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OBSERVATIONS ON INTERACTIONS BETWEEN NATURALLY-COLLECTED BACTERIA AND SEVERAL SPECIES OF ALGAE

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Abstract

Interactions between a naturally-collected algal species and strains of bacteria with which it was closely associated were examined under controlled conditions. Three strains of bacteria, *Pseudomonas, Xanthomonas* and *Flavobacterium,* were isolated from *Oscillatoria.* These bacteria were grown in combination with axenic cultures of the *Oscillatoria* culture as well as with several additional algal species. *Oscillatoria* growth was stimulated by all of the bacteria, but other algal species varied in their response. Some were stimulated, but others were inhibited or unaffected by exposure to the bacterial strains. There were also observations indicating that some algae may be able to develop resistance to antagonistic bacteria. These data suggest that succession and dominance of individual algal species may be influenced by interactions with bacteria.

Introduction

It is evident from extensive studies that biotic as well as chemical and physical factors influence algal growth, and it is clear that an understanding of the ecology of algae requires information concerning these biotic relationships. Among the interactions of potential significance for algae are those with bacteria. Algae growing in natural situations are always in association with bacteria, and there is evidence that the algal and bacterial members of such an association may exhert considerable influence on one another. Observations by Provasoli (1958), Parker & Bold (1961), Nakamura (1963), Vance (1966), Berland, Banin & Maestrini (1970), and Ukeles & Bishop (1975) suggest that bacteria can enhance the growth of algae.

Lange (1967, 1970, 1971) has proposed that bacteria may be an important source of CO_2 for algal growth during periods of carbon limitation. Others (Safferman & Morris, 1962; Blasco, 1965; Bershova, Kopteva & Tantsyurenko, 1968; Wu, Hamdy & Howe, 1968; Stewart & Brown, 1969; Shilo, 1970, 1971; Daft & Stewart, 1971; and Berland, Bonin & Maestrini, 1972) have reported various cases in which bacteria appeared to be antagonistic to algae. There is evidence that bacteria may derive nutritional benefit from extracellular products released by algae (Bershova *et al.,* 1968; Bell & Mitchell, 1972) and there have been several reports of bacteria being inhibited by algae (Steelman Nielsen, 1955; Davidson, 1959; Burkholder, Burkholder & Almodovar, 1960; Gupta & Shrivastava, 1965; Duff, Bruce & Antia, 1966; Ramamurthy & Krishnamurthy, 1967; Berland *et al.,* 1972).

Although there is ample evidence of algal-bacterial interactions, there is little information on species-specific interactions in defined conditions. The present investigation was conducted in order to examine under controlled conditions some interactions between a naturally collected algal species and strains of bacteria with which it was closely associated. The alga used for this purpose was *Oscillatoria* sp. collected from a fish-hatchery pond where it dominated the algal-flora during June 1975. Three strains of bacteria isolated from the *Oscillatoria* filaments were grown in various combinations with axenic cultures of the *Oscillatoria.* Examinations of interaction between the bacteria and other species of algae were also conducted.

Materials and methods

Oscillatoria was collected at the United States Fish Hatchery in Fort Worth, Tarrant County, Texas. Collections

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were made twice during June, 1975. The samples were maintained in soil-water media (Pringshein, 1956; Starr, 1964) and cultured in Mineral Medium no. 11, (Hughes, Gorham, Zehnder, 1958) at 20°C. Cultures were kept under 8-hour light, 16-hour dark cycle.

The following cultures were obtained from the Indiana University Culture Collection: *Chlorella pyrenoidosa* IU no. 1230, *Chlamydomonas reinholdt* IU no. 89, *Anabaena flos-aquae* IU no. 1444, *Euglena gracilis* 'Z' IU no. 753, *Haematococcus locustriis* IU no. 16, *Botrydiopsis arhiza* IU no. 87. *Euglena, Chlorella, Haematococcus,* and *Botrydiosis* were maintained on Proteose agar slants (Starr, 1964). *Chlamydomonas* was grown on Soil Extract agar (Starr, 1964). *Anabaena* was maintained on Mineral Medium no. 11. All cultures were grown under the same light and temperature conditions as *Oscillatoria.*

Oscillatoria was rendered axenic by the addition of a Penicillin-Streptomycin solution (final concentration of 50 units/ml.). Addition of a drop of Tween 80 in some cases and mild sonication in others was used to aid in breaking up algal mats to help render them axenic. Algal cultures were kept in the antibiotic solution for three to four days and then transfered to fresh media. Samples were periodically grown on nutrient agar to test for the presence of bacteria.

