# MORPHOLOGY OF PLEUROPNEUMONIA-LIKE ORGANISMS AND BACTERIAL L FORMS GROWN IN LIQUID MEDIA

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ABSTRACT

WEIBULL, C. (Central Bacteriological Laboratory of Stockholm City, Stockholm, Sweden) AND BRITT-MARIE LUNDIN. Morphology of pleuropneumonia-like organisms and bacterial L forms grown in liquid media. J. Bacteriol. **85**:440–445. 1963.—Cells of *Mycoplasma hominis*, *M. laidlawii*, and two tissue-culture strains of pleuropneumonia-like organisms (PPLO) moving freely in liquid medium were photographed with an electronic flash as the light source. The photomicrographs thus obtained demonstrated that, in young cultures of high viability, the cells of these organisms were mainly filamentous or, in the case of *M. laidlawii*, coccoid. In old cultures of the same organisms containing predominantly nonviable cells, granular and vesicular elements were found. By the use of the same photographic technique, liquid cultures of a stable *Proteus* L form were studied. Although no filaments of uniform thickness were found, there were spherical bodies and some threadlike material connected with the spheres. When samples of PPLO cultures containing filamentous forms were transferred to agar blocks, the filaments were converted to more or less spherical bodies. This conversion could be prevented by fixing the PPLO with formaldehyde. The morphology of *Proteus* L forms was not noticeably altered by fixation with this reagent.

Since the discovery of the pleuropneumonia-like organisms (PPLO), numerous morphological investigations have been carried out on them. A controversial point, recently reviewed by Dienes (1960), Freundt (1960), and Liebermeister (1960), has been the extent to which the individual PPLO assume a filamentous shape. The main reason why this point has not been satisfactorily settled seems to be that PPLO are very easily distorted by mechanical forces. Moreover, they obviously change their appearance with the environmental conditions and with the age of the bacterial cultures. The present study (to eliminate the influence of mechanical forces) is primarily concerned with PPLO moving freely in liquid media. In addition, we paid special attention to the extent to which the PPLO studied were viable, i.e., whether they were able to divide and give rise to colonies. Some comparative studies on two stable *Proteus* L forms were also carried out.

## MATERIALS AND METHODS

*Organisms.* (i) *Mycoplasma hominis*, strain Campo (Smith, Peoples, and Morton, 1957); (ii) *M. laidlawii*, strain A (Laidlaw and Elford, 1936); tissue-culture strains (iii) AE and (iv) 8490 T of PPLO; (v) a stable *Proteus* L form, strain L 9, derived from *Proteus mirabilis*, strain 9 (Klieneberger-Nobel, 1956; Weibull and Lundin, 1961); and (vi) a stable *Proteus* L form, isolated from *Proteus mirabilis*, strain 18 (Tulasne, 1949; Weibull and Hammarberg, 1962) were used.

Organisms i, iii, and v were obtained from E. Klieneberger-Nobel, Lister Institute of Preventive Medicine, London, England, and organisms ii and iv from E. A. Freundt, State Serum Institute, Copenhagen, Denmark. Organism vi was obtained from M. Sensenbrenner, Institut de Chimie Biologique, Strasbourg, France.

*Growth conditions. M. laidlawii* was grown at 30 C in meat broth supplemented with 1% inactivated horse serum. The other PPLO strains were grown in an aqueous solution containing 2% Tryptose (Difco), 0.4% yeast extract (Difco), 0.001% deoxyribonucleic acid, and 10% inactivated human serum. The pH of the medium was adjusted to 7.8. All organisms were grown in

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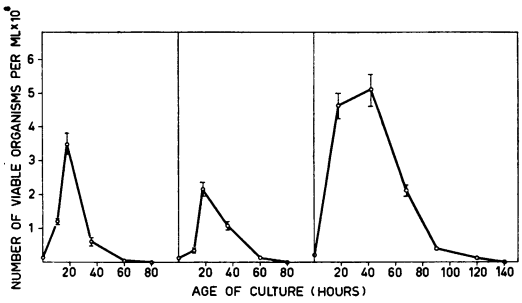


FIG. 1. *Viable counts of liquid cultures of PPLO strains AE (left), 8490 T (center), and Campo (right) as a function of the age of the cultures.*

200-ml Erlenmeyer flasks, each containing 50 ml of medium. Subcultures of the PPLO were made daily. The *Proteus* L forms were grown at 30 C in Abrams' (1955) liquid serum-free medium without penicillin.

