

**Bacterial diseases of eggs and yolk sac larvae of halibut
(Hippoglossus hippoglossus L.): Characterization and
experimental infection**

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ABSTRACT

Scanning electron micrographs of halibut eggs which were shown to have an epiflora dominated by a Flexibacter sp., revealed wounds colonized by large amounts of bacteria. The chorion was penetrated in most wounds, and the zona radiata was severely damaged. Infection experiments showed that exposure to these bacteria caused high mortality at hatching and early yolk sac stage. Eggs exposed to strains of Vibrio anguillarum and Vibrio fisheri showed a different mortality pattern, with low mortality at hatching, followed by a continuous high mortality throughout the yolk sac stage. Mortality in the uninfected control group was low throughout the experiment.

INTRODUCTION

Halibut (*Hippoglossus hippoglossus* L.) is an attractive candidate species for marine aquaculture, although so far high mortality at early life stages has been prevalent (Pittman et al. 1989, 1990 a,b, Skiftesvik et al. 1990 a,b). Bacterial infections have been suspected to be a major cause of mortality at early life stages of halibut (Blaxter et al. 1983, Jelmert and Mangor-Jensen 1987, Opstad and Bergh 1990, Pittman et al. 1990a, Rabben et al. 1986, Skiftesvik et al. 1990a,b). Several strains of bacteria suspected to be pathogenic have been isolated from halibut eggs and larvae and water in incubator tanks (Bolinches and Egidius 1987; Hansen and

Bergh, unpublished data; Hansen and Olafsen 1989; Pittman et al. 1990a).
Hitherto, however, no controlled infection experiments have been carried out.

It has long been known that bacteria are associated with fish eggs (Oppenheimer 1955) and it is now well established that the surface of marine fish eggs constitutes a habitat well suited for many epibiotic bacteria, some of which might cause damage to the chorion (Hansen and Olafsen 1989). Pathogenic bacteria infecting at the egg stage might persist and possibly proliferate at the egg surface until hatching, thereafter infecting the larva. Also, a possibility exists that the bacterial epiflora might cause damage to eggs by oxygen consumption or production of toxic metabolites.

In this study, we have exposed halibut eggs to defined amounts of selected strains of bacteria, and examined mortality and pathogenesis throughout the yolk sac stage.

MATERIALS AND METHODS

Bacteria

Five different bacterial strains were used in the infection experiment, two strains of *Flexibacter* sp., two different strains of *Vibrio anguillarum*: NCMB 6, isolated from cod (National Collection of Marine Bacteria, Aberdeen, U.K.), and *V. anguillarum* 651, isolated from halibut (Institute of Marine Research, Bergen, Norway) and one strain of *Vibrio fisheri* (ATCC 7744 (American Type Culture Collection, Rockville, Md, U.S.A.).

The *Flexibacter* sp. strains EKC001 and EKD 002 had been isolated from egg surfaces of two different halibut egg groups from the broodstock at the Austevoll Aquaculture Research Station during the 1989 spawning season. EKC 001 was isolated from the same egg group from which samples were taken for scanning electron microscopy. Similar strains have been isolated from other egg groups, larvae groups and incubator water during the 1989 and 1990 spawning seasons at Austevoll Aquaculture Research Station. A total of 35 isolates of this type have been characterized by means of biochemical and morphological tests (Hansen and Bergh unpublished data). The cells are Gram negative, Kovac's oxidase positive, pale yellow pigmented long, slender rods (0.5 by 2-20 μm) which occasionally grow to filaments with lengths of 70-100 μm . They show gliding motility. They do not produce acid from any of a wide range of carbohydrates, are able to degrade

gelatin, tyrosine, DNA and Tween-80, but are unable to degrade starch or cellulose. They possess catalase and nitrate reductase, are unable to grow under anaerobic conditions, and do not produce H₂S. The strains resembles *Flexibacter maritimus* (Wakabayashi et al. 1986), but differs in a range of characteristics, mainly absorption of Congo Red, growth in 30% seawater, utilization of sodium glutamate as a nitrogen source, and growth at 4° and 30°C. Further characterization is in progress, including hybridizing DNA from *Flexibacter* sp. with DNA from *Flexibacter maritimus* NCMB 2154^T and NCMB 2153 (Hansen, Bergh and Michaelsen, unpublished data).

