SIZE AND SHAPE OF PLEUROPNEUMONIA-LIKE ORGANISMS GROWN IN LIQUID MEDIA

C. WEIBULL[1] AND BRITT-MARIE LUNDIN

Central Bacteriological Laboratory of Stockholm City, Stockholm, Sweden

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ABSTRACT

WEIBULL, C. (Central Bacteriological Laboratory of Stockholm City, Stockholm, Sweden), AND BRITT-MARIE LUNDIN. Size and shape of pleuropneumonia-like organisms grown in liquid media. J. Bacteriol. **84**:513–519. 1962.—Samples of liquid cultures containing mainly nonaggregated cells of *Mycoplasma agalactiae* or *M. laidlawii* were transferred to agar blocks containing the same medium as the liquid cultures. By use of a phase-contrast microscope, photomicrographs were made of the slide cultures immediately after they had been prepared, and the dimensions of a large number of pleuropneumonia-like organisms (PPLO) were measured. These measurements indicated that, in young cultures (incubated for 24 to 48 hr), the size of the cells did not vary much more than that of ordinary bacteria; 95% of the cells had a width of 0.2 to 0.6 μ. The growth of individual PPLO was followed during incubation of the slide cultures. It was found that 80 to 100% of the cells present in liquid overnight cultures divided and gave rise to microcolonies within a few hours. Rod-shaped, ellipsoidal, and spherical cells were seen in these cultures. Liquid cultures incubated for several days contained mainly spherical cells. Fewer than 5% of the cells in these cultures showed any indication of growth during incubation in slide cultures for 5 days. Photomicrographs of cells of *M. agalactiae* moving freely in liquid medium were taken with an electronic flash as the light source. The photographs thus obtained directly demonstrated the existence of rod-shaped cells.

Several studies on the growth of pleuropneumonia-like organisms (PPLO) in slide cultures have been reported (Bartmann and Höpken, 1955; Kandler and Kandler, 1955*a*; Liebermeister, 1960). Detailed observations concerning the morphology, mode of division, and colony formation of PPLO were made in these investigations. In our study, special attention was paid to the relationship between the size and shape of the PPLO cells, on the one hand, and the viability of these cells, on the other.

Aggregates consisting of several individual cells are often found in liquid cultures of PPLO. In this study, organisms and media were chosen in such a way that nonaggregated cells were in the majority in the cultures investigated.

MATERIALS AND METHODS

Organisms. Mycoplasma laidlawii, strain A (Laidlaw and Elford, 1936), was obtained from E. A. Freundt, State Serum Institute, Copenhagen. The *M. agalactiae* strain used was obtained from E. Klieneberger-Nobel, Lister Institute of Preventive Medicine, London. It had been isolated from a goat ill with agalactia.

Growth conditions. M. agalactiae was grown at 37 C in meat broth supplemented with 10% inactivated horse serum. *M. laidlawii* was grown at 30 C in broth supplemented with 1% serum. Erlenmeyer flasks (200-ml), each containing 50 ml of medium, were used as culture vessels. The *M. laidlawii* cultures were incubated on a rotary shaker (100 rev/min). Subcultures of the PPLO were made daily.

Preparation of slide cultures and microscopy. The methods described by Weibull and Lundin (1962) were used in most experiments. A few photomicrographs of PPLO moving freely in liquid medium between slide and cover slip were taken with a Leitz Multiblitz-Mikro 300-w electronic flash as the light source. The box camera used in other photographic studies could not be used in these experiments, since insufficient light reached the photographic plate. Instead, a microcamera equipped with 35-mm Gevaert Duplo Ortho film was used. The techni-

[1] Present address: Department of Bacteriology, Medical School, University of Umeå, Umeå, Sweden.

cal quality of the photomicrographs thus obtained was slightly inferior to that obtained with the box camera. The final magnification of all photographs was 2,600 ×.

Measurements of respiration. The conventional Warburg technique was used for these determinations. To obtain measurable oxygen consumption, the bacterial cultures were concentrated about tenfold by centrifugation at 78,000 × *g* for 60 min and the sedimented cells were resuspended in fresh growth medium.