*Determination of total and viable counts.* A known volume of the liquid culture in question was spread uniformly over the surface of an agar plate. If necessary, the culture was diluted with growth medium before being spread. Usually, the agar plate contained the medium of the liquid culture supplemented with 0.8% agar (Difco). After drying the plate, about five slide cultures were prepared from each plate by cutting agar blocks from the plate, transferring these blocks to slides, covering the blocks with cover slips, and sealing the preparations with a mixture of Vaseline and paraffin. The slide cultures were immediately examined under a phase-contrast microscope using an oil-immersion objective. The number of individual PPLO in five to ten microscopic fields of view were counted. (Cell aggregates were each counted as one organism.) The slide cultures were then incubated for 24 hr, and the microcolonies formed were counted (the average size of the colonies was about 10 $\mu$). The total count, $T$, of the original culture was obtained from the formula

$$T = \frac{n_1 \cdot a_1 \cdot d}{a_2 \cdot v}$$

Here, $n_1$ is the average number of PPLO per microscopic field of view, $a_1$ the surface area of the agar plate, $a_2$ the area of the microscopic field of view, $d$ the degree of dilution of the sample spread on the agar plate as compared with the original liquid culture, and $v$ the volume of the sample spread on the agar plate.

The viable count, $V$, of the liquid culture was obtained by replacing $n_1$ in the formula with the average number of microcolonies per microscopic field of view, as determined from the incubated slide cultures.

*Photomicrography.* A Leitz 90 × phase-contrast objective and an 8 × compensating eyepiece were used. Photomicrographs of PPLO and L forms moving freely in liquid medium between slide and cover slip were taken with a microcamera equipped with 35-mm Gevaert Duplo Ortho film, with a Leitz Multiblitz-Mikro 300-w electronic flash as the light source. The magnification given by the negative was 330 ×. Photographs of PPLO and L forms in slide cultures were taken with a box camera, which provided a magnification at the negative of 950 X. Gevaert Graphic Ortho 05 plates were used.

## RESULTS

*Relationship between the viable count of PPLO cultures and their age.* Figure 1 shows the number of viable organisms in liquid cultures of PPLO strains AE, 8490 T, and Campo as a function of the age of the cultures. At zero time, growth was started by inoculating 50 ml of fresh medium with 2 ml of an overnight culture. At suitable time intervals, culture samples were spread on plates containing the same medium solidified with agar. Microcolonies were usually counted after 24 hr of incubation. Control counts made after 24 hr of additional incubation showed no significant increase in colony numbers.

The number of viable cells in cultures of PPLO strains AE and 8490 T decreases rapidly after

TABLE 1. *Viability of PPLO grown in liquid medium*

| Expt | PPLO strain | Age of liquid culture | Viability* |
|---|---|---|---|
| | | *hr* | % |
| 1 | AE | 12 | 46.6 ± 4.5 |
| 2 | AE | 12 | 75.0 ± 7.2 |
| 3 | AE | 20 | 58.5 ± 5.4 |
| 4 | 8490 T | 12 | 79.8 ± 7.7 |
| 5 | 8490 T | 12 | 93.8 ± 9.0 |
| 6 | 8490 T | 20 | 41.3 ± 4.2 |
| 7 | Campo | 18 | 84.8 ± 8.1 |
| 8 | Campo | 42 | 69.5 ± 6.7 |

* Standard error of the mean values indicated by the ± sign.

