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A *Pseudomonas* associated with disease in cultured rabbitfish *Siganus rivulatus* in the Red Sea

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ABSTRACT: Experiments were carried out to study the cause of a disease outbreak among rabbitfish *Siganus rivulatus* in the Saudi Arabian mariculture facility in the Red Sea and to investigate means of protecting fish against the disease. The causal agent of the disease proved to be a bacterium that was identified as *Pseudomonas putrefaciens*. When the bacterium was injected into healthy fish, it resulted in high mortality and produced the same clinical signs as those observed during the disease outbreak. A bacterin prepared by formalin killing was used to vaccinate fish. Fish vaccinated once by intraperitoneal injection suffered 17 % less mortality than control fish when challenged with the homologous bacterium. Fish vaccinated twice by the same method suffered 40 % less mortality than control fish. Fish were also vaccinated by direct immersion in the bacterin but when these fish and their unvaccinated counterparts were challenged by dipping into a suspension of the homologous bacterium they did not show any clinical signs nor did any mortality occur. The disease was thought to be stress-related.

INTRODUCTION

Rabbitfish *Siganus rivulatus* are indigenous to the Indo-Pacific and now occur in the Red Sea and Eastern Mediterranean, where they are sought by fishermen and anglers because they are readily accepted by consumers. They are herbivorous but grow well on a low protein diet (Popper & Gunderman 1975) and tolerate culture conditions (Thibaiti et al. 1984). Because of these characteristics, the Saudi Arabian Ministry of Agriculture and Water Resources has selected rabbitfish for initiation of a mariculture program in the Red Sea.

During spring 1985 a disease outbreak occurred and resulted in a high mortality in the sea cages. Following the initial epizootic, cases of the disease have frequently being diagnosed, the fish manifesting well-defined clinical signs (mentioned below). Primary isolation from all diseased fish resulted in substantial growth of bacteria whereas no parasites were evident on any fish. Bacterial infection was therefore thought to account for the disease.

This study was undertaken to identify the responsible bacterium and to investigate the possibility of immunizing fish against it.

MATERIALS AND METHODS

Fish serving as the source of the pathogen were obtained as diseased specimens from the mariculture facility. The chief clinical signs were discoloration, hemorrhagic necrosis on the body and mouth, frayed fins, and exophthalmia. All specimens were moribund and were extremely lethargic.

Bacterial isolates were obtained from liver, kidney, and spleen on Brain Heart Infusion Agar (BHI agar) containing 3 % salt (NaCl). Subcultures of the predominating bacterium were made on the same medium and were kept for further investigations. Maximum and minimum tolerance limits as well as optimum growth conditions for salinity, temperature, and pH were determined. Biochemical characteristics of the bacterium were determined at 35 °C using API 20 E according to the manufacturer's instructions (Analytical Profile Index 1983), as well as at 22 °C. All tests were also supplemented by standard tube and other

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tests including Gram stain and growth on McConkey agar.

To investigate the ability of the bacterium to produce disease in healthy fish, a saline suspension of the organism, equivalent to McFarland no. 6 turbidity standard, was inoculated intraperitoneally into healthy fish (1 ml fish^{-1}). Control fish were injected with 1 ml of sterile saline. Average weight of fish used was 50 g and all fish were held in fiberglass tanks supplied with continuous flowing seawater and aeration. Dead fish were removed, necropsies were aseptically performed, and bacteria were isolated from liver, kidney, and spleen on plates of salt-supplemented BHI agar. Bacterial identification was carried out as previously described.

To vaccinate fish, a bacterin was prepared by formalin-killing washed cells of the bacterium, harvested from BHI culture broth. The procedure of Saeed & Plumb (1986) was used. The bacterin was freeze-dried until used.

Fish for the vaccine experiments were caught by fish traps in the Red Sea. Their average weight was 60 g. Fish were acclimated to 600 l fiberglass tanks supplied with continuous flowing, aerated seawater. The water had salinity 38 ‰, pH 8.2, and temperature between 29 and 31 °C. Fish were fed a commercial sinking-type pelleted diet containing 30 % protein.

