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Morphology of *Mycoplasma laidlawii* Type A

I. Comparison of Electron Microscopic Counts with Colony-Forming Units

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ABSTRACT

ANDERSON, D. L. (University of Minnesota, Minneapolis), M. E. POLLOCK, AND L. F. BROWER. Morphology of *Mycoplasma laidlawii* type A. I. Comparison of electron microscopic counts with colony-forming units. J. Bacteriol. **90**:1764–1767. 1965.—Cells of *Mycoplasma laidlawii* A grown in dialyzing flask cultures were counted with the electron microscope by use of a spray technique which deposited mixtures of polystyrene latex of known concentration and *M. laidlawii* in discrete droplet patterns on specimen films. Glutaraldehyde and formaldehyde were effective in preserving gross morphology of cells in spray preparations. The standard deviation of the mean ratio of latex-*M. laidlawii* was 5.5% when 2,000 total particles were counted in 18 droplet patterns. Microscopic counts resembled counts of colony-forming units (CFU) at various culture ages when cells larger than 0.25 μ were enumerated. If small bodies ranging from 0.1 to 0.25 μ in diameter were included in microscopic counts of cultures older than 70 hr, these counts exceeded the numbers of CFU by 4 to 10 times.

Mycoplasma (pleuropneumonia-like organisms) have been quantitated by a variety of methods, but the relationship between numbers of viable cells in a population and actual numbers of morphological units has not been determined. Inasmuch as small and large *Mycoplasma* cell types presumably present stages in the life cycles of these organisms, a knowledge of the numerical relationship between viable cells and morphological entities is of utmost importance.

Numbers of colony-forming units (CFU) relative to actual numbers of cells of *M. laidlawii* A were determined in the present study by comparing colony counts on soy peptone-yeast extract-agar with electron microscopic counts.

MATERIALS AND METHODS

Culture. The organism used was *M. laidlawii* type A (ATCC 14089).

Growth media and enumeration of CFU. Soy peptone-yeast extract (SP-YE) broth and agar media were used for cultivation of *Mycoplasma* and enumeration of CFU (Pollock, 1965).

Materials for fixation, dialysis, and electron microscopy. Dialyzer tubing used for dialysis of cell suspensions was obtained from the Arthur H. Thomas Co., Philadelphia, Pa. (cellulose, no. 4465-A2, 5/8-inch diameter, inflated). Polystyrene latex (0.264-μ diameter) was supplied by the Dow Chemical Co., Midland, Mich. Stock buffer (10×) was prepared by dissolving 38.0 g of sodium acetate and 57.8 g of sodium barbital in 1 liter of distilled water. The *p*H of formaldehyde or glutaraldehyde (Union Carbide Chemicals, Chicago, Ill.) added directly to cell suspensions was adjusted to 7.3 by addition of 10× stock buffer. The dialyzing solution was 1× buffer containing either 3.3% glutaraldehyde or 9.2% formaldehyde; *p*H was adjusted to 7.3 with 1 N hydrochloric acid. The *p*H of the 0.2 M solution of ammonium acetate used for final dialysis was adjusted to 7.3 with 1 N sodium hydroxide. Coagulation tubes were purchased from Arthur H. Thomas Co. (no. 3467, 1 to 1.25 mm outer diameter). Copper specimen grids (3.05-mm diameter) were coated with 0.1% Formvar and reinforced with carbon.

Growth of cells. Experimental cultures were grown in dialyzing flasks as described by Pollock (1965). The membrane culture vessel contained 20 ml and the reservoir 320 ml of SP-YE broth. The flasks were brought to 37 C prior to use and the membrane culture vessel was inoculated with a diluted 4- to 18-hr culture in volumes ranging from 0.02 to 0.1 ml. The number of CFU in a given dilution could be estimated by reference to standard growth curves performed previously. Penicillin (E. R. Squibb & Sons, New York, N.Y., buffered penicillin G potassium for injection, USP) was added to the reservoir to a final concentration of 100 units per ml. Samples were taken from the vessels for determination of CFU at zero-time and the cultures were then incubated at 37 C in an Eberbach model 6250 water bath shaker oscillating at 115 cycles per min.

