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BARD

FINAL REPORT

PROJECT NO. US-1030-85

**Mollicutes that Cause Plant Disease:
Detection, Cultivation and Physiology**

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1990

Standard BARD Cover Page for Scientific Reports

Date September 10, 1990

BARD
P.O. Box 6
Bet Dagan, ISRAEL

BARD Project No. US-1030-85

Title of Research Project:

Mollicutes that cause plant disease: Detection, cultivation and physiology

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Cooperating Institution(s): The Hebrew University, Hadassah Medical School, Jerusalem

Project's starting date: September 20, 1986

Type of Report: 1st Annual 2nd Annual Final X

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ABSTRACT

Mycoplasmalike organisms (MLOs) cause hundreds of diseases worldwide, including many that damage crops in Israel and the United States. Nevertheless, the organisms have not been cultivated. In this cooperative project, advances were made that have led to improved detection of MLOs, to an improved understanding of their physiology, and to prospects for their eventual cultivation. Monoclonal antibodies were prepared against New Jersey aster yellows MLO, maize bushy stunt MLO, and the eastern X-disease MLO. Polyclonal antibodies and DNA probes were prepared against the walnut witches' broom MLO. Another group of plant/insect mollicutes (spiroplasmas) were used as models to study organismal physiology. Cell lysates of all spiroplasmas tested catalyzed phosphorylation of a major endogenous 57 kDa protein, which appeared to be highly conserved. Some evidence suggested that the protein kinase activity was linked (conceivably to assembly or disassembly) to fibrils associated with the spiroplasma membrane. The spiroplasmas possessed a membrane-bound ATPase activity, suggesting an important role in cell function. The data were consistent with a hypothesis that these organisms possess a proton translocating ATPase, which may act as an ion pump for extrusion of Na^+ and/or K^+ . The pumps may act in volume regulation. An active Na^+/H^+ antiport activity was detected at acidic pHs. At alkaline pHs, Na^+ was extruded by a primary $^{22}\text{Na}^+$ pump. The presence of both activities suggest that both protons and sodium ions may serve as coupling ions for secondary transport in spiroplasmas. Coupled mechanisms may drive the uptake of essential nutrients into the cells. In collaboration with D. Pollack, lysates of spiroplasma cells were examined for pathways of 67 enzyme activities; most activities were highly conserved within the genus. Twenty-seven cultivation trials for several MLO species, utilizing generations of media from past concepts of mollicute physiology, were negative. However, by monitoring cultures with monoclonal antibodies and DNA probes, some evidence was obtained for brief flushes of growth in early culture intervals. The apparent presence of antigens common to MLOs and achleplasmas, while concordant with recent placement of MLOs close to achleplasmas on the basis of molecular phylogenetic evidence, were more likely procedural artifacts. Cultivation through six passages of a previously uncultivable procaryotic nematode pathogen was obtained with media rationales deduced from MLO culture trials. The physiological data obtained in this study with other plant/insect mollicutes should lead to improved formulations tailored toward existing mollicute pathways and transport systems. Compilations of existing knowledge of MLO medium components, of mollicute physiology, and a detailed summation of reported MLO diseases worldwide were prepared.

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OBJECTIVES

1. Develop monoclonal antibody techniques for monitoring aster yellows MLO (AY-MLO) growth during cultivation attempts and for purification of MLOs to be used for production of polyclonal antibodies.
2. Develop gene probes and modify dot blot techniques for monitoring AY-MLO growth during cultivation attempts.
3. Develop cell culture systems and/or media appropriate for the cultivation of AY-MLOs.
4. Study ATP and phosphorylated sugar accumulation during energy metabolism in spiroplasmas and MLOs.
5. Study cell volume regulation in spiroplasmas and MLOs including the role of ATPase.
6. Determine if protein kinase is associated with intracellular fibril organization in spiroplasmas and MLOs.

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INTRODUCTION

Several members of the Class Mollicutes (wall-less prokaryotes: Razin and Freundt, 1984) are important plant pathogens, causing hundreds of millions of dollars in damage on a worldwide scale. These pathogens can be essentially divided into two groups: (i) helical, motile, cultivable spiroplasmas, and (ii) somewhat spherical, nonmotile, noncultivable mycoplasmalike organisms (MLOs), the "yellows" agents and their allies (McCoy et al., 1989). Both multiply in their plant hosts and leafhopper vectors. The MLOs alone cause disease in more than 100 plant species, including many crop plants, and cause major crop losses in Israel and the United States. Although the yellows diseases were recognized as early as 1902, it was 1967 before a team of Japanese researchers (Doi et al., 1967) showed that plants afflicted with yellows diseases (e.g., mulberry dwarf or aster yellows) consistently contained mycoplasmalike organisms in their sieve tubes and that remission of disease symptoms in plants could be induced by tetracycline antibiotics (Ishii et al., 1967). These discoveries prompted interest in MLOs as microbes and resulted in many attempts to cultivate them. While sporadic claims of cultivation of some of the agents have been made (Maramorosch, 1981; Skripal and Malinovskaia, 1984), other laboratories have been unable to repeat the results (Saglio and Whitcomb, 1979; Bové, 1984). Thus far, no claim has been made of fulfilling Koch's postulates, and none of the presumptive isolates has been deposited in any type culture collection.

Although it is possible that some of these organisms were MLOs which subsequently failed to multiply continuously in vitro because of inadequate culture media, others have been assumed to be common plant surface contaminants, such as Acholeplasma laidlawii.

Since culture attempts have been unsuccessful and only cumbersome bioassays were previously available for determining MLO presence or for monitoring their growth, microbiological research on these pathogens had essentially ceased in recent years. Two recent advances in mollicute research have now brought the study of yellows disease agents into a new era. These are:

(i) development of monoclonal antibodies specific for MLOs (Lin and Chen, 1985), and (ii) development of gene probes to detect mollicute infection in plants or insects (Kirkpatrick et al., 1987). Successful cultivation of intracellular, fastidious mollicutes such as the sex-ratio organism (SRO) of Drosophila in insect cell culture (Hackett et al., 1987) and development of sensitive means for monitoring progress in cultivation of MLOs together created an opportunity for renewed attempts at cultivation of these plant disease agents. The situation with spiroplasmas has been very different. All known plant-associated spiroplasmas are cultivable, and Koch's postulates have been fulfilled. One, Spiroplasma citri (Saglio et al., 1973), the causative agent of citrus stubborn disease, is a serious threat to citrus production in Israel and the United States. It has

been cultivated, characterized, and has now being used in a large number of basic microbiological studies (Bové et al., 1989). Similarly, the destructive corn stunt spiroplasma (S. kunkelii) was cultivated (Liao and Chen, 1975; Williamson and Whitcomb, 1975). A third plant spiroplasma, S. phoeniceum, has been similarly cultivated and characterized (Saillard et al., 1987). Although studies of spiroplasma nutrition and physiology have progressed considerably steadily during the past 10 years, much remains to be done.

Understanding an organism's physiology, and thus its means for adapting to the challenges of its habitat (Hackett and Clark, 1989), is important in the development of effective means for controlling the disease agents. Two major challenges confronting any organism are: (i) location of an adequate energy source and (ii) maintenance of osmotic homeostasis. Mollicutes have developed novel solutions to the latter problem. While turgor pressure in prokaryotes is generally maintained by the presence of a strong cell wall combined with the regulatory action of membrane-bound ion pumps, mollicutes, because they lack a cell wall, must rely on ion pumps and the osmotic stability of their environment.

Nonmotile MLOs and motile spiroplasmas appear to have evolved different strategies for locating suitable substrates. Because of their translational motility, spiroplasmas may have a

significant ecological advantage over MLOs in the same habitat, particularly if the habitat contains highly variable quantities of substrates or osmolarities. This advantage can be attained, however, only with a considerable expenditure of energy.

Alternatively, MLOs may be at an advantage in environments that are essentially homogeneous. In spite of these differences, plant pathogenic spiroplasmas and MLOs superficially appear to inhabit similar environments. A greater understanding of basic physiologic processes such as energy utilization and its relationship to motility and the mechanisms involved in maintaining osmotic homeostasis would contribute greatly to the understanding of the ecological roles of these two types of mollicutes, and could lead to eventual MLO cultivation. This rationale formed the backdrop for the collaborative work reported here.

OBJECTIVE 1. Develop monoclonal antibodies for monitoring AY-MLO growth during cultivation attempts, and for purification of MLOs to be used for production of polyclonal antibodies.

I. Develop monoclonal antibodies against MLOs.

EXPERIMENTAL PROCEDURES

Diseased plants and insect vectors. For the aster yellows agent (AY-MLO), lettuce plants infected with the New Jersey strain of aster yellows (NJ-AY) were used. The AY-MLOs were transferred from infected to healthy plants by the leafhopper Macrostelus fascifrons.

The maize bushy stunt agent (MBS-MLO) was kept in corn (Zea mays cv. Golden Bantam Cross). The leafhopper vector Dalbulus maidis was used for transferring the MLO from plant to plant.

Celery plants infected with the Harrow isolate of EX-MLO (Eastern X-disease) were used. The leafhopper vector Paraphlepsius irroratus was used to transmit the disease.

Antigen preparation. Partially purified MLOs were obtained either from MLO-carrying leafhopper vectors (e.g., AY-MLO) or from diseased plants. MLOs from leafhoppers were prepared according to the method of Lin and Chen (1985). Approximately 2000 inoculative leafhoppers (M. fascifrons) were allowed to feed on AY-infected lettuce for 3 days and subsequently maintained on rye plants for 2 weeks. Dissected salivary glands were homogenized in 0.02M PBS, and clarified by centrifugation (2000g). The supernatant was used as antigen.

Antigens were prepared from diseased plants following the methods of Jiang and Chen (1987). Briefly, diseased corn plants showing MBS symptoms were harvested, rinsed thoroughly in tap water, and cut into approximately 2 in. (1 in. = 25.4mm) pieces, which were then macerated in phosphate-buffered saline (PBS) (Jiang and Chen 1987), using the slow and gentle cycle of a tissue homogenizer (Omni-mixer, Sorvall) until most parenchyma tissue was broken. The macerated tissues were strained through a mesh screen and retained vascular tissues were placed in the isolation medium and subjected to further maceration by mortar and pestle. Extracted materials were centrifuged at 2600 X g for 6 min and the supernatant was centrifuged again at 27,000 X g for 30 min. The pellets were resuspended in suspending medium. Then 0.6 ml was layered over each of several tubes containing step gradients, prepared from 15, 30, and 60% iso-osmotic Percoll (Pharmacia Inc., Uppsala, Sweden). Discontinuous density-gradient centrifugation was carried out at 14,000 X g for 29 min. Antigen was collected near the interphase between 30 and 60% Percoll layers.

Immunization. Six-week-old female BALB/c mice were immunized with partially purified MBS MLOs. The protein content of the antigen preparation was predetermined to be 260 ug/ml (Bio-Rad protein assay; Bio-Rad Lab., Richmond, CA). Antigen (0.15 ml) was mixed with an equal volume of Freundt's complete adjuvant and was then administered by intraperitoneal injection into each mouse once a week for 4 weeks. On days 35 and 36 the same amount of antigen was used for intravenous injections, except adjuvant was not added. The mice were sacrificed on day 38.

Monoclonal antibody selection. The procedure for splenic cells and fusion with myeloma cells (p3/NS1/1-Ag4-1) was the same as that described by Lin and Chen (1985). Hybridomas grown in HAT selective medium + 15% fetal bovine serum supplemented with 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine) were screened for MBS MLO specific antibody production by using indirect ELISA with biotinylated antimouse immunoglobulin (Lin and Chen (1985)). ELISA plates were coated with preparations from both diseased and healthy corn plants (in 0.05 M carbonate buffer, pH 9.5) as described in Antigen Preparation, except that these preparations were not subjected to density-gradient centrifugation. Hybridomas that produced antibodies reacting with diseased and not with healthy plant preparations were selected for further monocloning by limiting dilution. Selected monoclonal antibody producing hybridoma cells were subcultured for antibody production and the cell lines were stored in liquid nitrogen.

B. RESULTS

1. Aster yellows. Seven monoclonal antibodies against NJAY MLO were obtained. These antibodies proved to be very specific and possess high affinity. They do not react with the agents of eight other MLO-induced diseases including the Western strain of aster yellows.

2. Maize bushy stunt MLO. Twenty-six hybridoma clones, producing antibodies specific to MBS-MLO but not healthy corn plants, were selected. A total of 48 monoclonal antibodies were obtained.

3. Eastern X-disease MLO. Eighteen hybridomas secreted antibodies that reacted only with diseased celery plants but not with healthy celery preparations and were therefore specific to EX-MLO. The monoclonal antibodies produced by these hybridomas did not cross-react with preparations from plants infected with other MLO diseases. However, the monoclonal antibodies to EX-MLO reacted with preparations from celery infected with 3 strains of Western X-disease.

II. Purification of MLOs.

1. Purification of MLOs from AY-infected lettuce. A technique was developed to partially purify mycoplasma-like organisms (MLOs) associated with the aster yellows (AY) disease. Young lettuce plants were inoculated with the AY agent by leafhopper transmission and kept in a greenhouse. Leaf veins from symptomatic lettuce were isolated and homogenized in an isolation medium consisting of D-mannitol, 3-(N-morpholino)-propane sulfonic acid (MOPS), polyvinylpyrrolidone, L-cysteine, and ethylenediamine tetraacetic acid, before low-(8 min at 1,500 g) and high-(30 min at 35,000 g) speed centrifugation. The pellet was resuspended in medium containing mannitol and MOPS and layered on a discontinuous Percoll density gradient of 15, 30, and 50% (v/v). After centrifugation at 20,000g for 20 min, the fraction formed at the interface of the 30 and 50% solutions was collected and diluted with suspending medium. Gradient material was removed by centrifuging the diluted fraction at 100,000g for

2 hr. Electron microscopy of thin sections made from purified preparations showed an extremely high concentration of MLOs with well-preserved cellular structures identical to those in the phloem of diseased plants. These MLO cells exhibited a trilaminar limiting membrane, cytoplasmic ribosomal granules, and a network of DNA.

2. Purification using affinity chromatography. Purification of AY-MLO from diseased lettuce using monoclonal antibodies in an affinity chromatography procedure was developed for purification of the mycoplasma-like organism (MLO) from lettuce with aster yellows (AY) disease. The affinity column consisted of Staphylococcus protein A covalently linked to a 6MB Sepharose matrix and coupled with monoclonal antibody specific against AY-MLO. AY-MLO was concentrated from clarified crude sap of diseased lettuce by differential centrifugation. After low-speed centrifugation, supernatants containing AY-MLO were loaded on the affinity column. Extraneous unbound plant materials from the crude sap were washed from the column after an incubation period. The aster yellows agent retained in the column was released by mechanical shaking and then eluted. The entire procedure took 3 hr to complete. Intact and undamaged cells were observed by electron microscopy, and the high purity of this preparation was revealed by electrophoretic protein profiles and the production of polyclonal AY-antiserum. In addition, the specificity of the monoclonal antibodies was confirmed by Western blots.

III. Production of polyclonal antibodies (PABs) using AY-MLO purified from affinity chromatography.

Preparation of purified AY-agent. AY-MLOs were concentrated from clarified sap of diseased lettuce by differential centrifugation. Monoclonal antibody (MAb) against AY-MLO was coupled to protein A-Sepharose 6MB in an acrylic plastic column and incubated 20 min at room temperature. After washing, the MAb-conjugated protein A-Sepharose column was loaded with AY-concentrated crude extract. After incubation, unbound materials were washed from the column and bound AY-MLO was separated from MAb by mechanical shaking and then eluted from the column. The AY-MLO was pelleted, resuspended in PBS and used as antigen.

Preparation of polyclonal antiserum. BALB/c mice were immunized with the AY-MLO purified from the affinity column. Each mouse was subjected to 3 intraperitoneal injections 10 days apart. Each injection contained 100 mg AY-MLO protein mixed with an equal volume of Freund's complete adjuvant. Mice were bled 5 days after a final injection containing 10 mg of AY-MLO protein in saline.

RESULTS

Immunoabsorption and electron microscopy. Electron micrographs of thin sections from pellets obtained after affinity chromatography from a diseased plant showed a very high concentration of AY-MLOs, indicating that the monoclonal

antibody-coupled affinity column was an efficient instrument for trapping intact cells of the organism. MLOs of various sizes and shapes were recovered. Most of the recovered cells maintained their cellular integrity, showing little or no damage. These cells exhibited a typical plasma membrane, ribosomal granules, and a fibrous network of DNA. No MLOs were found in eluates prepared from healthy control plants.

ELISA with polyclonal AY-antiserum. Previously it was found that polyclonal antisera from mice immunized with partially purified AY-MLO failed to distinguish between diseased and healthy lettuce plants because the sera cross-reacted with healthy plant proteins. In this study, the specificity of polyclonal AY-antiserum was increased with the use of highly purified AY-MLO antigen. In ELISA, antiserum discriminated between diseased and healthy lettuce plants. A_{490nm} values of diseased plants were about 7 times that of healthy plants. Similar results, but with much higher A_{490nm} values, were obtained with monoclonal antibody at comparable dilutions. The titer of polyclonal antiserum decreased with increasing dilutions.

ELISA with polyclonal antisera against walnut witches' broom MLO

EXPERIMENTAL PROCEDURES

MLO source. Leaves with severe symptoms of WWB were harvested from trees on the campus of Georgia Experiment Station at Griffin, Georgia.

MLO purification. The procedure of Hobbs et al. (1987) for MLO purification was used with minor modifications (Konai and Chang, 1990).

Production of polyclonal antiserum. One ml of the partially purified preparation from walnut or healthy leaves was mixed with 1 ml of Freund's incomplete adjuvant and injected intramuscularly into the hind leg of a New Zealand white rabbit. Five weekly injections were followed by five intravenous booster injections beginning 2 weeks after the final intramuscular injection. Bleedings were done 2 weeks after the last intramuscular injection and at weekly intervals before boosters.

Cross-absorption of antiserum. WWB-MLO antiserum was cross-absorbed at a 1:1 ratio with healthy walnut immunogen (Hobbs et al., 1987).

Preparation of gamma globulin and enzyme-conjugate. The procedure of Clark and Adams (1977) was modified for gamma-globulin purification (Konai and Chang, 1990). To prepare enzyme-conjugate, 1.0 ml of gamma globulin preparation was mixed with 2 mg alkaline phosphatase (P-5521, Sigma) and dialyzed in PBS pH 7.4 for 24 hr (with at least 4 changes) at room temperature. Glutaraldehyde (0.06% final concentration) was then added to the buffer and dialysis continued for an additional 4 hr. Excess glutaraldehyde was removed by dialysis for at least 24 hr. BSA at a final concentration of 5 mg/ml was added to the dialyzed enzyme-conjugated gamma-globulin and stored at 4° C.

ELISA procedure. The ELISA procedure of Clark and Adams (1977) was used with certain modifications (Konai and Chang, 1990).

RESULTS

The dilutions for the coating and enzyme-conjugated gamma-globulin at 1:800 and 1:200, respectively, were able to detect the WWB-MLO in preparations of diseased walnut tissue (Table 1). WWB-MLO was detectable in infected fresh leaves, petioles and roots of walnut trees as well as frozen leaves and petioles. The MLO was also detected in leaves of grafted seedlings that displayed WWB symptoms in the greenhouse. ELISA readings from the leaves of graft-infected seedlings and field-infected walnut trees were similar. Readings of healthy leaves from the field or greenhouse were much lower than readings of diseased leaves. Readings from healthy leaves were slightly higher than those of

Readings from healthy leaves were slightly higher than those of buffer controls, but were close to those from healthy petioles.

Table 1. ELISA values (410 nm) of different walnut tissues under different combinations of dilutions of coating and enzyme conjugated gamma globulin

Dilution of coating IgG	Healthy			WWB-diseased			Dilution of conjugate
	$1/10$	$1/20$	$1/40$	$1/10$	$1/20$	$1/40$	
1:200	0	0	0	0.20	0.15	0.14	1:200
	0	0	0	0.20	0.18	0.20	1:400
1:400	0	0	0	0.21	0.18	0.17	1:200
	0	0	0	0.12	0.08	0.07	1:400
1:800	0	0	0	0.42	0.32	0.22	1:200
	0	0	0	0.14	0.13	0.13	1:400
1:1600	0	0	0	0.18	0.14	0.13	1:200
	0	0	0	0.11	0.09	0.06	1:400

Samples collected from infected and healthy walnut trees during the fall were frozen, and later tested for the presence of WWB-MLO. In these tests, leaves and petioles were tested separately. In each case, ELISA values were significantly higher than those of healthy controls (Table 2). Average ELISA values from frozen samples, from fresh leaves of infected walnut leaves, and from grafted walnut seedlings were within the same range (0.54 to 0.60), although individual readings from each leaf source varied (0.40 to 0.75). In further experiments, there was no significant difference (0.544 versus 0.49) in average ELISA values from symptomatic and asymptomatic leaves of the same WWB-infected tree. In this case as well, individual asymptomatic leaves gave different readings (0.38 to 0.59). Healthy leaves gave average values close to the buffer control.

Table 2. ELISA values (410 nm) of different walnut tissues

Sample #	Fresh ¹	Leaves Fresh ²	Frozen	Petioles Frozen	Roots Fresh
1	0.59	0.67	0.64	0.42	0.58
2	0.60	0.75	0.54	0.64	0.44
3	0.60	0.67	0.62	0.45	0.45
4	0.58	0.58	0.51	0.35	0.53
5	0.63	0.63	0.46	0.75	0.48
6	0.53	0.58	0.54	0.42	0.43
7	0.50	0.66	0.59	0.44	—
8	0.60	0.57	0.44	0.38	—
AV.(D) ³	0.58	0.64	0.54	0.48	0.49
AV.(H) ⁴	0.32	0.34	0.31	0.25	0.24
Buffer	0.22	0.22	0.22	0.22	0.22

¹ Fresh leaves collected from WWB-diseased walnut trees.² Fresh leaves collected from WWB graft-infected walnut seedlings in the greenhouse.³ AV.(D) represents the average reading from eight diseased samples.⁴ AV.(H) represents the average reading from eight healthy samples.

To study the reactions of WWB-MLO with other mollicutes or presumed mollicutes, peach Western X-infected celery, aster yellows-infected periwinkle were tested. Peach Western X-infected celery gave a strong positive reaction to WWB-MLO. The highest reading was obtained with infected roots followed by infected leaves. However, there was no difference in readings between infected and healthy celery petioles (Table 3). None of the other organisms tested, including aster yellows, pecan bunch MLO, and Spiroplasma citri showed positive reactions to WWB-MLO.

Table 3. ELISA reaction of anti-WWB polyclonal antiserum with Peach Western X MLO-infected celery (values at 410 nm)

Samples #	Roots		Leaves		Petioles	
	D	H	D	H	D	H
1	1.06	0.34	0.56	0.30	0.25	0.28
2	0.93	0.33	0.43	0.27	0.29	0.26
3	0.79	0.35	0.42	0.35	0.40	0.29
4	0.60	0.35	0.50	0.36	0.39	0.42
AV.	0.85	0.34	0.48	0.32	0.33	0.31

Buffer Control 0.22

OBJECTIVE 2. Develop gene probes and modify dot blot techniques for monitoring AY-MLO growth during cultivation attempts.

EXPERIMENTAL PROCEDURES

Isolation of walnut witches' broom MLO DNA. Leaves showing severe witches' broom symptoms and healthy leaves were harvested in Georgia in late fall of 1989 and stored in a freezer until used. Frozen leaves or petioles were freeze-dried for three days and ground into powder before they were used for DNA extraction. About 0.5 to 1 g of tissue powder was added to 20 ml preheated CTAB buffer immediately after the addition of 0.5 ml mercaptoethanol. The extraction mixture was incubated at 65° C for 1 hr with occasional inversion, before an equal volume of chloroform-isoamylalcohol (24:1) was added. The components were mixed by inversion for 15 min before centrifugation at 6,500 rpm for 5 min. The pellet was washed in 500 ul 70% alcohol and transferred to a 1.5 ml Eppendorf tube. DNA was pelleted by centrifugation at 14,000 rpm for 5 min. The pellet was dissolved in 400 ul TE buffer. DNA was centrifuged in a CsCl gradient (Refractive Index = 1.3925) with 0.01 vol of 1 mg/ml bisbenzimidate at 42,000 rpm (Beckman SW55 Ti) for 48 hr. Under UV light, MLO DNA formed an extra band above the host DNA. MLO DNA was obtained by piercing the tube wall. The DNA was extracted twice with isopropanol, incubated at -20° C overnight and centrifuged at 14,000 rpm for 15 min. The DNA pellet was dissolved in 20 ul TE.

Cloning of WWB MLO fragments. WWB MLO DNA and pUC18 DNA were digested with HindIII and EcoR1 overnight before they were ligated with T4 ligase. The ligated DNAs were transformed into E. coli JM 83. WWB MLO DNA clones were screened with [³²P]-labeled cloned plasmids.

RESULTS

Comparison of WWB MLO with other MLOs. The WWB MLO was compared with other plant disease inducing agents. Using dot hybridization and Southern blotting techniques, at least one of the WWB MLO DNA clones (pWWB14 - 1.9 kb) hybridized strongly to DNA extracted from tissues of WWB infected trees, but only weakly to that from pecan bunch MLO infected trees, and not at all to blots of healthy walnuts and pecans or to periwinkle infected with Western X, BLTVA, eastern and severe western aster yellows

MLOs, and Spiroplasma citri. The presence of two to three visible bands, ranging from 0.9 to 5 kb, in agarose gels after electrophoresis of WWB MLO DNA, indicated that WWB MLO may contain plasmid-like DNA.

OBJECTIVE 3. Develop cell culture systems and/or media appropriate for the cultivation of AY-MLO

EXPERIMENTAL PROCEDURES

MLO sources. The following sources of inoculum were used in MLO cultivation trials (Beltsville and UC-Davis), gene probe development (UC-Davis), and antibody production (Rutgers): New Jersey aster yellows (NJAY) MLO-infected Macrosteles fascifrons 5th instar-adults; Eastern aster yellows (EAY) MLO-infected M. fascifrons adults (Canada); beet leafhopper transmitted virescence agent (BLTVA)-infected beet leafhoppers (UC-Davis); potato yellow leaf roll-infected leafhoppers (from A. Purcell, UC-Berkeley); NJAY-MLO-infected aster plants (Beltsville), severe aster yellows-, BLTVA-, or potato yellow leaf roll-infected celery plants (UC-Davis); NJAY-MLO-infected lettuce plants (Rutgers and Beltsville); and frozen NJAY-MLO-infected leafhoppers or aster laminae or petioles (Beltsville). The Beltsville strain of the soybean cyst nematode actinomycete (Pasteuria) sp. was used.

Extraction temperature and atmosphere. Extraction was usually done at ambient temperature, although mortar and pestles were usually kept on ice. For two experiments (25-26), extractions were done in a cold room with mortar and pestles kept on ice. Since oxygen may be harmful to MLOs, some extractions were performed in totally anaerobic environments. At the laboratory of J. Macy at UC-Davis, an anaerobic hood (Hood 1) with an atmosphere of 90% N₂, 10% H₂ (28°C) was used. At the laboratory in Beltsville, an anaerobic hood (Anaerobe Systems) (Hood 2) with an atmosphere of 90% N₂, 5% CO₂, and 5% H₂ (26°C) was used.