Fish were vaccinated by intraperitoneal injection or by direct immersion in the bacterin. Fish were intraperitoneally injected with either a single dose of bacterin (0.2 mg dry weight in 1 ml of 1 % saline) or with 2 doses of the bacterin (booster injection). For single injection, 3 groups of 30 fish each were used. Twenty of each group received the bacterin while the other 10 fish of each group were used as controls. Control fish were given an intraperitoneal injection of 1 ml of sterile 1 % saline. For booster injection, 30 fish were used: 20 received the bacterin on Days 0 and 7 and 10 received saline on Days 0 and 7.

Twelve d after the initial vaccination (5 d after the booster injection for fish vaccinated twice), samples of experimental and control fish were bled and their serum antibody titers were determined using formalin-killed cells of the bacterium.

Sixty fish were used in an experiment to assess the efficacy of vaccination by immersion. Forty fish were immersed in the bacterin while 20 fish (controls) were left unvaccinated. Fish were vaccinated by immersion for 5 min in a vaccine that contained 0.5 mg bacterin (ml seawater)$^{-1}$. Vaccinated fish were then washed by dipping into clear seawater before returning them to the experimental tank. Unvaccinated controls were immersed for 5 min into straight seawater. Fish were bled after 12 d and their antibody titers were determined as already described.

Fish vaccinated by injection were challenged 22 d after the initial vaccine injection by intraperitoneal injection of 1 ml of saline containing 2.3×10^{11} cells of the bacterium. Mortality of vaccinated fish was compared to that of the controls. Fish vaccinated by immersion were challenged by bath exposure. This was done by immersing both vaccinated and control fish in a suspension of the bacterium in seawater for 10 min. The suspension contained 5.75×10^{11} cells of the bacterium ml^{-1}.

RESULTS

The bacterium grew at temperatures between 15 and 42 °C and had an optimum growth temperature of 30 to 37 °C. It grew over a relatively wide range of salinity (0.85 to 9.00 % NaCl), with the optimum salinity for growth being between 1.0 and 3.0 %. Optimum pH was 8.9 but the bacterium tolerated pH values of 6.2 and 9.6 although it grew poorly and slowly at these pH extremes.

The bacterium was a Gram-negative rod and grew well on both Nutrient Agar and Brain Heart Infusion Agar media supplemented with salt. Biochemical characteristics are given in Table 1. Using the API 20 E system, the bacterium was identified as *Pseudomonas putrefaciens*.

Healthy fish injected with a suspension of the bacterium all developed the clinical signs typical of the disease and showed 80 % mortality within 48 h. The injected bacterium was readily recovered from liver, kidney, and spleen of freshly dead fish.

Table 2 shows the humoral antibody response of the fish following vaccination by intraperitoneal injection and by immersion, and gives the mortalities that resulted following challenge with *Pseudomonas putrefaciens*.

DISCUSSION

In the biochemical tests, the bacterium was positive in all of those reactions in which 90 % of known strains of *Pseudomonas putrefaciens* are positive. The identification of the bacterium as *P. putrefaciens* was therefore made with a high degree of confidence. The genus *Pseudomonas* is widely distributed in the sea because of its ability to use a variety of substances as food (Rheinheimer 1980). The species *P. putrefaciens* had been associated with fish disease in Singapore (Davy & Graham 1978) and was isolated from haddock (Levin 1968, 1972), cod (Lee et al. 1977) and many fish in southeast Queensland, Australia (Gillespi 1981). It has been associated with hydrogen sulfide spoilage in

Table 1 Biochemical characteristics of a bacterium isolated from rabbitfish *Siganus rivulatus* and identified as *Pseudomonas putrefaciens*. Characteristics were identical when determined at 22 °C and at 35 °C

