INFECTION AND IMMUNITY, Feb. 1982, p. 449–455
0019-9567/82/020449-07$02.00/0

Vol. 35, No. 2

Further Studies on Replication of Virulent *Treponema pallidum* in Tissue Cultures of Sf1Ep Cells

A. HOWARD FIELDSTEEL,* DAVID L. COX, AND RANDOLPH A. MOECKLI

Life Sciences Division, SRI International, Menlo Park, California 94025

Received 24 August 1981/Accepted 14 October 1981

A number of parameters aimed at optimizing culture conditions for both Sf1Ep cells and *Treponema pallidum* have been investigated. Optimum temperature for replication of *T. pallidum* ranged between 33 and 35°C. At 33°C, replication occurred in the presence of atmospheric oxygen concentrations of <0.3 to 10%, the optimum range being 1.5 to 5%. No replication occurred in the presence of 12.5% oxygen. When both temperature and oxygen concentrations were varied between 33 and 35°C and 1.5 to 5%, respectively, little differences in replication were noted. Although variation in the oxygen concentration within each temperature group had little or no effect on replication, it did have an effect on motility, which remained greater in the 5% oxygen concentration after 9 to 12 days of cultivation. Optimum concentration of fetal bovine serum in the culture medium was 20%, although replication occurred in concentrations ranging from 5 to 30%. If carefully screened, calf serum could be substituted for fetal bovine serum. Testis extract was an essential component of the culture medium. Although extract obtained from an adult rabbit—either normal or *T. pallidum* infected—was slightly superior, replication of *T. pallidum* occurred when rat or hamster testis extract was substituted.

We previously reported replication of the virulent Nichols strain of *Treponema pallidum* in tissue cultures of cottontail rabbit epithelium (Sf1Ep) cells (3). In a series of seven experiments, we were able to show that *T. pallidum* attaches and replicates on the surface of Sf1Ep cells growing in conventional monolayer cultures under an atmosphere of 1.5% oxygen at 33°C.

The extent of treponemal multiplication is dependent on the initial inoculum of *T. pallidum*; it is greatest when that inoculum is 10^6 and least when the inoculum is 10^8. However, for all of the inocula, the maximum ceiling of multiplication is 1×10^8 to 2×10^8. Thus, the fold increases of replicating *T. pallidum* decreases with increasing inoculum size. When the initial inoculum is 10^6, there is an average increase of 49-fold. When the inoculum is 10^7, the average increase is 11.8-fold. For the 10^8 inoculum, the greatest increase is only 2.4-fold, indicating little or no replication. It seems likely that the ceiling of multiplication is due to a combination of factors, including exhaustion in the medium of some essential components, the accumulation of toxic products, and exhaustion of oxygen, which both cells and treponemes need for survival and replication.

Although replication in our previous experiments was limited to some extent by the beginning of deterioration of the Sf1Ep cell sheet by the 12th day of incubation, it seemed possible that by optimizing the culture conditions for both cells and treponemes, the treponemal yield could be increased. Toward that end we have examined a number of parameters, including temperature, oxygen, serum, and rabbit testis extract requirements for the culture system.

MATERIALS AND METHODS

Animals. New Zealand white male rabbits (6 to 8 months old) that were free of treponemal infection, as determined by the Venereal Disease Research Laboratory test, were used for testicular passage of *T. pallidum*, as a source of treponemes for inoculation into tissue culture, and as a source of testis extract.

Young male rabbits (6 weeks old), adult Lewis rats, and adult Golden Syrian hamsters were also used as sources of testis extract.

***T. pallidum*.** The virulent Nichols strain was used throughout these studies and was passaged as described previously (2).

Tissue culture cells. An established cell line of cottontail rabbit epithelium (Sf1Ep) was used throughout this study. It was received from W. A. Nelson-Rees and was produced with support from the Division of Cancer Cause and Prevention, Biological Carcinogenesis Branch, National Cancer Institute, under the auspices of the Office of Naval Research and Regents of the University of California. Passage levels utilized in these experiments ranged from 73 to 85.

Sera. Fetal bovine serum (FBS), obtained from Flow Laboratories, Inc., Rockville, Md., was screened for suitability for use in tissue culture medi-

um as described earlier (4). Calf serum, obtained from Sterile Systems, Inc., Logan, Utah, was similarly tested for its suitability in this system and compared with a lot of FBS known to support replication of *T. pallidum*.

