Cultivation of *Phytomonas davidi* (Trypanosomatidae) in Serum-Free and in Chemically Defined Media

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ABSTRACT

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Phytomonas davidi (Trypanosomatidae), a flagellated protozoan found in latex vessels of plants in the Euphorbiaceae, was cultured in a new chemically defined medium (P201) as well as in complex undefined media in which serum was replaced by a mixture of lipid-extracted bovine serum albumin, cholesterol, fatty acids, phospholipids, hemin chloride, and Tween 80. Titers of the protozoan reached over 10^8 cells per milliliter from starting titers of 10^2–10^3 per milliliter. Keto acids (α-ketoglutarate and pyruvate) and nucleosides and bases promoted growth. Growth was inhibited by cysteine in some media. A strict requirement for hemin was observed. Chemically defined and serum-free media present new opportunities for research on nutritional requirements of *P. davidi*, and offer a new approach toward eventual cultivation of protozoans associated with plant diseases.

Additional key words: growth requirements, latex, parasite, plant disease, trypanosome.

Phytomonas davidi Lafont is a parasitic protozoan that inhabits the laticifers of plants in the Euphorbiaceae. Other plant parasitic protozoans include *P. elmassiani* Migone (an inhabitant of latex vessels in *Asclepias*), as well as the presumed plant pathogens *P. leptovasorum* Stahel and *P. staheli* McGhee & McGhee (inhabitants of phloem in *Coffea liberica* Hiern and in *Cocos nucifera* L., respectively) (2,11,12,15,21). Great interest has been stirred during the past decade by the discovery of uniflagellate protozoans associated with plant diseases (2,11–13,15,19–21). However, rigorous tests of the possible roles of these protozoans in plant disease have been hampered by inability to grow the suspected pathogens in pure culture. Of the latex vessel-inhabiting protozoans, few have thus far been obtained in pure culture (2,3,10,13). Studies of these cultivated strains could provide knowledge useful for cultivation in vitro of additional strains from latex vessels and perhaps eventually of plant pathogenic strains from phloem.

Media containing serum or blood have been employed in detailed studies of *P. davidi* (2,3,10,13) in culture, but use of media lacking serum, or use of chemically defined media, would be advantageous for research on this plant parasite. In this article, we briefly describe the removal of serum from a complex, chemically undefined medium and replacement of the serum with a mixture of lipids, Tween 80, bovine serum albumin (BSA), and hemin for culture of *P. davidi*. We further report culture of *P. davidi* in a chemically defined medium (6).

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TABLE 1. Serum-free medium P10 for cultivation of *Phytomonas davidi*

Ingredient	Amount per liter
Inorganic salt	
$MgSO_4 \cdot 7H_2O$	0.2 g
Amino acids	
L-Arginine	0.6 g
L-Asparagine	0.6 g
L-Glutamine	0.6 g
Methionine	0.4 g
Keto acids	
α-Ketoglutaric acid	0.4 g
Pyruvic acid	0.4 g
Carbohydrates	
Fructose	4.0 g
Sucrose	90.0 g
PPLO broth base (DIFCO)	12.0 g
Yeastolate (DIFCO)	1.5 g
HEPES (buffer)	15.0 g
Serum substitute	
Palmitic acid	14.8 mg
Linoleic acid	16.5 mg
Phosphatidic acid (egg yolk)	4.9 mg
Phosphatidylcholine (egg yolk)	7.0 mg
Lysophosphatidylcholine (egg yolk)	4.2 mg
Lysophosphatidylcholine (soybean)	4.2 mg
Cholesterol	9.8 mg
Tween 80	0.148 ml
Bovine serum albumin (lipid-free)	9.0 g
Hemin chloride	10.0 mg

