STUDIES OF FLAVOBACTERIUM PISCICIDA BEIN
I. GROWTH, TOXICITY, AND ECOLOGICAL CONSIDERATIONS

SAMUEL P. MEYERS, MORRIS H. BASLOW, SELWYN J. BEIN, AND C. EDITH MARKS

The Marine Laboratory, University of Miami, Miami, Florida

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Species of Flavobacterium are abundant in marine environments, occurring in sea water and sediments as well as in the slime and feces of various fish. The activity of flavobacteria in fish spoilage is not uncommon (Castell and Mapplebeck, 1952). Other than the studies of these latter workers, only the investigations of MacLeod and his associates (MacLeod et al., 1954, 1958) deal, to any extent, with the physiology and nutrition of marine flavobacteria.

In the latest treatment of Flavobacterium in Bergey's Manual of Determinative Bacteriology (Breed et al., 1957) 26 species are recognized, 13 of which occur in marine habitats. Eight species have been described originally from marine material, although only one, Flavobacterium piscicida Bein, has been diagnosed as toxic to fish.

F. piscicida was collected in 1951 from White-water Bay on the southwest coast of Florida, during an outbreak of mass mortality of fishes, commonly known as "red tide." Bein (1954) described the bacterium as a new species, and demonstrated its toxicity in laboratory tests. Since 1952, in conjunction with field investigations of marine fish mortalities, personnel of The Marine Laboratory have collected numerous flavobacteria from sea water and marine muds at areas along the west coast of Florida as far north as Apalachicola. The majority of the collections were made in inshore localities over an extensive geographic range, in phytoplankton blooms, with and without accompanying fish mortality; and in routine bacteriological analyses.

This present paper reports preliminary studies undertaken during the past year to investigate the physiology of F. piscicida and to establish the parameters of its toxicity. Included in our program is a study of possible relationships between the nutrition of F. piscicida and its role in fish mortalities and in general marine biological phenomena. The toxic properties of this species, together with its wide distribution in marine localities, suggest that the organism may be valuable in studies of the complex factors initiating fish mortalities and algal blooms.

MATERIALS AND METHODS

Cultures. F. piscicida Bein and other flavobacteria studied were taken from the microbiological culture collection of The Marine Laboratory. The isolates of flavobacterium, tentatively identified as strains or variants of F. piscicida, were collected at marine localities on the west coast of Florida, including San Carlos Bay, Pine Island, Captiva Island, Charlotte Harbor, Gasparilla Island, Venice and Tampa Bay.

Cultures were maintained on sea water agar slants and in broth, with 1.0 per cent peptone (Difco) as the sole added nutrient. Subcultures were made monthly.

Inoculum and growth conditions. The inoculum consisted of cultures, 2 to 5 days old, grown in 1 per cent peptone sea water broth. Approximately 0.1 ml of cell suspension was used for each inoculation. Control flasks, used in all of the growth tests, indicated that the amount of nutrients transferred with the inoculum was insufficient to affect the final turbidimetric readings. Unless noted otherwise, the peptone used in this work was specifically the Difco product.

The sea water for the stock cultures and experimental studies was taken from the Gulf Stream, filtered through an M-sintered glass funnel, and stored in 5-gallon carboys in the dark. No appreciable variation in total bacterial growth was noted when either aged (6 months or longer) or fresh sea water was used in the culture medium. Other than in certain designated
tests, peptone broth media were prepared with Gulf Stream sea water.

The majority of the growth determinations were made in 125-ml Erlenmeyer flasks containing 50 ml of the appropriate test medium. Cultures of flavobacteria used for toxicity tests were grown in 1-L Erlenmeyer and 2-L Fernbach flasks, with 200 and 500 ml, respectively, of 1.0 per cent peptone broth. The inoculum consisted of 10 and 20 ml, respectively, from stock broth cultures. These mass cultures were allowed to grow for 5 to 9 days before being tested. Nutritional and growth response experiments were checked daily and read usually at 60 to 65 hr. The pH of media used was adjusted to the range 7.0 to 7.3. Except for specific temperature experiments, cultures were incubated at laboratory room temperature, 27 to 31 C. During winter months, a 30 C incubator was used.

