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Comparison of Undefined Medium and Its Dialyzable Fraction for Growth of *Mycoplasma*

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In examining the medium used in cultivation of *Mycoplasma* for deoxyribonucleic acid isolation, it was found that an aggregate was present which sedimented with the organisms and which was ethyl alcohol-precipitated during deoxyribonucleic acid purification. To eliminate the contaminating material, a method was devised to obtain only the dialyzable constituents of the medium. This report describes the preparation of a dialysate of soy peptone-yeast extract. The medium, obtained by immersion of the encased dehydrated ingredients in sodium chloride solution for 5.5 hr at approximately 80 C, has been employed as the basal medium for cultivation of a number of *Mycoplasma* species. Comparative growth curves of two saprophytic strains and two parasitic species indicated that multiplication in dialysate, with suitable supplement, followed the pattern typical of the common eubacteria. Thus, by elimination of the sediment which occurred in nondialyzed medium, *Mycoplasma* could be concentrated without concomitant accumulation of contaminating macromolecules.

The cultivation of *Mycoplasma* for the purpose of obtaining large numbers of cells or large amounts of cellular components presents problems which have no counterpart in the study of most of the true bacteria. Defined media have been devised, but they support suboptimal growth of only a few species. Whereas saprophytic *Mycoplasma* grow in a mixture of peptones and nucleic acids, parasitic species require cholesterol and lipoprotein and multiply best when the latter are supplied in the form of a fraction of animal serum or ascitic fluid. The adequacy of various infusions, peptones, sera, and yeast extracts has been investigated and critically reviewed by Ogata et al. (5). The most commonly used basal media contain an infusion or enzymatic digest of animal tissue and yeast extract. However, a soy peptone-yeast extract medium (SP-YE) has been found (7) to be satisfactory as a basal medium for 15 strains representing 7 species of *Mycoplasma*. The use of plant peptone ensures that no animal protein products are present except those which are contained in the supplementary serum or serum fraction. This is an advantage in preparation of materials for antigenic studies, in that cross-reactions can be controlled more satisfactorily.

Most complex media contain material which is sedimentable at the high centrifugal force necessary for concentrating *Mycoplasma*. This was found to be a problem in the investigation of purified deoxyribonucleic acid (DNA) from a number of *Mycoplasma* species; it was shown that the flocculent matter in incubated, uninoculated medium could be carried through the isolation procedure to the final step. Because previous growth studies of *M. laidlawii* strains (6) had indicated that the dialyzable components of SP-YE supported active growth, the feasibility of preparing the dialysate in larger volume was investigated. Such a medium has now been employed successfully for the cultivation of both saprophytic and parasitic species.

All DNA preparations used for the characterization and hybridization studies (Peterson and Pollock, *in preparation*) were prepared from cells grown in dialysate. This report describes the method for preparation and gives evidence that growth of four *Mycoplasma* strains, representative of one saprophytic and two parasitic species, is comparable to that in the nondialyzed medium.

MATERIALS AND METHODS

Cultures. *M. laidlawii* types A and B were obtained from the American Type Culture Collection (ATCC 14089 and 14192, respectively). *M. hominis* was isolated from an established cell culture line derived from a human esophageal fistula (7) and was identified by

Wallace Clyde (*personal communication*). *M. pulmonis* 47, isolated from a New Zealand white rabbit (2), was obtained from G. E. Kenny.

Preparation of basal medium. The basal medium (SP-YE) contained (w/v): 2% soy peptone (Sheffield Chemical, Norwich, N.Y.; U.S.P. grade), 1% Difco yeast extract, and 0.5% sodium chloride in doubly deionized water. The *p*H was adjusted to 7.8 by addition of sodium hydroxide before autoclaving. For preparation of solid medium (SP-YE agar), sufficient Difco Noble agar was added to provide a final concentration of 0.85%.

