APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1983, p. 528–531
0099-2240/83/080528-04$02.00/0

Vol. 46, No. 2

Cyanobacterial Stimulation of Growth and Oxygen Uptake by *Legionella pneumophila*

GREGORY A. BOHACH AND IRVIN S. SNYDER*

Department of Microbiology, West Virginia University Medical Center, Morgantown, West Virginia 26506

Received 16 December 1982/Accepted 7 May 1983

A laboratory-adapted strain of *Legionella pneumophila* grew in coculture with *Fischerella*. Insoluble *Fischerella* slime contained carbohydrate and protein and was isolated from cultures on filters. Slime-free filtrates separated into three 280-nm absorbance peaks on Sephadex G-25. Peaks 1 and 2 contained protein and carbohydrate and stimulated *Legionella* respiration. Peak 3 and slime had no effect.

Tison et al. (15) showed in 1980 that *Legionella pneumophila* grows in an inorganic salts medium if cocultured with certain actively photosynthesizing cyanobacteria (blue-green algae). Growth in culture with the cyanobacterium *Fischerella* occurred at a rate similar to that obtained in complex media. This suggested that metabolites produced by *Fischerella* are utilized by *L. pneumophila*, and that growth requirements are not as strict as previously reported (4). Recently, Pope et al. (9) extended these results to other cyanobacteria and green algae. These findings, plus the fact that *L. pneumophila* is often found in the same environments as photosynthetic organisms (5, 15), provide a possible explanation for the widespread distribution of *Legionella*. In this note, we confirm and extend these findings in showing the growth of *L. pneumophila* in coculture with *Fischerella*. In addition, we show that specific fractions from a cyanobacterial culture supernatant stimulate oxygen uptake by *L. pneumophila*.

The identity and purity of a clinical isolate of *L. pneumophila*, serogroup 1, were confirmed by direct immunofluorescence (2), Gram staining, and plating on blood agar. Yeast extract broth (YEB [12]) was used for growth of *L. pneumophila*. The identity of *Fischerella* species (ATCC 29161) was confirmed by microscopic morphology, using the criteria of Rippka et al. (11). *Fischerella* sp. was grown in medium D (MD [1]) and was incubated at 37°C with shaking at 200 rpm and under 240 ft-c (2583 lx) of fluorescent or incandescent lighting.

For coculture, cells from late-exponential-phase cultures of *L. pneumophila* were washed and resuspended to a concentration of approximately 5×10^6 per ml in MD. *Fischerella* cells from a late-exponential-phase culture were washed in MD and resuspended to a turbidity of 100 Klett units. One liter of MD was inoculated with 3 ml of *Fischerella* cell suspension or 2.5 ml of *L. pneumophila* suspension or both. At appropriate times during incubation, samples from cultures containing *L. pneumophila* were homogenized and quantitated by indirect immunofluorescence, using antiserum prepared by the method of Cherry and McKinney (2) and fluorescein isothiocyanate-labeled goat anti-rabbit globulin (GIBCO Laboratories, Grand Island, N.Y.).

L. pneumophila grew and increased in number by approximately 0.4 log when cocultured with *Fischerella* (Fig. 1). *Legionella* grew for about 20 h before *Fischerella* began exponential growth. No detectable growth occurred in the control culture containing only *L. pneumophila*.

To isolate *Fischerella* slime, *Fischerella* cells from an axenic culture were pelleted by centrifugation. The culture supernatant was filtered through a 0.22-μm pore size membrane filter (Millipore Corp., Bedford, Mass.). *Fischerella* slime deposited on the membrane and was washed twice with 5 ml of distilled water. It was then scraped off the membrane, resuspended in distilled water, and sonicated until a uniform suspension was obtained. The sonicated *Fischerella* slime was dialyzed (12,000-molecular-weight cutoff) against several volumes of water for 24 h at 4°C.