Sterility: Antibiotics and filtration. To reduce the level of contaminants, antibiotics were generally added to the media (see media Tables). In some cases, filtration through 0.45 or 0.6 µm Millex filters was employed (see Procedures below).

Extraction procedures. Extractions for production of gene probes (Kirkpatrick et al., 1987) and monoclonal antibodies (Lin and Chen, 1985) have been described.

Extraction procedures (see Table 1) for MLO cultivation trials were the following:

Extraction procedure 1. Leafhoppers were placed in a state of torpor by cooling them for a few minutes in a freezer. 50 mg of infected *M. fascifrons* adults or diseased (0.1% sodium hypochlorite surface sterilized) plants were ground with a mortar and pestle in 1-4 ml extraction medium. Plant tissue was often diced before grinding and filtered through cheesecloth afterwards. All extracts were clarified by centrifugation in a clinical centrifuge for 5-10 minutes, and filtered through 0.45 (Expt. 1-7,10,27) or 0.65 (Expt. 19-22) μ m Millex filters. In Expt. 25-26, no filters were used. In Expt. 21, the ability of bacterial contaminants to pass through 0.22, 0.3, 0.45, 0.65, and 0.8 μ m Millex filters was compared.

Extraction procedure 2. This procedure was the same as Extraction Procedure 1, but a buffer was used rather than a medium. The extract was passed through 0.65 μ m Millex filters.

Extraction procedure 3. In this procedure (at Rutgers), NJAY-MLO-infected lettuce was cut, homogenized and strained through sieves to eliminate parenchyma tissue. Vascular tissue was then ground in the isolation medium, centrifuged twice in a clinical centrifuge, and purified using gradient centrifugation in 15, 30, and 50% Percoll. Purified MLOs were extracted from the interfaces.

Extraction procedure 4. At UC-Davis, approximately 30-50 infected leafhoppers were briefly frozen and (Expt. 13) ground in 3 ml of extraction medium with a glass tissue grinder or (Expt. 16) diced in 2 ml of medium in a glass plate. The resulting brei was added to 20 ml of extraction medium, centrifuged at low speed (3500 rpm) for 5 minutes, and stored on ice. After a high speed (11,000 rpm) centrifugation for 20 minutes, the pellets were resuspended in media and filtered through 0.45 μ m filters.

Extraction procedure 5. At UC-Davis, approximately 1 g of diseased celery tissue near the center of the plant (generally crown tissue) was cut into small pieces, placed in 500 ml 70% ethanol for 1.5 minutes to eliminate surface contaminants, and washed in distilled water. The celery tissue was then ground with a cold mortar and pestle in 10 ml of extraction medium, and the extract was added to 20 ml of BEM1, centrifuged at low speed (3500-4000 rpm) for 5 minutes, and stored on ice. After a high speed (11,000 rpm) centrifugation for 20 minutes, the pellets were resuspended in media and filtered through 0.45 μ m filters.

Extraction procedure 6. At Ottawa, Canada, 50 EAY-MLO-infected *M. fascifrons* adults were ground, by L. N. Chiykowski, in extraction medium (1/10 dilution, w/v), centrifuged (3000 rpm) for 10 minutes, filtered (0.45 μ m), diluted to 1/50 in culture

medium, divided into 0.2 ml portions, and stored in the refrigerator. Alternatively, 0.01 M phosphate buffered saline (PBS) was used for extraction and dilution.

Extraction procedure 7. Finely drawn glass needles were used to collect hemolymph from approximately 50 MLO-infected leafhoppers. Immediately upon collection, the hemolymph was added to a small glass tube containing 50 ul of culture medium, and kept on ice.

Extraction media. Extraction media are described in the media Tables 14-17 and in Appendix 1. Three strategies were used: (i) media used for cultivation were used for extraction; (ii) buffers (e.g., PBS and BEM1-2) were used; and (iii) hybrid buffers/media with large amounts of reducing agents were used (e.g., MEMEK1, M1D-SO₃, Percoll).

c. Conditions of Cultivation

Temperature/atmosphere. In general, the same temperature and atmospheric conditions that were employed for extraction were used for cultivation. In addition, cultivation was attempted in a GasPak system (95% CO₂, 5% H₂) in Expt. 13. To test the viability of MLOs under cooler conditions, refrigeration (4°C) was used in Expt. 17-19. In Expt. 19, mineral oil was added to the tops of the wells to limit oxygen.

Type of culture vessel. Culture was attempted in 24-well tissue culture (microtiter) plates, 3 ml plastic tubes, or on solid media. In Expt. 4, the yeast co-culture was placed in a well insert that had a pore diameter of 0.4 um.

Media Co-culture cells. Cultivation media were of two types, liquid or solid (0.8% agar), as described in Tables 14-17. In Experiments 1-4, 8, 15, 20, and 27, co-culture was attempted, using a yeast obtained from D. L. Williamson (Stony Brook) that had been successfully used to isolate the sex ratio spiroplasma of *Drosophila*, bacteria isolated in our investigations, or insect cells obtained from D. L. Lynn (DU-E, Tex2) or H.-T. Hsu (AC-20).

Means of determining growth. Four methods were used to determine the amount of MLO growth in culture systems: (i) microscopic enumeration under a dark-field microscope at 1250x; quantification through (ii) antibody/ELISA systems or (iii) gene probe dot blots; and (iv) bioassay. Microscopic enumeration was used primarily for estimating counts of spiroplasmas and yeasts used in the culture trials. In this case, color changing units (CCUs) were also used. However, structures were observed in MLO cultivation trials that may or may not have been MLOs; the morphologies and numbers of these cells were also recorded. Procedures for use of gene probes (Kirkpatrick *et al.*, 1987) and antibody/ELISA (Lin and Chen, 1985) have been described. For

gene probes, DNA was extracted by the alkaline lysis method (Maniatis et al., 1982) and the BRL HybridDot system was used for blots. rRNA probes were used (Kirkpatrick et al., 1987). For ELISA, samples (in EDTA and mercaptoethanol) were added to microtiter wells with a carbonate-bicarbonate, pH 9.6 coating buffer, and placed in a moist chamber at 37°C for 3 hours. 5% skim milk was used as a blocking solution. Washes were made with PBS-Tween 20. After addition of monoclonal or polyclonal antibody, biotinylated goat antimouse IgG and IgM conjugate (KPL) were added. Strepto-Avidin peroxidase conjugate (KPL) was added and reaction with horseradish peroxidase substrate was monitored by an ELISA reader. For bioassay, finely drawn glass needles were used to inject (approximately) 0.1 ul of MLO extract or culture between the sternites of 50-100 M. fascifrons 5th instar or adult leafhoppers. Insects were kept moist and cool until injection, which was performed under CO₂. They were then placed on rye plants. After an incubation period of 10 days, and until at least 28 days, leafhoppers were placed on aster plants for MLO transmission; exposed plants were observed for symptoms of yellows disease.

Spiroplasma Cultivation Experiments

Fastidious spiroplasmas. In an attempt to determine the impact of co-culture on nonfastidious (S. apis) and fastidious (S. kunkelii) spiroplasmas, as an index of what we could expect from MLOs in similar systems, we monitored spiroplasma growth in DCCM or AC-20M medium in co-culture with DU-E or AC-20 leafhopper cell lines, respectively, with or without yeast cells.

Experiment 11 - Spiroplasma apis. S. apis (strain B31) cultures in DCCM medium were added to microtiter wells containing DCCM medium containing the DU-E beetle cell line, the AC-20 leafhopper cell line, or Williamson's yeast cells. Each well contained approximately 7.5×10^4 insect cells, which were added fresh at the start of the experiment. Growth of spiroplasmas was monitored daily for 8 days by microscopic enumeration and color changing units (CCUs).

Experiment 12 - Spiroplasma kunkelii. The procedure was the same as for Experiment 11, but S. kunkelii (strain E275) cells were used.

MLO Cultivation Experiments

Experiment 1 - Co-culture with DU-E insect cells and Colorado potato beetle spiroplasmas. DU-E cells were set up as in Expt. 11. Cells were allowed to grow in the microtiter wells for one day prior to inoculation of MLO-containing extracts. Colorado potato beetle spiroplasmas (strain LD-1) were inoculated to a final concentration of 2×10^5 cells per ml. 0.2 ml of inoculum was added to 1.8 ml of medium in each well. Treatments were:

- o DU-E Cells/LD-1 Cells/DCCM Medium/NJAY-MLO Extract from M.f.
- o DU-E Cells DCCM Medium/NJAY-MLO Extract from M.f.
- o DU-E Cells DCCM Medium/Healthy Extract from M.f.

Experiments 2-3 - Co-culture with DU-E beetle cells. The procedure was as described in Experiment 1, no LD-1 cells were used. Treatments were as follows:

- o DU-E Cells/DCCM Medium/NJAY-MLO Extract from M.f.
- o DU-E Cells/DCCM Medium/Healthy Extract from M.f.

Experiment 4 - Co-culture with AC-20 leafhopper cells. The procedure was as described in Experiment 2, but no AC-20 leafhopper cells were used and log phase yeast cells were placed in 0.4 um pore "Minicell" inserts in the wells. Treatments were as follows:

- o AC-20 Cells/AC-20M Medium/yeast/NJAY-MLO Extract from M.f.
- o AC-20 Cells/AC-20M Medium/yeast/Healthy Extract from M.f.

Experiments 5-7 - Culture in M1D. These experiments were designed to determine the rate of transmission of injected leafhoppers, using extracts in M1D medium, with the reducing agent Na_2SO_3 added to the media used for plant extraction. Treatments were as follows:

- o Expt 5: M1D/Injected Leafhoppers as Inoculum
- o Expt 6: M1D- SO_3 /Fresh NJAY-Diseased Petioles as Inoculum
M1D- SO_3 /Fresh NJAY-Diseased Laminae as Inoculum
- o Expt 7: M1D- SO_3 /Shell-frozen NJAY-Diseased Petiole Inoculum

Experiment 8 - Co-culture with AC-20 Leafhopper cells with MLO cells trapped on 0.45 um filters. In this experiment, MLOs were extracted in a buffer and filtered through overlying 0.65/0.45um membranes. Presumably, most MLO cells would pass through the 0.65 um membrane and be retained by the 0.45 membrane. The 0.45 membrane was removed and placed in culture with AC-20 cells. The experimental set-up was similar to Experiment 1.

- o AC-20 Cells/AC-20M Medium/0.45 um/NJAY-MLO Extract from M.f.
- o AC-20 Cells/AC-20M Medium/0.45 um/Healthy Extract from M.f.

Experiment 9 - Percoll gradient purification and culture in C-3G/M1D media. For the purpose of obtaining high quality inoculum, lettuce was used to supply NJAY-MLOs extracted and purified on Percoll gradients. Because we suspected that Percoll, extraction medium, or contaminants were causing high mortality of injected leafhoppers, filtration through 0.45 um membranes was used in some trials. There were three parts to this experiment. In Parts A and C, enzymes were used to digest plant tissue, leaving only vascular material as the MLO source.

- o A: 10-50% Percoll gradient/Extract from lettuce C-3G Medium
- o B: Extract from lettuce in C-3G Medium
+/- Filtration
+ 0, 40, and 100% Percoll
- o C: Extract from lettuce/Percoll gradient (3 bands injected)

Experiment 10 - Survival and transmission efficiency of σ/ϕ 's. This experiment was conducted to determine the relative susceptibility and disease transmission potential of male and female leafhoppers. Since the extracts and resulting AC-20M cultures were tested for MLO survival, this was also a cultivation experiment.

- o Injected σ 's: NJAY-MLO in AC-20M
AC-20M Control
Noninjected Control
- o Injected ϕ 's: NJAY-MLO in AC-20M
AC-20M Control
Noninjected Control

Experiment 13 - BLTVA- & SAY-MLO culture in Chang medium under air or GasPak conditions. Experiments 13-16 were conducted by K. Hackett and B. Kirkpatrick at UC-Davis, in collaboration with C.-J. Chang, J. Macy, C. Kuske, M. Feldlaufer, D. Golino, D. Williamson, and A. Purcell. In these experiments, cultures were monitored by gene probes and polyclonal antibodies developed at the laboratory of B. C. Kirkpatrick. During these experiments, anaerobic conditions were used for the first time. Most cultures were incubated at 28°C.

In Experiment 13, leafhoppers were used as a source of BLTVA-MLO and celery was used for SAY-MLO. All extractions were done in Kirkpatrick's extraction buffer. Chang Medium was used. This medium, designed by C.-J. Chang, was based on celery extract. Treatments were as follows:

- o BEM1/BLTVA-MLO/Air
- o BEM1/BLTVA-MLO/GasPak
- o BEM1/SAY-MLO/Air
- o BEM1/SAY-MLO/GasPak

Experiment 14 - BLTVA- and PYLR-MLO culture in Chang based media under air or GasPak conditions. This experiment was similar to Experiment 13, but different organisms (PYLR) and media (DMCMH) were used, and an anaerobic hood (Hood 1) was used instead of a GasPak. The DMCMH medium was extremely complex, and was a compilation of DCCM, M1D, Chang, H-2, and H-1 Media. Treatments were as follows:

- o BEM2/BLTVA-MLO/Chang Medium/Air
- o BEM2/BLTVA-MLO/Chang Medium/Anaerobic Hood
- o BEM2/BLTVA-MLO/DMCMH Medium/Air
- o BEM2/BLTVA-MLO/DMCMH Medium/Anaerobic Hood
- BEM2/PYLR-MLO/Chang Medium/Air
- BEM2/PYLR-MLO/Chang Medium/Anaerobic Hood
- BEM2/PYLR-MLO/DMCMH Medium/Air
- BEM2/PYLR-MLO/DMCMH Medium/Anaerobic Hood

Experiment 15 - PYLR-MLO culture in P1, Chang, CBY, and M1D, under air or anaerobic hood conditions, +/- yeast, plant extract inoculum. This experiment differs from the previous two in that additional media were used (P1, CBY, M1D) and, of possible importance, all extractions were done in completely anaerobic environments. Aliquots of each extract were then left in the anaerobic hood or removed to an aerobic incubator. Also, the medium to be used for culture was used in the extraction. Media used in these trials were very complex. CBY medium was based on Chang medium, with added fresh brain extract, extracted by B. C. Kirkpatrick, and a yeast extract obtained from C. Kuske. Pig brain extraction was accomplished by using a tissue grinder to macerate neonate pig brains in cold 20 mM KCl/0.03 M HEPES buffer. The brei was centrifuged for 2 hours at 37,000 rpm, and the supernatant was filtered to remove contaminants. P1 Medium was the first medium designed by us to mimic plant phloem sap, and contained aster lipid extracts obtained from M. Feldlaufer. It also contained complex components such as pig brain and yeast extract. Yeast co-culture was attempted in half of the trials. The treatments were as follows:

- o PYLR-MLO/M1D Medium/Air
- o PYLR-MLO/M1D Medium/Air/Yeast
- o PYLR-MLO/M1D Medium/Anaerobic Hood
- o PYLR-MLO/M1D Medium/Anaerobic Hood/Yeast
- PYLR-MLO/Chang Medium/Air
- PYLR-MLO/Chang Medium/Air/Yeast
- PYLR-MLO/Chang Medium/Anaerobic Hood
- PYLR-MLO/Chang Medium/Anaerobic Hood/Yeast
- o PYLR-MLO/CBY Medium/Air
- o PYLR-MLO/CBY Medium/Air/Yeast
- o PYLR-MLO/CBY Medium/Anaerobic Hood
- o PYLR-MLO/CBY Medium/Anaerobic Hood/Yeast
- PYLR-MLO/P1 Medium/Air
- PYLR-MLO/P1 Medium/Air/Yeast
- PYLR-MLO/P1 Medium/Anaerobic Hood
- PYLR-MLO/P1 Medium/Anaerobic Hood/Yeast

Experiment 16 - PYLR-MLO culture in P1, Chang, CBY, and M1D, under anaerobic hood conditions, leafhopper inoculum. This experiment was similar to Experiment 15, except leafhoppers, obtained from A. Purcell, were used for PYLR inoculum. All cultures were maintained under the anaerobic hood. Treatments were as follows:

- o PYLR-MLO/Anaerobic Hood/M1D Medium
- PYLR-MLO/Anaerobic Hood/Chang Medium
- o PYLR-MLO/Anaerobic Hood/CBY Medium
- PYLR-MLO/Anaerobic Hood/P1 Medium

Experiments 17-18 - EAY-MLO culture in MIMEK1 and PBS. In Experiments 17 and 18, eastern (EAY) and western (WAY) strains of aster yellows were used by L. Chiykowski to compare survival of MLOs in the MEMEK1 extraction medium/MIMEK1 culture medium system

of K. Hackett to survival in 0.01 M phosphate buffered saline. The MEMEK1 extraction medium was designed to protect MLOs from oxygen. MIMEK1 was a medium that was designed to minimize MLO membrane damage, but without the strong buffers and reducing agents of MEMEK1. Although leafhopper extractions were carried out at ambient temperature, the cultures were immediately refrigerated for the duration of the experiments, which were 5 (Expt 17) and 4 (Expt 18) days. Treatments were as follows:

- o Expt 17: EAY/4°C/MEMEK1/MIMEK1
- o Expt 17: EAY/4°C/PBS
- Expt 18: WAY/4°C/MEMEK1/MIMEK1
- Expt 18: WAY/4°C/PBS

Experiment 19 - NJAY-MLO culture in PL-1 medium, under air or reduced oxygenation by oil, At 26°C or 4°C. Two innovations were introduced in this trial: a medium (PL-1) based on an extensive review of the literature on the composition of plant phloem sap, and through consultation with J. D. Pollack (Ohio State Univeristy) and J. G. Tully (NIH) (see Appendix 1); reduced oxygenation through the use of mineral oil overlays on cultures in wells. The effect of refrigeration on MLO survival was also reevaluated. Treatments were as follows:

- o NJAY-MLO/26°C/Air
- o NJAY-MLO/26°C/Oil
- o NJAY-MLO/4°C/Air
- o NJAY-MLO/4°C/Oil

Experiment 20 - Co-culture of NJAY-MLO with bacteria. Previous evidence had shown that minute (unidentified) "satellite" bacterial colonies occurred on solid media during MLO isolation attempts. Because of the metabolite retaining properties of solid media (see Appendix 1), the possibility exists that MLOs might grow better in this milieu than in liquid media. To study these factors, we conducted the following series of experiments (Experiments 20-23). In all experiments, NJAY-MLO-infected M. fasciifrons was used for inoculum. Either PL-1 or PL-2 (a double strength PL-1) media were used for extraction.

In Experiment 20, Luria, blood agar, and milk agar plates were used for culture; PL-1 medium (with Na₂SO₃) was used for extraction, and PL-1 medium was used for culture. A bacterium (Bact1) isolated during Experiment 19 from NJAY-MLO-infected leafhoppers was used for one series of co-culture attempts (this bacterial culture was presumed to have some viable MLOs). Other bacteria (BacMx) were obtained during isolation from NJAY-MLO-infected leafhoppers and plants. Extractions were similarly performed from healthy leafhoppers and plants (not shown in the treatment schema). Media were observed for bacteria and the presence of satellite MLO colonies. Treatments were as follows:

- o Bact1 & MLO/PL-1 Medium
- o Bact1 & MLO/Blood agar
- o Bact1 & MLO/Milk agar
- o Bact1 & MLO/Luria agar

- NJAY-MLO-Infected Leafhoppers/PL-1 Medium
- NJAY-MLO-Infected Leafhoppers/Blood agar
- NJAY-MLO-Infected Leafhoppers/Milk agar
- NJAY-MLO-Infected Leafhoppers/Luria agar
- NJAY-MLO-Infected Plants/Luria agar/Unfiltered Inoculum
- NJAY-MLO-Infected Plants/Luria agar/0.65 um Filtered Inoculum

Experiment 21 - To Determine if cultured bacterial contaminants pass through filters with MLOs. Evidence from previous experiments had suggested that small bacteria might be passing through 0.45 um filters. If so, filtration, which is known to significantly reduce MLO inoculum titer (even through 0.65 um pore filters) (R. F. Whitcomb, unpublished data), would not be a practical method of obtaining adequate MLO inoculum for culture trials. This experiment is included here because NJAY-MLO culture was attempted during the study. Essentially, a mixed bacterial culture (BacMx), containing MLO extract, was passed through 0.22, 0.3, 0.45, 0.65, and 0.8 um filters and plated on Luria agar.

- o NJAY-MLO/BacMx/Luria/Unfiltered
- o NJAY-MLO/BacMx/Luria/0.22
- o NJAY-MLO/BacMx/Luria/0.3
- o NJAY-MLO/BacMx/Luria/0.45
- o NJAY-MLO/BacMx/Luria/0.65
- o NJAY-MLO/BacMx/Luria/0.8

Experiments 22 - NJAY-MLO bacterial co-culture on solid medium under air and anaerobic hood conditions. This experiment was conducted, principally, to determine if MLOs might grow on solid medium under anaerobic conditions. Besides concentrating metabolites, solid media might act as a sieve trap for the limited inoculum. This experiment was also a co-culture experiment in that no antibiotics (and only 0.8 um filtration) was used in the extraction, which was done anaerobically. Penicillin discs were placed on the medium to provide a zone of bacterial inhibition, thus combining, in one experiment, the concepts of bacterial co-culture and bacterial-free isolation. Multiple PL-2A agar plates were used. Treatments were as follows:

- o NJAY-MLO/PL-2A/Air
- NJAY-MLO/PL-2A/Anaerobic Hood

Experiment 23 - NJAY-MLO culture on solid media, under air and anaerobic hood conditions, hemolymph inoculum. This experiment was similar to Experiment 22, but no bacterial co-culture was attempted; penicillin-G was used to eliminate bacterial contaminants on the plates (PL-2A) and liquid medium (PL-2). And, for the first time in our experiments, hemolymph from infected insects was used as inoculum. This was done to provide high quality inoculum free of toxins that are released during extraction of plant and insect material. The procedures should

also have reduced bacterial contamination. Treatments were as follows:

- o NJAY-MLO/PL-2A agar/Air
- o NJAY-MLO/PL-2A agar/Anaerobic Hood
- o NJAY-MLO/PL-2 Medium/Air
- o NJAY-MLO/PL-2 Medium/Anaerobic Hood

Experiment 25 - NJAY-MLO culture from lettuce in serum-free and serum fraction media Under air and anaerobic hood conditions.

Experiments 25 and 26 were conducted to test two concepts. One was that lettuce, because of the high quantities of infected tissue that can be processed, might be a better source of inoculum than leafhoppers. The second was that, since MLOs may be closely related to acholeplasmas, typical acholeplasma media, such as serum-free (SerFree) and serum fraction (SerFrac) media, might be effective growth media for MLOs. Levels of Tween 80 (from 0-0.4%), found to be important for acholeplasma cultivation, were employed. All extractions were performed in the cold room to reduce damage due to oxygenation. Cultures in both experiments were monitored by T.-A. Chen using monoclonal antibody/ELISA procedures developed during the course of this grant. Experiment 25 treatments were as follows:

- oo NJAY-MLO/Air/SerFrac Medium
- o NJAY-MLO/Air/SerFree0 Medium
- o NJAY-MLO/Air/SerFree1 Medium
- o NJAY-MLO/Air/SerFree2 Medium
- o NJAY-MLO/Air/SerFree4 Medium
- NJAY-MLO/Anaerobic Hood/SerFrac Medium
- NJAY-MLO/Anaerobic Hood/SerFree0 Medium
- NJAY-MLO/Anaerobic Hood/SerFree1 Medium
- NJAY-MLO/Anaerobic Hood/SerFree2 Medium
- NJAY-MLO/Anaerobic Hood/SerFree4 Medium

Experiment 26 - NJAY-MLO culture from lettuce In serum free, PL-1, and M1D media, under air and anaerobic hood conditions.

In this experiment, other promising media (PL-1 and M1D) were used in addition to serum free medium with 0.4% Tween80.

Treatments were as follows.

- o NJAY-MLO/Air/SerFree4 Medium
- o NJAY-MLO/Air/PL-1 Medium
- o NJAY-MLO/Air/M1D Medium
- NJAY-MLO/Anaerobic Hood/SerFree4 Medium
- NJAY-MLO/Anaerobic Hood/PL-1 Medium
- NJAY-MLO/Anaerobic Hood/M1D Medium

Experiment 27 - NJAY-MLO co-culture with an insect muscle cell line. During the course of these experiments, D. Lynn, one of our BARD collaborators, developed a cell line that had not previously been tried in mollicute co-culture. The intriguing aspect of this cell line was that it was the first insect muscle cell line ever developed. Importantly, it does not multiply; thus it might provide the advantages of co-culture for a slow

growing fastidious mollicute, without depriving the mollicute of nutrients [nutrient competition was shown to play a negative role in co-culture of fastidious spiroplasmas (Hackett et al., 1986)]. To study this, the muscle cell line (Tex2) was set up in two different media, Tex2M medium, normally used to grow the cell line, and DCCM medium, which had previously been used to cultivate fastidious spiroplasmas. All culture was attempted aerobically. Inoculum consisted of cultures (Experiments 23 and 25) that had tested positive by ELISA for acholeplasmas or NJAY-MLOs, and fresh NJAY-MLO extract. Treatments were as follows:

- o NJAY-MLO-Leafhopper Extract/DCCM Medium
- NJAY-MLO-Leafhopper Extract/Tex2M Medium
- o Expt 23 Culture in PL-1 Medium/Tex2 Cell Line/DCCM Medium
- Expt 23 Culture in PL-1 Medium/Tex2 Cell Line/Tex2M Medium
- o Expt 25 Culture in PL-1 Medium/Tex2 Cell Line/DCCM Medium
- Expt 25 Culture in PL-1 Medium/Tex2 Cell Line/Tex2M Medium

Pasteuria Cultivation Experiment

Experiment 24 - Determination of Pasteuria oxygen needs. Media designed for cultivation of MLOs, although unsuccessful for sustained cultivation of these microbes, when amended with components applicable to the "nematode pseudocoelomic microhabitat," were effective for cultivation of another, heretofore uncultivable, group of potentially important microbes, the plant nematode-parasitic actinomycetes, Pasteuria spp. Ironically, since Pasteuria spp. grow very slowly in these media, requiring 4-6 weeks between transfers, they might be excellent co-culture organisms for MLO culture. To begin the process of determining under which culture conditions they might be used, the oxygen requirements of Pasteuria were investigated. The treatments were as follows:

- o Pasteuria/Air
- Pasteuria/Anaerobic Hood

RESULTS AND DISCUSSION

Comparison of Methods for Determining Growth.