MATERIALS AND METHODS

A culture of *P. davidi* was kindly provided by R. E. McCoy (University of Florida Agricultural Research and Education Center, Fort Lauderdale). This culture is a subculture of ATCC 30287, the strain reported by McGhee and Postell (13) as isolated from laticifers in ethanol-washed stems of *Euphorbia cyathophora* Murray. We initially maintained the parasite by serial subculture in a serum-containing medium (LD8A) (8) at 30 C. To test growth-supporting ability of culture media, unless specified otherwise, 1.8- or 4.5-ml aliquots of broth media in 13-mm-diameter screw cap glass tubes were each seeded with 0.2 or 0.5 ml, respectively, of a 2- to 3-day-old culture of *P. davidi* diluted 10,000-fold in sterile 10% sucrose solution. This resulted in an initial titer of 10^2–10^3 trypanosomes (cells) per milliliter in medium formulations that were tested and a minimal carryover of growth factors into test formulations from media used for propagating the protozoan. The cultures were briefly stirred on a vortex mixer and incubated aerobically without shaking at 30 ± 0.5 C, unless specified otherwise. To determine temperatures permitting growth, portions of broth media were similarly seeded with a diluted culture of *P. davidi* and were incubated at 20, 25, 30, or 35 C. To determine titers of trypanosomes in cultures, 0.2-ml aliquots were removed from cultures, the trypanosomes killed and fixed by addition of 0.2–0.6 ml of 4% glutaraldehyde in 0.01 M phosphate buffer (pH 7), and then counted with the aid of a hemacytometer. The lower limit of titer directly determinable by this method was 10^4 trypanosomes per milliliter.

Media. A serum-free medium (P10) (Table 1) containing

TABLE 2. Composition of chemically defined medium P201

Ingredient	Amount	Ingredient	Amount	Ingredient	Amount
Amino acids (mg/L)		HEPES buffer[a] (g/L)	17.0	Cofactors and nucleotides (mg/L)	
L-Alanine	400			Coenzyme A	2.5
β-Alanine	100	Nucleosides and bases (mg/L)		NAD	2.5
L-Aspartic acid	1,000	Adenosine	45.0	NADP	2.5
L-Asparagine	600	Guanosine	30.0	FAD[b]	3.0
L-Arginine	600	Cytidine	30.0	UTP	2.0
L-Glutamic acid	1,800	Thymidine	30.0	Glutathione (reduced)	60.0
L-Glutamine	600	Uridine	30.0		
Glycine	400	Inosine	30.0	Lipids (mg/L)	
L-Histidine	400	5-Methylcytosine	4.5	Palmitic acid	14.8
L-Isoleucine	400			Linoleic acid	16.5
L-Leucine	1,000	Vitamins (mg/L)		Phosphatidic acid (egg yolk)	4.9
L-Lysine	400	Biotin	1.0	Phosphatidylcholine (egg yolk)	7.0
L-Methionine	400	Calcium pantothenate	2.0	Lysophosphatidylcholine (egg yolk)	4.2
L-Phenylalanine	500	Choline chloride	1.5	Lysophosphatidylcholine (soybean)	4.2
L-Proline	1,000	Folic acid	2.0	Cholesterol	9.8
L-Hydroxyproline	200	i-Inositol	1.5		
L-Serine	400	Niacin	2.0	Tween 80 (ml/L)	0.148
L-Threonine	200	Nicotinamide	2.0		
L-Tryptophan	200	*p*-Aminobenzoic acid	2.0	Bovine serum albumin (lipid-free) (g/L)	9.0
L-Tyrosine	200	Pyridoxine hydrochloride	2.0		
L-Valine	400	Pyridoxal hydrochloride	1.0	DL-Glycerophosphate (sodium salt) (mg/L)	400.0
		Pyridoxal phosphate	1.0		
Organic acids (mg/L)		Riboflavin	2.0	Glycerol (ml/L)	0.24
α-Ketoglutaric acid	400	Riboflavin-5-phosphate	0.5		
Pyruvic acid	400	Thiamine hydrochloride	1.0	Spermidine (mg/L)	1,000.0
		Thiamine pyrophosphate	2.0		
				Hemin chloride (mg/L)	10.0
Inorganic salts (mg/L)					
KH_2PO_4	400				
$MgSO_4 \cdot 7H_2O$	800				
NaCl	4,640				
$CaCl_2$	200				
Carbohydrates (g/L)					
Fructose	4.0				
Glucose	1.0				
Sucrose	35.0				

[a] HEPES = N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
[b] FAD = Flavin adenine dinucleotide.

