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Effect of *p*H on Human *Mycoplasma* Strains

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ABSTRACT

SHEPARD, MAURICE C. (U.S. Naval Medical Field Research Laboratory, Camp Lejeune, N.C.), AND CARL D. LUNCEFORD. Effect of *p*H on human *Mycoplasma* strains. J. Bacteriol. **89**:265–270. 1965.—The optimal reaction of culture media for the cultivation of T-strain *Mycoplasma* of human origin was investigated. By use of a recently modified tryptic digest medium, the optimal reaction in either agar or fluid medium was found to be *p*H 6.0. In contrast, human classic (large-colony) *Mycoplasma* could be cultivated in agar or fluid medium over a rather broad *p*H range, and the influence of the reaction of the medium appeared to be primarily species-dependent. *M. salivarium*, for example, grew best in agar from *p*H 5.5 through 6.5. *M. pneumoniae* (Eaton's agent) yielded largest colony numbers in agar and highest titers in broth at *p*H 8.0. In the case of T-strain *Mycoplasma*, both maximal colony numbers in agar and highest titers in fluid media were achieved at a reaction of *p*H 6.0. In addition, largest colony size of T-strain *Mycoplasma* was also achieved in agar at *p*H 6.0, and averaged 50 to 100% larger than that obtained by cultivation at *p*H 8.0 with the same medium. Although T-strains will develop in agar media over a *p*H range of from 5.0 through 10.0, the extremely small colony size and poor staining properties resulting from growth in an alkaline medium make their recognition in agar cultures difficult. Aerobic cultivation of T-strains was first achieved in agar adjusted to *p*H 5.5 to 6.0. In fluid medium, multiplication of T-strains occurred only within the limits of *p*H 5.0 through 8.0, with highest titers being reached at *p*H 6.0. Greater attention to the reaction of complete *Mycoplasma* media is stressed.

One of the distinguishing features which characterizes T-strain *Mycoplasma* [pleuropneumonia-like organisms (PPLO)] in primary cultures from nongonococcal urethritis patients is their very small colony size at maturity (Shepard, 1956, 1959, 1960; Ford, 1962; Ford, Rasmussen, and Minkin, 1962; Ford and MacDonald, 1963). This feature is most clearly appreciated when colonies of T-strain *Mycoplasma* are observed in primary cultures mixed with colonies of classic (large-colony) *Mycoplasma*. The very small size of T colonies can easily cause them to be overlooked by an inexperienced observer. This is particularly true in unstained primary agar cultures examined *in situ* by low-power microscopy. Any modification of present culture techniques that would result in development of larger T colonies on agar, therefore, would be of great help in the detection and recognition of T-strain *Mycoplasma* in primary cultures.

On several occasions, we noted a striking increase in size and density of T colonies which were developing adjacent to certain bacterial colonies—especially those of streptococci. This size increase was frequently 50 to 100% over the usual T-colony size of 10 to 15 μ in primary cultures, and was confined to a circular area of approximately 10 mm from a given bacterial colony. Repeated attempts to demonstrate an accessory growth factor from bacterial colonies exhibiting this effect on adjacent colonies of T-strain *Mycoplasma* were unsuccessful. Bacteria-free filtrates of broth cultures of two different strains of streptococci producing this size-enhancing response were tested, by use of a controlled agar-gel diffusion technique. We were unable to detect stimulation of T colonies by this procedure. Incorporation of such bacteria-free filtrates directly in the agar culture medium also failed to result in T-colony size increase. Stimulation could be achieved only by cultivation of the *Streptococcus* colonies directly on the surface of the agar medium and in close association with colonies of T-strain *Mycoplasma*. Colonies of classic (large-colony) human genital *Mycoplasma*, in our experience, have not shown this type of response in primary cultures from the male and female genitourinary tract. There is no evidence that T-strains ferment carbohydrates. However, since dextrose (0.1%) was available to the streptococci in the mixed primary cultures, the influence of *p*H on the growth of T-strain *Mycoplasma* was investigated.