Testis extract and tissue culture inocula. The preparation of the testis extract has been previously described in great detail (3). Briefly, infected testes were minced finely, and each testis was extracted with 5 ml of the modified basal reduced medium (BRMM) for 30 min at 33°C, followed by centrifugation at 500 × *g* for 5 min to remove gross particles. We then diluted that supernatant to the approximate desired concentration of treponemes, using fresh, infected-testis extract. The latter was prepared by centrifugation of a portion of the 500 × *g* supernatant at 12,000 × *g* for 10 min (the resulting extract is hereafter referred to as 12K extract). An accurate final count was then made on the diluted suspension, since the 12K extract contained a small but varying number of treponemes. Dilution with the 12K extract was such that 0.34 ml of the suspension contained the desired concentration of treponemes. Addition of the 0.34 ml of suspension to 10 ml of BRMM, the amount used in tissue culture bottles, resulted in a testis extract/BRMM ratio of 1:30. Testis extract from *T. pallidum*-infected rabbits is usually prepared on an empirical basis: one testis, irrespective of size, is extracted in 5 ml of BRMM. This procedure remains unchanged. However, when comparing the suitability of uninfected rabbit testes, as well as testis preparations from rats and hamsters, as a substitute for the extract from infected rabbits, we established a weight-volume basis as follows. The total weight of both testes from a normal 6-month-old rabbit was 4.67 g. These testes were extracted in 10 ml of BRMM by the method used to extract infected testes. On this basis, the weight-volume percentage of the uninfected mature rabbit testes was 46.7 g%. This, then, was the weight-volume percent basis for extracting testes from uninfected immature rabbits, mature rats, and mature hamsters. The uninfected 12K extracts, dilution of the inoculum, and inoculation of the bottles were the same as for infected 12K extract. These experiments were carried out at 33°C under an atmosphere of 1.5% O_2.

Trypsin-EDTA MEM. Trypsin-EDTA MEM is a solution of Ca^{2+}- and Mg^{2+}-free minimum essential medium (MEM) containing 10 mg of dithiothreitol, 20 mg of crystalline trypsin, and 20 mg of EDTA per 100 ml. This solution was used not only to disrupt the cell monolayer but also to separate the treponemes from the Sf1Ep cells. The latter were not removed from the suspension because they did not interfere with the counting of the treponemes.

Tissue culture procedures. The composition of BRMM and the procedure for setting up the Sf1Ep cultures have been described in detail elsewhere (3). Two days before infection with *T. pallidum*, 4-ounce (ca. 120-ml) prescription bottles were seeded with 5 × 10^5 Sf1Ep cells in Eagle MEM plus 10% FBS and incubated at 33°C. Before inoculation of the treponemes, the growth medium was removed and replaced by 10 ml of BRMM. The cultures were gassed with 5% CO_2–95% N_2 for 1 min at 3 liters/min and allowed to equilibrate for 4 to 5 h. Each flask was then inoculated with 0.34 ml of testis extract containing the desired number of treponemes. The cultures were then gassed for 1 min with a gas mixture containing the desired concentration of O_2, 5% CO_2, and the balance N_2 at 3 liters/min. Incubation was continued at 33°C, and cultures were sacrificed at various intervals up to 12 days. The procedures for harvesting and counting the treponemes have also been described in detail elsewhere (2, 3).

RESULTS

Effect of incubation temperature on replication of *T. pallidum*. We chose the temperature of 33°C for all of our previous experiments because the optimum temperature for survival of *T. pallidum* was thought to be 35°C or less (6, 7, 10). In attempts to experimentally determine the optimum temperature for replication of *T. pallidum*, we cultivated the organisms at temperatures ranging from 31 to 37°C (Table 1). Although minimal growth occurred at both extremes, it is

TABLE 1. Effect of incubation temperature on replication of *T. pallidum* in cultures of Sf1Ep cells[a]

Temp (°C)	Day of observation	Avg no. of treponemes (×10^7)	Avg % motile	Avg fold increase
31	5	0.65	60.2	1.3
	7	0.78	68.1	1.6
	9	0.95	69.5	1.9
	12	0.69	58.1	1.5
32	5	1.54	80.4	3.1
	7	2.75	87.6	5.6
	9	4.35	84.3	8.8
	12	4.74	71.3	9.7
33	5	2.23	89.1	4.5
	7	4.61	91.5	9.4
	9	7.89	90.2	16.1
	12	7.08	45.4	14.5
34	5	3.89	92.5	7.9
	7	6.43	87.9	13.1
	9	9.51	72.8	19.3
	12	5.54	22.6	11.3
35	5	4.80	93.7	9.8
	7	8.46	86.2	17.2
	9	9.47	59.4	19.3
	12	5.82	8.8	11.9
36	5	4.03	92.3	8.2
	7	6.33	77.4	12.8
	9	5.44	32.6	11.0
	12	2.98	0.6	6.1
37	5	2.16	75.4	4.3
	7	2.77	35.9	5.5
	9	1.45	3.6	2.9
	12	0.89	0.0	1.8

