APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1980, p. 456–459
0099-2240/80/02-0456/04$02.00/0

Vol. 39, No. 2

Growth of *Legionella pneumophila* in Association with Blue-Green Algae (Cyanobacteria)

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Legionella pneumophila (Legionnaires disease bacterium) of serogroup 1 was isolated from an algal-bacterial mat community growing at 45°C in a man-made thermal effluent. This isolate was grown in mineral salts medium at 45°C in association with the blue-green alga (cyanobacterium) *Fischerella* sp. over a pH range of 6.9 to 7.6. *L. pneumophila* was apparently using algal extracellular products as its carbon and energy sources. These observations indicate that the temperature, pH, and nutritional requirements of *L. pneumophila* are not as stringent as those previously observed when cultured on complex media. This association between *L. pneumophila* and certain blue-green algae suggests an explanation for the apparent widespread distribution of the bacterium in nature.

The bacterium *Legionella pneumophila,* the etiological agent of Legionnaires disease, has been isolated from a number of habitats, including air-conditioning cooling towers (5) and the water and sediments of a stream near the area of the Bloomington, Ind., outbreak (10). Isolation of *L. pneumophila* from aquatic habitats not associated with outbreaks of Legionnaires disease has recently been reported (7). This apparent widespread distribution of *L. pneumophila* raises questions as to the chemical and physical factors that enable this organism to grow and survive in nature, whereas in laboratory culture growth has been observed only under very restricted conditions (6). Generation times of 6 to 8 h at 35°C have been reported for *L. pneumophila* growing in complex media (W. A. Janssen, and R. G. Larson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, D63, p. 50) and of 7 h in a defined medium (W. J. Warren and R. D. Miller, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, D64, p. 50). Feeley et al. reported that *L. pneumophila* has a temperature optimum at 35°C and that it will not grow at 25 or 42°C in culture (6). They also reported a limited pH range of 6.9 ± 0.4 in culture (6).

(This paper is taken in part from a thesis submitted by D.L.T. in partial fulfillment of the requirements for the Ph.D in Biology at Rensselaer Polytechnic Institute.)

A survey of aquatic habitats at the Savannah River Plant near Aiken, S.C., was made for *L. pneumophila* by using antibodies conjugated with fluorescein isothiocyanate. Bacteria that were morphologically similar to *L. pneumophila* and reacted with a specific serogroup 1 fluorescent antibody conjugate were observed in the naturally occurring algal-bacterial mat communities in a thermal effluent ranging in temperature from 55°C to ambient (Tison and Fliermans, unpublished data). This mat community was composed of the blue-green algae *Fischerella* sp., *Phormidium* sp., and *Oscillatoria* sp. (Generic assignments were based on the taxonomic scheme for cyanobacteria of Rippka et al. [14].) Each of these algae was isolated in unialgal culture, along with their associated bacterial microbiota, in mineral salts Medium D at 45°C, as described by Castenholz (4). After several weeks of growth in culture, bacteria that were morphologically and antigenically similar to *L. pneumophila* of serogroup 1 as determined by direct immunofluorescence were observed in culture with *Fischerella* sp.

These bacteria were isolated and confirmed as *L. pneumophila* serogroup 1 by using methods previously described (7). By deoxyribonucleic acid homology studies the *L. pneumophila* isolate (SRP 6) showed a high level of relatedness to the Philadelphia 1 strain of *L. pneumophila.* This degree of relatedness is 76% at 60°C and 74% at 75°C. The divergence due to unpaired bases in the heterologous deoxyribonucleic acid duplex (i.e., deoxyribonucleic acid of Philadelphia 1 reassociated with deoxyribonucleic acid from SRP 6) is 2.3%. This degree of relatedness is indicative of the species level and is of the same order of relationship as are other strains of the various serogroups of *L. pneumophila* (D. J. Brenner, personal communication). *L. pneumo-*

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phila has subsequently been observed in unialgal cultures of *Oscillatoria* sp. and *Phormidium* sp. from the mat community (D. L. Tison, unpublished data).

