction V; BSA; in TTBS) and washed twice in TTBS. This was followed by a 1 hr incubation at room temperature (with agitation) in a 1:10,000 dilution of protein A-horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) in antibody buffer. Filters were then washed twice with TTBS and once with TBS (TTBS without the Tween 20) prior to color development by incubation at room temperature in a substrate mixture consisting of 0.5 mg/ml 4-chloro-1-naphthol (Sigma Chemical Co, St. Louis,MO), 16.6% v/v methanol, 83.3% v/v TBS and 0.05 ul/ml of a 3% H_2O_2 solution. After 30 min filters were washed with water and air dried without exposure to light. Positive clones, recognised by a blue-black spot on the filter, were picked from the agar plates and rescreened by streaking each onto new LB agar+Amp plates and repeating the above procedure. Clones again proving positive were picked and screened once more. Those clones expressing _S. citri_ antigens upon the third screening were grown in LB broth+Amp and a portion of each was frozen at -80C in 75% LB broth and 25% glycerol.

DNA Characterization

The fragments of cloned _S. citri_ BR3 DNA coding for the protein products recognized by the anti-_S. citri_ serum in the above immunoblots were partially characterized by size determination, restriction mapping and DNA/DNA hybridization.

Recombinant plasmids from positive clones were isolated and digested with the restriction endonucleases *Eco*RI and *Hin*dIII, which cut within the polylinker region of pUC19 on either side of the insert (effectively releasing the foreign DNA from the plasmid) and electrophoresed in 0.8% agarose submerged slab gels at 100 volts for 30 min. The DNA was stained with ethidium bromide and fragment sizes were compared with coelectrophoresed *Hin*dIII-cut lambda DNA standards. Recombinant plasmids were also analyzed by mapping with the restriction endonucleases *Pvu*II, *Kpn*I, *Sal*I and *Nar*I in addition to the previously used enzymes. Single digests were performed with *Pvu*II and *Kpn*I, while double digests were carried out with *Nar*I+*Sal*I and *Eco*RI+*Hin*dIII.

To further characterize the BR3 DNA harbored within these recombinant plasmids, hybridization experiments were done to determine which clones contain common DNA sequences. The steps taken were essentially as described by Maniatis, *et al*. (3). Plasmids were digested with both *Hin*dIII and *Eco*RI and electrophoresed in 0.8% agarose gels as described above, and photographs were taken. To aid in the transfer of the DNA, gels were subjected to acid depurination (0.25 M HCl) and alkali denaturation (0.5 M NaOH, 1.0 M NaCl) by gentle rocking in each solution for 15 min. Gels were rinsed with water and neutralized (0.5 M Tris-HCl, 3 M NaCl) for 15 min. The DNA was then transferred to nitrocellulose (BA85; Schleicher and Schuell Inc, Keene, NH) by stacking (from bottom up) paper towels, 3MM paper, agarose gel, nitro-

cellulose, 3MM paper, and more paper towels under a 600 gram weight using 20x SSC (2) as the transfer buffer. After 4.5 hr the nitrocellulose was peeled away from the 3MM paper and the agarose gel and soaked in 6x SSC for 5 min, drained, and baked for 2 hr at 75C under vacuum. Filters were then immersed in 6x SSC for 2 min and placed inside heat sealable plastic bags (which when sealed have 1/2 inch clearance on sides and bottom of filter and one inch on top). A prehybridization fluid (5x SSC, 0.5% SDS, 5x Denhardt's solution (2), 100 ug/ml denatured salmon sperm DNA, 45% formamide, 1 mM EDTA), for the purpose of blocking any nonspecific binding of nucleic acids, was added, and the bags were heat sealed and submerged in a 42C water bath for about 5 hr with periodic agitation to insure even coating of the filters.

Three of the clones were chosen for use as DNA probes. Prior to their addition to the hybridization bags, these recombinant plasmids were labeled with biotin-11 dUTP (BRL, Gaithersburg, Maryland) by nick translation. For each recombinant plasmid to be used as a DNA probe, the following reaction mixture was set up: 5 ul of 10x nick translation buffer (NTB; 0.5 M Tris-HCl, pH 7.2, 0.1 M $MgSO_4$, 1 mM dithiothreitol, 500 ug/ml BSA), 5 ul (about 1 ug) DNA, 1 ul each of unlabeled deoxynucleotide triphosphates dATP, dGTP and dCTP (1 mM stocks), 1 ul biotin-11 dUTP (0.4 mM stock), 30.5 ul water, 2.5 ul of a 1:5 dilution (in NTB) of E. coli DNA Polymerase I, and 3 ul DNase I (0.1 ug/ml stock). The reaction mixtures were incubated at 16C for about 1.5 hr,

followed by addition of 2 ul Ø.5 M EDTA and 1 ul 1Ø% SDS to stop each reaction. The nick translated plasmids were then recovered by two isopropanol/Na acetate precipitations (with addition of transfer RNA). Pellets were resuspended in 1ØØ ul TE buffer each and stored at 4C until use two days later.

After the filters had been submerged in the 42C water bath within heat sealed bags containing prehybridization fluid for about 5 hr, each probe was boiled for 5 min, chilled on ice for 2 min and then injected (using a 27 gauge needle) into the bags, which were then heat sealed again to exclude the needle puncture. Each of the three probes was hybridized with each recombinant plasmid isolated from positive clones. Bags were submerged in a 42C water bath with periodic agitation for 42 hr. Filters were then removed from the bags and washed at room temperature in a solution of 2x SSC and Ø.5% SDS for 5 min and transferred to fresh wash trays containing 2x SSC and Ø.1% SDS for 5 min. Then filters were washed in Ø.2x SSC with Ø.1% SDS for 3 min, then in Ø.16x SSC with Ø.1% SDS (preheated to 5ØC) for 15 min, followed by a brief rinse in room temperature 2x SSC with Ø.1% SDS.

DNA was detected in accordance with the instruction manual in the BRL detection kit. All reagents and buffer recipes were provided within the kit. Briefly, filters were incubated in a buffer containing BSA to block any non-specific binding of proteins, followed by incubation in streptavidin. They were then washed three times, incubated

in Poly (AP), washed four times, and incubated in the color development solution in darkness. When maximum color developed (about 1.5 hr) filters were washed in a stop solution (20 mM Tris-HCl, pH 7.5, 5 mM EDTA), air dried for 5 min and baked at 75C for 5 min under vacuum.