MATERIALS AND METHODS

Agar culture system. The agar medium employed in these studies was a modification of an earlier medium (Shepard, 1956). Details concerning the preparation of this modification (TDA-16) will be reported separately. A series of basal agars was prepared in 0.5 *p*H unit intervals from *p*H 5.0 through 10.0, with 1 N HCl or NaOH. The basal agars were enriched with pooled, sterile human plasma. The plasma enrichments were aseptically adjusted with sterile 1 N HCl or NaOH to match the *p*H of the respective sterile basal agars for which they were to serve as enrichment. The calculated volume of sterile acid or alkali required for 50 ml of sterile plasma was added to the melted basal agar instead of to the plasma, and then the plasma was added. This procedure avoided the development of precipitates which resulted in cloudy medium. All complete agar media contained 1,000 units per ml of penicillin. Measurements of *p*H were made with a glass electrode.

Procedure for agar cultures. Agar culture plates were used 48 hr after pouring to assure that agar surfaces were free from excess moisture. The agar plates (from *p*H 5.0 through 10.0) were uniformly inoculated with 0.05-ml drops of appropriately diluted broth culture of T-strain or classic *Mycoplasma* organisms. After the inoculum fluid was absorbed by the agar, the culture plates were inverted, sealed according to the Fortner (1928) technique, and incubated at 36 C for 48 hr. The agar medium described by Chanock, Hayflick, and Barile (1962) was employed for cultivation of *Mycoplasma pneumoniae* in these studies, and was incubated aerobically at 36 C for 7 days. Measurement of colony diameter was made under a magnification of 1,000× (oil) with a calibrated ocular micrometer. *Mycoplasma* colonies were measured to the nearest micron.

Fluid culture system. The fluid medium consisted essentially of the same basic formula as the agar medium, with the solidifying agent omitted. The fluid medium was enriched with sterile horse serum (20%) and, in addition, contained 1% yeast Supplement C (Difco). The reaction of the horse serum enrichment was adjusted in a manner identical to that employed for plasma for the agar medium.

Procedure for fluid cultures. Fluid medium was employed in 2.0-ml volumes in screw-capped culture tubes (13 × 100 mm). The culture tubes (from *p*H 5 through 10) were uniformly inoculated with 0.02 ml of fluid culture of T-strain or classic *Mycoplasma*. After inoculation, the caps were tightened, and the tubes were incubated at 36 C. T-strains of *Mycoplasma* were incubated for 18 hr; classic *Mycoplasma* strains were incubated for 48 hr, with the exception of *M. pneumoniae*, which was incubated for 7 days. After appropriate incubation, 10-fold serial dilutions of all cultures were prepared in *p*H 7.4 basal fluid medium (without serum), and 0.01-ml volumes from respective serial dilutions were plated in triplicate on the surfaces of standard TDA-16 agar culture plates. Total *Mycoplasma* colony counts per 0.01-ml drop were performed. Counts from triplicate drops were averaged, and the titers were expressed as colony-forming units (CFU) per milliliter of original fluid culture, at a specified *p*H.

Test organisms. The T-strain *Mycoplasma* were originally isolated in our laboratory from the urethrae of nongonococcal urethritis patients. Three of the T-strains were propagated in an all-agar system, the technique of which will be described in a separate report. The fourth T-strain was a fresh isolation in original exudate suspension. The history of the T-strains is summarized in Table 1.

For purposes of comparison, classic *Mycoplasma* species of human origin were also examined and represented serotypes 1, 2, 3, and 4 of Edward and Freundt (1956). The classic group organisms were *M. hominis*, type 1 (PG 21); *M. hominis*, type 2 (PG 27); *M. fermentans*, type 3 (PG 18); *M. salivarium*, type 4 (PG 20); and *M. pneumoniae* (Somerson, Taylor-Robinson, and Chanock, 1963), strain NMFRL.

TABLE 1. *History of the human T-strain Mycoplasma employed as test organisms*

T-strain culture no.	Date of original isolation	No. of agar passages
960*	November, 1961	30
K12	February, 1962	12–15
K42†	March, 1962	12–15
K71‡	May, 1962	0

* T-strain 960 represents our prototype strain. All T-strains (except K71) were isolated and propagated in an all-agar culture system.