**Measurement of growth.** Growth response was measured with a Bausch and Lomb Spectronic 20 colorimeter, set at 600 mč. Individual cultures were mixed thoroughly in a Waring Blender prior to each reading, because of the characteristic heavy pellicle which formed. Readings are expressed as per cent transmittancy (T), using the sterile medium as the reagent blank. Counts of the bacterial population at the different turbidimetric readings were made by the drop plate method (Reed and Reed, 1948).

**Poeciliid bioassay and preparation of toxic fraction.** A species of poeciliid top minnow, Gambusia sp., was selected as the bio-assay animal to determine the toxicity of the bacterial preparations. These fish, in addition to being small and plentiful, are quite hardy and able to tolerate readily the brackish water used to prepare the various toxic solutions.

One fish was used for each test, and most tests were run at least in duplicate. Female fish were used in the majority of our studies, since it was found that these are more hardy, and thus, gave a better evaluation of the potency of the toxin. The response of the animal was recorded in terms of the total time elapsed until the end point was reached. In these assays, the end point was established as the first convulsive state, immediately after which the fish loses complete control of its body movements.

The cultures used for the toxicity studies were mixed in a Waring Blender to break up the heavy orange pellicle. In some tests, cell-free filtrate was used, in which the homogenate was filtered successively through a no. 015 Selas filter candle and a no. 03 candle. The latter filter is rated as having a maximal pore radius of 0.60 μ. Aseptic transfer of drops of the clear filtrate to nutrient agar showed it to be bacteria free. In other experiments, the unfiltered bacterial culture was used. The toxicity tests were run in small aquaria containing 50 ml of 50 per cent sea water, to which was added 20 per cent by volume of either the bacterial filtrate or the untreated culture.

**Partial characterization of toxin.** Two duplicate samples, each 300 ml of cell-free filtrate, were placed in dialyzing tubing for 12 hr. One tube was suspended in the laboratory before an electric fan, and thus subjected to concentration by an evaporation process. After 12 hr, the volume of the sample was reduced to 170 ml. A second tube was placed in a closed container of distilled water. The distilled water was not changed during the dialysis process. Further treatment of this second sample consisted of vacuum distillation of the distilled water fraction at 60 C. The distillates were collected in a vessel cooled to approximately 0 C to −1 C. The toxicity of the dialyzates and distillates was tested with the poeciliid bio-assay.

**Salinity.** One per cent peptone broths of different salinities were prepared in which the proportions of distilled water and sea water were regulated to give the desired salinity. Salinities above 100 per cent sea water were obtained by an evaporation process at 70 to 80 C. To examine the effect of temperature on the salinity optima, a series of different salinities were run, with the temperature varied from 20 to 32 C.

**Artificial sea water.** In addition to Gulf Stream sea water, certain tests were conducted with an artificial sea water of the following composition: NaCl, 30 g; MgCl₂·6H₂O, 2.0 g; KCl, 500 mg; CaCl₂, 400 mg; KH₂PO₄, 10 mg; FeSO₄, 10 mg; trace metals, 30 ml (containing Co⁺², 0.1 mg per cent; Zn⁺², 0.5 mg per cent; Mn⁺², 5.0 mg per cent; Cu⁺², 0.1 mg per cent; all added as the Cl⁻ salt); and distilled water to make 1000 ml.

**RESULTS**

**General growth conditions.** *F. piscicida* exhibits excellent growth and pigmentation in 1.0 per cent peptone media without added carbohydrate. The development of the bacterium is correlated directly with the concentration of peptone in the medium. A compact pellicle, varying in color from yellow to deep orange, is characteristic of
growth of the organism in 0.2 to 1.0 per cent peptone broth.