Protein Characterization

All clones producing S. citri BR3 antigens, as judged by immunological screening, were further characterized by electrophoretic separation of proteins on SDS polyacrylamide gels, and Western blot analysis using anti-S. citri membrane serum (description of antiserum preparation in chapter two). Clones were streaked for isolation on LB+Amp agar and incubated at 37C. The following day colonies were picked and grown in LB+Amp broth at 37C with agitation to an OD_{600} of 0.60, at which time 5 ml of each culture was transferred into flasks containing 20 ml LB+Amp and 300 ug/ml isopropylthiogalactoside (IPTG), and incubated with agitation at 37C. IPTG is an analog of lactose and induces the lac operon, so that the expression of S. citri DNA which has been inserted into pUC19 in such a manner (described earlier) as to be under control of the lac promoter, will now be enhanced. After 2 hr, 1.5 ml of each sample was microcentrifuged and each pellet resuspended in 100 ul sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 0.1% bromphenol blue, 2.0% SDS, 2.0% B-mercaptoethanol, 1.0 mM phenylmethylsulfonyl fluoride; PMSF, and 1.0 mM N-

ethylmaleimide: NEM), boiled for 5 min and stored at $^{-}20C$. PMSF and NEM are protease inhibitors, used to minimize the possibility of degradation of the foreign proteins by bacterial proteases. Samples were loaded onto 1.5 mm SDS slab gels containing 15% acrylamide, and the proteins electrophoretically separated at 60 mA until dye front was 1/2 inch from the bottom. Western blots were performed as described in chapter two. Positive (BR3 membranes) and negative (JM107 cells containing the plasmid, but no *S. citri* DNA) controls were treated similarly.

In the event that certain BR3 proteins might not react with the antiserum due to denaturation during the Western blot procedure, a dot blot was also performed. The same cultures that were grown for Western blotting were used for the dot blot procedure. Instead of boiling in sample buffer, 7.5 ul of each culture was spotted onto a nitrocellulose membrane in 2.5 ul aliquots, allowing for drying between applications (2.5 ul total of BR3 membrane was used as a positive control). After this point the blot was performed the same as the original immunological screening with the exception that anti-*S. citri* membrane serum was used this time.

Monospecific Antibodies to Selected Surface Proteins

The main purpose of these recombinant DNA experiments was to clone some of the genes responsible for the expres-

sion of _S_. _citri_ surface proteins. The procedures described thus far could identify clones producing _S_. _citri_ antigens, but would not be able to distinguish whether or not these antigens were located on the surface of _S_. _citri_ cells. In order to do this, antisera reacting specifically with _S_. _citri_ surface proteins would be required. To this end, _S_. _citri_ proteins (prepared from whole cells) were separated by SDS-PAGE, along with prestained molecular weight standards (Diversified Biotech, Newton Centre, Ma). Several proteins, newly identified as having surface components by protease treatments and/or surface immunoprecipitation (described in chapter two), were extracted from the gels. With the aid of the prestained standards and previous Western blots, the location of several surface proteins was estimated. Strips of gel believed to contain predominantly the proteins of interest were removed with a razor blade. Each strip was placed into a test tube with a solution of TE buffer and 0.1% SDS (just enough to cover the gel strip), crushed with a spatula, frozen at $^{-}20$C, thawed at 37C and refrigerated at 4C for 2 days. The liquid was drawn off and saved (used for first injection). Another method (performed by Jacqueline Fletcher and Amy James) was later used in which the gel strips were crushed in TE buffer and homogenized by passing through increasingly narrower hypodermic needles (used for subsequent injections). The antisera were prepared by Jacqueline Fletcher as described in chapter two. The proteins used for this procedure were those of 29, 58, 77 and

86/89 KDa. The specificity of each antiserum was tested by performing surface immunoprecipitations followed by Western blots.

Clones which had reacted with the anti-*S. citri* membrane serum were rescreened with the new antisera to check for evidence of surface location of these antigens. SDS-PAGE and Western blotting of each of these clones was performed as before, except that a 1:400 dilution of each antiserum (all mixed together) was used.

Results

Immunological Detection of E. coli Transformants Expressing S. citri Antigens

Two libraries of *S. citri* DNA were constructed by incorporating either mechanically sheared or *Sau*3A digested BR3 DNA into the plasmid vector pUC 19. The mechanically sheared DNA was ligated into the *Sma*I site of pUC 19, and the products used to transform *E. coli* JM107, while the *Sau*3A-cut DNA was ligated into the *Bam*HI site of the vector, and these products were used to transform *E. coli* strain TB-1. Original screening of these libraries detected about 30 clones/library expressing *S. citri* antigens, however, upon rescreening, 12 clones (hereafter called SD 1-12) from the library containing mechanically sheared fragments and 5 clones (hereafter called 8f, 9f, 11e, 16f and 28b) from the library containing *Sau*3A cut fragments, were found to still test positive.

Restriction Endonuclease Analysis of Recombinant Plasmids

Recombinant plasmids were isolated from positive clones and digested with *Hin*dIII and *Eco*RI, releasing the BR3 insert from the plasmid. The sizes of the inserts were compared with lambda DNA cut with *Hin*dIII on electrophoresed, ethidium bromide stained, 0.8% agarose submerged slab gels (Figure 6, Appendix B).

These gels reveal a wide size range of BR3 DNA fragments, from approximately 700-6,000 bp. Several of the BR3 inserts contain restriction sites for either *Hin*dIII or *Eco*RI or both. No plasmid could be reisolated from clone 9f, so it was not used.

The plasmids were also cut with *Pvu*II, *Nar*I and *Sal*I. Plasmids 8f, 11e, 16f and 28b were also cut with *Kpn*I (Figure 7, Appendix B). This was done to better evaluate the size of each insert and also to see if sites for these enzymes were present.

The results of each digest were evaluated and compiled in Table II (Appendix A). Estimates of insert sizes were judged by these and other, similar gels, as well as those of the uncut plasmids (data not shown). The data compiled in Table II are rough estimates. Some results which were not reproducible are marked with question marks. SD 5 and SD 12 may be clones of more than one genotype each, as gels of the uncut plasmids reveal multiple bands. However, since *E. coli* JM107 is *rec*+, these additional bands may be dimers and

trimers.

When reporting on the HindIII/EcoRI digests, it is impossible to distinguish which one of these enzymes is responsible for cutting within the BR3 insert; single digests would clear the matter up. The NarI/SalI digests were performed under assay conditions for SalI, which are similar to those for NarI except that 150 mM NaCl is added and the pH is slightly higher. It was thought that both would function properly, but the results suggest that only one enzyme was active, as most of the plasmids were simply linearized. Because of this, if an additional band was seen, it was assumed that the enzyme (probably SalI) cut within the insert. With each of the digestions, it is also possible that additional bands could be missed due to comigration with others of similar molecular weight.