Halibut husbandry

For each experiment, eggs from one single egg batch of a female were fertilized with sperm from one single male. All parents belonged to the broodstock of Austevoll Aquaculture Research Station. The eggs were reared as described by Bergh et al. (1989) and Pittman et al. (1990a) until further processing as described below.

Infection experiment

Eggs were transferred to polystyrene multiwell dishes (NUNC, Roskilde, Denmark) 5 days before hatching (Jelmert and Naas 1990). Each well had 11 ml of sterile 70% seawater. Four days before hatching, 200 ul of an accenic culture of one of the strains of bacteria was added to each well, thus obtaining a final concentration of apporoximately $2-3 \times 10^6$ bacteria * ml⁻¹ in the wells. Each bacterial strain had previously been accenically cultivated in Marine Broth (Difco Laboratories, Detroit, Michigan, U.S.A.) which had been diluted to 10%, at 10°C. A total of 60 wells were used for each bacterial strain. In addition one group consisting of 60 wells was not infected by any of the strains. At Day 1 after hatching, the wells were washed, i.e. 10 ml water was carefully removed with a pipette, together with remainings of the eggshell, without damaging the larva. The washing procedure was designed to avoid cross-contamination of bacteria from different strains, thus separate sterile pipettes were used for each group of 60 wells. Thereafter, 10 ml 70% autoclaved seawater was added. Mortality was recorded from hatching until the end of experiment at Day 37 after hatching.

Electron microscopy

Eggs were washed in sterile filtered sea water (0.22 um), and fixed in formaldehyde-glutaraldehyde (final concentrations 2.5 and 2.0 % (vol/vol), respectively) in 0.05 M cacodylate buffer at pH 7.2. The eggs were postfixed in 1% osmium tetroxide in cacodylate buffer, dehydrated in ethanol, critical point

dried and coated with gold-palladium. Scanning electron microscopy was performed with a JEOL JSM-6400 microscope.

RESULTS AND DISCUSSION

Cumulative mortality is shown in Figure 1. Three different mortality patterns can be distinguished. The groups infected with *Flexibacter* sp. showed very high mortality at hatching. Out of 60 larvae in each group, 40 and 49 died in the EKD 002 and EKC 001 group, respectively. Mortality was also high between Day 1 and Day 9 after hatching. Thereafter, mortality was low. However, the number of larvae left alive at this stage was very low, 13 and 4, respectively. We are not aware of reports showing similar mortality patterns. However, it is well documented that the genus *Flexibacter* comprises several fish pathogens, including the marine *Flexibacter maritimus* (Bernadet et al. 1990, Wakabayashi et al. 1986).