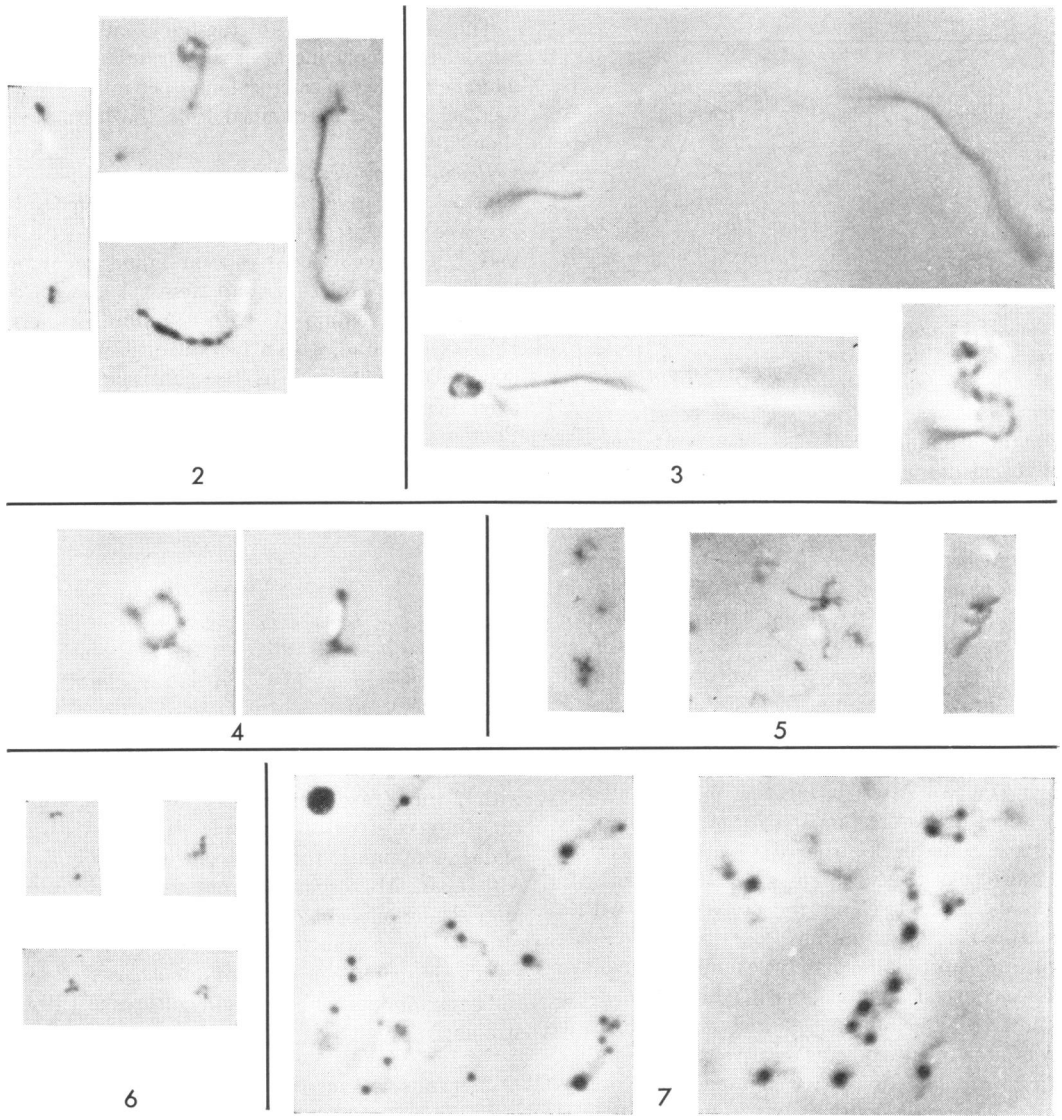


FIG. 2 to 7. *Freely moving cells from liquid cultures of PPLO and a Proteus L form. A drop of each culture was placed between slide and cover slip, and the cells were photographed under a phase-contrast microscope with an electronic flash as the light source. Magnification in all figures: 1,700 ×. Figure 2 (cluster at upper left) shows a 12-hr culture of PPLO strain AE; Fig. 3 (upper right) shows a 12-hr culture of PPLO strain 8490 T; Fig. 4 (middle left) shows a discoid cell from a 12-hr culture of PPLO strain 8490 T (photographed at different moments); Fig. 5 (middle right) shows an 18-hr culture of M. hominis, strain Campo; Fig. 6 (lower left) shows an 18-hr culture of M. laidlawii, strain A; Fig. 7 (lower right) shows a 24-hr culture of Proteus mirabilis, strain L 9.*

approximately 18 hr of incubation (Fig. 1). The cells in cultures of the Campo strain evidently retain their viability considerably longer.

*Viability of young PPLO cells.* In the counting experiments described above, plates containing human serum were used. In such plates, a precipitate always appeared. The microscopic particles constituting this precipitate could easily