DNA/DNA Hybridizations

Recombinant plasmids isolated from clones producing *S. citri* antigens were digested with HindIII and EcoRI, electrophoresed in 0.8% agarose submerged slab gels and transferred to nitrocellulose filters. Plasmids from clones SD 3, SD 7, and SD 10 were labeled with biotinylated dUTP and allowed to hybridize with each of the others. Figure 8 (Appendix B) contains photocopies of an ethidium bromide stained agarose gel before transfer of the DNA onto nitrocellulose and the corresponding nitrocellulose filter after color development. When first developed the blots were dark,

however, after baking they became much lighter. Therefore, only one representative which remained dark enough to photocopy well is shown.

The results of all three hybridizations are summarized in Table III (Appendix A). The blots were not subject to clear interpretation, so the data in this table must be considered preliminary. Probe SD 3 appears to hybridize with all of the other clones to varying degrees. Neither probe SD 7 nor SD 10 hybridized with clone 11e, even though it contains the largest BR3 insert. Probe SD 10 (containing the least amount of BR3 DNA of all the clones) did not hybridize with SD 1, SD 3 or 11e.

Western and Dot Blot Analysis

All clones expressing *S. citri* antigens were further characterized by electrophoresis on 15% polyacrylamide gels and subjected to Western blot analysis using anti-*S. citri* membrane serum (Figure 9, Appendix B). Only 4 of the 17 clones produced proteins which reacted with this antiserum. SD 2 exhibited one band of very low molecular weight. SD 8, SD 9, and 28b all displayed bands of similar molecular weight to spiralin as judged by comparison to *S. citri* membrane proteins which were coelectrophoresed. In addition, SD 8 exhibited two faint bands below this.

These same samples were used for a dot blot procedure in which they were spotted onto nitrocellulose and lysed before reacting with anti-*S. citri* membrane serum (Figure

10, Appendix B). The schematic shows where each sample was spotted. When viewing the blot, it is possible to distinguish a background spot from a positive reaction by comparison with the negative control (JM107 harboring pUC with no BR3 insert). The same samples which were positive on the dot blot (SD 2, SD 8, SD 9 and 28b) were also positive on the Western blot, indicating that the failure of the remaining clones to react with the antiserum is not due to denaturation caused by the SDS-PAGE and Western blot procedures.

Monospecific Antisera

Several S. citri surface proteins (29, 58, 77 and 89 KDa) were extracted from SDS polyacrylamide gels and used to elicit monospecific polyclonal antibodies. The specificity of each antiserum was assessed by performing surface immunoprecipitations followed by Western blot analysis. The blots (not shown) revealed that the 29, 77 and 89 KDa proteins were precipitated by each of the antisera, however, bands were darkest for each protein when the immunoprecipitation was performed with antiserum prepared against that protein. The 58 KDa protein could not be detected.

A mixture of the new antisera (1:400 dilution of each) was used to rescreen the clones testing positive on the previous Western and dot blots (SD 2, SD 8, SD 9 and 28b) along with the same negative control (data not shown). As a positive control, a duplicate Western blot was performed

using the anti-S. citri membrane serum. The positive control displayed the same bands as previously seen, while the blot performed with the new antisera only displayed one band (in SD 8) and it was faint enough to make it difficult to distinguish from background noise.

Discussion

Two libraries of S. citri BR3 genomic DNA have been constructed in E. coli cells using the plasmid vector pUC 19. One contains BR3 fragments of approximately 2,000 bp in length and the other of 10,000 bp average length. Immunological detection of E. coli cells harboring recombinant plasmids and producing S. citri antigens revealed about 60 clones expressing proteins which reacted with anti-S. citri (whole cell) antiserum. Out of these, only one, SD 8, exhibited preliminary evidence that the S. citri protein it produces is a surface protein, by reacting with a mixture of antisera directed against specific surface proteins. BR3 DNA insert sizes of each clone reacting with anti-S. citri membrane serum were estimated and DNA hybridization experiments were performed to identify clones carrying common S. citri DNA sequences.

The BR3 DNA library containing fragments of approximately 2,000 bp in length was constructed in such a manner as to increase the chances of obtaining a B-galactosidase fusion protein. One of the reasons for this is that although foreign proteins are often unstable in E. coli cells, fusion

proteins may have more stability (6). It is also possible that the method of fragmentation employed might have removed the S. citri promoter from the rest of the gene, necessitating insertion next to an E. coli promoter to obtain expression.

Small fragments of DNA were used in this library for several reasons. First, to obtain a fusion protein there must be no stop codons within the foreign DNA, and this is more likely with a smaller fragment. Second, the smaller size increases the chance of cloning a gene devoid of its promoter. This is desirable because it is uncertain whether all S. citri promoters are functional in E. coli, although Mouches et al. (4) have conclusively demonstrated that an S. citri DNA sequence did act as a promoter for the spiralin gene within E. coli. Third, there is a possibility that a sequence functioning as an amino acid codon in S. citri may be read as a stop codon in E. coli, as Yamao et al. (10) have shown that UGA codes for tryptophan in the mollicute Mycoplasma capricolum. Use of small DNA fragments narrows the chances of cloning such a sequence. However, this possibility may pose problems in expressing some S. citri genes in E. coli. To date, spiralin is the only S. citri gene reported to be expressed in E. coli cells; it contains no tryptophan (9). In case some of the isolated clones contain truncated genes, an alternate library was constructed that could be used to isolate full-length genes by hybridization.

The first screening of both BR3 genetic libraries revealed about 60 clones (out of about 18,000) which reacted with the anti-S. citri (whole cell) serum. Upon the third screening of these clones, only 17 tested positive. It is possible that in some cases the proper colonies were not picked off the agar plates. Another factor may have been that some of the S. citri proteins being produced may have been toxic to the E. coli cells, causing instability of those clones.

Strain JM107 was used partially because it has a mutation in the gene coding for the protein responsible for repressing the lac operon, the repressor being continually produced. In the event that the cloned BR3 sequence coded for a protein that was toxic to E. coli, it was thought that this protein would not be produced unless the cells were treated with an inducer, and therefore the cells could be maintained without harm. However, inclusion of XGAL (which turns blue in the presence of B-galactosidase) in the growth medium revealed blue colonies even without addition of an inducer, demonstrating that the cloned DNA was not prevented from being expressed. This may be due to the fact that pUC19 is a high copy number plasmid, possibly preventing even an elevated level of repressor from completely stopping expression.

