Comparative Growth and Primary Isolation of Spiroplasmas in Media Based on Insect Tissue Culture Formulations

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ABSTRACT

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The corn stunt organism (CSO) and *Spiroplasma citri* can be cultivated in a cell-free medium based on Schneider's medium for cultivation of *Drosophila* cells. Because Schneider's medium is largely synthetic, the influence of certain constituent factors on spiroplasma growth could be studied. Growth rates and primary isolation of four CSO strains were deleteriously affected by deletion of organic acids or by reduction of the osmolality of the medium from 540 to 300 mOsm. Inclusion of α-ketoglutaric acid in this medium was especially important for optimal CSO growth. In contrast, the growth rate of four strains of *S. citri* was reduced markedly by deletion of the defined amino acid constituent, but was not severely reduced by omission of factors that limited CSO growth. Thus, the sufficiency or insufficiency of spiroplasma medium formulations based on insect tissue culture media could be explained in terms of their content of components from Schneider's *Drosophila* medium.

Additional key words: mycoplasma, citrus stubborn disease.

The report of Doi et al. (7) that plant "yellows diseases" may be caused by mycoplasmas started a massive effort to cultivate the presumed causal agents. Preliminary reports of success have appeared, but most are subject to various criticisms (12). For example, some experiments could not be repeated in other laboratories, or in some cases, cultures were not available to others or were not deposited in type culture collections, or, if distributed to other laboratories, turned out to be cultures of known mycoplasmas, such as *Acholeplasma laidlawii*, for which plant pathogenicity could not be demonstrated. Eventually, in 1971, a helical mycoplasma, the incitant of citrus stubborn disease, was cultivated (9, 16) and later named *Spiroplasma citri* (17). However, the cultivation of other plant and insect mycoplasmas continued to be an elusive goal. Among the intractable agents was the corn stunt organism (CSO), for which the trivial term "spiroplasma" had been first proposed (6). Although Chen and Granados (1) maintained CSO infectivity for 43 days in primary isolates, repeated subculture could not be accomplished (5). Eventually, some workers concluded that the CSO posed a special cultivation problem (4, 19). However, in 1975, two groups, using very different approaches, achieved continuous cultivation of CSO. Chen and Liao (2) used a medium similar in many respects to earlier formulations (1), but Williamson and Whitcomb (26) developed a formulation, the M1 medium, based on media used for tissue culture of *Drosophila* cells.

The difficulty encountered in cultivation of CSO, compared to the ease with which *S. citri* was cultivated, raised many basic questions, including: What are the differences in cultural requirements of the two spiroplasmas? Can media for *S. citri* now be improved, using clues from the successful cultivation of CSO? Is there a simple medium which permits good growth of both spiroplasmas? What medium factor enhances primary isolation? In this paper we attempt to answer these questions, by comparing the ability of *S. citri* and CSO to grow in modifications of the M1 spiroplasma medium.

MATERIALS AND METHODS

Spiroplasmas.—All spiroplasmas were maintained in M1A medium. The Moroccan (Maroc) strain of *S. citri*, obtained from J. G. Tully, was in the 173rd passage at the start of our experiments. In early experiments we used isolate B of CSO in the 26th passage. Later, a filter-cloned B isolate (ATCC No. 27953) was employed. The Israel (13) and C-189 (9) isolates of *S. citri* were supplied by P. Saglio and were in their 112th and 103rd passages,

respectively. The M435 strain of *S. citri* was supplied by A. Granett and was in its seventh passage. The G strain of Mississippi CSO was supplied in stunted corn by L. R. Nault, and was in its ninth passage after isolation from plants. The Y isolate of Rio Grande corn stunt was in its sixth passage after isolation from hemolymph of infected *Dalbulus elimatus* Ball leafhoppers (23), and the G3N isolate of Rio Grande corn stunt was in its 10th passage after reisolation in the final step of fulfillment of Koch's postulates (26).

