APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1978, p.791-796
0099-2240/78/0035-0791$02.00/0

Vol. 35, No. 4
Printed in U.S.A.

Interactions Between the Diatom *Thallasiosira pseudonanna* and an Associated Pseudomonad in a Mariculture System

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Received for publication 12 October 1977

The marine diatom *Thallasiosira pseudonanna* (3H) and several bacteria associated with it were isolated from batch cultures at the University of Delaware mariculture facility. The interaction between the algae and each of the bacteria was investigated. One of the isolates, T827/2B (*Pseudomonas* sp.), was incapable of surviving in f/2 culture medium unless the algae were present. When the algae and T827/2B were grown together in the f/2 medium, the bacterial growth was stimulated and the algal growth was inhibited. Bacterial filtrate had a similar effect on the algae, indicating that the bacterial effect is an indirect one most likely resulting from the excretion of a harmful compound into the medium. Preliminary characterization of the material excreted by the bacteria indicates that it is proteinaceous in nature. The interaction observed does not fit into any single category of interactions but can be explained as a combination of competition and indirect parasitism.

The marine diatom *Thallasiosira pseudonanna* (3H) is a widely used alga in the culture of bivalve mollusks (36). This alga is routinely used at the University of Delaware for feeding larvae of the American oyster (*Crassostrea virginica*), where it is grown in large-batch unialgal but nonaxenic cultures. In recent years a phenomenon has been noted in which apparently healthy cultures of the diatom become moribund and a significant number die within a 1- to 2-day period. This phenomenon, which we term an algal crash, was associated with large cultures in late exponential or early stationary phase.

We report here our studies of the mechanism of the crash phenomenon. Our results indicate that the crash is likely the result of a complex interaction between the algal culture and one of the bacterial species associated with that culture.

Interactions between microbial species have been reported in several aquatic systems. Numerous workers (8, 11, 31) have found viruses in freshwater habitats capable of infecting and lysing blue-green bacteria. Species of *Bdellovibrio* (7), *Myxobacter* (39), *Cytophaga* (41), *Cellvibrio* (20), and *Bacillus* (34) have also been found that are antagonistic to blue-green bacteria. In marine systems, Jorgensen and Steeman-Nielsen (26), Sieburth (40), and Duff et al. (13) have studied the ability of various marine algal species to produce antibacterial substances. Droop and Elson (12) found a correlation between the growth phase of batch cultures of marine diatoms and the numbers of bacteria in the culture, with bacterial numbers increasing as the algal cultures aged. They were not able to determine, however, whether the bacteria affected the growth characteristics of the algae or were present in increased numbers as a result of the algae having entered stationary phase. Mitchell (29) has reported on a marine pseudomonad that is a predator of the diatom *Skelotonema*. The mechanism of this interaction, however, is not presented.

MATERIALS AND METHODS

Algal cultures. Cultures of Guillard's 3H strain of *T. pseudonanna* were obtained from the University of Delaware Mariculture Station. The algae were freed from bacterial contamination by streaking onto f/2 agar, picking isolated colonies, and restreaking. They were maintained on f/2 medium (21) made with Indian River water (Indian River, Del.) as a base. The Indian River water was pretreated with ultraviolet light to remove organic matter. Its salinity ranged from 15 to 20%. Cultures were grown at room temperature with constant illumination.

Bacterial cultures. Bacteria from cultures of *T. pseudonanna* populations undergoing the previously described crash phenomenon were isolated by direct plating onto marine agar 2216 (Difco Laboratories, Detroit, Mich.). Cultures of the three numerically dominant bacteria chosen on the basis of colony morphology were maintained on marine agar and used for this study. Bacteria were transferred to marine broth (Difco) or f/2 medium 24 h before all experiments.

Preparation of bacterial filtrate. Twenty-four-hour-old cultures of bacteria in f/2 medium containing

approximately 2×10^8 cells/ml were filtered through a 0.45-μm Nalgene filter (Sybron Corp., Rochester, N.Y.) to remove the bacterial cells. The filtrate was tested for sterility by microscopic examination and by plating onto marine agar. The filtrate was stored at 4°C in the dark until needed.

