Cultivation of Mycoplasmas on Glass

R. H. PURCELL, J. R. VALDEUSO, W. L. CLINE, W. D. JAMES, AND R. M. CHANOCK

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014

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Eight Mycoplasma species of human origin were successfully cultivated on glass. Complement-fixing (CF) antigens prepared from glass-adherent mycoplasmas were potent, specific, and free from anticomplementary activity. PPLO broth medium supplemented with 1 to 5% PPLO serum fraction (bovine), 2.5% fresh yeast extract, and 1% glucose (glycolytic species) or 1% arginine (arginine-utilizing species) supported moderate to luxuriant growth of mycoplasmas on glass. The potency of CF antigens prepared from glass-adherent mycoplasmas varied with the species of Mycoplasma tested and the duration of incubation. When the potency of CF antigens prepared from glass-adherent mycoplasmas was compared with that material sedimented from the broth phase of the same culture, three patterns of growth were observed: M. hominis and M. orale type 2 grew preferentially in the broth phase; M. salivarium, M. orale types 1 and 3, M. pneumoniae, and M. liphophilum preferentially adhered to the glass; and M. fermentans was biphasic. The growth of mycoplasmas on glass provides a simple means of concentrating and purifying such organisms for immunological and biochemical studies.

In recent years, the identification, classification, and epidemiological study of mycoplasmas has become increasingly dependent on serological methods. This is so because serological techniques permit differentiation of many organisms which are similar in biological and biochemical properties. Mycoplasma antigens are difficult to purify because the organisms are small and grow, in most cases, only in a complex medium rich in protein. Contamination of antigens with medium components causes difficulties in performance and interpretation of certain serological tests, notably complement fixation (CF) and gel diffusion. The problem of producing relatively pure preparations of Mycoplasma pneumoniae was partially solved when Somerson et al. (18) successfully cultured this mycoplasma on a glass surface. The organisms could be purified and concentrated in one simple operation by repeatedly washing the adherent cell sheet, and then scraping it into the desired volume of diluent. Such glass-grown preparations of M. pneumoniae were quite potent when used as CF antigens. Moreover, unlike suspensions prepared from organisms grown in broth, they were not anticomplementary. Subsequently, glass-grown organisms were successfully used for detailed immunochimical analyses of M. pneumoniae antigens (11, 17) and for biochemical studies of hydrogen peroxide production by this mycoplasma (4).

Attempts to grow other Mycoplasma species on glass have met with variable success. Warren et al. grew M. gallisepticum on glass and prepared a vaccine from the glass-adherent organism (25). Similarly, Robillard (personal communication) cultured on glass several mycoplasmas of animal origin and used antigens prepared from them to produce hyperimmune sera in donkeys. Taylor-Robinson and Manchee reported the cultivation on glass and plastic of several mycoplasmas of human and animal origin but found considerable variation among strains within species in their ability to adhere to such surfaces (22).

In this report, we describe (i) a standard method which was successful for cultivation of a number of mycoplasmas of human and animal origin on glass and (ii) the use of such organisms for the preparation of potent specific CF antigens.

MATERIALS AND METHODS

Media. Hayflick's broth medium was described in detail previously (3, 9). Briefly, it consisted of seven parts Difeo PPLO broth, two parts unheated horse serum, and one part 25% fresh yeast extract. In addition, it contained 1,000 units of penicillin G/ml and 0.05% thallium acetate. For most experiments in the present study, the medium was supplemented with 0.002% phenol red and 1% glucose (fermenting strains) or 1% L-arginine-HCl (arginine-utilizing strains). The glucose and arginine media were ad-
CULTIVATION OF MYCOPLASMAS ON GLASS

Antigen fractions. Mycoplasma antigen which remained adherent to the glass bottle after washing was termed the "glass-adherent fraction." Mycoplasma antigen which was sedimented by high-speed centrifugation from the broth medium phase was termed the "sedimentable fraction"; the supernatant fluid from such a centrifugation was designated the "soluble fraction." All such antigen fractions were stored at -20°C and were frozen and thawed at least once before being tested for CF activity. Glass-grown and sedimentable antigens were harvested and tested as 10X concentrates referable to the volume of the starting medium; soluble antigen was tested unconcentrated. After testing, the observed CF titers of mycoplasma antigens were adjusted so that the potency of the three antigen preparations could be directly compared.

RESULTS

Growth of mycoplasmas on glass in HS medium. Attempts were made to grow M. hominis, M. orale types 1 and 2, M. salivarium, and M. fermentans on glass in HS medium under aerobic conditions and in vacuo. Under such conditions, growth of these mycoplasmas on glass was variable and appeared to be influenced by unidentified substances in the horse serum. However, when 1% PPLO serum fraction (Difco) was substituted for the 20% horse serum in the medium, all of the Mycoplasma strains not only grew but adhered tenaciously to the glass under both aerobic and anaerobic conditions.

