



Cathelicidin antimicrobial peptides in cod and salmon

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Abstract

Cathelicidins are a family of antimicrobial peptides that have been well researched in mammals, where they have been described to have an important role in immunity. Cathelicidins are ubiquitous among vertebrates having been found in fish, amphibia, reptiles, birds and mammals including marsupialia, thereby demonstrating their conserved position throughout evolution. The Atlantic cod *Gadus morhua* is an economically important fish in Iceland. In this thesis the focus is on the cod cathelicidin „codCath“. It was in the course of this project isolated from cod and then characterized regarding its activity and regulation.

The cathelicidin antimicrobial peptide was isolated from cod head kidney by high-performance liquid chromatography. The mature peptide was sequenced at the N-terminus and a total size of 67 amino acids was deduced. The peptide has a predominance of the amino acids serine, arginine and glycine, a positive charge of +15 and is considered a novel and unique cathelicidin. Both the isolated and the synthetic peptide demonstrated antibacterial activity. The synthetic peptide helped to identify a salt sensitivity of this peptide with reduced activity at high salt concentrations. Our results indicated that the mode of activity of codCath was rapid lysis of bacterial cells, while it was selectively active towards microbial but not eukaryotic membranes. The peptide was degraded and inactivated by extracellular products of pathogenic bacteria.

Cod cathelicidin transcripts were found in eggs and larvae of Atlantic cod and in larvae levels were responsive to environmental stimuli. In a salmonid cell line cathelicidin was significantly upregulated through Gram-negative bacteria and flagellin and in a gadoid cell line through *Lactobacillus* sp. and poly(I:C). The responsiveness through microbial PAMPs indicates signaling through TLRs or other receptors. The inhibition of the PI3K further indicated that this protein has an inhibitory role on

receptor mediated signaling and is involved in the signaling cascade to cathelicidin upregulation in the salmon cell line.

In conclusion, a novel cathelicidin from a teleost fish was isolated and inducibility of expression by microbial PAMPs as well as antimicrobial activity of the peptide indicate that cathelicidin in cod plays a role in immunity.

Útdráttur

Cathelidins eru fjölskylda örverueyðandi peptíða sem hafa verið rannsökuð mikið í spendýrum og gegna mikilvægu hlutverki í ónæmiskerfinu. Þau hafa fundist meðal helstu flokka hryggdýra; fiska, froskdýra, skriðdýra, fugla og spendýra meðtaln pokadýr sem gefur vísbendingu um varðveislu í þróun og mikilvægt hlutverk. Þorskurinn (*Gadus morhua*) hefur megin efnahagsþýðingu fyrir Ísland. Í ritgerðinni og rannsóknunum var áherslan á cathelidín í þorski sem er nú kallað „codCath“. Í verkefninu var virkt cathelidín peptíð þorska einangrað, virkni greind og tjáning athuguð.

Virka cathelidín peptíð þorska var einangrað úr framnýra með háþrýstivöskvilju (HPLC). Amínósýruröð virka peptíðsins var ákvörðuð með raðgreingu á amínóenda og þar með stærðin ákvörðuð 67 amínósýrur. Áberandi amínósýrur í peptíðinu voru serín, argínín og glýsín og við hlutlausar aðstæður er jákvæð hleðsla +15. Peptíð líktist engu öðru einangruðu peptíði og er því einstakt meðal cathelidína. Bæði einangrað peptíð og samsvarandi peptíð hannað við tilraunaaðstæður höfðu bakteríudrepandi virkni. Hannaða peptíðið hjálpaði til við virknigreiningu og sýnt var fram næmni fyrir salti sem minnkaði virkni peptíðsins mikið. Peptíðið reyndist virka á himnu baktería og framkalla rof en hafði ekki áhrif á himnu heilkjörnunga. Bakteríusýklar reyndust seyta hindrum sem klipptu peptíðið og óvirkjuðu.

Genið fyrir cathelidín þorska er tjáð í hrognum og lirfum þorska og er tjáningin háð umhverfisþáttum. Í frumulínu frá laxfiskum reyndist tjáning á cathelidín örðuð af Gram-neikvæðum bakteríum og flagellín prótíninu. Í frumulínu frá þorski reyndist örvunin önnur og mest vegna *Lactobacillus* sp. og poly(I:C). Þetta munstur bendir til tjániga örvunar um viðtaka eins og TLR (Toll líkir viðtakar) sem þekkja einmitt munstursameindir örvera sem einkenna þær. Hindrun á PI3K kínasanum benti til að tjáningin á

cathelicidin væri bæld af þessum megin kílnasa frumunnar í frumlínu laxfiskanna.

Samantekið í meginatriðum var ný gerð varnarpeptíða einangruð úr þorski og skilyrði fyrir örvaða tjáningu greind. Virkni peptíðins og örvun bendir til mikilvægs hlutverks í ónæmiskerfi fiskanna.

*Dedicated to my grandmother Irma
Braakmann (1925-2012) and Bebi
(2010-2012).*

List of papers

- I. **Broekman DC**, Frei DM, Gylfason GA, Steinarsson A, Jornvall H, Agerberth B, Gudmundsson GH, Maier VH: Cod cathelicidin: isolation of the mature peptide, cleavage site characterisation and developmental expression. *Developmental and comparative immunology* 2011, 35(3):296-303.
- II. **Broekman DC**, Zenz A, Gudmundsdottir BK, Lohner K, Maier VH, Gudmundsson GH: Functional characterization of codCath, the mature cathelicidin antimicrobial peptide from Atlantic cod (*Gadus morhua*). *Peptides* 2011, 32(10):2044-2051.
- III. **Broekman DC**, Gudmundsson GH, Maier VH: Differential regulation of cathelicidin in salmon and cod. *Manuscript*.

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1. Introduction

1.1. Fish evolution

The first fish appeared on earth in the Cambrian period about 510 million years ago. Today more than 25000 different species of fish are known. The bony fishes in evolution became and have remained the dominant group of fishes throughout the world. Only about 800 species belong to the elasmobranchs. Other fish groups exist only in insignificant numbers of species [1, 2]. A discussion about the possibility of several genome duplications during the evolution of fish is based on the presence of hundreds of gene duplicates in teleost fish genomes [3]. Fish have a high variability in genome size and chromosome number, indicating a high genomic plasticity. This might have played an important role in speciation [3].

1.2. Atlantic cod

1.2.1. Biology

The family of Gadidae can be found in the Arctic, Atlantic and Pacific ocean. The Atlantic cod *Gadus morhua* (Linnaeus, 1758) is a marine species and is distributed along the shores of North America, Greenland, Europe to the Barents Sea. It can live at any salinity from nearly fresh water to high salt oceanic water and in a temperature range from nearly freezing to 20°C [4]. It is mostly found on continental shelves between 150-200 m depth but can also live in depth of more than 600 m. During the day the fish form compact schools that swim between 30 and 80 m above the bottom, and scatter at night. Some individuals or group of cod perform migrations of about 5 km per day. Cod living around Greenland have been shown to perform migrations of over 1000 km in total. The fish migrate to reach feeding or spawning grounds. Young cod remain on the nursery grounds until they join

the mature fish to make their first full spawning migration. They can reach maturity between 2-4 years of age. Cod are considered one of the most fecund fish, as a female can produce on average 1 million eggs. The number of eggs increases with the size of the female and the highest production recorded was 9 million eggs of a 34 kg female. The eggs as well as the larvae are pelagic and at about 2.5 months of age the postlarvae settle to the bottom. North Atlantic cod spawn between December and June, depending on the subpopulation. Spawning usually takes place in depth of less than 50 m and never beyond 200 m. The growth rate is different for the subpopulations. On average, a three year old male fish is 56 cm in length and a five year old male is 81 cm. The females are slightly longer. Cod can reach an age of 20 years and a maximum length of about 1.8-2 m [2]. They are omnivorous demersal feeders. Larvae and postlarvae feed on plankton, juveniles on invertebrates and older fish on other fish, including young cod, polychaetes, echinoderms or seaweed. Feeding occurs at dusk and dawn with the exception of fish of less than 20 cm that feed continuously [4].

1.2.2. Economic importance

Atlantic cod is a very popular food fish in Europe, North America and Brazil. It has been exploited in Europe since the beginning of fisheries [4]. It is one of the most commercial fish species in Northern Europe and North America's Eastern coast [5]. The worldwide catch has been declining steadily from the 1970s onwards, from 3.9 million tonnes in 1968 to 0.9 million tonnes in 2010 [4]. Atlantic cod is caught throughout the year and the countries with the largest catches are Iceland and Norway. In the 1980s production of cod juveniles in seawater enclosures was begun in Norway. From 2000 on another wave of cod aquaculture started mainly in Norway but also in the USA, Canada, UK, Iceland and other countries. Large hatcheries and modern technologies were developed and light manipulation allowed year-round egg production and delay of sexual maturation. Global cod aquaculture has increased from 205 tonnes in 1987 to 22000 tonnes in 2010. Aquaculture now offers the possibility for stable fish delivery throughout the year .

1.2.3. Diseases

Albeit cod being an economically important species, the susceptibility to diseases has not been fully elucidated since cod aquaculture has not progressed to an advanced and successful stage until recently. What can be said however is that many of the pathogens of cod are targeting the larval or juvenile stages and lead, among other reasons, to important losses during those early stages.

Vibriosis is considered as the most important bacterial disease in Atlantic cod and the Gram-negative bacterium *Vibrio (Listonella) anguillarum* is dominant among the pathogens isolated in connection with this disease. Especially serious mortalities have been reported from the serotype 02 β [6]. The main clinical signs are fin erosion and haemorrhages in the head region, especially in and around the eye [7]. Both typical and atypical *Aeromonas salmonicida* have been found in wild and cultured cod, and are considered to be among those bacteria that pose the greatest threat to cod [6]. This pathogen causes furunculosis and, in naturally infected fish caused mainly widespread granuloma formation with and without internal bacteria. Experimental challenges caused mortalities and haemorrhages, early stages of granuloma formation as well as necrotic changes were found in several organs. [7]. Bacteria of the genus *Tenacibaculum/Flexibacter/Cytophaga* cause the yellow pest in cod for which yellow nodules are typical. The clinical manifestation is severe ulcerative destruction of the integument, of cartilage and of bones, especially of the jaws. This disease occurs in early winter and spring [7]. The facultative intracellular bacteria of the genus *Francisella*, showing most similarity to *Francisella philomagia*, can lead to systemic granulomatous inflammatory disease. Moribund fish showed extensive gross lesions with moderate to massive occurrence of white partly protruding nodules in the spleen, heart, kidney and liver and numerous granulomas in these organs[7]. Several other bacteria like *Streptococcus paraubis*, *Mycobacterium sp.*, which is reported to mainly affect fish in display aquaria [6] and *Vibrio salmonicida* have been associated with pathology or mortalities [7].