After removal of slime, the culture supernatant was concentrated 100 to 200-fold by evaporation at 50°C on a Buchi Rotovapor R (Fisher Scientific Co., Pittsburgh, Pa.) and then was clarified by centrifugation. A 15-ml sample of the concentrated supernatant was applied to a bed (2.6 by 27 cm) of Sephadex G-25 (Pharmacia, Uppsala, Sweden). Fractions were eluted by a descending flow rate of 128 ml of 0.01 M potassium phosphate buffer (pH 7.2) per h, using a peristaltic pump. Column effluent was monitored by absorbance at 280 nm. Three peaks

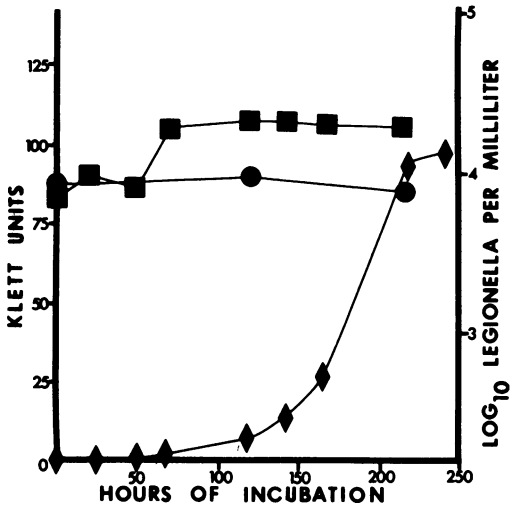

FIG. 1. Growth of *L. pneumophila* in coculture with *Fischerella* sp. Symbols indicate growth of *Legionella* cells incubated in MD (●) and in coculture with *Fischerella* sp. (■), and *Fischerella* growth in MD (◆).

resulted (Fig. 2). Peak 1 was eluted from the column in the void volume. A yellow-brown pigment in concentrated supernatants separated into two distinct bands (peaks 2 and 3). The fractions under each peak were pooled and dialyzed in a model 402 ultrafiltration cell with a UM05 Diaflo R membrane (500-molecular-weight cutoff; Amicon Corp., Lexington, Mass.) by sequential addition of at least five volumes of distilled water. Protein concentrations were determined by the method of Lowry et al. (7), with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard. Total carbohydrate content was measured by the phenol sulfuric acid method (3), with a glucose (Fisher) standard.

Protein and carbohydrate were detected in all samples except peak 3 (Table 1). The pigment in peaks 2 and 3 was not dialyzable by means of a membrane with a 6,000- to 8,000-molecular-weight cutoff.

Oxygen uptake by *L. pneumophila* was tested by using a Gilson differential respirometer (Gilson Medical Electronics, Inc., Middleton, Wis.). Mid-log phase *L. pneumophila* cells from YEB were washed once in K36 buffer (17) (0.1 M KCl and 0.015 M NaCl in 0.05 M potassium phosphate buffer [pH 6.9]). The pellet from 50 ml of culture was suspended in 15 ml of K36. The main compartment in each manometer flask received 1.0 ml of cell suspension and 1.0 ml of K36 buffer supplemented with 2 mM $MgSO_4$. The sidearm was filled with 1.0 ml (200 μg of protein) of test substrate or YEB. To determine endogenous respiration levels, K36 was added instead of the substrate. NaOH was used to trap CO_2. After equilibration by shaking at 37°C for 30 min, the sidearm contents were emptied into the main compartment. Manometer readings were recorded at 10-min intervals during incubation at 37°C.

Results from several respirometry experiments were pooled, and average oxygen uptake readings were plotted against time (Fig. 3). *L. pneumophila* respiration increased threefold over endogenous levels with YEB as substrate. There was no significant difference from endogenous levels when the bacteria were incubated in either peak 3 or sonicated *Fischerella* slime. Of the *Fischerella* supernatant fractions, peak 1 stimulated the greatest amount of oxygen uptake. Peak 2 and whole supernatant enhanced respiration, but to a lesser degree than peak 1. The data were analyzed by analysis of variance, using the Statistical Analysis System with a general linear model for split plot design (14). YEB, peak 1, peak 2, and whole supernatant

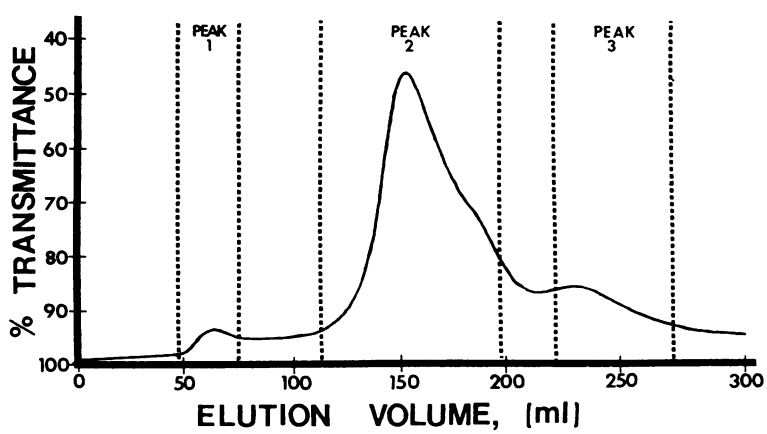

FIG. 2. Fractionation of *Fischerella* culture supernatants by gel filtration chromatography. Sephadex G-25 column effluent was monitored for the percentage of transmittance at 280 nm. Void volume was 53.5 ml.