Growth of algal and mixed algal-bacterial cultures. Algae at a final concentration of approximately 2×10^5 cells/ml were inoculated into 9 ml of f/2 medium, placed on a Bellco tube roller (Bellco Glass Co., Vineland, N.J.), and cultured under constant illumination at room temperature. Bacterial cells or filtrate from bacterial cultures were added to the algae in varying times and amounts as indicated.

The number of live *T. pseudonanna* was determined at 12-h intervals by counting 0.1-ml samples in a hemocytometer. Evans blue stain was used to differentiate live from moribund or dead cells (10). The number of live bacteria in a sample was determined by counting the number of colonies on marine agar spread plates. Competition between the algae and the bacteria was assayed by adding supplemental nutrients to the cultures in a modification of the marine algal assay procedure (14).

Dialysis experiment. A culture apparatus was designed to allow the alga and bacterium to be grown in such a way that cell-to-cell contact between the two populations was prevented while the exchange of small-molecular-weight compounds (<12,000) was allowed. This apparatus consisted of a dialysis tubing sac containing 3 ml of medium (Fisher, King of Prussia, Pa.) attached to a small piece of glass tubing. The dialysis sac was suspended in a 250-ml twin-necked, round-bottom flask containing 100 ml of f/2 medium in such a way that the glass tubing projected through the rubber stopper used to seal one neck of the flask. The glass tubing and the second neck of the flask were stoppered with cotton plugs. After the system was autoclaved, a sample of bacteria in marine broth was aseptically injected into the dialysis sac via the glass tube. Algae were added directly to the flask via the second of the two necks. Numbers of live algae were monitored as described previously. After 3 days of growth, the dialysis sac was aseptically cut and the two cultures were allowed to mix.

Radioisotope experiments. Algal production of extracellular products (ECP) was measured by using ^{14}C-labeled sodium bicarbonate (New England Nuclear Corp., Boston, Mass.). The procedure followed was a modification of that described by Anderson and Zeutschell (4). Bacterial uptake of labeled ECP was determined by the procedure of Herbland (23). All samples were counted on a Beckman LS230 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.), using PCS (Amersham/Searle, Arlington Heights, Ill.) as the counting cocktail.

RESULTS

Screening of bacterial cultures for antialgal activity. Of the three bacterial cultures tested, only T827/2B (*Pseudomonas* sp.) showed antialgal activity that might account for the crashes observed at the mariculture facility. The presence of this bacterium in relatively low numbers was sufficient to cause a drastic decline in the number of viable *T. pseudonanna* in the batch culture (Fig. 1).

The other two bacteria tested, T827/3 (*Micrococcus* sp.) and T827/2A (*Flavobacterium* sp.), had a slight effect on the growth rate of the algae. In no instance, however, did the presence of either of these two bacteria result in a decrease in the number of viable algae present. Therefore, these two isolates were ruled out as possible causes of the algal crashes, and all further experiments were limited to bacterium T827/2B.

Growth of mixed cultures. The algae demonstrated typical batch culture kinetics in f/2 medium (Fig. 2). The bacteria in pure culture were unable to grow in this medium (Fig. 2). When the algae and bacteria were mixed (Fig. 3), the bacteria were able to grow. The growth curves for the individual populations in mixed culture were out of phase with each other, with an increase in one population being associated with a decrease in the other.