Preparation of medium dialysate (SP-YE-D). For each liter of doubly deionized water, 20 g of soy peptone and 10 g of yeast extract were placed in dialysis tubing (Union Carbide, 1.75 inch, flat width) knotted at one end. A 90-ml amount of water was added and the tubing was tied to make the total length about 10 inches (25.4 cm). The bags were kneaded briefly to hasten solution of the dry ingredients; they were then placed in a flask containing 910 ml of water per bag and heated to 75 to 80 C. Six to seven liters could be processed at one time in a 12-liter Florence flask. The sodium chloride concentration was 0.5% (w/v) of the total volume of water but was added only to the dialyzing solution. Dialysis at 75 to 80 C was continued for 5.5 hr, except where otherwise noted. The medium was cooled to ambient temperature in an ice bath, and the *p*H was adjusted to 7.8 by addition of 1.0 N NaOH; it was then sterilized by autoclaving at 121 C for 30 min. The bags containing nondialyzable material were discarded.

Preparation of complete medium. For *M. laidlawii* strains A and B, media were used either without supplementation or with 1% Difco serum fraction. For *M. hominis*, the medium was supplemented with 15% human serum (SP-YE-HS or SP-YE-D-HS) which had been inactivated at 56 C for 30 min. Medium for *M. pulmonis* 47 contained 15% noninactivated horse serum (SP-YE-HOS or SP-YE-D-HOS). The content of serum was the same whether or not the medium contained agar. For the saprophytic *M. laidlawii* A and B, agar was not supplemented.

Inoculation and incubation. The size of the inoculum used for growth studies was based upon previous determinations of colony-forming units (CFU) in logarithmically growing cultures of known age. Cultures used for inoculation were prepared by 4- to 10-fold dilution of an 18 to 24 hr culture and were incubated 4 to 8 hr before use. Populations at zero-time were usually in the range of 10^8 to 10^9 per ml. Incubation was in air, with the exception of *M. hominis* which was placed in an atmosphere of 5% CO_2 in air. All cultures were held at 37 C.

Measurement of growth. The course of growth was determined by enumerating CFU. At intervals during incubation, samples were removed for serial dilution; 0.01 ml of diluted culture was placed on the surface of agar. Plates were then incubated in an atmosphere of 5% CO_2 in air until colonies were large enough to count at a magnification of 12 to 25×. The number of CFU in the original culture was calculated as described previously (6). Generation time during the logarithmic phase was determined according to the formula:

$$G = (0.301 \times T)/(\log_{10} B - \log_{10} A)$$

where A = CFU at a point in early log phase, B = CFU after lapse of time (T).

RESULTS

SP-YE-D, prepared as described, was a satisfactory basal medium for two saprophytic strains and two parasitic species of *Mycoplasma*. Pilot studies, using *M. laidlawii* A and B, indicated that a 5.5-hr dialyzing period resulted in a medium which gave slightly better growth than that dialyzed for shorter periods. For examination of the effect of dialysis time on the adequacy of the medium, uniformity of preparation was ensured by following the Klett reading with a no. 42 filter; medium obtained after 5.5 hr measured approximately 360 Klett units before sterilization. Klett readings with a no. 66 filter were found to be helpful in following cultures, once turbidity had appeared. Unsupplemented SP-YE-D (5.5-hr dialysate), sterilized and ready for use, gave values of 11 to 12 units when read against water. It was possible to predict that when cultures reached 5 to 6 net Klett units, the cells were still actively dividing; this was useful in determining time of harvest when actively growing cells were desired. A linear increase in Klett reading occurred from very late log phase well into stationary phase; this was of interest but appeared too late in growth to aid in estimating cell numbers.

Curves of both *M. laidlawii* A and B in SP-YE were typical of the bacterial course of growth. *M. laidlawii* A (Fig. 1) grew equally well in SP-YE-D up to 10^8 per ml whether dialysis time was

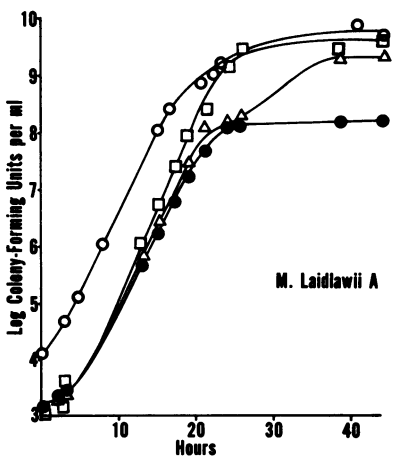

FIG. 1. *Growth of M. laidlawii type A in SP-YE* (○), *SP-YE-D, 2.5 hr* (●), *SP-YE-D, 5.5 hr* (△), *and SP-YE-D, 5.5 hr + serum fraction* (□).