Medium from algal cultures containing *L. pneumophila* was inoculated into log-phase cultures of *Fischerella* sp. that were free from all known serogroups of *L. pneumophila* as measured by direct immunofluorescence. Cultures were incubated under cool-white fluorescent illumination at an intensity of 100 μEi m^{-2} s^{-1} without shaking. Experiments were performed in which duplicate cultures were sampled at 2-h intervals over a 10-h period. Controls included those incubated in the dark, cultures to which 2 $\times 10^{-5}$ M 3-(3,4-dichlorophenyl) 1-dimethyl urea was added to inhibit photosystem II activity (12), and *L. pneumophila* inoculum in Medium D incubated in the light without *Fischerella* sp. The total numbers of *L. pneumophila* serogroup 1 were determined by direct immunofluorescence (7). Total bacterial numbers were determined by fluorescein isothiocyanate staining and direct epifluorescence microscopy (8). The amount of dissolved organic carbon and the pH of each culture at the time of sampling were also measured. *Fischerella* sp. numbers were not determined, since we have shown previously that this cyanobacterium has a doubling time of >20 h under these experimental conditions (D. L. Tison and D. H. Pope, unpublished data).

The results of these experiments (Fig. 1) show that *L. pneumophila* grew exponentially for 6 h. A mean doubling time of 2.7 h was obtained from linear regression analysis of the results of the two experiments. This growth rate of *L. pneumophila,* presumably on algal extracellular products, is more than twice as rapid as that previously reported for growth on complex or defined media. Growth of *L. pneumophila* at 45°C has not been previously reported (6, 15).

When the photosynthetic activity of *Fischer-*

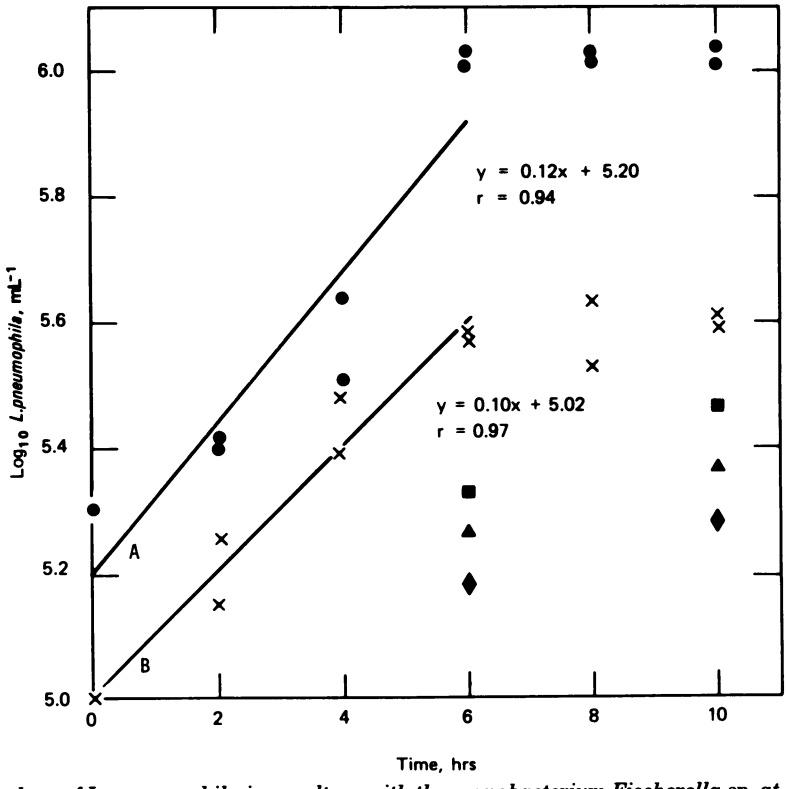

FIG. 1. *Numbers of L. pneumophila in coculture with the cyanobacterium Fischerella sp. at 45°C. A and B represent the lines indicated by the equations generated by linear regression analysis of the data from duplicate experiments with different L. pneumophila inoculum concentrations. The coefficient of correlation (r) for each regression line is shown. Controls corresponding to the data associated with regression line A are also shown. Each point represents the mean of triplicate counts from cultures sampled at the time indicated. Symbols:* (●) *L. pneumophila in experiment A;* (×) *L. pneumophila in experiment B;* (▲) *L. pneumophila in 3-(3,4-dichlorophenyl) 1-dimethyl urea control;* (■) *L. pneumophila inoculum without Fischerella sp.; and* (◆) *L. pneumophila in dark control.*

ella sp. was inhibited by dark incubation or by the addition of 3-(3,4-dichlorophenyl) 1-dimethyl urea, only a slight increase in *L. pneumophila* numbers was seen after 10 h. No growth of *L. pneumophila* was observed in the mineral salts medium without *Fischerella* sp. These results indicate that growth of *L. pneumophila* is dependent upon active photosynthesis by *Fischerella* sp. and presumably the extracellular release of substrates and possibly cofactors.