Although we used many techniques (light microscopy, bioassay, gene probes, monoclonal antibody/ELISA) for monitoring MLOs during these studies, bioassay is the only one that measures viability. Problems, however, ensued in using this technique. Unexpectedly, the NJAY-MLO appears to be pathogenic for M. fascifrons. Also, certain media promise to be toxic. In general, high mortality often made estimation of viability by disease production unreliable. For this reason, future experimentation should focus on use of gene probes or ELISA to monitor MLO titer by time course studies. Some progress toward this end was achieved.

In collaboration with B. Kirkpatrick at UC-Davis, we were able to observe an increase in gene probe intensity (e.g., Experiment 14: Table 2, 0→3 on intensity rank for PYLR-MLO cultures in DMCMH medium in anerobic hood; Experiment 15: Table 3, 2→5 for PYLR MLO in M1D medium) in dot blots of DNA extracted culture samples. The increase tended to peak at 1-2 days, followed by a slow decline. The gene probe results therefore suggest that the MLOs were increasing under the conditions of cultivation, but were unable to sustain growth.

ELISA, at Rutgers and UC-Davis, also indicated a 1-2 day burst of growth, followed by detection of the antigen for more than a month. For example, in Experiment 14 (Table 2), the optical reading in Chang medium increased from .38→.50 at Day 1.

These results, taken together, show promise for both methods in the monitoring of MLO growth.

Some questions remain to be answered. For example, in many experiments, e.g., Experiment 15 (Table 5, Without Yeast, Air, Passage 1, 10⁰), it is not clear, when starting with large amounts of inoculum (i.e., high optical readings), whether there has been a steady decline in the titer of organisms, or whether, some limited growth was obscured by the presence of large amounts of antigen. That some growth did occur is suggested by 10⁻¹ subculture (of these same cultures), which showed an increase in optical reading from Day 0→1→2→3, with a leveling off at Day 6. (It should be noted that during these cultivation attempts, fresh medium is typically added at an amount about 1/5 of the culture volume every 2 days. In the cases discussed here, much higher dilutions were made, with ATP and fresh plant extract as amendments. Maintenance of apparently stable values may therefore indicate growth.)

Although many different types of pleomorphic structures were seen in MLO culture attempts, none could be definitively shown to be MLOs. Therefore, dark-field microscopy is probably an unreliable method for determining the presence of MLOs. This conclusion is not, however, categorical. Pleomorphic, budding structures were present in cultures that tested positive by ELISA for acholeplasmas and NJAY-MLO in Experiments 23 and 25. More work, particularly electron microscopy, needs to be done to verify whether these structures are MLOs. If they are, dark-

field microscopy could be a valuable tool in monitoring MLO titers.

Comparison of Cultivation Components

1. Sources of Inoculum. The sources of inoculum used in these experiments were infected leafhoppers (hemolymph or macerates of whole insects) and diseased plants (celery, aster, lettuce). MLO isolates that exhibited an initial burst of growth in the culture systems were obtained from whole leafhopper macerates (Experiments 17, 18, 19) and celery (Experiments 13, 14, 15). In Experiment 15, fresh celery extracts, when added to aerobic (but not anaerobic) cultures at Day 2, appeared to result in an increase in PYLR-MLO growth by Day 3 (Table 3). This was particularly true for primary cultures that had been diluted 10-fold (Table 5: Without Yeast, Air, Passage 1, 10^{-1}). There was some evidence for isolation of MLOs from leafhopper hemolymph as well (Experiment 23, Table 13, slight ELISA reaction of NJAY-MLO pass 4 to NJAY-MLO-specific antisera.). However, the preponderance of evidence suggests that either whole leafhopper macerates, or macerates of highly diseased celery, were the best sources of inoculum used in these experiments.

2. Extraction Protocols. Initial survival of MLOs was accomplished using many extraction protocols, including (i) maceration of leafhoppers in air at ambient temperature (Experiments 3, 4, 5, 10, 13, 16), (ii) on ice with refrigerator storage (Experiments 17, 18), or (iii) on ice in a cold room (Experiments 25, 26), (iv) maceration of leafhoppers in an

anaerobic hood (Experiments 16, 22), and (v) maceration of celery in an anaerobic hood (15). Various extraction media proved efficacious, particularly the highly reducing medium, MEMEK1, designed for these studies (Experiments 17, 18), and various bacterial culture media (Experiments 14, 15, 16: P1, Chang, DMCMH, M1D, CBY) and insect tissue culture media (Experiments 3, 4, 10: DCCM, AC-20M). Use of traditional extraction solutions, such as phosphate buffered saline (Experiments 17, 18: Table 10) or sulfite-containing-media (Experiments 6, 7: Table 1) or buffers (Experiments 8-9: Table 1), with the exception of BEM2 (Experiment 14: Table 2) and, possibly, BEM1 (Experiment 13: Table 2), were not nearly as effective.

3. Culture Conditions.

The two principal comparisons made in these studies were between isolation and culture (i) at ambient vs. cool or cold temperatures, in (ii) aerobic vs. anaerobic environments. Two types of anaerobic system were used: GasPaks, in which isolations were accomplished in air, with transferral of isolates to the GasPaks; anaerobic hoods, in which isolations were usually performed inside the hoods. Two additional means of controlling the amount of oxygen available to the MLO cells were to use a mineral oil layer over the cultures, and to use tubes instead of microtiter wells.

Isolation on ice, with maintenance of cultures in the cold (at 4°C) was clearly superior with regards to AY-MLO survival (Experiments 17-19: Tables 1, 10). Although it is not likely that such frigid conditions would be suitable for MLO culture, this method might nevertheless be a method for maintaining MLOs until toxins (from extracted leafhoppers or plants) dissipate or the MLOs can produce metabolites necessary for their growth. In general, there is every reason to believe that maintenance of cool conditions during isolation is preferable. However, the optimal temperature for culture cannot be deduced from these studies. Increases in MLO titers occurred in cultures incubated from 4°C (Experiments 17, 18: Table 10) to 28°C (Experiments 13-15: Tables 2-4, 7), the complete span of temperatures tested.

Cultures were able to increase in air in tubes at 4°C (Experiments 17, 18: Table 10) or microtiter plates at 28°C

(Experiments 13, 14, 15: Tables 2-4, 7). As shown in Table 5 (Experiment 15), growth occurred in both aerobic and anaerobic environments. However, there was a striking difference between titers obtained from aerobic vs. anaerobic culture, with, as detected by ELISA, significantly higher titers of PYLR-MLOs in air (Tables 5-6: Day 1), but perhaps less continued maintenance of MLO titers in air (Table 5-6: Day 6). Counterintuitively, there was evidence (by gene probe) of a burst of growth in air after addition of fresh celery extract at Day 2 (Table 3). It is generally believed that oxygen would exacerbate damage by plant toxins; however, if the MLOs require oxygen, and plant extracts have nutrients needed for MLO culture, the observed growth is entirely explainable. Certainly, evidence accumulated in later experiments (Experiments 22-23, 25-26) do not support the contention that NJAY-MLO grows better in anaerobic environments. Also, oxygen-excluding mineral oil layers appeared to decrease, rather than increase, NJAY-MLO survival (Experiment 19: Table 1). With possible MLO affinity to the aerobic acholeplasmas (Sears et al., 1989), this is not surprising. Perhaps future experiments should be undertaken in systems, such as tissue culture flasks (with or without solid media) that allow easy penetration of oxygen. Aeration of cultures should also be considered.

4. Cultivation Systems. Together with the somewhat enigmatic increase in AY-MLOs at 4°C in MIMIK1 (Experiment 18, Table 10) and PL-1 (Experiment 19, Table 1) media, two cultivation systems especially showed promise: (i) cultivation of PYLR-MLO, extracted

from celery, in various media (particularly P1, Chang and M1D media), under aerobic conditions at 28°C (Experiments 14, 15); (ii) cultivation of NJAY-MLO, extracted from leafhoppers, in various media (particularly PL-1, SerFrac, and SerFree0), under aerobic conditions at 26°C (Experiments 22-23, 25-26). Evidence for the success of the former systems was obtained through time course monitoring of cultures by ELISA and gene probe; evidence for the success of the latter systems was obtained through microscopic and ELISA monitoring of cultures for long time periods, over many passages.

These methods suggest several conclusions:

1. All three monitoring systems (bioassay, gene probe, ELISA) used in these studies were adequate, but provided different clues (viability, genome multiplication, and cell growth, respectively) as to the progress of the cultures. Light microscopy may yet be found to have application, but much work needs to be done in this regard with confirmation of cultured organisms by electron microscopy.

2. Cultivation of several, somewhat unrelated, MLOs may be achievable (including, PYLR-MLO, BLTVA, and aster yellows MLOs from New Jersey, Canada, and the western United States). However, the importance of MLO strain in cultivation should not be overlooked as a possibly critical factor in success.

3. Whole leafhoppers (or possibly their hemolymph) or diseased celery appear to be suitable sources of inoculum for cultivation trials.

4. While extraction should be performed on ice, preferably in a cold room, cultivation at 28° is probably satisfactory. Whether cultivation at 26°C or 30°C would be better cannot be ascertained from these studies. However, poor survival of AY-MLO at temperatures above 32°C suggest that higher temperatures should be avoided.

5. Efforts should be made to increase oxygenation in culture systems. This does not, however, rule out the possibility that extractions might be less harmful to MLOs if carried out under totally anaerobic conditions. Perhaps, after a period of adaptation under anaerobiosis or refrigeration in which toxins are metabolized or inactivated or essential metabolites are allowed to accumulate under anaerobiosis, cultures could be gradually weaned to oxygen atmospheres and higher temperatures.

6. Media shown to be useful for cultivation of MLOs (i) were based on components found in phloem saps (P1, PL-1), (ii) had plant extract additives (Chang, or fresh celery extract), (iii) were based on a knowledge of insect hemolymph (M1D), or (iv) were media proven to be successful for cultivation of acholeplasmas (SerFree, SerFrac). A successful strategy might be one in which a medium with a large number of chemically defined components (e.g., P1, PL-1: Table 15) is supplemented with plant extracts and other complex nutrient sources (such as has been done in the past in formulation of Chang and M1D media, respectively).

7. None of the co-culture systems (Experiments 1-4, 8, 15, 20-21, 27: DU-E beetle cells, AC-20 leafhopper cells, yeast,

20-21, 27: DU-E beetle cells, AC-20 leafhopper cells, yeast, bacteria) appeared to improve survival of MLOs. The yeast, as utilized here, may have been detrimental (Experiment 15: Table B, D). However, the striking success in use of these systems for cultivation of other fastidious mollicutes (including spiroplasmas and mycoplasmas), suggests that this line of research should not be abandoned.

Studies on Biology of the New Jersey Aster Yellows Agent

Some features of the vector relationship of the NJAY MLO were determined. After a 4 day acquisition access by 1st instar Macrosteles fascifrons nymphs, followed by a latent period of 14-25 days (mean = 17.9), 59-66% of adult leafhoppers transmitted the pathogen for 9-46 days; maximum transmission occurred from 25 to 39 days after the acquisition access period. Plant symptoms appeared 2-4 weeks after infected leafhoppers were placed on young healthy plants. Symptoms were characteristic of aster yellows (AY), and included floral virescence. In contrast to classical strains of AY, however, NJAY MLO appeared (Fig. 1) to be pathogenic to M. fascifrons ($p < 0.05$ in Mann-Whitney and Wilcoxon Ranked Sum tests; infected $n = 23$, noninfected $n = 12$). Similar survivorships were observed for males and females. Also, the two sexes appeared to transmit the disease agent at similar rates: 33.3% (female), and 23.7% (male). Observation of pathogenicity for the vector suggests that, unlike many strains of AY agents in the eastern U. S. transmitted principally by

Schematic plot of longevities of healthy Macrosteles fascifrons leafhoppers, and of leafhoppers that transmitted NJAY MLOs to aster. Ordinate = longevity in days. The mean longevities of the two classes of leafhoppers were significantly different statistically (see text).

M. fascifrons, NJAY may be transmitted by another vector in the field. The failure of NJAY MLO monoclonal antibodies to react with other AY strains may, therefore, reflect basic biological differences in field maintenance.

Fig. 2. Titers of S. apis in three cell-assisted media for MLO cultivation

Comparison of Co-culture Systems

Our preliminary studies with Spiroplasma apis and S. kunkelii suggest (on the basis of analysis of microscopic enumeration data) that the nature of cells used in co-culture systems (DU-E or AC-20 insect cells, or yeast cells) affects mollicutes differentially. For S. apis (Fig. 2), yeast > AC-20 > DU-E cells; for S. kunkelii, AC-20 > yeast > DU-E cells. Since S. apis is a relatively fast growing species, it is not surprising that it is able to take advantage of fast-growing co-culture yeast cells. AC-20 co-cultures may be more suitable for slower growing mollicute cells, such as S. kunkelii. Since S. kunkelii, like the MLO, is a phloem/leafhopper-associated

Fig. 3. Titters of *S. kunkelii* in three cell-assisted media for MLO cultivation

organism, and grew preferentially in the leafhopper-derived AC-20 co-culture system, this system may be superior to yeast systems for co-culture of MLOs. However, it should be noted that a slow growing spiroplasma, the group II sex ratio organism of *Drosophila*, was more consistently isolated in yeast co-cultures than in insect cell (lepidopteran) co-cultures.

These results, when considered together, suggest that important principles may be revealed from continued attempts to cultivate other fastidious spiroplasmas or other mollicutes in cell lines from different sources.

Table 1. Cultivation Trials

Expt Inoculum			Extraction Procedure			Culture System			Assay Results		
Type	Source	Type	Medium	Temp/Atm	Medium	Cells	Vessel	Temp/Atm	Day	% Symp	ELISA Probe
01	NJAY	M. f.	1 DCCM	Amb/Air	DCCM	DU-E	24-well	26°C/Air	Bio	0-7	0
						DU-E/LD-1			Bio	0-7	0
02	NJAY	M. f.	1 DCCM	Amb/Air	DCCM	DU-E	24-well	26°C/Air	Bio	0-4	0
03	NJAY	M. f.	1 DCCM	Amb/Air	DCCM	DU-E	24-well	26°C/Air	Bio	0	18%
									Bio	2-7	0
04	NJAY	M. f.	1 AC20M	Amb/Air	AC20M	AC-20	24-well	26°C/Air	Bio	0	33%
						& yeast in insert					
05	NJAY	M. f.	1 M1D	Amb/Air	M1D		Tube	Amb/Air	Bio	2-4	0
06	NJAY	Ast.-Pet. 1	M1D-SO ₃	Ice/Air	M1D		Tube	Amb/Air	Bio	0	4%
		Ast.-Lam.							Bio	0	0
07	NJAY	Ast.-Pet. 1	M1D-SO ₃	Ice/Air	M1D		Tube	Amb/Air	Bio	0	0
		(petioles were shell frozen before extraction)									
08	NJAY	M. f.	2 Gly-Mg	Ice/Air	AC20M	AC-20	24-well	26°C/Air	Bio	0-2	0
09	NJAY	Lettuce	3 percoll	Amb/Air	C-3G		Tube	Amb/Air	Bio	0	0 ^M
10	NJAY	M. f.	1 AC20M	Amb/Air	AC20M		Tube	Amb/Air	Bio F	0	33%
									Bio M	0	24%
11	S. a.	Culture			DCCM	DU-E	24-well	26°C/Air	Microscope	(see Fig. 1)	
					DCCM	Yeast					
					AC20M	AC-20					
12	S. k.	Culture			DCCM	DU-E	24-well	26°C/Air	Microscope	(see Fig. 2)	
					DCCM	Yeast					
					AC20M	AC-20					

Expt Inoculum	Extraction Procedure			Culture System			Assay Results		
	Type	Source	Type Medium	Temp/Atm	Medium Cells	Vessel	Temp/Atm	Day	%Symp ELISA Probe
25	NJAY	Lettuce 1	SerFrac SerFree0 SerFree1 SerFree2 SerFree4 SerFrac SerFree0 SerFree1 SerFree2 SerFree4	Cold/Air	SerFrac	Tube	Amb/Air	ELISA (see Table)	
							Amb/Hood2		
26	NJAY	Lettuce 1	M1D SerFree4 PL-1 M1D SerFree4 PL-1	Cold/Air	M1D	Tube	Amb/Air	ELISA (see Table)	
				Cold/Air	M1D	Tube	Amb/Hood2	ELISA	
27	NJAY	M. f. 1	DCCM	Ice/Air	Tex2M DCCM	Tex2	24-well 26°C/Air	ELISA (see Table)	
		Culture Expt 23	PL-1/Solid/26/Ar		Tex2M DCCM				
		Culture Expt 25	SFC-SFR0/26/Ar		Tex2M DCCM				

Legend for Table 1.

24-well = Twenty four well microtiter plate
AC-20 = AC-20 leafhopper cell line
Amb = ambient (24-28°C)
Ast.-Lam. = aster laminae
Ast.-Pet. = aster petioles
Atm = atmosphere
Bact1 = small motile bacillus associated with leafhoppers
BacMx = mixture of bacteria associated with leafhoppers
Bio = bioassay
BLTVA = beet leafhopper transmitted virescence agent
ELISA = enzyme linked immunosorbent assay
Cold = extraction was done in cold room on ice
DU-E = Diabrotica undecimpunctata cell line
EAY = eastern strain of aster yellows
Expt = experiment
Ice = extraction was performed on ice
F= female
GasPak = anaerobic jar containing 95% CO₂, 5% H₂
Hood1 = anaerobic hood containing 90% N₂, 10% H₂
Hood2 = anaerobic hood containing 90% N₂, 5% CO₂, 5% H₂
Leafhp = leafhoppers
M = high mortality of test insects
M = male
M. f. = Macrosteles fascifrons
Micr = observation and enumeration by dark field microscopy
NJAY = New Jersey strain of aster yellows MLO
Pastr = Pasteuria penetrans (a nematode pathogen)
PYLR = potato yellow leaf roll strain of Western-X MLO
Probe = gene probe
SAY = severe strain of western aster yellows MLO
SFC-SFRO = serum fraction and serum free (no Tween 80) MLO cultures
Solid = solid agar plate
Symp% = % plants that developed aster yellows disease after
 inoculated leafhoppers had fed on them
Temp = temperature
Tex2 = hymenopteran parasite cell line
Tube = 12 x 75 mm culture tube
Yeast = yeast supplied by D. L. Williamson
Also, see: List of Media; Extraction Procedures

Table 2. Growth of PYLR in Chang and DMCMH Media, as Determined by Polyclonal Antibody/ELISA & [Gene Probe ranked by intensity of dot on X-ray film] (Experiment 14)

	Day 0	Day 1	Day 2	Day 17
Culture in Chang Medium				
Air	.38	.50	.50	
GasPak	.38	.48	.51	[2]*
Culture in DMCMH Medium				
Air	.29	.30	.32	
GasPak	.29	.31	.31	[1]*
-> Anaerobic Hood				
			[3]**	
Chang Medium Control				
Air			.01	
GasPak			.01	
DMCMH Medium Control				
Air			.01	
PYLR Celery Control	1.21			
Healthy Celery Control	.03			
[Gene Probe Control]				[0]

* = Pass 2, 1:10

** = Pass 3, 1:10; transferred to Anaerobic Hood on Day 7

Table 3. Growth of PYLR in Chang, M1D, CBY, and P1 Media, as

Determined by Reaction with an rRNA Gene Probe to PYLR
(ranked by intensity of dot on X-ray film) (Expt. 15)

	Day 0	Day 1	Day 2	Day 3	Day 6
<u>With Yeast Co-Culture</u>					
Culture in Chang Medium					
Air	1	1	0	0	0
Anaerobic	1	1	1	1	1
Culture in M1D Medium					
Air	1	3	1	2	1
Anaerobic	1	2	2	1	1
Culture in CBY Medium					
Air	1	0	0	0	0
Anaerobic	1	0	0	0	0
Culture in P1 Medium					
Air	3	3	1	2	2
Anaerobic	3	4	3	2	0
<u>Without Yeast Co-Culture</u>					
Culture in Chang Medium			ATP	FPE	ATP
Air	4	4	1 ↓	2 ↓	0
Anaerobic	5	4	3 ↓	1 ↓	2
Culture in M1D Medium					
Air	2	5	1 ↓	7 ↓	0
Anaerobic	?	4	3 ↓	1 ↓	2
Culture in CBY Medium					
Air	1	1 ↓	1 ↓	3 ↓	0
Anaerobic	4	1 ↓	1 ↓	2 ↓	ND
Culture in P1 Medium					
Air	8	7 ↓	1 ↓	2 ↓	0
Anaerobic	10	5 ↓	3 ↓	3 ↓	ND
<u>Media Controls</u>					
With Yeast					
Air					
M1D		0			0
Anaerobic					
M1D		0			0
P1		0			0
Without Yeast					
Air					
M1D		0			0
P1		0			0
<u>Healthy Plant Extract Controls</u>					
In M1D Medium		0			0
In P1 Medium		0			?

ND = not determined;

↓ = ATP or Fresh plant extract added to culture

Table 4. Detection/Growth of PYLR, Extracted From Leafhoppers, In Anaerobic Hood, as Determined by Reaction with an rRNA Gene Probe to PYLR (ranked by intensity of dot on X-ray film) (Experiment 16)

	Day 0	Day 7
Chang Medium		
PYLR Diseased Celery	1	
Healthy Celery Control	0	
M1D Medium		
PYRL Diseased Celery	1	
CBY Medium		
Healthy Celery Control	0	
P1 Medium		
PYLR Diseased Celery	1	0
Healthy Celery Control	0	

Table 5. Growth of PYLR in Chang, M1D, CBY, and P1 Media, as Determined by Reaction with Polyclonal Antibody/ELISA (Experiment 15) [aveages of two values]

	Day 0	Day 1	Day 2	Day 3	Day 6
<u>With Yeast</u>					
Air					
Passage 1 (10^{-1})					
Chang Medium	.143	.208*	.280*	.263*	.177
M1D Medium	.202	.115	.443*	.076	.013
CBY Medium	.137	.184	.278*	.280*	.279*
P1 Medium	.140	.207	.154	.168	.136
Anaerobic Hood					
Passage 1 (10^{-1})					
Chang Medium	.164	.226	.276	.250	
M1D Medium	.143	.063	.051	.050	
CBY Medium	.112	.169	.156	.166	
P1 Medium	.140	.174	.158	.168	
<u>Without Yeast</u>					
Air					
Passage 1 (10^0)					
Chang Medium	.798	.731	ATP .719	FPE ↓ .508(.45)	ATP ↓ .419
M1D Medium	.686	.635	.607	↓ .454(.37)	↓ .207
CBY Medium	.583	.540	↓ .516	↓ .376(.32)	↓ .322
P1 Medium	.615	.650	↓ .603	↓ .510(.29)	↓ .422
Passage 1 (10^{-1})					
Chang Medium	.124	.124	.138	↓ .152	↓ .149
M1D Medium	.117	.136	.142	↓ .128	↓ .091
CBY Medium	.104	.101	↓ .105	↓ .121	↓ .125
P1 Medium	.132	.187	↓ .182	↓ .185	↓ .174
Anaerobic Hood					
Passage 1 (10^0)					
Chang Medium	.798	.677	.694	↓ .533(.43)	↓ .504
M1D Medium	.686	.558	.563	↓ .446(.35)	↓ .437
CBY Medium	.583	.469	↓ .465	↓ .359(.29)	↓ .336
P1 Medium	.615	.503	↓ .479	↓ .419(.30)	↓ .410
Passage 1 (10^{-1})					
Chang Medium	.135(.15)	.161	.180	↓ .194	↓ .217
M1D Medium	.156(.14)	.123	.137	↓ .118	↓ .125
CBY Medium	.075(.12)	.113	↓ .113	↓ .113	↓ .121
P1 Medium	.112(.12)	.146	↓ .142	↓ .102	↓ .042
Passage 2 (10^{-1})					
Chang Medium	.339	.344		.365	
M1D Medium	.319	.293		.277	
CBY Medium	.231	.218		.205	
P1 Medium	.272	.254		.248	

* = precipitate in well, from yeast growth, may have interfered with measurements; ↓ = ATP, or Fresh plant extract added

Table 6. Comparison of PYLR Titers in Air and Anerobic Hood Without Yeast, Passage 1, as Determined by Reaction with Polyclonal Antibody/ELISA (Experiment 15)

	<u>Air</u>	<u>Anaerobic Hood</u>
DAY 1		
Chang Medium	.731	.677
M1D Medium	.635	.558
CBY Medium	.540	.469
P1 Medium	.650	.503
DAY 6		
Chang Medium	.419	.504
M1D Medium	.207	.437
CBY Medium	.322	.336
P1 Medium	.422	.410

Table 7. Growth Titers of Selected PYLR (Experiment 14), BLTVA (Experiment 13), and SAY (Experiment 13) Cultures in Chang And DCMCH Media, as Determined by Reaction with Polyclonal and Monoclonal Antibody/ELISA

POLYCLONAL Ab

Celery Inoculum	
SAY (Pass 2, Chang Medium)	.077
PYLR	
(Pass 1, Chang Medium, GasPak)	.394
(Pass 2, DCMCH Medium, Air)	.106
PYRL Celery Control	1.251
Leafhopper Inoculum	
BLTVA (Pass 2, Chang Medium)	.069
BLTVA Celery Control	.071

MONOCLONAL Ab

Celery Inoculum	
SAY (Pass 2, Chang Medium)	.065
PYLR	
(Pass 1, Chang Medium, GasPak)	.344
(Pass 2, DCMCH Medium, Air)	.113
PYRL Celery Control	1.314
Leafhopper Inoculum	
BLTVA (Pass 2, Chang Medium)	.068
BLTVA Celery Control	.057

Table 8. Detection/Growth of PYLR, Extracted From Leafhoppers, In Anaerobic Hood, as Determined by Reaction with Polyclonal and Monoclonal Antibody/ELISA (Expt. 16)

	Day 0	Day 1	Day 4	Day 6
Chang Medium				
PYRL (10^{-1})	.050	.045	.039	
Healthy LH Control	.034	.038	.036	
Medium Control (Anaer.)				.026
M1D Medium				
PYRL (10^{-1})	.036	.034	.033	
Healthy LH Control	.023	.028	.025	
Medium Control (Air)				.016
Medium Control (Anaer.)				.021
CBY Medium				
PYRL (10^{-1})	.041	.034	.032	
Healthy LH Control	ND	ND	.013	
Medium Control (Air)				.017
Medium Control (Anaer.)				.021
P1 Medium				
PYRL (10^{-1})	.028	.025	.025	
Healthy LH Control	.024	.026	.024	
Medium Control (Air)				.016
Medium Control (Anaer.)				.017

ND - not determined; LH = leafhopper; Anaer. = anaerobic hood

Table 9. Growth Titers of BLTVA, SAY, and PYLR in Chang and DMCMH Media, as Determined by Reaction with Polyclonal and Monoclonal Antibody/ELISA (Expt. 13 & 14)

	Day 9	Day 15
Healthy Leafhopper Control (ave. of 4)		.024
Media Control (ave. of 4)		.020
BLTVA		
Pass 2, Air, Chang Medium		.026
DMCMH Medium		.017
Pass 2, GasPak, Chang Medium		.024
SAY		
Pass 2, Air, Chang Medium		.024
Pass 2, GasPak, Chang Medium		.029
Anaerobic Hood, Chang Medium	.031	
PYLR		
Pass 2, GasPak, Chang Medium		.086
DMCMH Medium		.108
Anaerobic Hood, Chang Medium	.347	

Table 10. Maintenance of AY-MLO in MIMEK1 cf. PBS, at 4°C, in Air, as Determined by % Disease Transmission of Injected Leafhoppers (Experiments 17 & 18)

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
SAY-MLO						
MIMEK1 Medium	16	42	10			0
PBS	3	0	0			0
WAY-MLO						
MIMEK1 Medium	20	4	0	38	50	
PBS	47	0	0	0		

Table 11. Growth of NJAY-MLO in PL-1, M1D, SerFree, and SerFrac Media, as Determined by Reaction in ELISA with a Mixture of 6 NJAY-MLO-specific Monoclonal Antibodies and Acholeplasma-specific Polyclonal Antibodies (Experiment 25)

	<u>Antibody</u>			
	<u>NJAY</u>	<u>A.ax</u>	<u>A.gr</u>	<u>A.ld</u>
CONTROLS				
PBS Control	.000	.000	.000	.000
SerFree0 Medium Control	.025	.073	.062	.000
SerFrac Medium Control	.000	.005	.004	.284*
Healthy Lettuce Control		.061	.122	.104
NJAY-MLO Control	>2	.062	.051	.103

Also, Healthy Rabbit Serum reacted with NJAY-MLO at 0.084, with Healthy Lettuce at .055.

