Bacterial pathogens associated with early life stages of marine fish

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ABSTRACT

Bacteria are intimately associated with all life stages of marine organisms. In aquaculture, high population densities and suboptimal rearing conditions often make conditions ideal for opportunistic pathogens, with high mortalities being the result. Knowledge of the different ecological relations between bacteria and the different cultivated species is essential if we are to ensure increased survival. Studies of the pathogenic or mutualistic bacteria of early life stages have been hampered by the lack of adequate protocols for challenge experiments and methods for isolation and detection of bacteria associated with eggs and larvae. New methods, including the rearing of the larvae exposed to different bacteria in multiwell dishes, and the detection of bacteria by means of immunohistochemical protocols designed for eggs and larvae, have thus been developed. The studies described in this paper are focused on the Atlantic halibut *(Hippoglossus hippoglossus)* and Atlantic cod *(Gadus morhua),* on bacteria that are pathogenic to the eggs and early larvae, primarily *Flexibacter ovolyticus,* and on the implications of such bacteria on egg and larval quality and survival.

Introduction

It has long been known that the surface of fish eggs is a habitat for bacterial colonisation and growth [13]. As the egg hatches, the yolk sac larva become exposed to the bacteria present on the egg surface, as well as those in the surrounding environment. Exposure of yolk sac larvae to opportunistic pathogens may result in disease and mortalities. Among the bacteria associated with the early life stages of Atlantic halibut there are strains which are probably mutualistic [4] as well as opportunistically pathogenic strains [2]. As both of these categories of associated bacteria may be of crucial importance to survival, increased knowledge of the factors governing which bacteria occupy the niches associated with fish eggs and larvae is of great advantage to the aquaculturist. The present paper presents challenge models for eggs and yolk sac larvae of the Atlantic halibut *(Hippoglossus hippoglossus)* and the Atlantic cod *(Gadus morhua)*. *Flexibacter ovolyticus* [10] is pathogenic to eggs and yolk sac larvae of the Atlantic halibut [2] but has not so far been isolated from any other species or habitats. A comparative challenge experiment with *F. ovolyticus*, and two other fish pathogens *Vibrio anguillarum* and *Aeromonas salmonicida* subsp. *salmonicida* was carried out, as well as two dose-response challenge experiments with *F. ovolyticus* infecting cod and halibut eggs.

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Materials and methods

Forty-three batches of cod eggs were screened for the presence of *F. ovolyticus.* The eggs and sperm were collected from wild fish captured on two different research cruises in the Lofoten/Vesterålen area off the North-Norwegian coast in 1992 and 1993. Eggs were fertilised at sea and brought live to the laboratory within 24 h in 1 L polystyrene containers in seawater from the sampling site.

Eggs were homogenised in a glass homogeniser and a dilution series was made in 25 ppt. sterile seawater. Samples were plated on Difco 2216 Marine Agar plates and incubated at 10°C. Representative isolated strains were checked with respect to colony morphology and pigmentation, Gram staining (Bacto Gram stain set, Difco) , oxidase (API 7046 kit, BioMerieux, France) , and catalase reaction (3% H_20_2). The following tests were carried out as described by Hansen and Sørheim [9], with modifications according to Bergh [4]: oxidative/fermentative metabolism of glucose; aerobic acid production from fructose, sucrose, mannitol, mannose, glycerol, ribose and N-acetyl-glucosamine; gelatinase, nitrate reductase, Voges-Proskauer and methyl red; production of indole from tryptohane, degradation of Tween-80, growth on tryptone citrate bile salt agar (Oxoid, U.K.), and utilization of citrate. The same tests were used in order to check isolates from moribund larvae in the challenge experiments.

Challenge experiments were performed in darkness at 6°C using protocols modified from Bergh et al. [2]. Two different challenge experiments were performed on halibut eggs. Surface disinfection of the eggs was not carried out. These experiments were conducted in sterile multiwell dishes containing 10 mL sterile seawater and one egg per well. Sixty eggs were used in each treatment and control. Eggs were obtained from three egg batches from the halibut production line at Austevoll Aquaculture Research Station. In all challenge tests, differences in mortality were tested for statistical significance using χ^2 analysis, followed by comparison of groups with the Tukey-type multiple comparison of proportions [17], assuming the data to be binomially distributed. A level of probability $P \leq 0.05$ was considered to be significant.

In Experiment 1, three different isolates of *F. ovolyticus,* NCIMB 13127, 13128, and 13129 [10], were compared to a strain of *Aeromonas salmonicida* subsp. *salmonicida* and a strain of *Vibrio anguillarum* (As55 and HI 10448, respectively, Institute of Marine Research, Bergen Norway). All strains were grown in Difco Marine Broth at 10ºC and added to the wells in amounts equivalent to approximately 10^6 CFU mL^{-1}. In the control group, nothing was added to the wells. The challenge was done three days before hatching. One day after hatching, the remains of the eggshells were removed, the wells and larvae gently were rinsed with sterile seawater, and 10 mL of sterile seawater was added. Larval mortality was assessed every second day until day 27 post-hatch.