[a] Combined data from three experiments. Inocula ranged from 4.78 × 10^6 to 5.18 × 10^6 (average = 4.92 × 10^6) treponemes per bottle. Bottles contained an atmosphere of 1.5% O_2–5% CO_2–93.5% N_2.

TABLE 2. Effect of atmospheric oxygen concentration on replication of *T. pallidum* in cultures of Sf1Ep cells[a]

Atmospheric oxygen concn (%)[b]	Day of observation	Avg no. of treponemes ($\times 10^7$)	Avg % motile	Avg fold increase
<0.3	5	1.91	85.1	3.6
	7	3.68	88.7	6.9
	9	5.63	78.9	10.6
	12	4.11	46.2	7.7
1.0	5	2.12	89.2	4.0
	7	4.91	91.2	9.2
	9	8.14	90.4	15.2
	12	8.03	59.5	15.1
1.5	5	2.18	88.5	4.4
	7	5.00	91.4	10.1
	9	7.06	79.9	14.2
	12	12.43	59.0	25.0
2.0	5	2.09	91.4	3.9
	7	4.80	90.9	9.0
	9	8.92	92.6	16.7
	12	12.15	81.1	22.8
2.5	5	1.71	87.3	3.2
	7	4.68	93.6	8.8
	9	7.80	92.4	14.6
	12	12.70	81.5	23.8
3.0	5	1.76	80.9	3.3
	7	4.79	93.5	9.0
	9	8.05	91.8	15.1
	12	11.95	89.6	22.4
3.5	5	1.96	83.1	3.7
	7	4.68	92.2	8.8
	9	7.47	88.7	14.0
	12	12.95	83.6	24.3
4.0	5	1.72	83.5	3.2
	7	3.94	93.8	7.4
	9	7.55	92.6	14.2
	12	13.50	85.6	25.3
4.5	5	1.63	85.8	3.1
	7	4.06	88.9	7.6
	9	7.77	89.0	14.6
	12	11.95	87.5	22.4
5.0	5	1.54	83.8	3.1
	7	4.28	89.3	8.6
	9	7.88	90.8	15.9
	12	13.77	81.6	27.7
7.5	5	1.22	58.4	2.9
	7	2.51	80.6	5.9
	9	4.32	90.4	10.2
	12	8.58	66.8	20.2
10.0	5	0.43	41.5	1.0
	7	1.04	66.2	2.5
	9	1.06	72.5	2.5
	12	2.09	65.5	4.9
12.5	5	0.27	0	0
	7	0.21	0	0

[a] Inocula ranged from 4.24×10^6 to 5.48×10^6 in three separate experiments. Temperature of incubation was 33°C.

[b] Gas mixture contained the indicated percent oxygen and 5% CO_2, the remainder being N_2.

apparent that the optimum temperature for replication ranged from 33 to 35°C.

Effect of atmospheric oxygen concentration on replication of *T. pallidum*. We previously had determined, in gradient cultures of Sf1Ep cells, that in the area in the gradient containing the greatest number of treponemes, the dissolved oxygen concentration is 1.5%. Therefore, we used that concentration throughout our studies without attempting to determine whether that concentration of oxygen was optimum in the present system in which *T. pallidum* was replicating. Experiments were carried out to determine the optimum concentration of oxygen for replication of *T. pallidum* at 33°C. Replication was examined in concentrations of oxygen ranging from <0.3 to 12.5% (Table 2). Some multiplication was observed in all concentrations except 12.5%. However, the concentrations of oxygen between 1.5 and 5% appeared to be within the optimum range for replication of *T. pallidum*; increases in numbers at those concentrations ranged from 22.4- to 27.7-fold.