Fixation for electron microscopy. Hydrated

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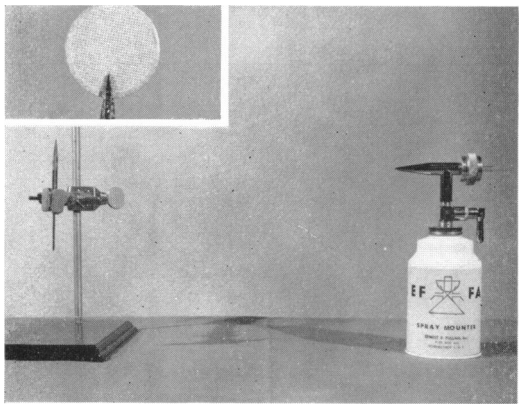

FIG. 1. *Apparatus used in the spray technique. The specimen grid is held with tweezers (insert) at a distance of 18 inches from the EFFA spray mounter. Reduced approximately 7.3 times.*

dialyzer tubing was doubly knotted on one end. In most experiments, a 0.5-ml sample was removed from the culture vessel and mixed with 0.5 ml of polystyrene latex at a concentration of 3.46×10^{10} per milliliter; to this suspension was added glutaraldehyde or formaldehyde to final concentrations of 3.3 or 9.2%, respectively. The mixture was placed in dialyzer tubing, and, after exclusion of air, the tubing was doubly knotted and the bag was placed in 500 ml of diluted fixative. The mixture was dialyzed for 24 hr on a magnetic mixer at room temperature. Dialysis was continued against 1× Veronal-acetate buffer lacking fixative for 6 hr, followed by a final 2-hr dialysis against 0.2 M ammonium acetate.

Preparation of grids. A coagulation tube was filled with the *Mycoplasma*-latex mixture, placed in an EFFA spray mounter, and the mixture was sprayed on films for electron microscopy as demonstrated in Fig. 1. The grids were shadowed with uranium at an angle of 20 to 30 degrees and observed in a Siemens Elmiskop 1A electron microscope.

Droplet patterns were photographed at magnifications of × 1,000 to 4,000 and enlarged approximately three times for counting.

RESULTS

Spray technique. Cells from a 39-hr culture of *M. laidlawii* A fixed in 3.3% glutaraldehyde were sprayed on specimen grids from distances of 15, 28, 46, and 61 cm to determine the optimal distance for obtaining discrete droplets. Nearly all supporting films were broken when a 15-cm distance was used; at 28 cm, films were intact but most of the droplets were too large, in many instances covering entire grid openings. Distances of 46 and 61 cm provided discrete droplets for electron microscopy; droplets were larger and more numerous on grids sprayed at 46 cm, hence this distance was used in subsequent experiments.

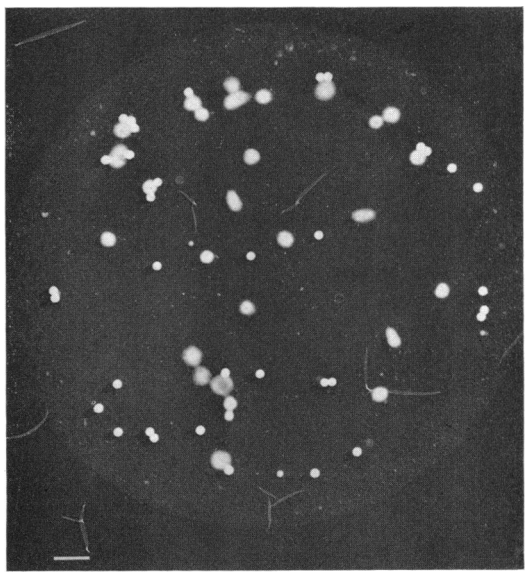

FIG. 2. *Representative droplet pattern obtained by spraying a mixture of 0.264-μ polystyrene latex and cells of a 64-hr culture of Mycoplasma laidlawii.*

Statistical variation of counts among droplet patterns. Cells from a 42-hr culture of *M. laidlawii* fixed in 3.3% glutaraldehyde were mixed with latex and sprayed on specimen films. Approximately 2,000 total particles were counted in 18 droplet patterns to evaluate statistical fluctuations of counts among individual droplets. The standard deviation of the mean ratio of reference latex-*Mycoplasma* (1.54) was 0.084 or 5.5%. A similar 5.5% standard deviation of a mean ratio of 1.3 was calculated when 1,100 particles in 11 droplet patterns of a 64-hr culture were counted. A representative droplet pattern is shown in Fig. 2.