RESULTS

Occurrence of cell aggregates and nonaggregated cells in liquid cultures of M. agalactiae and M. laidlawii. When *M. laidlawii* was grown in broth supplemented with 10% horse serum, cell aggregates were predominant in the cultures. How-

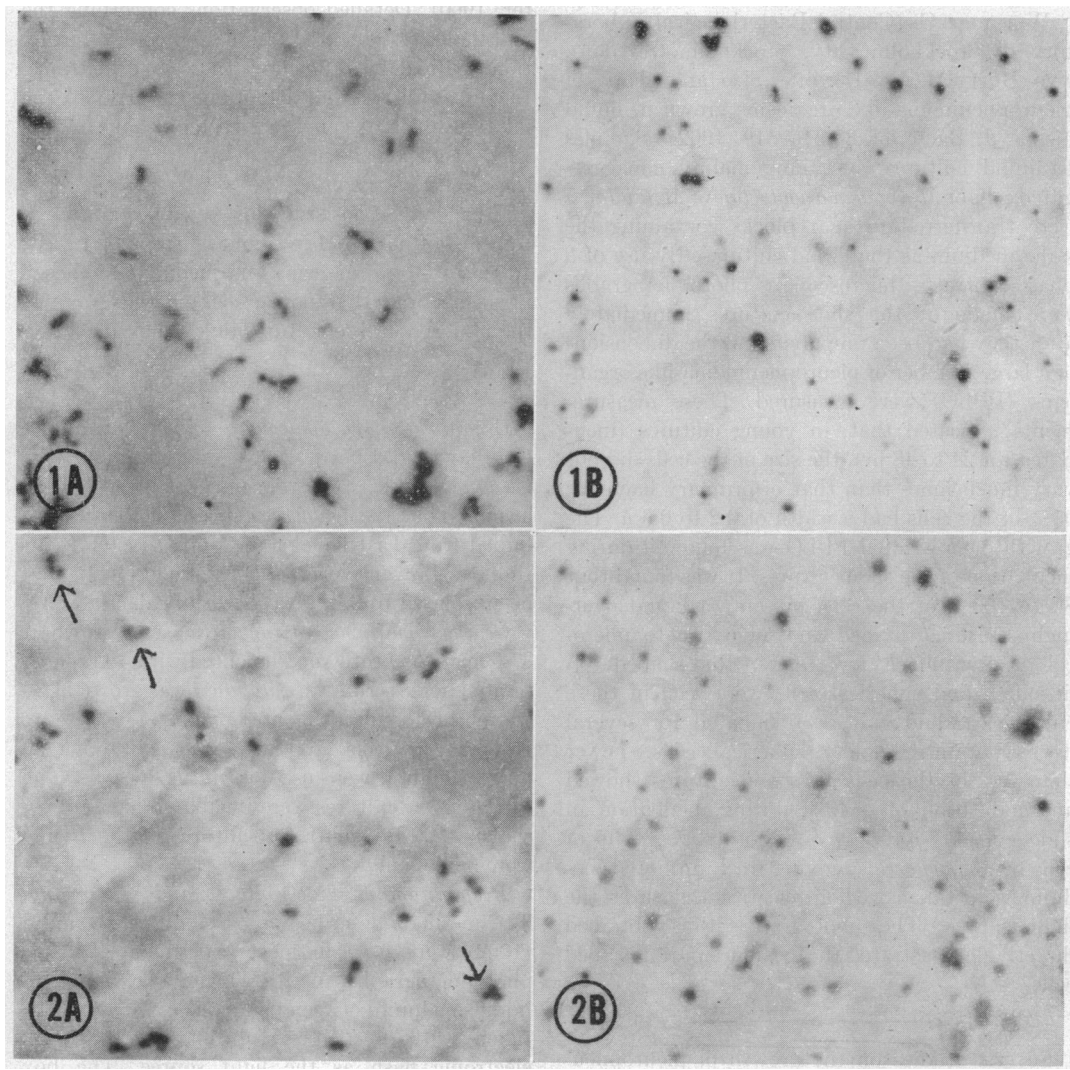

FIG. 1. *(top) Slide cultures of Mycoplasma agalactiae, photographed immediately after inoculation with cells from 24-hr (left) and 250-hr (right) liquid cultures of this organism.*

FIG. 2. *(bottom) Slide cultures of Mycoplasma laidlawii, photographed immediately after inoculation with cells from 24-hr (left) and 100-hr (right) liquid cultures of this organism.*

ever, when the serum content of the medium was diminished, the number of nonaggregated cells increased. Thus, in a medium containing 1% serum, one to three cell aggregates per ten nonaggregated cells were usually found. Each aggregate contained three to ten individual cells. Agitation of the culture vessels during incubation did not diminish appreciably the number of cell aggregates in a culture of *M. laidlawii*.