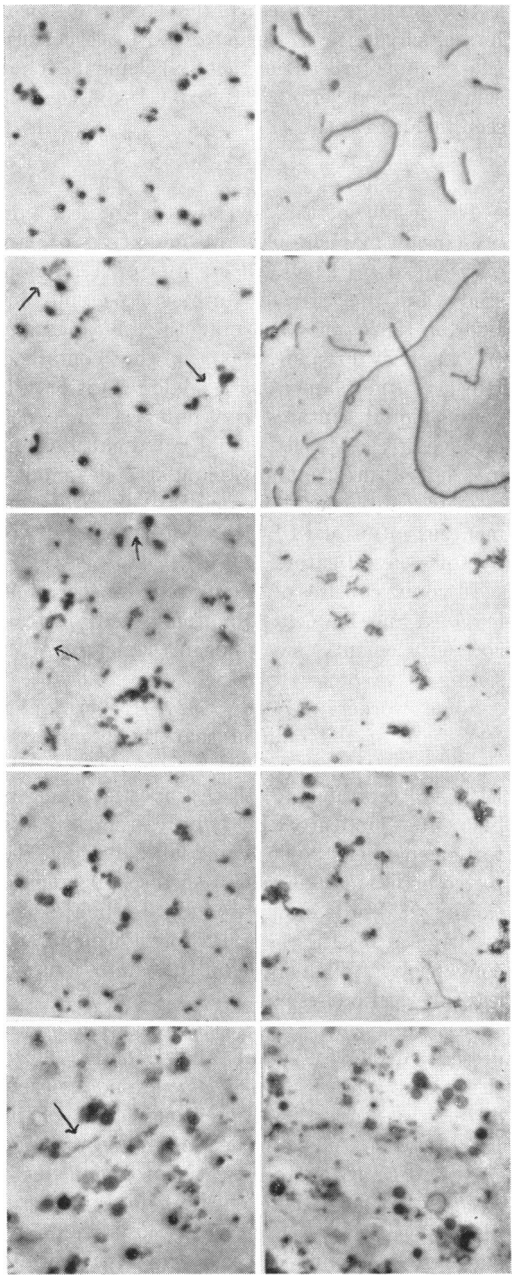

FIG. 8 to 12. *Cells of PPLO and Proteus L forms grown in liquid media after transfer to agar blocks containing the same growth medium. The cells were photographed under a phase-contrast microscope immediately after the transfer. The cells in all of the photographs on the right were fixed in the liquid medium with formaldehyde; the two lowest photographs on the left represent unfixed specimens. Magnification in all figures: 1,250 ×.*

be taken for individual PPLO cells (but not for microcolonies). Consequently, no adequate total counts could be made. For total counts, plates containing horse serum were used. In such plates, no precipitates appeared, even when samples of liquid cultures containing human serum were spread on the plates. Viable counts were made concurrently on the horse serum plates, and the viability of the PPLO was calculated. Table 1 shows the results of experiments with PPLO grown in a medium containing human serum and spread on horse serum plates. It can be seen that figures for between 40 and 90% viability were obtained. Probably, however, growth conditions on horse serum plates are not ideal for these strains, and the figures obtained should therefore be considered a low estimate of their viability. As a rule, the viability of the PPLO cells decreased with age.

*Morphology of PPLO and L forms moving freely in liquid medium.* Cells of *M. agalactiae* were photographed with an electronic flash as the light source (Weibull and Lundin, 1962*b*). By this technique, it could be clearly demonstrated that viable cells of this organism grown in liquid culture often have a rodlike shape. In the present study, the same technique has been applied to young cultures of *M. laidlawii*, *M. hominis*, and the tissue-culture strains AE and 8490 T. As has been shown in the preceding paragraphs and in a previous investigation (Weibull and Lundin, 1962*b*), such cultures contain mainly viable cells. Figures 2 to 5 show cell types frequently found in 12- to 18-hr cultures of the parasitic PPLO strains. Filamentous cells of various lengths can be seen, but also discoid and starlike forms. Figure 6 shows photographs of a culture of *M. laidlawii*. Obviously, the cells of this organism are coccoid rather than rod-shaped. Cells consisting of three coccoid elements forming a kind of triangle are often seen.

Photomicrographs of a 24-hr liquid culture of L 9, the stable *Proteus* L form, taken with an electronic flash as the light source, are shown in Fig.

*Fig. 8. (top row) shows a 12-hr culture of PPLO strain AE; Fig. 9 (second row) shows a 12-hr culture of PPLO strain 8490 T; Fig. 10 (third row) shows an 18-hr culture of M. hominis, strain Campo; Fig. 11 (fourth row) shows a 100-hr culture of PPLO strain AE (left) and PPLO strain 8490 T (right); Fig. 12 (bottom row) shows a 24-hr culture of Proteus mirabilis strain L 9 (left) and 18 (right).*

7. As can be seen, spherical elements of various sizes are present in this culture. This is in accord with earlier observations on the same L form (Weibull and Beckman, 1960; Weibull and Lundin, 1961; Weibull and Lundin, 1962*a*). In addition, threadlike structures appear. However, these threads are thinner and of less regular thickness than the filamentous PPLO shown in Fig. 2 to 5. Moreover, the L form threads are always connected to one or several spherical elements.