undefined components was formulated by modifying the *Spiroplasma* culture medium LD8A (8). The modification consisted of deletion of cysteine, reduction of HEPES concentration, addition of $MgSO_4 \cdot 7H_2O$ (0.2 g/L), and replacement of serum with a serum substitute containing BSA (code no. 81-066-2, Miles Laboratories, Inc., Elkhart, IN), cholesterol, fatty acids, phospholipids, Tween 80, and hemin chloride. The composition of P10 is similar to that of *Spiroplasma* culture medium LD57 (I.-M. Lee and R. E. Davis, *unpublished*) except for the absence of cysteine and the presence of hemin chloride in P10. All components except keto acids, supplemental amino acids, lipids, and BSA were dissolved in distilled water, the pH adjusted to 7.4 with 2 N NaOH, and sterilized by autoclaving. The remaining components were prepared as separate stock solutions sterilized by filtration through 0.2-μm pore diameter membrane filters. Lipids and BSA were prepared as described (7). BSA was defatted by five extractions with excess (20-fold [v/w]) chloroform:methanol (2:1), as described previously (7).

For preparation of chemically defined medium P201, medium LD82, previously described for culture of *Spiroplasma* (7), was modified to yield the formulation given in Table 2. BSA used in the chemically defined medium was defatted as above.

RESULTS

P. davidi grew well in serum-containing medium LD8A at 30 C, reaching titers of over 10^7 trypanosomes per milliliter in repeated experiments. Subsequently, experiments were conducted to determine the influence of temperature from 20 to 35 C on growth of the parasite in this medium and in two modified versions of the medium, one (LD8A-AA) lacking all five supplemental amino acids found in LD8A and the other (LD8A-CA) lacking cysteine and arginine. *P. davidi* grew at 20, 25, and 30 C, but little or no growth was observed at 35 C (Table 3). Growth was heaviest at 30 C. In one of four experiments, however, turbid growth developed in one of two cultures incubated at 35 C; subcultures from this tube grew well at 30 and 35 C. Subsequent experiments directed toward culture of the organism in serum-free medium and in chemically defined medium were performed at 30 C. In the first series of these experiments, serum was replaced in medium LD8A by a mixture of lipids, Tween 80, BSA, and hemin chloride.

Requirement for hemin. A strict requirement for hemin was observed in serum-free medium P10 (Table 4). Similar results were obtained in three separate experiments. Titers of *P. davidi* reached over 10^7 cells per milliliter when supplied with 0.001% hemin chloride. No detectable growth occurred without hemin or in the presence of 0.000001 or 0.00001% hemin chloride. Growth occurred at 0.0001, 0.001, and 0.01% hemin chloride and was similar at 0.001 and 0.01%. Serum-free media and chemically defined media subsequently were supplemented with 0.001% hemin chloride, because the higher concentration (0.01%) appreciably darkened the media. No growth of *P. davidi* was observed in chemically defined media unless media were supplemented with hemin.

Keto acid, yeastolate, and amino acid supplements in serum-free medium. Results from one of two separate experiments are given in Table 5. Removal of yeastolate or of supplemental amino acids had relatively little effect on growth in the serum-free medium P09 (= P10 plus 0.6 g of cysteine per liter) containing undefined components. However, deletion of pyruvate and α-ketoglutarate from the medium resulted in greatly reduced growth. From initial titers of 1.1×10^2 cells per milliliter, titers reached nearly 10^8 trypanosomes per milliliter after 6 days in medium containing the keto acids, but reached only about 10^6 per milliliter in medium lacking the keto acids.