One of the main viral pathogens in cod is the nodavirus which is a single stranded RNA virus. The disease it causes is referred to as either viral encephalopathy and retinopathy (VER) or viral nervous necrosis (VNN). The main target for the nodavirus is the central nervous system including the brain, spinal cord and retina, where it causes extensive cellular vacuolation and neuronal degeneration. This virus particularly affects larval and juvenile stages and leads to mass mortalities [6]. Transmission routes are not fully elucidated but in theory vertical or horizontal transmission is possible. Infectious pancreatic necrosis virus (IPNV) is a member of the Birnaviridae [8]. Screenings have shown that IPNV is present in cod and outbreaks of IPN in cod fry have been reported [7]. Upon examination, abnormally pale spleen, kidneys, liver and heart are revealed and ascitic fluid might have accumulated in the body cavity. Vertical and horizontal transmission is considered possible [7], but trials with juveniles did not show any susceptibility [6]. The viral haemorrhagic septicaemia virus (VHSV) has been isolated from cod and in experimental trials susceptibility was demonstrated, however only after injection [6]. Clinical signs are the presence of exophthalmia and ascites. Only juvenile cod were reported to be susceptible while it was not possible to infect adult cod horizontally through bath challenge or cohabitation [6]. The primary cause of the cod ulcer syndrome is suggested to be an iridovirus [9]. But also large amounts of secondary *Listonella anguillarum* could be isolated from the ulcers. In this disease various skin lesions are observed all over the fish that start as papules and develop into ulcers. This disease is widely distributed in all ages [7]. Atlantic cod was found to be resistant to infectious salmon anaemia virus (ISAV) [6].

Fungal diseases reported from cod are e.g. Saprolegniaceae fungi which were found in larvae but not in adults. Further, microsporidia have been described from the gills of cod. The impressive number of 107 named parasites, including ecto- and endoparasites were listed for Atlantic cod [6], which, although they can infest the fish in high number and cause serious pathologies, shall not be discussed here.

1.3. Chinook salmon

1.3.1. Biology

Chinook salmon *Oncorhynchus tshawytscha* (Walbaum, 1792) is the largest of the Pacific salmon species and is distributed from California, over Canada to Alaska in Northern America and from Japan to Russia in Northeastern Asia. They possess a complex and diverse life history strategy and have been suggested diverging into stream-type Chinook salmon that reside in freshwater for a year or more after hatch and ocean-type Chinook salmon that migrate to the ocean within their first year. 10-18% are reported to be stream-type, while 82-90% are ocean-type [10]. A significant process in juvenile life-history is smoltification, the physiological and morphological transition from a freshwater to a marine existence. Pacific salmon are generally anadromous (with the exception of 10-12% of juvenile males): egg incubation and hatching takes place in fresh water, the juveniles then migrate to the ocean where maturation is initiated, and they return to freshwater to complete maturation and to spawn. They have a strong homing ability and usually return to their natal streams for spawning [2]. Fecundity ranges from 2000-17000 eggs per female. The size of a nest is approximately six square meters in average and females dig shallow depressions, deposit a pocket of eggs and cover it with gravel. Over the course of one to several days the female deposits four or five pockets of eggs [10]. All anadromous individuals die after spawning [2]. Chinook salmon remain at sea 1-6 years, more commonly 2-4 years. Outmigrating juveniles are opportunistic feeders with food items such as aquatic insect larvae and adults, *Daphnia*, amphipods and *Neomysis*. They grow into predators of fish and crustaceans. Fry and fingerlings of ocean-type Chinook salmon reside in estuaries for some time, while stream-type fish move quickly through the estuary into coastal waters and the open ocean [10]. The vertical distribution in the ocean varies with season, ranging from 0-100 m and the vertical distribution of Chinook salmon is typically deeper than the distribution of other salmonids. The size of spawning adults range from 75-80 cm. The maximum recorded size was 150 cm.

The average spawning adult weight is 45 kg with highest weight recorded 61.4 kg. The adults tolerate water temperatures of 3.3-19.4°C, while 21.1-22.2°C were reported lethal [10].

1.3.2. Economic importance

Chinook salmon is caught on sea but is also an important game fish. The global capture has been declining, with catches in 1989 being approximately 20000 tonnes and in 2010 being 8000 tonnes. It is mostly caught in the USA and the Russian Federation. Chinook salmon aquaculture became relevant in 1984 with a quantity of 135 tonnes and in 2010 amounted to 13000 tonnes [11]. Accordingly, the amount of chinook salmon farmed is now higher than the amount caught in the wild. Canada and New Zealand are among the main producers of Chinook salmon with New Zealand accounting for about half of the global production [12].

1.3.3. Diseases

Among the bacterial diseases affecting salmon is typical furunculosis which is caused by the Gram-negative bacterium *Aeromonas salmonicida*. The disease is characterised by furuncle-like swelling and ulcerative lesions on the skin and in the musculature. The acute form is found in growing fish and usually leads to death within 2-3 days, which does not allow for furuncles to develop. In the chronic form, which affects older fish and has low mortality, furuncles are likely to be observed, while other signs are found to a lesser degree. Shedding of *A. salmonicida* into the environment by infected fish leads to horizontal transmission [13]. Vertical transmission however is not considered as a significant route of infection [14]. Another important bacterial pathogen is the non-motile Gram-positive bacterium *Renibacterium salmoninarum* which is the causative agent of bacterial kidney disease (BKD). It affects the liver where focal necrosis can be found and causes a chronic bacteraemia. The kidney is enlarged and contains granulomatous lesions. Evidence exists that BKD is transmitted vertically from infected adults via

the egg, and horizontally through viable bacteria in the water and faeces of infected fish. Intraovum infection resulted in subclinically infected offspring that did not show mortalities [15]. Enteric redmouth is caused by the Gram-negative bacterium *Yersinia ruckeri*. Subcutaneous haemorrhaging leads to reddening of throat and mouth, hence the name, which can progress to erosion of the jaw and palate. Internal signs are congested blood-vessels, haemorrhages on the liver, pancreas, swim-bladder, muscles and adipose tissue and fluid may be in the stomach and intestine. The main way of infection is considered the horizontal transmission [16].

A significant viral pathogen of salmonids is IHNV (infectious haematopoietic necrosis virus), which has been isolated from Chinook salmon. It is a rhabdovirus [17] and typically causes necrosis of the haematopoietic tissues and often mortality. In chinook salmon, fry may have a subdermal haemorrhagic area immediately behind the head. The inner organs of fry are pale due to anaemia. Older fish are less susceptible to IHNV, while yolk-sac fry and fish up to 2 months are highly susceptible. Vertical and horizontal transmission takes place. IPNV (infectious pancreatic necrosis virus) is an aquatic birnavirus and is a non-enveloped double stranded RNA virus. IPNV causes mortality in young salmonids and rarely affects yearlings or older fish. External signs include hyperpigmentation, pop-eye and petechial haemorrhage on the ventral surface. The disease is transmitted vertically from previously infected brood-fish and horizontally through virus-containing faeces and urine [18]. ISAV (infectious salmon anaemia virus) likely belongs to the orthomyxoviridae and is an enveloped single stranded RNA virus. [19]. Internally, ascites, changes and damage to the liver and spleen, damage in the visceral fat and sometimes congestion of the foregut can be observed. In cohabitation experiments, horizontal transmission has been shown. Skin, mucus, faeces and urine can be the transmitting vectors and gills and skin injuries are the probable routes of entry. No evidence of vertical transmission has been found [20]. The pathogen causing VHS (viral haemorrhagic septicemia) belongs to the rhabdoviridae and contains a single stranded RNA molecule. Internally, the kidney is enlarged and darkened or even necrosed

and pale. The liver is pale and shows haemorrhages. Horizontal transmission has been demonstrated [21].

Fungi and parasites affecting Chinook salmon are members of the genus *Dermocystidium*, *Saprolegnia* and *Ichthyophonus* [22, 23].

The fishes Atlantic cod and Chinook salmon were chosen for this study on cathelicidin antimicrobial peptides because of their economic importance and the lack of knowledge of the role of cathelicidin in these species.

1.4. Disease management in aquaculture

1.4.1. Atlantic cod

The main diseases currently affecting wild and farmed cod are francisellosis, listonellosis (vibriosis), atypical furunculosis and viral nervous necrosis (VNN). Juveniles are routinely vaccinated against vibriosis, while a vaccine against atypical furunculosis is being developed. No vaccine is available against francisellosis, so broodstock screening and avoidance of high water temperature are measures of prophylaxis [5]. Against listonellosis, francisellosis and atypical furunculosis oral administration of antibiotics is used. Countermeasure against the parasitic diseases gyrodactylosis and trichodinidosis is bath treatment in formaldehyde, while against lomosus no measure is described [5].

The immune system of cod does not contain MHC II [24] which in vertebrates is usually responsible for bacterial and parasitic antigen presentation. This can have implications for vaccine efficacy. However, vaccination of cod is possible [25], but does not seem to be as easy to achieve as in other fish like salmonids due to a weak specific antibody response [26]. Opposed to this, cod have been shown to have high titers of so called natural antibodies that are present in the circulation without prior stimulation, have a broad but characteristic specificity [27] and have demonstrated increased levels after vaccination [28].

1.4.2. Salmon

Many viral diseases are affecting salmonids like ISA, VHS and IPN. Vaccines against these diseases are not yet available although they are partly in development. Vaccination is available against the Salmon Pancreas Disease virus. For prophylaxis or treatment of the bacterial diseases furunculosis, winter ulcer and enteric redmouth vaccines or antibiotics are being used. Against bacterial kidney disease broodstock screening and good management is applied. Against the fungal disease saprolegniasis bronopol/formalin bathing is applied with the latter being similarly applied against freshwater protozoa *Ichthyobodo*, *Trichodina* and *Ichthyophthirius*. Sea lice infections are being treated chemically by bathing or oral medication. Similarly chemical treatment/medication exists for tapeworm infections. Gill amoebas are being combated with freshwater baths.

In salmonids, vaccination is in general possible and successful. High titers of specific antibodies are observed around 6-12 weeks post-vaccination and are described as being correlated to survival [29]. However, still some confusion exists concerning the role of specific antibodies in protection from disease in fish after vaccination and the contribution of other humoral or cellular factors to protection is considered a possibility [30].

1.5. Fish immune system

The lymphoid organs are responsible for the generation of cellular immune factors which include T cells, B cells, cytotoxic cells, macrophages and polymorphonuclear leukocytes [31, 32]. The lymphoid organs in fish are thymus, spleen and kidney. The thymus consists of a thin layer of lymphoid tissue of a defined oval shape that is located on the insides of the opercula. It contains mostly macrophages that support the production of T-cells. Also, myeloid cells and eosinophilic granular cells can be present in the thymus. In zebrafish, the thymus develops from neural crest cells of the neuroectoderm [31].