The following experiments were performed to determine the optimal conditions for production of glass-grown CF antigen.

Comparison of Povitsky bottles and prescription bottles for production of CF antigens. The substitution of inexpensive disposable prescription bottles for Povitsky bottles in the production of antigens would offer certain advantages, among them convenience, economy, and versatility. To determine whether such bottles were suitable, cultures of M. hominis, M. salivarium, M. orale type 1, and M. fermentans were incubated in prescription bottles of 2- to 16-oz sizes. Glass-adherent organisms prepared in such bottles were as potent as antigens prepared in a similar manner in Povitsky bottles.

Effect of the concentration of serum fraction in the medium on yield of glass-adherent CF antigens. When mycoplasmas are cultured for antigen production, it is desirable to keep extraneous proteins in the medium at the lowest concentration consistent with luxuriant growth of the mycoplasma. Medium containing 1% PPLO serum fraction (BSF medium) has been used for the growth of many mycoplasmas. However, other investigators (5, 10) found that better growth and antigen yield were obtained from

Vol. 21, 1971

justed to pH 7.8 or 7.0, respectively, with 1 N HCl (14, 23). These media will be referred to as "horse serum" (HS) media. Bovine serum fraction (BSF) media were prepared by substituting 1 or 5% bovine serum fraction for the 20% horse serum (16). This medium was supplemented as described above.

Mycoplasmas. Thrice-cloned high-passage pools of each of the following Mycoplasma species were employed (strain designation in parentheses): M. hominis (PG-21), M. salivarium (PG-20), M. orale type 1 (CH-19299), M. orale type 2 (CH-20247), M. orale type 3 (DC-333), M. fermentans (PG-18), M. pneumoniae (FH), and M. l lipophilum (Jones). The origin of these strains was described previously (6, 8, 19, 20; DelGiudice, Bacteriol. Proc., p. 67, 1968). Each strain was passaged three to five times in BSF medium prior to use.

Conditions of cultivation. Cultures of mycoplasmas were incubated at 36 to 37°C in an atmosphere of room air or under 40 mm of vacuum. Sterile 2-liter Povitsky bottles (tetanus toxoid culture bottles, Corning glass) and 2-, 8-, and 16-oz (ca. 60-, 240-, and 480-ml) prescription bottles (Foster-Forbes Glass Co.) were used to culture mycoplasmas on glass. Povitsky bottles were washed with green soap, extensively rinsed with distilled water, and autoclaved prior to use. New prescription bottles were used as they were received, and then discarded. The ratio of medium volume to bottle volume varied between 1:4 and 1:10; however, all bottles used in any one experiment contained the same relative volume of medium.

Centrifugation. Broth cultures were centrifuged at 19,000 to 20,000 rev/min for 1 hr in a Spinco 21 angle-head rotor [35,000 to 40,000 × g (average)].

Diluent. Phosphate-buffered saline (PBS), pH 7.2, was used for washing of glass-adherent and brothgrown organisms and for suspension of final antigens.

Removal of mycoplasmas from glass. Glass-adherent mycoplasmas were scraped from glass with a rubber policeman.

1 CF. Methods for CF were described previously (24); 1.7 exact units of complement was used, with overnight fixation at 4°C.

Quantitation of viable mycoplasmas. Decimal dilutions of mycoplasmas were prepared in broth medium; 0.3 ml of each dilution was inoculated into two vials of broth medium containing phenol red and the appropriate substrate. The vials were observed daily until color change (pH change) of the medium was no longer progressive. The viability titer was expressed in color-changing units, 50% (CCU50), that is, the highest dilution of mycoplasmas which produced a color change in 50% of the vials inoculated (23).

Preparation of hyperimmune sera. Rabbits were hyperimmunized with antigens grown in rabbit muscle infusion supplemented with rabbit serum and emulsified in Freund's adjuvant as described previously (24).

Nitrogen determinations. Total nitrogen was determined by a micro-Kjeldahl method in the analytical chemistry laboratory of W. Alford, Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases.
M. pneumoniae when this mycoplasma was grown on glass in medium containing 5% serum fraction.

To determine whether the yield of antigen from other mycoplasmas was similarly increased by a higher concentration of PPLO serum fraction, M. hominis, M. salivarium, M. orale types 1, 2, and 3, M. fermentans, and M. lipophilum, as well as M. pneumoniae, were grown in shallow culture in medium containing 1 or 5% serum fraction. After 3 to 5 days of incubation the glass-adherent mycoplasmas were washed three times and harvested. The antigen preparations were tested against 8 units of antiserum in one CF test. M. hominis and M. pneumoniae yielded more potent glass-adherent CF antigens when they were grown in medium supplemented with 5% serum fraction. The other mycoplasmas tested grew equally well in both media or better in medium containing 1% serum fraction than in medium supplemented with 5% serum fraction. In other experiments, the growth of M. hominis was suppressed when medium containing 10 or 20% serum fraction was employed.