Bacteria were isolated from non-axenic *Oscillatoria* by drawing washed filaments through semi-soft nutrient agar in a sterile petri dish. Bacteria were isolated from these dishes and cultured on fresh nutrient agar at 37°C. Bacteria were classified by using standard bacteriological methods: gram stain, general and colony morphology, motility, sugar reduction, gas and indole production. All bacteria cultures were maintained on nutrient agar slants at 37°C. Three strains of bacteria were isolated and grown singly and in combination with axenic samples of algae.

Growth of algae in response to bacteria was assayed by a modification of the paper disc-agar plate method of Ukeles & Bishop (1975). The appropriate algal growth medium containing 1.5% Bacto-Agar was sterilized and poured into pre-sterilized polyethylene petri dishes partitioned into quadrants. Each quadrant was seeded with equal quantities of the test algae.

Inocula of the three strains of bacteria used for experiments were standardized by the following procedure: bacteria were subcultured from agar slants into nutrient broth and incubated until the suspension reached an optical density of 0.67 when read on a Baush and Lomb Spectronic 20 at 520 nm. Aliquots of this suspension were used in making dilution plate counts. Suspensions used to charge the paper discs had average concentrations of: *Pseudomonas* sp. 1.95 x 10^7/ml., *Xanthomonas* sp. 2.15 x 10^7/ml., *Flavobacterium* sp. 2.71 x 10^7/ml.

Paper discs were prepared from Millipore filter support paper (cat. no. AP100-4750, Millipore Corp., Bedford, Mass.) with a standard paper punch. Discs were sterilized in a steam autoclave, charged with 20 lambda of the standardized bacterial suspension and placed in quadrant I of the algal-seeded petri dish. Quadrant II received a disc prepared as described above but which had subsequently been subjected to two hours in a dry heat oven at 180°C. A third set of discs were charged with 20 lambda of a sterile bacterial broth medium. A sterile paper disc without any additives was placed in the fourth quadrant.

Filtrates of bacterial suspensions, prepared by passing through 0.45 micron pore diameter filter paper, were also tested for their effects on algal growth. The filtrate was collected aseptically and charged onto paper discs (20 lambda). Several combinations of mixed bacteria and mixed bacterial filtrates were also tested.

All tests were run in triplicate with each algal species. Algal test plates were incubated at 20°C under 8-hour light, 16-hour dark cycle. Three times a week for 8 weeks the plates were examined for signs of inhibition or enhancement of algal growth. Inhibition was noted as a clear zone around the test disc. Growth was judged stimulatory when any one of the following conditions were met: 1. an excess of growth on or under the disc; 2. a heavy ring of growth surrounding the disc; 3. heavy growth or spots of growth on the disc. All results were judged relative to the sterile disc.

Results

Three species of bacteria were isolated from natural cultures of *Oscillatoria.* All three bacteria were short, gram-negative rods. None of the bacteria were lactose-fermentors. Type I bacterium formed white circular colonies with lighter margins. Type II bacterium had yellow to orange pigmentation and punctiform colonies which were raised with entire margins. Type III bacterium developed as smooth colonies, flat with entire margins. Results of the biochemical tests with these bacteria are summarized in Table 1. Type I bacterium has been identified as a species of *Pseudomonas* and Type II, a species of *Xanthomonas.* Type III bacterium, difficult to maintain after primary isolation, has been identified as a species of *Flavobacterium.* All three types of bacteria are

Table 1. Biochemical reactions of three strains of bacteria isolated from *Oscillatoria* (+ denotes a positive result)

	Bacteria		
Test	Type I	Type II	Type III
Glucose	+	+	–
Sucrose	–	–	–
Lactose	–	–	–
Manitol	–	–	–
Sacchrose	–	–	–
Maltose	–	–	–
Citrate	+	–	–
Indole	–	–	–
Motility	+	–	–
H_2S Prod.	+	+	+
Oxidase	+	–	–

common water inhabitants and *Pseudomonas* and *Xanthomonas* are known plant pathogens.