In liquid cultures of *M. agalactiae*, a maximum of one cell aggregate per ten nonaggregated cells was found.

Morphology of PPLO in liquid cultures of various ages. Photomicrographs of typical cells from 24-hr liquid cultures of *M. agalactiae* and *M. laidlawii* are shown in Fig. 1 and 2. It can be seen that these cells were spherical, ellipsoidal or rod-shaped. Most of the rods had swollen ends. Several cells consisted of two rodlike segments, forming an angle with each other (such cells are indicated by arrows in Fig. 2).

In PPLO cultures incubated for several days, almost all cells were spherical (Fig. 1 and 2).

Filtration experiments have been carried out to establish the size of the smallest viable elements of PPLO (Elford, 1938; Sabin, 1941; Kellenberger, Liebermeister, and Bonifas, 1956; Klieneberger-Nobel, 1956). It was deemed of interest to compare the results of these experiments with the dimensions of PPLO as determined microscopically. Therefore, the maximal width (in the case of spherical cells, the diameter) of a large number of *M. agalactiae* and *M. laidlawii* cells was determined by measurements

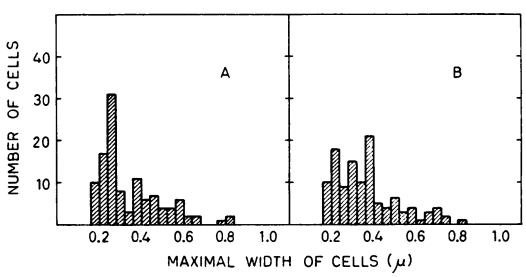

FIG. 3. *Maximal width of cells present in liquid cultures of Mycoplasma agalactiae. The cells were transferred to agar blocks, photographed, and the width of the cells was measured from the photographic prints. (A) Cells from 24-hr cultures. Pooled data from two different cultures. (B) Cells from 250-hr cultures. Pooled data from two different cultures.*

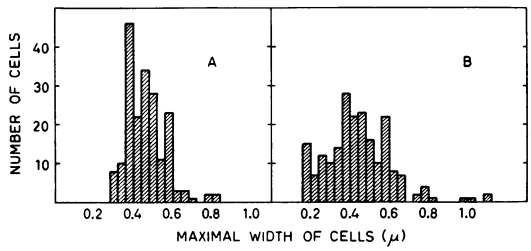

FIG. 4. *Maximal width of cells present in liquid cultures of Mycoplasma laidlawii. (A) Cells from 24-hr cultures. (B) Cells from 100-hr cultures. The measurements were made as in Fig. 1.*

on photomicrographs of agar blocks inoculated with these organisms. The majority of the PPLO cells investigated had a width or diameter ranging from 0.2 to 0.6 μ (Fig. 3 and 4). In the case of *M. laidlawii*, the width of cells from old cultures varied slightly more in size than that of cells from young cultures. The average width of *M. agalactiae* cells in 24-hr cultures was 0.36 ± 0.014 μ (the ± sign indicates sample standard error; Snedecor, 1956). The corresponding figures for *M. laidlawii* were 0.46 and 0.007 μ.

It is well known that the shape of PPLO can be distorted by mechanical forces. This point was emphasized by Liebermeister (1960). However, we have not found any appreciable difference in appearance between PPLO moving freely in liquid medium between slide and cover slip and those lying immobile on the agar surface in slide cultures. To demonstrate this, a drop of a liquid culture of *M. agalactiae* was placed between slide and cover slip, and the organisms were photographed with an electronic flash as the light source (Fig. 5). It can be seen that the cells shown in Fig. 5 and in Fig. 1 (left) have practically the same appearance. It is especially noteworthy that rod-shaped cells can be seen in both photomicrographs.

