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Growth of *Legionella pneumophila* in Continuous Culture

JAMES D. BERG,[1]† JOHN C. HOFF,[2] PAUL V. ROBERTS,[3] AND ABDUL MATIN[4]

Rio Linda Chemical Co., Sacramento, California 95814[1]; Drinking Water Research Division, Municipal and Environmental Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268[2]; and Departments of Civil Engineering[3] and Medical Microbiology,[4] Stanford University, Stanford, California 94305

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A method was developed to grow *Legionella pneumophila* in continuous culture. A chemostat was used to simulate nutrient-limited, submaximal growth in the natural environmental and to provide a precisely controlled growth regimen. Cultures grew under forced aeration under conditions yielding up to 38% saturation of dissolved oxygen; supplemental CO_2 (5%) at the same gas flow rates as ambient air had no effect on culture growth. Pleomorphism was observed during growth under all conditions. Pigment was produced only at $D < 0.03$ h^{-1}. Catalase was produced at higher growth rates but not at higher temperatures. The pathogenicity was unaffected by altering either the growth rate or the growth temperature.

Ecological studies have demonstrated the ubiquity of *Legionella pneumophila* (4–7, 9, 10, 17–19, 22, 24, 29, 32). Among these habitats there exist a wide range of physical and chemical characteristics, such as dissolved oxygen levels (0 to 10 mg liter^{-1}), temperatures (0 to 70°C), and pH (5 to 8.5) (9). The physiology and morphology of *L. pneumophila* grown in the laboratory (24, 25, 33) have been shown to change drastically under differing growth conditions. Pine et al. (21) observed changes in cell size, shape, and surface-to-volume ratio as a function of temperature, aeration, and nutrient limitation in batch culture. Moreover, there are some contradictory observations regarding nutrient requirements [e.g., iron(III)] and biochemical characteristics (e.g., catalase production) of *L. pneumophila* grown in laboratory batch culture (8, 20, 25, 27).

The aforementioned variability in phenotype may occur as a result of environmental parameters that cannot be adequately controlled during batch culture. These parameters include nutrient concentration and composition and redox potential which have been shown in our laboratory (16; J. D. Berg, Ph.D. dissertation, Stanford University, Stanford, Calif., 1984) and by others (12) to affect the physiology, growth rate, and gross morphology of other bacteria. The use of a chemostat circumvents many of the problems associated with batch culture. Therefore, the objective of this study was to develop a procedure for the growth of *L. pneumophila* in a precisely controlled manner in continuous culture to investigate some cursory physiological characteristics that have been in question, including nutritional requirements, cell morphology, catalase activity, pigment production, and pathogenicity.

(This work represents a portion of the Ph.D. dissertation research of J.D.B. completed at Stanford University, Stanford, Calif., 1984. The results were presented in part at the Second International Symposium on *Legionella*, June 1983, Atlanta, Ga.)

The chemostat growth medium contained (per liter) yeast extract (Difco Laboratories), 10 g; L-cysteine, 0.2 g; and starch, 0.25 g. The medium was not supplemented with iron.

* Corresponding author.
† Present address: Division of Hydraulic and Sanitary Engineering, Norwegian Institute of Technology, University of Trondheim, 7000 Trondheim, Norway.

Subcultures of *L. pneumophila* serogroup 1, Philadelphia strain 1 were stored at −70°C in 20% Trypticase soy broth (BBL Microbiology Systems) in glycerol and distributed in 0.5-ml ampoules. These subcultures were streaked onto CYE (1) agar slants and incubated for 48 to 72 h at 37°C. The growth on the slant was then washed into a 250-ml flask containing 100 ml of chemostat medium and incubated in a shaking water bath at 37°C for 24 to 48 h. To start the chemostat culture, this culture was transferred into the 2-liter chemostat vessel which had been purged with nitrogen. The culture was then diluted with fresh medium to an optical density at 660 nm (OD_{660}) of >0.15. Once the culture grew to an OD_{660} of ca. 0.40 (after 4 to 6 days), positive aeration and nutrient pumping were started. Steady-state cultures were harvested after five volume changes (i.e., 2 to 7 days depending on the dilution rate [D] h^{-1}).