Algal species varied in their response to the three bacteria (Table 2). All three bacteria stimulated the growth of *Oscillatoria.* In all cases growth in the quadrants with bacteria exceeded growth in the quadrants with the sterile disc. Growth of *Oscillatoria* was greater in quadrants with *Xanthomonas,* covering nearly twice the area on the agar as that resulting from growth with the other two bacteria. Growth in quadrants with *Pseudomonas* was equal to the growth with *Flavobacterium.* Mixed bacteria were stimulatory to *Oscillatoria* in all combinations (Table 3).

Flavobacterium had little effect on *Chlorella, Anabaena* or *Euglena,* the growth being equal to the sterile disc. *Flavobacterium* did, however, stimulate the growth of *Chlamydomonas* and 'spotting' (dense, small spots of growth) occurred with *Haematococcus* and *Botrydiopsis.*

This secondary growth was transfered to a new agar plate and inoculated with *Pseudomonas* as in the original plate. No initial inhibition was observed and no clear zone appeared around the disc. Growth was actually slightly stimulatory with the algal growth greater than that around the sterile disc. It was also noted that if *Chlorella* was allowed to grow into a thick algal mat (5-8 days), then inoculated with *Pseudomonas,* no inhibition resulted.

Initial observations of axenic liquid cultures of *Oscillatoria* suggested that the presence or absence of bacteria might influence colony morphology. When first collected, the *Oscillatoria* was in the form of a loose mat of long filaments. Following antibiotic treatment, the filaments coiled into tight, compact spheres. Inasmuch as Schwabe and Mollenhouer (1967) have reported that the presence of bacteria was essential for the formation of normal colonies in *Nostoc spericum,* an effort was made to determine if this *Oscillatoria* population has a similar requirement. It was determined, however, that colony morphology was influenced more by the nature of the culture vessel than by the presence or absence of bacteria. Both axenic and bacterized cultures formed tight coils

Table 2. Responses of algal cultures to bacteria isolated from *Oscillatoria.*

	Bacteria		
Algae	*Pseudomonas*	*Flavobacterium*	*Xanthomonas*
Oscillatoria	Stimulation; thick growth around disc	Stimulatory	Stimulatory
Chlorella pyrenoidosa	Inhibition followed by 2° growth	No response	Stimulatory; growth heavy on disc
Chlamydomonas reinholdt	Stimulatory; dark ring a around disc	Stimulatory; dark growth on disc	Inhibition; small zone around disc
Anabaena flos-aquae	Stimulatory; dense ring around disc	No response	No response
Euglena gracilis "Z"	Inhibition; some 2° growth	Inhibition; small zone around disc	Inhibition; no mat growth
Haematococcus locustriis	No response	Stimulatory; spotting on disc	Stimulatory; slight darker growth on disc
Botrydiopsis arhiza	Inhibition; small zone-fine mat growth	Stimulatory; spottong on disc	Stimulatory growth heavy on bottom of disc

Table 3. Responses of algal cultures to mixed populations of bacteria.

	Bacteria		
	Pseudomonas Flavobacterium	*Pseudomonas Xanthomonas*	*Pseudomonas Xanthomonas Flavobacterium*
Oscillatoria	Stimulatory; dark growth around disc	Stimulatory	Stimulatory
Chlorella pyrenoidosa	Inhibition followed by 2 growth	Inhibition followed by 2 growth	Inhibition followed by 2 growth
Chlamydomonas reinholdt	Inhibition	Inhibition	Inhibition
Anabaena flos-aquae	Stimulatory; heavy spots of growth on disc	Stimulatory	Stimulatory
Euglena gracilis "Z"	Stimulation; slow growth	Slight Stimulation	Stimulation; dark growth on disc
Haematococcus locustriis	No response	No response	No reponse
Botrydiopsis arhiza	No response	No response	No response

when grown in 14 x 150 mm test tubes whereas similar cultures grown in 250 ml. Erlenmeyer flasks exhibited the loose mat typical of naturally occurring colonies. These findings were more like those of Kantz & Bold (1969) who found no significant differences in the colony morphology of axenic and non-axenic strains of *Anabaena* and *Nostoc* which they studied.

Xanthomonas stimulated the growth of *Chlorella, Botrydiopsis,* and to a lesser extent, *Haematococcus. Chlamydomonas* and *Euglena* were inhibited by the growth of *Xanthomonas.*

Pseudomonas stimulated the growth of *Anabaena* with dark rings of growth forming around the disc. In a similar manner, *Pseudomonas* stimulated the growth of *Chlamydomonas* by a darkening of the disc and a slight growth ring around the disc. *Haematococcus* and *Botrydiopsis* showed also little reaction or were slightly inhibited by *Pseudomonas.*

Mixed bacteria were inhibitory to *Chlamydomonas,* but stimulatory to *Anabaena* and *Euglena. Haematococcus* and *Botrydiopsis* were not responsive to mixed cultures, their growth being equal to that of the sterile disc.