Medium preparation.—The basal spiroplasma medium (Table 1), designated M1A, differed from the M1 medium developed for the isolation and maintenance of the CSO (26) in its content of fetal bovine serum (FBS; 16.7% rather than 20%) and by the omission of trehalose. Schneider's *Drosophila* medium (18) was obtained from Grand Island Biological Company (GIBCO), Grand Island, New York, or was prepared in our laboratory. The M1A medium or variations of it were prepared from stock solutions of yeastolate (YATE) (Difco Laboratories, Detroit, Michigan), amino acids, $CaCl_2$, $NaHCO_3$, and organic acids combined with the remaining inorganic salts. All of these solutions were filter-sterilized through 0.22 μm pore size Millipore filters. Peptone and tryptone (Difco), PPLO broth base (Baltimore Biological Laboratories, Cockeysville, Maryland), and carbohydrates were dissolved in water and the pH adjusted to about 7.8 with 1N NaOH before autoclaving. Filtered stock solutions or Schneider's medium from GIBCO, and cooled autoclaved solutions were then combined. Phenol red (0.5%) and fresh yeast extract (FYE) (Microbiological Associates, Inc., Bethesda, Maryland) then were added. The FBS (Flow Laboratories, Rockville, Maryland), after being heated at 56 C for 1 hr, was cooled and added as the final ingredient. In some experiments, FBS was replaced with bovine serum fraction (BSF) supplied by GIBCO. The pH of the final medium was usually about 7.4, but was adjusted with small volumes of NaOH or HCl when necessary. The osmolality of the M1A medium, determined by the freezing-point method with a Model 31LAS osmometer (Advanced Instruments, Inc., Needham Heights, Massachusetts), was about 545 mOsm. Grace's insect tissue culture medium, and three media used for culture of vertebrate cells [Medium 199, NCTC-135, and CMRL-1066 (GIBCO)], were used as supplements in certain experiments. Difco lactalbumin hydrolysate (LH) solutions were prepared by dissolving 5.2 g/100 ml of warm water and filter sterilizing through 0.22 μm pore size Millipore filters. The osmolar concentrations of media were adjusted with a sterile 60% sorbitol solution.

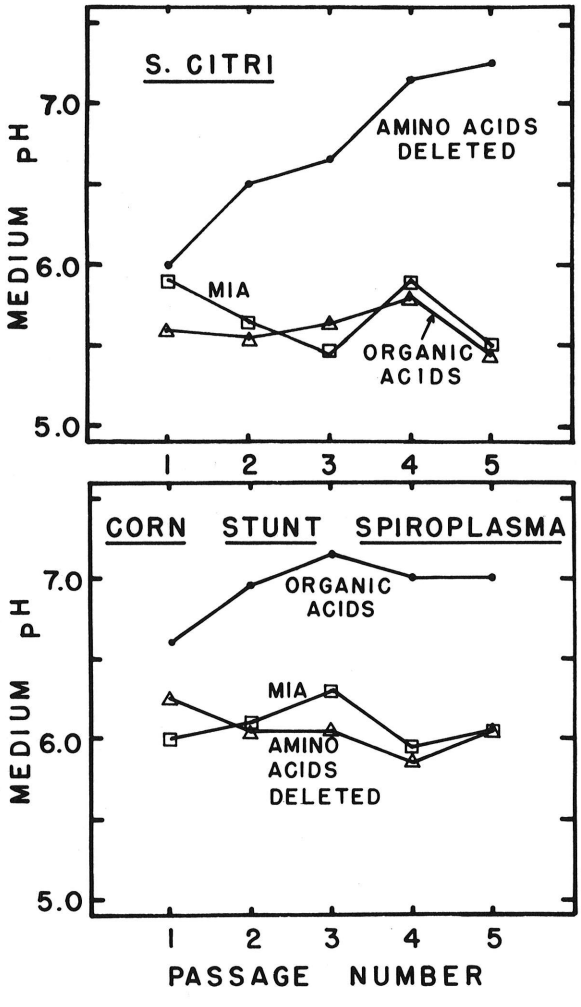

Fig. 1. Change in medium pH through five passages of *Spiroplasma citri* and of the corn stunt spiroplasma in M1A spiroplasma medium and in M1A media with the amino acids fraction or the organic acids fraction omitted. All media initially were adjusted to about pH 7.3.

Passage procedure.—The media, tubed in 5-ml aliquots, were inoculated with 0.1 ml of a log phase *S. citri* culture or 0.4 ml of a CSO culture. In later tests, 0.2 ml of inoculum was used for both organisms. Unless stated otherwise, cultures of *S. citri* were transferred at 3-day intervals and those of CSO at 4-day intervals. Tubes were incubated aerobically at 32 C and 29 C for *S. citri* and the CSO, respectively. Five passages in the experimental media were made before the estimation of growth.