The growth of *F. piscicida* was examined in media containing proteinaceous compounds other than peptone (Difco). The nitrogen sources used were tryptone, proteose peptone, yeast extract, trypicase, casamino acids, animal protein factor, urea, beef extract, and polypeptone, all prepared at a 0.1 per cent concentration in sea water. Although growth occurred on all of these substrates, except urea, only the proteose peptone and polypeptone supported the typical pigmentation of the organism. Bein (1954) observed that *F. piscicida* develops readily, with strong pigmentation, on such proteinaceous material as fish flesh, marine snails, and concentrated mixed plankton.

In the growth of the bacterium, toxicity developed in 1.0 per cent peptone broth, but was not detected in cultures grown in 0.1 per cent peptone concentration. Ray and Wilson (1957), in laboratory tests of *F. piscicida*, failed to duplicate the earlier work of Bein in which the toxicity of the organism was established initially. However, this may be due in part, or entirely, to their use of 24-hr cultures grown in 0.1 per cent peptone concentration.

*Salinity and phosphate tolerance.* Twenty-four-hr cultures of *F. piscicida* exhibited approximately equal amounts of growth at salinities from 30 to 100 per cent sea water, whereas 65-hr cultures showed equal growth from 15 to 200 per cent sea water (figure 1). In 24-hr cultures, growth is inhibited below 30 per cent sea water, whereas in older cultures, a salinity tolerance of 10 to 15 per cent sea water is evident.

Pigment production also increases directly with the salinity of the culture medium, from nonpigmented growth at 30 per cent sea water through gradations of yellow and yellow-orange pigmentation at sea water concentrations from 30 to 200 per cent.

Subminimal amounts of phosphate, rather than salinity directly, may inhibit the growth of 24-hr cultures at 30 per cent sea water. An addition of 2 mg per cent each of K$_2$HPO$_4$ and KH$_2$PO$_4$ to the 30 per cent sea water flasks stimulated as much growth as occurred in the flasks of 60 per cent sea water. The addition of the phosphate salts to the 20 per cent sea water flasks gave only a slight growth stimulus, whereas similar amounts of phosphate added to the 0 to 10 per cent sea water flasks had no effect on growth.

The salinity response was similar over the entire temperature range (20 to 32 C) tested. No shift in salinity optima with different temperatures was observed as is reported for certain isolates of marine fungi (Ritchie, 1957).

*Temperature and pH.* *F. piscicida* grows well over a range of temperatures from slightly below 20 to 35 C. The upper temperature limit has not been established. While good growth occurs from 20 to 35 C, optimal growth and pigmentation is around 30 C. Below 20 C, development is poor, nonpigmented, and distributed throughout the culture broth. This distributed growth is characteristic of shake cultures of the organism. At 25 C, a yellow pigment is present, whereas at 30 and 35 C, most of the bacterial growth occurs in a compact, deep orange pellicle. *F. piscicida* tolerates a wide pH range, from 6.2 to 8.7 with the optimal growth occurring under slightly alkaline conditions.

*Response to calcium.* CaCl$_2$, adjusted to give 0 to 400 mg per cent Ca$^{2+}$, was added to artificial sea water containing 1 per cent peptone, in concentrations of from 0 to 400 mg per cent Ca$^{2+}$. The normal concentration of Ca$^{2+}$ in sea water is approximately 40 mg per cent.

Development of the bacterium was correlated
with the level of Ca++, increasing proportionally over the entire range tested. An increase in pigmentation also was associated with more vigorous growth at the higher calcium concentrations. The development of the organism in peptone broth cultures is characterized by an encrustment of CaCO₃ along the sides of the vessel at the periphery of the pellicle.

A spectrographic analysis, using a Bausch and Lomb Dual Grating Spectrograph, of cells of *F. piscicida*, grown in 1.0 per cent peptone broth, showed a concentration of calcium 100 to 1000 times that present in the cell-free effluent.