Immunological screening of the 17 remaining clones revealed only four capable of reacting with the anti-S. citri membrane serum. It may be that those which were nonreactive

were still producing _S. citri_ antigens, but not ones that are recognized by this antiserum. It is also possible that the proteins originally produced were unstable in _E. coli_. Another screening of these clones with the whole cell antiserum would help clear this up.

To determine whether any of the clones are producing proteins that are exposed to the surface of _S. citri_ cells, antisera against several of the newly identified surface proteins were produced. However, when the specificity of these antisera was evaluated by surface immunoprecipitations and Western blot analysis, crossreactivity was observed. These procedures were only performed once for each antiserum, however, and it is possible that reduction in the amount of antiserum used in the immunoprecipitations would help. Cross adsorption of each antiserum with the other proteins, particularly spiralin, which is extremely antigenic, would also help. When these antisera were used to screen the four clones reacting with the anti-membrane serum, only one appeared to react, however, this procedure was only done twice.

There is clearly much more work to be done in this area, and the research described in this thesis has opened up many exciting avenues to travel. Further screening of the libraries may identify genes coding for surface proteins which could later be sequenced. With this knowledge, amino acid sequences can be predicted, possibly providing clues as to the arrangement of these proteins within the membrane, as

well as gaining information relating to their function by comparison with proteins of similar structure. To date, the function of proteins exposed to the surface of S. citri cells is unknown.

The fact that S. citri cells are capable of growing in three different environments (artificial media, insect and plant hosts) raises the question of whether its surface proteins are expressed equally in each of these circumstances. Once the DNA sequences coding for these proteins are identified within the genetic libraries, they will be easy to retrieve by restriction endonuclease digestion. The genes can then be used as probes in hybridization reactions with RNA extracted from plant and insect tissue as well as from whole cells grown in culture medium. This will provide information as to the level of expression in each environment.

Isberg and Falkow (2) identified a Yersinia pseudotuberculosis gene responsible for invasiveness of that pathogen by constructing a genetic library of Y. pseudotuberculosis DNA in E. coli and screening this library for the ability to invade animal tissue culture cells. It is feasible that our libraries could be used in similar experiments, as spiroplasma cells have demonstrated the ability of binding and entry into cultured insect cells including the Drosophila melanogastor cell line, Dm-1 (7).

Agglutination experiments could be performed with clones expressing S. citri surface proteins in efforts to

identify any functioning as adhesins. Once the monospecificity of the last antisera to be described has been attained, they can be used to detect blockage of agglutination, and of attachment to insect tissue culture cells. This would provide information as to which surface proteins are involved in attachment.

The surface proteins of _S_. _citri_, because of their location, are available for interaction with their plant hosts and insect vectors, and it is therefore feasible that they may be involved in the infection process. Cloning the genes which code for these proteins would be a step toward understanding this interaction.

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spiroplasmas. Ann. Micro. 135A:73-82.

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APPENDIX A

TABLES

TABLE I

IDETIFICATION OF SURFACE PROTEINS OF *SPIROPLASMA CITRI* BY PROTEASE TREATMENTS OR BY SURFACE IMMUNOPRECIPITATION

Method	Molecular Weight of Surface Proteins (KDa)								
	89	86	77	61	58	54	45	37	29
Protease									
Trypsin				+	+		+		
Chymotrypsin					+	+	+	+	
Proteinase K	+	+		+	+				+
Papain									
Immuno-precipitation	+	+	+		+				+

TABLE II

RESTRICTION ENDONUCLEASE ANALYSIS
OF RECOMBINANT PLASMIDS

Clone	Est. Insert Size (Kbp)	# of Internal R. E. Sites HindIII/EcoRI	NarI/SalI	PvuII	KpnI
SD 1	2.0	0	0	0	-
SD 2	1.7	0	0	1	-
SD 3	2.7	1	0	0	-
SD 4	0.7	0	0	0	-
SD 5	1.5	?	1	0	-
SD 6	2.7	1	1	0	-
SD 7	1.7	0	0	0	-
SD 8	1.8	1	0	0	-
SD 9	2.2	1	0	0	-
SD 10	0.7	0	?	0	-
SD 11	2.1	1	0	?	-
SD 12	?	?	1	1	-
8f	1.5	0	0	0	0
11e	10.0	2	1	0	1
16f	2.5	1	0	1	0
28b	3.9	2	0	0	0

TABLE III

DNA/DNA HYBRIDIZATION ANALYSIS OF RECOMBINANT PLASMIDS

Clone	# of Bands Hyb. with (other than Vector) Probe SD 3	SD 7	SD 10	# of Bands Present (other than Vector)
SD 1	1	1	0	1
SD 2	2	2	(?)1	2
SD 3	2	(?)1	0	2
SD 4	1(?)2	1(?)2	2	2
SD 5	2	3	1	5
SD 6	2	(?)1	(?)1	2
SD 7	(?)2	2	1	2
SD 8	2	2	2	3
SD 9	1(?)2	1(?)2	1	3
SD 10	2	1(?)2	2	2
SD 11	2	2(?)3	2	3
SD 12	4	4	3(?)4	5
8f	2	2	2	2
11e	1	0	0	3
16f	2	2	2	4
28b	3	-	3	6

