A Pigment-Producing Pseudomonad Which Discolors Culture Containers of Embryos of a Bivalve Mollusk

CAROLYN BROWN
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Middle Atlantic Coastal Fisheries Center
Milford, Connecticut 06460

ABSTRACT: A recurring pink discoloration found at the bottom of polyethylene buckets containing embryos of hard clams (Mercenaria mercenaria) is produced by a red-pigmented pseudomonad. The pink discoloration itself is not a problem, but the number of bacterial cells present is cause for concern. This study showed that at concentrations below $10^5$ cells per milliliter of culture of clam embryos the bacterium could produce the discoloration without being detrimental to the clams. Above this concentration, however, either embryonic development decreased or mortality occurred. Chloramphenicol and neomycin were used effectively in preventing the growth of the pigment-producing bacterium and the concomitant adverse effects on clam embryos.

Introduction

Although many advances in culture techniques have been made and it is now feasible to rear some species of bivalve mollusks commercially, problems still arise in both research and commercial shellfish hatcheries. One significant problem is the high incidence of fatal epizootics among larval and juvenile mollusks (Tubiash, Chanley, and Leifson, 1965). Two genera of bacteria, Pseudomonas and Vibrio, have been identified as bivalve pathogens (Guillard, 1959; Tubiash et al., 1965). This study was prompted by the periodic finding, at the Milford laboratory, of a pink discoloration at the bottom of polyethylene buckets containing embryos and larvae of bivalve mollusks. The discoloration has been found both in cultures of clams, Mercenaria mercenaria, and oysters, Crassostrea virginica.

This study was undertaken to determine the cause of the discoloration, whether it was harmful to clam embryos and, if so, what control measures could be employed.

Materials and Methods

ISOLATION AND IDENTIFICATION OF A PIGMENT-PRODUCING BACTERIUM

The organism was isolated from a 15-liter polyethylene bucket which had contained 1-day-old clam larvae. The pink discoloration was wiped with a sterile swab and the swab streaked on plates of an isolation medium consisting of 0.1% yeast extract (Difco), 0.1% trypticase (BBL), 1.5% agar (Difco), and aged, Millipore membrane-filtered seawater. The plates were incubated at 22–24 C for three days after which time the suspected colony was subcultured on plates of an isolation medium consisting of 0.1% yeast extract (Difco), 0.1% trypticase (BBL), 1.5% agar (Difco), and aged, Millipore membrane-filtered seawater. The plates were incubated at 22–24 C for three days after which time the suspected colony was subcultured in seawater broth containing 0.1% yeast extract and 0.1% trypticase in aged, membrane-filtered seawater. The procedures employed by Murchelano and Bishop (1969) were used to determine morphological and physiological characteristics and the bacterium was identified to genus using the taxonomic scheme of Murchelano and Brown (1970).

TEST FOR DISCOLORATION

The ability of the suspected bacterium to discolor two types of containers, 15-liter polyethylene buckets and 1-liter polypropylene...
beakers, was tested using cultures of clam embryos. Each culture contained approximately 30 embryos per milliliter of filtered, ultraviolet-treated seawater. The cultures were maintained in a constant temperature water bath at 26°C for two days. Duplicate cultures were used for each experiment, as well as for the untreated controls. The culture containers were checked for the presence of the stain after 24 and 48 hours.

The ability of the suspected bacterium to cause the pink discoloration was tested in four different ways: broth culture, broth culture filtrate, washed bacteria, and heat-killed bacteria.

**BROTH CULTURE.** Inocula of 1.0, 0.5, 0.1, and 0.01-ml of a 24-hour broth culture were used per liter of culture of clam embryos.

**BROTH CULTURE FILTRATE.** Fifty milliliters of a 24-hour broth culture were centrifuged at 5,500 RPM for 6 minutes. The supernatant was decanted and the bacteria re-suspended in 50 ml of sterile seawater. The supernatant was then membrane-filtered and 1.0 ml was inoculated per liter of culture.

**WASHED BACTERIA.** The bacteria were centrifuged at 5,500 RPM for 6 minutes and twice re-suspended in 50 ml of seawater. The washed bacteria were then re-suspended with a vortex mixer to break up clumps and 1.0 ml was added to each liter of clam culture.

**HEAT-KILLED BACTERIA.** A 24-hour broth culture was heated at 65°C for 30 minutes, cooled to room temperature and 1.0 ml added per liter of clam culture.

**TEST FOR PATHOGENICITY**

Following the procedures described above, the effects of the bacterium upon the development of clam embryos were determined in 1-liter beakers containing 30 embryos per milliliter. Each experiment was conducted either in duplicate or quadruplicate with similar controls. After 48 hours the clam embryos, which had developed into larvae, were collected on a 36µ Nitex screen, re-suspended and a representative aliquot obtained from each beaker and fixed with 5% neutral formalin. The samples were examined with a compound microscope in a Sedwick-Rafter counting cell and the number of live-normal, dead-normal, live-abnormal, and dead-abnormal larvae counted.

**BACTERIAL COUNTS**

For determination of bacterial numbers, 1.0 ml of a 24-hour broth culture was used to prepare dilutions of $10^{-5}$, $10^{-6}$, and $10^{-7}$ in sterile, membrane-filtered seawater; 0.1 ml of the three dilutions was then plated on seawater agar plates with an alcohol-flamed glass rod. The plates were incubated at room temperature for one week and counted using a Quebec colony counter.

**EFFECTS OF ANTIBIOTICS**

Sensitivity tests with chloramphenicol (Sigma) were performed using 50 mg of antibiotic per liter of clam culture and 0.5 ml of a 24-hour bacterial broth culture per liter; sensitivity tests with neomycin (Sigma) were conducted with 100 mg of antibiotic per liter of culture. Duplicate or quadruplicate cultures were used for each antibiotic, as well as for the controls.

To determine the time required for each of the two antibiotics to suppress the growth of the test organism, four beakers were filled with 1.0 liter of filtered, uv-treated seawater containing 30 embryos per milliliter and the following additions made, using the concentrations described above: bacteria and chloramphenicol, bacteria and neomycin, bacteria, and untreated controls. Every hour for 8 hours samples were taken of each of the beakers and plated on seawater plates, using dilutions of $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$. The plates were incubated for a week at room temperature, colonies counted, and their morphology noted.

**Results**

**DISCOLORATION**

Plates streaked from discolored areas of the polyethylene buckets showed large numbers of red-pigmented bacterial colonies. From the initial tests, it was found that 1.0 ml of a 24-hour broth culture of the red-pigmented bacterium contained $1.2 \times 10^8$ cells per milliliter and when 1.0 ml was added to a 1 liter clam culture, the polyethylene buckets became discolored. The same concentration of washed bacteria also discolored the polyethylene buckets. It was found, however, that neither the broth culture nor the washed bacteria was capable of discoloring the polypropylene beakers. Further testing showed that as few as