The kidney in fish can be equalled with the bone marrow in vertebrates due to its haematopoietic role. From a young age it already produces red blood cells and granulocytes. B-cells are present that produce IgM. The anterior kidney (head-kidney) consists of a network of reticular fibres that support the lymph tissue. The cells with the highest abundance in the head-kidney are macrophages that form into melanomacrophage centers and lymphoid cells which are mostly mature B-cells. Reticular cells within this organ support the function of lymphoid and endothelial cells. The sinusoids in the kidney are the main structures for filtering the blood through endocytosis [31].

The spleen contains melanomacrophage centers, lymphoid tissue and ellipsoids. The macrophages phagocytose antigen from the blood and may retain them or the specific antibodies against them for long periods of time which plays a role in immune memory. The spleen is further an important haematopoietic organ [31].

The immune system is customary divided into innate and adaptive immunity. Innate immunity is considered the main branch of immune defense in fish, due to limitations of adaptive immunity like low antibody variety and slow proliferation, maturation and memory of the lymphocytes, which is also attributed to low body temperature/poikilothermia [33].

The following factors constitute innate immunity. When a pathogen comes into contact with a fish, the surface epithelia like skin, gills and intestines are the first barriers to pathogen entry. The mucus on those epithelia contains lectins, pentraxins, lysozymes, complement, antimicrobial peptides and immunoglobulin. In the epidermis, lymphocytes, macrophages and eosinophilic granular cells are present to defend the fish [31, 33]. Antimicrobial peptides secreted onto the integument have the ability to inactivate bacteria, viruses, fungi, and protozoa [34, 35]. Phagocytosis is mainly effected by neutrophils and macrophages and is opposed to other immune factors rather unaffected by temperature. These phagocytotic cells produce reactive oxygen species through respiratory burst which can kill ingested pathogens. Myeloperoxidase, lysozyme and other enzymes in the

lysosomes also aid in killing off the ingested pathogens. Lysozyme is widely distributed in the body like in serum, secretions, mucous membranes and in tissues like kidney and intestine and mainly in monocytes/macrophages and neutrophils, but also in intestinal eosinophilic granular cells. Lysozyme hydrolyzes the peptidoglycan of bacterial cell walls which leads to cell lysis of both Gram-positive and Gram-negative bacteria [31]. Complement is another important component of the fish immune system and can be triggered in three different ways: through the binding of antibody to the cell in the classical pathway, through the direct binding of foreign microorganisms in the alternative pathway, or through the binding of lectin attached to the bacterial cells in the lectin pathway [31]. The cytokines TNF- α and - β in fish have been shown to activate macrophages. Interferons have been shown to induce antiviral genes and therefore play a role in non-specific antiviral defense. IL-1 β has been detected in 13 teleost fish species so far. Its role is to regulate immunity through the stimulation of T-cells and it is upregulated through microbial PAMPs. IL-6, just like TNF- α and IL-1 β induces inflammatory responses against Gram-negative bacteria in fish. Also, the cytokines granulocyte colony stimulating factor (CSF), macrophage-CSF and IL-7 have been identified in fish. Further, chemokines are also present in high numbers in some fish species [31]. Protease inhibitors are present in the circulation or other body fluids to inactivate proteolytic enzymes secreted by pathogens. Natural antibodies which are produced by B cells without stimulation through PAMPs, are found in high numbers in serum and are considered key components of nonspecific immunity. C-reactive protein has been found in several fish species and is upregulated due to tissue injury, trauma or infection. It can activate the classical complement pathway and the removal of apoptotic cells through opsonization [31].

Fish, agnathans excluded, are the earliest class of vertebrates to possess the elements of both innate and adaptive immunity, including immunoglobulin-producing B-cells. Specific antibodies are part of the adaptive immune system. Antibodies are found in skin, intestine, mucus, bile and serum and specific Ig can be generated locally without a systemic response [31]. The most

abundant immunoglobulin (Ig) in bony fish is IgM which is usually tetrameric. Teleost B-cells are further expressing IgD and IgT, the latter of which is mostly found in mucosa [36]. In salmonids, specific antibody production takes at least 4-6 weeks, which indicates why innate immunity is important since many pathogens can kill the fish within a few days [37]. Cod does not produce a strong specific antibody response to vaccines that produce prominent responses in other species, although vaccination against vibriosis and furunculosis has shown some success [25]. However, the levels of Ig in cod (5-20 mg/ml) were higher than in other species (1-3 mg/ml). Further, the absence of Major Histocompatibility Complex class II and TLR 5 demonstrate that cod has a unique immune system [24, 38].

Environmental factors can influence the immune response of fish. Temperature affects the rate of physiological processes through modulation of enzyme activity. Increasing light exposure was shown to decrease the number of circulating leucocytes, while less light led to an increase. Increase of photoperiod can increase the activity of lysozyme and the circulating IgM. Low oxygen levels decreased the respiratory burst in macrophages and the levels of circulating IgM, while high oxygen concentration increased the IgM levels. High levels of suspended solids increased lysozyme activity and IgM levels. Elevated salinity increased the activity of lytic enzymes, the respiratory burst of macrophages and IgM levels. The stress in intensive culture system is said to increase cortisol levels which in turn downregulates innate and adaptive immunity [31].

Immunostimulation via the food, bath or injection is an established means of temporarily strengthening the immune defense of fish. It has been shown that nucleotide supplementation increased the resistance of salmonids against pathogens as well as improving the effectiveness of vaccination by influencing activity of macrophages, natural killer cells, lymphocytes and Ig production, increasing complement activity and production of superoxide anions in phagocytes. Various forms of β -glucans are used for immunostimulation of fish, either in the food, as injection or as vaccine adjuvant. It potently activates

nonspecific defenses by increasing the activity of macrophages, complement and levels of lytic enzymes and improves the efficiency of vaccination [31].

Probiotics influence the microbial composition on body surfaces by suppressing the spread of pathogenic microbes in the intestines. The probiotics employed belong to the genus *Lactobacillus*, *Bacillus* or to the yeasts, but also other microbes are used. They improve the health status of the fish and may augment the innate responses through TLRs or other receptors [31].

1.6. Ontogeny of immunity

The defense systems of freshly hatched fish larvae are not yet fully developed. In the first 2-3 month after hatch, cod larvae might rely solely on innate immune parameters, which include phagocytes, complement, lectins, lytic enzymes, AMPs and proteinase inhibitors as well as a variety of pattern recognition receptors including the TLRs [39]. In cod larvae it was found that IgM and C-reactive protein were not present in the first 57 days after hatch [40]. B-cells and Ig were detected in cod head-kidney 58 days post hatch. The order of appearance of lymphomyeloid organs during the ontogenesis of marine fish is in general: kidney, spleen, thymus. B-cells and immunoglobulin appear later in marine fish than in freshwater species [41]. For cod it was reported that lymphocytes appeared in the thymus 28 days post hatch, and in the spleen 51 days post hatch. In zebrafish, B cells and Ig in the head-kidney as well as in the thymus appeared three weeks post hatch, while T cells in the thymus were already found 4 days post hatch. Head-kidney and thymus have been observed to be lymphoid before hatching in rainbow trout and Atlantic salmon [42]. Full immunocompetence is less defined by the presence of lymphoid organs and lymphocytes but rather by their functioning [43]. The timing until full immunocompetence is developed varies between different fish species, even closely related ones, and is also influenced by environmental conditions. Full maturation of the zebrafish immunesystem is reached at 1-2 months of age. Full immune responses in rainbow trout were observed from an age of 3 months [43].

Fish larvae also derive some protection from maternally derived defense proteins [42]. It has been shown that maternal Ig is transferred from mother to offspring which may protect both the embryo in the egg as well as the larvae before autologous Ig is produced at 2-3 months post hatch [33]. Antibodies raised in the maternal circulation are incorporated into the oocytes and then move from the larval yolk sac into the larval circulation and have the ability to recognize antigens and thereby defend immunity in the offspring. Not in every case however is the immunity of an adult transferred to the offspring, suggesting another role of maternal Ig. Also, maternal complement component C3 has been reported to be transferred to the offspring, as well as protease inhibitors and lysozyme [42].

1.7. Antimicrobial peptides

Within the last few decades, antimicrobial peptides have been recognized to be essential components of innate immunity in animals and the number of newly discovered and characterized peptides is constantly growing. From plants 272 peptides have been described, 157 from bacteria, 810 from amphibia, 60 from human and 60 from fish [44]. Antimicrobial peptides (AMPs) have first been recognized for their antimicrobial activity which gives them the role of innate antibiotics. Next to AMPs able to kill bacteria, there are AMPs that are antifungal, antiviral, or antiprotozoal [34, 35]. Antimicrobial mature peptides are very heterogenous regarding their sequence and size but most of them contain between 12-50 amino acids [45]. They are usually cationic and amphipathic and are either constitutively expressed or upregulated through stimuli. AMPs are categorized according to their secondary structure. These categories are α -helical peptides, peptides with a β -sheet structure and linear peptides with a predominance of certain amino acids. Multicellular organism tend to have a variety of different peptides from those structural groups in their tissues [34].

The antimicrobial action of AMPs is selective for microbial cell membranes, while not being as harmful for eukaryotic cell

membranes. The eukaryotic cell membrane surface of plants and animals is neutral and negatively charged phospholipids in the membrane are facing the inside of the cell. The cholesterol, which is present in animal membranes, is further thought to stabilize the membrane in the face of an interaction with AMPs. The bacterial cell membrane however contains many negatively charged phospholipids on the surface. The positive charge of cationic AMPs is thought to enter into an electrostatic interaction that attracts the peptides to the bacterial membranes. The interaction of the peptides with the membranes will lead to a disturbance in the lipid bilayer. This will either allow the peptides to migrate through the membrane to the interior of the cell where they will disturb intracellular targets, or will destabilize the membrane to a degree that will lead to cell lysis [34].

AMPs are expressed as inactive (pre-)pro-peptides and require processing to release the antimicrobial mature peptide part. This is why expression of respective proteases is required and their presence or absence is a means of regulation of AMP activity. It was also found that AMPs can be developmentally regulated, with constitutive expression at specific sites in early life stages that decrease with maturation of the individual. Induction of AMP expression is often a response to microbial ligand binding by pattern recognition receptors or through stimulation by cytokines [46].

Increasing evidence has suggested that AMPs not only kill microbes but also exert an array of additional effects that aid in the resolution of infection and inflammatory processes, thereby complementing the antimicrobial activity [46]. Among these additional functions is chemotactic activity [47]. This includes the direct recruitment of leucocytes to the site of infection or injury and the induction of chemokine and cytokine expression which indirectly promotes the recruitment of effector cells. AMPs can further inhibit TLR dependent responses [48], seen in the inhibition of proinflammatory gene expression. Another facet of the ability to modulate inflammatory responses is the ability of AMPs to block the binding of LPS to macrophages [49], which is thereby inhibited to trigger inflammation [46]. Another important

role of AMPs is their ability to promote wound healing [50] and angiogenesis [51], which is effected through the stimulation of migration and proliferation of endothelial cells, thereby improving the regeneration after an infectious or injurious insult [46]. In summary, AMPs can alert, mobilize and amplify innate and adaptive immune responses of the host.