Growth in chemically defined medium. Initially, a chemically defined medium (LD82) designed for culture of *Spiroplasma* spp. was modified by the addition of hemin chloride (10 mg/L). The new medium was termed P200. Medium P200 was further modified by deletion from the medium of five different amino acids singly or in groups of four or five, and the resulting media, including medium P200, were tested for ability to support growth of *P. davidi*. Table 6 gives data from one of two separate

TABLE 3. Influence of temperature of incubation on growth of *Phytomonas davidi* in three serum-containing broth media

Temperature of incubation (C)	Medium[a]	*P. davidi* cells ($\times 10^{-4}$) after incubation (no./ml)[b] 2 Days	4 Days
20	LD8A	3	44
	LD8A-AA	3	52
	LD8A-CA	4	74
25	LD8A	9	631
	LD8A-AA	11	667
	LD8A-CA	35	690
30	LD8A	100	1,725
	LD8A-AA	172	3,679
	LD8A-CA	189	2,112
35	LD8A	1	1
	LD8A-AA	1	2
	LD8A-CA	1	1

[a] Medium LD8A contains five supplemental amino acids (arginine, asparagine, cysteine, glutamine, and methionine). All five deleted to yield LD8A-AA; deletion of cysteine and arginine from LD8A yielded LD8A-CA.

[b] Average of two cultures. Initial titer of each culture was 3.9×10^2 cells per milliliter. Volume of each culture 5 ml.

TABLE 4. Influence of hemin on growth of *Phytomonas davidi* in serum-free medium P10 at 30 C

Hemin chloride (%)	*P. davidi* cells ($\times 10^{-4}$) after incubation[a] (no./ml) 3 Days	4 Days
0.01	1,900	3,750
0.001	1,000	3,930
0.0001	880	2,250
0.00001	2	0
0.000001	0[b]	0
None	0	0
Serum-containing control[c]	3,680	4,120

[a] Initial titer was 1.6×10^3 cells per milliliter in each medium.

[b] 0 Indicates no *P. davidi* cells observed in hemacytometer. Titer less than 10^4 cells per milliliter.

[c] Serum-containing control medium consisted of medium LD8A supplemented with 10% fetal bovine serum, but with supplemental amino acids deleted.

TABLE 5. Influence of keto acids, yeastolate, and supplemental amino acids on growth of *Phytomonas davidi* in serum-free broth medium P09 at 30 C

Component deleted from medium P09[a]	Cells ($\times 10^{-4}$) after incubation[b] (no./ml) 4 Days	6 Days
None	2,900	5,100
Pyruvate, α-ketoglutarate	66	150
Yeastolate	1,400	6,500
5 Amino acids[c]	2,700	8,100
Arginine	1,900	6,000
Asparagine	860	3,400
Cysteine	1,400	9,400
Glutamine	1,500	8,600
Methionine	2,200	8,900

[a] Medium P09 consisted of medium P10 plus cysteine (0.6 g/L). All media above contained 0.001% hemin chloride.

[b] Titer at time 0 was 1.1×10^2 cells per milliliter in each medium.

[c] Deletion of all five supplemental amino acids (arginine, asparagine, cysteine, glutamine, and methionine) from medium P09.

experiments that gave similar results. Little growth occurred in medium P200 without deletions of certain amino acids. Appreciable growth occurred when arginine, asparagine, glutamine, and cysteine were deleted from the medium, but greatest growth occurred when cysteine alone was deleted. Titers in the defined medium lacking cysteine (termed medium P201) reached 10^8 trypanosomes per milliliter (Table 6).

Chemically defined medium P201, which lacks cysteine, was then employed for extended subcultures of *P. davidi* in order to determine usefulness of the medium for long-term culture of the parasite. At the same time, variations in the medium were examined by deletion of nucleotides and bases or by reducing the level of cholesterol in the medium by one-half. The resultant media, P217 (equivalent to P201 without nucleotides and bases), medium P219 (equivalent to P201 with cholesterol content reduced to 4.9 mg/L), and medium P201 supported the growth of *P. davidi* for no less than 60 serial subcultures (Table 7). Titers of close to 10^7 trypanosomes per milliliter in medium P217 and of close to or more than 10^8 trypanosomes per milliliter in media P201 and P219 were consistently reached within 2–4 days of incubation (Table 7). The pH of cultures decreased from an initial value of 7.5 to a final pH of about 4.8 by the seventh day of incubation. Growth in medium P201 was heaviest at 30 C, with little growth at 35 C (Table 8).

Growth curves of *P. davidi* in media P201, P217, and P219 are shown in Figure 1. Maximum titers (over 10^8 cells per milliliter) and growth rates of the protozoan were similar in P201 and P219, but *P. davidi* reached only slightly more than 10^7 cells per milliliter in medium P217. Average doubling times during log phase increase in media P217, P219, and P201 were about 6, 5, and 5 hr, respectively.