The fifth passage was chosen for estimating growth because spiroplasmas passed in suboptimal media often produced large amounts of acid during the first two or three passages. However, by the fifth passage, growth and acid production had ceased or decreased to a lower, more stable, level. This concept is illustrated using results from a typical experiment (Fig. 1). In the first passage, *S. citri* lowered the pH of M1A spiroplasma medium, and the pH of a similar medium lacking the amino acids fraction, to about pH 6. In subsequent passages, the pH in M1A was reduced to below pH 6 while in medium without the amino acids, the pH increased gradually until no change in medium pH occurred after a 3-day incubation period. When the organic acids fraction was deleted, the CSO initially lowered medium pH, but by the third passage pH became more stable. Acid production therefore indicated the ability of the organisms to persist through continued passage in a given medium, as well as growth in the fifth passage alone. Such estimates were often confirmed by microscopic estimation of numbers of helical bodies viewed by dark-field microscopy (23).

TABLE 1. Composition of M1A and other spiroplasma media based on insect tissue culture media[a] and fifth-passage acid production by the B clone 2 isolate of the corn stunt spiroplasma and the Moroccan strain of *Spiroplasma citri*

Components	Insect tissue culture media components			
	M1A medium (mg/liter)	Grace's insect cell medium (mg/liter)	Mitsuhashi's leafhopper medium (mg/liter)	Chiu and Black's AcTc medium (mg/liter)
Inorganic salts:				
$CaCl_2$	300	375	109[d]	300
KCL	800	2,050	160[d]	800
KH_2PO_4	225			225
$MgSO_4 \cdot 7H_2O$	1,850	1,390	40[d]	1,850
$MgCl_2 \cdot 6H_2O$		1,140	40	
NaCl	2,716.6[b]	1,666.6[b]	4,160[d]	2,716.6[b]
$NaHCO_3$	200	175	50	200
Na_2HPO_4	350			350
$Na_2HPO_4 \cdot H_2O$		506.5	105[d]	
Organic acids:				
α-ketoglutaric acid	100	185	100	100
Fumaric acid	50	27.5	50	50
Malic acid	50	335	50	50
Succinic acid	50	30	50	50
Amino acids:				
α-alanine			10[c,d]	
β-alanine	250	100		
L-Alanine		112.5		
L-Arginine	200			
L-Arginine HCl		350	14[d]	
L-Asparagine		175		
L-Aspartic acid	200	175	12[c,d]	
L-Cysteine	30			
L-Cysteine HCl			.02[d]	
L-Cystine	50	11	4[d]	
L-Glutamic acid	400	300	27[c,d]	
L-Glutamine	900	300	20[d]	
Glycine	125	325	10[d]	
L-Histidine	200	1,250		
L-Histidine HCl			4[d]	
L-Hydroxyproline			2[d]	
L-Isoleucine	75	25	8[c,d]	
L-Leucine	75	37.5	24[c,d]	
L-Lysine HCl	825	312.5	14[d]	
L-Methionine	400	25	6[c,d]	
L-Phenylalanine	75	75	10[c,d]	
L-Proline	850	175	8[d]	
L-Serine	125	550	10[c,d]	
L-Threonine	175	87.5	12[c,d]	
L-Tryptophan	50	50	4[c,d]	
L-Tyrosine	250	25	8[d]	
L-Valine	150	50	10[c,d]	
Lactalbumin hydrolysate			5,200	6,500
Carbohydrates:				
Glucose	1,333.3	1,333.3	1,800[d]	1,333.3
Fructose	333.33	1,200		333.3
Sucrose	3,333.3	13,340		3,333.3
Sorbitol[e]	23,333.3	15,333		23,333.3
Other components:				
Tryptone	3,333.3	3,333.3		3,333.3
Beef heart infusion (Solids)	2,000[b]	2,000[b]		2,000[b]
Peptone	6,000[b]	6,000[b]	5,200	8,533.3[b]
Fresh yeast extract	33.3 ml	33.3 ml	33.3 ml	33.3 ml
Yeastolate	1,000	4,000	2,000	5,000
Phenol red (0.5% sol)	4 ml	4 ml	4 ml	4 ml
Penicillin (250,000 units/ml)	8.3 ml	10 ml	10 ml	10 ml
Fetal bovine serum	166.6 ml	166.6 ml	166.6 ml	166.6 ml

TABLE 1. (continued)

Components	Insect tissue culture media components			
	M1A medium (mg/liter)	Grace's insect cell medium (mg/liter)	Mitsuhashi's leafhopper medium (mg/liter)	Chiu and Black's AcTc medium (mg/liter)
Fifth-passage acid production by:				
Corn stunt spiroplasma	14.4[f]	5.1	0	5.6
Spiroplasma citri	16.0	0.7	3.8	15.5

[a]For compositions of insect tissue culture media, see references 3, 10, 14, and 18.