APPENDIX B

FIGURES

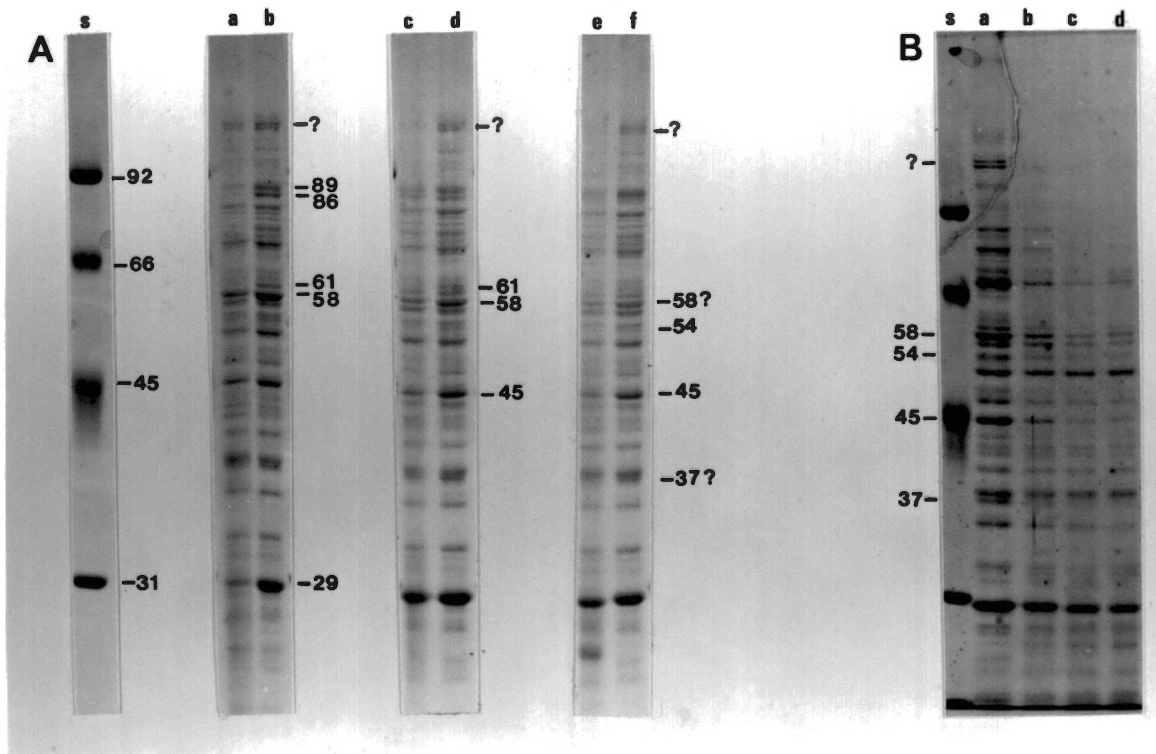


Figure 1. Protease treatment of intact S. citri strain BR3 cells. Treated cells were pelleted to collect the unaffected proteins which were then subjected to SDS-polyacrylamide gel electrophoresis beside molecular weight standards. The positions and estimated molecular weights of those surface proteins consistently identified from experiment to experiment are indicated to the left of each panel. Prominent bands that gave ambiguous results are labeled with question marks (?). **(A)** A Coomasie blue stained gel shows typical results obtained using proteinase K (lane a),

trypsin (lane c), or chymotrypsin (lane e). Untreated controls for each experiment are also shown (lanes b, d aand f). The molecular weight standards from the gel are shown in lane s. (B) A Coomasie blue stained gel from an experiment using chymotrypsin illustrates the effects of using varying amounts of protease, this case chymotrypsin (lane a, Ø mg/ml; lane b, Ø.Ø5 mg/ml; lane c, Ø.25 mg/ml; lane d, Ø.5Ø mg/ml).

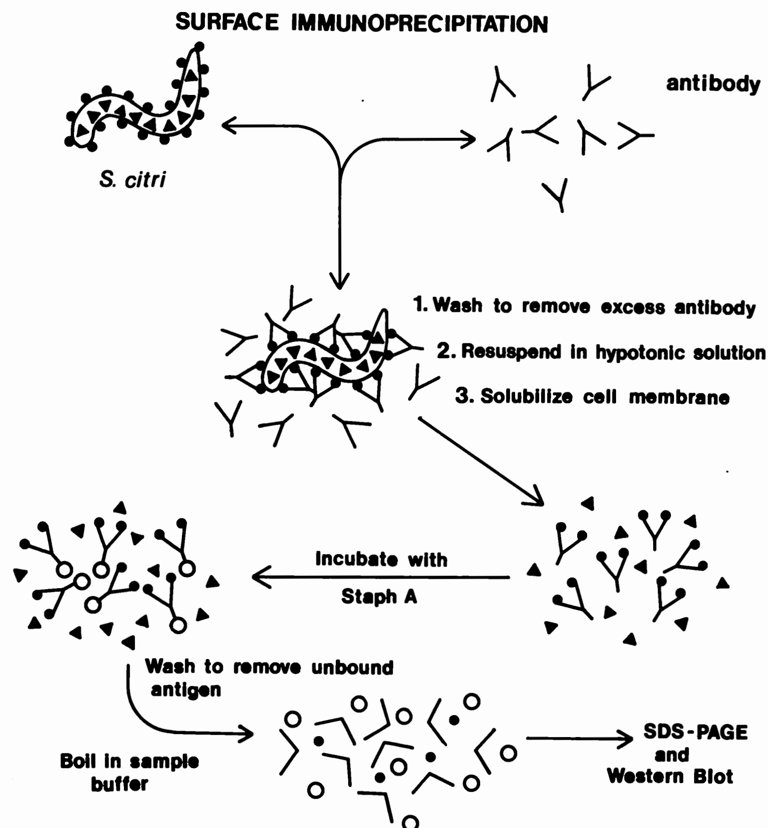


Figure 2. A schematic representation of the surface immunoprecipitation technique used to collect proteins from the surface of spiroplasma cells. Intact cells are exposed to antibodies produced against S. citri and the excess antibodies are washed away. The membranes are then solubilized using detergents and those surface proteins that are bound to antibodies are collected with Staph A, leaving cytoplasmic antigens in the supernatant. The antigen-antibody-Staph A complexes are finally analyzed by Western blot analysis using the same antibody. (See Materials and Methods for details).