TABLE 6. Influence of selected amino acids on growth of *Phytomonas davidi* in chemically defined medium P200 at 30 C

Amino acid(s) deleted from medium P200[a]	Cells ($\times 10^{-4}$) after incubation[b] (no./ml)		
	3 Days	5 Days	8 Days
None	16	18	22
Arg, asp, cyst, glut, meth	6	6	22
Asp, glut, cyst, meth	2	6	20
Arg, glut, cyst, meth	0	10	32
Arg, asp, glut, meth	0	4	10
Arg, asp, cyst, meth	0	8	26
Arg, asp, glut, cyst	50	440	2,100
Arginine	6	10	48
Asparagine	6	10	28
Cysteine	180	2,800	10,000
Glutamine	4	6	20
Methionine	2	4	4
Control[c]	750	4,300	8,800

[a] Medium P200 consists of medium P201 plus cysteine (0.6 g/L). Arg, arginine; glut, glutamine; asp, asparagine; cyst, cysteine; meth, methionine.
[b] Initial titer was 1.1×10^2 cells per milliliter in each medium.
[c] Serum-containing control medium LD8A with deletion of five supplemental amino acids (arginine, asparagine, glutamine, cysteine, and methionine).

TABLE 7. Serial subculture of *Phytomonas davidi* in chemically defined media[a]

Medium designation	Subculture no.	Duration of incubation (days)	Cells ($\times 10^{-5}$) after incubation at 30 C (no./ml)
P219[b]	20	2	818
	30	4	1,120
	35	2	613
	50	4	1,120
	55	4	1,290
	60	4	1,210
	65	2	890
	70	3	1,100
	75	3	1,120
P201	21	2	1,150
	29	3	2,910
	36	1	896
	40	2	1,560
	50	2	1,260
	60	2	1,120
	65	2	1,000
	70	2	970
	75	2	1,110
P217[c]	14	2	68
	15	3	122
	16	2	93
	40	4	250
	45	2	262
	50	4	230
	55	4	240
	60	4	200
	63	4	257

[a] Organisms were subcultured at 2- to 4-day intervals by passing 0.05 or 0.1 ml of culture into 1.8 ml of sterile medium in 13-mm-diameter culture tubes. Titers were determined at time of subculturing.
[b] P219 contained half the concentration of cholesterol in P201.
[c] Deletion of nucleosides and bases from P201 yielded P217.

Growth curves of *P. davidi* in media P201, P217, and P219 are shown in Figure 1. Maximum titers (over 10^8 cells per milliliter) and growth rates of the protozoan were similar in P201 and P219, but *P. davidi* reached only slightly more than 10^7 cells per milliliter in medium P217. Average doubling times during log phase increase in media P217, P219, and P201 were about 6, 5, and 5 hr, respectively.

DISCUSSION

The plant parasitic *P. davidi* ATCC 30287 was cultured in the absence of serum. The new media developed for this culture are of two types. One is simply termed serum-free; its components include yeastolate and PPLO broth base, a major undefined ingredient containing beef heart infusion and peptone. The other is chemically defined. Serum-free medium P10 and chemically defined media P201 and P219 are now being used in our laboratory for routine subculture and nutritional investigations of *P. davidi*.

In this study, a serum-containing medium (LD8A) designed for culture of *Spiroplasma* spp. (8) was modified principally by deletion of cysteine, reduction of HEPES concentration, addition of $MgSO_4 \cdot 7H_2O$, and by replacement of serum with a mixture of BSA, cholesterol, fatty acids, phospholipids, Tween 80, and hemin chloride to yield the medium termed P10. The mixture of BSA, lipids, and Tween 80 had the same composition as that in a chemically defined medium (LD8A) for culture of *Spiroplasma* (7). However, the addition of hemin to the serum substitute was a critical requirement for the growth of *P. davidi*. When hemin chloride was present in concentrations of 0.001% or greater, titers of the protozoan reached more than 10^8 cells per milliliter, from initial titers of 10^2–10^3 trypanosomes per milliliter.