This relationship between the growth characteristics of the two populations in mixed culture correlates well with the pattern of occurrence of T827/2B in samples taken from *T. pseudonanna* cultures. Whereas T827/2B was one of the dominant bacteria isolated from crashing or immediately precrash algal cultures,

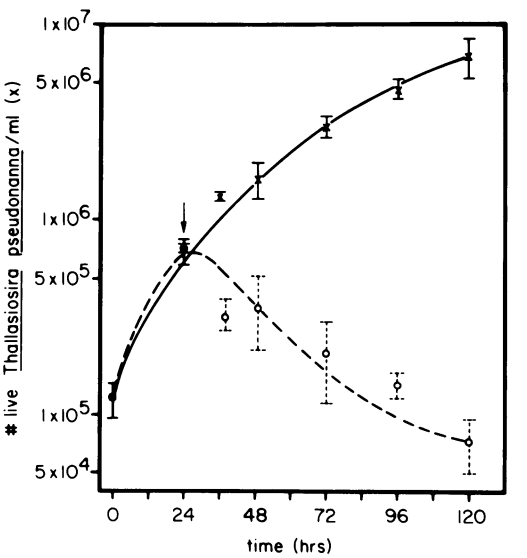

FIG. 1. *Influence of bacterium T827/2B on the growth of T. pseudonanna. Control cultures* (×) *were supplemented with 0.5 ml of marine broth at 24 h. Experimental cultures* (○) *received 0.5 ml of a* 2.0×10^8 *culture of T827/2B in marine broth at 24 h. The bars indicate the 95% confidence intervals. The arrow indicates time of addition of bacteria.*

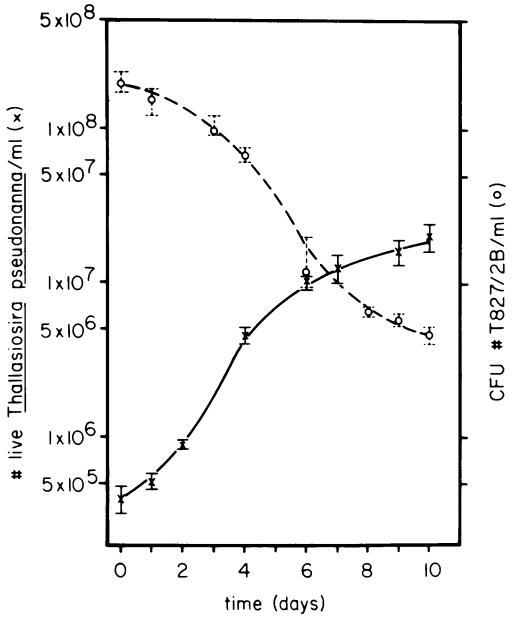

FIG. 2. *Growth of T. pseudonanna* (×) *and T827/2B* (○) *in single cultures in f/2 medium. The bars indicate the 95% confidence intervals.*

it was rarely isolated from healthy algal cultures.

Microscopic examination of mixed cultures of algae and bacteria revealed a progressive increase in the number of moribund *T. pseudonanna* cells in the presence of the bacteria. Also, an association between the bacterium and the alga was noted, with high concentrations of bacterial cells being found in close proximity to moribund algal cells (Fig. 4). No such association was seen between the bacterium and healthy algal cells.

The addition of supplemental f/2 nutrients to a mixed culture of T827/2B and *T. pseudonanna* was able to partially reverse the inhibition of the alga (Fig. 5). Similar nutrient additions to control cultures containing the alga alone had no effect.

Dialysis experiment. Enclosure of actively growing bacteria within a dialysis sac capable of preventing the exchange of large-molecular-weight substances and whole cells between the two populations completely prevented the antagonistic effect of the bacterium on the alga (Fig. 6). When the sac was cut, however, and the two populations were allowed to mix, the algal population declined in number immediately, showing a typical crash pattern.

Activity of bacterial filtrate. Bacterial filtrate was an effective inhibitor of the algae causing a 20% decrease in the growth rate. Microscopically, *T. pseudonanna* cultures grown in the presence of bacterial filtrate resembled cultures grown in the presence of T827/2B. There was a significant increase in the number of moribund and lysed algal cells in the cultures with the bacterial filtrate over control cultures, in which the number of dead cells was always quite small. The inhibition of the *T. pseudonanna* cultures resulting from the addition of bacterial filtrate was not as pronounced as when bacterial cells rather than filtrate was added to the culturs. Neither autoclaving the filtrate nor adding low levels of f/2 nutrients to the filtrate was individually capable of completely overcoming the inhibition of the algae. This inhibition was overcome when both autoclaving and nutrient supplements were used together, indicating that a combination of effects was responsible for the inhibition.