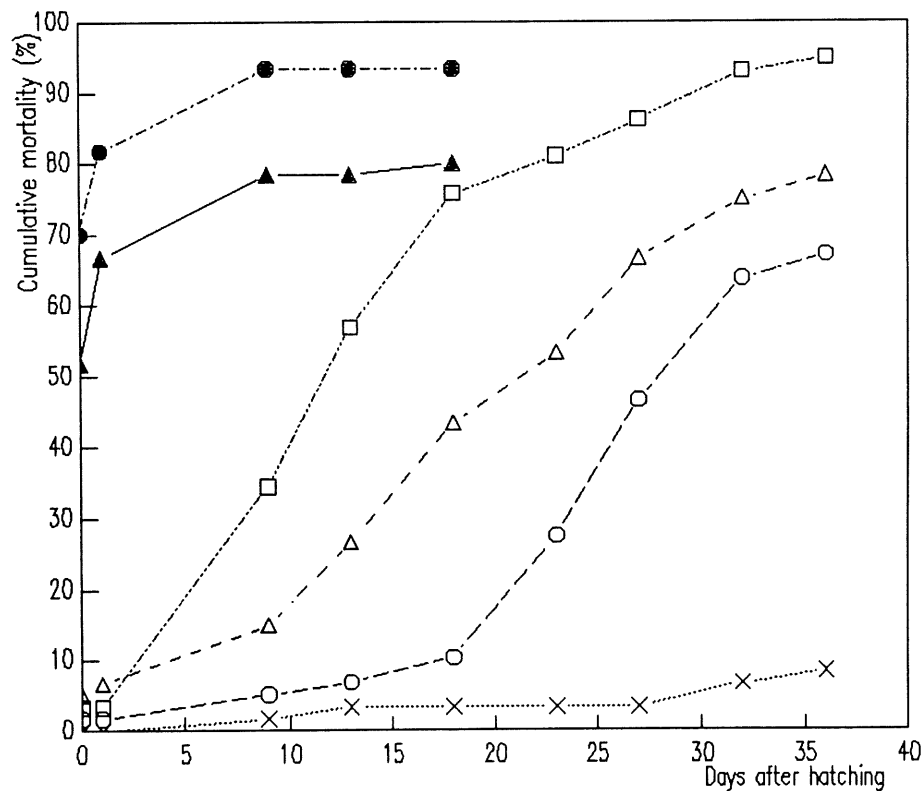


Figure 1. Cumulative mortality (in %) of the six groups in the infection experiment. The six groups are: larvae exposed to strain EKC 001 (filled circles), EKD 002 (filled triangles), NCMB 6 (open squares), ATCC 7744 (open triangles), *V. anguillarum* 651 (open circles) and the control group (crosses).

Neither the control group nor the *Vibrio*-infected groups showed significant mortality at hatching. The *Vibrio*-infected groups showed an almost even distribution of mortality throughout the experiment. At the end of the experiment, cumulative mortality of these groups had raised to 78% (ATCC 7744), 67% (*V. anguillarum* 651) and 95% (NCMB 6). As *V. fisheri* and *V. anguillarum* have previously been shown to occur in relatively large numbers in incubators for halibut yolk sac larvae (Bolinches and Egidius 1987), this might partly explain the high mortalities reported so far (Bergh et al. 1989, Opstad and Bergh 1990, Pittman et al. 1989, 1990 a,b, Skiftesvik et al. 1990 a,b).

In the control group, mortality was low throughout the experiment, as only 5 out of 60 larvae were dead at Day 37 after hatching, which is well into the window of first feeding (Skiftesvik et al. 1990b). This indicates that from the outset, the eggs were healthy, and that pathogenic bacteria were not abundant. It also supports the statement of Jelmert and Naas (1990) that rearing of halibut yolk sac larvae in multiwell dishes is an adequate way of obtaining healthy larvae for experimental purposes.

The ultrastructure of the halibut egg shell was investigated by Lønning et al. (1982) and Helvik (1988), who found that a typical chorion had a thickness of about 0.2 μm , whereas the thickness of the zona radiata, consisting of 18 electron dense lamellae, was 9.5 μm . However, the halibut is a batch spawner, and Helvik states that the thickness of these layers was variable, in such way that late egg batches tended to have thinner eggshells compared to earlier batches. Thus, late egg batches should be more sensitive to pathogenic bacteria, compared to early batches. This might, at least in part, explain why mortalities throughout the yolk sac stage tend to increase at the end of the season (I. Opstad, Austevoll Aquaculture Research Station, pers. comm.)

The scanning electron micrographs revealed wounds, where the chorion was completely penetrated. The wounds were colonized by large amounts of large rod-shaped bacteria morphologically similar to *Flexibacter* sp. In some wounds, the zona radiata was severely damaged. It is not possible from the micrographs to deduce whether the zona radiata was completely penetrated. Although one can never deduce from a scanning micrograph that a bacterium belongs to a certain species, it is likely that the bacteria really are *Flexibacter* sp. as these bacteria comprised more than 99% of the viable counts of homogenized eggs from the same egg batch (Hansen, Bergh and Michaelsen, unpublished data). A gradient could be distinguished between areas where only chorion was damaged, areas where

destruction of zona radiata had begun, and areas where zona radiata was severely damaged. One micropyle was observed heavily colonized by *Flexibacter*-resembling bacteria. It was not possible to deduce from the micrographs whether eggs were intra-ovularily infected, as has often been shown for salmonids (Sauter et al. 1987).

It has been shown before that bacteria are able to damage chorion, probably by exoproteolytic activity (Hansen and Olafsen 1989). However, this is to our knowledge the first evidence of complete destruction of the chorion and severe destruction of the zona radiata by bacteria.

CONCLUSIONS

Several bacterial strains which are representative of those associated with the rearing of early life stages of halibut in aquaculture, are opportunistic pathogens. When present in sufficient amounts, they may cause mass mortality of eggs and yolk sac larvae. Our findings emphasize that efficient antibacterial treatment procedures should be included in a production line for halibut eggs and yolk sac larva.

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