AIR

SerFree0, Pass 1, 10-1	.330	.167	.022	.063
10-2	.083	.211	.000	.071
10-3	.000	.152	.000	.047
SerFree1, Pass 1, 10-2	.022	.000	.000	.000
SerFree2, Pass 1, 10-2	.000	.000	.000	.000
SerFree4, Pass 1, 10-1	.073	.055	.151	.063
SerFrac, Pass 1, 10-1	.287	.173	.008	.238
10-2	.054	.187	.000	.219
10-3	.057	.148	.000	.414

ANAEROBIC HOOD

SerFree0, Pass 1, 10-2	.087	.047	.450	.309
SerFree1, Pass 1, 10-2	.000	.000	.003	.000
SerFree2, Pass 1, 10-2	.055	.000	.009	.071
SerFree4, Pass 1, 10-2	.000	.051	.186	.155
Pass 2, 10-1	.026	.019	.051	.017
SerFrac, Pass 1, 10-2	.028	.000	.006	.344

A.ax = Acholeplasma axanthum; A.gr = A. granularum;

A.ld = A. laidlawii

Table 12. Growth of NJAY-MLO in PL-1, M1D, SerFree, and SerFrac Media, as Determined by Reaction in ELISA with a Mixture of 6 NJAY-MLO-specific Monoclonal Antibodies and Acholeplasma-specific Polyclonal Antibodies (Experiment 26)

	<u>Antibody</u>			
	<u>NJAY</u>	<u>A.ax</u>	<u>A.gr</u>	<u>A.ld</u>
CONTROLS				
PBS Control	.000	.000	.000	.000
SerFree1 Medium Control	.000	.000	.005	.453*
M1D Medium Control	.000	.000	.002	.256*
PL-1 Medium Control	.000	.000	.000	.143*
Healthy Lettuce Control		.061	.122	.104
NJAY-MLO Control	>2	.062	.051	.103
Also, Healthy Rabbit Serum reacted with NJAY-MLO at 0.084, with Healthy Lettuce at .055.				
AIR				
SerFree1, Pass 1, 10-2	.015	.060	.020	.313
SerFree4, Pass 1, 10-2	.000	.000	.008	.418
M1D, Pass 1, 10-2	.000	.000	.049	.191
Pass 2, 10-1	.002	.000	.000	.164
PL-1, Pass 1, 10-1	.071	.000	.000	.347
10-2	.055	.000	.000	.264
ANAEROBIC HOOD				
SerFree1, Pass 1, 10-2	.050	.000	.000	.350
SerFree4, Pass 1, 10-2	.007	.000	.000	.350
M1D, Pass 1, 10-2	.019	.201	.000	.121
PL-1, Pass 1, 10-1	.038	.094	.007	.268
10-2	.048	.006	.000	.385

Table 13. Growth of NJAY-MLO in PL-1 Medium, as Determined by Reaction in ELISA with a Mixture of 6 NJAY-MLO-specific Monoclonal Antibodies and Acholeplasma-specific Polyclonal Antibodies (Experiments 22 & 23)

	<u>Antibody</u>			
	<u>NJAY</u>	<u>A.ax</u>	<u>A.gr</u>	<u>A.ld</u>
CONTROLS				
PBS Control	.000	.000	.000	.000
PL-1 Medium Control	.000	.000	.000	.143*
Healthy Lettuce Control		.061	.122	.104
NJAY-MLO Control	>2	.062	.051	.103
Also, Healthy Rabbit Serum reacted with NJAY-MLO at 0.084, with Healthy Lettuce at .055.				
AIR				
<u>Experiment 22: From agar ---> liquid medium at pass 2.</u>				
PL-1, Pass 4, 10-2	.018	.001	.264	.489
Pass 5, 10-1	.021	.009	.000	.007
<u>Experiment 23: From agar ---> liquid medium at pass 2.</u>				
PL-1, Pass 3, 10-2	.000	.013	.000	.424
Pass 4, 10-1	.012	.026	.000	.308

Table 14. MLO Cultivation Media			
Component	H-1	DCCM	M1D
<u>Inorganic salts and buffers</u> (mg/liter)			
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	95	66	350
$\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$		0.04	
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$		0.2	
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$		0.55	
Hepes	16,000		
KCl	180	2,450	852
KH_2PO_4	285		240
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$		156	
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	95	838	1,972
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$		0.02	
NaCl	2,350		2,786
NaHCO_3			213
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$			704
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	285	1,370	
$(\text{NH}_4)_6\text{MO}_{24} \cdot 4\text{H}_2\text{O}$		0.04	
$\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$		0.06	

Amino Acids (mg/liter)			
L-Alanine	380		
β -Alanine	95		266
L-Arginine·HCl	1,900	800	213
L-Asparagine	550	1,225	
L-Aspartic acid	950	1,000	213
L-Cysteine·HCl	550		32
L-Cystine·2HCl		109	53
L-Glutamic acid	1,400	1,300	416
L-Glutamine		3,000	960
Glycine	380	400	133
L-Histidine	760	200	213
L-Hydroxyproline	450	800	
L-Isoleucine	450	500	80
L-Leucine	900	400	80
L-Lysine·HCl	380	700	879
L-Methionine	380	1,000	416
L-Phenylalanine	950	1,000	80
L-Proline	950	600	906
L-Serine	380	600	133
L-Threonine	200	200	187
L-Tryptophan	380	100	53
L-Tyrosine	380	250	266
L-Valine	380	500	160

<u>Nucleic acid precursors (mg/liter)</u>			
Adenosine	45		
Cytidine	45		
Guanosine	45		
Inosine	45		
Thymidine	45		
Uridine	45		
2'-Deoxyadenosine	45		
2'-Deoxycytidine	45		
2'-Deoxyguanosine	45		
5-Methyldeoxycytidine	2.0		
Inosine monophosphate	2.0		
Uridine 5'-triphosphate	4.5		
D-(-)-Ribose	9.0		
2'-Deoxy-D-ribose	9.0		

<u>Cofactors (mg/liter)</u>			
Coccarboxylase	4.5		
Coenzyme A	4.5		
Flavin adenine dinucleotide	4.5		
Nicotinamide adenine dinucleotide	4.5		
Nicotinamide adenine dinucleotide phosphate	4.5		
<u>Vitamins and reducing agents (mg/liter)</u>			
Ascorbic acid	30		
d-Biotin	0.45	0.12	
Calcium pantothenate	0.85	0.12	
Choline chloride	0.65	250	
Folic acid	0.45	3.6	
Glutathione	25		
i-Inositol	0.65	10.0	
Niacin	0.45	0.24	
Niacinamide	0.45		
p-Aminobenzoic acid	0.45	0.12	
Pyridoxal·HCl	0.45		
Pyridoxine·HCl	0.85	0.3	
Riboflavin	1.30	0.12	
Thiamine·HCl	0.45	0.12	
Vitamin B ₁₂		1.0	

<u>Carbohydrates, Sugars, Sugar alcohols (mg/liter)</u>			
Fructose	900		333
Glucose	4,800	5,500	1,400
Maltose		1,000	
Mannitol		20,000	
Sodium glucuronate	2.0		
Sorbitol			23,333
Sucrose	1,900		3,333
Trehalose	950		
Xylose	100		
<u>Organic acids (mg/liter)</u>			
Fumaric acid			53
α -Ketoglutaric acid			107
Malic acid			53
Succinic acid			53
<u>Lipids (mg/liter)</u>			
Cholesterol	20		
Oleic acid	10		
Palmitic acid	10		
Glycerol		2,000	
DL- α -Glycerophosphate $\cdot$ 6H ₂ O		1,000	
Sphingomyelin	20		
Tween 40 (ml/liter)	0.1		
Tween 80 (ml/liter)	0.1		

<u>Proteins</u> (mg/liter)			
Bovin serum albumin	11,750		
<u>Undefined components</u> (mg/liter)			
Brain heart infusion			2,000
Fetal bovine serum		9%	17%
Liver digest	750		
Peptone	1,250		2,700
Tryptone			5,000
Yeast autolysate	5,000		2,733
<u>Other components</u> (mg/liter)			
N-acetyl-D-Glucosamine		1,000	
Polyvinylpyrrolidone K90		100	
Penicillin G (10^5 U/ml)	10		
Phenol red (0.2%) (ml/l)	10		
pH	7.35	6.3	7.4
mOsM	440	400	500

Table 15. MLO Cultivation Media.			
Component	PL-1	P-1	H-2
<u>Inorganic salts and buffers (mg/liter)</u>			
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	10	340	480
$\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$	0.02		0.01
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	1.0		0.05
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.8		0.13
H_3BO_4	0.02		
Hepes	15,000	1,600	
KCl	1,000	2,900	2,875
KH_2PO_4	1,500	280	60
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$			40
MgO	100		
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	100	2,000	2,100
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$			0.005
MnSO_4	1.0		
NaCl	20	1,400	670
NaHCO_3	100	220	230
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$		730	675
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$		30	340
Na_2MoO_4	0.02		
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$			0.01
$\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$	0.01		0.015

<u>Amino Acids</u> (mg/liter)			
L-Alanine	250	260	115
β -Alanine		285	170
L-Arginine·HCl	50	190	600
L-Asparagine	500	1,000	480
L-Aspartic acid	250	315	480
L-Cysteine·HCl	50	90	10
L-Cystine·2HCl		55	60
L-Glutamic acid	200	580	430
L-Glutamine	500	3,000	1,290
Glycine	50	180	460
L-Histidine	150	300	1,350
L-Hydroxyproline		45	200
L-Isoleucine	520	125	170
L-Leucine	400	170	160
L-Lysine·HCl	50	940	710
L-Methionine	50	480	380
L-Phenylalanine	330	175	345
L-Proline	110	1,000	550
L-Serine	1,500	180	735
L-Threonine	360	210	185
L-Tryptophan	5	100	90
L-Tyrosine	40	310	155
L-Valine	600	210	215

<u>Nucleic acid precursors</u> (mg/liter)			
DNA, herring sperm	10		
RNA, torula yeast	2.5		
Adenine	25		
Adenosine	50	200	
Adenosine monophosphate	5		
Adenosine triphosphate	2.5	200	
Cytidine	25	50	
Cytidine monophosphate	5		
Cytidine triphosphate	2.5		
Guanine	50		
Guanosine	100	50	
Guanosine monophosphate	5		
Guanosine triphosphate	2.5		
Hypoxanthine	25		
Inosine	50	50	
Inosine monophosphate	5	0.2	
Thymidine	50	50	
Thymidine monophosphate	5		
Thymidine triphosphate	2.5		
Thymine	25		
Uracil	25		
Uridine	50	50	
Uridine monophosphate	5		
Uridine 5'-triphosphate	2.5	5	
Xanthine monophosphate	5		
2'-Deoxyadenosine	50	50	
2'-Deoxyadenosine monophosphate	5		
2'-Deoxycytidine		50	
2'-Deoxycytidine monophosphate	5		

2'-Deoxyguanosine	100	5	
2'-Deoxyguanosine monophosphate	5		
2'-Deoxyuridine monophosphate	5		
5-Methyldeoxycytidine		0.2	
Phosphoribosyl pyrophosphate	50		
D-(-)-Ribose	250	100	
Ribose-1-phosphate	50		
2'-Deoxy-D-ribose	250	100	

<u>Cofactors (mg/liter)</u>			
Coccarboxylase	2.25	5	
Coenzyme A	2.25	5	
Flavin adenine dinucleotide	2.25	5	
Nicotinamide adenine dinucleotide	2.25	5	
Nicotinamide adenine dinucleotide phosphate	2.25	5	
<u>Vitamins, Reducing agents (mg/liter)</u>			
Ascorbic acid	10	100	
p-Aminobenzoic acid	0.05		0.04
d-Biotin	0.5	0.05	0.035
Calcium pantothenate		0.6	0.04
Choline chloride	see Carb.	0.07	see Carb.
Folic acid	0.1	0.05	1.0
Glutathione	25	2.5	
i-Inositol	see Carb.	0.07	see Carb.
Niacin (Nicotinic acid)	0.5	0.5	0.07
Niacinamide	0.2	0.05	
Pyridoxal·HCl		0.05	
Pyridoxal-5-phosphate	0.5		
Pyridoxine·HCl	0.5	0.09	0.09
Riboflavin	0.5	0.13	0.05
Thiamine·HCl	0.4	0.5	0.05
Vitamin B ₁₂	0.1		0.25

<u>Carbohydrates, Sugars, Sugar alcohols, Phosphorylated Sugars, Osmoregulators (mg/liter)</u>			
Choline chloride	50		65
Erythrose	500		
Fructose	1,000	90	285
Fructose-6-phosphate	400		
Glucose	1,000	1,600	2,100
Glucose-6-phosphate	400		
Glycerol-3-phosphate	40		
i-Inositol	50	100	2.5
Maltose			250
Mannitol			5,000
Sodium glucuronate		0.2	
Sorbitol		65,000	5,830
Sucrose	30,000	65,000	14,175
Trehalose		1,200	
Xylose		10	
<u>Organic acids (mg/liter)</u>			
Acetic acid	100		
Fumaric acid		55	40
α -Ketoglutaric acid	250	110	210
Malic acid	150	55	350
Mevalonic acid	100		
Oxalacetic acid	10		
Pyruvic acid	25		
Shikimic acid	50		
Succinic acid		55	45

<u>Lipids</u> (mg/liter)			
Campesterol	0.8	50	
Cholesterol	7.7	2.0	
Sitosterol	0.8	50	
Stigmasterol	0.8	50	
Linoleic acid	0.8		
Oleic acid	3.8	50	
Palmitic acid	3.8	50	
Glycerol	100		500
DL- α - Glycerophosphate $\cdot 6H_2O$	0.8	50	250
Phosphatidic acid	0.8		
Phosphatidylcholine	1.5		
Phosphatidylcholine, di-oleoyl	0.8		
Phosphatidylcholine, di-palmitoyl	0.8		
Phosphatidylethanolamine	0.8		
Phosphorylcholine $\cdot HCl$		50	
Phosphorylethanolamine	0.8	50	
Sphingomyelin	1.5	2.0	
Tween 40 (ml/liter)	0.08	0.1	
Tween 80 (ml/liter)	0.3	0.1	

<u>Polyamines</u> (mg/liter)			
Putrescine	0.5		
Spermine	12.5		
Spermidine	0.5		
<u>Proteins</u> (mg/liter)			
Bovin serum albumin	11,600	6,500	
<u>Undefined components</u> (mg/liter)			
Brain extract (ml/l)		50	
Brain heart infusion			500
Fetal bovine serum (ml/l)			110
Lactalbumin hydrolysate			1,500
Liver digest		75	
Peptone		2,500	675
Phytone	5,000		
Tryptone		2,500	1,250
Yeast autolysate		1,600	2,200
Yeast extract (Kuske) (ml/l)		30	
<u>Other components</u> (mg/liter)			
N-acetyl-D-Glucosamine			250
Polyvinylpyrrolidone K90			25
Penicillin G (10^5 U/ml)		1.0	
Phenol red (0.2%) (ml/l)		1.0	
pH	7.0	7.5	
mOsm	440	N.D.	

Table 16. MLO Cultivation Media.			
Component	Schneiders Drosophila Medium	CMRL Media-1066	Media 199
<u>Inorganic salts and buffers (mg/liter)</u>			
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	600 Anh	200 Anh	200 Anh
$\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$			
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$			
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$			
$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$			0.72
H_3BO_4			
Hepes			
KCl	1,600	400	400
KH_2PO_4			
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$			
MgO			
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	3,700	200	200
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$			
MnSO_4			
NaCl	2,100	6,800	6,800
NaHCO_3	400	2,200	2,200
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	1,321		
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$		140	140
Na_2MoO_4			
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$			
$\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$			

<u>Amino Acids</u> (mg/liter)			
L-Alanine		25	50
β -Alanine	500		
L-Arginine·HCl	400	70	70
L-Asparagine			
L-Aspartic acid	400	30	60
L-Cysteine·HCl	60	260	0.11
L-Cystine·2HCl	100	20	20
L-Glutamic acid	800	75	150
L-Glutamine	1,800	100	100
Glycine	250	50	50
L-Histidine	400	20	22
L-Hydroxyproline		10	10
L-Isoleucine	150	20	40
L-Leucine	150	60	120
L-Lysine·HCl	1,650	70	70
L-Methionine	800	15	30
L-Phenylalanine	150	25	50
L-Proline	1,700	40	40
L-Serine	250	25	50
L-Threonine	350	30	60
L-Tryptophan	100	10	20
L-Tyrosine	500	40	40
L-Valine	300	25	50

<u>Nucleic acid precursors (mg/liter)</u>			
DNA, herring sperm			
RNA, torula yeast			
Adenine			10
Adenosine			
Adenosine monophosphate			0.2
Adenosine triphosphate			1.0
Cytidine			
Cytidine monophosphate			
Cytidine triphosphate			
Guanine			0.3
Guanosine			
Guanosine monophosphate			
Guanosine triphosphate			
Hypoxanthine			0.3
Inosine			
Inosine monophosphate			
Thymidine		10	
Thymidine monophosphate			
Thymidine triphosphate			
Thymine			0.3
Uracil			0.3
Uridine			
Uridine monophosphate			
Uridine 5'-triphosphate		1.0	
Xanthine			0.3
Xanthine monophosphate			
2'-Deoxyadenosine		10	
2'-Deoxyadenosine monophosphate			
2'-Deoxycytidine		10	

2'-Deoxycytidine monophosphate			
2'-Deoxyguanosine		10	
2'-Deoxyguanosine monophosphate			
2'-Deoxyuridine monophosphate			
5-Methyldeoxycytidine		0.1	
Phosphoribosyl pyrophosphate			
D-(-)-Ribose			0.5
Ribose-1-phosphate			
2'-Deoxy-D-ribose			0.5

<u>Cofactors (mg/liter)</u>			
Coccarboxylase		1.0	
Coenzyme A		2.5	
Flavin adenine dinucleotide		1.0	
Nicotinamide adenine dinucleotide		7.0	
Nicotinamide adenine dinucleotide phosphate		1.0	
<u>Vitamins, Reducing agents (mg/liter)</u>			
Ascorbic acid			0.5
p-Aminobenzoic acid			0.05
d-Biotin			0.01
Calciferol			0.1
Calcium pantothenate			0.01
Choline chloride			0.5
Folic acid			0.01
Glutathione (reduced)		10	0.05
i-Inositol			0.05
Menadione			0.01
Niacin (Nicotinic acid)			0.025
Niacinamide			0.025
Pyridoxal·HCl			0.025
Pyridoxal-5-phosphate			
Pyridoxine·HCl			0.025
Riboflavin			0.01
Thiamine·HCl			0.01
α-Tocopherol phosphate			0.01
Vitamin A			0.14
Vitamin B ₁₂			

<u>Carbohydrates, Sugars, Sugar alcohols, Phosphorylated Sugars, Osmoregulators (mg/liter)</u>			
Choline chloride			
Erythrose			
Fructose			
Fructose-6-phosphate			
Glucose	2,000	1,000	1,000
Glucose-6-phosphate			
Glycerol-3-phosphate			
i-Inositol			
Maltose			
Mannitol			
Sodium glucuronate		4.2	
Sorbitol			
Sucrose			
Trehalose	2,000		
Xylose			
<u>Organic acids (mg/liter)</u>			
Acetic acid		83	50
Fumaric acid	100		
α -Ketoglutaric acid	200		
Malic acid	100		
Mevalonic acid			
Oxalacetic acid			
Pyruvic acid			
Shikimic acid			
Succinic acid	100		

<u>Lipids</u> (mg/liter)			
Campesterol			
Cholesterol			0.2
Sitosterol			
Stigmasterol			
Linoleic acid			
Oleic acid			
Palmitic acid			
Glycerol			
DL- α - Glycerophosphate $\cdot 6H_2O$			
Phosphatidic acid			
Phosphatidylcholine			
Phosphatidylcholine, di-oleoyl			
Phosphatidylcholine, di-palmitoyl			
Phosphatidylethanolamine			
Phosphorylcholine $\cdot HCl$			
Phosphorylethanolamine			
Sphingomyelin			
Tween 40 (ml/liter)			
Tween 80		5	20

<u>Polyamines</u> (mg/liter)			
Putrescine			
Spermine			
Spermidine			
<u>Proteins</u> (mg/liter)			
Bovin serum albumin			
<u>Undefined components</u> (mg/liter)			
Brain extract (ml/l)			
Brain heart infusion			
Fetal bovine serum (ml/l)			
Lactalbumin hydrolysate			
Liver digest			
Peptone			
Phytone			
Tryptone			
Yeast autolysate	2,000		
Yeast extract (Kuske) (ml/l)			
<u>Other components</u> (mg/liter)			
N-acetyl-D-Glucosamine			
Polyvinylpyrrolidone K90			
Penicillin G (10^5 U/ml)			
Phenol red (0.2%) (ml/l)		20	20
pH			
mOSM			

Table 17. Complex media used in MLO cultivation trials.

AC20M

200 ml Schneider's Drosophila Medium
20 ml Medium 199
10 ml CMRL 1066
60 ml Fetal bovine serum (Hyclone)
180 ml Histidine buffer (pH 6.2; = 2.15 g histidine)
pH = 6.35

BEM1 (from B. Kirkpatrick)

BEM2 (from B. Kirkpatrick)

45 mM Glycine
45 mM HEPES (pH 7.8, 0.5 M)
4.5 mM MgCl₂
9% Sucrose
1% Bovine serum albumin
0.03 M Ascorbic acid
pH 7.3 (adjusted with KOH)

Blood - Blood agar plates.

C-3G (Modified)

17 g Mycoplasma Broth Base (BBL)
91 g Sucrose
10 % Fetal bovine serum (Hyclone)
11 ml Penicillin-G (705 mg)
6 ml Phenol red (0.5%, w/v)

CBY

92 ml Chang Medium
5 ml Pig brain extract (Kirkpatrick)
3 ml Yeast extract (Kuske)

Chang - This medium is proprietary until published.

DCCM - see Table 1.

DMCMH Medium

2 parts DCCM Medium
1 part M1D Medium
2 parts Chang Medium
1 part H-2 Medium
2 parts H-1 Medium

Gly-Mg

0.3 M Glycine
0.03 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$
pH 7.3
mOsmol 915 (adjusted with Sucrose)

Luria - Luria plates.

M1D - see Table 1.

M1D-SO₃ - M1D & 0.01 M Na_2SO_3

MEMEK1 Medium

50 ml DCCM Medium
1 mM EDTA, disodium
30 mM MOPS
4 mM L-Cysteine
0.3% Polyvinylpyrrolidone
0.5 ml Spermine
0.05 Glycine
0.01 MgCl_2
0.5% Bovine serum albumin
10 mM L-Glutamine
7 g Sucrose
11 ml 1N KOH
50 ml Distilled water
pH 7.1
mOsmol 915

Milk - Milk agar plates.

MIMEK1 Medium

50 ml DCCM Medium
0.5 ml Spermine
5 mM L-Glutamine
5 mM L-Arginine
0.2% Bovine serum albumin (Hyclone)
2.5 mM MgCl_2
6.8 g Sucrose
0.3 ml 2N KOH
4 ml Distilled water
pH 7.2
mOsmol 935

MIMEK1P Medium - MIMEK1 & Penicillin-G

P1 - see Table 2.

Percoll - Chen's isolation media with 40-100% Percoll.

PL-1 - see Table 2.

PL-2 - 2x PL-1

PL-2A - PL-2 plates.

R-1 Medium - This Pasteuria medium is proprietary until published.

SerFree (Serum Free Media)

Serum Free Base:

865 ml Distilled water
20 g Mycoplasma Broth Base (BBL)
100 ml Yeast extract (25%)
5 ml L-Arginine·HCl (42%)
10 ml Glucose (50%)
600 mg Penicillin-G (= 1000 U/ml final)
120 mg Polymyxin B (= 1000 U/ml final)
20 ml Phenol red (0.1%)
pH 7.4
mOsmol 330

SerFree0:

1000 ml Serum Free Base

SerFree1:

1000 ml Serum Free Base
0.1 ml Tween 80

SerFree2:

1000 ml Serum Free Base
0.2 ml Tween 80

SerFree4:

1000 ml Serum Free Base
0.4 ml Tween 80

SerFrac (Serum Fraction Medium)

900 ml Distilled water
21 g Mycoplasma Broth Base (BBL)
100 ml Yeast extract (25%)
10 ml Serum Fraction (Difco)
5 ml L-Arginine·HCl (42%)
10 ml Glucose (50%)
600 mg Penicillin-G (= 1000 U/ml final)
120 mg Polymyxin B
20 ml Phenol red (0.1%)
pH 7.4
mOsmol 320

Tex2M = Excel400 - D. Lynn medium for Tex2 cell line (proprietary).

Objectives Studied in the Jerusalem Laboratory

Several objectives were studied mainly in the Laboratory of Ultrastructure and Membrane Research at the Hasassah Medical School of Hebrew University. These objectives were:

OBJECTIVE 4. Study ATP and phosphorylated sugar accumulation during energy metabolism in spiroplasmas and MLOs.

OBJECTIVE 5. Study cell volume regulation in spiroplasmas and MLOs including the role of ATPase.

OBJECTIVE 6. Determine if protein kinase is associated with intracellular fibril organization in spiroplasmas and MLOs.

Work on these objectives is described in the following section.