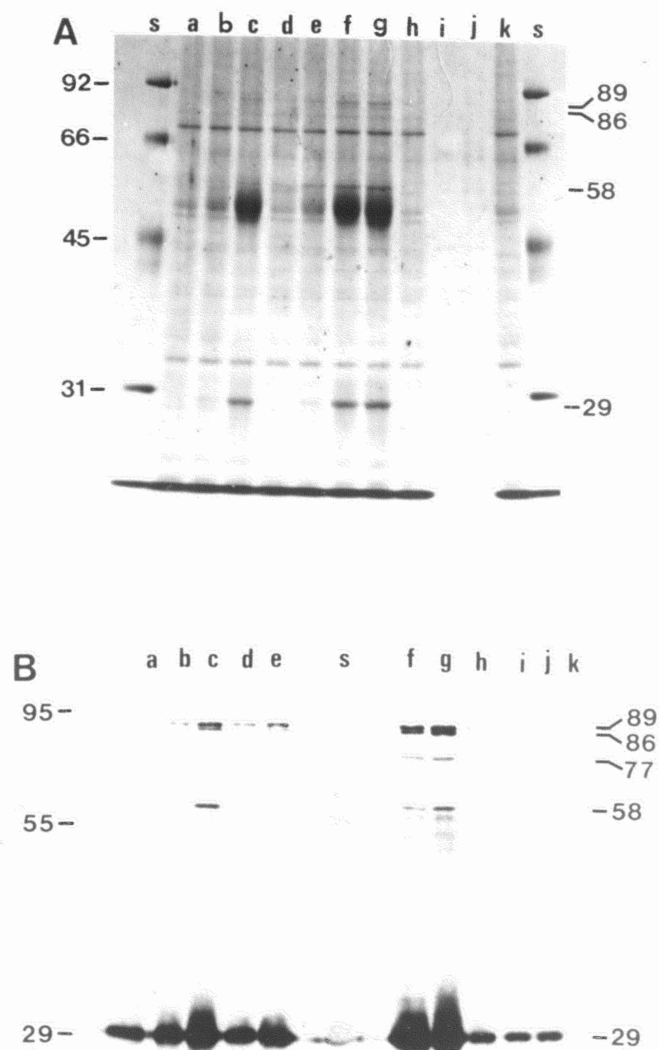


Figure 3. Surface immunoprecipitation of proteins from S. citri BR3. Varying amounts of intact spiroplasma cells were incubated with varying amounts of anti-BR3 membrane serum. The resulting antigen-antibody-Staph A complexes were subjected to SDS-polyacrylamide gel electrophoresis. (A) A Coomasie blue stained gel from a typical experiment. The sizes and positions of molecular weight standards are labeled to the left of the panel, and those corresponding to S. citri surface proteins are indicated to the right. Other, unlabeled bands are Staph A proteins or light or heavy chains of the antibody. Descriptions of individual lanes:

Lane	Antibody (ul)	Cells (ml)	Staph A
a	Ø.5	1	+
b	2.5	1	+
c	1Ø.Ø	1	+
d	Ø.5	3	+
e	2.5	3	+
f	1Ø.Ø	3	+
g	12.Ø	6	+
h	Ø.Ø	1	+
i	Ø.Ø	1	Ø
j	2.5	1	Ø
k	2.5	Ø	+

(B) Western blot analysis of the immunoprecipitated surface proteins using the same anti-BR3 membrane serum as the second antibody. The contents of the gel lanes in this experiment are identical to that in panel A.

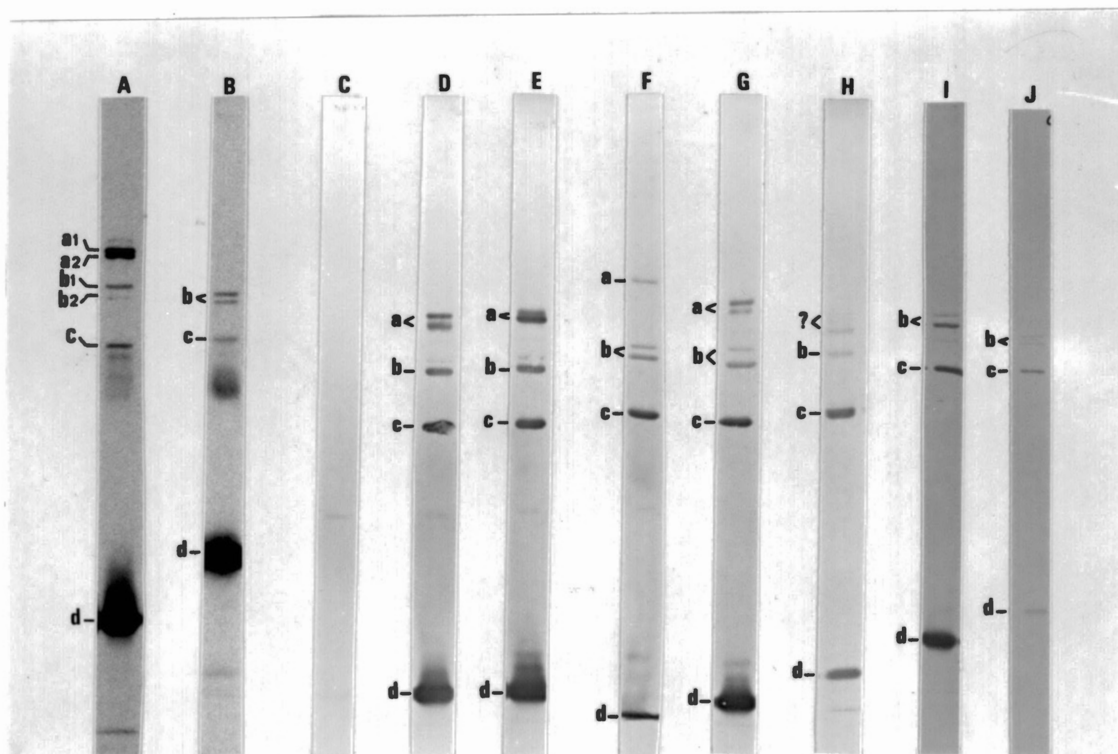


Figure 4. Comparison of several strains of _S_. _citri_ and three other species of spiroplasmas by surface immunoprecipitation. Bands corresponding to surface proteins are labeled, and those located in the same position relative to the standards are designated with common letters (a_1=89 KDa, a_2=86 KDa, b=77 KDa, c=58 KDa, d=29 KDa). Several gels were used for this analysis, thus proteins of common size are not aligned. (A) _S_. _citri_ BR3, (B) _S_. _citri_ R8A2, (C) _S_. _floricola_ 23-6, (D) _S_. _citri_ Ex Calavan citrus, (E) _S_. _citri_ BR6, (F) _S_. _citri_ Iran, (G) _S_. _citri_ BR18, (H) _S_. _melliferum_ AS576, (I) _S_. _citri_ ASP-1, and (J) _S_. _kunkelii_ CSS.