Effect of temperature and oxygen variations on *T. pallidum* replication. The results of the previous experiments indicated that optimum growth of *T. pallidum* occurs at temperatures between 33 and 35°C and at oxygen concentrations between 1.5 and 5%. We therefore carried out experiments at 33, 34, and 35°C, utilizing oxygen concentrations of 1.5, 3, and 5% (Table 3). It is apparent that there was not much difference among the groups in *T. pallidum* replication. The maximum fold increases ranged from 18.9 in the 33°C group incubated under 5% oxygen to 26.6 in the 34°C group incubated under 3% oxygen. Variation in the oxygen concentration within each temperature group had little or no effect on replication. It did, however, affect motility, which invariably remained greater in the 5% oxygen concentration than in lower concentrations after 9 to 12 days of cultivation.

Effect of FBS concentration on *T. pallidum* replication. We had previously established in experiments carried out in gradient cultures that

TABLE 3. Effect of various temperature and atmospheric oxygen concentrations on replication of *T. pallidum* in cultures of Sf1Ep cells

Incubation temp (°C)	Atmospheric oxygen concn (%)[a]	Day of observation	Avg no. of treponemes ($\times 10^7$)[b]	Avg % motile	Avg fold increase
33	1.5	5	1.60	88.1	3.7
		7	4.29	92.7	9.3
		9	8.15	92.3	17.6
		12	10.75	42.8	23.3
	3.0	5	1.51	85.8	3.5
		7	4.15	88.9	9.0
		9	9.03	91.8	19.5
		12	10.50	42.2	22.7
	5.0	5	1.40	92.0	3.3
		7	3.66	88.8	7.9
		9	8.74	91.1	18.9
		12	8.68	66.0	18.8
34	1.5	5	3.35	88.2	7.3
		7	6.85	88.0	14.8
		9	10.45	39.8	22.6
		12	7.95	4.4	17.2
	3.0	5	3.22	87.8	7.0
		7	7.73	92.7	16.7
		9	12.30	63.2	26.6
		12	8.81	9.3	19.1
	5.0	5	2.98	87.3	6.5
		7	7.93	92.1	17.2
		9	12.20	83.0	26.4
		12	10.72	35.6	23.2
35	1.5	5	3.69	89.7	8.0
		7	10.89	89.1	23.6
		9	11.10	57.2	24.0
		12	6.92	5.3	15.0
	3.0	5	4.26	92.6	9.2
		7	10.26	93.1	22.2
		9	11.75	68.4	25.4
		12	8.20	12.6	17.7
	5.0	5	3.18	93.7	6.9
		7	8.17	93.3	17.7
		9	11.02	81.5	23.9
		12	9.94	30.8	21.5

[a] Culture flasks contained the indicated concentrations of oxygen and 5% CO_2, the remainder being N_2.
[b] Average of two experiments. Inoculum was 4.62×10^6 *T. pallidum* per flask.

a 20% concentration of FBS in the BRMM is optimum for survival of *T. pallidum* (2). In the present system, in which both tissue culture cells and treponemes were replicating and competing for this protein source, it was important to reinvestigate the requirements of the system for FBS. The results are shown in Table 4. Although replication of *T. pallidum* occurred in all serum concentrations tested, the optimum concentration in this system was also 20%.

In addition to FBS, we also investigated calf serum for its ability to support replication of *T. pallidum*. The results were not unlike those we obtained in earlier studies which entailed screening FBS for its ability to support survival of *T. pallidum* (4). We tested eight lots of calf serum, two of which did not support replication of *T. pallidum*. In three lots, increases ranged from 4.7- to 8-fold. In two other lots, the increases were 9.1- and 9.8-fold. The eighth lot gave

TABLE 4. Effect of FBS concentration in culture media on replication of *T. pallidum*[a]

FBS concn (%)	Day of observation	Avg no. of treponemes ($\times 10^7$)	Avg % motile	Avg fold increase
5	5	1.23	71.0	2.6
	8	1.89	48.9	4.0
	12	4.29	68.4	9.1
10	5	1.14	78.5	2.4
	8	3.69	66.1	7.9
	12	4.80	69.6	10.2
15	5	1.74	89.4	3.7
	8	3.92	73.1	5.3
	12	7.51	69.6	16.0
20	5	1.89	88.5	4.0
	8	5.15	86.9	11.0
	12	9.65	53.3	20.5
25	5	1.93	85.3	4.1
	8	3.68	79.3	8.1
	12	6.44	55.4	13.7
30	5	1.58	90.0	3.4
	8	3.89	80.0	8.3
	12	6.36	55.1	13.5

[a] The culture media were BRMM containing the indicated concentrations of FBS. Incubation was at 33°C in an atmosphere of 1.5% O_2–5% CO_2–93.5% N_2. Inoculum was 4.70×10^6 *T. pallidum*.

results comparable to those of the FBS control serum, the average increase in treponemes being 13.7-fold in three experiments, compared with 16.5-fold in the FBS control.