2.5 or 5.5 hr. However, the longer dialyzing period appeared to favor a greater final yield, in spite of the fact that a temporary lag interrupted the logarithmic phase at about the point where growth in 2.5-hr dialysate ceased. This apparent diauxy phenomenon was found to be reproducible, but has not been studied further. The addition of 1% serum fraction to 5.5-hr dialysate resulted in restoration of the classical sigmoidal curve. Generation time varied from 0.9 to 1.1 hr.

In our experience, *M. laidlawii* B has regularly exhibited a longer generation time than has type A, regardless of medium employed. Growth of this strain is shown in Fig. 2. Generation times in SP-YE and in SP-YE-D supplemented with serum fraction were 1.6 and 1.8 hr, respectively, while doubling time in unsupplemented dialysate was extended to 2.6 hr.

Both *M. hominis* and *M. pulmonis* 47 were shown to grow in either SP-YE or SP-YE-D supplemented with 5% serum fraction (data not included here). However, whole serum supported growth more satisfactorily and, as shown in Fig. 3, yields of *M. hominis* from supplemented SP-YE and SP-YE-D differed by less than twofold at termination of the experiment. The yield of *M. pulmonis* 47 (Fig. 4) was not significantly different in the two types of media, although growth in SP-YE supplemented with horse serum appeared to be more erratic. Generation time was 1.3 hr for both species.

DISCUSSION

The need for a medium from which macromolecular components could be excluded first became apparent when electron microscopic studies of the saprophytic *M. laidlawii* were undertaken (1). A method was devised for cultivating these organisms in a dialysate of SP-YE which contained (as additions) only a defined mixture of salts, amino acids, and vitamins (6). The immersion of the culture in a large reservoir of SP-YE allowed the organisms to grow to high concentration and made possible fixation and preparation for electron microscopy without centrifugation. The present work, which represents an extension of the effort to exclude macromolecular constituents without necessarily increasing yield, was developed in the course of cultivating a number of *Mycoplasma* species for isolation of DNA. The elimination of aggregated medium components became desirable when it was found that the sediment from incubated, uninoculated, nondialyzed medium yielded alcohol-preciptable material, following the same general procedure which was used to obtain DNA from the cells. Although

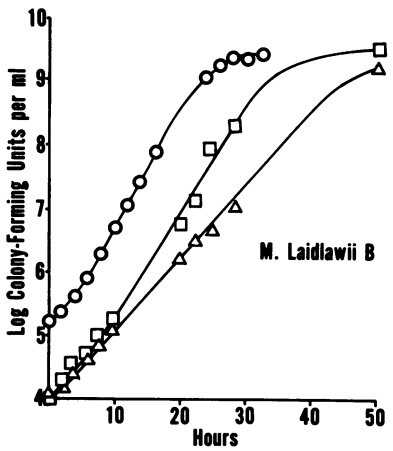

FIG. 2. *Growth of M. laidlawii type B in SP-YE* (○), *SP-YE-D, 5.5 hr* (△), *and SP-YE-D, 5.5 hr + serum fraction* (□).

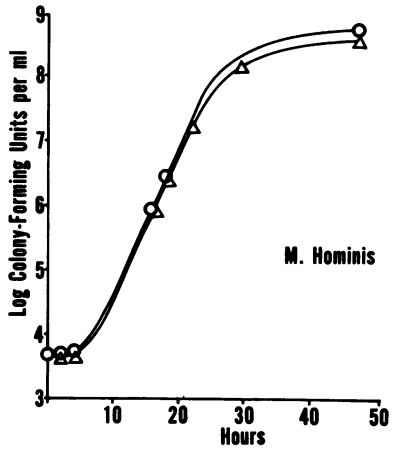

FIG. 3. *Growth of M. hominis in SP-YE-HS* (○) *and SP-YE-D-HS* (△).