TABLE 1. Chemical characterization of *Fischerella* culture supernatant, fractions, and slime

Culture component	Carbohydrate/protein ratio		
	Mean	Range	No. of tests
Sonicated *Fischerella* slime	3.87	2.95–5.24	10
Whole filtered supernatant	0.67	0.51–0.82	2
Peak 1	0.90	0.76–1.04	2
Peak 2	0.26	0.21–0.31	2
Peak 3	0	ND[a]	2

[a] ND, Not detected.

linear regression lines were significantly elevated over endogenous levels, whereas peak 3 and sonicated *Fischerella* slime were not ($\alpha = 0.05$).

In this report, we confirmed the findings of Tison et al. (15) and Pope et al. (9) and showed that a laboratory-adapted clinical isolate of *L. pneumophila* can grow in coculture with *Fischerella*. Growth of *L. pneumophila* occurred before the exponential phase of *Fischerella* growth. In addition, culture supernatants from *Fischerella* sp. and fractions obtained from the supernatant were oxidized by *L. pneumophila*. These data may explain in part the growth of *Legionella* in coculture with *Fischerella* sp.

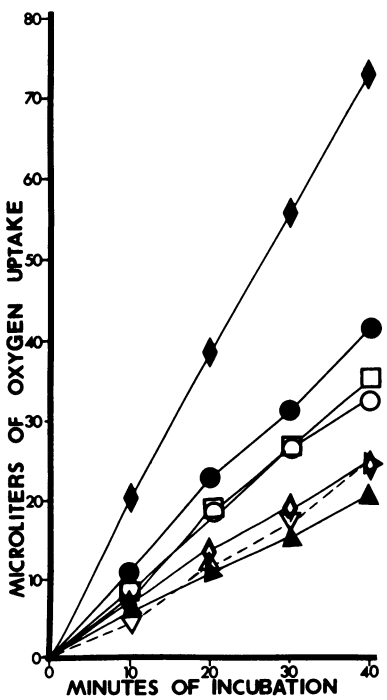

FIG. 3. Effect of extracellular *Fischerella* growth products on *L. pneumophila* oxygen uptake. *L. pneumophila* cells were incubated in K36 buffer (◇), yeast extract broth (◆), peak 1 (●), peak 2 (□), peak 3 (◀), whole filtered *Fischerella* culture supernatant (○), and *Fischerella* slime (◁). All substrates tested, except for peak 3, were adjusted to a concentration equivalent to 200 μg of protein per ml. Values shown at each time point are the averages of between 3 and 10 separate experiments.

Iron-binding macromolecular pigment-peptide complexes have been isolated from cultures of cyanobacteria (16). One of the peaks (peak 2) obtained by fractionation of culture supernatant contained a yellow-brown pigment and protein. The same or a similar pigment was found in the third peak, which contained neither protein nor carbohydrate and which did not stimulate oxygen uptake. Since growth of *L. pneumophila* is enhanced by iron or other metals (10), it is tempting to speculate that the pigmented fraction, if it binds iron, might play a role in nutrition of *L. pneumophila*. Although the nutrient requirements of *Legionella* in nature are not known, laboratory-adapted strains use amino acids (6, 8, 13). The culture supernatant and the fractions stimulating oxygen uptake both contained protein as the major component as well as carbohydrate. Carbohydrates do not appear to be important for growth, but they can be metabolized under some conditions (13, 17).

Additional characterization of the components stimulating oxygen uptake is necessary to further understand the *Legionella*-cyanobacteria relationship.

G.A.B. was the recipient of a West Virginia Medical Corporation Fellowship. The research was supported by a grant from the West Virginia Dental Corporation.

We thank William Thayne and Mohd Zainud-Din Hassan for their help in statistical analysis of the respirometry data.

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