AMPs are present in different tissues each of which can have a different environment, is exposed to different pathogens and harbors a specific resident microflora. Their place of expression defines their activity spectra, expression pattern and regulation [52]. In humans, AMPs are expressed in the skin and sweat glands. This way, the skin, which is a potential site of entry for bacteria, is equipped with a chemical barrier to infection. In the case of injury there is rapid production and release of AMPs. The epithelia of the oral cavity, the stomach and the intestines express AMPs in a constitutive fashion. Further, AMPs are present in the epithelia of the respiratory system and the urogenital system, the kidney and liver, the eye surface, some bodily fluids and in lymphocytes [52].

1.8. Antimicrobial peptides in fish

Fish are the earliest class of vertebrates that possess innate and adaptive immunity. Due to the shortcomings of fish adaptive immunity in comparison with that of higher vertebrates, the fish innate immunity therefore has an important role in immune defense of fish and might even be the more important branch of immunity. AMPs are a crucial component of mammalian innate immunity and as more fish AMPs are identified this brings more evidence that they are important effectors in fish immune defense, too. The discovered fish AMPs have shown similar antimicrobial activities *in vitro* as those from mammals, which indicates an important role in immune defense. Further, as immunomodulatory functions of fish AMPs are reported [53], this suggests a conserved role and function of AMPs.

Many of the first AMP isolations from fish derived the peptides from the skin mucus. The body surface that includes the skin, the

gills, the ocular surface and the intestines is the portal of entry for pathogens and therefore needs to provide protection against microbes. It has since been shown that fish contain different AMPs in the surface mucus. A considerable part of the known fish AMPs have not been directly isolated from tissues, but have been predicted from nucleotide sequences (Table 1).

Pleurocidin was isolated from the epithelia of winter flounder. It was not inhibited by physiological salt concentrations, was active against both Gram-positive and negative bacteria and was found to be located in the mucus cells of the epithelia [54]. Pardaxin was isolated from glands along the dorsal anal fins of Red Sea Moses sole in secretions that were reported to be toxic to fish and to repel sharks. It is an acidic peptide [55] and demonstrated potent activity against Gram-positive and -negative bacteria [56]. Parasin was isolated from the mucus of injured skin from catfish and was not found in uninjured skin. It showed strong antimicrobial activity towards Gram-negative and -positive bacteria and fungi, while not being hemolytic. MICs were in the range of 1-4 µg/ml, which make it one of the most potent AMPs found [57]. An AMP identified as histone H2A was isolated from rainbow trout mucus and skin epithelium. It had potent activity towards Gram-positive bacteria and demonstrated sensitivity towards salt. Occasionally, as in this case, newly discovered AMPs turn out to be known proteins or protein fragments not previously expected to have antimicrobial properties. Histones have also elsewhere been shown to have antimicrobial activity [58]. A hepcidin AMP was isolated from the gills of hybrid striped bass, but was subsequently also found to be upregulated in the liver following bacterial challenge. It was active against *E. coli* [59]. Another study found antimicrobial peptides in rainbow trout erythrocytes that displayed antimicrobial activity [60].

Defensins are present in fungi, plants, invertebrates and vertebrates and their ancestor has been traced to be a bacterial defensin-like peptide. β -defensins are the largest family of vertebrate defensins and have been initially isolated

Table 1. Selected antimicrobial peptides in fish. I = isolated peptide, C = cloned nucleotide sequence.

Peptide name	Fish species	pI (pre-pro-protein)	Mol. weight (kDa) (pre-pro-protein)	Source	Reference
pleurocidin	winter flounder (<i>Pseudopleuronectes americanus</i>)	6.8	7.6	I	[54]
pardaxin	red sea moses (<i>Pardachirus marmoratus</i>)	8.59	3.6	I	[55]
moronecidin	white bass (<i>Morone saxatilis</i>)	6.39	9.2	I	[61]
chrysopsin 1-3	red sea bream (<i>Chrysophris major</i>)	12.3, 11.7	12.5, 2.9, 2.3	I	[62]
oncorhyncin II	rainbow trout (<i>Oncorhynchus mykiss</i>)	10.97	20.8	I	[63]
piscidin 1,3	hybrid striped bass (<i>Morone saxatilis</i> , <i>M. chrysops</i>)	10.9	8.4	I	[64]
misgurin	loach (<i>Misgurnus anguillicaudatus</i>)	11.8	2.5	I	[65]
hepcidin	gilthead seabream (<i>Sparus aurata</i>)	8.09	9.7	C	[66]
hepcidin	tilapia (<i>Oreochromis mossambicus</i>)	6.05	9.7	C	[67]
hepcidin	hybrid striped bass (<i>Morone chrysops</i> , <i>M. saxatilis</i>)	6.05	9.5	I	[59]
gcLEAP-2	Chinese grass carp (<i>Ctenopharyngodon idella</i>)	9.2	10.4	C	[68]

continued

Table 1. Selected antimicrobial peptides in fish. I = isolated peptide, C = cloned nucleotide sequence (continued).

saBD	gilthead seabream (<i>Sparus aurata</i>)	7.55	7.1	C	[69]
epinecidin	orange-spotted grouper (<i>Epinephelus coioides</i>)	8.02	7.7	C	[70]
β -defensin	mandarin fish (<i>Siniperca chuatsi</i>)	8.9	7.3	C	[71]
β -defensin	common carp (<i>Cyprinus carpio</i>)	8.2	7.2	C	[72]
Cathelcidins					
HFIAP-1,-3	Atlantic hagfish (<i>Myxine glutinosa</i>)	5.02, 4.4	20, 18.2	I	[73]
aCATH	ayu (<i>Plecoglossus altivelis</i>)	9.49	19.6	C	[74]
codCath	Atlantic cod (<i>Gadus morhua</i>)	10.5	20.5	C, I	[75] [76]
rtCath-1,2	rainbow trout (<i>Oncorhynchus mykiss</i>)	9.4, 8.96	23.6, 22.7	C	[77, 78]
asCATH_1,2	Atlantic salmon (<i>Salmo salar</i>)	9.82, 9.36	22.9, 21.9	C	[78]
btCath 1,2	brook trout (<i>Salvelinus fontinalis</i>)	10.1, 10	23.9, 16.8	C	[79]
cathelcidin	grayling (<i>Thymallus thymallus</i>)	8.9	20.1	C	[79]
csCath	chinook salmon (<i>Oncorhynchus tshawytscha</i>)	8.76	17.9	C	[80]
btrCath	brown trout (<i>Salmo trutta fario</i>)	9.5	22.3	C	[79]
acCath	Arctic char (<i>Salvelinus alpinus</i>)	9.5	13.6	C	[75]

from the neutrophils of cattle [81]. The precursor consists of a signal peptide and a mature peptide and the genes consist of two exons and one intron. An evolutionary relationship between β -defensins and big defensins, which are present e.g. in horseshoe crab, mussels and amphioxus, has been demonstrated in that the C-terminal part of big defensins corresponds to β -defensins, containing the typical 6 cysteines. It is hence suggested that vertebrate β -defensins originate from ancestral big defensins. β -defensins have been identified in several fish species including zebrafish, fugu, green spotted pufferfish, rainbow trout, medaka, olive flounder, orange spotted grouper and mandarin fish and antimicrobial activity was reported [71].

1.9. Cathelicidin

The AMP family of cathelicidins is restricted to vertebrates and the most elementary vertebrate showing the presence of these peptides so far is the Atlantic hagfish [73]. It has been suggested that there is a common ancestral origin of cystatins (cysteine protease inhibitors) and cathelicidins [76]. Cathelicidins were first discovered in mammals in myeloid cells and neutrophils, where they are stored as inactive proforms [82]. They are secreted by exocytosis where they are thought to encounter the protease that cleaves off the mature peptide and thereby activates it. Cathelicidins from different species have in common that the protein consists of three domains: the N-terminal signal sequence that is cleaved off co-translationally, a very conserved propeptide, the so-called cathelin domain containing 4 cysteine residues, and a very variable C-terminal mature peptide domain. Most cathelicidins are encoded by four exons (Fig. 1).

The conserved cathelin domain has the ability to inhibit the cysteine protease cathepsin-L, which is the reason for the naming [46]. The precursor cathelicidin protein consisting of the propeptide and the peptide part does not show antimicrobial activity. Through enzymatic processing intra- or extracellularly, the peptide part is liberated and thereby becomes antimicrobially active [46]. The function of the liberated propeptide has not been

fully elucidated, but has been ascribed some antimicrobial and protease inhibitory activity [83].

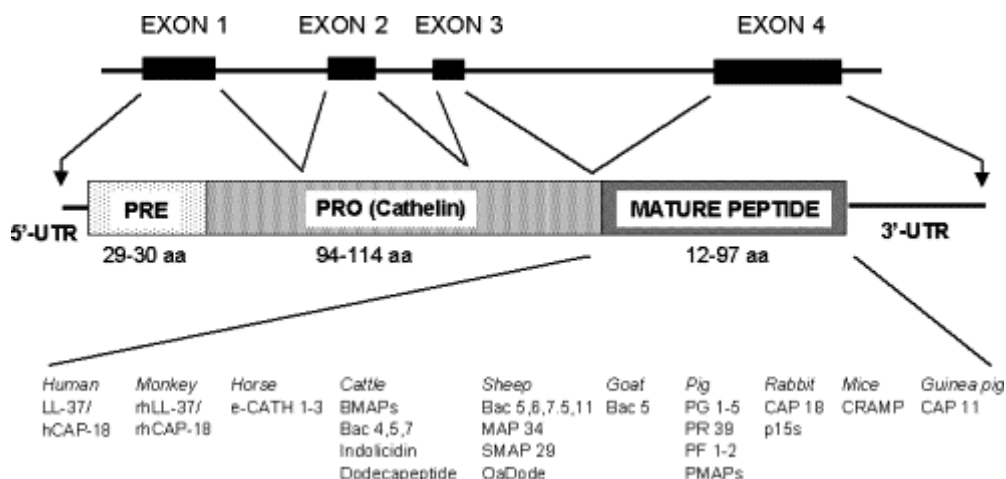


Fig.1. General gene structure of cathelicidins, picture taken from [84]. The cathelicidin gene consists of four exons that encode a pre-pro-peptide. Examples of different mammalian mature peptides are given.

In the case of the human cathelicidin, one of the processing enzymes that cleaves off the mature peptide part from the propeptide is proteinase 3. In many other mammals elastase has been indicated. Kallikreins can process the mature cathelicidin in humans into smaller derivatives. These derivatives have been reported to have a different spectrum of activity [46]. Humans and mice have been reported to have only one cathelicidin, while other mammals have several [85]. Within and between species the secondary structures of cathelicidin vary. These can be linear structures rich in certain amino acids, β -sheet structures with disulfide bonds, and α -helical structures.