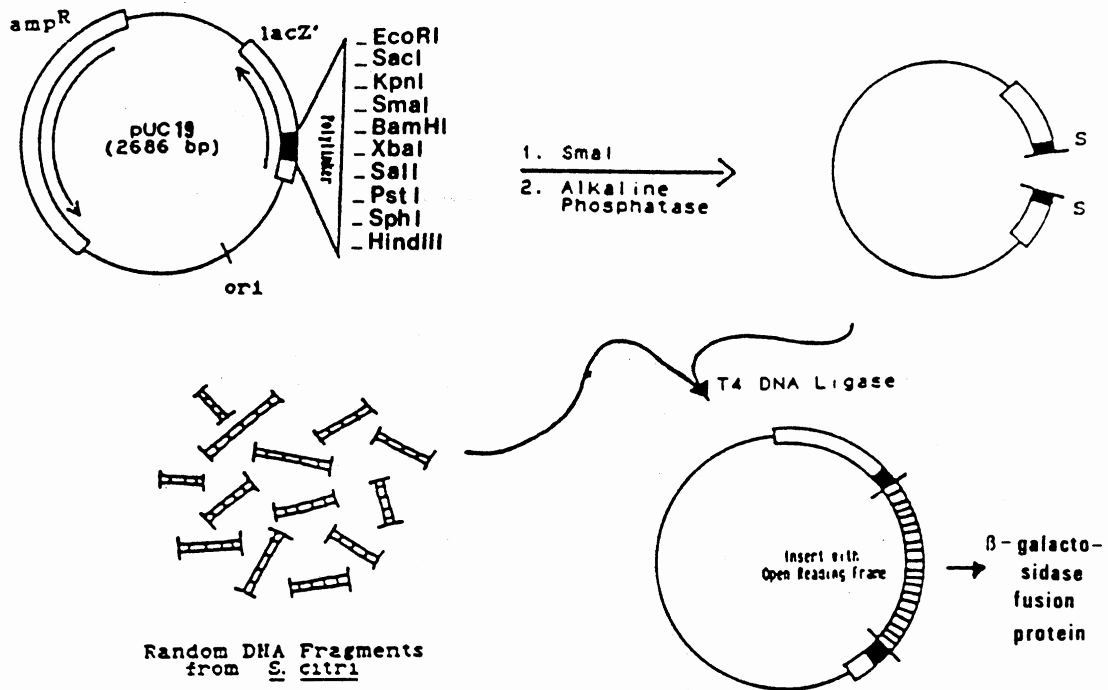


Figure 5. Construction of BR3 genetic library that promotes expression of B-galactosidase fusion proteins. Ori = origin of DNA replication; ampR = ampicillin resistance gene; lacZ' = B-galactosidase coding sequence.

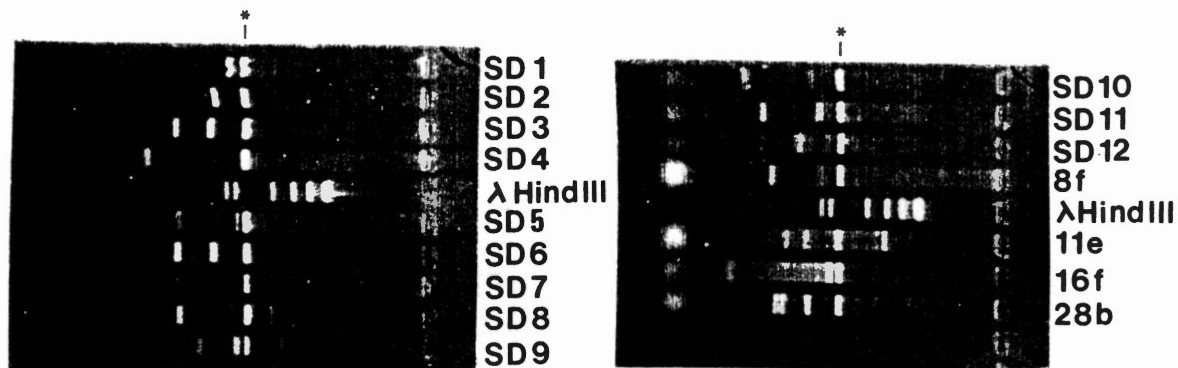


Figure 6. HindIII/EcoRI digests of recombinant plasmids, electrophoresed on Ø.8% agarose gels, stained with ethidium bromide. Labels are at the top of each gel. * = pUC 19.