† T-strain K42 produced "TR"-type colonies (Shepard, 1956) on original isolation.

‡ T-strain K71 was an original broth suspension of urethral exudate (frozen).

RESULTS

Agar culture system. With the exception of strain 960, all T strains examined produced recognizable colonies on TDA-16 agar throughout the *p*H range of 5.0 through 10.0. T-strain 960 failed to grow at *p*H 5.0. Average T-colony size was 12 μ in the alkaline range from *p*H 7.5 through 10, and colonies generally were poorly stained with the wet-stained agar block technique (Dienes et al., 1948). Average T-colony size progressively increased with increasing acidity, and largest size was reached in agar cultures of *p*H 5.5 and 6.0. In addition, increase in colony size was paralleled by increase in colony numbers, which reached maximum at *p*H 5.5 and 6.0 (Table 2 and 4). The average increase in T-colony size at *p*H 5.5 and 6.0 over that observed in the alkaline

TABLE 2. *Influence of pH on colony size of human T-strain Mycoplasma in an agar culture system**

Initial *p*H of complete agar medium†	Test organisms									Mean colony size over all T-strains
	960			K12			K42			
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	
5.0	NG	NG	NG	14	2.2	10–18	14	3.2	9–20	14
5.5	21	4.1	13–28	20	3.0	14–27	25	2.4	19–28	22
6.0	22	4.3	13–28	20	2.4	13–24	26	2.2	24–32	23
6.5	17	2.4	13–21	16	1.5	13–18	25	3.8	14–30	19
7.0	16	3.0	9–20	17	1.4	15–20	18	1.8	14–21	17
7.5	10	1.7	6–13	13	2.0	9–16	12	1.7	8–14	12
8.0	14	1.9	11–18	14	1.9	10–18	15	1.6	12–18	14
9.0	9	2.6	5–13	14	1.7	11–16	13	2.7	10–19	12
10.0	7	0.7	6–8	11	1.4	8–13	14	1.6	12–17	11

* Values are mean colony diameter of 20 random colonies measured in microns. NG = no growth.
† TDA-16 medium containing 1,000 units per ml of penicillin.

TABLE 3. *Influence of pH on colony size of human classic Mycoplasma in an agar culture system**

Test organisms (*Mycoplasma*)	Initial *p*H of complete agar medium†								
	5.0	5.5	6.0	6.5	7.0	7.5	8.0	9.0	10.0
M. hominis-1	NG	52	50	47	44	45	45	45	42
M. hominis-2	NG	NG	44	72	49	51	46	45	42
M. salivarium	NG	35	57	52	53	43	43	35	NG
M. fermentans	NG	NG	47	46	38	32	31	24	27

* Values are mean colony core diameter of 20 random colonies measured in microns. NG = no growth.
† TDA-16 medium containing 1,000 units per ml of penicillin.

range was approximately two-fold. Similar results were observed when horse serum was substituted for human plasma as enrichment in the agar medium. A slight but definite drop in T-colony size at *p*H 7.5 remains unexplained. It was accompanied by a similar reduction in colony numbers.

The five classic *Mycoplasma* species of human origin presented a somewhat variable response to changes in reaction. None of the classic species grew in agar cultures of *p*H 5.0, and three (*M. hominis*, type 2; *M. fermentans;* and *M. pneumoniae*) failed to grow at *p*H 5.5. *M. pneumoniae* also failed to grow at *p*H 6.0. With the exception of the latter species, all of the classic *Mycoplasma* species examined produced colony growth throughout the *p*H range from 6.0 through 9.0. A reaction of *p*H 10 proved to be completely inhibitory for *M. salivarium* and *M. pneumoniae*. For the classic *Mycoplasma*, relative colony numbers (Table 4) appeared to be a more reliable measure of response to changes in reaction than colony size (Table 3). In contrast to growth of T-strain *Mycoplasma* in agar cultures, classic *Mycoplasma* exhibited wide variation in colony size, which appeared to be primarily related to the degree of crowding.