Toxicity tests and description of syndrome. Using the poccilid bio-assay, we have found that the toxin either is absent or present in sublethal concentrations during the initial development of the organism. After approximately 5 days, the toxin is present in appreciable quantities and increases thereafter. Cultures 6 to 8 weeks old still exhibit toxicity, approximating that of 1-week cultures. The toxin does not accumulate in the medium of the older cultures. The relationship between pH, number of viable cells, and toxicity in min, during a 9-day growth period, is recorded in table 1.

Other cultures of *F. piscicida* tested after 8 days of growth showed a toxicity end point of 5 min. All 10 of the flavobacteria examined exhibited toxicity, with an end point of from 14 to 26 min. The average end point was about 15 to 16 min. The amount of growth of these isolates in the 1.0 per cent peptone broth was approximately equal.

Similar tests of cultures of nonchromogenic marine bacteria failed to demonstrate toxicity.

### Table 1

<table>
<thead>
<tr>
<th>Age of Culture (days)</th>
<th>pH</th>
<th>No. of Cells (× 10⁵)</th>
<th>Toxicity or End Point (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.2</td>
<td>17.5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>7.4</td>
<td>98.5</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>7.6</td>
<td>172.0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>7.6</td>
<td>220.0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>7.7</td>
<td>1,774.0</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>7.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>7.9</td>
<td>10,400.0</td>
<td>15.5</td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>8.0</td>
<td>12,000.0</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Controls of 1.0 per cent peptone, and 50 per cent sea water in 50-ml aliquots, with and without artificial aeration, were nontoxic to the fish.

Figure 2 notes the response of serial dilutions of the filtered media on *Gambusia* sp., using the convulsive state as the end point. In figure 2, the per cent of toxic material was prepared in the following manner, using 20 per cent concentration as an example: (a) 10 ml of filtrate, (b) 15 ml of 100 per cent sea water, and (c) 25 ml of distilled water, giving a total concentration of 50 per cent sea water.

The concentration of the toxic material is proportional to the end point. Previous tests have shown that the minimal end point is reached at 50 per cent concentration, and even at 100 per cent level, the end point is not less than 4 to 5 min. It is possible that this represents the minimal time required for the toxin to penetrate the gill membrane and affect the neurological processes of the fish.

Although the exact nature of the toxic material has not been established as yet, the activity syndrome suggests that the central nervous system of the fish is affected (Cutting et al., 1959). The characteristic syndrome may be described in the following sequence: (a) onset 2 to 30 min, followed by apparent loss of some senses, al-

![Figure 2. Toxicity of serial dilutions of Flavobacterium piscicida to Gambusia sp.](http://jb.asm.org/ on September 23, 2017 by guest)
though swimming and equilibrium are maintained; (b) aimless swimming, usually in small circles, with a rising to the surface of the aquaria; (c) disturbance of equilibrium, followed by spasms, convulsions, and tetanic bending of the trunk; (d) paralysis and resultant death. Affected fish may be revived from the early convulsive state if placed in uncontaminated brackish water. Recovery occurs usually in 10 to 30 min.

The similarity between the action of this toxic material and that of some compounds with tranquilizing activity is striking in that a fish removed from the test aquaria during the confused state will remain for some time sufficiently docile to be handled easily. Coordination is apparently normal and the animal cannot be differentiated visually from those in the control tanks.

**Nature of the toxin.** The sample, concentrated by exposure of the dialyzing tube to evaporation in the laboratory for 12 hr, was no longer toxic. Apparently, the toxic component is a small molecule and volatile, being able to pass through the walls of the dialyzing tube. The duplicate sample, dialyzed in distilled water for 12 hr, showed toxicity in the fraction both within and outside the dialyzing tube, thus confirming the observation that the toxin is a small molecular weight compound.