1.10. Fish cathelicidin

The cathelicidins are among the most well researched AMPs in mammals and have been found in other vertebrate groups like birds [86], reptiles [87], amphibia [88] and fish [78]. In fish several cathelicidins have been discovered most of which are derived from salmonid fish so far. Both mammalian and fish cathelicidin genes contain four exons and three introns, with a few exceptions. The first three exons encode the pre-pro-part which includes the signal sequence and the cathelin domain, while the cleavage site and the antimicrobial peptide domain are encoded by the fourth exon. In comparison with mammalian cathelicidins, fish cathelicidins have a shorter signal peptide [79].

The first fish cathelicidins were isolated from the intestines of Atlantic hagfish (*Myxine glutinosa*) and called HFIAP-1 and -2 (hagfish intestinal antimicrobial peptide) which are encoded by the genes *MgCath37* and *MgCath29*, respectively, and the sequence identity between the genes was said to be 96%. The mature peptides contain bromotryptophan which is an unusual amino acid with a post-translational modification. They were localized in the haematopoietic tissue around the intestines but not in the epithelial layer of the gut. They were described to be potently active against a variety of Gram-positive and Gram-negative bacteria and not against a fungus [73].

The salmonid cathelicidins can be divided into two classes due to the mature peptide sequences which includes two cysteine residues in the peptide region of the type 1 gene, while the type 2 genes lack these residues. Not in all salmonids genes of both types are found [75].

In the Atlantic salmon and rainbow trout two cathelicidins have been found (Atlantic salmon asCath_1; asCath_2, rainbow trout rtCath_1, rtCath_2). In general there is high sequence homology in the first three exons between these four salmonid cathelicidins while there is strong variation in exon 4. It was shown that rtCath_2 was constitutively expressed in gill, head kidney, intestine, skin and spleen, while rtCath_1 was only expressed after

a bacterial stimulus in gill, head kidney and spleen, but not in intestine and skin. The constitutive rtCath_2 expression increased locally after a bacterial stimulus especially in gill, head kidney and intestine. Synthetic peptides of rtCath_1 and rtCath_2 derived from predicted cleavage sites were strongly active against Gram-negative bacteria, showed less prominent activity against Gram-positive bacteria, and the two peptides differed in their activity spectra. Sensitivity of rtCath_1 to the divalent cations calcium and magnesium but not to monovalent sodium was demonstrated [77].

Two cathelicidin genes were found in brook trout (*Salvelinus fontinalis*), while one each was found in grayling (*Thymallus thymallus*) and brown trout (*Salmo trutta fario*). Exon 3 in cathelicidin 2 Cath2_Salfo of brook trout is not present and the gene generates a functional transcript where exon 2 is joined to exon 4 [79]. The cathelicidin 1 of brown trout was expressed in the head-kidney, trunk-kidney, spleen and to a lesser degree in brain, stomach, skin and gills of uninfected fish. Expression in immunologically relevant tissues like head-kidney and spleen as well as tissues that are the interface with the environment indicate a role in immune defense [79]. One cathelicidin gene was found and cloned in Chinook salmon (*Oncorhynchus mykiss*). Bacterial DNA and protein upregulated the gene expression [80]. The grayling, Arctic char and Chinook salmon cathelicidins have been assigned to the cathelicidin 2 group by phylogenetic analysis [79]. Another salmonid that was discovered to have an exon three deletion in the conserved region of its cathelicidin gene was the Arctic char (*Salvelinus alpinus*) [75]. This peptide (acCath) was initially cloned from the gill tissue and is suggested to be a member of the group 2 cathelicidins. The charr cathelicidin acCath was found to be expressed constitutively in all tissues studied with the most prominent expression in spleen and kidney and was also observed in skin. Expression was also further upregulated through infection [75].

From Atlantic cod (*Gadus morhua*) spleen a cathelicidin pre-pro-peptide of 193 amino acids was described whose predicted mature peptide codCath1 was rich in arginine (21%), serine

(25%) and glycine (35%) residues with four repeats of the sequence GSRGS. Further searches showed two more putative cathelicidin sequences in cod. The Atlantic cod cathelidins are said to form a separate group due to their evolutionary distance from the salmonid cathelidins and their unique sequence giving a very cationic mature peptide. The expression of codCath1 was found to be constitutive in spleen and kidney, with weaker expression in gills, pyloric caeca and the intestines. Upregulation due to a bacterial stimulus was observed in the liver and pyloric caeca and to a lower extent in the gills. No expression of cathelicidin was observed in the skin of cod unless the fish were infected [75].

The latest cathelicidin reported from fish has been found in liver of ayu (*Plecoglossus altivelis*). Upon infection aCath was expressed in liver, spleen, head-kidney, gill, intestine and muscle [74]. It had a high constitutive expression in liver, head-kidney and spleen. The full pro-peptide had no antimicrobial activity, while the synthetic mature peptide demonstrated activity against Gram-negative and not a Gram-positive bacterium, indicating a role in immune defense [74].

1.11. AMPs in disease prevention

Fish are affected by viruses, bacteria, fungi and parasites. In the intensive production systems in aquaculture, diseases can occur often and cause high economic losses. With increasing understanding of the important role of AMPs in immunity the question arises how this knowledge can be used for disease prevention in aquaculture. The usual strategies to prevent economic losses due to fish diseases are prophylactic measures like vaccination, antibiotic and chemical treatment, and the prevention of spreading of diseases from infected animals.

New avenues taken are e.g. to transfect fish cells with foreign AMP genes which has shown that the peptides expressed by these cells are functional [89]. Transgenic medaka possessing a foreign gene for cecropin were shown to be more resistant to a pathogen

challenge than non-transgenic fish [89]. It remains to be shown how effective the generation and subsequent use of transgenic fish in aquaculture is in terms of disease resistance and the prevention of disease outbreaks, since such foreign genes might have other additional effects. Another study has demonstrated that the continuous application of a synthetic AMP derived from either insects or fish significantly increased the survival of coho salmon during an infection challenge. This study therefore demonstrated *in vivo* efficacy of antibacterial activity of synthetic AMPs and mimicked the physiological situation in a transgenic fish that expresses a foreign AMP [90]. It should be noted however, that a longterm exposure of a microbe to an AMP can eventually lead to the development of resistance [91]. If such a resistance would spread it could have serious consequences. Also selection for naturally resistant pathogens could occur which would then expose populations to a higher disease risk.

Recently it was suggested to measure the tissue concentration of AMPs to obtain an indication of the chronic stress level of fish, since stress has been shown to downregulate several immune factors [92]. This would be an innovative strategy to manage the disease risk in fish and a warning system for impending disease problems, which can then be counteracted through improvements in rearing conditions. Another strategy for disease prevention or treatment involving AMPs is the controlled upregulation of the animal's AMP expression, which has the potential to be a powerful tool to enhance immune defense. This can be especially useful before or during stressful events like transfers, handling or husbandry changes. Given that AMP responses are usually rapid with upregulation happening a few hours or up to a day following stimulation, the stimulus can be applied shortly before the event to increase disease resistance. Also during disease treatment a controlled upregulation of AMPs might improve the outcome [92]. Well-known stimuli are LPS and oligosaccharides from yeast cell walls (e.g. β -glucans). Many other microbial components including live or attenuated microbes are also thinkable stimuli. Since AMPs have been shown to be antibacterial in fish, a strategy of controlled upregulation is likely to protect against disease. Lastly, selective breeding with the target of genetic resistance traits can be a successful route to decrease disease problems in

aquaculture. Regarding AMPs, it can be of interest to select for high constitutive expression levels since such a trait might confer better resistance to pathogens. Further, prominent responsiveness translated into a high amplitude and/or duration of upregulation can be another desirable trait to be selected for. Also a resistance to stress-related down-regulation of AMPs, which would keep an individual from becoming more vulnerable to infection, is a desirable trait. It is further thinkable to select for individuals that possess a protein variant that is particularly active. Respective genomic tools need to be developed and traits of interest need to be localized in the genome in order to use this strategy for aquaculture disease control [92].

2. Aims

The general aim of this project was to isolate a cathelicidin AMP from a teleost fish species and to investigate its role in immune defense.

The specific aims were:

- 1) To isolate cathelicidin from Atlantic cod (*Gadus morhua*) in order to determine the mature peptide.
- 2) To characterize the newfound peptide with regard to its antimicrobial activity.
- 3) To investigate cathelicidin expression in early life stages of Atlantic cod.
- 4) To investigate the cathelicidin response towards different stimuli and characterize the underlying molecular mechanisms of expression in salmon and cod.

3. Results summary and discussion

Here I will review and summarize my work and discuss methodology and avenues taken that have not been proven successful, that have not been mentioned in the publications/manuscript.

3.1. Paper I

Cathelicidins are synthesized as prepropeptides consisting of the signaling sequence, the cathelin propeptide and the mature peptide region. The signaling peptide is usually cleaved off co-translationally, while the mature peptide part is cleaved off when activity is needed, either constitutively or following a stimulus. The necessary protease that is stored in a separate compartment is released together with the proform resulting in cleaving off of the mature peptide [85].

Since the proteases responsible for the cleaving between propeptide and peptide part are not determined in fish, it is not possible so far to define with certainty the cleavage site of new fish cathelicidins and only predictions can be made. The salmonid cathelicidins described by Chang *et al.* [78] have been suggested to be cleaved by elastase since this is a protease cleaving many mammalian cathelicidins. The rainbow trout mature cathelicidin peptide predicted by Chang *et al.* demonstrated antimicrobial activity, thereby supporting the hypothesis that their predicted elastase cleavage site is the *in vivo* cleavage site. However, elastase has to the author's knowledge not been described in rainbow trout. Hence, it is necessary to isolate the peptide from tissues in order to have certainty regarding the definition of the cleavage site, which we set out to do in this project.

Other antimicrobial peptides have been isolated from fish before, however no cathelicidin had earlier been isolated from teleost fish, making our isolation the first isolation of a cathelicidin from teleost fish.

We set out to isolate cathelicidin from the kidney tissues of Atlantic cod, since RNA levels had been shown to be the highest in the kidneys in unstimulated fish. The kidney is the main haematopoietic organ in fish and contains structural tissues as well as high amounts of blood cells, making it a rather heterogenous tissue with a multitude of different proteins. Separating cathelicidin, which seemed to be present in only low amounts in the tissue, from a vast array of other proteins proved to be a challenge initially. We opted for an initial purification of the kidney protein extract with Oasis HLB cartridges to enrich the sample in proteins and remove non-proteinaceous compounds and salts. The next steps were effected with high performance liquid chromatography (HPLC). We used an antibody that was raised against an N-terminal region of the mature peptide in order to identify the HPLC fractions that were positive for the peptide with the help of western blotting. The first HPLC step was cation-exchange chromatography and the next and final step was reversed-phase chromatography. Since we could recover cathelicidin in only low amounts from the tissue, we did not consider it as feasible to add another purification step to the process in order to prevent loss of material. With this 3-step-purification process we succeeded in obtaining a sample that was sufficiently pure for peptide sequencing.