**Effect of substrate on yield of glass-adherent CF antigens.** All of the recognized large-colony mycoplasmas of human origin with the exception of M. pneumoniae metabolize arginine to ammonia (1,12,13). M. pneumoniae lacks this metabolic pathway but does possess a glycolytic enzyme system, whereas M. fermentans has the ability to metabolize both arginine and glucose. To determine whether arginine or glucose added to the media stimulated the adherence of mycoplasmas to glass, the following experiments were performed.

M. fermentans was grown aerobically in replicate prescription bottles containing BSF medium with 1% arginine, 1% glucose, or no added substrate. Each day the glass-adherent mycoplasma sheet from one bottle containing each medium was harvested. On the second day, the sedimentable broth antigens were also harvested. The glass-adherent organisms grown in glucose-containing medium were found to possess higher CF activity than did organisms grown in arginine-supplemented medium or unsupplemented medium (Table 1). In contrast, the CF activity of antigens sedimented from the broth phase of the culture after 2 days of incubation was the same (1:16) for both glucose- and arginine-supplemented media, whereas sedimentable antigen from unsupplemented medium had no demonstrable CF activity at a dilution of 1:2. From this, we conclude that both glucose and arginine stimulated the growth of M. fermentans or its production of CF antigen, or both, but only glucose stimulated adherence of the mycoplasmas to glass.

To determine whether glucose potentiated the adherence to glass of mycoplasmas which do not metabolize this substrate, M. salivarium and M. hominis were grown aerobically in prescription bottles in BSF medium supplemented with glucose or arginine or with neither of these substrates. The glass-adherent M. salivarium organisms were harvested on the second day of incubation; glass-adherent M. hominis organisms were harvested from replicate cultures after 1 to 3 days of incubation. The CF potency of these glass-adherent antigens was potentiated by arginine but not by glucose (Table 1).

In another experiment, production of glass-adherent M. salivarium antigen was only marginally improved by the addition of arginine when this mycoplasma was grown in vacuo (data not shown).

**Effect of duration of incubation on potency of CF antigens.** To determine the optimal time for harvest of mycoplasma CF antigens, the following experiments were performed.

Sets of twelve 16-oz prescription bottles were inoculated with 10^7 to 10^8 CFU_10^5 log-phase mycoplasmas of one of the following species: M. hominis, M. salivarium, M. orale type 1, M. orale type 2, M. orale type 3, M. pneumoniae, M. lipophilum, and M. fermentans. Six bottles of each set were loosely capped and incubated in a desiccator jar in vacuo. The other six bottles were not evacuated but were tightly capped. Each day one evacuated bottle and one aerobic bottle were removed from the incubator, and the glass-adherent, sedimentable, and soluble fractions were harvested. All of the antigen preparations from one set of 12 bottles were tested for CF activity in one test.

### Table 1. Effect of glucose or arginine on production of glass-adherent mycoplasma antigen

| Mycoplasma species | Day of harvest | CF titer (reciprocal) |
|--------------------|----------------|-----------------------|
|                    | Glucose, 1%    | Arginine, 1%         | Neither (control) |
| M. fermentans      | 4              | 256                   | <2 <2             |
| M. salivarium      | 2              | 4                     | 64 4              |
| M. hominis         | 3              | 2                     | 32 2              |

* Each of the three media was adjusted to the same pH (7.8 for M. fermentans; 7.2 for M. salivarium and M. hominis).
Three patterns of CF antigen production were observed, based upon whether or not the CF titer of glass-adherent antigen exceeded the titer of sedimentable antigen. *M. salivarium, M. orale* types 1 and 3, *M. pneumoniae*, and *M. lipophilum* demonstrated similar patterns of growth. *M. salivarium* and *M. orale* type 1 grew luxuriantly on the glass; data from a typical experiment with *M. salivarium* are shown in Fig. 1. Glass-adherent antigen usually reached its maximal potency on the second or third day; the potency then remained the same or declined. The CF activity of the glass-adherent antigen usually exceeded the activity of sedimentable antigen after the first day. Soluble antigen reached high levels of CF activity by the second day and remained thus throughout the experiment. *M. salivarium* and *M. orale* type 1 grew somewhat better under aerobic conditions than in vacuo, but the difference was not great.