Relationship between the age of PPLO cells and their viability. Figures 6 and 7 show slide cultures of *M. agalactiae* and *M. laidlawii* before and after incubation for about 15 hr. Cells grown overnight in liquid medium were used as inoculation material. It can be seen that all of the individual cells shown in these figures gave rise to microcolonies during incubation, i.e., the viability of these cells was 100%. When similar studies were made with cells of *M. agalactiae* that had been grown in liquid medium for 10 days, microcolonies

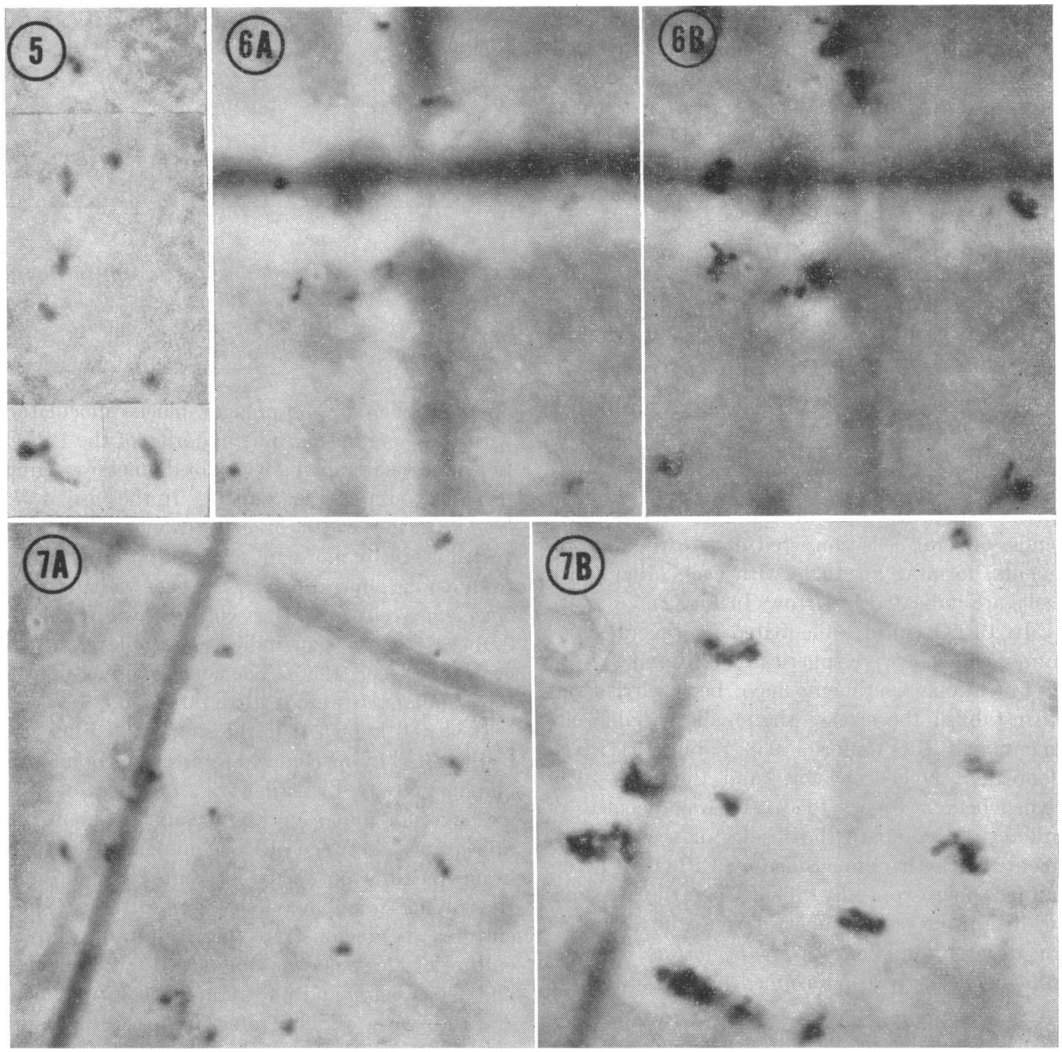

FIG. 5. *(top, left) Freely moving cells in a 24-hr liquid culture of Mycoplasma agalactiae. A drop of the culture was placed between slide and cover slip, and the cells were photographed with electronic flash as the light source.*

FIG. 6. *(top, center and right) Slide culture of Mycoplasma agalactiae before (center) and after (right) 12 hr of incubation at 37 C. The slide culture was inoculated with cells from a 24-hr liquid culture of M. agalactiae.*

FIG. 7. *(bottom) Slide culture of Mycoplasma laidlawii before (left) and after (right) 20 hr of incubation at 30 C. The slide culture was inoculated with cells from an 18-hr liquid culture of M. laidlawii.*

were only occasionally found in the microscopic fields of view. Most of the individual PPLO cells did not change their appearance during incubation for 2 days in slide cultures. Similar results were obtained with cells of *M. laidlawii* grown in liquid medium for 4 to 5 days.