Initially, cod cathelicidin was considered to be cleaved by elastase and this prediction led to a theoretical peptide of 69 amino acids [75]. The sequencing of the mature peptide we had isolated however demonstrated that the cleavage site was two residues more towards the C-terminus than predicted, giving a peptide of the size of 67 amino acids and a positive charge of +15 (Fig. 2). We did not however see the proform which can be due to the N-terminal residues of the mature peptide region, against which our antibody was raised, being covered by the propeptide in spite of the denaturing environment during SDS-PAGE.

MTTQMRLLCFAAVTLLAEAQMIPDPFIFPLKNFRPLLDQLRVETVYP
 EGVDLSTMSVRKMTFPAQELDCSQVNTSM PGQQCPLKENGKMMN
 CNFTLSYINQDADIQGFQFNCDAAIKEATLTRVRRSRSGRSGKGG
RGSGRGSSGSRGSKGPSRGSSGSRGSKGSRGGRSGRGSTIA
GNGNRNNGGTRTA

Fig. 2. Atlantic cod cathelicidin full sequence. Italics demonstrate the signal sequence, the peptide region is in bold. Dark grey region: predicted cleavage site, light grey region: in vivo cleavage site.

We further set out to determine the presence of cathelicidin transcripts in cod eggs and freshly hatched larvae. Transcripts were present in both eggs and larvae and in the larvae the levels seemed to be very responsive to outer stimuli. We had two groups of larvae that were reared on two different feeding regimes, and it seemed that the food regime had an influence on cathelicidin transcripton, possibly due to microbial compounds in the feeds.

3.2. Paper II

For the second paper we characterized the peptide we had isolated to approach its possible role in immune defense.

Initially we were surprised to find that the peptide we had isolated, and that we subsequently had synthesized, was salt-sensitive while Atlantic cod is a marine fish. It is however very common that AMPs show salt sensitivity, and several AMPs from marine organism have earlier been shown to be salt-sensitive [93]. Subsequently, in a low salt environment the peptide demonstrated potent broad spectrum activity with an average MIC of 5-10 μ M.

Aeromonas hydrophila, *A. salmonicida* and *Vibrio anguillarum* extracellular products (ECPs) led to the peptide being degraded and its antimicrobial activity being abolished. This was likely due to proteases secreted by these pathogenic bacteria and can be interpreted as a way to protect themselves from the activity of AMPs. *E. coli* (D21) on the other hand, which is not a fish

pathogen, was not able to degrade the peptide, thereby supporting the theory of it being a specific resistance mechanism of pathogens.

We also investigated the mechanism of killing of cod cathelicidin by following the integrity of bacterial cells over time under influence of the peptide. Cod cathelicidin after addition caused a steep decline in optical density (600 nm) of a bacterial suspension which suggested killing through cell lysis. Disturbance and disruption of the cell membrane is regarded as a very prevalent mode of AMP action.

We investigated selectivity of activity of cod cathelicidin and found no damage to fish cells up to a concentration of 40 μM , while our results had earlier shown that the peptide was active against bacteria at 5 μM . Selectivity of activity was further confirmed when cathelicidin was tested on microbial and eukaryotic membrane mimetics. Hence, cod cathelicidin was active against prokaryotic but not eukaryotic membranes.

The salt sensitivity of cod cathelicidin called into question its role in microbial killing during disease and makes it uncertain that the peptide would be antimicrobially active in the normal physiological environment. It can be suggested that this peptide fulfills other roles. In view of this it would have been interesting to actually localize the peptide on the cellular level. Should the peptide be localized to a salt-regulated environment like lysosomes or the glycocalyx, this would suggest a role in microbial killing in these special environments where less salt induced inhibition of activity would be expected.

We had set out to localize the peptide in tissues of infected fish by immunohistochemistry. While it was promising initially to see a difference in staining intensity between healthy and infected tissues, suggesting an increased expression in the latter, a more thorough investigation revealed that the staining was localized on the bacteria in the infected tissues. Since the mature peptide that we likely detected on the bacteria in the tissue was secreted and not localized intracellularly we were not able to identify the

source location. The proform could not be detected by our antibody, which can be due to the epitope of the antibody being hidden by the propart. The epitope of the antibody we were working with was very close to the propart and likely not accessible for the antibody. Stronger unmasking/antigen retrieval techniques did not improve the outcome. Finding the proform that is uncleaved and therefore likely unsecreted might facilitate the discovery of the place of synthesis or storage. An option that might have led to more success is the raising of an antibody against a more C-terminal region of the mature peptide or against the propart which was not done in this project.

3.3. Paper III

In the third project we set out to determine the underlying molecular mechanisms of cathelicidin upregulation in salmon and cod and screened several compounds to elicit that upregulation.

The salmonid cathelicidins had been described before to demonstrate a constitutive mode of expression for the cathelicidin 2 group and an induced mode of expression for the cathelicidin 1 group. The only cathelicidin identified in the chinook salmon cell line (CHSE-214) was grouped previously into the salmonid cathelicidin 2 group. So it might not seem surprising that in an attempt to investigate cathelicidin upregulation in this cell line, we initially had to battle constitutive expression in the cells. This seemed to be related with the passage number and the length of time the cells had been in use, i.e. the longer they were in use, the more they moved towards constitutive expression. It took a long time to settle the issue of constitutive expression and it was overcome when we found a strain of CHSE-214 that exhibited a more stable expression pattern.

We found that cathelicidin in the chinook salmon cell line was significantly upregulated by dead Gram-negative bacteria. Assessment of the temporal expression pattern demonstrated that the maximal RNA level was reached at 24 h post stimulation (p. s.). Following the peak at 24 h p. s. was a steep decline in

cathelicidin RNA levels at 30 h p. s. with RNA levels then recovering and demonstrating the second highest peak at 48 h p, which might indicate a second slower response. The low RNA levels at 30 h could be due to a negative feedback loop to prevent too high peptide levels.

In general the speed of upregulation was fast (within hours) and transient.

Different components were screened for induction of upregulation in the salmonid cell line and flagellin from *P. aeruginosa* was the only component to significantly upregulate the peptide.

Using signaling inhibitors in order to elucidate the pathways leading to cathelicidin upregulation, we found significant downregulation with brefeldin A (Golgi inhibitor) and nocodazole (microtubuli inhibitor) and significant upregulation with wortmannin (PI3K inhibitor). The first two inhibitors indicate that signaling proteins or even receptors like TLRs are localized on the structures inhibited by these compounds. Wortmannin has been described as an inhibitor of the PI3K, which in turn has been suggested to be a negative regulator of TLR signaling. The significantly increased response with wortmannin indicated an inhibitory role of the PI3K in cathelicidin signaling in salmonids.

In the cod cell line, the killed *Lactobacillus* sp. was the only microbe that significantly upregulated expression. Since Lactobacilli are not necessarily pathogens, the effect can be interpreted as the effect of a probiotic that stimulates TLR mediated responses without being an actual threat to the organism.

From the different components screened, it was poly(I:C) that significantly upregulated expression in cod. It has been reported elsewhere that the cod immune system is likely to be very responsive to viral PAMPs [24] thereby supporting our results. We further found indications that stimulated cod cells secreted the cathelicidin peptide into the growth medium, since growth medium extracts harvested from high expressing cells

demonstrated antibacterial activity and were sensitive to proteases. The sensitivity to proteolytic degradation indicated the antibacterial component to be a protein/peptide.

4. Conclusions

The research done for this thesis has led to the following conclusions:

- 1) The mature cod cathelicidin AMP was isolated, its sequence was determined and the cleavage site defined.
- 2) Cod cathelicidin exhibited salt sensitivity, antimicrobial activity indicating a role in immunity, selectivity of activity, a lytic mode of killing and was degraded and inactivated by proteases of pathogenic bacteria.
- 3) Cathelicidin RNA was present in freshly hatched cod and levels very responsive to environmental stimuli, indicating a role in immunity.
- 4) Several non-pathogenic components were shown to upregulate cathelicidin expression in salmon and cod, and insights were gained about receptors and a signaling molecule involved.

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Cod cathelicidin: Isolation of the mature peptide, cleavage site characterisation and developmental expression

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ABSTRACT

Cathelicidin antimicrobial peptides are multifunctional peptides that are important in the innate immune system of mammals. Cathelicidins have been identified in several fish species. In this study we have isolated cathelicidin from Atlantic cod (*Gadus morhua*) and identified the cleavage site from the cathelin propeptide. This is the first isolation of a cathelicidin from teleost fish. The mature cathelicidin was found to be a 67-residue peptide, highly cationic with a pI of 13. Reversed phase chromatographic fractions containing the purified peptide had pronounced antimicrobial activity and the activity of the mature peptide was confirmed using a synthetic peptide. We examined the expression of cathelicidin during cod larvae early development using real-time PCR and detected expression that varied in the course of the first 68 days post hatching (dph). Two groups of larvae having a different food regime were compared. Cathelicidin expression was found to differ between the two groups and this could be linked to their food input. The presence and rapid adjustment of cathelicidin expression in the larvae indicate that the immune system of cod is active from early on in development and responds to external stimuli by the production of antimicrobial peptides.

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1. Introduction

The innate immune system is the first line of defence against pathogens. Antimicrobial peptides (AMPs) are an important part of this defence system and act as natural antibiotics. Due to the cationic and often amphipathic character of these peptides, they attack the negatively charged microbial cellular membrane (Zasloff, 2002). AMPs have been intensively studied in mammals and have been found, in addition to their direct antimicrobial action, to also be involved in many other processes in infection and inflammation, such as promotion of wound healing and initiation of adaptive immune responses (Lai and Gallo, 2009). Cathelicidins are a class of antimicrobial peptides that have so far only been identified in vertebrates. They are produced as prepropeptides with a conserved

cathelin-like N-terminal domain (cathelin propeptide) and a diverse C-terminal antimicrobial domain (Fig. 1), reviewed in (Tomasinsig and Zanetti, 2005). In mammals, the cathelin part is encoded mainly by exons 1–3 and the sequence identity of this region is approximately 40% between species. The mature antimicrobial peptide is encoded by exon 4 and it is hypervariable with respect to size and sequence (Tomasinsig and Zanetti, 2005). This variability in the antimicrobial region is thought to have evolved after gene duplication through sequence remodelling by point mutations rather than exon shuffling (Zhu, 2008; Zhu and Gao, 2009).

Cathelicidins have been identified in several fish species and their mRNA expression was found to increase after bacterial infection (Chang et al., 2005, 2006; Maier et al., 2008a,b), indicating a role of cathelicidins in fish innate immunity. The fish cathelicidin genes identified so far have the typical four-exon structure, but interestingly a cDNA for cathelicidin in the *Salvelinus* genus lacks exon 3 (Maier et al., 2008a; Scocchi et al., 2009), deleting a part of the cathelin region of the proform. In mammals the antimicrobial peptide-coding sequence and the 3'-untranslated region are located in exon 4 with the processing site of the peptide close to the N-terminus of exon 4 (Tomasinsig and Zanetti, 2005). This is thought to be true also for fish cathelicidins (Fig. 1). The amino acid identity of fish cathelicidins found so far is approximately 30%, both

Abbreviations: HPLC, high performance liquid chromatography; RP, reversed phase; TFA, trifluoroacetic acid; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; kDa, kilodalton; CFU, colony forming units; dph, days post hatch.