Radioisotope experiments. Axenic cultures of *T. pseudonanna* were found to be capable of converting ^{14}C-labeled sodium bicarbonate into labeled ECP. After 2 days of incubation during exponential and stationary phase, approximately 10% of the added label was found in the form of labeled non-bicarbonate material in the media. This material could have been the result of excretion of carbonaceous material by algal cells or it may have arisen from cell lysis. No attempt was made to determine which of these two possibilities was the source of the material.

When T827/2B was inoculated into f/2 medium enriched with labeled *T. pseudonanna*

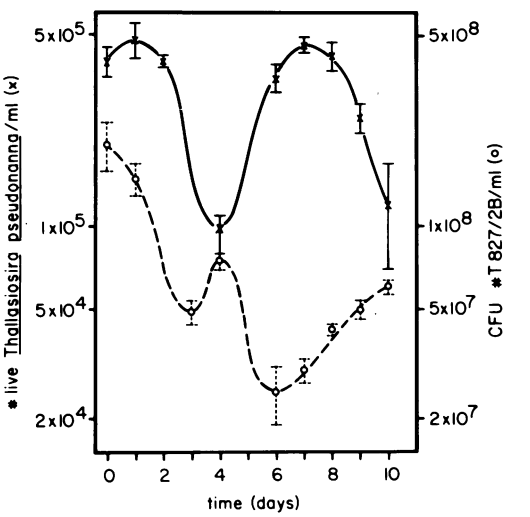

FIG. 3. *Growth of mixed bacterial-algal cultures. Growth of T. pseudonanna in the presence of T827/2B* (×); *growth of T827/2B in the presence of T. pseudonanna* (○). *Growth of the mixed cultures was in f/2 medium. The bars indicate the 95% confidence intervals.*