Experiment 2 was a dose-response challenge experiment with *F. ovolyticus* strain NCIMB 13127. Three different challenge doses (High, Medium and Low) were used, equivalent to a batch challenge concentration in wells of approximately 10^6, 10^5 and 10^4 CFU mL^{-1}, respectively. Control groups were treated in the same way with exception of the addition of bacteria. Three different egg batches from three different pairs of broodstock fish were used. The eggs were challenged at five days post-hatch, and the wells and larvae were washed one day after hatching as described above. Larval mortality was assessed every second day until day 23 post-hatch.

Experiment 3 was a dose-response challenge experiment of cod eggs with *F. ovolyticus* strain NCIMB 13127. The two batches of cod eggs which were used were obtained from the cod production line at Austevoll Aquaculture research station. In this experiment, 24-well multiwell dishes were used, with 2 mL sterile seawater and one egg per well. Seven day post-hatch eggs were exposed to the same challenge doses as used in experiment 2. Seventy two eggs were used in each of the challenge doses and the controls. One day after hatching the eggshells were removed, the larvae and the wells rinsed with sterile seawater, and 2 mL of sterile seawater added. Larval mortality was determined daily until 10 days post-hatch.

In all three challenge experiments, parallel multiwell dishes were set up in order to provide larvae for re-isolation of pathogens and immunohistochemical protocols. Moribund larvae from these multiwell dishes were homogenised in 25 ppt sterile seawater, and plated out from a dilution series on Difco 2216 Marine Broth agar (MBA) and Tryptone Soya Agar (TSA). Strains suspected to be *F. ovolyticus* due to colony morphology and the pale yellow colony pigmentation were compared to the type strains by the biochemical tests listed above. For immunohistochemical studies, larvae were treated as described by Bergh et al. [5]. As primary antibodies, polyclonal rabbit antisera against *F. ovolyticus* and *V. anguillarum* and a monochlonal antibody against *A. salmonicida* subsp. *salmonicida* were used.

Results

Of a total of 901 bacterial isolates from the wild cod eggs, none of the isolated colonies had the pale yellow colour typical of *F. ovolyticus* and none could be identified as *F. ovolyticus* on the basis of the tests that were used.

In Experiment 1 only the two *F. ovolyticus* strains NCIMB 13127 and 13128 caused mortality significantly different from the control group before hatching. From Day 7 after hatching, there was significantly higher mortality of larvae exposed to all five strains (Fig.1A). *Flexibacter ovolyticus* and *V. anguillarum* were re-isolated from moribund larvae, whereas *A. salmonicida* subsp. *salmonicida* was not, and this bacterium could not be detected in larval tissue using the immunohistochemical approach. *F. ovolyticus* was found on egg surface and skin of larvae. *V. anguillarum* could be detected on the skin, in connective tissues, and in the intestine of affected larvae. There was a significant dose response effect of *F. ovolyticus* on halibut eggs in experiment 2 (Fig.1B). At the highest exposure level there was less than 10% survival to hatch and complete mortality in all groups by 7 days post-hatch. At the medium exposure level, survival was prolonged with a maximum mortality of 80% seen at the end of the experiment. At the lowest exposure level, mortality remained relatively low (<15%) throughout most of the experiment with an increase in the mortality rate occurring at 21 to 23 days post-hatch. Mortality in the control group was relatively low (< 10%) throughout the experiment.

Damage to the egg surfaces and larvae caused by *F. ovolyticus* was evident in the all three batches of eggs exposed to the high and medium doses. The presence of *F. ovolyticus* at the sites of the damage was confirmed by immunohistochemical analyses (data not shown).

