GROWTH CHARACTERISTICS OF VIBRIO PARAHAEMOLYTICUS

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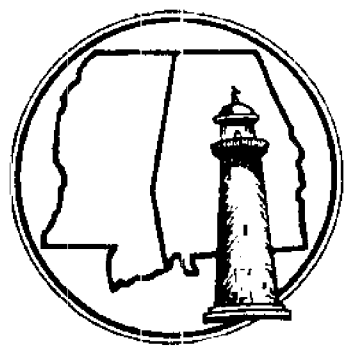

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INTRODUCTION

Vibrio parahaemolyticus is a normal inhabitant of the coastal marine environment and may be seen associated with human disease as well as pathogenic conditions of fish and shellfish. Factors effecting the virulence of the organism influence the "die off" during food processing, the toxin levels in consumer products, and the ability of the bacteria to produce tissue damage to fish in normal populations. Accumulation of data on optimal growth conditions for both organism multiplication and toxin production may help correlate estuary conditions to massive fish infections during the year.

One specific example of pathogenic effects on fish is fin rot. This disease is common in mullet found off the Mississippi Gulf Coast, and appears to vary in its severity from season to season as well as geographically. The overall contributions of pollution to this disease may indicate either effects on the organism or its toxin producing potential or an increased host susceptibility due to stress in polluted waters.

This study was designed to consider only the effect of oil on survival of the bacteria under varying conditions of temperature and pH, as well as a crude assay on cytoxicity of *Vibrio* toxin under *in vitro* testing conditions.

MATERIALS AND METHODS

Media

a. Trypticase Soy Agar (Broth) with 3% NaCl (TSA) (TSB)

Ingredients:

Phytone BBL	5.0 g
Trypticase BBL	15.0 g
NaCl	30.0 g
Agar	15.0 g
Bile Salt #3	1.0 g
H_2O	1.0 liter

b. Mineral Salt Medium

Ingredients:

1.0 g KNO_3

0.38 g K_2HPO_4

0.29 g $MgSO_4.7H_2O$

0.05 g $FeCl_3.6H_2O$

1.0 l H_2O

c. Oil Medium

Ingredients:

1.0 liter Mineral Salt Medium

10 ml oil (Empire Mix Crude)

Boil 15 minutes before autoclaving

Organism

Vibrio parahaemolyticus ATCC 17750 was obtained from American Type Culture Collection and propogated routinely on TSA.

Mammalian Cells

Porcine kidney cells (PK-15) were obtained from the Veterinary Diagnostic Laboratory and maintained in Eagles Medium.

Cell Culture and Cytotoxicity Assay

PK-15 cells were propogated in 75 cm^2 Falcon Tissue Culture Flasks as monolayer cultures. The cells were subcultured by trypsinization using 0.25% trypsin in phosphate buffered saline (PBS). Cell counts were carried out with a hemocytometer, and 5 x 10^6 viable cells were used for each treatment. A constant volumn of medium (Eagles Medium + 5% Calf serum) containing varying amounts of culture supernatant was incubated with cells for 2 hours prior to viability counting. After incubation, the cells were removed from mololayer, mixed with Eagle's medium + 5% calf serum, diluted 1:10 with PBS + .1% neutral red, and viability determined.

Cytotoxic Supernatant

PBS was added to 24 hour TSA slants of Vibrio parahaemolyticus, and the cells aspirated and suspended. One ml of this supernatant was used to innoculate 50 ml of either minimal salts medium + 3% NaCl + 1 g glucose/liter, or oil medium + 3% NaCl. These cultures were incubated for 24 hours at 25^0 C, dialysed for 24 hours with PBS, and filtered through 0.22 μ Millipore filters. This supernatant was then diluted with Eagle's medium and tested for cytotoxicity.

Heat Inactivation

Vibrio parahaemolyticus was grown in TSB for 24 hours at 25^0 C, and serial dilutions of 10^{-1}, 10^{-2}, and 10^{-3} were made in PBS. These dilutions were atempered at 60^0 C for 5 minutes and the time 0 sample

taken for innoculation of fresh medium. Successive 10, 20, and 30 minute samples were then taken, loop transferred to fresh TSB, and incubated for 24 hours at 25^{0} C. Growth was noted by simple observance of turbulance in the innoculated tubes.

pH

TSB was adjusted with 0.1 N NaOH or 0.1 N HCl to pH ranges from 1 to 10. Fresh 24 hour cultures of bacteria were then used to loop innoculate each of the 10 tubes. These cultures were then incubated for 24 hours at 25^{0} C and turbulence was noted in each.

