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# *Flexibacter* infection in cultured marine fish in Japan

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ABSTRACT: *Flexibacter* infection is common in red sea bream *(Pagrus major)* and black sea bream *(Acanthopagrus schlageli)* cultured in floating net cages. The disease occurs most frequently among the fry and causes significant mortalities. Externally the diseased fish show eroded mouths, frayed fins and tail rot. Lesions contain large numbers of long, thin rods giving a pale yellow appearance to the infected tissue. The morphological, physiological and serological characteristics of 15 strains isolated from diseased fish during the epizootics at selected locations in Japan were studied. The organism failed to grow on cytophaga medium even when NaCl was added, but grew on cytophaga medium prepared with sea water. All isolates were Gram negative, flexible rods (approximately 0.5 µm × 2–30 µm) which exhibited gliding motility on wet surfaces. They did not utilize agar, cellulose or chitin. The mol % G + C base ratio of 8 strains selected for study ranged from 31.3–32.5 %. The bacterial strains examined appeared to be a new species of the genus *Flexibacter*. Serological comparisons by agglutination test revealed no serological differences between the isolates studied. Attempts were made to infect both species of juvenile sea breams by topical application of the bacterial cultures on the skin. The experimentally infected fish displayed essentially the same clinical signs as those naturally infected.

## INTRODUCTION

Red sea bream *(Pagrus major)* and black sea bream *(Acanthopagrus schlageli)* are among the most commercially important marine fish in Japan. 26567 tons were taken in the fishery and 18243 tons were cultured in floating net cages during 1981. They are artificially propagated and the larvae are reared to "seedling size" in the hatchery. Some seedlings are released along the coast, in the hope of augmenting the fishery, and the remainder are sold to fish farmers for culture to marketable size. Propagation is carried out in tanks supplied with flowing or recirculated sea water during April to June. After about 20 days following hatching, when the larvae are about 10 mm long, they are transferred to floating net cages anchored in a calm bay. Until they attain seedling size (2 to 10 cm), the fry are fed a diet of minced fish and/or shrimp.

*Flexibacter* infection was first described by Masumura & Wakabayashi (1977) as the cause of mortalities (20 to 30 %) among the fry (15 to 60 mm in length) reared at a hatchery in Hiroshima Prefecture. Affected fish had eroded mouths, frayed fins and tail rot. In the lesions large numbers of long, slender bacterial rods were observed giving the infected tissue a pale yellow appearance. The organisms isolated from the diseased fish were characterized by Hikida et al. (1979). This type of epizootic has become a common problem among hatchery-reared fry of red and black sea bream at certain locations since

1976. Furthermore, *Flexibacter* infection has also caused mortalities among older cultured fish in winter. In older fish, the lesions occur initially as greyish-white cutaneous foci on the fins, head and trunk. On the skin, the lesion become eroded and shallow ulcers are produced. The purpose of this study was to characterize the causative organisms, to compare strains isolated from fish at various locations, and to examine their ability to cause the disease.

## MATERIALS AND METHODS

Isolation of bacteria was accomplished by streaking material from external lesions and/or kidney tissues on cytophaga agar (Anacker & Ordal, 1959) containing 70 % sea water. After the plates were incubated at 25 °C for 2 to 3 days, colonies appeared which were pale yellow, flat and irregular with uneven edges. The isolates were maintained for

Table 1. History of isolates used in this study

| Strain No. | Date isolated | Host species | Location |
|---|---|---|---|
| B1, B2 (NCMB2513) | 26 July 76 | black sea bream | Ondo, Hiroshima |
| R4 | 20 Nov. 76 | red sea bream | Ondo, Hiroshima |
| R5 | 24 Dec. 76 | red sea bream | Ondo, Hiroshima |
| R6 | 18 Jan. 77 | red sea bream | Ondo, Hiroshima |
| R1 | 30 Jun. 77 | red sea bream | Ondo, Hiroshima* |
| R2 (NCBM2514) | 01 July 77 | red sea bream | Ondo, Hiroshima |
| R3 | 07 July 77 | red sea bream | Ondo, Hiroshima |
| B3 through B17 | 20 July 78 | black sea bream | Notojima, Ishikawa |
| R7 through R21 | 11 Oct. 78 | red sea bream | Ondo, Hiroshima |
| R22 through R30 | 26 Dec. 78 | red sea bream | Tsubaki-domari, Tokushima |
| R31, R32, R33 | 10 Jan. 79 | red sea bream | Arari, Shizuoka |
| P1 | 16 Feb. 79 | rock bream | Goto, Nagasaki |
| R34 | 04 July 82 | red sea bream | Arari, Shizuoka |
| R35, R36, R37, R38 | 29 Jun. 82 | red sea bream | Kainan, Tokushima |

* These fish were hatched at Hakatajima, Ehime, and transfered to Ondo, Hiroshima on 29 June 1977

further study. The isolates were suspended in cytophaga broth containing both 70 % sea water and 10 % glycerol, and stored at −80 °C to preserve their natural properties. Bacteria were isolated from diseased fish at selected locations in Japan (Table 1).