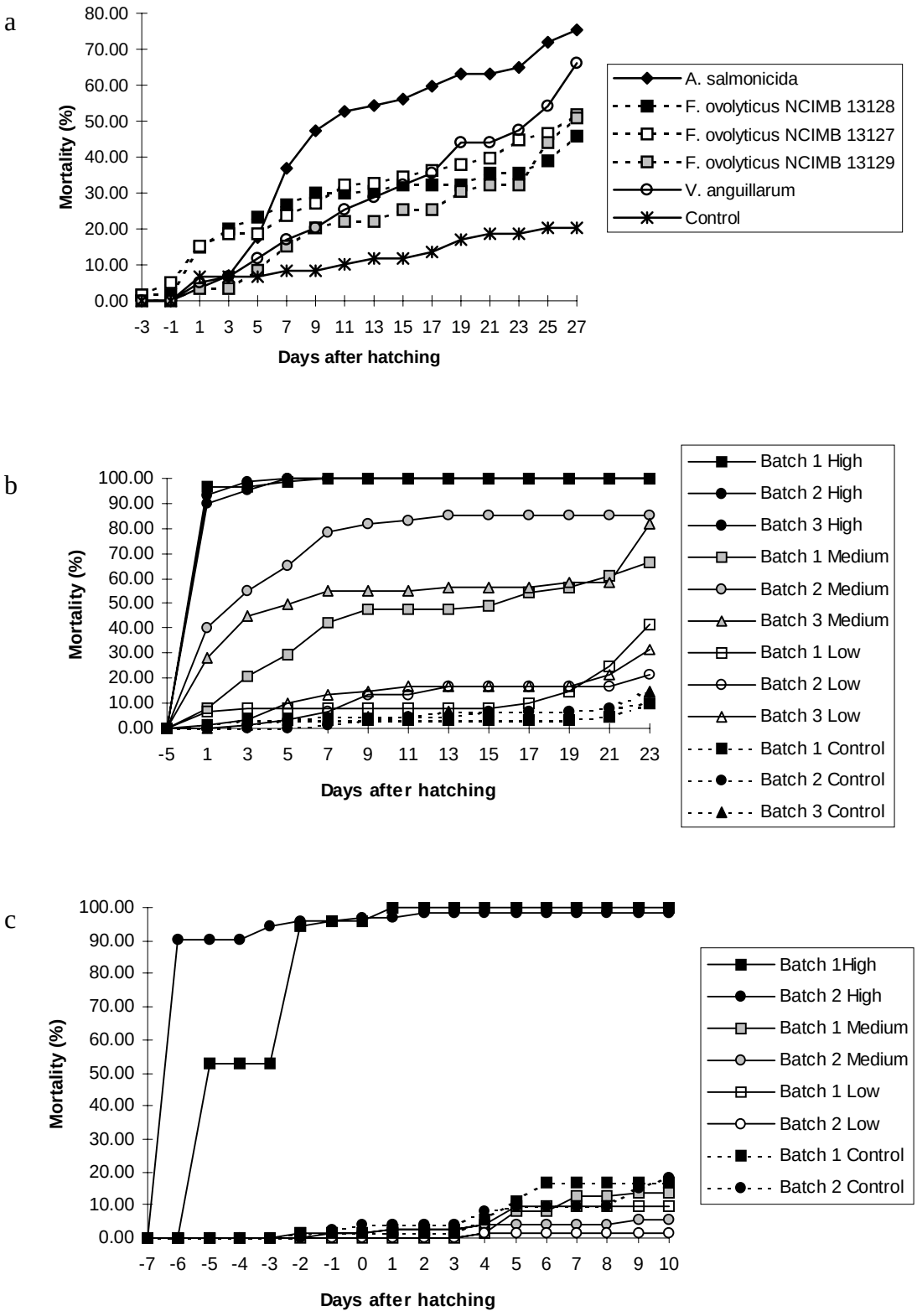

Fig 1. Mortality (%) of eggs and larvae during the egg/larval development in the three challenge experiments: Experiments 1 (a), 2 (b), and 3 (c).

Exposure of cod eggs to the highest dose of *F. ovolyticus* resulted in 100% mortality by 1 day post-hatch (Fig. 1C). At the lower doses there was no significant difference in mortality from that of the control group.

Discussion

Flexibacter ovolyticus is able to cause mortality of halibut as well as cod at the egg stage, by penetrating the eggshell. *V. anguillarum* and *A. salmonicida* subsp. *salmonicida* did not cause significant mortality to halibut at the egg stage, but probably infected the larvae post-hatch causing significant mortalities. The different mortality patterns reflect different ecological strategies of the bacteria involved. The ecological strategy of *F. ovolyticus*, which is directly pathogenic to eggs, would demand the enzymatic capability to penetrate or at least damage the *zona radiata* and *zona pellucida* of the eggshell, or the ability to accumulate in such numbers at the egg surface that the exchange of oxygen or other metabolites falls beyond the critical level. In contrast, the strategy represented by *V. anguillarum* and possibly *A. salmonicida* apparently do not demand the capability to penetrate the eggshell. These bacteria may however adhere to the eggshell and infect the larva post-hatch, causing mortality to later life stages from yolk sac larvae onwards. Bacteria following such strategies may be successfully fought by applying suitable egg surface disinfection procedures. Pathogenic intraovulary bacteria, if present, would eventually represent a third strategy, as they could not be fought by surface disinfection. They may cause mortality directly to the egg stage, or represent a means through which the pathogen is vertically transmitted to later life stages of the host.

