

Liquid Medium for Growth of *Legionella pneumophila*

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The medium described is a simple yeast extract broth capable of growing large numbers of *Legionella pneumophila*, the causative organism of Legionnaires disease. Filtration was chosen as a means of sterilization, since medium that was autoclaved did not support growth without the presence of Norite A. The filtered medium gave rapid cell growth and maintained the initial antigen production. The observed generation time was 99 min with a maximum cell population of 2×10^9 colony-forming units per ml in approximately 40 h.

Legionella pneumophila (1) is a gram-negative, pleomorphic bacterium that is highly fastidious. On the enriched laboratory media presently used, growth requires a large inoculum; single colonies do not reach countable size for 3 to 10 days. Primary isolation growth is slow or may not occur at all.

Experiments in bacterial physiology, virulence, and immunology require large numbers of organisms in well-defined stages of growth. Currently, slow growth on solid media prevents definition of these stages and yields low numbers of organisms.

Several investigators have recently described broth media for the growth of *L. pneumophila* (4-6). These media all have extended generation times, which make them somewhat difficult to use.

The objective of this work was to formulate a liquid medium in which small inocula would grow rapidly and give high concentrations of bacteria without loss of antigenicity. The lag, logarithmic, stationary, and death phases are easily determined and reproducible.

MATERIALS AND METHODS

Bacterial species. The strain used was the Philadelphia 3 isolate of *L. pneumophila*. It was obtained from the Center for Disease Control, Atlanta, Ga.

Plating medium. The charcoal-yeast extract (CYE) agar developed by Feeley et al. (3) was used as the plate count medium. Its formula per liter of medium was: 10 g of yeast extract (lot no. 566385, Difco Laboratories, Detroit, Mich.), 0.4 g of L-cysteine-HCl-H₂O (Sigma Chemical Co., St. Louis, Mo.), 0.25 g of ferric nitrate (Fisher Chemical Co., Silver Spring, Md.), 1.5 g of Norite A (Sigma), 17 g of agar (Difco), and 980 ml of distilled water. All ingredients except L-cysteine-hydrochloride and ferric nitrate were autoclaved at 121°C for 15 min and then cooled to 50°C in a water bath. L-Cysteine-hydrochloride (0.4 g in 10 ml of distilled water) and ferric nitrate (0.25 g in 10 ml of distilled water) were membrane sterilized sepa-

ately. The cysteine solution was added to the medium first. The complete medium was adjusted to pH 6.9.

Broth medium. The formula per liter of medium was: 10 g of yeast extract (Difco), 0.4 g of L-cysteine-HCl-H₂O (Sigma), 0.25 g of ferric pyrophosphate (Biological Products Division, Center for Disease Control, Atlanta, Ga.), and 1,000 ml of distilled water. The ingredients were all added to the distilled water and then membrane filter sterilized (0.45 μm, Millipore Corp., Bedford, Mass.). The pH was adjusted to 6.9 after sterilization. This medium will be referred to as yeast extract broth (YEB).

Control broth medium was formulated as follows: control 1 was 10 g of yeast extract (Difco) and 980 ml of distilled water to which a cellophane bag containing 1.5 g of Norite A was added and autoclaved at 121°C for 15 min and then cooled to room temperature. L-Cysteine-hydrochloride (0.4 g in 10 ml of distilled water) and ferric pyrophosphate (0.25 g in 10 ml of distilled water) were membrane sterilized separately. The cysteine solution was added to the medium first. The complete medium was adjusted to pH 6.9. Control 2 was formulated as above except that the Norite A was not added.

Dilution blanks. All dilutions were made in normal saline which contained 0.1% tryptose. The pH was adjusted to 7.0.

Inoculation of medium. Stock cultures were maintained in hen yolk sac membrane homogenates stored at -70°C.

Stationary-phase cells for inoculation were obtained by inoculating 500 ml of YEB in a 1-liter Erlenmeyer flask with 1 ml of stock culture and incubating for 60 h. The incubation was carried out in a controlled-environment incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C at approximately 100 oscillations per min without CO₂. At the stationary phase, 1 ml was removed and transferred to fresh YEB for the physiological studies presented here. The inoculated broth was incubated as described above; samples were taken at 2-h intervals for the first 8 h. The sampling times thereafter were as follows: 16, 24, 30, 34, 40, 48, 50, 52, 54, and 62 h. Samples were diluted in tryptose-saline; 0.2 ml was inoculated onto plates and spread. Plates were prepared in triplicate.

Samples were withdrawn for pH determinations

and microscopic observations. Due to the slow growth of *L. pneumophila*, colonies on the dilution plates were counted at 72 h.

Microscopic and other procedures. Cells were observed and photographed with a Zeiss Epi-illuminated fluorescence microscope and Olympus OM-2 camera. Surface antigen production was demonstrated by staining directly with fluorescein isothiocyanate conjugate prepared against group I *L. pneumophila* (2); rabbit hyperimmune serum was obtained from the Biological Products Division, Center for Disease Control. The pH of all media and samples was measured with a Beckman Zeromatic pH meter standardized with a Beckman pH standard buffer (25°C).

RESULTS

Growth parameters in YEB. Figure 1 shows the growth curve of *L. pneumophila*. Results obtained by taking samples every 2 h for the first 8 h indicate that the strain has a lag phase of approximately 8 h. Logarithmic growth proceeded from 8 to 40 h, attaining a maximum cell population of 2×10^9 colony-forming units per ml. The average generation time was 99 min. The stationary phase began at 40 h and proceeded through 62 h. When the experiment was terminated at 62 h, the cell population had decreased to 10^9 colony-forming units per ml. The pH showed little variation from the initial 6.9. The final pH at 62 h was 7.04.