Effect of various kinds of testis extract on replication of *T. pallidum*. Nelson (7) showed that extracts from rabbit testes—either normal or *T. pallidum*-infected rabbits—or from bull testes are an important factor for in vitro survival of *T. pallidum* in a cell-free system. We also found that infected-rabbit testis extract is essential for prolonged survival of *T. pallidum* in gradient cultures of Sf1Ep cells (2). We therefore utilized it in the medium in our initial replication studies. However, we did not previously determine whether it was essential or whether other extracts could be substituted. Table 5 summarizes the results of such recent experiments. It is clear that all of the testis extracts tested—*T. pallidum*-infected adult rabbit, normal adult rabbit, normal immature rabbit, adult rat, and adult hamster—were capable of supporting replication of *T. pallidum*. When no extract was added, the number of treponemes increased 10.7-fold, as compared to the 25.6-fold increase observed when extract from infected adult rabbit testes was added. This probably reflects the fact that the inoculum, because it is derived from rabbit testes, can not be eliminated entirely from the BRMM. In these experiments, the final dilution of the extract in the no-extract group was 1:537, compared with 1:30 for all the other groups.

DISCUSSION

We have now confirmed and extended our earlier observation that *T. pallidum* replicates in cultures of Sf1Ep cells. The purpose of the current investigation was to determine the optimum conditions for in vitro replication of *T. pallidum*.

The optimum temperature for replication of *T. pallidum* has been the subject of numerous investigations. Turner and Hollander (9) concluded that *T. pallidum* could multiply in vivo in the range of 30 to 38°C but that the optimum temperature level was probably 35 to 38°C. For in vitro survival in cell-free cultures, the optimum temperature has been variously reported to be between 25 and 35°C (6, 7, 10). In survival studies of *T. pallidum* in tissue culture, consideration had to be given to temperatures that would favor growth of the tissue cultures as well as the treponemes. Thus, temperatures between 33 to 36°C have been previously utilized (2, 5, 8). In the present studies, the optimum temperature for replication of *T. pallidum* was found to be between 33 and 35°C. Although little or no multiplication occurred at 31°C, 58% of the treponemes were surviving at the 12th day of incubation. At 37°C, the maximum increase occurred on the 7th day of incubation, but by the 12th day, none of the treponemes had survived.

Before 1974, *T. pallidum* was considered to be an anaerobic organism. Therefore, in most attempts to cultivate the organism, rigorous efforts were made to exclude oxygen, because its presence was considered to be inimical to survival and growth. However, in 1974, Cox and Barber (1) demonstrated that *T. pallidum* not only utilized molecular oxygen but also contained an operating cytochrome oxidase system. Subsequently, various concentrations of oxygen were included in tissue culture systems employed in attempts to cultivate or obtain prolonged survival of *T. pallidum*. We have now shown that *T. pallidum* is apparently a microaerophilic organism, replicating in atmospheres containing between <0.3 and 10% oxygen but failing to survive in 12.5% oxygen. The optimum oxygen concentration for replication at 33°C ranged from 1.5 to 5%. However, in a series of experiments in which both temperature and oxygen concentrations were varied, it appeared that the optimal condition for replication of *T. pallidum* was incubation at 34°C under an atmosphere containing between 3 and 5% oxygen.

TABLE 5. Effect of various kinds of testis extract on replication of *T. pallidum*

Extract prepared from[a]:	Day of observation	Avg no. of treponemes ($\times 10^7$)	Avg % motile	Avg fold increase
Adult rabbit, infected	0	0.51		
	5	2.51	88.5	4.9
	8	7.82	93.1	15.3
	12	13.05	71.4	25.6
Adult rabbit, normal	0	0.47		
	5	2.07	90.6	4.4
	8	8.60	93.8	18.3
	12	10.85	65.2	23.1
Immature rabbit, normal	0	0.45		
	5	1.82	85.4	4.0
	8	6.51	90.9	14.5
	12	9.22	67.5	20.5
Adult rat	0	0.48		
	5	1.83	88.0	3.8
	8	6.12	92.7	12.8
	12	7.89	69.2	16.4
Adult hamster	0	0.46		
	5	1.83	89.3	4.0
	8	4.40	90.2	9.6
	12	9.21	79.2	20.0
No added extract[b]	0	0.43		
	5	0.94	83.1	2.2
	8	2.10	91.2	4.9
	12	4.48	67.7	10.7

[a] Each 12K extract was prepared from the testes of indicated animals. The inoculum for each group was diluted from the original infected-rabbit testis extract such that the final concentration of each 12K extract in the culture medium was 1:30.