[b]NaCl, peptone, and beef heart infusion supplied in 7,000 mg/liter mycoplasma broth base included in these calculations.

[c]Amino acids were DL forms.

[d]Part or all of these components were from Medium 199 with Modified Earle's Salts used at 20%. Medium 199 also contains vitamins, nucleic acid precursors, and lipid precursors.

[e]Sorbitol also was used to adjust the osmolality as required.

[f]Milliliters of 0.01 N HCl required to lower the pH of 10 ml of noninoculated medium to the pH measured at the end of the fifth passage.

Growth estimates.—Whenever feasible, growth was assessed by preparing serial tenfold dilutions in M1 or M1A spiroplasma media of fifth-passage cultures harvested at the normal passage intervals. The endpoint was defined as the greatest dilution at which an acid pH shift occurred. The titer of the culture was expressed in color changing units (CCU), where one CCU is defined as the $\log_{10}$ (reciprocal of endpoint). The CCU method was chosen because solid media have been inconsistent for the enumeration of the CSO by counting of colony-forming units.

When CCU titration was not feasible, we relied on the amount of acid produced by cultures in the final passage as a measure of the ability of the spiroplasma to multiply in a given medium. Acid production was determined by titrating 10-ml aliquots of noninoculated medium with 0.01 N HCl and computing the amount of acid required to lower the medium to the pH value measured at the end of the fifth passage. Because of variation from experiment to experiment due to batch-to-batch variability in the complex media and in the growth of different isolates of the two spiroplasmas, the data from some experiments were transformed to allow for comparisons between treatments. The data were transformed by determining the ratio A_{d5}/A, where A_{d5} is the fifth-passage acid production in the deficient medium, and A is acid production in the complete M1A medium. Thus, a figure of 1.00 would indicate acid production equal to that in complete medium.

RESULTS

Substitution of other insect tissue culture media.—The M1A spiroplasma medium (Table 1) is based on Schneider's *Drosophila* medium. We wished to determine if other insect tissue culture media could be used to develop or improve spiroplasma media. Grace's insect tissue culture medium (10), AcTc medium (3), and Mitsuhashi's medium (14) were tested by substituting them for Schneider's *Drosophila* medium in the basic M1A formulation (Table 1). *Spiroplasma citri* grew poorly in media based on Mitsuhashi's medium, but CSO failed to grow in this medium. Growth of *S. citri* in the AcTc-based medium was about the same as in M1A medium, but CSO grew poorly. The CSO grew more rapidly than *S. citri* in a medium based on Grace's insect cell medium, but CSO growth was slower than in M1A medium.

Deletion of organic acids.—A consistent difference between *S. citri* and CSO was their response to deletion of organic acids from M1A medium. Although deletion of all organic acids greatly reduced growth of CSO (Table 2, experiments 1 and 2), retention of α-ketoglutaric acid alone, but not succinic acid, permitted normal growth. On the other hand, growth of *S. citri* was not diminished by the deletion of all organic acids and was not stimulated when α-ketoglutaric acid or succinic acid was added singly. Increases of *S. citri* growth in the absence of organic acids (Table 2, experiments 1 and 2) were inconsistent (Table 5).

Deletion of amino acids.—Growth of both spiroplasmas was reduced when the defined amino acid fraction was eliminated from the M1A medium (Table 2, experiments 1 and 2). For CSO, the decrease in fifth-passage acid production or titer of viable organisms was small, but growth of *S. citri* was markedly reduced. These results suggest that media designed for *S. citri* should be rich in free amino acids if optimum growth is to be achieved.

Replacement of amino acids with lactalbumin hydrolysate.—Lactalbumin hydrolysate (LH) is used in many insect tissue culture media as the source of amino acids (22). We therefore attempted to substitute LH for the amino acid mixture in M1A medium. To allow more time for adaptation to the LH-containing media, the passage intervals were increased to 4 and 5 days for *S. citri* and CSO, respectively. Acid production by the CSO cultures in the fifth passage was equivalent to 14.4, 13.5, 1.8, 0, and 2.0 ml of 0.01 N HCl/10 ml of culture media with 0, 3.25, 6.5, 10.1, and 13.0 g/liter LH, respectively. Acid production by *S. citri* in corresponding media was 16.0, 14.7, 13.0, 1.5, and 3.6 ml of 0.01 N HCl/10 ml culture. Thus, growth of CSO and *S. citri* in medium with 3.25 g/liter LH was similar to their growth in M1A medium, but CSO growth was reduced at LH concentrations of 6.5 g/liter or above, and growth of *S. citri* was reduced at 10 g/liter LH or higher.