EXPERIMENTAL PROCEDURES

Organisms and the Preparation of Cell Fractions. *Spiroplasma citri* (Maroc R8A2), *S. melliferum* (BC3), *S. floricola* (BNR1), *Spiroplasma* sp. MQ1 (Serogroup VII), *S. apis* (B32) and *Spiroplasma* sp. PPSI were grown for 24-72 h at 32°C in a modified Saglio medium (Saglio et al., 1971), supplemented with 4-10% horse serum. For *in vivo* protein phosphorylation, 1 μ Ci/ml of [32 P] Na₂HPO₄ (500 Ci/nmol) was added to the growth medium. For labelling of membrane lipids, 0.004-2 μ Ci of either [3 H]- palmitate or [3 H] oleate (14-60 Ci/nmol) were added to the growth medium. The growth medium was inoculated with a 2-5% frozen inoculum and incubated vertically at 32°C for 18-36 h. Growth was monitored by measuring the A₆₄₀ and pH changes of the growth medium. Cells were harvested by centrifugation at 12,000 xg for 10 min, washed, and suspended in isoosmotic solutions of either sodium chloride (0.25 M), or choline chloride (0.25 M). For swelling measurements, cells were harvested at the late exponential or stationary phase of growth. The viability of the cell suspensions was measured by the colony-counting technique.

For the preparation of cell fractions, cells were osmotically lysed after preloading with glycerol, as previously described (Razin and Rottem, 1976). Briefly, the washed cells (5-10 mg cell protein per ml) were incubated in 2 M glycerol for 10 min at 37°C and injected into a 50-fold volume of a solution containing 1 mM dithiothreitol (DTT), 10 μ g/ml RNase I and 1 mM phenylmethylsulfonyl fluoride (PMSF) in a 5 mM Tris-Hepes buffer, pH 8.0 and incubated for 15 min at 37°C. Unbroken cells were removed by centrifugation at 4,000 xg for 15 min and membranes were separated from the soluble fraction by centrifugation at 37,000 xg for 45 min. The membranes were washed twice and resuspended in 25 mM Tris-Hepes buffer, pH 7.5 containing 5 mM DTT and 5 mM PMSF. The soluble fraction was concentrated to 20 mg protein/ml by freeze drying and centrifuged at 100,000 xg for 2 h to remove residual membrane fragments and ribosomes. The membrane and soluble fractions were stored at -70°C until used.

Triton insoluble fraction was obtained by extracting washed intact cells or total cell lysates with Triton X-100. In brief, washed cells containing 20-30 mg cell protein were added to 50 ml of a 4 M glycerol solution in 10 mM Tris-HCl buffer (pH = 7.5) at room temperature. Triton X-100 was then added to 1% (wt/vol) and the mixture was stored at room temperature for 2 h. The Triton X-100 insoluble material was collected by centrifugation at 80,000 xg for 3 h at 15°C, washed twice in 1% Triton X-100 in 10 mM Tris-HCl pH 7.5 buffer, resuspended in the Tris-HCl buffer and kept at -20°C until used.

Measurement of Intracellular Water Volume. To determine intracellular volume, cells were incubated in 2.4 ml of a solution containing 250 mM NaCl, 25 mM Tris-MOPS (morpholinopropanesulfonic acid) buffer (adjusted to the appropriate pH), ³H₂O (4.3 μ Ci/ml), [14 C] polyethylene glycol (PEG; 0.1 μ Ci/ml) and a 10-fold excess of unlabelled PEG. In most experiments, 10 mM glucose was added. After incubation at 37°C for 15 min, 1-ml samples, in

duplicate, were layered onto the surface of silicone oil (0.3 ml) in 1.5-ml plastic microfuge tubes and centrifuged at 12,800 $\times g$ for 2 min. Under these conditions, the cells pass through the silicone oil, forming a pellet at the bottom of the tube, with the aqueous phase remaining above the silicone oil (0.3 ml). After samples of the aqueous phase were withdrawn for counting of radioactivity, the aqueous and silicone phases were removed by suction, the tips of the centrifuge tubes (containing the cell pellets) were cut off and the radioactivity was counted. The 3H counts were taken as a measure of total pellet water, and [^{14}C] PEG readings were taken as a measure of extracellular space. In most experiments, the PEG space was approximately 20% of the total pellet water. The intracellular water volume was calculated by subtracting the PEG space value from the total water space value.

Measurements of pH and . For the measurement of pH, the distribution of either ^{14}C -labelled benzoic acid (0.04 $\mu Ci/ml$) or ^{14}C -labelled methylamine was determined. For the measurement of membrane potential, the distribution of 3H -labelled tetraphenylphosphonium ([3H]TPP; 0.03 $\mu Ci/ml$) was determined (Rottem et al., 1981). In brief, cells (1.5 mg of cell protein per ml) were incubated in a solution containing 250 mM NaCl, 10 mM glucose, and 25 mM Tris-MOPS for 10 min at 37°C. Samples (1 ml) were then layered onto the surface of silicone oil in the 1.5-ml microfuge tubes and treated as described above. Intracellular and extracellular concentrations of labelled benzoic acid, methylamine, or TPP were determined, and the pH and ψ were calculated (Rottem et al., 1981).

Acridine Orange Fluorescence Measurements. Primary sodium-pumping activity was assayed by monitoring the membrane potential-driven uptake of protons in cells in the presence of an uncoupler. The formation and maintenance of pH due to the uptake of protons was estimated by determining the fluorescence quenching of the dye acridine orange (Dimroth, 1987). Fluorescence studies were done in a Perkin-Elmer spectrofluorometer with excitation of 492 nm and emission at 530 nm. Cells (final concentration, 30 μg of protein per ml) suspended in 2.5 ml of buffer (reaction medium, consisting of 250 mM NaCl, 2.5 mM $MgCl_2$, 10 mM Tris hydrochloride [pH 8.5], and 5 μM acridine orange) were made permeable to protons by the addition of 0.4 μM SF6847 (provided by Y. Shahak, Weitzmann Institute). Fluorescence quenching was initiated by the addition of an energy source (10 mM glucose) and followed at 37°C.

$^{22}Na^+$ Efflux. $^{22}Na^+$ efflux measurements were performed with preloaded *S. floricola* cells and efflux was initiated by the addition of glucose. Cells (6 mg/ml) were loaded with $^{22}Na^+$ by incubation for 1 h at 32°C in 0.4 M sorbitol solution containing 25 mM NaCl, 40 mM $MgCl_2$, 40 mM Tris-MES buffer (pH 7.5), and 3.0 μCi of $^{22}Na^+$ per ml. The loaded cells were diluted 1:200 into a reaction mixture containing 110 ml of either 0.25 M choline chloride, 0.25 M NaCl, or 0.5 M sorbitol solution in the presence and absence of 10 mM glucose. The reaction mixtures were incubated at 32°C, and at the indicated time intervals, 10-ml portions were withdrawn and filtered through fiberglass filters (25 mm GF/C, Whatman, Inc.) under negative pressure (filtration time, 3 sec).

The filters were washed twice with a 10-ml volume of cold 0.25 M NaCl and the radioactivity retained on the filters was determined. Over 95% of the cells were retained on the filters, as judged from measuring the retention of [^3H] palmitate-labelled cells.

Immunoblotting Assay. Membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were electroblotted onto nitrocellulose paper in a solution containing 20 mM Tris hydrochloride, 100 mM glycine, and 12% (vol/vol) methanol at 700 mA at 12 °C. The nitrocellulose paper was washed several times in PBS solution (136 mM NaCl, 2.6 mM KCl, 16.2 mM Na_2HPO_4) containing 0.2% (vol/vol) Tween 20. It was then incubated for 90 min at room temperature with rabbit monospecific polyclonal antibodies directed against either the β -subunit of the *E. coli* F_1F_0 -ATPase (Rott and Nelson, 1981), or by the yeast plasma membrane ATPase (both kindly provided by N. Nelson, Roche Institute of Molecular Biology). This incubation was followed by a further incubation with ^{125}I -labelled protein A. The papers were washed again as above, and the immune complexes were detected by autoradiography.

Protein Phosphorylation. *In vitro* phosphorylation was assayed in a reaction mixture (final volume of 50 μl) containing 20 mM Tris-maleate buffer (pH = 6.0), 1 mM DTT, 50 mM NaF, 2 mM CaCl_2 , 10-40 μg of spiroplasma cell protein and 1 mM γ - ^{32}P -ATP (5-20 Ci/mol, The Radiochemical Center, Amersham, UK). In some experiments, the reaction mixture was supplemented with the soluble fraction derived from *A. axanthum* cells (25 μg protein). The reactions were carried out for 30-120 sec at 37°C and were terminated by the addition of 50 μl of a denaturing SDS solution (6% SDS in 200 mM Tris-HCl buffer pH = 6.8, 1% 2-mercaptoethanol, 4% glycerol and 0.005% bromophenol blue) and boiling for 3 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), using 10% acrylamide gels for 2 h at 60 mA at 4°C. The gels were stained with Coomassie Blue R-250 in methanol:acetic acid:water (12.5:2.5:10 by vol) at 50°C and destained for 5 h with frequent changes of the same solvent. The gels were then dried and exposed to X-Omat (Kodak) at -70°C for autoradiography. Radioactivity in the polypeptide bands was quantified by excising the appropriate Coomassie Blue stained bands and counting them in a scintillation fluid. Treatment of the gels with NaOH, HCl, trichloroacetic acid, ribonuclease or pronase was performed as described before (Platt et al., 1988). In certain experiments, the phosphorylated proteins were separated from excess radioactive ATP and P_i by descending paper chromatography on DEAE-cellulose (Whatman) using 0.22 M ammonium formate (Platt et al., 1988). Protein spots at the application points were detected by ninhydrin spray reagent (Sigma), ATP was detected by uv light and P_i by phosphorus spray reagents.

Protein Acylation. For determining the extent of acylation among spiroplasmal membrane protein, membranes were isolated from cells grown in the presence of either [^3H] palmitate or [^3H] oleate. To remove non-covalently bound lipids, membranes were extracted 4 times, each with 1.5 ml chloroform/methanol (2:1, vol/vol). The residual protein pellets were solubilized in SDS and analyzed on

12.5% polyacrylamide slab gels by the method of Laemmli (1970). Fluorography was performed after treating the gels with Amplify (Amersham, UK).

Preparation of sealed membrane vesicles. Sealed membrane vesicles were prepared by fusing spiroplasma membranes with preformed lipid vesicles. The fusion was induced by the freezing and thawing procedure. The lipid vesicles were prepared by extensively mixing 50 mg asolectin and the desired amount of cholesterol (i.e., 20 mg. cholesterol for 40 mol %), dried from chloroform solutions in 1 ml of 10 mM potassium phosphate buffer, pH 7.4, on a Vortex mixer. The milky solution was clarified by sonication in a Branson B12 bath type sonicator (USA). The resultant lipid vesicle preparation was mixed with M. gallisepticum membranes at the desired ratio of asolectin to membrane protein. Fusion was effected by freezing the mixture with gentle shaking in liquid nitrogen. The frozen mixture was thawed at room temperature and sonicated for 30 sec. Any additions to be trapped in the fused preparations were added before the freezing step.

Solute retention by fused membrane-lipid vesicles was determined in vesicles prepared in the presence of radio-labelled solutes carrier-free [^{14}C]-labelled glucose, galactose or malonate were present at 20,000 to 50,000 cpm/ml). The vesicles were separated from the untrapped solute by passing 100- μl samples through Sephadex G-50 (Sweden) in 1-ml tuberculin syringes. The vesicles were incubated for 1-2 h at 37°C. The retention was calculated from the residual radioactivity after passing the vesicles through a second Sephadex column. For sucrose density gradient analysis of membrane vesicles, samples (0.1 ml) of isolated membranes, lipid vesicles or isolated membranes plus lipid vesicles were layered over a 1-ml linear sucrose gradient (5-40%). The membranes were labelled with [^3H] palmitate, and [^{14}C] malonate was added to the samples to measure trapped volume. All samples were frozen and thawed and filtered through G-50 Sephadex columns before the sucrose gradient analysis. The gradients were centrifuged for 2 h at 100,000 $\times g$ (Beckman SW 41 rotor, USA) and fractions (0.25-0.5 ml) were collected from the bottom of the tube and assayed for radioactivity. For fluorescence dequencing analysis, Spiroplasma membranes were labelled with octadecyl-rhodamine-B-chloride (R18), as described before (Citovsky et al., 1985). Briefly, 5 μl of 6 mg/ml ethanolic solution of R 18 were rapidly injected into 400 μl of isolated membranes (2 mg protein/ml in NT buffer). The mixture was incubated for 15 min at room temperature in the dark and centrifuged in an Eppendorf (FRG) microfuge for 2 min, and the pellet was washed twice and resuspended in the NT buffer to give a final protein concentration of 2 mg/ml. The labelled membranes were incubated with lipid vesicles, and the degree of fluorescence was determined (excitation at 560 nm emission at 590 nm). Fluorescence in the presence of 0.1% Triton X-100 was taken to represent 100% dequenching. Fluorescence measurements were carried out with a Perkin-Elmer MPF-44A (USA) spectrofluorometer, with 520-nm-high pass filter and narrow excitation slits to reduce light scattering.

Arginine Transport Assay. Spiroplasma membrane vesicles obtained by the fusion procedure were diluted by a factor of 30-40 at pH 6.0

with 50 mM K_2HPO_4 and 500 μ M ornithine. After 2 h of incubation at 32°C, the membrane vesicles were washed and rapidly diluted into 50 mM K_2HPO_4 pH 6.0 containing radioactive arginine. At indicated time intervals, samples were filtered (0.45 μ m glass fiber (GF/C) filters). The filters were washed twice with 0.1 M LiCl and the radioactivity retained on the filters was measured.

Materials. [7- 14 C] benzoic acid and 125 I-labelled protein A were purchased from New England Nuclear Corp. (Boston, MA, USA). [14 C] PEG 4000, 3H_2O , $^{22}NaCl$, [9, 10-($\underline{m}$)- 3H] palmitate, and [γ - ^{32}P] ATP tris salt) were the products of The Radiochemical Centre (Amersham, UK). [3H] TPP (bromine salt) was purchased from the Nuclear Research Center-Negev (Beersheba, Israel). Glycylglycine chloride, Tris, MES, MOPS, valinomycin, carbonyl cyanide $\underline{m}$ -Chlorophenyl-hydrazone (CCCP), Na_2 -ATP (vanadate free), Tris-ATP (vanadate free), sodium azide, oligomycin, sodium orthovanadate, dicyclohexylcarbodiimide (DCCD), $\underline{N}$ -ethylmaleimide (NEM), DNase I, phenylmethylsulfonyl fluoride, DTT, bovine serum albumin (fraction V), potassium cholate, and acridine orange were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Horse serum was obtained from Bio-Lab (Jerusalem, Israel). Silicone oil (550:556 grade [1213] [vol/vol] was obtained from Dow Corning Corp. (Midland, MI, USA). Diethylstilbestrol (DES) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Zwittergent 3-12 was obtained from Calbiochem.

Analytical Methods. Protein content was measured by the method of Bradford (1976). ATPase activity in membrane preparations was determined as previously described (Razin and Rottem, 1976). Phosphoamino acids were identified as described by Wang and Koshland (1978). In brief, the phosphorylated protein band was excised from the gel and hydrolyzed by heating at 110°C for 2 h in 6 M HCl. The acid hydrolysate was dried, redissolved in deionized water and analyzed by cellulose thin layer electrophoresis for 60 min at 1 kV in acetic acid/pyridine/ H_2O (50:5:945 by vol). The ^{32}P -amino acid was detected by autoradiography and identified by comparing its migration characteristics to those of standard phosphoamine acids that were identified by the ninhydrin spray reagent (Sigma).

RESULTS

PROTEIN PHOSPHORYLATION AND PROTEIN KINASE ACTIVITY IN SPIROPLASMAS.

Cell lysates were prepared from exponential phase cells of Spiroplasma species and subjected to the in vitro phosphorylation reaction using (γ - 32 P)-ATP as a phosphate donor. Fig. 1 shows typical gels of several Spiroplasma species. As is apparent, the

Fig. 1. A phosphorylated protein in Spiroplasma species.

Capital letters - Coomassie Blue stained gels; small letters, autoradiogram of the gels after 24 h exposure. (A,a) - A. laidlawii; (B,b) - M. capricolum; (C,c) - S. citri; (D,d) - S. melliferum (BC3); (E,e) - S. floricola (BNRI); (F,f) - Spiroplasma sp. strain (PPSI).

cell lysates of all the Spiroplasma species actively catalysed phosphorylation of a major endogenous protein that was readily detected after short exposure periods (12 h). Similar phosphorylation was observed in cell lysates of Spiroplasma sp. strain MQ1 and S. apis (B32) (data not shown). The apparent molecular weight of the phosphorylated bands were 57 kDa. No radiolabelled protein bands were detected with cell lysates of A. laidlawii or M. capricolum. Long autoradiographic exposure of the

phosphorylated cell lysate of S. melliferum (BC3) allowed the detection of at least five additional phosphorylated protein bands with molecular weights ranging from 14 to 72 kDa (data not shown). Densitometry analysis of the autoradiograms revealed that the 57 kDa major phosphorylated protein band accounts for $75 \pm 4\%$ of the total radioactivity. Among the minor phosphorylated bands, about 18% of the total radioactivity incorporated was associated with a 51 kDa protein band. Trypsin digestion of the 57 kDa protein of S. melliferum, S. floricola, S. citri, and S. apis generated a very similar peptide pattern (data not shown) and monoclonal antibodies raised against the S. floricola phosphorylated protein cross reacted with the soluble protein fractions of all the various Spiroplasma species tested (Fig. 2).

Fig. 2. Immunostaining of the soluble fraction of Mycoplasma, Acholeplasma, and Spiroplasma species with MABS raised against the 57 kDa phosphorylated protein of S. floricola.

The endogenous 57 ka protein kinase substrate was not phosphorylated by exogenous kinases from eukaryotic origins, such as the cAMP-dependent protein kinase, smooth muscle myosin kinase, light chain kinase, casein kinase I, casein kinase II, or protein kinase C, but was efficiently phosphorylated by a protein kinase present in the soluble fraction of A. axanthum (data not shown).

No phosphorylation was obtained when washed isolated membranes were

utilized as the sole cellular protein in the reaction mixture (Fig. 3, lane A2). Nevertheless, when the soluble fraction of A. axanthum was added to a reaction mixture containing either native or heat denatured washed S. melliferum membranes, a 57 kDa phosphorylated protein band was apparent (Fig. 3, lane A4). Comparing the labelling intensity of the phosphorylated band obtained from the heat denatured membrane preparation (Fig. 3, lane A4) with that obtained from heat denatured soluble preparation (Fig 3, lane B4) revealed that about 25% of the 57 kDa protein substrate was associated with the membrane fraction. About 80% of the radioactivity was released from the phosphorylated native membranes upon a single washing in 25 mM Tris-Hepes buffer, pH = 7.5, whereas only 7% of the membrane-bound ATPase activity was released by the same treatment.

Fig. 3. Phosphorylation of the 57 kDa protein in the soluble and membrane fraction of S. melliferum.

A) Phosphorylation was carried out with isolated membranes. B) Phosphorylation was carried out with the soluble fraction. (1) Coomassie-Blue stained gel prepared from the complete reaction mixture. (2) Autoradiogram of (1) after 24 h exposure. (3) Autoradiogram of a gel prepared from a reaction mixture containing heat denatured membrane or soluble protein (80°C, 10 min. (4) Autoradiogram of a gel prepared from a reaction mixture described in (3), supplemented with 25 µg of native soluble fraction of A. axanthum.

As the bacterial protein kinases described so far do not phosphorylate the protein substrates utilized by eukaryotic protein kinases to a significant extent (Cozzzone, 1986), it was of interest to determine the ability of the protein kinase of S. melliferum to

phosphorylate common phosphate acceptor proteins. Our results show that the protein kinase of S. melliferum did not significantly phosphorylate histone, smooth muscle myosin, or kemptide. However, ^{32}P was incorporated to a large extent into phosvitin (Fig. 4) and, to a much lesser extent, into casein (data not shown). Similar results were obtained when phosphorylation was assessed following chromatography on DEAE-cellulose paper.

Fig. 4. In vitro phosphorylation of phosvitin and of a 57 kDa spiroplasma protein by A. axanthum.

Reaction mixture without an exogenous substrate. Coomassie-Blue stained gel (1), and an autoradiogram of (1) after 7 days of exposure (2). (B) Reaction mixtures with phosvitin (100 µg). Autoradiograms of gels after 12 h exposure obtained from a reaction mixture containing native (1) or heat-inactivated (80°C, 10 min) A. axanthum soluble fraction (2). (C) Autoradiogram (24 h exposure) of a reaction mixture with heat inactivated S. melliferum cell extract.

When the phosvitin and casein bands were cut out from the gels and counted, the radioactivity in the casein band was only 10 - 15% of that in the phosvitin bands. The phosvitin preparation, when run on SDS-PAGE, and stained with Stains-All, contained a cluster of phosphoproteins dominated by 28 kDa and 43 kDa BANDS (Wallace and Morgan, 1986). All three major phosphoproteins were phosphorylated by the S. melliferum protein kinase. Phosphorylation of the exogenous protein substrates was not affected by cyclic AMP (1 - 10 mM), spermine (1 mM), protamine (1 mM), or calmodulin (6 µM). Heparin (100 ng), however, inhibited the phosphorylation of phosvitin and casein by 90 - 95%.

The phosphorylation of the 57 kDa protein of S. melliferum was linear, under conditions employed (50 mM NaF), for about 2 min. Longer incubation periods resulted in the decrease in the phosphorylation level depending on the concentration of NaF in the reaction mixture. Decreasing the NaF from 50 to 60 mM resulted in a 50% decrease in the level of phosphorylation within 8 min and in a reaction mixture without NaF, phosphorylation could not be detected (Fig. 5). The phosphorylation of the 57 kDa protein was pH-dependent with highest levels of phosphorylation obtained in the pH range of 5.5 - 6.0. Little or no phosphorylation was observed in the more alkaline pH range. As the phosphorylation reactions were carried out in a crude cell extract containing kinase, the substrate, and, probably, a phosphoprotein phosphatase, it is impossible to determine the extent of phosphorylation on a molar basis.

^{32}P -incorporation into the 57 kDa S. melliferum protein was found with γ - ^{32}P ATP, but not with α - ^{32}P ATP. During the course of preparing the soluble fraction, we discovered that the kinase activity was considerably more stable in extracts where DTT was present at 1 mM and, therefore, we included DTT in all buffers. Table 1 shows that the kinase activity was very sensitive to inhibition by sulphhydryl blocking reagents, suggesting that the enzyme requires cysteine-SH groups for its activity and/or stability. The kinase activity was not stimulated by phosphoenolpyruvate nor glycolytic intermediates, and neither was it affected by cyclic AMP, nor by calmodulin with or without Ca^{2+} (Table 1). The phosphoryl

Fig. 5. Effect of NaF on the phosphorylation of the 57 kDa protein. Phosphorylation in vitro was determined following SDS-PAGE and autoradiography. (O), No NaF; (□), 10 mM NaF; (Δ) 50 mM NaF.

moiety of the 57 kDa S. melliferum protein was completely released by pronase treatment (50 µg/ml for 1 h at 37°C), or by incubating the phosphorylated gels in 1 M NaOH at 60°C for 15 min. The phosphoryl moiety was unaffected by ribonuclease treatment (25 µg/ml for up to 2 h at 37°C), was stable during staining and destaining of the gels at 50°C and only slightly affected by boiling in 1 N HCl for 15 min, suggesting that the phosphorylated protein contains an acid-stable phosphoamino acyl residue. To identify the phosphoamino acid residue, total hydrolysis of the ³²P-labelled protein was performed and the hydrolysate was subjected to electrophoresis. The autoradiogram of the electrophoretogram revealed that the phosphorylated 57 kDa protein contained phosphoserine, but not phosphothreonine or phosphotyrosine residues (Fig. 6).

Fig. 6. Phosphoamino acid analysis of the 57 kDa phosphoprotein of S. Melliferum.

Hydrolysis of the phosphorylated protein and electrophoresis were performed as described in Materials and Methods. Dashed circles in the autoradiogram indicate the position of standard phosphoamino acid detected by ninhydrin spray.

The phosphorylation of the 57 kDa S. melliferum protein was completely inhibited by 2 mM EDTA, suggesting an essential

requirement for a divalent cation for the protein kinase activity. Quantitative evaluation of the requirement for divalent cations revealed that the protein kinase was best activated by low concentrations of Mg^{2+} (0.1 - 1.0 mM) and, to a lesser extent, by Mn^{2+} and Ca^{2+} (Fig. 7). Ni^{2+} , Sn^{2+} , Fe^{2+} and Zn^{2+} had no

TABLE 1. EFFECT OF INHIBITORS ON THE PHOSPHORYLATION OF THE 57 kDa PROTEIN OF S. MELLIFERUM.

Inhibitor ^a	Control	Level of Phosphorylation ^b (% of control)
None	-	100
EDTA	2 mM	10
Cyclic AMP	10 mM	96
Calmodulin	6 μ M	92
+1 mM $CaCl_2$		94
+4 mM $CaCl_2$		90
HgCl ₂	0.1 mM	4
	1 mM	<1
p-chloromercuribenzoate	1 mM	70
	10 mM	25
N-ethylmaleimide	5 mM	92
	20 mM	78

^a The soluble fraction was incubated for 15 min in the presence of the various inhibitors prior to the addition of (γ - ^{32}P) ATP.

^b Phosphorylation was carried out for 1 min at 37°C.

effect on the phosphorylation reaction. High concentrations of Mg (>1 mM) markedly inhibited phosphorylation. No cooperative effects were observed when Ca^{2+} , Mn^{2+} , or Mg^{2+} were added simultaneously with either Zn^{2+} , Fe^{2+} , Co^{2+} , or Ca^{2+} or when Ca^{2+} and Mg^{2+} were combined (data not shown). Monovalent cations (Na^+ , K^+ , Li^+ or NH_4^+) added to the reaction mixtures at concentrations of up to 25 mM had no effect on the rate or extent of

phosphorylation (data not shown).

Fig. 7. The effect of divalent cations on the phosphorylation of the 57kDa protein of *S. melliferum*.

Phosphorylation was carried out in a reaction mixture containing various concentrations of: CaCl_2 (Δ), MgCl_2 ($\square$), or MnCl_2 ($\circ$). The 57 kDa protein bands were excised from the SDS gels and radioactivity was counted.

To determine whether the phosphorylation of the 57 kDa protein is reversible, dephosphorylation was followed in the ^{32}P -labelled soluble fraction of *S. melliferum* cells. After excess [γ - ^{32}P]ATP, as well as other low-molecular-mass constituents were removed from the reaction mixture by membrane filtration, the time course of the loss of ^{32}P label from the phosphorylated 57 kDa protein was studied. The results show that the rate of dephosphorylation of the 57 kDa phosphoprotein was high at pH 8.5, but very low at pH 6.0. The dephosphorylation rate at pH 6.0 was accelerated to a large extent by inorganic phosphate ($T < 10\text{s}$) and to a much lesser extent by pyrophosphate ATP or ADP (10 mM of each). Similar fast rates of dephosphorylation were observed with either unlabelled or labelled (5 mM, 0.5 mCi/mol) inorganic phosphate present in the reaction mixture. The half time of dephosphorylation elicited by arsenate (5mM) was

similar to that elicited by inorganic phosphate. The rate of dephosphorylation in the presence of 10 mM inorganic phosphate was inhibited by NaF or HgCl₂, but not by N-ethylmaleimide (10 mM of each). The reversibility of the phosphorylation reaction was completely eliminated by heating the phosphorylated soluble fraction at 100°C for 3 min.