*Appearance of PPLO and L forms grown in liquid media after transfer to agar plates.* It has been shown (Weibull and Lundin, 1962*b*) that spherical elements, coccoid bodies, and short rods are present in young cultures of *M. agalactiae* and *M. laidlawii*. In similar preparations from old cultures, predominantly spherical bodies were seen. In slide cultures prepared from 12- to 18-hr cultures of PPLO strains Campo, AE, and 8490 T, approximately spherical bodies and small rodlike elements were seen (Fig. 8a, 9a, and 10a). Long filamentous elements, such as those predominant in liquid cultures before transfer to the agar plates (Fig. 2 to 5), were not found. Sometimes threadlike structures were seen (indicated by arrows in Fig. 9a and 10a), but these threads seemed to represent distorted spherical bodies. In old cultures, spherical bodies and vesicular elements were predominant (Fig. 11a, b).

When samples from young liquid cultures of the parasitic PPLO strains were fixed for 1 to 2 hr with formaldehyde (final concentration in the fixed preparation, 4%), samples of the fixed cultures were spread on agar plates, and the agar surface was examined microscopically. Threadlike elements were predominant, as in liquid cultures not spread on agar (Fig. 8b, 9b, and 10b). Evidently, the fixing process efficiently preserved the filamentous forms, which otherwise were converted into more or less spherical bodies when transferred to an agar surface. In old cultures, only a few threadlike elements were found after fixation (Fig. 11a, b).

In connection with the discussion of morphological relationships between L forms and PPLO, it was deemed of interest to investigate whether filamentous forms appeared in young L form cultures to which formaldehyde had been added. Filaments of regular thickness did not appear in such preparations (Fig. 12a, b), only some threadlike material (indicated by an arrow in Fig. 12a, b).

It should be mentioned that the addition of formaldehyde to L form and PPLO cultures caused some lysis of the bacterial elements. Thus, some spherical "ghosts" (lysed L bodies) can be seen in Fig. 12a, b.

## DISCUSSION

The results of this investigation indicate the presence of filamentous forms in cultures of various parasitic PPLO. The results of a previous study by the present authors (Weibull and Lundin, 1962*b*) and of several other investigations (reviewed by Freundt, 1960) are thus confirmed. However, the filaments are predominant only in young liquid cultures containing a high percentage of viable cells. In old liquid cultures and in solid cultures, mainly granular and spherical elements are seen. This may explain why filamentous forms of PPLO have not been observed by some investigators.

In young cultures of the saprophytic species *M. laidlawii*, no filaments are found but, instead, coccoid elements, singly or in structures consisting of two or several cocci.

Some workers (Dienes, 1960; Liebermeister, 1960) are of the opinion that the filamentous shape is not a characteristic property of the PPLO. According to these investigators, the filaments found in PPLO cultures represent distorted spherical bodies. Undoubtedly such distorted bodies may be seen in microscopic preparations. Our results, however, indicate the frequent occurrence of the opposite phenomenon: the conversion of filamentous PPLO into more or less spherical bodies.

According to a previous investigation by the present authors (Weibull and Lundin, 1962*b*), the rodlike cells of *M. agalactiae* are not so easily distorted as are the filamentous forms of the PPLO strains studied in the present investigation.

According to our observations on cultures of *Proteus* L forms, these cultures do not contain filaments similar to those present in young PPLO cultures. In young L form cultures, the predominant morphological elements are spherical bodies of various sizes. Some threadlike material is also present, but this material can hardly be confused with the filaments found in PPLO cultures. These threads are not clearly revealed in slide cultures prepared from liquid L form cultures (Weibull and Beckman, 1960; Weibull and Lundin, 1961; Weibull and Lundin, 1962*a*), even

after fixation with formaldehyde. It could be added that the spherical elements found in L cultures differ very much in size and shape from the coccoid elements present in cultures of the saprophytic PPLO (*M. laidlawii*).

In our opinion, the results of the present investigation favor the view that important differences exist between L forms and PPLO. In young, liquid cultures containing predominantly viable cells, the morphological differences are striking. The elements present in old liquid cultures of PPLO and L forms, on the other hand, are not easily distinguished from each other. The same is true for the elements present in solid cultures of PPLO and L forms.

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