The Components of *Mycoplasma salivarium* and its Growth Medium that are Responsible for Film Formation

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(Received 8 November 1978)

Studies on film production by mycoplasmas revealed that film was produced by completely disintegrated mycoplasma cells on Noble agar in the presence of horse serum. Film production was due to an enzymic reaction between mycoplasma lipase, possibly phospholipase A, and phospholipid in serum.

INTRODUCTION

Since the first description of film and spot production by some mycoplasma species by Edward (1950*a, b*), this phenomenon has been widely used as an aid for the classification and identification of mycoplasma species (Rottem & Razin, 1964; Aluotto *et al.*, 1970). Edward (1954) found that the film contained cholesterol and phospholipid and postulated that it might be produced by the action of lipases which digest phospholipid. Razin & Rottem (1963) pointed out that granules and needles resembling the film appeared when a drop of pancreatic lipase solution was placed on plates of growth medium. Fabricant & Freundt's (1967) report that film production was enhanced by addition of 10% egg yolk emulsion or lecithin to the growth medium suggested that the component of the solid medium required for film production might be phospholipid.

During experiments on mycoplasmacin (Nakamura *et al.*, 1975; Nakamura, 1976), we discovered that film was produced by mycoplasmacin derived from mycoplasma cells that had been inactivated by ultrasonication and ultraviolet irradiation.

No detailed investigations of film production by mycoplasmas have been made. This paper describes a simple experimental system for studying factors affecting film and spot production, and some biochemical properties of mycoplasma cells and culture medium essential for the production of film.

METHODS

Strain. *Mycoplasma salivarium*-C Hup 127, maintained in our laboratory, was used throughout the experiments.

Medium. For growth of mycoplasma cells, enriched PPLO broth was used; this was composed of 70 vol. PPLO broth (Difco), 20 vol. horse serum and 10 vol. 25% (w/v) freshly prepared yeast extract, supplemented with 0.0125% (w/v) thallium acetate (Wako Co., Osaka, Japan) and 1000 U penicillin G ml⁻¹ (Meiji Co., Tokyo, Japan). Enriched PPLO agar contained 70 vol. PPLO agar (Difco) instead of PPLO broth, and it was distributed in 7 to 8 ml amounts into Petri dishes (65 mm diam.). When components were omitted from PPLO agar, they were replaced by an equal volume of phosphate-buffered saline. For 20% horse serum/Noble agar, 20 ml horse serum was added aseptically to 80 ml of a sterilized solution of 1.5% (w/v) Noble agar (Difco) in water.

Sera. Horse, bovine, calf, goat and rabbit sera were filtered through membrane filters (pore size 0.22 µm; Seitz or Millipore), heated at 56 °C for 30 min, and then stored at -20 °C.

Medium for studying the effect of lipids on film production. Lipid (40 mg) was added to 40 ml 1.5% Noble agar, and this mixture was autoclaved and then emulsified by ultrasonication (19.5 kHz, 3 A, 10 min). For

experiments with horse serum lipid, 3.2 ml lipid dissolved in chloroform were added to 100 ml Noble agar. The homogenized agar was distributed in 7 to 8 ml amounts into Petri dishes.

Observation of film production. For the intact mycoplasma, a single colony was observed under a microscope ($\times 40$) at intervals in the course of film production. Inactivated cells or extracts were either directly inoculated on to solid medium, or 0.1 ml samples were placed in wells (diam. 9 mm) in the solid medium. Inoculated plates were incubated at 37 °C.

Preparation of cell extract. Mycoplasma cells (about 10^9 colony-forming units ml^{-1}) grown in liquid medium for 4 d at 37 °C were collected by centrifugation at 9900g for 60 min. The cells were washed three times with distilled water, and then resuspended in distilled water (1/50 of the original culture volume) for ultrasonication (19.5 kHz, 3 A, 10 min). The supernatant obtained by ultracentrifugation (54000g, 60 min) of the ultrasonicated cell suspension still contained some viable mycoplasma cells. In order to inactivate these completely, samples were poured into a Petri dish (diam. 87 mm) and irradiated with ultraviolet light (germicidal lamp, 15 W-G; Toshiba Electric Co., Tokyo, Japan) from a distance of 20 cm for 30 to 60 min. These samples, which we refer to as 'cell-free extracts', were lyophilized and stored in a refrigerator.