Fig. 8. Phosphorylation of a 57 kDa protein associated with *S. melliferum* fibrils.

Phosphorylation was carried out as described in Materials and Methods. A) Coomassie Blue stained gel of total cellular protein fraction. B) Coomassie Blue stained gel of the Triton X-100 insoluble fraction. C) Autoradiogram of a gel obtained after SDS-PAGE of the complete reaction mixture containing the total cellular protein fraction. D) Autoradiogram of a gel after SDS-PAGE of the Triton X-100 insoluble fraction obtained by extracting the reaction mixture described in (C). E) Autoradiogram of a gel after SDS-PAGE of the Triton X-100 insoluble fraction first extracted and then phosphorylated in a reaction mixture containing 25 µg of *A. axanthum* soluble fraction.

A purified fibril preparation was prepared from the Triton-insoluble material obtained by extracting intact *S. melliferum* cells with 1% Triton X-100, as previously described (Townsend et al., 1980). Fig. 8, lane B, shows that the fibrils contain a major protein band with an apparent molecular weight of 57 kDa, as revealed by one-dimensional SDS-PAGE. When the fibril preparation was the sole protein added to the reaction mixture, no phosphorylation was

detected (not shown). Nevertheless, when the fibrillar protein was added to a reaction mixture together with A. axanthum soluble fraction, the 57 kDa protein band was intensively labelled (Fig. 8, lane E). When the whole cell lysate was phosphorylated prior to the Triton extraction, relatively little labelling was obtained in the Triton insoluble fraction (Fig. 8, lane D).

TABLE 2. BINDING OF SOLUBLE PROTEINS TO S. MELLIFERUM MEMBRANES.

Soluble Proteins Added	Amount added (μ g/mg protein)	Soluble protein bound (cpm/mg protein)	% bound
125 I-labelled	900	9790 $\pm$ 1200	6.5 $\pm$ 0.8
	450	3220 $\pm$ 450	4.2 $\pm$ 0.6
32 P-labelled	900	630 $\pm$ 250	1.3 $\pm$ 0.5
	450	390 $\pm$ 120	1.6 $\pm$ 0.4

Isolated membranes (1 mg/ml) were incubated for 15 min at 37°C with equal volumes of the soluble protein fraction (2 mg/ml) labelled by either 125 I-chloramine T procedure, or by the in vitro phosphorylation. The resulting membrane-soluble protein complex was collected by centrifugation (37,000 xg for 45 m), washed once, and resuspended in the reaction buffer and the amount of soluble protein bound to the membranes was estimated by measuring the respective isotope.

When the total soluble fraction of S. melliferum was 125 I-labelled with Chloramin-T and incubated with the isolated membrane fraction, about 5% of the 125 I-labelled protein was bound to the membrane fragments (Table 2). As the addition of NaCl (final concentration 0.5 M) to the binding media strongly inhibited the binding, and washing the membrane soluble protein complex with 1 M NaCl released over 85% of the bound soluble proteins, it appears that the soluble proteins bind to the membrane fragments almost exclusively by

electrostatic bonds. When the ^{32}P -labelled soluble fraction was incubated with the isolated membrane fraction, the binding of the ^{32}P -labelled protein to the membrane fragments was found to be only about 20% of that of the ^{125}I -labelled protein. As with the ^{125}I -labelled proteins, the ^{32}P -labelled proteins were readily released by washing with 1 M NaCl.

VOLUME REGULATION IN SPIROPLASMAS.

Changes of Cell Volume in Response to External Osmolarity. Lacking a cell wall, Mycoplasma and Acholeplasma species behave as osmometers (Razin, 1978), responding to changes in the external medium with changes in their internal cell volume. Table 3 shows volume as the

TABLE 3. VOLUME CHANGES OF S. FLORICOLA, M. GALLISEPTICUM AND A. LAIDLAWII IN RESPONSE TO EXTERNAL OSMOLARITY

NaCl (mM)	Intracellular Water Volume ($\mu\text{l}/\text{mg}$ protein)		
	<u>S. floricola</u>	<u>M. gallisepticum</u>	<u>A. laidlawii</u>
100	-	2.8	3.3
150	-	N.D.	N.D.
200	2.3	2.5	2.9
250	2.3	2.4	2.7
375	2.3	2.3	2.5
500	2.3	2.1	1.8

Cells were grown to mid-exponential phase of growth. The cells were harvested, washed in 0.25 M NaCl and resuspended in solutions of various NaCl concentrations containing $^3\text{H}_2\text{O}$ or in ^{14}C -polyethylene glycol. The suspensions were incubated at 37°C for 10 min and intracellular volume was determined.

concentration of NaCl varied were, however, observed with S. floricola cells de-energized by prolonged incubation (1-2 h) in an

energy-free isoosmotic salt solution at 37°C (not shown).

Swelling in isoosmotic Salt Solutions. The process of swelling was further investigated by the following changes in the absorbance of de-energized *S. floricola* cells suspended in isosmotic salt solutions (Fig. 9). As a pronounced and fast swelling was observed in NaCl, KCl, choline chloride, or lysine chloride solutions, it seems that all these ions permeate into the cells, most likely through a non-specific transport system. A somewhat lower swelling rate was observed in an isoosmotic sorbitol solution, but not in a sucrose solution. Swelling was inhibited in the presence of 10 mM glucose, fructose, sucrose, or arginine, but was not affected by lactose, sorbitol or α -methyl glucoside (Fig. 10). In the absence of added glucose, the intracellular ATP concentration fell from 1.4 mM to 0.2 within 30 min, 0.12 within 60 min, and 0.04 within 120 min at 37°C.

Figure 9. Swelling of *S. floricola* cells in various isoosmotic salt solutions.

Cells were incubated at 32°C in a solution containing 25 mM Tris-HCl buffer (pH=8.0) and 225 mM of either NaCl, (O); KCl, (Δ); choline chloride, ($\square$); or lysine chloride, ($\diamond$) solutions.

Figure 10. The effect of energy sources on swelling of *S. floricola*.

Cells were incubated in an isoosmotic NaCl solution containing 10 mM of glucose, (●); D-fructose, (▲); L-arginine, (▼); sorbitol, (Δ); α-methylglucoside, (▽); or lactose, (□) without added energy source (O).

The cell volume was maintained at a constant level during the first 60 min of incubation and only when the ATP levels fall to very low levels (0.04 mM after 120 min), swelling begins. The age of the cells has very little effect on cells swelling. Similar rates of swelling was observed with early logarithmic phase ($A_{640}=0.08-0.12$ pH=7.4) or late logarithmic phase ($A_{640}=0.18-0.20$ pH=6.2) cells. Somewhat lower swelling rates were obtained with stationary phase cells ($A_{640}=0.24$ pH=5.4).

Effect of Inhibitors on Cell Swelling. If the ATPase known to be present in these cells is an ion translocating mechanism involved in volume regulation, then dicyclohexylcarbodiimide (DCCD), an inhibitor of ATPase should stimulate *S. floricola* cell swelling even in the presence of glucose. Fig. 11 shows that DCCD (100 μ M) did not induce cell swelling in isoosmotic NaCl solution at 37°C.

Figure 11. The effect of inhibitors on swelling of *S. melliferum*.

Cells were incubated in an isoosmotic NaCl solution with (open symbols) or without (closed symbols) 10 mM glucose. (○, ●), no inhibitor was added; (△), 10 μM CCCP; (Δ), 100 μM DES; (◇), 10 μM gramicidin; (□), 100 μM DCCD; (⊙), 5 μM nigericin.

Several ionophores were then utilized to selectively dissipate either the chemical or electrical components of the proton motive force. The K^+ ionophore valinomycin collapsed the electrical potential in the presence of high external potassium (200 mM), nigericin, by promoting electroneutral K-H exchange collapsed the pH gradient in the presence of external K^+ (50 mM). Neither nigericin nor valinomycin induce swelling of *S. floricola*, an isosmotic salt solution containing glucose. The proton ionophore carbonyl cyanide m-chlorophenylhydrazone R (CCCD 10 μM) collapses only the chemical component of the proton motive force and failed to cause swelling in the presence of glucose and NaCl. Gramicidin (10 μM), a non-specific cation channel, and diethylstilbesterol (DES) (100 μM) induce rapid swelling and cell lysis of energized *S. floricola* cells suspended in isoosmotic NaCl solution. The swelling induced by DES was obtained at very low DES concentrations (see Fig. 21) even with cells suspended in isoosmotic choline chloride or sorbitol solutions (not shown). These findings are consistent with the view that DES either alters the permeability barrier per se, or affects the intracellular fibrillar system that plays a major role in

maintaining the unique spiral structure and stability of these organisms (Williamson, 1974). DES did not affect the production of ATP by glycolysis (not shown) and only partially inhibited the ATPase activity of S. floricola membranes (see Table 4).

Effect of External pH and Temperatures on Swelling. S. floricola cells swell much more rapidly at pH 8.5 than at pH 7.5 or 6.5 in 0.25 M NaCl in the absence of glucose (Fig. 12). Glucose completely prevented swelling at pH 7.5 and 8.5, but only partially at pH 6.5.

Figure 12. The effect of pH on swelling of S. floricola cells.

Cells were incubated in an isoosmotic NaCl solution with (open symbols) or without (closed symbols) 10 mM glucose at pH=6.5, (○, ●); pH=7.5, (□, ■); or pH=8.5, (△, ▲).

Fig. 13 shows that the swelling rate of S. floricola in 250 mM NaCl solution was increased with the increase in the temperature of the suspending medium from 24°C to 37°C. No swelling was observed during an 8 h period at 18°C. Presumably the cells are relatively impermeable to the ions at this temperature.

²²Na efflux from S. floricola cells. ²²Na⁺ extrusion was studied by loading S. floricola cells with ²²Na⁺ and following the efflux under various conditions. Fig. 14 shows the kinetics of ²²Na⁺ efflux. In either the absence of external Na⁺ or the presence of 250 mM NaCl in the external medium. ²²Na⁺ efflux was

TABLE 4. CHARACTERIZATION OF *S. FLORICOLA* MEMBRANE PREPARATIONS

Preparation	Protein (mg/500 ml)	Lipid to Protein ratio (cpm/mg protein)	NADH ^a dehydrogenase activity	ATPase ^b activity
Intact cells	11.7	1.25×10^5	3.7	1.3
Membranes obtained by osmotic lysis	4.9	2.03×10^5	1.3	2.3
Membranes obtained by ultrasonic treatment	3.2	2.35×10^5	0.4	1.5
Membranes obtained by DES treatment	4.6	2.25×10^5	0.7	3.4

Organisms were grown in 500 ml volumes of a medium containing 0.012 μ Ci of ³H-palmitate per ml. ^aNADH dehydrogenase activity was expressed as 340 per min per mg protein. ^bATPase activity was expressed as μ mole Pi released per mg protein.

Figure 13. The effect of temperature on the swelling of *S. floricola* cells.

Cells were incubated in an isoosmotic NaCl solution containing 10 mM glucose at: 37°C, (○); 34°C, (□); 24°C, (Δ); or 18°C (▽).

glucose dependent, rapid and linear with time over a period of about 4 min until about 80% of the ²²Na⁺ was released. The efflux has no sharp pH optimum over the pH range of 6.0 to 8.0 (not shown).

Figure 14. Efflux of $^{22}\text{Na}^+$ from *S. floricola* cells.

Efflux was carried out in a medium containing either 250 mM NaCl (0,0) or 0.1 mM NaCl ($\square, \square$) solutions with (open symbols) or without (closed symbols) 10 mM glucose.

The effect of inhibitors on the glucose-dependent $^{22}\text{Na}^+$ efflux was studied in cells that were suspended in isoosmotic NaCl or choline chloride (250 mM) solution and adjusted to various pH's over the range of 6.0 to 8.0. Fig. 15 shows that uphill $^{22}\text{Na}^+$ efflux (efflux into 250 mM NaCl solution) was inhibited by CCCP (10 μM) at the acidic pH range (pH=6.0-7.0), but only partially inhibited at the more alkaline pH range (pH=7.5-8.5). Downhill $^{22}\text{Na}^+$ efflux (efflux into a Na^+ poor choline chloride solution) was not affected by the uncoupler CCCP (not shown). The efflux was markedly inhibited by DCCD, but very little affected by DES.

Figure 15. The effect of inhibitors on the efflux of $^{22}\text{Na}^+$ from *S. floricola*.

Efflux was carried out in a medium containing 250 mM NaCl and 10 mM glucose at pH=6.5. Symbols: ($\square$) without inhibitor; CCCP, ($\bullet$); DCCD, (Δ); DES, ($\square$).

Valinomycin (10 μM) or tetraphenylphosphonin bromide (TPP^+) (100 mM) had no effect on $^{22}\text{Na}^+$ efflux at the acidic pH range. At pH-

8.0, valinomycin partially inhibited efflux, whereas TPP^+ enhanced $^{22}\text{Na}^+$ efflux (not shown). These observations are consistent with a non-electrogenic sodium-proton exchange mechanism operated at the acidic pH range and with the premise that the alkaline pH range $^{22}\text{Na}^+$ is extruded electrogenically. Under conditions in which the membrane is permeated by valinomycin, K^+ is released generating $\Delta\psi$ that has an inhibitory effect on Na^+ extrusion. In the presence of TPP^+ , the inhibitory effect of $\Delta\psi$ is reduced.

A Cation/Proton Antiport Activity. To study further whether the driving force for the energy-dependent sodium transport is a proton electrochemical gradient, an imposed ΔpH was generated in intact cells by equilibrating the cell preparations with NH_4Cl and diluting them into a buffer containing 250 mM choline chloride, adjusted to pH 8.0. The ΔpH was produced by the dissociation of NH_4^+ to NH_3 and H^+ and the downhill diffusion of NH_3 out of the cells. The ΔpH was visualized from the quenching of the weak base acridine orange. The addition of either NaCl (25 mM) or KCl (25 mM) to *S. floricola* cells, after a substantial ΔpH was generated, markedly affected the fluorescence intensities (Fig. 16). In a control experiment performed with *S. melliferum*, NaCl or KCl did not induce the quenching of acridine orange. As expected, nigericin induced rapid dequenching. These results suggest that under the experimental conditions described (low intracellular pH, high NH_4^+ concentrations), there is a detectable Na^+/H^+ and K^+/H^+ antiport activity in *S. floricola*.

Figure 16. The effect of cations on the imposed pH in intact *S. floridicola* cells.

Δ pH was imposed by the ammonium prepulse technique. NaCl or KCl was added to a final concentration of 2.5 mM to initiate the cation/H⁺ exchange.

Effect of CCCP on Growth of *S. floridicola* in High-Salt Media. A typical growth curve for *S. floridicola* in a medium containing 124 mM NaCl and 4% horse serum is presented in Fig. 17. The increase in absorbance was accompanied by a decrease in the pH of the medium from pH 7.8 at the early exponential phase of growth to pH 5.5 at the late exponential phase of growth. When CCCP (100 μ M) was added to the growth medium at the early exponential phase of growth, cells continued to grow until the pH of the external medium approached 7.3, at which point growth slowed down and ceased. When the pH of the external medium was raised to pH 7.6, growth resumed. The membranes of *S. floridicola* were completely permeable to protons at the concentration of CCCP used (100 μ M) (not shown).

Evidence for an Electrogenic Na⁺ Extrusion. The ability of cells to generate $\Delta\psi$ by the primary extrusion of Na⁺ was studied indirectly by assaying for Δ pH in the presence of an uncoupler. Generation of $\Delta\psi$ by the extrusion of this ion should drive H⁺ uptake, acidifying the cell interior and resulting in quenching of acridine orange fluorescence. Proton uptake into sodium-loaded cells

Figure 17. Effect of CCCP on growth of *S. floricola*.

Growth was monitored in a medium containing 4% horse serum in the absence (open symbols) or presence (closed symbols) of CCCP (100 μ M). The A_{640} of the culture (solid lines) and pH of the culture (broken lines) were recorded. At time a, CCCP (100 μ M) was added to part of the growth medium ($\bullet$). At time b, the pH of part of the growth medium containing CCCP was adjusted to pH 7.6 and the A_{640} was recorded ($\blacksquare$).

occurred only in the presence of both SF 6847 (0.4 μ M) and glucose (10 mM) (Fig. 18). Glucose could be replaced by fructose, sucrose, or arginine, but not by galactose or lactose (Fig. 19). When KCl (25 mM) were added to collapse $\Delta\psi$, fluorescence was dequenched (Fig. 19). Furthermore, when KCl was added prior to the addition of glucose or SF6847, no quenching of the fluorescent signal was observed (not shown). The results suggest that KCl (25 mM) was able to collapse $\Delta\psi$ and that 0.4 μ M SF6847 was sufficient to make the membranes permeable to protons.

The extent of dequenching of the fluorescent signal depends on the intracellular Na^+ concentration. Thus, the dequenching was pronounced with cell pre-incubated with Na^+ for 1.5-3 h, less with cells pre-incubated for 0.5 h, and very little with cells which were not pre-incubated (Fig. 19). Very little or no quenching of the

Figure 18. Uncoupler-dependent proton uptake by *S. floricola*.

Proton uptake by *S. floricola* was initiated by the addition of SF 6847 (0.8 μ M) and monitored by the acridine orange fluorescence technique. The energy requirements were met by 10 mM of either glucose (I), sucrose (IV), arginine (V), fructose (VI), lactose (VII), or galactose (VIII). Control without an energy source (II); control without SF 6847 (III). The pH generated was collapsed by 20 mM KCl.

Figure 19. The effect of intracellular NaCl on the uncoupler-dependent proton uptake by *S. floricola*.

The experimental conditions are as described in the legend to Fig. 18. The cells were loaded with Na^+ by incubating them in 250 mM NaCl solution for 0, 0.5, 1.5 and 3.0 h.

fluorescent signal was observed when the assay was performed with cells pre-incubated for 1.5 h with choline chloride to deplete the intracellular concentration of Na^+ (not shown). Fig. 20 shows that the dequenching of the fluorescent signal was most pronounced at the alkaline pH range (8.0-8.5), and much less at pH=7.0. These

results are consistent with an electrogenic ion pump specific for Na^+ that operates at the alkaline pH range.

Figure 20. The effect of pH on the uncoupler-dependent proton uptake by *S. floricola*.

The experimental conditions are as described in the legend to Fig. 18. The acridine-orange fluorescence was monitored at the various pHs as indicated in the figure.

Isolation of Spiroplasma Membranes by DES-Induced Lysis. The effect of DES concentration on lysis of *S. floricola* cells in an isoosmotic NaCl solution is shown in Fig. 21. The linear increase in the maximal swelling rate, with the increase in DES concentration up to 100 mM, shows that the swelling of the cells

Figure 21. Lysis of *S. floricola* cells induced by DES.

Washed mid-exponential cells were incubated at 32°C in 225m NaCl solution containing 25 mM Tris-HCL buffer, pH=7.5. containing 10 mM glucose and various concentrations of DES. Symbols: 100 μM (○), 50 μM (Δ), 10 μM (□), or 5 μM (◇); without DES (●).

obeys first order kinetics with respect to DES over the concentration range of 10-100 mM. The sensitivity of the cells to DES leads to

developing a simple procedure to prepare membranes from S. floricola cells. The cells were lysed in an isoosmotic solution of NaCl by DES. Table 4 shows various properties of membranes obtained by DES lysis, osmotic lysis, and ultrasonic treatment. The lipid to protein ratio was higher in membranes prepared by DES lysis than in membranes obtained by osmotic lysis, as reflected by the radioactivity in membrane lipids per mg of protein. The lipid to protein ratios were consistent with the results of sucrose density gradient analysis. After centrifugation in a sucrose gradient, membranes prepared by DES lysis appeared as a sharp band having a density of 1.178 g/ml. The membrane prepared by ultrasonic treatment, however, formed a more diffuse band centered at 1.186 g/ml. The membrane preparations prepared by DES showed very little contamination by the cytoplasmic enzyme NADH dehydrogenase, as compared to membranes obtained by osmotic lysis and retained most of the membrane bound ATPase activity. We propose, therefore, that cell lysis by DES is a potentially powerful method for membrane isolation from Spiroplasmas. This procedure is more gentle than ultrasonic irradiation, its efficiency in terms of membrane yield is about 90%, and the membranes were formed without appreciable contamination by unlysed cells or cytoplasmic components, as compared to osmotically lysed cells. This lysis occurring in an isoosmotic NaCl solution was independent of the age of the culture and not affected by Mg^{2+} (10 mM).

Immunological Evidence for an Active F_1F_0 -ATPase in Spiroplasmas.

We have utilized immunological cross-reactivity to determine the conservation of the β subunit of the F_1F_0 -ATPase in spiroplasmas. Triton X-100 solubilized proteins were separated by

SDS-PAGE, and the protein bands blotted onto nitrocellulose paper. The bands were reacted with various anti- β subunit antisera followed by treatment with ^{125}I -labeled protein A-Sepharose beads. Fig.22 shows that the monospecific polyclonal antisera raised against the β subunit fraction of E. coli F_1F_0 -ATPases reacted with a protein present in the Triton X-100 solubilized cells of the spiroplasmas tested. When antiserum raised against the β subunit fraction of yeast or spinach chloroplasts were utilized, a similar immunoreactivity was obtained. However, no cross-reactivity was obtained with rabbit antisera raised against the α or β subunits of yeast F_1F_0 -ATPase. In some experiments, additional bands, apparently representing degradation products of the β subunit, appeared. The appearance of such bands could be prevented by increasing the levels of protease inhibitors during the preparation of the cell extracts. The proteins that reacted with the anti- β subunit antibodies had an apparent MW of $52 \pm \text{kDa}$.

Figure 22. Cross-reactivity of a spiroplasmal protein with the β subunit of Escherichia coli ATPase.

Cell proteins separated on 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis were transferred to nitrocellulose and immunoreacted with rabbit anti-E. coli β subunit antisera, followed by treatment with ^{125}I -labelled protein A. A) S. citri, B) S. floridcola, C) S. melliferum, arrow = 52 kDa.

DISCUSSION

The spiroplasmas tested in our study possess a membrane-bound ATPase activity suggesting an important role in cell function. Our data that the membrane-bound ATPase of several Spiroplasma species interacts specifically with the β -subunit (which harbors the catalytic site) of E. coli F_1F_0 -ATPase would support the notion that, as with other mycoplasmas, spiroplasmas possess a proton translocating ATPase. In A. laidlawii and M. gallisepticum (Jinks et al., 1978; Shirvan et al., 1989_a, 1989_b) it was suggested that the ATPase also functions as an ion pump for the extrusion of Na^+ and, perhaps, K^+ . Such mechanism would be consistent with our observation that the addition of glucose to S. floricola leads to extrusion of $^{22}Na^+$ from the cells, whereas DCCD, an inhibitor of F_1F_0 -ATPases, inhibits such extrusion (Fig. 15). One of the important physiological functions of ion pumping in cells without cell walls is volume regulation. In the absence of such pumps, solutes and water enter the cells due to the colloid osmotic effect of intracellular macromolecules (Wilson, 1954). In S. floricola, energy depleted cells swelled when suspended in NaCl solution. The swelling was prevented by the addition of glucose. This observation is consistent with the hypothesis that the energy is required to operate the pumping of Na^+ . Nevertheless, as glucose prevented swelling of S. floricola cells in isoosmotic solutions of a variety of salts (other than Na^+ salts), and, in all cases, swelling was not stimulated by the ATPase blocker DCCD, it seems that in Spiroplasmas, energy is required to maintain an intracellular structure, rather than to operate a Na^+ pump. It is suggested that

such a structure stabilizes the cells, preventing them from swelling and lysis. The possibility is very intriguing that the intracellular filaments shown previously to be present in many spiroplasmas (Williamson, 1974) are components of such structures. It was shown that these filaments play a role in determining cell shape and motility (Townsend and Markham, 1980). A stabilizing intracellular structure can also be deduced from the observation that whereas Mycoplasma and Acholeplasma cells behave as ideal osmometers, varying the osmolarity of the medium did not affect the volume of Spiroplasma cells (Table 3).

Several possible mechanisms for sodium extrusion were examined, beginning with a possible Na^+ extrusion by an Na^+/H^+ antiporter. The results indicate that an active Na^+/H^+ antiport activity is detectable in S. floricola cells at the acidic pH range. This conclusion is based on the following evidence: 1) A pronounced inhibition of $^{22}\text{Na}^+$ efflux was observed in uphill $^{22}\text{Na}^+$ efflux after treatment of the cells with an uncoupler such as CCCP (Fig. 15). 2) A Na^+/H^+ exchange was observed in cells after imposition of a pH gradient (acidic inside) by the ammonium chloride dilution procedure (Fig. 16). Similarly, generation of ΔpH by the same procedure drove $^{22}\text{Na}^+$ uptake in sealed membrane vesicles (data not shown). At the neutral-alkaline pH range, the activity of the Na^+/H^+ antiporter activity was very low. At this pH range, S. floricola cells were able to grow and remain viable in a high-salt containing medium, even in the presence of CCCP, suggesting that the Na^+/H^+ antiport activity is not required under physiological growth conditions. Furthermore, the swelling of energized

S. floricola cells suspended in isoosmotic NaCl solution was not enhanced by CCCP (Fig. 11). These results suggest that, at the more alkaline pH range, Na⁺ movement is neither driven by $\Delta \mu_{H^+}$ nor inhibited by the collapse of $\Delta \mu_{H^+}$, the proposed driving force. It is suggested, therefore, that at the alkaline pH range, Na⁺ is extruded from S. floricola by a primary ²²Na⁺ pump. The first evidence showing primary Na⁺ pumping in Spiroplasma is shown in our study by following the internal acidification of energized cells loaded with Na⁺ in the presence of a proton uncoupler. The presence of an outward-directed Na⁺ pump could act to maintain the extracellular/intracellular Na⁺ ratio higher than expected by the Donnan distribution. The effect would be to osmotically balance the colloid osmotic effect and work against osmotic lysis. As a result, S. floricola cells were able to grow even in the presence of uncouplers, indicating that a sodium electrochemical gradient is sufficient for cell growth. The presence of a primary sodium pump, as well as a Na⁺H⁺ antiporter activity, suggest that both proton and sodium may serve as coupling ions for secondary transport in Spiroplasmas. Coupled mechanisms may drive the uptake of essential nutrients, such as amino acids and carbohydrates, into the cells. Similar couplings are known in eukaryotic and prokaryotic systems. However, whereas eukaryotic cells and marine bacteria generally use Na⁺ coupling (Krulwich, 1983; Rosen, 1986; Skulachev, 1985), non-marine bacteria use proton coupling. It seems that S. floricola use both.