The assay for L-cysteine (3, 34) was an adaptation of the methods of Roberts and Rouser (26). Catalase activity was determined by the standard assay with 5% hydrogen peroxide at room temperature (11). The presence of soluble, brown pigment was determined spectrophotometrically at 392 nm (24). The pathogenicity was tested by intraperitoneal injection of a culture sample (5×10^7 CFU ml^{-1} contained in 1 ml) into guinea pigs and observing for successful infection (13). The rationale for the development of the medium and methodological details are presented elsewhere (Berg, Ph.D. dissertation).

Chemostat growth medium. The salient feature of the yeast extract-based media used in previous work with batch culture (8, 24, 27) has been the inclusion of an excessive amount of the amino acid L-cysteine, which has been shown to be required for growth (8, 21). A cysteine concentration equal to 0.2 g/liter, one-half the concentration recommended by the Centers for Disease Control, ultimately was used in the chemostat medium. It provided a medium conducive for the initial, sensitive, batch culture phase and for maintaining stable growth in the continuous phase. Yet subsequently, when medium containing 0.1 g/liter was fed to an established culture (OD = 1.45) at D = 0.15 h^{-1} and 37°C, the OD remained unchanged. Therefore, the cysteine concentration that was employed in the study was not limiting but appeared essential for the start-up of the chemostat. The 0.1-g/liter concentration yielded eight times the cell mass in continuous culture under positive aeration that had been

TABLE 1. Characteristics of *L. pneumophila* grown in continuous culture by using a complex yeast extract medium supplemented with L-cysteine[a]

D (h^{-1})	Growth temp (°C)	Steady-state yield (OD_{660})	Predominant morphology[b]	Catalase activity[c]	Pigment	Pathogenicity
0.01	44	1.45	SR	−	+	ND[d]
0.03	44	1.50	SR	−	+	ND
0.06	44	1.60	LR, F	+	−	ND
	37	1.70	LR, F	++	−	+
0.15	44	1.20	LR, F	−	−	+
	37	1.30	LR, F	++	−	+
0.20	44	1.40	SR	++	−	ND
	37	1.25	SR	++	−	ND
0.30	44	Washout				
	37	0.84	SR	++	−	ND

[a] For comparison, results in batch culture at 37°C after 24 h were: OD_{660} = 0.70; catalase, absent; pigment, absent. Morphology was predominantly large rods.
[b] LR, Large rods; SR, small rods; F, filaments.
[c] ++, Strong; +, weak; −, absent.
[d] ND, Not determined.

attainable in the batch-culture mode with 0.4 g of cysteine per liter. This strongly suggests that the cysteine is not required for growth in the excessive concentrations that had been determined from the results of previous batch culture experiments. The cysteine may serve some catalytic function, perhaps in the transport of iron(III) (15, 24).

A reaction stoichiometry between cysteine and oxygen of 4:1 in the presence of iron(III) was proposed by Taylor et al. (28). The reaction is zero-order with respect to both reactants. Using this relationship, we can estimate the amount of dissolved oxygen required to oxidize the cysteine in the medium and the time required to do so. Assuming a cysteine concentration of 3.3×10^{-4} M, a dissolved oxygen concentration of 8.25×10^{-5} M (2.64 mg/liter or 38% of saturation at 37°C) will permit the complete oxidation of the cysteine in less than 60 min. Therefore, a reducing environment is essential to maintain a cysteine concentration that will support growth in the presence of iron(III). This could explain partially the difficulty of growth in continuous culture where even the slightest overaeration would effectively oxidize the cysteine.

Growth characteristics in the chemostat. In the literature there is disagreement as to whether *L. pneumophila* is catalase positive or negative (8, 21). The data on the growth of *L. pneumophila* at steady state in the chemostat indicate that the enzyme activity is subject to variation by the growth environment (Table 1). Catalase was inactive or absent at higher temperatures (44°C) and at the slower growth rates ($D < 0.03$ h^{-1}). This may partially explain the variability that has been observed by others in the determination of catalase activity, since the growth conditions in batch culture can be highly variable.