[b] Contained only extract present in inoculum. Dilution of the inoculum was in the BRMM rather than in the testis extract. The final dilution of the extract was 1:537, compared with 1:30 for other groups.

In our earlier studies on survival of *T. pallidum* in gradient cultures of Sf1Ep cells, we established that the FBS concentration which was optimum for treponeme survival was 20%. In the current experiments, *T. pallidum* replicated in all FBS concentrations between 5 and 30%. We also demonstrated that the optimum concentration for multiplication as well as survival was 20%. Because of the increasingly limited supplies of FBS, it was important to test other sources of serum. We found that when properly screened, calf serum was just as suitable as FBS for cultivation of *T. pallidum*.

One of the more important ingredients in the BRMM has been the rabbit testis extract. In all of our early experiments, this was prepared from infected testes, which were also used as the source of inoculum. The question then arose as to whether the extract had to be prepared from infected testes, adult testes, or indeed from rabbit testes at all. It was quite clear from the results of our current experiments that whatever was responsible for supporting replication was present in all rabbit, rat, and hamster testis preparations and was unrelated to either infection or animal species utilized. The extract prepared from the infected-rabbit testes appeared to be slightly more effective than the other preparations. All of the extracts except that from infected testes were prepared on the same weight-volume basis and therefore can be properly compared. The infected testes, irrespective of weight, were extracted in 5 ml of BRMM. Since these testes were at least twice as large as uninfected testes, the extract was generally more concentrated. Even in the absence of added testis extract, we observed a 10.7-fold increase in treponemes, probably because the cultures contained extract from the inoculum.

It is possible that extracts of tissues other than testis also help to support the growth of *T. pallidum* in vitro. Other tissues have not been investigated because rabbit testes are more susceptible to *T. pallidum* infection than are other

tissues, and because infected rabbit testes were readily available at the time of the experiment.

In this report, we define conditions for in vitro cultivation of *T. pallidum*. Our results indicate that *T. pallidum* is not as fastidious as was formerly believed, since replication occurs under fairly wide ranges of temperature and oxygen concentrations.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant RO1 AI-15113 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Cox, C. D., and M. K. Barber.** 1974. Oxygen uptake by *Treponema pallidum*. Infect. Immun. **10**:123–127.
2. **Fieldsteel, A. H., F. A. Becker, and J. G. Stout.** 1977. Prolonged survival of virulent *Treponema pallidum* (Nichols strain) in cell-free and tissue culture systems. Infect. Immun. **18**:173–182.
3. **Fieldsteel, A. H., D. L. Cox, and R. A. Moeckli.** 1981. Cultivation of virulent *Treponema pallidum* in tissue culture. Infect. Immun. **32**:908–915.
4. **Fieldsteel, A. H., J. G. Stout, and F. A. Becker.** 1981. Role of serum in survival of *Treponema pallidum* in tissue culture. In Vitro **17**:28–32.
5. **Fitzgerald, T. J., R. C. Johnson, J. A. Sykes, and J. N. Miller.** 1977. Interaction of *Treponema pallidum* (Nichols strain) with cultured mammalian cells: effects of oxygen, reducing agents, serum supplements, and different cell types. Infect. Immun. **15**:444–452.
6. **Kimm, G. E., R. H. Allen, M. J. Morton, and J. F. Morgan.** 1962. Studies on the *in vitro* survival of virulent *Treponema pallidum*. Am. J. Hyg. **75**:339–346.
7. **Nelson, R. A., Jr.** 1948. Factors affecting the survival of *Treponema pallidum in vitro*. Am. J. Hyg. **48**:120–132.
8. **Sandok, P. L., H. M. Jenkin, H. M. Matthews, and M. S. Roberts.** 1978. Unsustained multiplication of *Treponema pallidum* (Nichols virulent strain) in vitro in the presence of oxygen. Infect. Immun. **19**:421–429.
9. **Turner, T. B., and D. H. Hollander.** 1957. Biology of the treponematoses. WHO. Monograph Series no. 35. World Health Organization, New York.
10. **Weber, M. M.** 1960. Factors influencing the *in vitro* survival of *Treponema pallidum*. Am. J. Hyg. **71**:401–417.