Deletion of inorganic salts.—A major difference between Saglio's medium for *S. citri* (16, 17) and the M1A medium for CSO is the absence in Saglio's medium of a defined inorganic salt component comparable to that in

Schneider's *Drosophila* medium. Thus, M1A medium is comparatively rich in inorganic ions, especially divalent cations and phosphate. It was, therefore, of interest to study the importance of the inorganic salt constituents of M1A medium. There was a moderate diminution of CSO growth when all salts were deleted, but growth of *S. citri* was not affected (Table 2, experiments 1 and 3). The deletions of $MgSO_4$ or KH_2PO_4, but not $CaCl_2$ and $NaHCO_3$ had a similar effect.

Deletions of peptone tryptone and mycoplasma broth base.—Both organisms could be cultivated in media with mycoplasma broth base, peptone, or tryptone removed, or with any two of these components removed (Table 2, experiments 4 and 5). However, both organisms grew slowly when all three components were deleted. In experiment 4, deletion of tryptone and peptone reduced *S. citri* but not CSO growth. As might be expected, the deletion of mycoplasma broth base, a complex mixture, from media already enriched with other complex ingredients, gave somewhat variable results.

Growth in media with modified yeast components.—We wished to determine if higher concentrations of YATE or FYE would substitute for both yeast derivatives in M1A medium. Growth of the CSO and of *S. citri* (Table 3) was poor when FYE was omitted from medium containing 1 g/liter YATE, but growth was often better than in M1A medium at YATE concentrations of 2-6 g/liter. Optimal growth of *S. citri* was noted in media with 99 ml/liter of FYE, but no YATE. However, CSO required YATE for optimal growth. In summary, our data suggest that YATE might substitute for FYE in media for CSO and *S. citri,* but that FYE at any concentration was unable to replace YATE for optimal CSO growth.

Growth in serum fraction media.—As a possible prelude to detailed physiological experiments we tested the effect of replacing the FBS in M1A spiroplasma medium with 5% BSF and the additional effect of deleting FYE and YATE from the medium. Also, enrichment of the simplified serum and yeast-deficient media was attempted by adding 20% (v/v) of Medium 199, NCTC-135, or CMRL-1066, or 7% each of all three tissue culture media. Cultures of *S. citri* were passed at 4-day intervals and CSO at 5-day intervals.

Even with the addition of the tissue culture media, the replacement of FBS with 5% BSF resulted in suboptimal or inadequate media for spiroplasma growth (Table 4, group 1). Growth of the spiroplasmas was reduced further when FYE and YATE were omitted from the 5% BSF medium (Table 4, group 2). Growth in M1A media without yeast supplements was reduced less than in media from which yeast supplements and FBS had been removed (Table 4, group 3 vs. groups 1 and 2). Medium 199 appeared to be a more satisfactory supplement for

TABLE 2. Growth of the corn stunt spiroplasma (CSO) and *Spiroplasma citri* in selectively deficient M1A spiroplasma media[a]

Experiment	Deletion	CSO		*S. citri*	
		Acid production[b]	Titer	Acid production[b]	Titer
1	None	7.85	9	9.15	8
	None	8.60	9	4.20	8
	Amino acids	6.80	9	0	5
	All organic acids	2.05	7	18.35	8
	All salts	2.50	8	17.75	9
	$CaCl_2$, $NaHCO_3$	6.15	9	15.20	9
2	None	8.20	nd	9.80	nd
	Amino acids	5.60	nd	1.37	nd
	All organic acids	4.60	nd	16.20	nd
	All organic acids except:				
	α-ketoglutaric acid	10.20	nd	12.20	nd
	succinic acid	1.20	nd	13.60	nd
3	None	9.0	nd	12.80	nd
	KH_2PO_4	3.2	nd	17.00	nd
	$MgSO_4 \cdot 7H_2O$	5.5	nd	13.5	nd
4	PPLO broth base	8.60	8	14.55	nd
	Tryptone and peptone	7.40	8	1.05	nd
	PPLO broth base and peptone	6.85	8	10.85	nd
	PPLO broth base and tryptone	4.60	9	11.35	nd
	PPLO broth base, peptone and tryptone	1.50	7	.80	nd
5	None	7.85	9	9.15	8
	PPLO broth base	7.40	9	5.15	7
	Tryptone	8.60	9	14.7	8
	Peptone	9.90	8	7.15	8

[a]Osmolalities of the treatments ranged from 438 to 555 mOsm. See Table 1 for composition of M1A spiroplasma medium.
[b]Milliliters of 0.01 N HCl required to lower the pH of 10 ml of noninoculated medium to the pH measured at the end of the fifth passage. The symbol nd = not done.

simplified media than NCTC-135, particularly for growth of CSO. None of the media variations was equivalent to M1A medium (Table 4, group 4) for spiroplasma growth.