Fluid culture system. Growth of T-strain *Mycoplasma* occurred in fluid cultures over the *p*H range of 5.0 through 8.0 for all strains examined. However, highest titers were reached at *p*H 6.0 (Fig. 1). Fluid media of *p*H greater than 8.0 were uniformly inhibitory, and growth of T-strains has never been observed in fluid medium above *p*H 8.0. Average titers over all T-strains tested, at *p*H 5.0, 6.0, 7.0 and 8.0, respectively, were 1.7×10^5, 5.9×10^7, 1.3×10^6 and 9.5×10^3 CFU per milliliter.

The five classic *Mycoplasma* species grew in fluid cultures over a broad *p*H range (Fig. 2). Three species (*M. hominis*, type 1; *M. hominis*, type 2; and *M. salivarium*) reached highest titers in the region of *p*H 6 to 7. Growth of these three species occurred, however, over a *p*H range of 5 through 9. The remaining two species (*M. fermentans* and *M. pneumoniae*) were unable to grow in fluid medium of *p*H 5.0 and, in contrast, reached highest titers at *p*H 8.0. These were the only two classic species of human origin examined that also fermented dextrose. None of the classic

TABLE 4. *Influence of pH on relative colony numbers of human T-strain and classic Mycoplasma in an agar culture system**

Test organisms (*Mycoplasma*)	Initial *p*H of complete agar medium†								
	5.0	5.5	6.0	6.5	7.0	7.5	8.0	9.0	10.0
T-strain 960.....	NG	3+	4+	3+	2+	1+	2+	2+	2+
T-strain K12.....	3+	4+	3+	3+	3+	2+	3+	2+	2+
T-strain K42.....	2+	4+	3+	3+	3+	2+	3+	2+	2+
M. hominis-1.....	NG	2+	3+	4+	4+	4+	4+	4+	4+
M. hominis-2.....	NG	NG	2+	2+	2+	2+	2+	2+	2+
M. salivarium.....	NG	3+	4+	4+	1+	1+	1+	1+	NG
M. fermentans.....	NG	NG	1+	1+	1+	2+	2+	2+	1+
M. pneumoniae	NG	—	NG	—	3+	—	4+	3+	NG

* Results expressed as relative colony numbers. Large numbers = 4+, moderate numbers = 3+, small numbers = 2+, scant numbers = 1+, no growth = NG, and — = not done.

† TDA-16 medium containing 1,000 units per ml of penicillin.

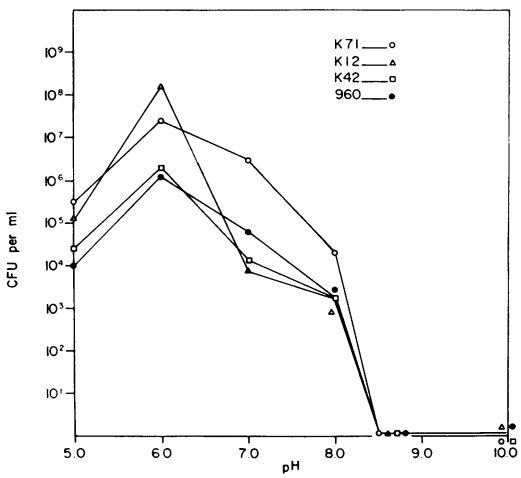

FIG. 1. *Influence of initial pH on multiplication of human T-strain Mycoplasma in a fluid culture system.*

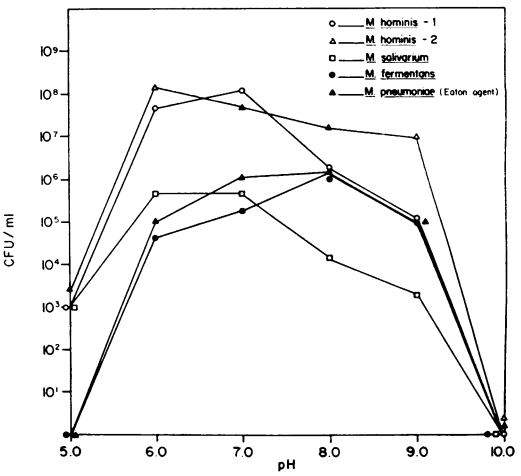

FIG. 2. *Influence of initial pH on multiplication of human classic Mycoplasma in a fluid culture system.*

human *Mycoplasma* was able to grow in fluid medium of *p*H 10.