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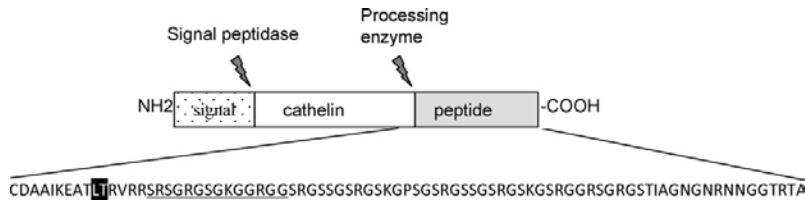


Fig. 1. Cathelicidins are produced as prepropeptides with a signal sequence, cathelin propeptide and a mature antimicrobial peptide. The activating processing enzyme releases the antimicrobial peptide from the conserved cathelin region. The amino acid sequence of the predicted cod cathelicidin in the indicated region is shown and one aim of this study was to determine the actual cleavage site of the peptide. The shaded black amino acid residues (LT) represent the exon 3/4 boundary, which has been confirmed by sequencing (Maier et al., 2008a). The cleavage site of the processing enzyme was expected near the N-terminal boundary of exon 4. The cod cathelicidin antibody used in this study is raised against the underlined amino acid sequence.

for the cathelin and the mature peptide region. The relatively high sequence identity at the antimicrobial peptide region is unusual and is likely to reflect a conserved functional significance (Maier et al., 2008a), provided by positive selection as has been found in the mature peptides of primate cathelicidin (Zeletzsky et al., 2006). The cathelicidin peptides in fish can be divided on their putative secondary structure into peptides forming a disulphide bond and peptides forming an extended structure (Maier et al., 2008a). Examples of the latter are the newly identified Atlantic cod cathelicidins, with predicted mature peptides rich in arginine, glycine and serine (RGS). Little is known about the function of cathelicidins in fish, but *in vitro* studies using a synthetic fragment of a predicted rainbow trout cathelicidin have shown it to have antimicrobial activity (Chang et al., 2006). However, no mature teleost cathelicidins have been isolated to homogeneity or studied in detail.

The aims of this study were to purify cod cathelicidin, identify the proteolytic *in vivo* cleavage site of the proform (Fig. 1), and examine the mRNA expression during the development of cod larvae. Cod larvae have a very high mortality rate during the first 4–8 weeks after hatching, with a typical hatchery survival of 10–20%. In aquaculture this is partly due to rearing problems, such as fish density and food (Steinarsson, 2004), but infections also play an important part. Natural antibodies (IgM) are present in fish serum independent of antigenic stimulation and are thought to have an important role in the innate immune system of Atlantic cod (Magnadottir et al., 2009). Studies on newly hatched cod larvae did not detect any immunoglobulin producing cells until at least 58 dph (Schroder et al., 1998) and no IgM was detected in the first 2 months of the larvae (Magnadottir et al., 2004). Similarly, the pentraxin CRP (C-reactive protein), a lectin important in the acute phase response, could not be detected at this stage (Magnadottir et al., 2004). These findings suggest that during the first 2 months after hatching the larvae depend mainly on other innate immune factors for their defence against pathogens.

In the present study we have isolated the mature cod cathelicidin from head kidneys, structurally characterised it and shown it to exhibit antimicrobial activity. The antimicrobial activity, as well as its increased transcription during infection, suggests a role of cathelicidin in fish immunity. We show that cathelicidin transcription is detected in cod eggs and larvae, which indicates that antimicrobial peptides provide a protection from infection in the early stages of development, before the appearance of other immune factors.

2. Materials and methods

2.1. Collection of cod material

For the infection study ten juvenile cod (60–120 g) were injected intraperitoneally (i.p.) with 10^6 CFU of *Aeromonas salmonicida* ssp. *achromogenes* (Asa) and as control 10 additional cod were injected

with PBS as described earlier (Maier et al., 2008a). Twenty-four hours after infection the fish were dissected, samples were taken from various organs and immediately snap frozen in liquid nitrogen. Atlantic cod eggs were either taken from female cod in December (called immature eggs) after dissection of the fish or in April from running females ready to spawn (called mature eggs) and snap frozen immediately. For the protein extraction cod head kidneys were collected from adult, cultured cod and tissues were immediately snap frozen in liquid nitrogen.

In order to compare the effect of different feeding regimes on cod larvae, rearing trials were conducted at the Marine Research Institute's Experimental Station, Stadur, Grindavik, Iceland, in the spring of 2009. Two groups of larvae (groups 1 and 2) in commercial hatchery tanks were reared on different start-feeding protocols. They were monitored from the egg stage, through the larval stage and well into the pre-juvenile stage. Rearing conditions were identical for both groups. Eggs were incubated at 7 °C and sterilized with Pyceze (Novartis Animal Vaccines Ltd.) at –8 and –2 days pre-hatch using 100 parts per million (ppm) for 30 min. Upon completed hatching, two hatchery tanks (3200 L) were stocked with 170,000 larvae each. Antibiotics (25 ppm Lincomspectin, Pfizer Ltd.) were routinely administered on 1, 4 and 7 dph. Light was provided at 3 dph (150 lux at surface) and increased incrementally to 700 lux from 10 dph onwards. The photoperiod was initially fixed at a 18:6 ratio (Light:Dark hours), but adjusted to 24:0 from 22 dph onwards. Rearing temperatures were held at 8 °C until 7 dph and gradually increased to 12–13 °C from 34 dph onwards. The water flow was 3.5 L/min at 3 dph and increased incrementally to 13 L/min from 45 dph onwards. Concentrated *Nannochloropsis* algae (Instant algae, Reed Mariculture) were added for shading twice daily (60–80 mL per day).

The group 1 larvae were fed enriched (Algamac 3000, Aquafauna Bio-Marine) rotifers (*Brachionus plicatilis*) from 3 to 30 dph (26–75 millions per day), *Artemia* (*Artemia salina*) nauplii and metanauplii from 16 to 50 dph (0.5–60 millions per day) and dry feed (Gemma Micro Diamond, Skretting) from 20 dph onwards. The group 2 larvae received the same rotifer protocol but were not fed *Artemia*. Dry feeding commenced at 14 dph in this group (Fig. 6C).

Larvae were collected directly into RNAlater (Ambion) at the days indicated in Fig. 6. The number of larvae was different at various sampling timepoints, with 50 larvae for the first dph, approximately 10–20 larvae until day 40 and thereafter 2–4 pre-juveniles for each timepoint.

2.2. RNA isolation, cDNA synthesis and quantitative real-time PCR

Atlantic cod larvae were homogenized with a Pellet Pestle Cordless Motor (Kimble-Kontes) and RNA was extracted using TRI Reagent (Ambion). For the infection and egg studies approximately 50 mg of frozen tissue was ground with a pestle in a mortar and total RNA was extracted using TRI Reagent. RNA was pre-

pared using manufacturer's instructions and dissolved in 30 μ L RNase-free water. To minimise contamination with DNA, 10 μ g of RNA were treated with DNase (New England Biolabs), followed by ethanol precipitation and the resulting RNA pellet was dissolved in 20 μ L RNase-free water. Quantity and quality of the resulting RNA was assessed using NanoDrop ND-1000 UV/Vis-Spectrophotometer (NanoDrop Technologies). The integrity of the RNA from approximately half of the samples was evaluated by agarose gel electrophoresis.

For the following real-time analysis cDNA was prepared from 300 ng of RNA using the First Strand cDNA synthesis kit (Fermentas), according to manufacturer's protocol. The absence of genomic DNA was confirmed by preparing several samples without reverse transcriptase in the reaction. The cDNA was diluted 10-fold (infection study) and 100-fold (larvae study) in water for further use in quantitative real-time PCR. The RNA (1000 ng) extracted from cod eggs was reverse transcribed into cDNA and amplified by PCR with cathelicidin and β -actin specific primers as described previously (Maier et al., 2008a).

Real-time PCR was performed in 96 well-PCR plates on an ABI 7500 real-time PCR System (Applied Biosystems) using Power SYBR green PCR Master Mix as recommended by the manufacturer (Applied Biosystems) with the exception of using 10 μ L final reaction volume. Reactions were run in duplicates. Real-time primers for cathelicidin were designed in PerlPrimer (Marshall, 2004) and the sequence for the forward primer used was: 5'GGTGAAGACTGTCTATCCAGAGG3' and the reverse primer 5'AACCTCTGTGCAGGAATGTC3'. The relative expression of cathelicidins was measured against one or two reference genes encoding ubiquitin and ribosomal protein S 9 (RPS 9) as these genes had been shown to have stable expression levels in both adult cod (Olsvik et al., 2008) and larvae (Saele et al., 2009). The sequences of the primers were obtained from these studies. Efficiencies for all primers and tissues were calculated and shown to lie within the 90–110% required in order to employ the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) for the calculation of the fold differences in expression. For the infection study the expression detected in skin from healthy fish was set as value 1 and all other tissues were compared to this value. One representative experiment is shown, corresponding to one healthy and one infected individual. In the larvae analysis the reference gene encoding ubiquitin was used and the expression detected on the hatching day (0 dph) was set as value 1. Other measurements of mRNA expression were compared to this value. Statistical differences between tissues and treatments were assessed from three cod for each condition, with a one-tailed *t*-test unequal variance. Values below 0.05 were considered significant.

2.3. Peptide isolation

Head kidney tissue from healthy farmed cod was collected in liquid nitrogen and kept at -80°C until used. Frozen tissue (approximately 6 g) was ground with a pestle and mortar and proteins were extracted from the sample by overnight shaking in 60% acetonitrile/1% TFA at 4°C . Supernatants were subsequently collected by centrifuging for 40 min at $10,000 \times g$ and lyophilised. Oasis HLB cartridges (Waters) were used for desalting and enrichment of peptides and proteins in the supernatants. The cartridges were activated with 100% acetonitrile and then equilibrated with three washes of 0.1% TFA. The samples were dissolved in 0.1% TFA and applied to the cartridges. The cartridges were rinsed with 0.1% TFA and then with 10% acetonitrile in 0.1% TFA. Proteins were eluted from the cartridges with 80% acetonitrile in 0.1% TFA and the samples were lyophilized before further processing. Subsequently a 5 mL HiTrap CM FF Sepharose column (GE Healthcare) was employed for cation exchange chromatography. The samples

were dissolved in starting buffer (0.2 M acetic acid) and elution took place in a segmented gradient of 1.5 M ammonium acetate and a flow rate of 0.5 mL/min. Eluted fractions were lyophilised, reconstituted in water and an aliquot of each fraction was screened by Western blot analysis for the presence of cod cathelicidin. Lyophilised fractions positive for cathelicidin were pooled, dissolved in starting buffer (2% acetonitrile/0.1% TFA) and subjected to reversed phase chromatography. This was carried out on a Discovery C18 HPLC column (Sigma–Aldrich) in a linear gradient of 80% acetonitrile in 0.1% TFA and a flow rate of 1 mL/min. Aliquots of eluted and lyophilised fractions were screened by Western blotting.