Morphological changes in deficient media.—Cells growing in inadequate media often appeared distorted when examined by dark-field microscopy. Helical morphology of the organisms was less distinct in media of varying osmolality or salt composition; some turns of the helices tended to open, permitting more elongated configurations. In some media, there were large numbers of bodies without helical morphology; occasionally, relatively high CCU titers were observed from such cultures. The appearance of large numbers of helices, or growth to normal titers of 8 to 9 CCU in M1A medium, always was accompanied, so far as we were able to determine, by strong acid production.

Comparative growth of the corn stunt spiroplasma and Spiroplasma citri in deficient media.—Because Moroccan *S. citri* and the B clone 2 strain of CSO responded differently to certain modifications of M1A spiroplasma medium, we wished to determine if other strains of each agent, particularly isolates in early passage, would respond similarly. Three additional isolates of each spiroplasma were tested in 11 selected media (Table 5). To compare treatments, the results were standardized by dividing the acid production for each treatment by the acid production for that isolate in complete medium. The data for each spiroplasma were subjected to an analysis of variance with isolates as the replications.

Removal of the salt fraction, or KH_2PO_4 and $MgSO_4$ alone, from M1A medium influenced CSO growth less than in previous experiments, and was inconsistent in discriminating between spiroplasma isolates. However, all CSO isolates grew fairly well in M1A medium without the defined amino acid component, whereas *S. citri* isolates grew poorly in such media. Deletion of the organic acids, or all organic acids except succinic acid, or reduction of the osmolality of the complete medium to 300 mOsm was detrimental to growth of all the CSO strains, but was not detrimental to *S. citri* strains. Growth of CSO was comparable to that in complete M1A medium if only α-ketoglutaric acid alone, rather than the entire organic acids complement was used. In summary, several consistent differences were demonstrated in the cultural requirements of spiroplasma isolates of the *S. citri* and the CSO groups.

Primary isolations in deficient media.—A series of the deficient M1A medium variations from Table 5 were tested for their ability to support primary isolation of CSO from infected insects. Approximately 1-2 μliter of hemolymph were withdrawn from infected *Dalbulus elimatus* Ball leafhoppers after leg severance. One droplet from each insect was transferred to 5 ml of deficient or complete M1A medium. Primary isolations were obtained only in complete media, or in media with α-ketoglutaric acid (Table 6).

TABLE 3. Fifth-passage acid production by the corn stunt spiroplasma (CSO) and *Spiroplasma citri* in M1A spiroplasma medium containing different concentrations of fresh yeast extract and yeastolate[a]

Yeastolate (grams/liter)	Fresh yeast extract (ml/liter)	Acid production	
		CSO	*S. citri*
1[b]	33	8.2[c]	9.8
1	0	2.6	3.8
2	0	11.6	9.6
4	0	10.6	11.4
6	0	11.0	11.2
8	0	8.5	11.2
0	33	6.2	12.8
0	66	4.2	13.5
0	99	6.3	20.7
0	132	3.3	16.6

[a]Osmolality of the treatments ranged from 517 to 554 mOsm. See Table 1 for composition of M1A spiroplasma medium.
[b]Complete M1A spiroplasma medium.
[c]Milliliters of 0.01 N HCl required to lower the pH of 10 ml of noninoculated medium to the pH measured at the end of the fifth passage.