No growth occurred in serum-free or in chemically defined media unless supplemented with hemin chloride. In other work, *P. davidi* grew in medium LD8A with serum when five supplemental amino acids were deleted from that medium (R. E. McCoy, *personal communication*). In the present work, deletion of supplemental amino acids from serum-containing medium LD8A or from serum-free medium P10 influenced the growth of *P. davidi* only minimally. However, deletion of the supplemental keto acids (α-ketoglutarate and pyruvate) from the serum-free medium markedly reduced growth of *P. davidi*, indicating that keto acids are important for growth of this protozoan in P10.

TABLE 8. Influence of temperature of incubation on growth of *Phytomonas davidi* in chemically defined medium P201

Temperature of incubation (C)	Cells ($\times 10^{-4}$)[a] (no./ml)	
	4 Days	7 Days
20	5	34
25	41	5,040
30	1,135	9,330
35	1	1

[a] Each value is the average from two separate cultures. Initial titer of each culture was 1.8×10^2 cells per milliliter.

Serum-free medium P10 offers an alternative to use of serum-containing media for culture of *P. davidi*. Although we and others have found *P. davidi* grows well in several media supplemented with serum (2,9,10), some disadvantages may be associated with the use of serum in media for cultivation of this parasite. Similar disadvantages could apply to attempts to culture in vitro other plant parasitic trypanosomatids. For example, lots of sera may differ in growth supporting abilities; and high-quality serum occasionally can be difficult to obtain or unavailable in some parts of the world. In addition, animal sera contain components inhibitory to growth of some microorganisms requiring serum (5), are sometimes contaminated with microorganisms or viruses (1,14), and could contain components that tend to inhibit the growth of fastidious microbes that do not naturally occur in vertebrate hosts. Potential disadvantages of sera suggest that the use of media lacking sera could also provide an important alternative approach toward eventual cultivation in vitro of plant parasitic protozoans that are more fastidious than the strains of *P. davidi* now in culture. The phloem inhabitants *P. leptovasorum* and *P. staheli* are principal among the plant parasitic protozoans for which establishment of in vitro cultures would greatly aid the progress of research. It must be noted, however, that even in the case of latex inhabitants, few successful cultures have been obtained from infected plants, in spite of numerous attempts to establish new isolates of this parasite in culture (2,3,13).

It should also be noted that the identity of ATCC 30287 as a *Phytomonas* sp. and the authenticity of its original isolation from the interior of a plant by McGhee and Postell (13) have been questioned by Dollet (2). Unpublished similarities in some properties between ATCC 30287 and an unspecified, unnamed insect *Herpetomonas* have been cited in support of these questions (2). However, little is known about the characteristics of plant parasitic protozoans, and no data supporting the contention that strain ATCC 30287 is other than a *Phytomonas* have been published. Until recently, only strain 30287 has been available for extensive characterization. Strains in coconut, oil palm, coffee, and manioc have not yet been characterized, nor have *Phytomonas* isolates obtained in culture from *E. pinea* or *E. characias* (3). Detailed definition of the taxonomic characteristics of *Phytomonas* thus awaits further study. The conclusion by McGhee and Postell (13) that strain ATCC 30287 was isolated from the interior of their source plant is supported by the fact that the plant tissue was washed with 70% ethanol before isolation of the protozoan in culture (13).

In the present study, *P. davidi* was grown in several chemically defined media (P217, P219, and P201) as well as in chemically undefined media in which serum was replaced by a defined mixture of BSA, lipids, and hemin. The titers reached in these media were comparable to or greater than titers reported for this protozoan in media containing serum (4,9,10). Although *P. davidi* was noted to grow previously in a chemically defined medium, the work was preliminary, and data on this organism were not specified (4). Fish et al (4) included *P. davidi* among 26 species of trypanosomatids tested for growth in a chemically defined medium of Steiger and Steiger (17,18). Nineteen species, including *P. davidi*, grew for 10 subcultures, but specific data on growth rates, peak titers, or nutritional requirements were not provided. Titers of *P. davidi* apparently reached at least 10^7 cells per milliliter from a starting titer of 10^6 per milliliter in that work (4), but titers of *P. davidi* in the present work reached more than 10^8 cells per milliliter from starting titers of only about 10^2 cells per milliliter in chemically defined media P201 and P219.