Cell morphology was recorded from light microscope observations of Gram stained smear preparations. Motility was determined by examining wet mounts with a phase contrast microscope. Microcyst formation was tested by the method of Dworkin & Gibson (1964).

Optimum growth temperature was determined by use of a temperature gradient incubator Model TN-3 (Toyo Kagaku Sangyo Co.). The ability of the strains to grow in the following media was tested: cytophaga broth containing 0, 1, 3, 5, 7 and 10 % NaCl; cytophaga broth containing 0, 10, 30, 70 and 100 % sea water. Effect of inorganic salts on growth of the bacterium was determined by inoculating media containing various

combinations of NaCl, KCl, $MgCl_2$, $MgSO_4$ and $CaCl_2$. The basal medium used in this experiment consisted of 0.1 % bacto-tryptone (Difco), 0.1 % casamino acids technical (Difco) and 0.02 % yeast extract (Difco).

The following physiological characteristics were determined by the methods described by Pacha (1968), Pacha & Porter (1968) or Levin & Lounsbery (1969): degradation of agar, cellulose, chitin, starch, esculin, casein, gelatin, tributyrin and tyrosine; utilization of tryptone, casamino acids, yeast extract, sodium glutamate, ammonium sulfide and potassium nitrate as a nitrogen source; production of ammonium, hydrogen sulfide, indole and catalase; reduction of nitrate; lysis of dead cells of *Escherichia coli, Edwardsiella tarda* and *Aeromonas hydrophila*. Cytochrome oxydase was detected by use of commercially prepared test paper (Nissui Co.). Production of acids from carbohydrates was determined by the method of Leifson (1963). Congo red adsorption was tested by flooding the colonies with 0.01 % aqueous solution of the dye (Johnson & Chilton, 1966). Basal medium used in these tests was cytophaga medium containing 70 % sea water, and in some tests TCY medium: 1.0 g bacto-tryptone, 1.0 g bacto-casamino acids technical, 0.2 g bacto-yeast extract, 31.3 g sodium chloride, 0.7 g potassium chloride, 10.8 g magnesium chloride $6H_2O$, 1.0 g calcium chloride $2H_2O$, 1000 ml distilled water (pH 7.0–7.2) was also used.

DNA was extracted from bacterial cells by the method of Marmur (1961). Guanine plus cytosine (G + C) content was determined by thermal denaturation (Marmur & Doty, 1962).

Pigment was extracted from bacterial cells by the method of Lewin & Lounsbery (1969). The extract was examined spectrophotometrically in Hitachi recording spectrophotometer Model 124.

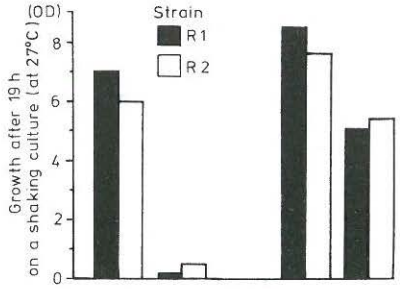
Immune sera were obtained from rabbits which had been injected subcutaneously with 20 mg of doubly washed, formaline killed cells in Freund's complete adjubant, and 2 weeks later, intramuscularly with saline suspension of cells in successive doses of 1, 2 and 4 mg on every 3rd day. The rabbits were bled from the carotid artery 3 weeks after the last injection. The sera were heat-inactivated at 56 °C for 30 min, and stored at −80 °C. The microtiter technique was used to examine the serological relations among the strains. The antigen suspension consisted of doubly washed saline suspension of cells adjusted to a density of 10 mg/ml.

Three methods were used to test pathogenicity. Bacteria from 48 h cultures were injected intramuscularly into juvenile red and black sea breams, applied topically on the mouth, trunk and tail using an inoculation loop, or they were added to the aquarium water. Materials from external lesions and/or kidney tissues of fish that died were cultured on cytophaga agar containing 70 % sea water for reisolation of the bacteria.