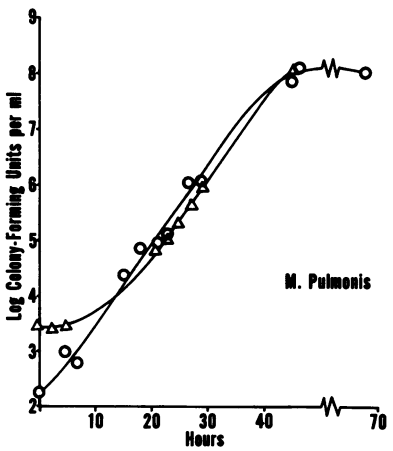

FIG. 4. *Growth of M. pulmonis 47 in SP-YE-HOS* (○) *and SP-YE-D-HOS* (△).

the medium components could easily be distinguished from DNA, they were not so readily detected and eliminated in the presence of DNA.

A dialysate of soy peptone-yeast extract either without additions or supplemented with 1% serum fraction or 15% serum has since been employed successfully for cultivation of saprophytic and parasitic *Mycoplasma*. Dialyzing time at 75 to 80 C has been varied from 1.5 to 5.5 hr. Although statistically significant data are not available, cultures in 5.5-hr dialysate have appeared to grow more satisfactorily. Because absolute temperature control has been difficult, termination of dialysis was originally based on Klett readings with a no. 42 filter; this made possible the preparation of uniform lots of medium.

The growth of four strains of *Mycoplasma* has been shown to be similar to that of the eubacteria. For the saprophytic *M. laidlawii* strains, the dialysate could be used in place of the complete, complex medium. However, growth comparable to that in nondialyzed medium was obtained only if Difco PPLO Serum Fraction was added. When used without supplement, medium dialyzed for 5.5 hr gave a higher yield of *M. laidlawii* A than that dialyzed for 2.5 hr, but an apparent diauxy phenomenon occurred prior to the end of the logarithmic phase. It is not known whether some other effect, for example, clumping, may alter the course of growth. However, the phenomenon was reproducible in studies of the A strain; it was less apparent in the growth of strain B.

Although the size of inocula shown here was not the same for all media, no significant differences in generation time or yield have resulted, even when CFU at zero-time have varied from 10^2 to 10^5. If initial cell number is greater than 10^6, the logarithmic phase is too short to permit accurate determination of generation time.

Studies employing the parasitic *M. hominis* and *M. pulmonis* 47, which require a medium supplemented with serum, also indicated that the 5.5-hr dialysate provided a satisfactory basal medium. Although the parasitic species did not grow to as high a maximal yield as did the saprophytic species, logarithmic growth continued well past 10^8 CFU per ml.

Because the turbidity of *Mycoplasma* cultures is so slight, there is no satisfactory method for concurrent estimation of growth in broth during the logarithmic phase. However, experience has shown that cultures which have reached late log phase give readings of 5 to 6 net Klett units at 660 nm. This corresponds to populations of 2 to 5 $\times$ 10^8 per ml (by CFU). At such low turbidity, the Klett reading is only a rough estimation of cell number, but readings have been helpful in determining time for harvest of cultures still growing actively.

It has not been desirable to scale up the preparation of dialysate for our purposes. However, Gerhardt and Schultz have described systems for dialysis culture by means of which much greater populations can be obtained (3). Their design might well be adapted to the procedure given here for dehydrated culture media. The importance of excluding sedimentable medium components from *Mycoplasma* cultures lies in the need for high centrifugal force to concentrate these relatively small organisms. Whereas the macromolecular components may not be objectionable for some purposes, their presence in purified DNA could lead to misinterpretation of physical and chemical studies. The composition of the aggregated material is not known, but the fact that at least some of it is ethyl alcohol-precipitable during DNA purification indicates that it probably contains polysaccharide and (or) oligonucleotides.

Dialsyates of other dehydrated media have not been tested for their adequacy. However, all *Mycoplasma* species cultivated in this laboratory have grown satisfactorily in SP-YE-D with suitable supplement. Although our purpose for using dialysate as a basal medium was to produce cells free of contaminating medium constituents, such a medium might well be used to obtain extracellular products of bacteria, as discussed by Herold and co-workers (4). For most such studies, the medium of choice may be one for which less laborious methods are available.

ACKNOWLEDGMENT

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