Filtrates of *Pseudomonas* and *Flavobacterium,* and combination of the two had no effect on the growth of algae. Growth patterns were uneven and the same as the sterile disc.

Pseudomonas had an interesting effect upon *Chlorella* and *Euglena.* Initially the bacteria inhibited the algal growth, evidenced by a large, clear zone around the disc. After 10-14 days, a secondary growth of algae appeared around the disc inside the original zone of inhibition. The secondary growth appeared to be stimulated by bacteria and within two weeks overtook the clear area. *Chlorella* was more successful in overtaking the inhibition than was *Euglena.* Tests with mixed bacterial cultures also showed similar effect. All combinations that included *Pseudomonas* showed this initial inhibition followed by a secondary growth.

Discussion

It is necessary to exercise caution in the application of data obtained under controlled laboratory conditions to natural situations. Nevertheless, the laboratory approach can provide insight into the complex interactions that occur between organisms in their microenvironments. This investigation has provided evidence that growth of an algal population can be influenced by bacteria with which it actually co-exists in nature. In the case of the *Oscillatoria* population studied here, its satellite bacteria clearly enhanced its growth rate under the experimental conditions established in this investigation. Other species of algae were stimulated, inhibited or unaffected by exposure to these same strains of bacteria indicating that algal-bacterial interactions may be species-specific. This variation in algal response could have a strong influence on the succession and dominance of individual forms.

The complexity of algal-bacterial interactions is further illustrated by the assays involving more than one bacterial strain. Some species of algae responded in a similar manner to single- and multiple-member bacterial cultures, but other species did not. For example, *Pseudomonas* or *Flavobacterium* alone stimulated the growth of *Chlamydomonas,* whereas in combination they were inhibitory to the alga. Inasmuch as algae and bacteria occur in multi-species associations in nature, simple laboratory tests involving two species may not necessarily indicate the manner in which a particular pair of organisms affect one another in natural situations.

Of particular interest in this investigation was the observation that *Chlorella* and to lesser extent *Euglena* were able to overcome initial inhibition by *Pseudomonas.* This resistance to inhibition by the *Chlorella* population appeared to be permanent and might represent the induction of some detoxifying system or a selection for naturally resistant individuals. Whatever the mechanism, the ability to resist bacterial inhibition would seem to impart a selective advantage to algae that frequently encountered antagonistic bacteria. Somewhat similar observations were made by Bershova *et al.* (1968) in their study of the influence of over 2000 strains of naturally isolated bacteria on four species of blue-green algae. They found that, after prolonged incubation, some bacteria which were formerly inhibitory to algae lost this property, and in some cases even became stimulatory to the algae. These findings have led Whitton (1973) to propose that with at least some pairs of species in nature interactions may be either mutualistic or antagonistic, according to environmental circumstances.

Results from tests with heat-killed bacteria and bacterial filtrates showed that any effect on algal cultures resulted from viable bacteria. Other investigations have shown that filtrates of bacterial cultures have little effect on algal growth (Hamburger, 1958; Ukeles & Bishop, 1975; Machlis, 1973). Various explanations have been suggested as to the nature of the action of the live bacteria. Ukeles & Bishop (1975) attribute enhancement to the

release of growth factors from the bacterial hydrolyses of agar. Parker & Bold (1961) suggest that the decomposition by bacteria of nitrogenous substrates release simplified products available to algae as a nitrogen source. There also exists the possibility that the bacteria could supply necessary CO_2 (Lange, 1967, 1970, 1971; Keuntzel, 1969), alter the pH (Parker & Bold, 1961) or even remove autotoxic algal metabolites (Hamburger, 1958). Due to the close physical association to algal cells, bacteria may also aid the cell in absorption of essential nutrients.

It is evident from the data presented here and those of others that under natural conditions a close and complex relationship must exist between bacteria and algae. These interactions are poorly understood and need much additional study. A combination of controlled laboratory experiments in conjunction with field observations will probably provide greatest insight into the ecological and physiological relationships that exist between microorganisms in natural environments.

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