Test	Reaction
Gram stain reaction	–
Catalase	+
Cytochrome oxidase	+
TSI (Triple Sugar Iron agar)	K/Ag (H_2S)
ONPG (O-nitrophenyl-B-D-galactopyranoside)	–
ADH (Arginine Dihydrolase)	–
LDC (Lysine Decarboxylase)	–
ODC (Ornithine Decarboxylase)	+
CIT (Citrate)	+
H_2S (Hydrogen Sulfide)	+
URE (Urease)	–
TDA (Tryptophane Deaminase)	–
IND (Indole)	–
VP (Voges-Proskauer)	–
GEL (Gelatin Liquefaction)	+
GLU (Glucose)	–[a]
MAN (Mannitol)	–
INO (Inositol)	–
SOR (Sorbitol)	–
RHA (Rhamnose)	–
SAC (Sucrose)	–
MEL (Melibiose)	–
AMY (Amygdalin)	–
ARA (Arabinose)	–
NO_3 (Nitrate reduction)	+
N_2 (Nitrogen production)	–
MOB (Mobility or motility)	+
OF-O (Oxidation of Glucose)	–[b]
OF-F (Fermentation of Glucose)	–
Growth on McConkey agar	+

[a] Sometimes weakly positive
[b] Sometimes positive

haddock fillets and with green discoloration of fresh meat (Hugh & Gilardi 1974).

The antibody response of the fish to the injected bacterin was higher in booster-immunized fish than in fish receiving a single bacterin injection. In addition, booster immunization resulted in superior protection. Lack of a humoral antibody response in fish vaccinated by immersion may have been due to a lack of uptake of the antigen or to insufficient uptake of antigen. However, lack of humoral antibodies does not exclude the possibility of a secretory immune response. If such a response occurred, however, its protective effect was not measurable because of the inadequate challenge method used.

Our inability to infect fish experimentally by immersing them in a heavy suspension of *Pseudomonas putrefaciens* suggests that the pathogen is of low virulence and that the disease in the seawater-caged fish was stress-related. It is well known that stress is conducive to disease. For example, channel catfish subjected to a combination of environmental stressors suffered higher mortalities than fish held under optimal conditions when the fish were challenged with an intraperitoneal injection of the bacterium *Aeromonas hydrophila* (Walters & Plumb 1980). In addition, Rasheed & Plumb (1984) were unable to infect Gulf killifish with a water-borne challenge of *Streptococcus* sp. unless the fish were first injured by scratching the skin with a razor blade.

The grow-out cages of the mariculture facility are located in a backreed lagoon which is closed to major water currents. The water turnover rate at the facility is therefore low. Feed remains accumulate under the cages. These high densities of fish in an environment enriched through feeding may result in deterioration of water quality and predispose the fish to this pathogen.

In this area, the Red Sea temperature fluctuates between a high of 42 °C and a low of 19 °C (Behairy & Abdulrahman 1985). This temperature range favors year-round growth of the bacterium and may make this disease a constant threat to the mariculture facility.

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Table 2. Antibody response and survival of 60 g rabbitfish *Siganus rivulatus* vaccinated with *Pseudomonas putrefaciens* and challenged with *P. putrefaciens*

Vaccination method	No. of fish in experiment[a]	No. of fish bled	Average agglutination titer[b]	No. of fish challenged	Method of challenge	Percent mortality[c]
One injection	60 (30)	15 (9)	64 (1.3)	60 (40)	Injection	83.3 (100)
Two injections	20 (10)	5 (3)	512 (0)	20 (10)	Injection	60 (100)
Immersion	40 (20)	10 (10)	0 (0)	40 (20)	Immersion	0 (0)

[a] Numbers in parentheses refer to control fish; other numbers refer to vaccinated fish
[b] Reciprocal of highest serum dilution that agglutinated *P. putrefaciens*. Titers in parentheses refer to control fish; other titers refer to vaccinated fish
[c] Differences between mortality of vaccinated and control fish in once-injected and twice-injected fish are both significant ($p = 0.05$ in Fisher's exact test of independence). Difference between mortalities in once-injected and twice-injected fish is significant ($p = 0.05$ in χ^2 test)

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