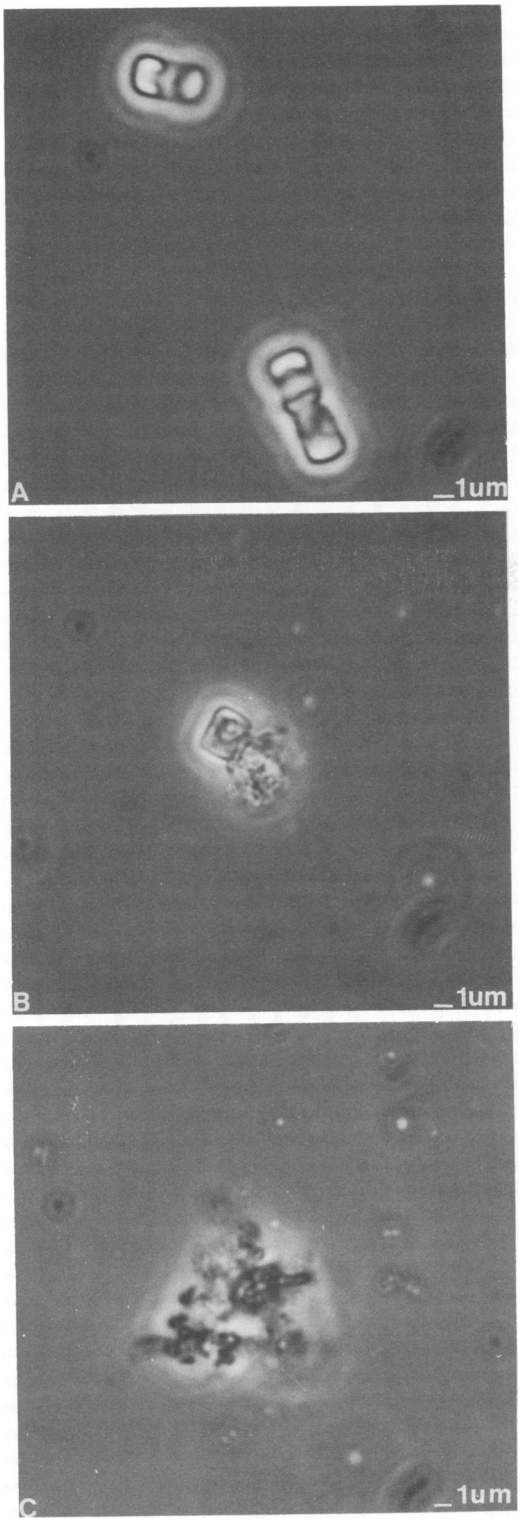

FIG. 4. *Association between. T. pseudonanna (3H) and Pseudomonas sp. T827/2B. (A) T. pseudonanna in axenic culture. (B) T. pseudonanna and Pseudomonas sp. (T827/2B) in early stages of interaction showing a close association between the bacteria and moribund alga. (C) T. pseudonanna and Pseudomonas sp. (T827/2B) in late stages of interaction showing a close association between the bacteria and dead alga. In (B) and (C) the moribund and dead algae were distinguished from debris by their ability to take up Evans blue stain.*

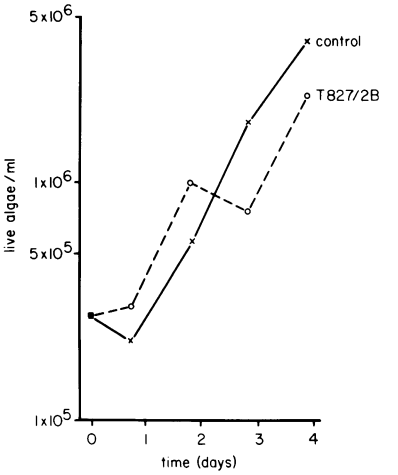

FIG. 5. *Effect of added f/2 nutrients on the algal-bacterial interaction. Control cultures (×) had no bacterial additions but were supplemented with nutrients on day 3. Experimental cultures (○) had 0.5 ml of a 2.0 × 10^8 culture of T827/2B added on day 2 and f/2 nutrients added on day 3.*

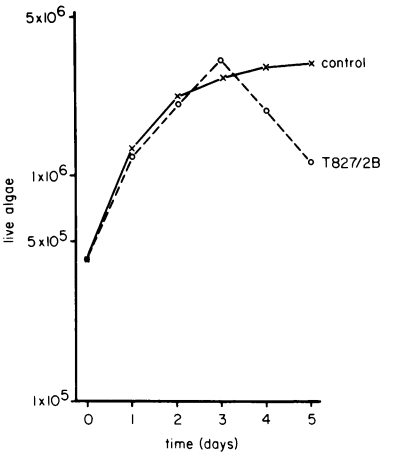

FIG. 6. *Dialysis experiments. The apparatus used for the experiment is described in the text. The control flask (×) contained a dialysis sac with sterile marine broth inside it. The experimental flask (○) contained a dialysis sac with a culture of T827/2B in marine broth inside it. Both dialysis sacs were aseptically cut on day 3.*

ECP, 15% of the labeled ECP was found in association with the particulate fraction of membrane-filtered bacterial cultures within a 0.5-h time period. Whether or not this association represents bacterial utilization of the ECP or a passive adsorption of the ^{14}C-labeled material to the bacterial cells is not known.

DISCUSSION

The relationship between *T. pseudonanna* and T827/2B can be explained in terms of a combination of interactions, which results in the algal crashes. The competitive aspects of this interaction can be seen in the ability of added f/2 nutrients to partially overcome the inhibition of the algae by the bacterium. However, competition alone cannot explain the lack of inhibition when the algae and bacteria are separated by a dialysis sac or the inability of added nutrients to completely reverse the inhibition. T827/2B cannot survive in f/2 medium alone, and if the interaction is simply competition for one of the nutrients in f/2 medium, either organism should be capable of surviving in f/2 medium and its growth in the medium should be better alone than when the other organism is present (1, 37, 42).