Microscopic observations. Throughout the

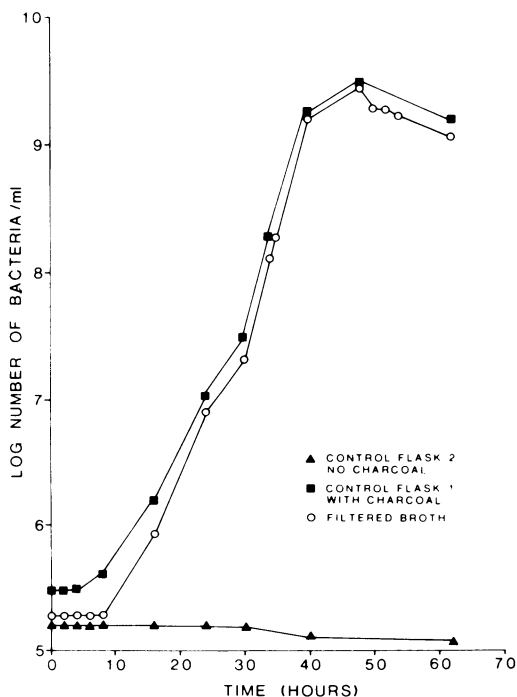


FIG. 1. Growth of *L. pneumophila* L-3 in YEB.

growth of *L. pneumophila* in YEB, varying quantities of extracellular material were observed. At 24 h there was an abundance of this material, so much so that individual cells were difficult to distinguish. This material gradually decreased; at 40 h, there were only a few cells clumped together (Fig. 2). At 62 h, there appeared to be more cell clumping than at 40 and 48 h. The nature of this extracellular debris is presently being investigated.

Fluorescent microscopy was performed throughout the growth cycle. Staining characteristics remained the same. Broth-grown cells at 40 h gave a 3+ fluorescence. CYE agar-grown cells at 72 h also gave 3+ staining.

DISCUSSION

There are numerous factors, such as temperature, medium phase, nutrients, and inhibitors, known to influence the growth of bacteria. Failure to remove inhibitor substances from growth media has been a problem with some organisms. *L. pneumophila* has fallen into this category; recently there have been some improvements. Charcoal and starch have both been used successfully to promote the growth of *L. pneumophila* (3). These compounds promote growth by absorbing a substance(s) that is toxic to the organism. There is speculation that the toxic substances are fatty acids. Pine et al. have shown that oleic acid inhibits the growth of this organism in the absence of starch (4). These inhibitors could be inherent in the broth or formed during autoclaving or during growth of the organism.



FIG. 2. Appearance of *L. pneumophila* in YEB at 40 h. $\times 1,000$.

Our studies have shown that YEB autoclaved in the absence of Norite A will not support the growth of *Legionella*. By filter sterilizing the medium, the need for autoclaving was eliminated, and the charcoal could be removed. Since the primary function of charcoal is that of an absorbant, it was assumed that an inhibitory compound(s) was produced and removed by the charcoal. If toxic substances were present in the broth before autoclaving, it is unlikely that they would be removed by filtering. Further investigation is in progress to determine the nature of this inhibition.

To date, 14 strains of *L. pneumophila* have been grown in YEB. Their growth rates have not been calculated, but they are approximately the same as the L-3 strain used here. Ferric nitrate has been used in place of ferric pyrophosphate with similar results, but was discontinued because it produced cloudy medium.

YEB provides a means of producing large numbers of *L. pneumophila* in a short period of time. It also provides a system for investigation of antigens and possible toxins. The addition of agar or agarose to YEB has provided a clear medium for immunological studies. It is hoped that further investigation with filtered media will produce a less complex medium, so that further physiological investigations can be completed.

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LITERATURE CITED

1. Brenner, D. J., A. G. Steigerwalt, and J. E. McDade. 1979. Classification of the Legionnaires' disease bacterium: *Legionella pneumophila*, genus novum, species nova, of the family Legionellaceae, familia nova. *Ann. Intern. Med.* **90**:656-658.
2. Cherry, W. B., B. Pittman, P. P. Harris, G. A. Hebert, B. M. Thomason, L. Thacker, and R. E. Weaver. 1978. Detection of Legionnaires disease bacteria by direct immunofluorescent staining. *J. Clin. Microbiol.* **8**:329-338.
3. Feeley, J. C., G. W. Gorman, and R. J. Gibson. 1978. Primary isolation media and methods, p. 108-117. In G. L. Jones and G. A. Hebert (ed.), "Legionnaires," the disease, the bacterium and methodology. Center for Disease Control, Atlanta, Ga.
4. Pine, L., J. R. George, M. W. Reeves, and W. K. Harrell. 1979. Development of a chemically defined liquid medium for growth of *Legionella pneumophila*. *J. Clin. Microbiol.* **9**:615-626.
5. Wang, W. L. L., M. J. Blaser, J. Cravens, and M. A. Johnson. 1979. Growth, survival, and resistance of the Legionnaires' disease bacterium. *Ann. Intern. Med.* **90**: 614-618.
6. Warren, W. J., and R. D. Miller. 1979. Growth of Legionnaires disease bacterium (*Legionella pneumophila*) in chemically defined medium. *J. Clin. Microbiol.* **10**:50-55.