During the course of the growth experiment, the pH of the culture medium increased from pH 6.9 to a maximum of pH 8.1 after 10 h (Fig. 2). After about 6 h, when growth of *L. pneumophila* ceased, the pH of the cultures was near the maximum permissible pH of 7.6 recently reported for *L. pneumophila* in a defined medium (Warren and Miller, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, D64, p. 50). Even though *L. pneumophila* apparently ceased active growth after 6 h under these conditions, the cells remained viable for several weeks at a pH of 8.0 to 8.5. This was shown by observing that when the bacteria were transferred from 12-week cultures along with the cyanobacteria to fresh media, growth resumed (Tison, unpublished data). The survival of *L. pneumophila* in pond water for periods of >250 days has been recently reported (Janssen and Larson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, D63, p. 50).

Both the amount of dissolved organic carbon (Fig. 2) and the total numbers of bacteria (data not shown) in the cocultures increased for about 4 h. The decrease in dissolved organic carbon concentrations may be a result of decreased dissolved organic carbon production by *Fischerella* sp. or increased utilization of algal extracellular products by the associated heterotrophic microbiota after an initial lag period. A similar lag has been reported for heterotrophic utilization of algal extracellular products in both cultures and natural communities (11). A decrease in total bacterial numbers after 4 h appeared to have been the result of cell lysis, since increasingly larger amounts of cellular debris were observed during the course of the experiment using direct epifluorescence counts. This may have been due to accumulation of toxic products in the cultures. There was no apparent lysis of *L. pneumophila* cells, since no cellular debris was observed by direct immunofluorescence.

Limitation of bacterial growth due to the accumulation of end products or availability of nutrients is not likely to occur in natural habitats to the extent observed in culture, since, in nature, toxic products may be removed by diffusion or utilized by other associated microbiota. It has been documented that algal cells produce organic substrates, some of which may be excreted and utilized by bacteria (16, 17). Growth of pathogenic bacteria and bacterial indicators of fecal pollution on algal extracellular products has also been reported (9). The amount of photosynthetic products released extracellularly by the mat community used in our studies ranged from <1 to 6% of the total amount of CO_2 fixed photosynthetically (particulate plus excreted), which is similar to values obtained by other workers studying other thermophilic algal-bacterial mat communities (1, 2). Additionally, algal photosynthetic activity provides oxygen that can be utilized in aerobic respiration, which in turn produces CO_2, which may be available for algal photosynthesis. This mutualistic association between algae and cyanobacteria and bacteria may

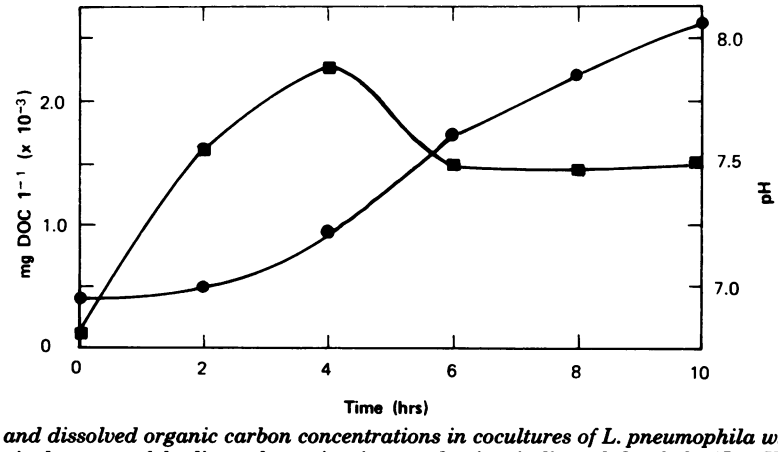

FIG. 2. *pH and dissolved organic carbon concentrations in cocultures of L. pneumophila with Fischerella sp. Each point is the mean of duplicate determinations at the time indicated. Symbols:* (●) *pH;* (■) *dissolved organic carbon.*

occur in natural planktonic communities (3, 13).