The presence of *T. pseudonanna* cells in a mixed culture with T827/2B stimulates the growth of the bacterium with a corresponding decrease in the algal population. The algae or their metabolic products can be seen as supplying the organic compounds necessary to supplement the f/2 medium, thereby allowing the bacteria to grow. The ability of the bacteria to cause a decline in the algal population argues against this being a simple case of commensalism, such as the interaction between *Phaeodactylum* and bacteria reported by Raymont and Adams (33). It may indicate that this aspect of the interaction is more closely related to predation or parasitism, with the bacterium in some way killing the algae and thus obtaining its nutritional requirements.

Frederickson (19) has noted that the terms "predation" and "parasitism" are usually applied to direct interactions such as the predation of *Bdellovibrio* on *Escherichia coli* (35). He has proposed the term "indirect parasitism" to cover situations in which one of the interacting populations releases a substance into the medium that results in the lysis of organisms in the second population (19). The interaction between T827/2B and *T. pseudonanna* exhibits characteristics of indirect parasitism.

The antialgal activity of filtrate from cultures of T827/2B demonstrates that cell-to-cell contact is not required for inhibition, indicating that the bacteria are excreting a toxic compound that kills the algae. The preliminary physical characterization of the material produced by T827/2B indicates that it has a relatively high molecular weight, is heat labile, and is probably a protein.

Even though cell-to-cell contact is not required for inhibition, microscopic examination of the mixed algal-bacterial cultures reveals that there is a close physical association between T827/2B and *T. pseudonanna* in crashing cultures. This association may represent an attraction of the bacteria to dead algal cells similar to that proposed by Bell and Mitchell (6), who observed an attraction between a marine bacterial isolate and the diatom *Skelotonema,* with the bacterium exhibiting no effect on the growth of the alga. Growth of their bacterial isolate was enhanced by the presence of the diatom.

The interaction between T827/2B and *T. pseudonanna* is interesting in light of recent speculation concerning the biological conditioning of water and the role of ECP in aquatic systems. Many authors have speculated that dissolved compounds released by bacteria or algae into the water could have profound effects in determining the microorganisms capable of surviving in a body of water (3, 9, 24, 27, 32). Thus, the organic materials produced by one type of microorganism could be responsible for either stimulating or inhibiting the growth of other microorganisms. This effect of one microorganism on the biological and chemical quality of a body of water can be termed biological conditioning.

The ability of freshwater and marine algae to excrete organic material into the environment has been well documented (16, 22). Measurements of photosynthetically fixed carbon excreted by aquatic algae have ranged from 15 to 50% of the total carbon fixed (2, 5, 15, 16, 18, 22, 27). Such compounds include amino acids, keto acids, fatty acids, polypeptides and polysaccharides (17, 18).

Sharp (38) has recently proposed that the excretion of ECP by species of *Thallasiosira* is normally limited to old or physiologically stressed cells. Crashes of *Thallasiosira* were observed when the cultures were in late exponential or stationary phase of growth. Since the culturing of algae for mariculture at the University of Delaware is thus far limited to batch cultures, which differ significantly from natural populations (25), the aging of the culture may play an extensive role here not seen in nature.

The role of ECP in aquatic systems has not been established clearly. Many workers have implied that this material is utilized by bacteria as a supplemental food source (24, 28). Such inferences have usually been based on the failure of researchers to find increasing concentrations of ECP associated with algal blooms (17, 24).

Herbland (23) has recently demonstrated an association between ^{14}C-labeled ECP and bacterial populations in marine samples. Our data also indicate that such an association between the ECP from *T. pseudonanna* and the pseudomonad T827/2B does occur. This association between the ECP and the bacterial cells does not unequivocally indicate utilization of ECP by the bacterium. Additional support for the theory that T827/2B utilizes the algal ECP comes from the inability of the bacteria to survive in f/2 medium unless *T. pseudonanna* is also present. The presence of the algae in some way conditions the medium to allow for the growth of T827/2B.

ACKNOWLEDGMENTS

We thank Pamela Franklin and Benjamin Blutstein for their invaluable assistance.

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