## RESULTS

### Morphological, colonial, and growth characteristics of bacterial isolates

Cells of the bacterium were Gram negative, flexible, slender rods measuring 0.5 μm × 2–30 μm in length. Occasionally cells up to 100 μm long were observed. They had no flagella, but exhibited gliding motility. As the cultures aged, the cells tended to become somewhat shorter. No formation of microcyst occurred on any of the culture media.




| Combination of inorganic salts | | A | B | C* | D | E | F* |
|---|---|---|---|---|---|---|---|
| NaCl | 0.5132M | 0 | 0 | 0 | 0 | 0 | 0 |
| KCl | 0.0098M | 0 | 0 | 0 | 0 | 0 | 0 |
| $MgCl_26H_20$ | 0.0525M | 0 | | | 0 | | 0 |
| $MgS0_47H_20$ | 0.0216M | 0 | | | | 0 | |
| $CaCl_22H_20$ | 0.0037M | 0 | 0 | | 0 | 0 | |

Fig. 1. Influence of inorganic salts on the growth of *Flexibacter* sp. A combination of inorganic salts (0) was added in a basal medium which contained 0.1 % tryptone, 0.1 % casamino acids and 0.02 % yeast extract. The total molar concentration was adjusted with additional NaCl. *: After 7 days of incubation, there was growth in media C and F also, but no growth in media excluding NaCl or KCl

Though spherical cells were found in older cultures, they were not capable of germinating in a fresh medium. Colonies were flat, thin, light yellow with uneven edges. In liquid medium, surface growth formed a pellicle. The organisms failed to grow anaerobically.

The bacteria grew at temperatures between 15 and 34 °C, and best at about 30 °C. There was no growth on cytophaga medium prepared with the addition of NaCl instead of sea water, and at least 30 % sea water in the medium was required. Growth occurred in 6 of 16 media with various combinations of five inorganic salts. The results showed that the organisms required KCl as well as NaCl for growth. It was also found that $Ca^{++}$ enhanced growth of the bacterium, while $SO_4^{--}$ was slightly inhibitory (Fig. 2).

## Physiological characteristics

Results of the physiological tests carried out on 15 selected strains are given in Table 2. With the exception of lysis of dead bacterial cells, all strains tested were uniform in their physiological characteristics. They produced catalase, cytochrome oxidase and ammonium, and hydrolysed casein, gelatin, tributyrin and tyrosine. Nitrogenous compounds such as tryptone, yeast extract and casamino acids were utilized as sources of carbon and nitrogen for growth. Sodium glutamate, ammonium sulfide and potassium nitrate were not used by this bacterium. Nitrate was reduced to nitrite. None of 24 carbohydrates tested were utilized. All of the cultures were negative for production of hydrogen sulfide and indole, and degradation of agar, cellulose, chitin, starch and esculin. Congo red test was positive.

The DNA base compositions of 8 strains ranged from 31.3 to 32.5 mol % G + C.

The absorption curve of pigment-solution in n-hexane had three peaks at 425, 450

Table 2. Physiological characteristics of *Flexibacter* sp. examined in this study. + = positive, − = negative, d = diverse (no. of strains positive/no. of strains tested)

| Characteristic | 15 isolates* | Characteristic | 15 isolates* |
|---|---|---|---|
| Degradation of | | Nitrogen source | |
| Agar | − | Tryptone | + |
| Cellulose | − | Casamino acids | + |
| Chitin | − | Yeast extract | + |
| Starch | − | Sodium glutamate | − |
| Esculin | − | Ammonium sulfide | − |
| Casein | + | Potassium nitrate | − |
| Gelatin | + | Production of | |
| Tributyrin | + | Ammonium | + |
| Tyrosine | + | Hydrogen sulfide | − |
| Carbohydrate utilization | | Indole | − |
| Glucose | − | Catalase | + |
| Galactose | − | Cytochrome oxidase | + |
| Lactose | − | Reduction of nitrate | + |
| Sucrose | − | Congo red test | + |
| Sorbose | − | Lysis of dead cells | |
| Inulin | − | *Escherichia coli* | d (4/15) |
| Sorbitol | − | *Edwardsiella tarda* | + |
| Inositol | − | *Aeromonas hydrophila* | + |

* Strains B1, B2, B3, R1, R2, R3, R4, R5, R6, R8, R10, R22, R28, R31 and P1 were tested