Effects of fixation on morphology and microscopic counts. Several fixation procedures were tested for preservation of cells subjected to the spray mounting technique. Osmium tetroxide (1%) in Palade's buffer was added to cells sedimented from the culture fluid by centrifugation at 8,000 × *g* for 30 min or to cells washed twice with Hanks' solution lacking calcium and magnesium salts and sedimented each time by centrifugation and suspended in 0.1 M ammonium acetate. After osmium fixation, however, sedimented cells could not be dispersed to yield homogeneous suspensions. Since homogeneous mixtures were needed for microscopic counts, osmium tetroxide was not used subsequently. The applicability of aldehyde fixation was de-

TABLE 1. *Comparison of electron microscopic counts with colony counts of Mycoplasma laidlawii A*

Expt	Age of culture	Fixation	No. of droplet patterns	Total latex	Total *Mycoplasma* (PPLO)	Mean ratio (latex-PPLO)	SD of mean ratio	Coefficient of variation	Electron microscopic count	CFU
	hr							%		
10	20	G*	6	549	99	6.30	1.32	20	5.5×10^9	5.6×10^9
	20	F	5	611	90	6.73	0.56	8	5.1×10^9	5.6×10^9
	28	G	5	295	169	1.73	0.23	13	2.0×10^{10}	1.6×10^{10}
	28	F	6	932	222	4.27	0.48	11	8.1×10^9	1.6×10^{10}
12	24	G	5	247	123	2.27	0.42	18	1.5×10^{10}	1.6×10^{10}
	27	G	5	291	140	2.31	0.61	26	1.5×10^{10}	2.0×10^{10}
	30	G	5	365	311	1.22	0.29	23	2.8×10^{10}	3.9×10^{10}
	33	G	5	379	250	1.58	0.19	12	2.2×10^{10}	3.9×10^{10}
	36	G	5	287	168	1.86	0.31	16	1.9×10^{10}	3.4×10^{10}
	39	G	5	437	457	1.05	0.26	24	3.3×10^{10}	3.2×10^{10}
14	95	G	5	365	211	1.76	0.11	6	2.0×10^{10}	2.3×10^{10}
	119	G	6	404	170	1.99	0.34	17	1.7×10^{10}	1.8×10^{10}

* G denotes glutaraldehyde; F, formaldehyde.

termined by adding 37% formaldehyde or 25% glutaraldehyde directly to suspensions of cells grown in dialysis flasks to give final concentrations of 9.2 and 3.3%, respectively. Cells were grown to sufficient titer in dialysis flasks to make concentration by centrifugation unnecessary (Pollock, 1965). Latex reference particles were added to the cell suspensions prior to dialysis to eliminate the possible effects of volume changes on the counts. In most instances, it was not possible in shadowed preparations to distinguish formaldehyde-fixed cells from glutaraldehyde-fixed organisms.

A 28-hr culture was fixed with glutaraldehyde at 4 C and at room temperature to determine the effect of temperature on fixation. No differences between preparations fixed at the two temperatures were detected.

Comparison of electron microscopic counts with CFU. Numbers of CFU were compared with morphological entities, as observed in the electron microscope with cultures of various ages (Table 1). In most instances, the electron microscopic counts resembled closely the counts based on colony-forming ability; in no instance do the counts differ by more than a factor of two. Also, counts of organisms from a single population fixed with formaldehyde or glutaraldehyde do not differ substantially.

Culture age over the indicated ranges apparently did not significantly affect the close agreement between CFU and morphological entities. However, small bodies varying in size from 0.1 to 0.25 μ were detected in cultures approximately 28 hr old and gradually increased in number with time. Microscopic counts exceeded the numbers of CFU by 4 to 10 times in cultures 70 hr or older when small bodies were included in the counts.