DISCUSSION

For the past 27 years, since the first report by Dienes and Edsall (1937) of the isolation of *Mycoplasma* from a human genital lesion, the optimal reaction of culture media for the isolation and cultivation of these organisms has been considered by the majority of investigators to be *p*H 7.8 to 8.0. Adjustment of reaction was generally confined to the basal agar or basal broth. Native protein enrichments, such as ascitic fluid, serum, or plasma, were usually added without prior *p*H adjustment. Certain yeast additives, which are called for in many formulas for *Mycoplasma* media, are quite acid (*p*H 5.1, for example). Without prior *p*H adjustment of such materials, the final *p*H of such media may be considerably lower than is generally assumed. In certain cases, the improvement attributed to yeast factors may be the result of lowering the reaction of the medium to a more favorable value.

In our early studies of T-strain *Mycoplasma* isolated from the genitourinary tract of men with nongonococcal urethritis, the agar media then employed (Shepard, 1954, 1956) were adjusted to *p*H 7.8 in the customary manner described above. Ford (1962) compared the growth of T-strain *Mycoplasma* in agar media adjusted to *p*H 7.0, 7.4, and 7.8, incubated under a gaseous mixture of 10% carbon dioxide and air. He observed no

significant differences in growth and therefore selected an initial pH of 7.8 for continued use in his laboratory.

The present studies of T-strain *Mycoplasma* evolved from a study of the stimulatory effect of certain *Streptococcus* colonies on colonies of T-strain *Mycoplasma* in mixed primary cultures. No evidence was found that this was a growth factor response. The possibility that this stimulation was a pH effect is supported by the present findings. Growth of T-strain *Mycoplasma* experimentally in agar cultures of pH 5.5 to 6.0 essentially duplicated all of the observations made in mixed primary cultures in the presence of certain *Streptococcus* colonies. The relatively acid reaction of pH 5.5 to 6.0 is considered optimal for the primary isolation and cultivation of T-strain *Mycoplasma* in an agar system. Adjustment of the agar medium to this value permitted the first successful cultivation of T-strains in an aerobic atmosphere. Growth of T-strains at pH 6.0 in a microaerophilic environment (Fortner, 1928), however, resulted in much improved growth. In contrast, satisfactory growth of classic human *Mycoplasma* occurred over rather wide pH limits and was considered primarily species-dependent, as exemplified by three of the human classic species—*M. salivarium*, *M. fermentans*, and *M. pneumoniae*.

The cultivation of *Mycoplasma* in a fluid medium provided a more precise quantitative measure of the influence of reaction than that obtained in an agar system. In general, the findings in fluid cultures were similar to those obtained in agar cultures for classic *Mycoplasma* and T-strains. A definite preference was shown by T-strain *Mycoplasma* for a reaction of pH 6.0 in fluid medium. The limiting alkaline reaction proved to be pH 8.0, and a reaction of pH 8.5 or more was completely inhibitory. This observation is in general agreement with Ford (1962), who reported that a reaction of pH 8.0 was lethal to T-strains in fluid medium.

The optimal reaction for growth of T-strain *Mycoplasma* (pH 5.5 to 6.0) in either agar or fluid cultures was not confined to TDA-16 medium alone. Six different agar media prepared from commercial sources, enriched as described with human plasma or horse serum, were substantially improved in their ability to support growth of T-strain *Mycoplasma* when their reaction was adjusted to pH 6.0. With TDA-16 medium (adjusted to have a final pH of 6.0 as described), the successful propagation of T-strain *Mycoplasma* beyond primary isolation is no longer a difficult problem. It can now be reliably accomplished in an all-agar system. This medium has been in routine use in our laboratory for over 2 years. It is suggested that a final reaction of pH 7.8 to 8.0 may not be the optimal reaction for the cultivation of all species of human classic *Mycoplasma*. It is realized that reaction of the medium is only one factor in its successful performance. However, in the case of *T-strain Mycoplasma* this factor is an important one.

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