Our results indicate that *L. pneumophila* is able to grow on substrates such as algal extracellular products which would normally be present in natural habitats, and they indicate also that the temperature and pH ranges of *L. pneumophila* are wider than previously reported. The relatively rapid growth rates of *L. pneumophila* with these natural substrates as nutrients, and the ability of *L. pneumophila* to grow and survive under physical conditions not previously observed, may explain the apparently widespread distribution of this organism in natural and man-made habitats.

We thank R. M. McKinney, G. A. Hebert, and P. P. Harris for the fluorescent antibody conjugates, C. W. Moss for analyzing cellular fatty acids by gas-liquid chromatography, and D. J. Brenner for the deoxyribonucleic acid homology data. L. H. Orrison gave technical assistance.

D.L.T. was supported in part by an Oak Ridge Associated Universities Laboratory Graduate Participation Fellowship. This paper was prepared in connection with work done under Contract no. AT(07-2)-1 with the U.S. Department of Energy.

LITERATURE CITED

1. **Bauld, J., and T. D. Brock.** 1974. Algal excretion and bacterial assimilation in hot spring algal mats. J. Phycol. **10**:101-106.
2. **Belly, R. T., M. R. Tansey, and T. D. Brock.** 1973. Algal excretion of ^{14}C-labeled compounds and microbial interactions in *Cyanidium calderium* mats. J. Phycol. **9**:123-127.
3. **Caldwell, D. E., and S. J. Caldwell.** 1978. A *Zoogloea* sp. associated with blooms of *Anabaena flos-aquae*. Can. J. Microbiol. **24**:922-931.
4. **Castenholz, R. W.** 1969. Thermophilic blue-green algae and the thermal environment. Bacteriol. Rev. **33**:476-504.
5. **Center for Disease Control.** 1978. Isolation of organisms resembling Legionnaires' disease bacterium—Georgia. Morbid. Mortal. Weekly Rep. **27**:415.
6. **Feeley, J. C., G. W. Gorman, R. E. Weaver, D. C. Mackel, and H. W. Smith.** 1978. Primary isolation media for Legionnaires disease bacterium. J. Clin. Microbiol. **8**:320-325.
7. **Fliermans, C. B., W. B. Cherry, L. H. Orrison, and L. Thacker.** 1979. Isolation of *Legionella pneumophila* from non-epidemic related aquatic habitats. Appl. Environ. Microbiol. **37**:1239-1342.
8. **Fliermans, C. B., and E. L. Schmidt.** 1975. Fluorescence microscopy: direct detection, enumeration, and spatial distribution of bacteria in aquatic systems. Arch. Hydrobiol. **76**:33-42.
9. **McFeters, G., S. A. Stewart, and S. B. Olson.** 1978. Growth of heterotrophic bacteria and algal extracellular products in oligotrophic waters. Appl. Environ. Microbiol. **35**:383-391.
10. **Morris, G. K., C. M. Patton, J. C. Feeley, S. E. Johnson, G. Gorman, W. T. Martin, P. Skaliy, G. F. Mallison, B. D. Politi, and D. C. Mackel.** 1979. Isolation of Legionnaires' disease bacterium from environment samples. Ann. Inter. Med. **90**:664-666.
11. **Nalewajko, C.** 1976. Primary productivity, extracellular release and heterotrophy in two lakes in the ELA, NW Ontario. J. Fish. Res. Bd. Canada **33**:219-226.
12. **Neilson, A. H., W. B. Blankley, and R. A. Lewin.** 1973. Growth with organic carbon and energy sources, p. 275-285. *In* J. R. Stein (ed.), Handbook of phycological methods: culture methods and growth measurements. Cambridge Univ. Press, Cambridge.
13. **Paerl, H. W.** 1976. Specific associations of the blue-green algae *Anabaena* and *Aphanizomenon* with bacteria in freshwater blooms. J. Phycol. **12**:431-435.
14. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111**:1-61.
15. **Weaver, R. E.** 1978. Cultural and staining characteristics, p. 39-43. *In* G. L. Jones and G. A. Hebert (ed.), "Legionnaires'": the disease, the bacterium and methodology. Center for Disease Control, Atlanta, Ga.
16. **Wiebe, W. J., and D. F. Smith.** 1977. Direct measurement and DOC release by phytoplankton and incorporation by microheterotrophs. Mar. Biol. **42**:213-223.
17. **Wright, R. T., and J. E. Hobbie.** 1965. The uptake of organic solutes in lake water. Limnol. Oceanogr. **10**:22-28.