DISCUSSION

The spray technique used in this study was patterned after the method of Backus and Williams (1950). These investigators found that the standard deviation of the mean ratio of bushy stunt virus-latex was 2 to 4% when 1,000 pairs of particles were counted in 20 or more droplet patterns. In the present study, the standard deviation of the mean ratio of latex-*Mycoplasma* was about 5.5% when 1,100 and 2,000 particles were counted in 11 and 18 droplet patterns. Recognition of *Mycoplasma* in many instances involved subjective judgment on the part of the investigator due to the tremendous size variation of *M. laidlawii* cells and the appearance of small bodies during the stationary phase of growth; judgment concerning 0.1 to 0.25 μ structures was most difficult. Also, cells of *M. laidlawii* are often observed in stages suggesting division; hence differentiation of adjacent but separate organisms from a single dividing unit in a late stage of separation was difficult or impossible. Cells larger than the 0.264-μ polystyrene latex were arbitrarily counted as *Mycoplasma*. Small bodies, 0.1 to 0.25 μ in size, were enumerated separately.

Regardless of the apparent difficulties in performing microscopic counts, the technique provides a means for comparing structural entities of *Mycoplasma* with colony-forming ability during specific phases of growth. Good agreement of

microscopic and colony counts was obtained when cells larger than 0.25 μ were counted, hence most small bodies of the size range 0.1 to 0.25 μ apparently do not contribute to the number of CFU of *M. laidlawii* unless a high proportion of large cells cannot form colonies. This in no way refutes the possibility that small bodies have the potential to develop into cells able to form colonies under suitable environmental conditions. Furthermore, it must be recognized that colony-forming ability may not be an absolute index of viability. The answer to the viability of small bodies lies obviously in studies of purified preparations of these units.

It is essential to point out that the youngest cells used in the present studies were in late logarithmic or early stationary phases of growth. To observe cultures during logarithmic growth, it is necessary either to concentrate cells by physical means or to inoculate dialyzing flask cultures with large numbers of cells, thus shortening the logarithmic growth phase.

Of importance in assessing the advantages of the dialyzing flask technique is that cells can be consistently grown to titers of 2×10^{10} to 5×10^{10} per milliliter, thereby eliminating the need for concentration of cells by physical means. Centrifugation of *Mycoplasma* has disadvantages in that the organisms are easily disrupted when pellets are dispersed, and small bodies are lost unless the centrifugation speed is high.

Osmium tetroxide was not used as a fixative in the spray technique because of the inability to disperse clumps of cells after fixation. Formaldehyde and glutaraldehyde were effective in preserving gross morphology of *M. laidlawii*.

The spray count technique should find particular application in studies of the life cycles of *Mycoplasma*. The life cycles of these organisms have been a matter of controversy because of morphological pleomorphism due to lack of a cell wall, dependence of gross morphology on the nature of the suspending medium, and lack of definitive information on cell division and reproduction. Terms such as "elementary body" and "granule" have been used to describe minimal reproductive units which presumably represent a stage in the developmental cycle of these organisms. Morowitz and Tourtellotte (1962) reported at least four morphological cell types of *M. laidlawii*, largely on the basis of size, which may represent stages in the life cycle of this organism. By use of filters of defined pore size, the smallest units of *M. laidlawii* were found to be between 0.1 and 0.2 μ in diameter. Density-gradient centrifugation was used to separate the small "elementary bodies" from larger cell types, some of which were greater than 1 μ in diameter. Klieneberger-Nobel (1962) reported that the smallest particles of agalactia of sheep were 120 to 180 mμ in diameter on the basis of filtration through gradocol membranes. Viability of cells in filtrates was measured in terms of ability to produce growth in broth. Definitive information as to the viability of these small bodies is of great importance in understanding the life cycles of *Mycoplasma*. The count technique should be of value in defining the proportion of purified small bodies able to initiate colony formation or growth in broth. Use of the spray technique to count cells of *M. laidlawii* growing in SP-YE in dialyzing flask cultures has also indicated that *Mycoplasma* can be counted, even though they have not been highly purified.

ACKNOWLEDGMENTS

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