Our data demonstrate that protein phosphorylation mediated by a protein kinase is present in all of the Spiroplasma species tested,

representing 5 different serogroups (I-1, I-2, III, IV and VII) within the family Spiroplasmataceae. A major protein substrate with an apparent molecular weight of 57 kDa was identified in all the spiroplasmas. As judged from the labelling intensity of the major 57 kDa phosphorylated band, the level of phosphorylation in the in vitro system containing cell-free extracts was similar among the various Spiroplasma species tested, but 2-4 fold higher than the levels described in M. gallisepticum (Platt et al., 1988), or M. Mobile (Fischer et al., 1988). As the peptide pattern generated by trypsin digestion of the 57 kDa phosphorylated protein obtained from the various spiroplasmas was very similar, and all the clones of monoclonal antibodies raised against the 57 kDa protein of S. floricola (strain BNR 1) cross-reacted with the soluble protein fractions of the various Spiroplasma species employed, it is conceivable to assume that the phosphorylated protein is highly conserved within the Spiroplasma species.

The acid stability of the endogenous phosphoprotein of S. melliferum, and its sensitivity to alkali, strongly suggest that, as with M. gallisepticum (Platt et al., 1988), the phosphoryl moiety is neither linked as an acylphosphate, nor as a polyphosphate or phosphoamidate, but is linked to an amino group as a phosphomonoester. Indeed, acid hydrolysis of the 57 kDa phosphorylated protein, followed by separating the phosphoamino acid by electrophoresis, revealed that, as in other bacteria (Cozzone, 1984), serine is almost the exclusive (>95%) phosphate acceptor. The finding that the phosphorylated protein could only be detected in reaction mixtures containing NaD, a nonspecific phosphatase

inhibitor, suggested the presence of protein phosphatase activity in the system. Phosphatase activity was directly demonstrated by following the loss of ^{32}P -label from the phosphorylated 57 kDa protein. The loss of label was greater at alkaline pH and was markedly facilitated by inorganic phosphate. Two possible mechanisms may account for the rapid loss of the label from the phosphorylated 57 kDa protein following addition of inorganic phosphate: (a) exchange of the radiolabelled phosphoryl moiety in the protein with unlabelled inorganic phosphate, or (b) activation by inorganic phosphate of a phosphoprotein phosphatase. Our observation that the rates of dephosphorylation with either labelled or unlabelled inorganic phosphate were identical, and the finding that no significant radiolabelling of the 57 kDa protein was observed upon addition of $[\text{}^{32}\text{P}]\text{P}_i$ to the 57 kDa protein which was previously phosphorylated with unlabelled ATP, suggest that inorganic phosphate activates an enzymatic dephosphorylation reaction rather than an exchange with a phosphoryl group already bound to the protein.

Earlier failures to demonstrate protein phosphorylation in prokaryotes were attributed to the use of exogenous substrates of eukaryotic origin, widely used for assaying eukaryotic enzymes, for the kinase reaction. Thus, the protein kinases investigated in coliform bacteria did not phosphorylate such substances (Wang and Koshland, 1981; Cozzone, 1985). Our finding that the cell-free extract of S. melliferum is capable of phosphorylating phosvitin is, therefore, of great interest. Although the involvement of the same protein kinase in the phosphorylation of phosvitin and of the 57 kDa protein is not proven, the reduced level of the 57 kDa radiolabelled

protein band upon the addition of phosphatidylcholine supports such a view. The identification of phosphatidyl kinase activity in S. melliferum may open the way to further characterization of the kinase and phosphatase involved in the reversible phosphorylation of the 57 kDa protein in this organism.

The observation that no phosphorylation was obtained when washed isolated membranes were utilized as the sole cellular protein in the reaction mixture led us to assume that both the 57 kDa substrate and the protein kinase involved are soluble proteins. Nevertheless, our findings, that when the isolated membrane preparations were treated with the protein kinase preparation of A. axanthum (Platt and Rottem, 1988), a phosphorylated 57 kDa protein band appeared, suggest that the phosphoprotein substrate, at least in its unphosphorylated form, may be attached to the cell membrane. Our findings that, after the isolated S. melliferum membrane preparations were phosphorylated, radioactivity was easily removed by washing the membranes under conditions that did not affect other membrane proteins (e.g., ATPase activity) suggest that the phosphorylated and unphosphorylated 57 kDa proteins differ in their affinity to their membrane receptors. Such differences may be due to a conformational change that affects binding to membrane fragments. Further support of this notion was obtained by showing that the binding of the soluble phosphorylated 57 kDa protein to membrane fragments was lower than the binding of the total soluble protein fraction. Differences in the affinity of phosphorylated and dephosphorylated heavy meromyosin to F-actin were recently demonstrated (Szeczesna et al., 1987).

The physiological role of the protein kinase in spiroplasma will be understood when the phosphorylated protein is identified and when the effect of phosphorylation on the functional properties of the cell have been elucidated. Although spiroplasmas possess a phosphoenolpyruvate-dependent sugar phosphotransferase system, the involvement of the 57 kDa phosphoprotein in the control of this system is unlikely in view of the finding that phosphorylation was not stimulated by phosphoenolpyruvate nor by glycolytic intermediates. The possibility that protein kinase is associated with the unusual morphology and motility of this organism is intriguing. Questions concerning the structural basis for the unique morphology have been raised in several previous investigations of spiroplasmas and intracellular fibrils that might play a role in determining morphology and motility have been detected by electron microscopy in various Spiroplasma species (Williamson, 1974); Townsend et al., 1980). It was suggested that if anchored to the cell membrane, these fibrils could play a major role in motility and shape maintenance (Williamson, 1989). The regulatory mechanisms that govern assembly-disassembly of such fibrils, as well as their attachment to the cell membrane, are still unclear. Our findings that the Triton X-100 insoluble material of S. melliferum, which contains the intracellular fibrils (Townsend et al., 1980), was phosphorylated by the protein kinase preparation of A. axanthum, and that radioactivity was found exclusively in the major 57 kDa fibrillar protein (identical to the 55 kDa protein described by Townsend et al., (1980), is, therefore, of great interest. The fibrillar 57 kDa protein is apparently a potential target for the endogenous protein

kinase, and its phosphorylation has an effect on its association with the cell membrane. Whether the reversible phosphorylation of the 57 kDa fibrillar protein of S. melliferum affects assembly-disassembly of the fibrils as well is not yet clear. Nevertheless, as a reversible phosphorylation was recently shown to be involved in controlling the assembly of intermediate desmin and vimentin filaments (Inogaki et al., 1985; 1988), it is conceivable that the assembly-disassembly of spiroplasma fibrils is regulated by a similar process. Establishing a stable and purified phosphorylation system will allow further biochemical analyses of the assembly-disassembly process.

GENERAL DISCUSSION

The diverse approaches utilized in our studies underscore the many ways that advances in seemingly unrelated scientific areas reinforce and augment one another. Detailed consideration of the problems in cultivating MLOs make it clear that a general understanding of microbial physiology will probably be necessary if we are to eventually succeed in cultivating these agents.

It is true that in the past seemingly impenetrable barriers to cultivation of fastidious spiroplasmas have been relatively easily breached, as in the case of Spiroplasma kunkelii (Chen and Liao, 1975; Williamson and Whitcomb, 1975), S. mirum (Tully *et al.*, 1977), the Colorado potato beetle spiroplasma (Hackett and Lynn, 1985), and the Drosophila sex ratio organism (Hackett *et al.*, 1987). In the case of MLOs, although the results presented herein are promising, none of the special procedures that succeeded for these fastidious spiroplasmas, including use of co-cultivation systems and/or anaerobiosis, were successful with MLOs.

In such a case, the advances made in basic understanding of mollicute physiology are of special importance. The expertise of D. Pollack was of prime importance in developing a vastly improved picture of intermediate metabolism of the spiroplasmas. An understanding of the enzymatic pathways universally present in this taxon, together with work done previously by Pollack's group on acholeplasmas and mycoplasmas, should lead to vastly improved culture media and cultural conditions for fastidious mollicutes. In Appendix 1, we have given in detail the rationale used in developing the generations of MLO media that are in use today.

Detailed considerations of the physiology of cultured organisms makes it clear that presence of nutrients in media does not guarantee that they are transported. The conditions under which nutrients are transported, and the interactions of these processes with cell volume regulation, are no doubt of equal or greater importance in MLOs, in comparison with other mollicutes. The contributions reported herein to the understanding of transport and cell volume regulation could therefore play a vital future role in designing sophisticated new cultivation systems for MLOs.

Many of the results reported herein have considerable value, even in the absence of MLO cultivation. The development of gene probes, and monoclonal and polyclonal antisera to MLOs, now gives scientists the means to identify MLOs in diseased tissue, and to monitor MLO presence in experiments. Indeed, both systems were used to monitor the MLO cultivation trials reported herein.

Demonstrating again the cross-fertilization of research efforts, work on MLO nucleic acids performed in other laboratories during the tenure of this grant (Lim and Sears, 1989; Sears et al., 1990; Kirkpatrick et al., 1990;) gave impetus and direction to our cultivation trials. These workers showed that MLOs could be placed in the Acholeplasma-Anaeroplasma clade in mollicute phylogenetic classifications. This revelation, of great philosophic interest (Woese, 1987) to microbiologists in general, is of the utmost practical significance to agricultural scientists. Now that we know which microbial groups are the closest actual relatives of MLOs, we have substantial clues as to which sets of physiologies to explore most intensively.

The interfaces described here have already led to the establishment of new collaborations and new working teams on MLOs. Under the auspices of the International Research Program on Comparative Mycoplasmaology of the International Organization for Mycoplasmaology, a concerted effort is now being made to coordinate the comparative studies of mollicutes from around the world. The immense assemblage of disease conditions (McCoy et al., 1989) throughout the world demands that the effort be intensified and focused on the vital issues concerning these organisms. Cultivation of the organisms would bring these studies to a new, higher phase of research in which laboratory understanding could be brought to bear in the field to achieve disease management.

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COOPERATION

Cooperative ties in this project were extensive. Visits of Dr. Rottem to the United States and of Dr. Whitcomb to Israel facilitated transatlantic cooperation. The research team assembled at the 1988 Vienna and 1990 Istanbul meetings of the International Organization of Mycoplasmaology. At these meetings, ongoing research was discussed, and many valuable suggestions were exchanged. Cooperation among the United States collaborators was especially intense. Research materials were routinely shipped among the collaborators, or, in the case of Beltsville and Rutgers, hand-carried from one laboratory to the other. Collaborative ties extended to several groups not formally included in the grant. Three laboratories in particular, the plant pathology laboratories at Davis California (Dr. Bruce Kirkpatrick, UC Davis, and Dr. Deborah Golino, ARS), the microbial physiology laboratory of Dr. Dennis Pollack, Ohio State University, Columbus, and the vector laboratory of L. N. Chiykowski, Canada Agriculture, Ottawa, made major collaborative contributions to the quality and quantity of work reported herein. Research on the Western X-disease MLO was done entirely at Davis by Dr. Hackett and Dr. Kirkpatrick. Dr. Chang prepared special media and made them available for the Davis experiments. The final series of cultivation experiments performed at Beltsville were monitored by NJAY-MLO ELISA tests performed at Rutgers University. Finally, the continuing collaboration of D. L. Lynn (Beltsville) was vitally important.

MAIN ACHIEVEMENTS

A. Studies on MLOs resulted in major advances in detection and cultivation protocols:

1. Monoclonal antibodies were produced against two additional MLOs, the agents of Eastern X-disease, and the maize bushy stunt MLO.
2. Polyclonal antibodies were produced, by two widely different strategies, to the the NJAY- and WWB-MLOs.
3. Cultivation technologies developed for MLO cultivation enabled cultivation of a fastidious, previously uncultivable nematode pathogen, and could lead eventually to commercial production of a biocontrol agent.
4. An early growth flush of MLOs in cell-free media was demonstrated, giving hope to eventual prospects of continuous cultivation.

B. Studies on the physiology of another group of plant/insect mollicutes (spiroplasmas) led to vastly improved insight into their adaptive strategies:

5. Sixty-seven enzyme systems were studied in ten spiroplasmas.
6. Cell lysates of all spiroplasma species tested catalyzed phosphorylation of a major endogenous 57 kDa protein, which appeared to be highly conserved, and is suspected to play a role in spiroplasma motility.
7. An active Na^+/H^+ antiport activity was detected at acidic pHs. At alkaline pHs, Na^+ was extruded by a primary $^{22}\text{Na}^+$ pump. The presence of both activities suggests that both protein and sodium may serve as coupling ions for secondary transport in spiroplasmas.
8. Chapters in Volume V of The Mycoplasmas on mollicute detection, mollicute physiology, the corn stunt pathogen, and a detailed listing of MLO occurrences were prepared.

PUBLICATIONS

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APPENDIX 1. RATIONALE FOR PHLOEM-BASED MEDIA

This series of media utilized the "Golden Elixir Approach", in which a defined base medium was produced and undefined components added to it. The basic approach in medium formulation in this series involved mimicking sap. A modification of this approach involved the use of components that might be taken up more readily than the analogs present in the phloem. It was considered possible that compounds present in low concentrations might be the most important. Such components are present in sufficient concentration for MLOs because they are present at low levels in the sap as a result of plant metabolism. An example of this approach involved supplementation with erythrose, a readily translocated primer, rather than amino acids that derive biosynthetically from erythrose. In general, sugars (erythrose, fructose, glucose-6-phosphate, ribose), because they are more easily transported, could possibly be more efficacious than amino acids such as phenylalanine, tryptophan, or tyrosine.

In any event, experience with A. laidlawii suggests that glutamine and asparagine are extremely important nutrients. These nutrients can, in some metabolisms, yield NH_3 which might then be fixed via the carbamoyl phosphate pathway.

SPECIFIC MEDIUM COMPONENTS

Amino Acids

All amino acids were supplied at a level of at least 5 mg/100 ml. In many cases glutamine and asparagine were supplied in a separate fraction.

<u>Component</u>	<u>Suggested concn. (mg/100ml)</u>	<u>Notes</u>
L-Alanine	25	Product of photosynthesis; formed from pyruvate; high in oats and barley
Arginine	5	Often low in sap, but moderate in oats and barley; may not be important natural metabolite, but <u>S. citri</u> and <u>S. kunkelii</u> utilize this compound for energy
Asparagine	50	High in apple sap; with glutamine, totals 75% of N; Moderate concentrations in oats and barley; Direct transfer from xylem; can replace (17 mg/100ml) yeastolate for <u>Spiroplasma citri</u> , and is essential for <u>Spiroplasma melliferum</u>
Aspartic acid	25	Moderate concentration in apple sap; Dominant in corn sap; moderate in oats and barley; can be used to form

AMP from IMP; D. Pollack has suggested that high aspartate in sap may explain low level of IMP there; a basic building block in the synthesis of asparagine, methionine, threonine, isoleucine, or lysine; Because it is highly acidic, it must be preneutralized or the entire amino acid fraction must be neutralized

Cysteine	5	Rare or absent in plant sap, but may be rare because it is so essential that it is constantly consumed. Thus, it may be very important. Formed from serine; possibly essential for <u>Spiroplasma melliferum</u> . In contrast to phloem, it is abundant in xylem
Glutamic acid	20	Occurs in moderate concentration in apple and other saps; high in oats and barley; dominates in corn; A product of photosynthesis, it is a basic building block of glutamine, proline, and arginine.
Glutamine	50	High in apple and other saps, e.g., corn; required by <u>Spiroplasma melliferum</u> ; may act as a glutamate(-) donor to hold H(+) ions during K(+) influx during osmoregulation; May also be a direct source of ATP (Glm + Mg + ADP = Glu + ATP); Directly transferred from xylem
Glycine	5	Low in oats, barley, and other saps; Formed from serine; Nevertheless, is a major requirement for certain L-phase variants
Histidine	15	Moderate concentrations in saps, including apple, but low in oats and barley
Hydroxyproline	-0-	Not reported from sap
Isoleucine	52	Abundant in apple and other saps; may not be taken up readily
Lysine	5	Low in many saps, but high in oats and barley; formed from aspartate
Methionine	5	Very low (e.g., oats and barley) or absent in saps; formed from aspartate

Phenylalanine	33	Moderate concentrations in some saps, but low in apple sap; a product of lignin digestion; a building block for tyrosine; some mycoplasmas and acholeplasmas are able to synthesize this amino acid. However, it may not be transported
Proline	11	Low in plant saps; formed from glutamate; gram-positive bacteria import this amino acid to regulate osmolarity, or, in some cases, synthesize it from glutamate; this amino acid is not readily taken up
Serine	150	Very high in saps, including poaceous crops such as corn, oats and barley; One of the first products of photosynthesis, it is a basic building block for cysteine and glycine
Threonine	36	Occurs in moderate concentration in saps, including apple; a building block for isoleucine; transferred directly from xylem
Tryptophan	0.5	Although there is a low content in apple and grain saps, tryptophan is absent in most saps; a product of lignin digestion; interaction with light may produce toxic compounds; uptake of this amino acid may be poor
Tyrosine	4	Low in all saps, including apple, oats and barley; a product of lignin digestion; light intermediated reactions may produce toxic compounds; taken up poorly
Valine	60	Occurs in moderate concentrations in apple and other saps; direct transfer from xylem

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*Concentrations in the phloem are usually in the 1,000 mg range, although some may be as high as 42,000 mg. Some important values are 520 (Ricinus), 3,000 (Vigna), and 10,000 (cucurbits). Phloem sap is also abundant in metabolic precursors of amino acids, e.g., glutamate, aspartate, serine, and in the subprecursors phenylalanine and threonine. It is also high in the 3 amino acids formed from pyruvate, i.e., alanine, valine, and leucine, and moderate in histidine (which is formed from ribose-5-phosphate).

Buffers

Buffers are important in optimizing yields of mollicutes. HEPES seems to supply stronger buffering capacity than $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, $\text{KH}_2\text{PO}_4/\text{NaOH}$, or Tris-HCl. Spiroplasma yields are generally, but not always higher in the HEPES buffer; however, it is not known whether the growth curves are steeper in non-HEPES buffered media. Concentration of buffer is important; higher or lower concentrations are inhibitory.

Component	mg/100ml	Notes
HEPES	1500	Buffers media from effects of acid production; inhibits protective effect of Mn^{++} /bicarbonate
NaHCO_3	10	Should be furnished as a separate fraction as a primer Terrestrial animals are high, aquatic animals are low in bicarbonate; with Mn^{++} , protects <u>Lactobacillus</u> from peroxide; inhibited by HEPES, inorganic phosphate, PPI ; stimulated by amino acids

Cofactors

Cofactors are nonessential for S. melliferum and S. floricola if nicotinic acid and riboflavin are provided. In these variations, quantities similar to those used in previous defined media, e.g., H-1 were chosen.

Components	mg/100ml	Notes
Coccarboxylase	0.45	Coenzyme for aldehyde transfer, decarboxylation of α -keto acids and formation or degradation of α -ketols
Coenzyme A	0.45	Carrier of acyl groups, e.g., in fatty acid oxidation, synthesis
FAD	0.45	Electron transfer
NADH	0.45	Electron transfer; Not toxic; not transported by acholeplasmas A necessary component for the "incognita" strain of <u>Mycoplasma fermentans</u> ;
NADPH	0.45	Lipid biosynthesis and electron transfer; probably not toxic; not transported by acholeplasmas

Emulsifiers and stabilizers

Component	mg/100 ml	Notes
Bovine serum albumin	1200	1200 mg/100ml in H-1 spiroplasma medium
Coconut milk (see undefined)		Protects plant membranes from phospholipases
Polyamines (see polyamines)		
Serum (see redox)		Provides lipids; Functions in lipid transport
Tween 40 (palmitate)	0.01ml	0.1 ml/l in CC494 and LD82
Tween 80 (oleate)*	0.04ml	0.04% recommended for <u>A. florum</u> 0.01 ml/100ml in CC494, H-1, LD82 spiroplasma media

* Classical acholeplasmas and A. axanthum use 0.01% [0.01 ml/100ml] Tween 80 [Sigma]. A. florum requires 0.04% Tween 80 (with 0.5% albumin). 0.1% Tween 80 is toxic to most acholeplasmas, but some strains can tolerate up to 0.06%. Oleic acid could not substitute for Tween 80. Some sterol-requiring strains are sensitive to fatty acids.

Hormones

Any culture attempt using a plant rationale must take hormones into account. Although it is unlikely that MLOs utilize hormones per se, they may utilize certain metabolites (e.g., tryptophan, acetate, isopentenyl PPI, adenine, geranylgeranyl PPI) that are needed by plants to make hormones or other products (cholesterol or carotenoids). In either case, plant hormone precursor depletion would likely occur. Ruminant Lactobacillus spp. that are pneumotoxic produce 3-methylindole from IIA, anaerobically. Some Clostridia, Pseudomonas and Rhizobium species also metabolize hormones or their metabolites. Tween 80 is removed from the medium when testing IAA metabolism. Pyruvate acts as a Schiff base cofactor (rather than biotin, etc.). Some important intermediates are: tryptophan --> auxin; acetate --> isopentenyl PPI --> geranyl PPI --> cholesterol --> alkaloids; geranyl PPI --> geranylgeranyl PPI --> gibberillin; geranylgeranyl PPI --> carotenoids; acetate --> isopentenyl PPI + adenine --> cytokinins

Component	mg/100ml	Notes
Absciscic acid		106 x 10 ⁻⁴ mg/100ml in <u>Ricinus</u> sap
Auxin		Plant cell culture media have 10 ⁻⁷ to 10 ⁻⁶ M indole-3-acetic acid, indole-3-butyric acid or a-napthaleneacetic acid; 11 x 10 ⁻⁴ mg/100ml in <u>Ricinus</u> sap; 0.02% IAA used for ruminal lactobacilli
Cytokinin		Plant cell culture media has 10 ⁻⁷ to 10 ⁻⁵ M benzyladenine or N6 isopentenyladenine; 11 x 10 ⁻⁴ mg/100ml in <u>Ricinus</u> sap
Giberellin		Prepared fresh for plant cell culture media at 10 ⁻⁸ to 10 ⁻⁶ M; 2 x 10 ⁻⁴ mg/100ml in <u>Ricinus</u> sap

Inorganics

Suggested Medium Additives

Major

Component	mg/100ml	K+	Na+	Mg++	Ca++	Cl-	PO4=	SO4=
KCl*	120	63				57		
NaCl**	2		1			1		
KH2PO4***	100	28					70	
MgSO4.7H2O****	36			3				15
CaCl2.2H2O*****	1				0.3	0.4		

Trace

Component	mg/100ml	Zn++	Mn++	Fe++	Cu++	Co++	Mo++	B
ZnSO4.7H2O	0.8	0.2						
MnSO4.H2O	0.15		0.05					
FeSO4.7H2O	0.7			0.14				
CuCl2.2H2O	0.1				0.04			
CoCl2.6H2O	0.002					0.0005		
Na2MoO4.2H2O	0.002						0.001	
H3BO3	0.005							0.001

*Inhibitory to A. laidlawii and Mycoplasma mycoides at >300-440 mg/100 ml in some studies, but in other studies M. gallisepticum and Acholeplasma laidlawii grew at concentrations > 1040 mg. Can be as high as 370 mg/100ml without inhibitory effects for spiroplasmas.

** Inhibitory to spiroplasmas; may interfere with glycolysis. Provided as trace contaminants of other components.

*** Essential for certain spiroplasmas. Growth throughout range of 1-100 mg/100ml tested, with a tentative optimal at 7-34 mg

**** 300 mg/100ml is inhibitory to mollicutes. 185 mg/100ml in M1D is needed for spiroplasmas. Can be supplied as Mg oxide (artificial seawater); can assist in anionic adjustment if $\text{SO}_4^{=}$ is too high.

***** 64 mg/100ml is inhibitory to spiroplasmas. Not required for spiroplasmas.

Ion Importance

Ca++ - membrane stability; exclusion important for sieve element formation; not required by plant spiroplasmas; may regulate virulence genes.

Cl- - second largest anion in sap

Fe++ - required for catalase (but this enzyme is lacking in clostridia and mollicutes; may be required by NAD dehydrogenase in A. laidlawii)

Co++ - endo- and exonuclease poison; value unclear

Cu++ - needed by NAD dehydrogenase (see Fe++)

K+ - osmoregulation; ATP pump; principal cation in phloem; Important; can be supplemented by adding K2 or KH_2PO_4 to avoid problems with Cl-

Mg++ - membrane stability; required by some spiroplasmas

Mn++ - with bicarbonate, disproportionates H_2O_2 ; may protect cells in absence of catalase (e.g. Lactobacillus); greatly augmented by presence of amino acids; inhibited by HEPES, inorganic phosphate, PPi

Na+ - antiporter may need (Na^+/H^+) in Acholeplasma laidlawii; may be relatively unimportant or inhibitory

$\text{PO}_4^{=}$ - when supplied as monobasic salt, causes less divalent cation precipitation; predominant anion in sap, involved in many energy pathways

$\text{SO}_4^{=}$ - sulfur is usually present in organic form in sap

Zn++ - endo- and exonuclease poison; value unclear

Since metals are usually determined by ashing, the amounts may be misleading; in nature, they are probably mostly bound to organic compounds.

Lipids and Membranes

Except in cucurbits, lipid scarcity is the rule in phloem. Theoretically, mollicute media should contain free sterol, free fatty acids, and ready-made phospholipids. Most of these components are present in balanced proportions in serum; 20% FCS or HS will satisfy most needs. The question is whether MLOs, with their particular phylogenetic history, will prove to be typical mollicutes. One emphasis on possible failures to grow in conventional media is that the lipids supplied may be too rich or inhibitory for other reasons. One possible approach would be to supply unsaturated fatty acids, but at proportions no more than 5 ug/ml (0.5%). Cocktails are dangerous because of toxicity; even with nonfastidious acholeplasmas, replacement of the serum component by albumin and lipids can present problems. A balanced additive would be: (1) 10 ug/ml oleate, 10 ug/ml palmitate; (2) 10-20 ug/ml sitosterol, spinasterol or cholesterol; (3) phospholipid mixture of sphingomyelin and phosphatidylcholine; and (4) a detoxifying agent such as 1-2% albumin.

Previous work (Kahane and Razin, 1977) is of some guidance, at least for conventional mollicutes. The percentage of cholesterol taken up by M. hominis exceeded that of phosphatidylcholine by a factor of 3-5; acholeplasma did not take up phosphatidylcholine; M. hominis took up 3x as much cholesterol as acholeplasma; dispersions with high cholesterol to dipalmitoyl phosphatidylcholine ratios were better cholesterol donors; mycoplasmas cannot hydrolyze cholesteryl esters; cholesterol was required at concentrations of at least 5 ug/ml, but 30 ug/ml was inhibitory to M. hominis. The optimal concentration was about 15 ug/ml. There are alternative strategies for supplying lipids. It may be desirable to supply sterols in an aggregated form (e.g., by attaching them to starch (the basis for Dubois' oleic acid medium for Mycobacterium), or by use of liposomes, to decrease the toxicity of lipids.

Many media contain albumin. There are some problems with supplying this material, since it is undefined, and because proteases may cleave it in the medium. In defined fractions, it might be desirable to use PVP (in part for supplying fatty acids, but also as an osmotic agent).