Cultivation of *P. davidi* in a chemically defined medium offers unprecedented opportunities for investigation of nutritional requirements of this parasite. Our studies of *P. davidi* demonstrating a requirement for hemin, promotion of growth by pyruvate and α-ketoglutarate in serum-free medium, and inhibition of growth by cysteine in chemically defined medium, encourage further investigations of components in defined medium P201. The formulation of medium P201 conceivably can be simplified considerably in the development of a minimal medium for study of growth requirements of *P. davidi*.

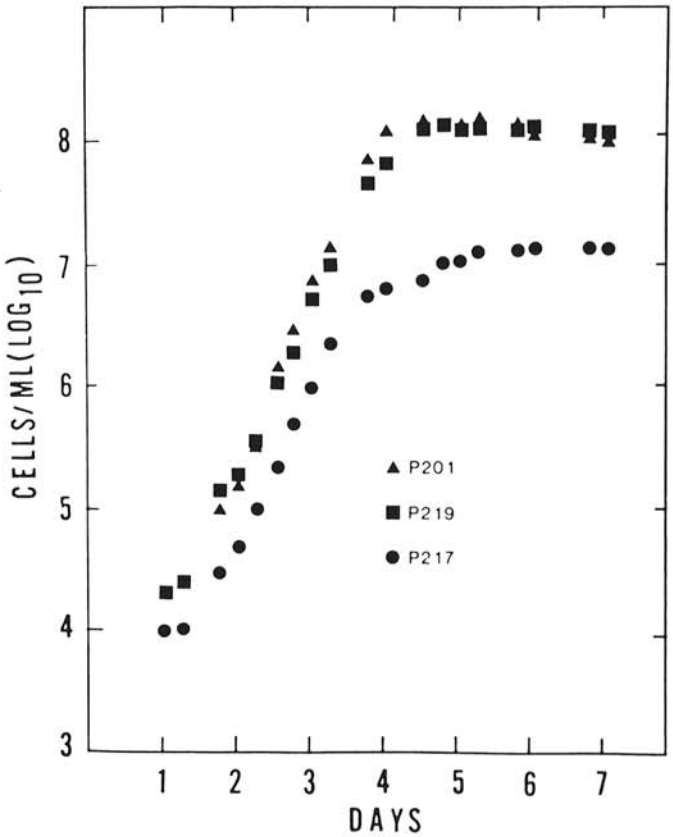

Fig. 1. Growth of *Phytomonas davidi* in chemically defined media P201, P219, and P217. Each point represents the mean from two separate cultures incubated aerobically at 30 C. Starting titer in all cultures was 3×10^2 cells per milliliter.

Failure of *P. davidi* to grow consistently at 35 C in our work was unexpected in light of reports by McCoy (9,10). Our culture was derived from that reported by McCoy (9,10) to grow well at 35 and 37 C. This difference in experimental results possibly could be attributable to selection, through culture at 30 C, of organisms less well adapted for growth at 35–37 C. Our observation of the occasional growth of *P. davidi* at 35 C is consistent with this hypothesis. Another factor possibly contributing to differences in temperatures permitting growth could be the use of culture media differing in composition. Work with other protozoans as well as with *P. davidi* has shown that medium composition can determine permissible temperatures for growth (4,16). McCoy (9,10) used a serum-containing medium at 37 C, whereas we employed a different serum-containing medium and a serum-free undefined medium as well as chemically defined media, and we maintained cultures at 30 C unless temperature ranges were being tested. For the original isolation of the *P. davidi* used in this and other studies (9,10), McGhee and Postell (13) incubated cultures at 28 ± 1 C.