L. pneumophila has been found to produce a soluble brown pigment in the stationary phase of batch culture (21, 24). In the present study, pigment was produced only at the slowest growth rates used ($D < 0.03$ h^{-1}) (Table 1). Since

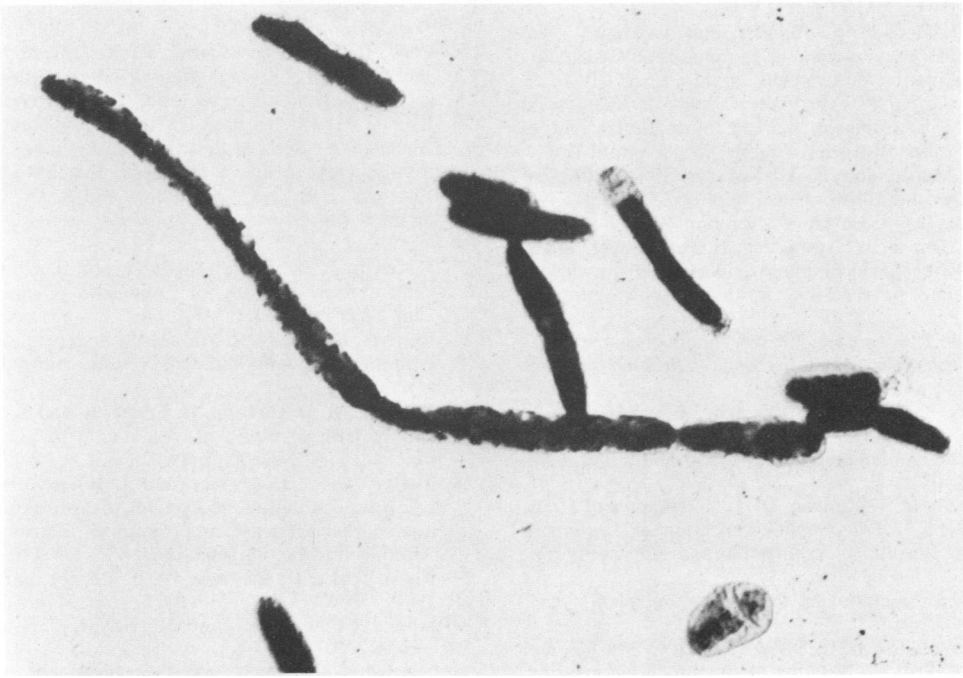

FIG. 1. Pleomorphism of *L. pneumophila* in the chemostat. D = 0.06 h^{-1} at 44°C. ×2236.

other environmental factors in the chemostat were controlled, pigment production appears to be influenced by changes in the growth rate alone. This conclusion cannot be reached from batch culture observations alone.

Morphology. The pleomorphic behavior of *L. pneumophila* has been described by Pine et al. (22), who observed distinct morphologies (filaments, large and small bacilli) that occurred only under specific sets of growth conditions in batch cultures. In this study, the simultaneous occurrence of all three forms was observed at steady state, with different forms (Fig. 1) predominating at different times (Table 1). Since the conditions in the chemostat at steady state are theoretically uniform with respect to the limiting nutrient, etc., the occurrence of the different morphologies must not be induced exclusively by difference in type of substrate, as previously thought (21).

The pathogenicity was not affected by the growth rates (the control represents maximum batch growth; $D = 0.15$ h^{-1} represents a slow rate) nor by the growth temperature tested (37 and 44°C). This observation is consistent with field data in which infectivity was shown not to be a function of the different environments that had been sampled (30).

The continuous culture technique described here represents a valuable tool for providing a precisely controlled growth environment to study microorganisms. Physiological functions (e.g., catalase activity) can be studied unambiguously, and responses of the organism to a simulated natural growth environment can be observed in the laboratory. Similar studies to test the sensitivity of *L. pneumophila* to disinfectants as a function of growth conditions are presented elsewhere (1; J. D. Berg, J. C. Hoff, P. V. Roberts, and A. Matin, Conference on Water Chlorination: Environmental Impact and Health Effects, June 1984, Williamsburg, Va., in press).

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