Table 3. Comparison of different methods of infecting fish using a culture of *Flexibacter* sp. (strains B2) with juvenile red and black sea bream (25–70 mm in total length)

| Group | Method of infection* | Species of sea bream | No. of fish used | No. of deaths | Mean time to death (days) |
|---|---|---|---|---|---|
| Experiment | injection | red | 10 | 0 | – |
| | | black | 10 | 2 | 2.5 |
| | application (mouth) | red | 11 | 4 | 2.3 |
| | | black | 10 | 9 | 1.2 |
| | (tail) | red | 10 | 3 | 2.0 |
| | | black | 10 | 10 | 1.5 |
| | bath | red | 9 | 0 | – |
| | | black | 10 | 1 | 2.0 |
| Control | injection | black | 10 | 0 | – |
| | application (mouth) | black | 10 | 0 | – |

* Injection: Fish were injected intramuscularly with 0.02 ml of the culture (experimental group) or saline (control group). Application: Fish were topically applied with the culture (experimental group) or saline (control group). Bath: Fish were exposed to bacterial suspensions for 2 h in static water with aeration and then held in pathogen free flowing water at 23–24 °C

and 475 nm. The highest peak was at 450 nm, which indicated the presence of carotenoid-like pigments.

### Serological characteristics

Three strains were selected and used to immunize rabbits. The three strains chosen were B2, R2 and R8. Agglutination titers of homologous antisera for these strains were 1:256. The heterologous titers of these antisera were also 1:256. The titers for the rest of the isolates listed in Table 1 were determined using anti-R8 serum, and they were in the range of 1:128 to 1:256. Thus all of the isolates tested are very similar serologically.

### Pathogenicity

Results of experimental infections of the bacteria in juvenile red and black sea bream are given in Table 3. Mortality among experimental fish varied widely according to the different methods used to infect these animals. Fatal infection occurred most frequently when fish were exposed to topical application of the culture on the surface of the mouth or the tail using an inoculation loop. The infected fish displayed essentially the same clinical signs as the naturally infected ones. However, death rarely occurred among fish injected intramuscularly with the bacteria or among those exposed to waterborne organisms.

## DISCUSSION

The strains examined were found to represent a very homogeneous group of bacteria thought to belong to a single species. The organism consisted of long, slender, Gram negative rods displaying gliding motility, which could not attack agar, cellulose and chitin, or form fruiting bodies or microcysts. Its DNA base composition was in the range of 31 to 33 mol % G + C. On the basis of the 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974), the bacterium described here belongs to the genus *Flexibacter*. However, it is different from all *Flexibacter* species described in the current edition of the manual.

There are reports of gliding bacteria associated with diseases of fish in sea water. Borg (1960) described an undetermined genus of gliding bacteria from diseased pink salmon *(Oncorhynchus gorbuscha)* held in sea water. Wood (1968) reported *Sporocytophaga* from salmonids in Washington State hatcheries having a sea water supply. From an epizootic in rainbow trout *(Salmo gairdneri)* reared in sea water in Scotland, Anderson & Conroy (1969) isolated long, slender, gliding bacteria which produced round bodies but distinct in appearance from the *Sporocytophaga* sp. Sawyer (1976) reported *Flexibacter* sp. from an epizootic in coho salmon *(Oncorhynchus kisutch)* reared in an estuary in the State of Maine, USA. The present organism is morphologically and physiologically similar to that reported by Sawyer, but differs from it in its obligate requirement for sea water for growth. The sea water requirement could be replaced by NaCl in the case of the bacterium Sawyer reported.

The fact that the isolates examined had a common antigen regardless of various sources would suggest that it may be possible to use serological tests as a means for rapid identification of the bacterium. Before reliance can be placed on such a procedure, further investigations will have to be carried out to provide required evaluation.

The disease occurred one or two weeks after transferring from hatchery tanks to inshore net cages, and rarely affected fish of more than 60 mm in body length; the younger the fish the more severe the clinical signs were. Stress imposed by transferring from hatchery to inshore no doubt predisposes hatchery raised fry to *Flexibacter* infection. Although increased water temperature is preferable for growth of the bacterium, there has been no occurrence of the disease in the summer and fall. Red and black sea bream are found in warmwater inshore locations in southern and central parts of Japan. These fish are not very active below about 15 °C, and waters where temperature decreases below 10 °C are not suited for sea bream culture. Red sea bream are more sensitive to low temperature (10 °C) than black sea bream. This seems to be the reason why winter outbreaks of the epizootic have occurred among red sea bream, but not among black sea bream.

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