TABLE 4. Fifth-passage acid production by the corn stunt spiroplasma (CSO) B clone 2 and the Moroccan strain of *Spiroplasma citri* in M1A spiroplasma medium variations enriched with other tissue culture media

Medium variation		Acid production[b]	
		CSO	*S. citri*
Group 1: Deletion of fetal bovine serum and addition of 5% bovine serum fraction plus:			
Medium 199 (20%)		5.40	3.66
NCTC-135 (20%)		3.62	2.62
CMRL 1066 (20%)		4.71	3.14
None		4.00	4.00
	Mean	4.43	3.36
Group 2: Deletion of fresh yeast extract, yeastolate, and fetal bovine serum; addition of 5% bovine serum fraction plus:			
Medium 199 (20%)		1.33	3.00
NCTC-135 (20%)		0.00	2.00
CMRL 1066 (20%)		1.37	3.33
7% of each TC medium		0.71	2.00
	Mean	0.85	2.58
Group 3: Deletion of fresh yeast extract and yeastolate and the addition of:			
Medium 199		10.77	8.80
NCTC-135		7.80	6.80
	Mean	9.29	7.80
Group 4: Complete M1A spiroplasma medium		14.4	16.0

[a]Osmolalities of the media ranged from 507 to 579 mOsm. See Table 1 for composition of M1A spiroplasma medium.
[b]Milliliters of 0.01 N HCl required to lower the pH of 10 ml of noninoculated medium to the pH measured at the end of the fifth passage.

DISCUSSION

Indications that specific medium deletions resulted in serious cultural deficiencies for spiroplasmas were confirmed by (a) repetition of experiments; (b) testing four strains of each spiroplasma in the deficient media; and (c) by assessing the impact of these deletions on primary isolation of CSO. Differences between the growth rates of *S. citri* and CSO in a number of deficient media were significant when tested by statistical methods (Table 5).

The procedures for passing the organisms favored media to which spiroplasmas could rapidly adapt. Had we delayed passage, more cultures may have adapted to the modified media. Adaptation of spiroplasmas during extended passage in vivo and in vitro is well documented. For example, transfer of spiroplasmas in *Drosophila* hemolymph in vivo was accompanied by gradual increase of the achieved numbers of organisms (24, 25). Prolonged adaptation usually resulted in eventual loss of the ability of spiroplasmas to complete the natural biological cycle. Adapted isolates, however, retained the ability to multiply and reduce the longevity of insects (23). The ability of plant spiroplasmas to multiply after injection into insects of wide phylogenetic position (23), most of which have no evident relevance to the natural ecology of the organism, points to the composition of insect hemolymph as a principal clue for the composition of spiroplasma media. The prominent role of Schneider's *Drosophila* medium in the development of media for culture of leafhopper cells (3), and the adaptation of CSO to *Drosophila* hemolymph (25, 26) led to the formulation of M1 spiroplasma medium (26).

We now show that the M1 and M1A media contain several factors that contribute to adequacy for primary isolation and continuous growth of CSO, including the presence of organic acids, a favorable salt balance and osmolality, and the presence of yeastolate. Also, fetal

TABLE 6. Primary isolation of the corn stunt spiroplasma from insect hemolymph droplets transferred into complete and deficient variations of the M1A spiroplasma medium[a]

Medium variation	Primary isolations in: Deficient medium	Complete medium
Deletion of amino acids	0/9[b]	6/7
Lactalbumin hydrolysate (6.5 g/liter), amino acids deleted	0/8	1/3
No organic acids	0/8	7/9
α-ketoglutaric acid only	7/8	7/8
Succinic acid only	0/8	6/8
Fresh yeast extract (99 ml/liter), yeastolate deleted	0/10	6/6
Deletion of KH_2PO_4	0/4	3/4
Deletion of $MgSO_4 \cdot 7H_2O$	0/9	3/4
Deletion of inorganic salts	0/9	5/5
300 mOsm adjusted with sucrose	0/7	7/7

[a]See Table 1 for composition of M1A spiroplasma medium. Osmolality of complete medium was 541 mOsm.

[b]The numerator in the ratios is the number of successful isolations; the denominator is the number of isolations attempted. One hemolymph droplet of 1-2 μliter was transferred from each insect into 5 ml of a deficient and of a complete medium. Successful isolations were detected by indicator change and confirmed by dark-field microscopy.

TABLE 5. Fifth-passage acid production by four isolates of *Spiroplasma citri* and four isolates of the corn stunt spiroplasma in M1A spiroplasma medium variations selected for possible differentiation of the two spiroplasmas[a]