The failure to isolate *F. ovolyticus* from cod eggs, in comparison to the relatively high prevalence of this bacterium previously found on halibut eggs [9], may indicate differences in the surface characteristics of the eggs of these two fish species. It could be hypothesised that the bacterium may attach to some surface determinants that are present on halibut eggs but not on cod eggs, although the differences in waters in which the broodstock were held must be taken into account. However, the present data do not indicate that there are differences in the pathogenicity of the bacterium to the two fish species, once the bacteria are established on the egg surface in sufficient amounts.

The place on the parasitism-mutualism axis where a relationship between a fish egg of a given species and an associated bacterium inhabit may be highly variable, reflecting the varying degree of overlap of interests between the genes of the bacterium and their host i.e. the varying *desiderata list*. According to Dawkins [6], the key variable affecting this degree of overlap is the extent to which the parasite is dependent upon the host for its survival. If it is highly dependent, the shared desiderata list will be relatively large. When parasite reproduction is through an entirely separate channel from host reproduction, the shared desiderata list will be relatively short, and the parasite will be malignant. This hypothesis may elucidate the observed low or non-existing correlation between occurrence of intraovulary bacteria and survival of eggs and larvae found by Sauter et al. [14] and Barker et al. [1]. Following the hypothesis of Dawkins [6], the genes of a pathogenic bacterium specialised for the *intra ovum* niche, would have a relatively great overlap of interest with the genes of their host, as its survival would be dependent on the reproductive success of the host. In contrast, the genes of an opportunistically pathogenic bacterium, of which *V.*

anguillarum is a typical example, would have a relatively lower overlap of interests, as the bacterium would be able to survive and reproduce in the absence of the host.

In this study *A. salmonicida* subsp. *salmonicida* could not be re-isolated from moribund larvae. Bergh et al. [5] was unable to re-isolate this species from the larvae of halibut and turbot, *Scophthalmus maximus*. Furthermore they were unable to detect it in halibut by immunohistochemical means. It is possible that the mortality found in their experiment, as well as in the present study , was due to toxic products from the bacterium that were released in the wells.

Due to the protection of the embryo by the eggshell and the perivitelline membrane, it is possible to disinfect eggs with significantly higher doses of disinfectant than what is possible after hatching. Application of iodophor [3] or glutaraldehyde [11] to disinfect halibut eggs has been demonstrated to increase the output of functional larvae. In these studies determination of egg survival and hatching rate alone were not found sufficient for measuring either beneficial effects of halibut egg disinfection, or the possible toxic effects which may become detectable later during the development of the larvae [3]. This conclusion is supported by the findings of Harboe et al. [11], who found no differences in survival at hatching or during the yolk sac stage after disinfecting halibut eggs with glutaraldehyde. However in their study there was a significantly higher feeding incidence among larvae from disinfected groups when compared to the controls. Removal, by disinfection, of the bacterial epibiota that normally accumulates during the development of the eggs may be beneficial to the development of the larvae post hatching, a conclusion which demonstrates the importance of the bacterial community in which the larvae hatch and develop. It is also possible that non-pathogenic bacteria growing on the surface of eggs may have a mutualistic relationship with their hosts, as their physical presence *per se* may reduce the ability of pathogens present in the environment to colonise the egg. An ability to inhibit growth of pathogens has been shown for bacteria isolated from the surface of eggs of the American lobster, *Homarus americanus* [7], and fish [16]. It is likely that similar relationships exist between egg surface bacteria in at least some other species.

The term *egg quality*, which is commonly used in fisheries and aquaculture research has been defined by Kjørsvik et al. [12] as *the egg's potential to produce viable fry*. According to this definition, egg quality should be determined when the eggs have been spawned and fertilized. Although not discussed by Kjørsvik et al. [12], the presence of pathogenic bacteria on the egg surface or intra ovum would clearly influence the egg quality. In this study we have demonstrated that the presence of bacterial epibiota on fish eggs may strongly influence egg viability as seen by reduced survival of infected eggs. Large differences in the composition of the epibiota of halibut eggs between different batches have been reported [8, 4]. This adhered microbiota also influences larval behaviour and thereby indirectly the ability of the larvae to feed [15]. Thus, the bacterial microbiota associated with eggs and larvae is variable between batches, but it would influence any measurement of the egg's potential to produce viable fry, hence, egg quality. Thus, as all measurements of egg and larval viability or behaviour may be highly influenced by the epibiota, it is hard to see how it is possible to measure egg quality by the definition by Kjørsvik et al. [12], except maybe in an axenic or gnotoxenic system, i.e. in a highly artificial microbial environment which is well experimentally controlled. Another possibility is the use of multiwell systems as in this study, ensuring that a high number of eggs or larvae are reared individually, by a

method that makes it possible to treat the survival of individual eggs and larvae as events that are independent of each other, thereby ruling out most of the effects of random blooms of opportunistic pathogens.

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