Treatment of cell-free extracts. The pH stability of the film-producing ability of cell-free extracts was investigated in the pH range from 3 to 9 [pH 3 to 8 in McIlvaine's buffer (0.1 M-citric acid, 0.2 M- Na_2HPO_4), pH 9 in Michaelis' veronal buffer (0.1 M-sodium barbiturate, 0.1 M-HCl)] for 60 min at room temperature. The heat stability of samples suspended in water was tested by heating at 37, 56, 65, 80 and 100 °C for 30 min. The effect of dialysis against water for 18 h at 4 °C was tested. In all tests, lyophilized cell-free extract was suspended at 20 mg ml^{-1} and 0.1 ml of the treated suspension was immediately added to a well in 20% horse serum/Noble agar. Loss of film-producing ability was observed microscopically during 14 d incubation at 37 °C.

Extraction of lipids from horse serum. The method employed was based on that of Bligh & Dyer (1959). Horse serum (125 ml) was shaken with 470 ml chloroform/methanol (1:2, v/v) and centrifuged at 890g for 30 min. The sediment was extracted with 580 ml chloroform/methanol/water (1:2:0.8, by vol.) and centrifuged. The combined supernatants were mixed with 2.5 vol. chloroform. An equal volume of distilled water was then added and mixed. The chloroform fraction obtained by centrifugation was mixed with an equal volume of benzene. Horse serum lipid was obtained from this mixture by rotary evaporation, and dissolved in 10 ml chloroform.

Analysis of lipids. Phospholipids, total cholesterol and neutral lipids were measured by enzymic methods (Takahashi, 1974; Hess, 1964; Ansell, 1965).

Enzymes. Pancreatic lipase (unknown activity; Wako Co., Japan), wheat germ lipase (6 U mg^{-1} ; Sigma), phospholipase A_2 (minimum 100 U mg^{-1} , from snake venom; Boehringer-Mannheim), phospholipase C (1 to 2 U mg^{-1} , from *Clostridium perfringens*; P-L Biochemicals) and phospholipase D (0.5 U mg^{-1} , from cabbage; P-L Biochemicals) were used.

Protein assay. Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin fraction V (Armour Pharmaceutical Co.) as a standard.

RESULTS AND DISCUSSION

Establishment of a simple experimental system for studying film production by mycoplasmas

Previous observations (Nakamura, 1976) indicated that film was produced by inactivated mycoplasma cells. To identify the film-producing agent of mycoplasmas, we examined the effects of sonication, ultracentrifugation and ultraviolet irradiation on film-producing ability. Completely disrupted mycoplasma cells still produced a film on enriched PPLO agar. Therefore, a cell-free extract was used for all further experiments. To determine what components of enriched PPLO agar were essential for producing film, horse serum and yeast extract were omitted from complete PPLO agar or were added to 1.5% Noble agar. Film was not produced on PPLO agar without horse serum but it was observed on 1.5% Noble agar containing 20% horse serum without yeast extract (Fig. 1).

Properties of film-producing ability of cell-free extract of M. salivarium

The film-producing ability of the cell-free extract was weakened by heating for 30 min at 65 °C and completely destroyed at 80 °C, but was not affected by changes in pH between 3 and 9 or by dialysis for 18 h at 4 °C against water. Therefore it seemed that the film-producing agent in the cell-free extract might be a protein. The film-producing abilities of

Fig. 1. Film production around a drop of cell-free extract of *M. salivarium* on the surface of 20% horse serum/Noble agar. Bar marker represents 0.25 mm.

Fig. 2. Relationship between film production and the lipid contents of different types of serum: ■, phospholipids; □, total cholesterol; ▨, neutral lipids. Film production was tested using cell-free extract of *M. salivarium*: + + +, film production started after 2 d incubation and expanded over the whole surface of the agar; + +, film production started after 3 d incubation and expanded over half the surface of the agar; +, film production started after 5 d incubation and was limited to about 1 cm diam.; —, no film production.