Medium variation	Acid production by corn stunt spiroplasma isolates[b] Miss G	Y4	B Clone 2	G3N	Mean ratio A_{d5}/A medium[c]	Acid production by *S. citri* isolates[b] Maroc	M435	Israel	C189	Mean ratio A_{d5}/A medium[c]
Deletion of amino acids	8.8	5.8	8.7	8.1	0.77	0.8	0.0	2.6	0.0	0.06**
Lactalbumin hydrolysate (6.5 g/liter), amino acids deleted	10.7	3.0	6.2	9.0	0.68	18.2	0.8	15.9	10.7	1.02
No organic acids	1.7	0.8	8.5	2.7	0.30**[d]	14.3	7.4	14.6	4.0	1.06
α-ketoglutaric acid only	12.6	10.6	13.2	10.3	1.16	14.3	5.3	14.2	8.0	1.08
Succinic acid only	1.0	0.5	8.0	7.4	0.38**	13.3	4.0	14.0	6.6	0.94
Deletion of KH_2PO_4	9.7	8.3	1.7	7.7	0.72	12.1	2.0	12.2	6.5	0.76
Deletion of $MgSO_4 \cdot 7H_2O$	8.6	7.0	12.1	8.8	0.89	13.8	8.4	14.0	6.3	1.18
Deletion of inorganic salts	8.1	4.4	9.8	7.6	0.71	11.6	4.5	12.2	6.5	0.90
300 mOsm adjusted with sucrose	0.7	0.5	7.0	2.0	0.22**	11.1	5.6	11.6	6.4	0.94
300 mOsm adjusted with sorbitol	0.4	0.2	6.2	1.6	0.18**	10.5	6.8	10.8	6.1	0.97
Complete M1A medium	11.1	7.2	12.3	10.6	1.00	14.5	4.4	14.5	6.9	1.00
LSD ($P = 0.05$)					0.35					0.45
LSD ($P = 0.01$)					0.47					0.61

[a]Osmolalities were adjusted to about 540 mOsm unless stated otherwise.

[b]Milliliters of 0.01 N HCl required to lower the pH of 10 ml of noninoculated medium to the pH measured at the end of the fifth passage.

[c]A_{d5} is the fifth-passage acid production in the deficient medium and A is acid production in M1A medium.

[d]Asterisks (**) indicate that the means differ significantly at $P = 0.01$ from the complete M1A medium.

bovine serum may be superior to horse serum for spiroplasma growth (Whitcomb, *unpublished*). Factors important to the growth of *S. citri* in this medium included free amino acids, fresh yeast extract, and osmolalities somewhat lower than that used by Saglio et al. (16, 17) in their medium. Fudl-Allah and Calavan (8) also noted the stimulatory effect of fresh yeast extract for *S. citri*.

The consistent reduction in CSO growth which occurred when organic acids were deleted from the medium is, as yet, unexplained. The effect may be largely attributable to α-ketoglutaric acid, since its addition to deficient media restored normal growth. The failure of succinic acid to replace α-ketoglutarate indicates that some of the tricarboxylic acid enzymes are probably absent. Although the presence of organic acids is critical to growth of CSO in M1 or M1A spiroplasma media, Liao and Chen (11) have cultivated CSO in a medium containing only horse serum, PPLO broth, and high concentrations of sucrose.

The free amino acid mixture from Schneider's *Drosophila* medium dramatically affected the growth of *S. citri* in M1A spiroplasma medium but exerted a lesser effect on CSO growth. At present, it is unknown whether a single amino acid or combinations of a few amino acids would stimulate growth. Lactalbumin hydrolysate at concentrations used in insect tissue culture media (22) could not be substituted for the free amino acid mixture in M1A medium, but *S. citri* particularly grew in media containing somewhat lower concentrations. This observation may be of considerable importance in attempts to cultivate other plant mycoplasmas (15).

It is important to stress that although our experiments demonstrate stimulation or depression of growth rate, they do not establish growth requirements for spiroplasmas. The concept that such requirements are easily determined (19) is erroneous. Demonstration of such requirements would best be done in defined or semisynthetic media; failing this, chemical analysis of the medium for the substance, and preferably for its possible precursor, would be necessary. Only in this manner could the significance of the substance be unequivocally demonstrated. Unfortunately, there may be a conversion of substances after fabrication of complex media, especially if active enzymes are present. Such complexities discouraged us, temporarily, at least, from attempting the rigorous techniques required to demonstrate the exact physiological role of amino acids or organic acids in spiroplasma metabolism.

The major focus of our study was practically oriented, aimed at a broad understanding of the role of definable components for spiroplasma growth, in the hope that such empirical knowledge may lead to successful cultivation of other fastidious mycoplasmas. In this regard, success already has been achieved. The cultivation of the spiroplasma causing the suckling-mouse cataract syndrome of rats and mice, a new class of vertebrate pathogen (21), which was noncultivable by previously available means, now has been achieved (20) in media based on concepts developed in this study. Perhaps other fastidious pathogens also may yield to cultivation in media based on approaches derived from our experiments.

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