Alternative lipids or lipid sources may be advantageous. Some of the possibilities include (i) ester lipids; (ii) defined lipids or lipid precursors in conjunction with ester lipids; (iii) Celex can replace serum for Spiroplasma citri; if this could be used in MLO media, it might provide an additional strategy for reducing the sodium content of media.

Fatty acids

In phloem, free fatty acids are 1.2-5.2% of lipid fraction, which itself is 0.13-0.54% of dry weight. Concentrations of free fatty acids should be kept at less than 10-15 ug/ml. Short chain

length and unsaturation enhance the fluidity of fatty acids and their derivatives; for example, there is a 6.5 C lower melting temperature for palmitic acid (C16) than for stearic acid (C18) (69.6 C). It is possible that high levels of unsaturated fatty acids or carotenoids (from plants) in MLO membranes may cause them to be sensitive to high temperatures.

Component	mg/100 ml	Notes
Acetate		Acholeplasmas synthesize lipids from acetate; compared to cholesterol, carotenoids produced from acetate cause low proton leakiness
Caprylic acid		Present in sap
Linoleic acid	0.1	Present in sap; apparently required by some spiroplasmas
Linolenic acid		Present in sap
Oleic acid	0.5	10-15 mg/l in CC494, LD82, H-1 spiroplasma media. Required by <u>S. citri</u> ; High oleate/palmitate ratio increases adenylate charge
Palmitic acid	0.5	10-15 mg/l in CC494, LD82, H-1 <u>S. citri</u> does not require this fatty acid, but it does promote growth; <u>Acholeplasma laidlawii</u> incorporates this fatty acid at 37C, but can synthesize it also; 10 ug/ml stimulates growth of <u>M. gallisepticum</u>
Triolein	0.2	Possibly may serve to supply requirements

Sterols and derivatives

AY-MLO membranes may be very fluid. If so, the fluidity could be due to incorporation of high amounts of unsaturated fatty acids from plants, plasma membranes (cf. tonoplast) have more saturated fatty acids and sterols than unsaturated fatty acids. Many physiological factors are affected by fatty acids amounts and relative compositions. For example, adenylate charge across membrane increases as oleate/palmitate ratio increases. If sterols are to be added to media, there is a wide choice of sterol species to select from. Sterols are specifically supplemented in several spiroplasma media. Concentrations of sterols have ranged from 0-20 mg/l in CC494, LD82, and H-1 spiroplasma media.

Component	mg/100 ml	Notes
<u>Bile Salts</u>		Although bile salts are harmful to many microbes, microbes are found in guts where bile salts occur. They enhance lipid solubility, but are destructive to many biological membranes. They are utilized by some clostridia, indicating possible linkages of these mollicute ancestors with sterol pathways. They require NAD as a coenzyme, and a pH of 6 - 7.5 as a substrate for <u>C. innocuum</u> .

Steryl esters

Steryl esters may be found in high levels in plants. For example, in apple sap, steryl esters (5 mg/100ml), together with sitosterol, were the major lipids. Although Smith (1954) felt steryl esters could substitute for sterols, the evidence is ambiguous.

Component	mg/100 ml	Notes
Cholesteryl laurate	0.3	0.01 mg/ml may serve as a substitute for cholesterol for some mollicutes
Cholesteryl acetate/ Oleate/Stearate/Palmitate	0	Cannot substitute for cholesterol
Cholesteryl linoleate		With protein, can penetrate membranes

Free sterols

Sterols can be prepared from plant sources. In one experiment, 15 g aster (live weight) yielded 11 mg crude total sterols (M. Feldlaufer, unpublished data).

Campesterol	0.05	Comprised 10% of rye sterols
Chlorophyll	0.1	Stabilizes highly unsaturated membranes
Cholesterol	-0-	Supplied by cholesteryl laurate; Stigmasterol or ergosterol cannot replace for <u>Spiroplasma citri</u> ; Very small amounts in some plant species; enhances ATPase function at lower temperatures.
Sitosterol	0.3	50% of rye sterols; Apple sap has 0.04 mg/100ml;

S. melliferum can use this sterol to replace cholesterol; May be supplied as sitosteryl acetate (Sigma)

Spinasterol	1.0	Aster lipids are 83% spinasterol; A delta-7-sterol; 42% of alfalfa sterols (M. Feldlaufer, unpublished results)
Stigmasterol	0.3	25% of rye sterols

Phospholipids and precursors

Biosynthesis

Phospholipids and precursors are biosynthesized in most organisms:

choline ---> phosphorylcholine ---> phosphatidylcholine
(a salvage pathway)

glycerol + serine + fatty acid ---> phosphatidylserine
+ ethanolamine ---> phosphatidylethanolamine

glycerol + ethanolamine + fatty ac. ---> phosphatidylethanolamine
+ methionine ---> phosphatidylcholine

Component	mg/100 ml	Notes
Choline chloride (see sugars/osmoregulation)		Not associated with coenzymes; Building block of phosphoglycerides; Synthesized by <u>S. floricola</u>
Glycerol (see sugars)		Precursor for phospholipids
Ethanolamine		Precursor for phospholipids
myo-Inositol (see sugars)		Not associated with coenzymes; Building block of phosphoglycerides
Methionine (see amino acids)		Precursor for phospholipids
Phosphatidic acid	0.1	Required by <u>S. citri</u>
Phosphatidylcholine	0.5	Present in sap and plasma membrane of plants; resembles sphingomyelin; Needed by phloem spiropasmas, but not <u>S. floricola</u>
Di-oleoyl P-choline	0.1	Needed by <u>S. kunkelii</u>
Di-palmitoyl P-choline	0.1	Needed by <u>S. kunkelii</u>

P-tidylethanolamine	0.2	Can replace dipalmitoyl phosphatidylcholine and dioleoyl phosphatidylcholine for <u>S. citri</u> , not for <u>S. kunkelii</u>
Phosphoglycerate	0.1	Present in sap; Although it could be important for microbial growth, there is little data on its transport
Phosphorylethanolamine	0.2	Present in sap and plasma membrane
Sphingelomyelin		Promotes growth of <u>S. mirum</u>

Carotenoids

These components are of special interest since the revelation that MLOs may have derived from the acholeplasma lineage.

Biosynthetic pathways are:

acetate ---> mevalonate ---> isopentenyl PPI --->
geranylgeranyl PPI ---> carotenoids (light protection)

Component	mg/100m	Notes
Acetate		(See organic acids)
Acetoacetate		Could possibly be used to replace early steps in biosynthesis
Mevalonate		Can substitute for acetate in acholeplasmas

Sugar Containing Components

The possible importance of components such as sphingosine, mannose, or sialic acids is unclear.

Nucleic acids

Component	mg/100ml	Notes
DNA	0.5	Whole DNA is often used in media; It occurs at 0.5 mg/ml in plant sap; Possible sources include salmon sperm, aster, or acholeplasmas
RNA	0.15	In plant sap RNA occurs at 0.16 mg/100ml); Mycoplasmas have an exo ds-RNase

Nucleic acid precursors

Although some plants have very unusual nucleosides or bases, (e.g., vicin in Vicia spp.), it is unlikely that the unusual bases would be required for MLOs such as AY, which are generalists.

Supplements of 50 mg/l of any base or nucleoside supply all required NAPS for many spiroplasmas (but not for S. melliferum), probably because of impurities in the reagents. But, including all RNA bases or nucleosides improved growth of all spiroplasmas tested. Nucleotide supplements were required for growth of Mycoplasma fermentans.

Nucleosides are transported better than nucleotides. Although nucleotides may not be transported by mollicutes, there are exceptions. High concentrations of nucleotides could exert a toxic influence by altering phosphorus metabolism. Although mycoplasma broth bases and yeast extracts have plenty of bases, they are low in nucleosides, so may be necessary to add these compounds to the elixir formulation. In general, experience suggested that a desirable ratio would be 10 mg nucleoside > 5 mg base > 1 mg monophosphate > .7 mg diphosphate > 0.5 mg triphosphate per 100 ml medium. As much deoxyriboside as riboside would seem optimal.

Component	mg/100ml	Notes
<u>Purines</u>		
ADE	5	Required by <u>M. pneumoniae</u> ; 5-12.5 mg/100ml in wheat, barley; High in <u>Robinia</u>
ADO	10	Acholeplasmas and mycoplasmas utilize this purine; it achieves a level of 3.6-4.5 mg/100ml in CC494, LD82, H-1 spiroplasma media; 10-30 mg/100ml in wheat, barley; very low amounts in dicots
AMP	1	Although some mycoplasmas and ureaplasmas import AMP, it may pose some problems in transport; Perhaps best used in low amounts as a primer
ATP	0.5	Occurs in very high levels in most saps (3.5-130 mg/100 ml); 50-90 mg in cereals; 0.5 mg/100 ml would fulfill a primer role

dADO	10	3.6-4.5 mg/100 ml in CC494 and H-1 spiroplasma media
dAMP	1	May be beneficial in a primer role
HPX	5	Potential intermediate ADE ---> GUA
INO	10	Potential intermediate ADE ---> GUA
IMP	1	Potential intermediate ADE ---> GUA; 0.2 mg/100 ml in H-1 spiroplasma media; 3 mg inosine in LD82
XMP	1	<u>S. floricola</u> has an enzyme to convert to GMP
GUA	5	Very hard to solubilize; Minimal precursor in <u>M. mycoides</u> ; Mollicutes are low G + C; should therefore be lower than A or T
GUO	10	Hard to solubilize; component of CC494, LD82, H-1 spiroplasma media
GMP	1	Some mycoplasmas and ureaplasmas import GMP
GTP	0.5	Occurs in plant sap; 0.5 would serve a function
dGUO*	10	Occurs in H-1 spiroplasma media
dGMP	1	
<u>Pyrimidines</u>		
URA	5	Because spiroplasmas do not have UDE phosphorylase, they may need URA; Minimal precursor for <u>M. mycoides</u>
URD	10	In CC494, H-1 spiroplasma media; Occurs in cereals, but at very low concentration in dicots
dUMP*	1	Some mycoplasmas and ureaplasmas import monophosphates; potential source of dR-1-P
UDP	1	Precursor of dUDP, dTMP
UTP	0.5	Occurs in plant saps: occurs at 2-4.5 mg/100ml in CC494, LD82, H-1 spiroplasma media; 0.5 mg/100ml as a primer; precursor of CTP

CDE	5	2-4.5 mg/100ml in CC494, LD82, H-1 media; Occurs in cereals, but is low in dicots
very		
CMP	1	
dCMP	1	Some mycoplasmas and ureaplasmas import monophosphates; potential source of dR-1-P
CTP	0.5	Occurs in plant sap; could be used at 0.5 mg/100 ml as a primer
THY	5	Minimal precursor for growth of <u>Mycoplasma mycoides</u>
THD	10	THD was supplied at levels of 3-4.5 mg/100ml in CC494, LD82, H-1
TMP	1	
TTP	0.5	Mycoplasmas scavenge to synthesize TTP; could be supplied at 0.5 mg/100ml as a primer; mollicutes lack a thymidylate synthetase
Sugar precursors		
PRPP (phosphoribosyl pyrophosphate)	10	Presumably an important component because all mollicutes can utilize it; (base + PRPP ----> NMP)
dRibose	50	Should be supplied at about 50 mg/100ml; Should be about 2x to concentration of bases, to drive reaction toward nucleosides; if too much ribose is supplied it would be toxic; Although supplied at 4.5-9.0 mg/100ml in CC494 and H-1 spiroplasma media it is not required by spiroplasmas
dRibose-1-Phosphate	10	May be important because mollicutes scavenge it, suggesting a need; however there is little evidence regarding its transport

Organic acids

Overall, there are low amounts of organic acids in sap; Furthermore, the Krebs cycle appears to be absent from Mollicutes. However, some organic acids have been shown to assist spiroplasma growth.

<u>Component</u>	<u>mg/100ml</u>	<u>Importance</u>
Acetate	10	Should be furnished at 10 mg/100ml as a possible lipid precursor
Acetoacetate	10	Could be used at 10 mg/100ml to bypass acetate for lipid synthesis; can be very toxic to some microorganisms
Citric acid		Present in most saps, abundant in apple sap (50mg/100ml); important to xylem bacteria; chelates metals; may do more harm than good in medium
Fumaric acid		Sometimes present in sap, but rare in apple sap; although it is a component in M1D, there is no evidence it would be needed
Gluconic acid		Rare in sap
a-ketoglutaric acid	25	10 mg/100ml improves growth of <u>S. kunkelii</u> , but not <u>S. citri</u> ; Present in many saps, but not apple May use up NADH ---> Glutamate; $\text{NH}_3 + \text{a-k-ga at pH 8} \rightarrow \text{glutamate (-)}$ in cell; this allows cell to retain H^+ to prevent pH drop even in face of K^+ influx
Malic acid	15	Present in most saps, abundant in apple sap (25 mg/100ml); stored in vacuoles in high concentrations; is a component of M1D spiroplasma media; it has been shown that spiroplasmas possess malate dehydrogenase, so they may be able to use malic acid as an energy/NADH source
Malonic acid		Rare in sap
Mevalonate		Used by acholeplasmas as lipid precursor
Oxalic acid		Occurs in some saps; some clostridia are anaerobic oxalate fermenters in rumens; these organisms require 1-2 mM acetate for carbon; Oxalate $^{2-}$ is antiported into some bacteria (anaerobically), with formate $^{1-}$ exported; this generates ATP via a H^+ pump

Oxaloacetic acid	1	Link to amino acids in acholeplasmas and mycoplasmas, but not in spiroplasmas; Rare in sap; Since aspartate is usually abundant, this may not be needed as a precursor for amino acids; very unstable
Pyruvic acid	25	In some saps; Some mutant mycoplasmas can use pyruvate and not glucose); 440 mg/100 ml inhibitory to spiroplasmas May be used in Schiff reaction for IAA-metabolizing ruminal lactobacilli; A hydrogen peroxide scavenger
Quinic acid		In some saps
Shikimic acid	5	Acholeplasmas may use to make aromatic amino acids; 4 mg/100ml in apple sap; Sink tissues use large amounts of shikimate (for aromatic amino acid synthesis), but they apparently import sugars to get the shikiimate skeleton
Tartaric acid		In some saps

Polyamines and ureides

Polyamines may be used as N-sources, membrane stabilizers, or DNA stabilizers. They are also suspected of being second messengers, intimately associated with cytokinin and giberrellic acid function. These may be free, positively charged, polyamines, or phenol conjugates, e.g., the cinnamoyls. The question of whether polyamines are translocated in plants is still unsettled, although they are clearly taken up by suspension cultures.

Potential toxicity could be a problem with these components.

Component	mg/100ml	Notes
Agmatine		Precursor of putrescine
Allantoic Acid	1	occurs at 30 mg/100ml in <u>Acer</u> , <u>Platanus</u> , <u>Aesculus</u> sap
Allantoin	1	Same concentration as allantoic acid in sap
Canavanine	5	Abundant in legume sap (30 mg/100ml) as N-source
Citrulline	5	Abundant in many plant saps (30mg/100ml), e.g. cucurbits, even algae

Ornithine	0.5	Precursor of putrescine
Putrescine	0.1	Moderate concentration in sap (1.5 mg/100ml); precursor of spermine; Can be formed from arginine via agmatine; 1 mg/100ml may be toxic; impedes DNA repair
Spermine		Apparently occurs in all microbes 0.5 AY-MLO maintenance was promoted by 1.0 mg/100ml
Spermidine	0.3	Apparently occurs in all microbes; Precursor of spermine
S-Adenylmethionine		Precursor of spermidine

Redox regulators

Intuitively, this is an important issue. Possible strategies include use of an anaerobic hood, GasPak and/or reducing agents. A 3-gas, small bottle fermentation system could also be used.

1. Characteristics of clostridial ancestors: C. ramosum, C. tertium and C. innocuum are trehalose-fermenting, gut-inhabiting clostridia; C. ramosum is an obligate anaerobe; C. tertium is aerotolerant; C. ramosum (meso-DAP in cell wall, 26% G + C), C. innocuum (no DAP, 43-44% G + C), C. tertium (no DAP, 24-28% G + C). C. ramosum and C. innocuum have B-D-glucosidase (like acholeplasmas, but unlike mycoplasmas), and (unlike most other clostridia) ferment trehalose, galactose, cellobiose, sucrose (ideally suited to plants and/or insects). They (like some other clostridia) also benefit by the presence of bile salts (cholesterol derivatives that help solubilize lipids) in the medium (an early phylogenetic association with cholesterol pathways). Bile salts are toxic to most microbes.

2. Redox regulation was an important factor for growth of the CPB spiroplasma. M. neurolyticum grows better in older media. Anaerobic conditions seem to enhance growth of fastidious mollicutes in poor media.

3. 293mV across sap membrane (pH 8.0). For each 10-fold decrease in the concentration of oxygen, there is a decrease of 15mV in the oxygen potential. Methane bacteria need $<-330\text{mV}$ ($=10^{-75}$ that of oxygen in the atmosphere). Some anaerobes are not killed at high potentials, but cannot grow until the medium is more reduced.

4. It is impossible to maintain very low redox potentials simply by removing oxygen; some reducing agents must be used (at minimal levels to reduce toxicity and to simulate the concentrations in most environments). Final concentrations of 0.02-0.05% of

reducing agent are noninhibitory; Hungate (1969) used 30 mg/100ml; cysteine HCl and 0.25 ml of H₂S gas per ml of medium. Or, he uses hydrogen, sodium thioglycollate, sodium sulfide, and dithionite. Reazurin (0.0001%) indicates redox >-0.042. Exposure to oxygen during medium preparation must be avoided or an excessive amount of reducing agent must be added (which must be added just before medium use, and must be kept under oxygen-free conditions). CO₂ is heavy and is easier to use in ridding broth of oxygen (which is not very soluble in water); may need to bubble 30-60 min. from heat labile media. Alternatively, growth of another organism can be used to reduce oxygen in medium. Some media, if prepared anaerobically, may be sufficiently reduced to start growth without adding reducing agents, particularly if inoculated heavily. Buffering capacity must be increased to reduce acid fermentation products.

5. Care should be taken in using CO₂ to reduce oxygen, because CO₂ can affect glucose and amino acid uptake by effects on membranes (perhaps by increasing membrane fluidity due to alteration of fatty acids). CO₂ is highly reactive with amines.

6. Ruminant lactobacilli that are pneumotoxic produce 3-methylindole from IAA anaerobically. This is also done by clostridia.

7. Oxygen is a definite requirement for at least some mycoplasmas, presumably to reoxidize NADH.

8. Reducing environment inside host cells causes breakage of disulfide bonds in Chlamydia spp., opening pores to allow nutrient exchange.

9. Whereas air is high in oxygen and low in carbon dioxide, air is scarce in water (carbon dioxide is about the same).

Component	mg/100ml	Notes
Ascorbic acid	1	10 mg/100ml in plant sap; 30 mg/l in H-1 spiroplasma media;
Cysteine HCl		Hungate (1969) used 0.03% (w/v) Not found in plant sap
Glutathione	2.5	25 mg/l in H-1 spiroplasma media; 18-31 mg/100ml in fruit phloem exudate; 31-107 in leaf tissue (animals have 15-310); these may be low estimates due to water dilution; Needed for K ⁺ entry into cell; Prevents oxidation of ascorbic acid; May only function in plants at neutral or acid pH, oxidized at alkaline pH

	(but phloem has glutathione reductase) Functions in amino acid transport; Needed in high concentration to total K ⁺ influx, for osmoregulation; Activator of enzymes
Mineral Oil	If this is to be used, a 10-20% overlay, with N ₂ or GasPak spurge
Serum, fetal bovine (see undefined)	Contains many antioxidants (tocopherol, ascorbic acid, glutathione, ceruloplasmin, transferrin, catalase, superoxide dismutase, glutathione peroxidase); peroxidases in horse serum influence batch quality
Sodium thioglycollate	All concentrations, from 0.01 up 100 mg/100ml hurt AY-MLO, 50 mg/100 survival was best

Sugars and osmolreegulators

Component	mg/100 ml	Notes
Choline	5	Needed for glycine-betaine formation for osmoregulation; phospholipid precursor
Erythrose	50	
Fructose	100	As a primer, may be required; 25 mg in apple sap; 1-4% in LD82 and H-1 spiroplasma media
Glucose	100	1-5 mg in apple sap; 1-8% in CC494, LD82, H-1 spiroplasma media; 900 mg/100ml ideal for spiroplasmas; 3% used for ruminal lactobacilli
Glutathione (see redox)		An osmoregulator
Glycerol	10	0.24 ml/l in LD82 spiroplasma medium <u>M. mycoides</u> may require in lieu of other sugars. Can also be an osmoregulator; May be used as phospholipid precursor
m-Inositol	5	10 mg/100ml in plant sap in autumn; 20 mg/100ml in apple sap, also in Asteraceae; apparently, cannot be

		synthesized, but is useful as a phosphorylated derivative
Mannitol		Abundant in trees, but not in AY-MLO hosts
Raffinose		Aster has <1%
Sorbitol		Abundant in trees, but not in AY-MLO hosts;
Sucrose	3000	Although decline and witches' broom agents appear to occur only in hosts with 15-25% sucrose, aster yellows agents are in asters (1%), <u>Datura</u> (1%), <u>Solanum</u> (6%); occurs at levels of 2-3.5% in LD82, H-1 spiroplasma media

An alternative approach is suggested by the work of Maloney et al. (1990) , who show importance of anionic sugars (e.g., glucose-6-phosphate) transport by starved Staphylococcus, Streptococcus; the organic anion replaces accumulated Pi in the microbial cell. This step could be useful or necessary as a primer. G6P and glycerol-3-P are particularly important, but other sugars may also participate in: 2dG6P, M6P, F6P, GluNH2P, R5P, MeG6P, F1P, Gal6P, G1P, G3P, PEP.

* Also, see amino acids, lipids, organic acids.

Undefined components

The number of undefined components suggested is legion. Some of those to be considered seriously are:

Peptones

May have a high undesirable fat content; plant-based peptones such as phytone may be most suitable.

Some peptones have almost no Na⁺ (e.g., proteose peptone), others have high K⁺/Na⁺ ratio (e.g., proteose peptone, neopeptone). A concentration of 3% proteose peptone was used for ruminal lactobacilli

Most contain significant amounts of Cl⁻, some Fe⁺⁺, ppm toxic inorganics, vitamins.

Acholeplasma parvum required phytone (high K⁺, high in some vitamins) or soybean extract.

Components used for Xylem-Limited Bacteria

Components have included: hemoglobin (10mg), hemin chloride (10-40mg), or FePPI (0.25g), isovitalex (10g), charcoal (2g), soluble starch (2g), Mueller-Hinton Agar (38g), cornmeal agar (17g), soytone (2-8g), PPLO broth base (10g), casitone (4-7g), yeast extract (10g), phytone (4g), trypticase (1g).

Yeast Products

Yeastolate and/or fresh yeast extract are important for S. kunkelii, and S. citri. The manufacturer and batch of fresh yeast extract are extremely important. Truly fresh extract, prepared in the laboratory, may be much better than commercial preparations (McGarrrity, 1990). Either product can be inhibitory at higher concentrations.

Low MW (<2000) fractions derived from these undefined products appear to be most important, i.e., NAPs, vitamins, amino acids.

Component	mg/100ml	Importance
Phytone	500	Required by <u>A. parvum</u> ;
Serum		Classical component of mycoplasma and spiroplasma media; One major function, that of sterol and lipid supply and transport must be solved in some fashion;
Yeast Products		Yeastolate may be less inhibitory than FYE; concentrations determined for spiroplasmas may be of some help, but MLOs may be much more sensitive to inhibitors

Vitamins

Component	mg/100ml	Notes
Biotin	0.05	<u>C. butyricum</u> is only clostridium requiring; Required by pyruvate dehydrogenase, which is abundant in acholeplasmas
Folic Acid	0.01	Stimulatory to gut clostridia
Nicotinic Acid	0.05	Fair amount in plant sap, but very low in corn; 0.05 mg/100ml in Murashige and Skoog plant cell formulations; One of 2 vitamins required by

		spiropasmas; Optimal is 4.87 nM (= 6 x 10 ⁻⁴ mg/100ml) for spiropasmas;
Pantothenic Acid		Present in sap as coenzyme A
PABA		0.01 mg/100 ml in Murashige and Skoog plant cell medium
Pyridoxal-5-PO ₄	0.05	Necessary for transaminases
Pyridoxine HCl	0.05	Occurs in plant sap; 0.05 mg/100ml in Murashige and Skoog plant cell media
Riboflavin	0.05	One of 2 vitamins required by spiropasmas; Optimal is 3.83 nM (= 1.4 x 10 ⁻³ mg/100ml) for spiropasmas; Stimulatory to gut clostridia; Sensitive to Fe ⁺⁺ , H ₂ O ₂ , and oxidizes substances in light;
Thiamine HCl	0.04	Occurs in plant sap; 0.04 mg/100ml in Murashige and Skoog plant cell media
Vitamin B12	0.01	Many clostridia utilize

PHYSICAL FAECTORS

Cultivation Systems for Oxygen-sensitive Organisms

The principal systems considered were use of an anaerobic hood under N₂/H₂ atmosphere, 0.0% O₂; (ii) Microaerophilic conditions; (iii) use of deep broth or deep agar; (iv) GasPak; and/or 10-20 % overlay of mineral oil.

Medium pH

The pH optima need to be determined empirically. Although values of 6.0 to 8.0 have been classically chosen, plant cell cultures are maintained at pH 5.6.

	Plant Sap	Other Tissues	Animal Cytoplasm
pH	7.2-8.5	Slightly acid	7-8

Some pH Values Relevant to MLO Culture

pH	MLO	MLO	<u>A. laidlawii</u>
6.0-6.25		24 h	
6.5-7.5	Best	48 h	<7.2-7.4 growth ceases
>7.5		Poor	
7.75-8.0		4 h	
8.5			Best

Sap pH	Host	MLO Disease	WtBr	Decline	AY	Phyl
6.8-7.4	<u>Cucurbita pepo</u>				JAY	
7.1-7.5	<u>Fraxinus</u> spp.	+		+		
7.0 (most)-8.7	<u>Robinia</u> spp.	+				
7.5-8.0	<u>Malus</u> spp.	AP				
8.0	<u>Beta vulgaris</u>	+				
8.0-8.1	<u>Cucurbita maxima</u>				EAY	EuCP

Conclusions are difficult to make.

MLOs may have different pH optima, but it is difficult to imagine that they are profoundly different from spiroplasmas, or that they are extremely exacting, given their residence in phloem, insect gut, hemolymph, and saliva. A neutral to slightly basic pH appears to be adequate.

Solid Phase Support

Selection of a solid-phase support, such as agar or a filter membrane may be important. Since a single bacterium has a mass of 1.5pg, there is a total mass of 15mg in one colony or 10^7 CFU/ml. This mass of bacteria is diluted almost 70 times in broth. In semisolid medium, drainage is slow, bacteria are surrounded by metabolites in high concentration.

Agar quality is important. Noble agar, for most other mollicutes, has been employed at 0.8 to 2.4%.

Temperature

Temperature optima vary among mollicutes depending on their habitats. Historically, AY organisms have been shown to be heat sensitive, but 28C appears to be a suitable temperature.