The photographic studies described above were confirmed by counting experiments. Cells from liquid PPLO cultures of various ages were used to prepare slide cultures of *M. agalactiae* and *M. laidlawii*. Before incubation, the number of cells in several microscopic fields of view was determined. The slide cultures were then incubated for 18 to 120 hr. The number of microcolonies

TABLE 1. *Viability of PPLO cells grown for various times in liquid medium**

Expt	Organism	Age of liquid inoculum cultures	Slide cultures		
			No. of cells per field of view before incubation	No. of microcolonies per field of view after incubation	Time of incubation
		hr			*hr*
1	*M. agalactiae*	24	16.7 ± 0.9	13.5 ± 0.6	24
2	*M. agalactiae*	250	24.5 ± 0.8† 1.3 ± 0.2‡	1.2 ± 0.2	48
3	*M. agalactiae*	250	34.5 ± 2.7† < 0.5‡	<0.1	120
4	*M. laidlawii*	24	16.3 ± 0.8	17.3 ± 0.8	18
5	*M. laidlawii*	48	16.9 ± 1.1	17.1 ± 0.8	18
6	*M. laidlawii*	72	19.9 ± 0.9	11.4 ± 0.6	24
7	*M. laidlawii*	72	19.4 ± 1.1	4.2 ± 0.3	24
8	*M. laidlawii*	96	24.1 ± 1.1	0.7 ± 0.1	24
9	*M. laidlawii*	250	9.9 ± 0.6	0.33 ± 0.06 0.35 ± 0.06	48 120

* Agar blocks were inoculated with samples of liquid PPLO cultures. The inoculated blocks were used for preparation of slide cultures, which were studied microscopically before and after incubation. The numbers of cells and colonies given below are the average numbers found per microscopic field of view. The ± sign indicates sample standard error.

† Number of spherical cells.

‡ Number of rod-shaped cells.

formed during incubation was determined in the same way as the number of cells in the unincubated slide cultures. The average size of the microcolonies was about 10 μ. Table 1 shows the results of these counting experiments. It can be seen that the viability of the young PPLO cells studied was 80 to 100%. On the other hand, cells from cultures that had been grown for several days in liquid medium before transfer to the slide cultures displayed a viability of less than 5%. The figures obtained from experiments 2 and 3 (Table 1) suggest that only rod-shaped cells are viable in old cultures of *M. agalactiae*. It could be argued that the semianaerobic conditions prevailing in the slide cultures might inhibit to some extent the growth of old PPLO. To test this possibility, samples of liquid cultures of PPLO were spread uniformly on agar plates. A portion of each plate was used to prepare a slide culture. The slide cultures and the plates were then incubated in parallel for the desired period of time (between 1 and 5 days). A second set of slide cultures was then prepared from the plates. Immediately afterwards, the number of colonies in each of the pairs of slide cultures was counted and compared. No significant difference was revealed in the number of colonies in the initial and final slide cultures in each pair. This shows that the cells of *M. agalactiae* and *M. laidlawii* formed colonies equally easily in the semianaerobic conditions of the slide cultures and in the fully aerobic agar plates.

TABLE 2. *Respiration of PPLO cells grown for various times in liquid medium**

Organism	Age of culture	Uptake of O_2
M. agalactiae	24	23
M. agalactiae	48	11
M. agalactiae	150	2.8
M. agalactiae	250	0.5
M. laidlawii	24	2.0
M. laidlawii	48	2.0
M. laidlawii	72	0.8
M. laidlawii	96	0.1

* The liquid cultures were concentrated about tenfold by centrifugation and resuspension of the sediment in fresh growth medium. The figures indicate the uptake of O_2 (μliters/hr) by 1 ml of the original culture. Each figure represents a mean value of data from two independent experiments.