RESULTS AND DISCUSSION

Heat inactivation studies indicated that cells grown in TSB were somewhat more resistant to heat inactivation than were cells grown in oil medium (TABLE 1, 2). Plate counts were not done on the 24 hour seed cultures to determine viability so the actual bacterial viable number for the diluted cultures is not known. This could explain the phenomenon if oil medium resulted in drastically lower viable organisms prior to incubation at 60^{o} C. One would actually expect that the oil grown cells would be more resistant to heat inactivation due to an insulating effect of crude oil. If the organism is "coated" with the nonpolar hydrocarbon, the diffusion of polar nutrients into the cell may also retard growth in short term culture. Any future experiment might warrant longer circulation time to test viability in the more dilute suspensions.

The effect of pH on Vibrio parahaemolyticus indicates a rather broad range of capability with only a small difference in the lower limits for both cultures. At pH's of less than 5, no growth could be detected, but the upper limit of 10 showed good growth in all experiments (TABLE 3, 4).

Studies on the cytotoxicity of cell supernatants on mammalian cells indicates that both supernatants from 24 hour cultures are cytotoxic to mammalian cells under the conditions employed (TABLE 5). If, in fact, the viable bacterial count is less in oil medium-grown cells, the cytotoxicity study indicates that a more potent toxicity for PK-15 cells exists in the supernatant from this culture. There is a clear indication that supernatants from cells grown in glucose-mineral salts medium, even

TABLE 1. Heat Inactivation of TSB Grown Cells[c]

Incubation Time[a]	Dilutions[b] 10^{-1}	10^{-2}	10^{-3}
0 min.	+	-	-
10 min.	+	-	-
20 min.	+	-	-
30 min.	+	-	-

[a]Time "0" is taken after 5 minutes atempering at 60° C.

[b]All dilutions were made in TSB.

[c]The results are a composite from 4 separate experiments.

+ = growth, - = no growth

TABLE 2. Heat Inactivation of Oil Medium Grown Cells[a]

Incubation Time	Dilutions 10^{-1}	10^{-2}	10^{-3}
0 min.	+	-	-
10 min.	-	-	-
20 min.	-	-	-
30 min.	-	-	-

[a]Above legend applies.

+ = growth, - = no growth.

TABLE 3. pH Tolerance of Cells Grown in TSB[a]

pH of TSB	Growth
1	-
2	-
3	-
4	-
5	-
6	+
7	+
8	+
9	+
10	+

[a]See MATERIAL AND METHODS.

+ = growth, - = no growth

TABLE 4. pH Tolerance of Cells Grown in Oil Medium[a]

pH of TSB	Growth
1	-
2	-
3	-
4	-
5	+
6	+
7	+
8	+
9	+
10	+

[a]See MATERIALS AND METHODS.

+ = growth, - = no growth.

TABLE 5. Cytotoxicity of Culture Supernatant

Medium[a]	Volume of Supernatant[b] 10 µl	100 µl	500 µl	1 ml	2 ml	3 ml
Glucose + Mineral Salts	4.5×10^6(c)	1.5×10^5	10^3	-	-	-
Glucose + Mineral Salts Control	3.0×10^6	2.8×10^6	2.9×10^6	3×10^6	4×10^6	3×10^6
Oil Medium	5.0×10^5	10^3	-	-	-	(cont)[d]
Oil Medium Control	4.0×10^6	3×10^6	2.5×10^6	2.9×10^6	2.0×10^6	1.8×10^6

[a]All control cultures were treated exactly as were innoculated cultures.

[b]Dialysed supernatant was added in a total of 5 ml with Eagles Medium.

[c]All numbers represent viable cells after 2 hours incubation of 5×10^6 cells.

[d]Contaminated.

though they are more resistant to heat inactivation, are less cytotoxic *in vitro* than parallel oil-grown cells. If these studies could be confirmed, this enhanced cytotoxicity on cell cultures may mimic the *in vivo* occurrence of rapidly spreading fin rot in fish populations grown in the presence of added hydrocarbons. Such an observation would certainly indicate a higher risk for fish populations in areas where water is contaminated with oil or other hydrocarbons.

The danger of food poisoning is not as great in this country as in others where raw fish is consumed almost daily, however, it is becoming more apparent in oyster, shrimp, and other shellfish from Gulf Coast estuaries. If conditions in the harvest areas result in higher levels of toxin production, the danger might be directly proportional to levels of hydrocarbons in the water. Such an obervation could shed some light on the relationship between the food-born illness in man and environmental conditions which enhance pathogenicity of the organism. Although these results have not been confirmed by further testing, we feel that they warrant further examination.