LITERATURE CITED

1. Barile, M. F., Hopps, H. E., and Grabowski, M. W. 1978. Incidence and sources of mycoplasma contamination: A brief review. Pages 35-45 in: Mycoplasma Infection of Cell Cultures. G. J. McGarrity, D. G. Murphy, and W. W. Nichols, eds. Plenum Publ. Corp., New York.
2. Dollet, M. 1984. Plant diseases caused by flagellate protozoans. Annu. Rev. Phytopathol. 22:115-132.
3. Dollet, M., Cambrony, D., and Gargani, D. 1982. Culture axénique in vitro de *Phytomonas* sp. (Trypanosomatidae) d'Euphorbe, transmis par *Stenocephalus agilis* Scop (Coreide). C. R. Acad. Sci. 295: Serie III, 547-550.
4. Fish, W. R., Holz, G. G., Jr., and Beach, D. H. 1978. Cultivation of trypanosomatids. J. Parasitol. 64:546-547.
5. Igwegbe, E. C. K. 1978. High concentrations of horse serum inhibit growth of corn stunt spiroplasma. Appl. Environ. Microbiol. 35:146-148.
6. Konai, M., Davis, R. E., and Lee, I.-M. 1985. Nutrition of the plant

parasitic protozoan, *Phytomonas davidi*, in serum-free and chemically defined media. Phytopathology 75:1380.
7. Lee, I.-M., and Davis, R. E. 1983. Chemically defined medium for cultivation of several epiphytic and phytopathogenic spiroplasmas. Appl. Environ. Microbiol. 46:1247-1251.
8. Lee, I.-M., and Davis, R. E. 1984. New media for rapid growth of *Spiroplasma citri* and corn stunt spiroplasma. Phytopathology 74:84-89.
9. McCoy, R. E. 1978. Growth of the plant parasitic protozoan *Phytomonas davidi* in mycoplasma media and in plant phloem sap. Phytopathol. News 12:217.
10. McCoy, R. E. 1983. Sensitivity of *Phytomonas davidi* to antimicrobial substances. Plant Dis. 67:855-857.
11. McCoy, R. E., and Martinez-Lopez, G. 1982. *Phytomonas staheli* associated with coconut and oil palm diseases in Columbia. Plant Dis. 66:675-677.
12. McGhee, R. B., and McGhee, A. H. 1979. Biology and structure of *Phytomonas staheli* sp. n., a trypanosomatid located in sieve tubes of coconut and oil palm. J. Protozool. 26:348-351.
13. McGhee, R. B., and Postell, F. J. 1976. Axenic culture of *Phytomonas davidi* Lafont (Trypanosomatidae), a symbiote of laticiferous plants (Euphorbiaceae). J. Protozool. 23:238-241.
14. Mollander, C. W., Paley, A., Boon, C. W., Kniazeff, A. J., and Imagawa, D. T. 1978. Studies on virus isolation from fetal calf serum. In Vitro 4:148.
15. Parthasarathy, M. V., van Slobbe, W. G., and Soudant, C. 1976. Trypanosomatid flagellate in the phloem of diseased coconut palms. Science 192:1346-1348.
16. Roitman, C., Roitman, I., and de Azevedo, H. P. 1972. Growth of an insect trypanosomatid at 37 C in a defined medium. J. Protozool. 19:346-349.
17. Steiger, R. F., and Steiger, E. 1976. A defined medium for cultivating *Leishmania donovani* and *L. braziliensis*. J. Parasitol. 62:1010-1011.
18. Steiger, R. F., and Steiger, E. 1977. Cultivation of *Leishmania donovani* and *L. braziliensis* in defined media: Nutritional requirements. J. Protozool. 24:437-441.
19. Thomas, D. L., McCoy, R. E., Norris, R. C., and Espinoza, A. S. 1979. Electron microscopy of flagellated protozoa associated with marchitez sorpresiva disease of African oil palm in Ecuador. Phytopathology 69:222-226.
20. Vanstein, M. H., Roitman, I., and Kitajima, E. W. 1984. "In vitro" cultivation of a flagellate (*Phytomonas* sp.) associated with a manioc (*Manihot esculenta*) disease - Programe e Resumos de Communicacoes, XI Reuniao anual Pesquisa Básica em Doença de Chagas, Caxambu, 20–22 Nov., p. 37. (Abstr.)
21. Waters, H. 1978. A wilt disease of coconuts from Trinidad associated with *Phytomonas* sp., a sieve tube - restricted protozoan flagellate. Ann. Appl. Biol. 90:293-302.