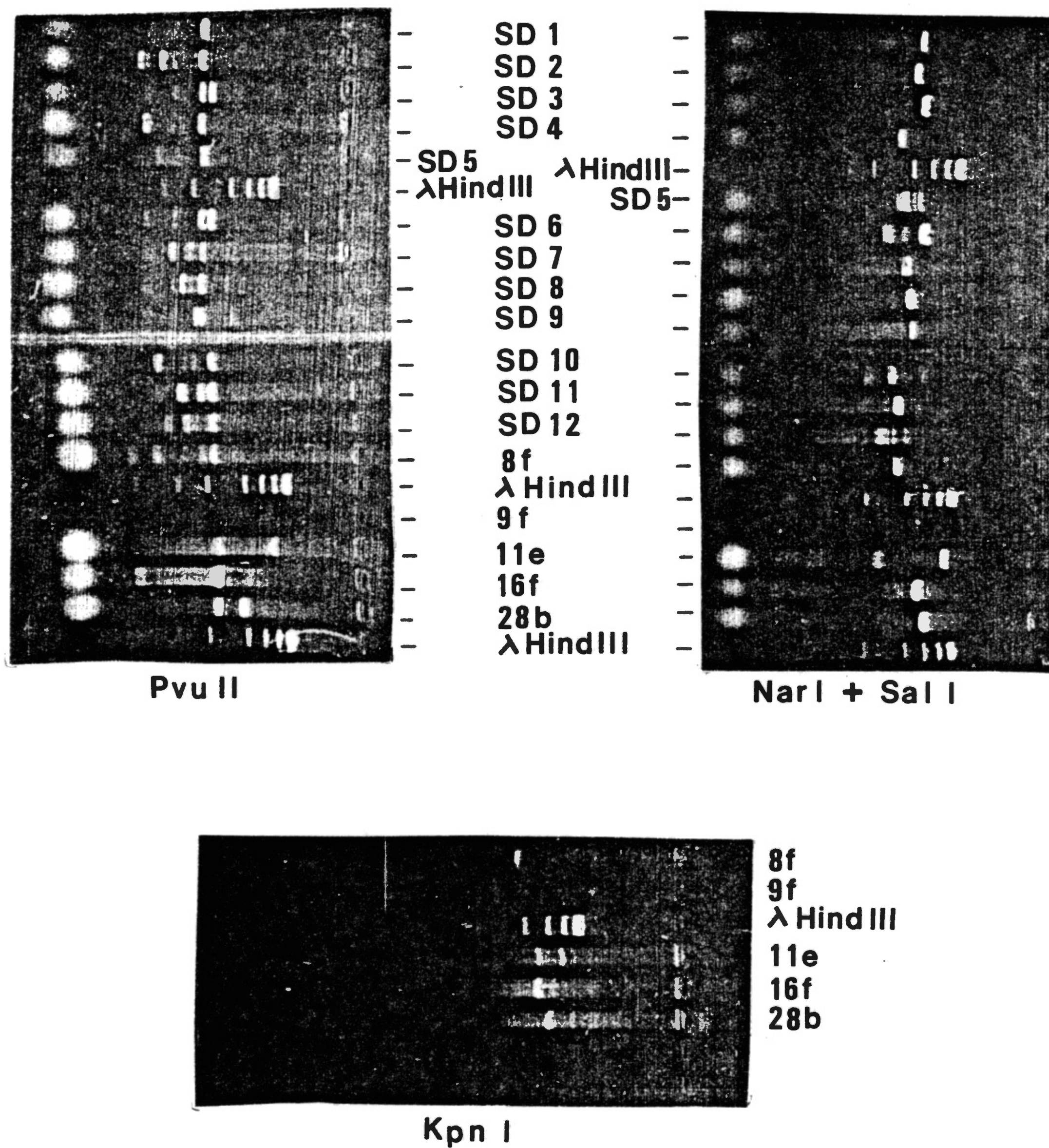


Figure 7. PvuII, NarI/SalI, and KpnI digests of recombinant plasmids (as in Figure 6).

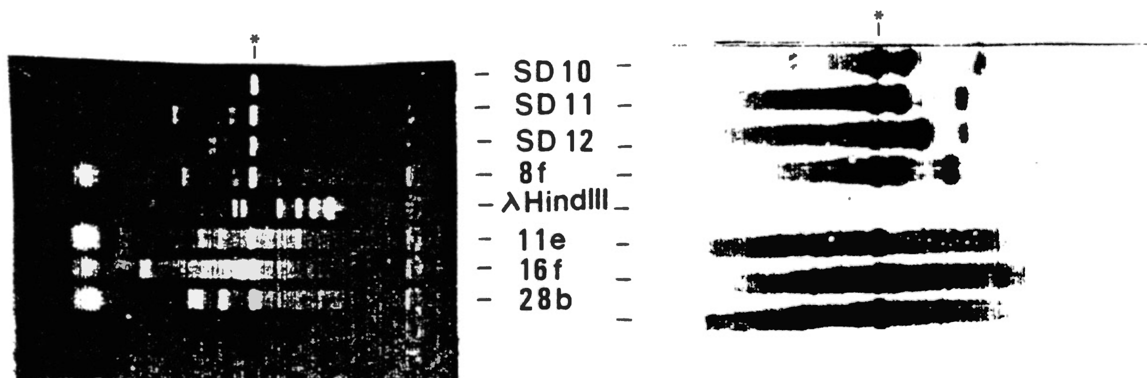
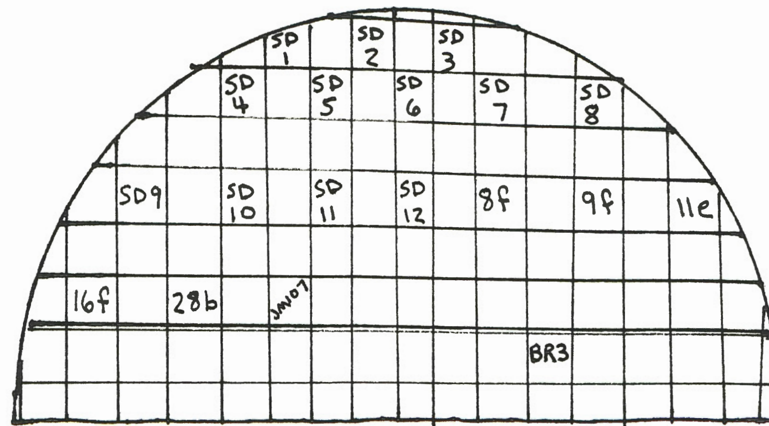
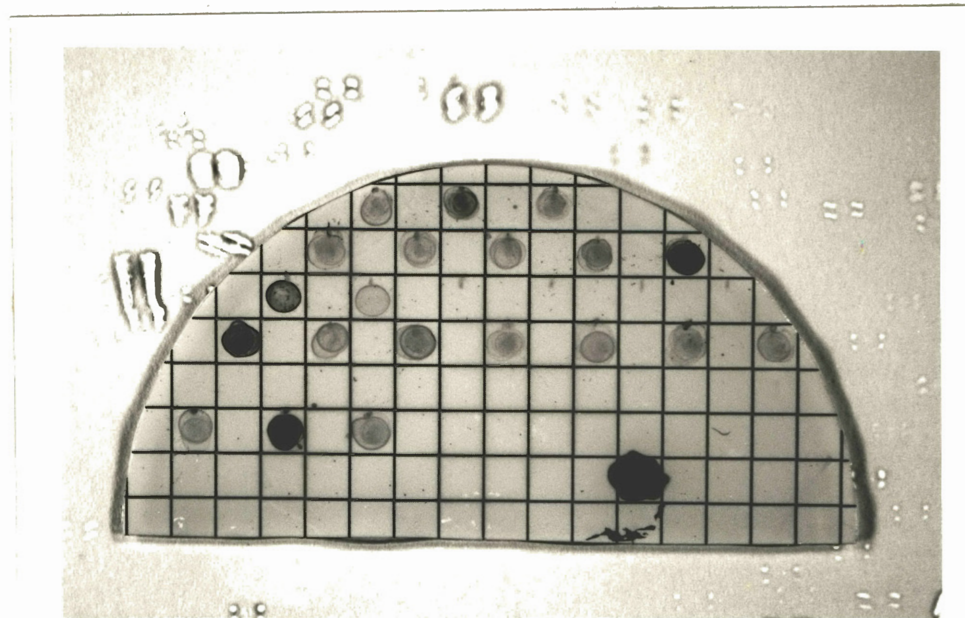


Figure 8. DNA hybridization of biotinylated recombinant plasmid SD 3 with HindIII/EcoRI cut recombinant plasmids SD 9-12, 8f, 11e, 16f, and 28b. * =pUC 19. Labels are at the top of the agarose gel and developed blot.

Figure 9. Western blot analysis of clones determined to be expressing S. citri antigens.



Schematic



Blot

Figure 10. Dot blot of identical samples used in Figure 9. Samples were spotted onto nitrocellulose, lysed, incubated with anti-membrane serum and S. citri antigens were detected by incubation with conjugate and substrate.

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