*M. pneumoniae* and *M. orale* type 3 grew in a manner similar to *M. salivarium* and *M. orale* type 1 except that none of the preparations of sedimentable antigen fixed complement. Thus, it appeared that *M. pneumoniae* and *M. orale* type 3 organisms preferentially adhered to the glass. However, the CF titer of the soluble antigen of *M. orale* type 3 equaled or exceeded the titer of glass-grown antigen throughout the experiment, whereas the CF titer of the soluble antigen of *M. pneumoniae* remained quite low throughout the experiment. *M. lipophilum* also preferentially adhered to the glass, but the growth of this mycoplasma was much less luxuriant than was the growth of the other mycoplasmas tested. *M. lipophilum* as well as *M. pneumoniae* and *M. orale* type 3 grew equally well under aerobic and anaerobic conditions.

In contrast to the growth patterns just described, *M. hominis* and *M. orale* type 2 grew preferentially in the broth phase. Figure 2 illustrates results obtained with *M. orale* type 2. Rarely did CF activity of the glass-adherent antigen equal or exceed the sedimentable antigen. The glass-adherent antigen reached maximal CF potency by the second or third day and its potency diminished thereafter. The CF activity of the
soluble antigen rose to high levels. There was little difference in the potency of CF antigens from aerobic and anaerobic cultures of *M. hominis* and *M. orale* type 2.

Finally, the growth of *M. fermentans* on glass was characterized by a pattern intermediate between those described above, in that the CF titer of sedimentable antigen reached an early peak (second day), and then diminished as the titer of glass-adherent antigen began to rise (Fig. 3). On the third day, the rising titer of the glass-adherent antigen equaled the titer of sedimentable antigen and thereafter exceeded it. The soluble antigen reached moderate levels of CF activity on the second day and fluctuated somewhat thereafter. Similar results were obtained when *M. fermentans* was cultured under anaerobic conditions.

As shown in Fig. 4, the relative potency of CF activity in the different antigen fractions of mycoplasma preparations was reproducible, although the number of days of incubation required to attain the maximal CF titer of the glass-adherent antigen varied somewhat, depending on the
Mycoplasma antigens grown on glass reacted with homologous and heterotypic antisera in the same manner as broth-grown antigens (24). Thus, glass-adherent antigens of mycoplasmas appear to be serologically similar to broth-grown antigens when tested by CF.

**DISCUSSION**

The ability of mycoplasmas to grow on glass appears to be a common characteristic of these organisms. However, such glass-adherent growth occurs irregularly when mycoplasmas other than *M. pneumoniae* or *M. gallisepticum* are grown in medium containing 10 to 20% horse serum (25). When the concentration of horse serum is decreased to 2 to 5%, several mycoplasmas of animal origin adhere to glass (Robillard, personal communication). Similarly, when horse serum is replaced with bovine serum fraction, the mycoplasmas of human origin adhere to glass. Thus, adherence of mycoplasmas to glass appears to be inhibited by certain components of horse serum.

Growth of mycoplasmas on glass occurred both aerobically and under conditions of reduced oxygen tension. Furthermore, such growth was potentiated, at least under aerobic conditions, by supplementation of the medium with glucose (fermenting species *M. fermentans* and *M. pneumoniae*) or arginine (arginine-utilizing species *M. salivarium* and *M. hominis*), and it is probable that the growth of other fermenting or arginine-utilizing species on glass will be similarly stimulated by these substrates. Antigens prepared from glass-adherent mycoplasmas were as serologically reactive and as specific as antigens prepared from broth cultures, and, in addition, were easier to prepare.

Senterfit (personal communication) has found *M. pneumoniae* glass-grown antigens to be free from serum components derived from the medium, even when such antigens were tested for by attempting to elicit an anaphylactic response in guinea pigs immunized with the antigen and subsequently challenged with bovine serum. Such a test is one of the most sensitive serological techniques for detecting the presence of contaminating proteins. Since *M. pneumoniae* grows as a tangled mass of organisms, both in broth and on glass (2), whereas the other mycoplasmas of human origin grow in a more dispersed manner, it is likely that contamination of glass-grown antigens with medium components is minimal.

Glass-adherent mycoplasma preparations are useful for various serological and biochemical procedures. Mycoplasmas grown on glass micro-

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**Fig. 5. Relationship between duration of incubation and "specific activity" (reciprocal of CF titer per millimole of nitrogen) of glass-adherent *M. salivarium* and *M. fermentans.**

- **SPECIFIC ACTIVITY (CF titer per mm nitrogen)**
  - **DAYS OF INCUBATION**
  - **M. salivarium**
  - **M. fermentans**
have found growth on glass to be a simple means of preparing such organisms for analysis of their proteins by acrylamide gel electrophoresis as described by Rotten and Razin (15).

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