several types of lipase were tested, because film production by pancreatic lipase was re-reported by Razin & Rottem (1963). All commercial lipases tested, (pancreatic lipase, wheat germ lipase, phospholipases A₂, C and D) produced film on 20% horse serum/Noble agar, but to different extents; the strongest activity was found in phospholipase A₂, which was as active as the cell-free extract. The film-producing ability of phospholipase A₂ was not inactivated by heating at 65 °C for 30 min, but was slightly inactivated at 80 and 100 °C, and was not affected by dialysis. It seemed that phospholipase A₂ might be useful as a model of the film-producing agent of *M. salivarium*. The minimum concentration of horse serum in Noble agar that gave film production with phospholipase A₂ was 5% (v/v)

(5.2 mg protein ml⁻¹). In 20 % horse serum/Noble agar, 1.5×10^{-2} units of phospholipase A₂ (11.3 ng protein) were able to produce the film.

Film production by commercial lipids and lipid extracted from horse serum

Fabricant & Freundt (1967) reported that egg yolk emulsion, which mainly consists of lecithin, stimulated film production. Therefore film production by commercial lecithin, palmitic acid, tributyrin, cholesterol, cholesterol stearate and lipid extracted from horse serum was tested, using cell-free extract of *M. salivarium* or pancreatic lipase as the agent for producing film. The cell-free extract only produced film on Noble agar containing lecithin and pancreatic lipase only produced film on Noble agar containing either lecithin or lipid from horse serum. Thus an essential factor for film production was lecithin digested by lipase.

Relationship between film production and different types of serum

The relationship between the contents of phospholipids in several types of serum and film production was studied, using cell-free extract of *M. salivarium* or pancreatic lipase as the film-producing agent. There was a parallel relationship between the extent of film production and the contents of phospholipids and cholesterol, but not neutral lipids (Fig. 2). Pancreatic lipase also produced film in horse and bovine sera.

It is concluded that film production by mycoplasmas is mainly due to a reaction between a bacterial phospholipase A and lecithin in the growth medium. Although bacterial phospholipases C and D cannot release free fatty acids and lysolecithin from lecithin, they did have slight film-producing ability. It is possible that commercial phospholipases C and D contained a small amount of diesterase which was able to degrade lecithin to free fatty acids and lysolecithin.

REFERENCES

- ALUOTTO, B. B., WITTLER, R. G., WILLIAMS, C. O. & FABER, J. E. (1970). Standardized bacteriologic techniques for the characterization of *Mycoplasma* species. *International Journal of Systematic Bacteriology* **20**, 35–58.
- ANSELL, G. B. (1965). The action of drugs on phospholipid metabolism. *Advances in Lipid Research* **3**, 139–170.
- BLIGH, E. G. & DYER, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911–917.
- EDWARD, D. G. FF. (1950a). An investigation of pleuropneumonia-like organisms isolated from the bovine genital tract. *Journal of General Microbiology* **4**, 4–15.
- EDWARD, D. G. FF. (1950b). An investigation of the biological properties of organisms of the pleuropneumonia group. *Journal of General Microbiology* **4**, 311–329.
- EDWARD, D. G. FF. (1954). The pleuropneumonia group of organisms. A review together with some new observations. *Journal of General Microbiology* **10**, 27–64.
- FABRICANT, J. & FREUNDT, E. A. (1967). Importance of extension and standardization of laboratory tests for the identification and classification of mycoplasma. *Annals of the New York Academy of Sciences* **143**, 50–58.
- HESS, R. (1964). Evaluation of drugs active against experimental atherosclerosis. *Advances in Lipid Research* **2**, 295–445.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- NAKAMURA, M. (1976). Mycoplasma growth inhibitory substance (mycoplasmacin) derived from mycoplasmas. *Proceedings of the Society for General Microbiology* **3**, 173.
- NAKAMURA, M., ITOH, T. & HIRATA, N. (1975). Mycoplasmacin: mycoplasma growth inhibitory substance produced by mycoplasmas. *Kurume Medical Journal* **22**, 219–221.
- RAZIN, S. & ROTTEM, S. (1963). Fatty acid requirements of *Mycoplasma laidlawii*. *Journal of General Microbiology* **33**, 459–470.
- ROTTEM, S. & RAZIN, S. (1964). Lipase activity of mycoplasma. *Journal of General Microbiology* **37**, 123–134.
- TAKAHASHI, J. (1974). The determination of serum triglyceride by the enzymatic hydrolysis. *Journal of Clinical Chemistry* **2**, 425–432.