The latter dialyzed fraction was vacuum distilled, and the first distillate, which came over at 46°C, contained the toxic material. A second distillate, as well as the residual material, was nontoxic. The presence of \( \text{NH}_3 \) was detected in the toxic material. The first distillate, pH 9, was neutralized with dilute HCl to pH 7 to determine if the \( \text{NH}_3 \) was the toxic entity. The neutralized material was still toxic. As a control, a sample of distilled water was brought to pH 8 with \( \text{NH}_4\text{OH} \) and tested for toxicity, with negative results. The toxin is stable when kept in either fresh water or sea water in the freezer compartment of a refrigerator.

**DISCUSSION**

Although more than half of the species of bacteria in the sea are chromogenic (Zobell, 1946), a paucity of information exists concerning their nutritional requirements and relationships to other marine organisms. Such knowledge is essential to a better understanding of the mechanisms of marine ecology, and especially, of the dynamics of epiphytotics in the sea.

The characteristics of catastrophic outbreaks of fish mortality, commonly known as "red tide," have been well described (Gunter et al., 1948). Along the west coast of Florida, a species of algal flagellate, *Gymnodinium brevis* (Davis, 1948), has been designated the causal organism of such outbreaks in that area. Other than the work of this laboratory and that of Ray and Wilson (1957), studies of the occurrence and activity of bacteria in mortality of fishes are notably absent. In view of the ubiquitous nature of bacteria, a consideration of their possible participation in such phenomena is pertinent. The toxicity of *F. piscicida*, and its occurrence and "bloom" in red tide areas, warrants an evaluation of the salient ecological features of this marine bacterium.

*F. piscicida* is a common benthic species along the west coast of Florida, tolerating a wide range of salinity. Temperatures of optimal growth in laboratory cultures, 28 to 30°C, are not uncommon during the summer in the coastal areas where the bacterium has been collected. Development of the organism is stimulated greatly by increased concentrations of phosphate and calcium. Indeed, the ability of *F. piscicida* to utilize and concentrate large amounts of calcium suggests that the species may be active in marine sedimentary processes. Thus, this bacterium is well adjusted for growth in the shallow, brackish, warmer waters in areas where land runoff of inorganic nutrients, especially phosphate, is common.

Toxicity tests indicate that the effect of *F. piscicida* on fish is quite similar to that of a neuromuscular toxin. The presence of compounds with potential pharmacological application has been demonstrated in various phyla of marine animals (Halstead, 1956), and, more recently, in antibiotic-producing microorganisms in sea water and in marine sediments (Grein and Meyers, 1958). *F. piscicida* and other marine flavobacteria may provide additional sources of interesting, biologically active, compounds.

Contrary to the report by Wood (1958), no attempts have been made to determine whether *F. piscicida* causes toxicity in shellfish, and therefore the statement "this toxicity affects the humans who eat these shellfish" is without support. However, the possible activity of a toxic bacterial flora in various types of fish poisoning, such as ciguatera (Randall, 1958), merits consideration. The latter worker discusses benthic
microorganisms, including bacteria, as possible producers of the toxic material.

To date, our nutritional tests, including a step-wise replacement of the peptone in the sea water media, indicate that *F. piscicida* is a fastidious species, and requires some component or components of peptone for optimal growth and toxin production. MacLeod et al. (1958), in studies of a marine species of *Flavobacterium*, noted a similar complexity in growth requirements.

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SUMMARY

Growth studies of *Flavobacterium piscicida*, including temperature and salinity optima, and response to Ca$^{++}$ and PO$_4$$^{3-}$, support the ecological evidence for the well adjusted euryhaline nature of this marine species. A standard bio-assay method, using poeciliid fishes as test organisms, has been developed to ascertain the toxicity of the bacterium. The biological implications of the toxicity and growth characteristics of the organism are considered, especially in relation to its role in mortality of fishes, commonly known as “red tide.”

REFERENCES