Respiration experiments. Kandler and Kandler (1955*b*) published data showing that cells from old cultures of a PPLO strain isolated from mice respired much less vigorously than cells from young cultures. Table 2 shows that the same is also true from *M. agalactiae* and *M. laidlawii*.

Thus, the loss in viability of the PPLO described in the preceding paragraphs is accompanied by a marked decrease in respiratory activity.

DISCUSSION

The pleomorphism of the PPLO has been emphasized by most workers who have studied these organisms. (The PPLO have recently been revised by Klieneberger-Nobel, 1962.) The PPLO investigated by us were studied under conditions that should minimize this pleomorphism. Firstly, cells grown in liquid media were used as the starting material for our investigations. This implies that all organisms in a particular culture were grown under practically identical environmental conditions. (This is never the case with organisms grown on solid media.) Secondly, mainly viable organisms were studied, since aging and degenerating forms of PPLO often are highly pleomorphic. Thirdly, media were chosen in which the nonaggregated cells outnumbered the cell aggregates.

According to our results, the dimensions of the PPLO studied do not vary much more than those of many ordinary bacteria under comparable conditions. The maximal width of the thinnest viable PPLO photographed by us was about 0.2 μ (*M. agalactiae*) or 0.3 μ (*M. laidlawii*). These figures are slightly larger than those obtained from filtration experiments designed to determine the smallest reproductive units of PPLO, namely, 0.12 to 0.25 μ (Elford, 1938; Sabin, 1941; Klieneberger-Nobel, 1956; Kellenberger, Liebermeister and Bonifas, 1956). The agreement can, however, be regarded as rather close, considering that the PPLO cells possess a certain degree of plasticity (Liebermeister, 1960). These cells might thus pass through filter pores in the form of thread-like structures having a smaller diameter than the PPLO in unfiltered cultures. On the other hand, our photomicrographs of *M. agalactiae* taken with an electronic flash as the light source show that cells of this organism are not deformed so easily that they will change their shape appreciably when they are transferred from a liquid culture to the agar block of a slide culture.

Cuckow and Klieneberger-Nobel (1956) and Klieneberger-Nobel (1956, 1962) published electron micrographs of several PPLO, among them *M. agalactiae* and *M. laidlawii*. Particles of various sizes were seen in the photographs. The authors concluded that the smallest of these particles, which were approximately spherical and had a diameter of approximately 0.1 μ, represented minimal reproductive units. However, it was not clearly proven that a significant number of these particles were viable before being fixed. In addition, the washing and fixing procedures used for the preparation of the electron-microscope specimens may have changed the size and shape of the PPLO particles present in the original cultures.

To our knowledge, comparisons between viable and total counts of PPLO cultures, other than ours, have not been made so far. In cultures of *M. agalactiae* and *M. laidlawii* grown for 24 to 48 hr, 80 to 100% of the cells were found to be viable. Our counting experiments, and photographs taken of slide cultures of PPLO before and after incubation, indicate that the PPLO studied do not possess any submicroscopic reproductive units. Some viable cells were spherical, but the majority of them were rod-shaped or ellipsoidal. In cultures several days old, most of the cells were spherical and few of them divided when transferred to slide cultures. Probably the only viable cells in these cultures were the rod-shaped ones. This suggests that the spherical cells present in PPLO cultures several days old do not represent significant reproductive units. Bartmann and Höpken (1955) drew the same conclusion from their photomicrographs.

Weibull and Lundin (1962) studied the size and shape of elements found in 24-hr cultures of a stable *Proteus* L form. Mainly spherical bodies were found, their diameters ranging from the resolving limit of the microscope used (about 0.2 μ) to about 1.5 μ. Only elements having a diameter $>$ 0.6 to 0.7 μ were able to grow in slide cultures. Thus, the elements of this L form exhibited an inhomogeneity, with respect to both size and viability, that was not encountered in the present study on PPLO cells from young (24 to 48 hr) liquid cultures. It should also be emphasized that the small, nonviable L elements (diameter $<$ 0.6 μ) respired vigorously; thus, there was a dissociation between respiratory and reproductive capabilities in these bodies. In this respect, too, the PPLO studied by us behaved differently; viable PPLO cells respired vigorously and nonviable cells showed at the most a weak respiratory activity. Thus, these results and those reported by Weibull and Lundin (1962) are

in accordance with the view that very significant dissimilarities exist between the PPLO and the bacterial L forms.

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