Chromatographic runs were monitored at 280 and 214 nm. The chromatograms and silver stained protein gels were used to assess purity of the eluted fractions.

2.4. Amino acid sequence analysis and mass spectrometry

A part of a chromatographic reversed phase fraction containing only the cathelicidin peptide, as determined by SDS PAGE, silver staining and Western blot analysis, was analysed for a mass value with MALDI-MS utilising an Applied Biosystems Voyager DE.PRO instrument.

For N-terminal sequence analysis the remainder of the above chromatographic fraction, was applied onto Applied Biosystems precise cLC sequencer (PE Applied Biosystems). The sequencing was confirmed using a second independent cathelicidin peptide isolation.

2.5. Immunoassays

An affinity purified antibody against a synthetic fragment of the cod cathelicidin putative peptide (sequence: SRSGRGS-GKGGRRG, underlined in Fig. 1) was generated in rabbit by GenScript. Lyophilised chromatographic fractions were reconstituted in water, mixed with NuPAGE LDS sample buffer (Invitrogen) containing 10% mercaptoethanol, heated at 70°C for 10 min and loaded onto NuPAGE Novex Bis-Tris gels (Invitrogen) Electrophoresis was performed at 90 V for 10 min and at 120 V for 50 min. The ColorPlus prestained protein marker (New England Biolabs) was used for all SDS-PAGE runs. Proteins in the gels were blotted onto polyvinylidene fluoride (PVDF) membrane (Millipore) for 120 min at 40 V. The blotted membrane was then blocked in 1% fat-free milk in PBS with 0.05% Tween 20 for 1 h at room temperature prior to incubating with the primary antibody (0.1 μ g/mL) overnight at 4°C in 0.1% fat-free milk. The next day after three 10-min washes in PBS containing 0.05% Tween 20 the membrane was incubated with an anti-rabbit secondary antibody conjugated with horse radish peroxidase (Sigma–Aldrich) (diluted 1:10,000) in 0.1% fat-free milk for 2 h at room temperature. The protein bands were visualised with ECL Plus Western Blotting Detection Reagent (GE Healthcare) on a Typhoon 9400 scanner (GE Healthcare).

For detection of cathelicidin in crude tissue, organs were extracted in 60% acetonitrile/1% TFA at 4°C overnight, desalted and concentrated using Oasis HLB cartridges (Waters Corp.) as described above. The resulting pellet after freeze-drying was reconstituted in water and the protein concentration was measured on the NanoDrop ND-1000 UV/Vis-Spectrophotometer (NanoDrop Technologies). Equal protein concentrations (10–30 μ g) were loaded onto NuPAGE Novex Bis-Tris Gels (Invitrogen). Blotting and visualisation was performed as described above.

2.6. Peptide synthesis and antimicrobial assays

The characterised 67-residue cod cathelicidin peptide (Table 1) was produced synthetically (GenScript) with over 95% purity. The synthetic cathelicidin peptide and reversed phase chromatogra-

Table 1

Biochemical characteristics of the cod cathelicidin antimicrobial peptide.

Amino acid sequence	SRSGRSGSGKGRGSGSSGSRGSKGPSGSRGSSGSRGSKGSRGSRGSGSTIAGNGNRNNGGTRTA
Number of amino acids	67
Positive charge	+15
Molecular weight measured by MS (Da)	6239
Molecular weight calculated (Da)	6235
Theoretical pI	13.04

phy fractions containing cod cathelicidin peptide were assayed for antimicrobial activity against *Bacillus megaterium* strain Bm11. Single colonies of Bm11 were picked and used to inoculate 20 mL Luria Bertani (LB) medium. Bacteria were grown at 37 °C until the optical density reached 0.6 (at 595 nm). Thin 1% agarose plates (1 mm) of LB media containing 3×10^4 bacteria/ml were poured and wells 3 mm in diameter were punched in the agarose layer. Three microlitres of the tissue extracts (10–20 µg) or the synthetic peptide dissolved in water at indicated concentrations were loaded into each well. The agarose plates were incubated at 37 °C and the following day inhibition zone diameters were measured.

The minimal inhibitory concentration (MIC) of the synthetic cathelicidin peptide was determined using colony counting assays as described elsewhere (van Dijk et al., 2007). Briefly, 10 µL of a log phase bacterial suspension of Bm11 or *Escherichia coli* strain D21 adjusted to approximately 10^6 bacteria/ml were mixed with 10 µL of a 2-fold serial dilution of the synthetic peptide ranging from 20 µM to 0.3125 µM. The incubation took place in diluted LB medium (1000-fold diluted in water) at 37 °C for 2 h and thereafter serial 10× dilutions of the suspensions were made and spread out on LB agar plates. Bacterial growth was assessed after an overnight incubation at 37 °C and the lowest concentration of synthetic cathelicidin peptide, which inhibited bacterial growth, was deemed the MIC.

3. Results

3.1. Expression of Atlantic cod cathelicidin mRNA

We have previously shown that bacterial infections of fish cause an increased expression of cathelicidins (Maier et al., 2008a). The fish pathogenic bacterium *Aeromonas salmonicida* ssp. *achromogenes* (Asa) causes atypical furunculosis, a systemic disease in many fish species (Gudmundsdottir and Bjornsdottir, 2007). In the present study cathelicidin expression in healthy and Asa infected cod was compared using real time PCR. Increased cathelicidin expression was observed in all seven tissues examined, after injection of the cod with bacteria, compared to fish injected with PBS alone (Fig. 2). Due to the high individual variation a representative experiment of one individual for each condition is shown. For the statistical analysis three healthy fish and three infected fish were compared. A one-tailed *t*-test with unequal variance showed a statistically significant increase in cathelicidin expression in all tissues ($p < 0.05$) analysed except for gills, when comparing infected to healthy fish. Constitutive expression was found to be most pronounced in kidney and spleen. The high basal expression of cathelicidin in kidney or spleen was found to be statistically significant ($p < 0.01$ for kidney and $p < 0.05$ for spleen), when compared to all other tissues. Due to the relative high expression of cathelicidin in uninfected kidney and due to the availability and size of the organ, this tissue was used in the subsequent isolation of the mature cathelicidin peptide.

3.2. Isolation of the mature peptide of cod cathelicidin

Proteins were extracted from healthy cod kidneys and initially separated by high performance liquid chromatography (HPLC)

using a cation exchange column (Fig. 3A). Fractions were screened for the presence of cathelicidin by Western blot analysis, with an antibody raised against a synthetic N-terminal fragment of the cathelicidin peptide (Fig. 1, underlined amino acids). The resulting positive fractions were pooled, separated by reversed phase (RP) HPLC (Fig. 3B) and cathelicidin was again detected by Western blot analysis. The presence of cathelicidin in the fractions was confirmed by peptide-mass fingerprinting using endoproteinase Lys-C: a positive fraction was separated by SDS-PAGE and silver stained. The band around 7 kDa, predicted to correspond to the mature cathelicidin peptide from the respective Western blot, was excised from the gel and digested with Lys-C. Three of the expected four fragments were identified by mass spectrometry (data not shown). The fourth band corresponding to the N-terminus of the predicted peptide could not be detected. To identify the exact N-terminal sequence of the isolated cod cathelicidin peptide and thereby the processing site, fractions containing cathelicidin were further analysed. Cathelicidin was eluted from the reversed phase column in fractions 19 and 20 (Fig. 3B). Although fraction 20 had larger amounts of cathelicidin protein, silver staining revealed that fraction 19 contained only one protein, while fraction 20 contained at least five additional proteins (data not shown). The pure protein in fraction 19 was analysed by MALDI-MS and gave a mass value of 6239 Da. Sequence analysis of the first 10 amino acid residues of the peptide in this fraction and from another independent preparation revealed the following sequence: SRSGRSGSGXG. This sequence showed that the N-terminus of the mature peptide started after the fifth amino acid in exon 4 (see Fig. 1), confirming a processing site between Arg 126 and Ser 127. A cleavage at this site results in a mature peptide with a predicted mass value of 6235 Da and this is in agreement with the measured mass value of 6239 Da. Key characteristics of the cod cathelicidin are summarised in Table 1.

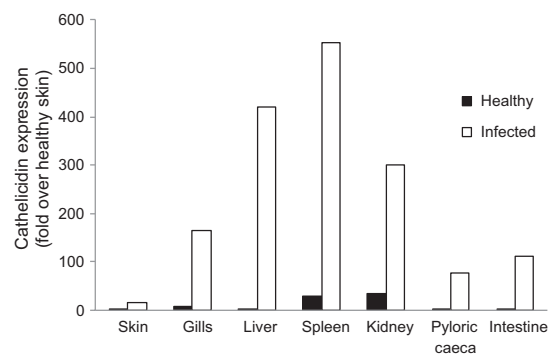


Fig. 2. Cathelicidin expression is increased during infection. Cod were injected with PBS (Healthy) or *Aeromonas salmonicida* ssp. *achromogenes* (Infected) and incubated for 24 h. Tissues were sampled as indicated. Relative cathelicidin expression was analysed using quantitative real time PCR and the fold induction was compared to expression of healthy skin. Ubiquitin and RPS9 were used as internal reference and a representative result from one individual fish for each treatment is shown.

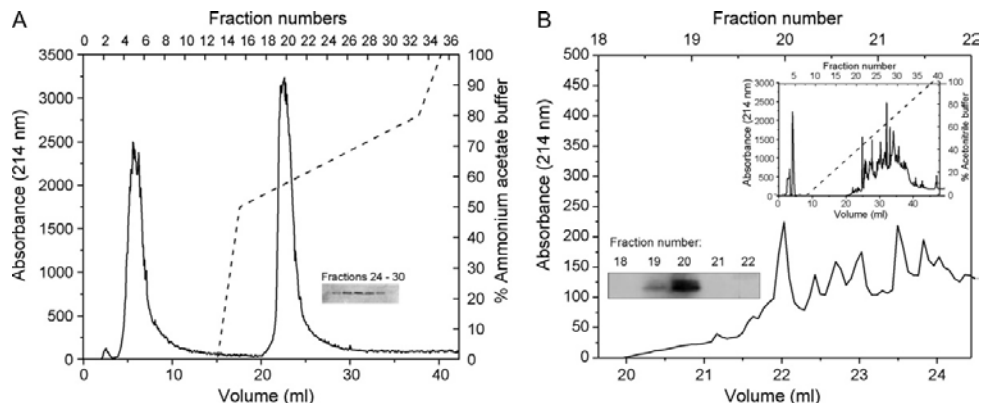


Fig. 3. Isolation of cod cathelicidin. (A) Extract of kidney tissue from non-infected cod was subjected to cation exchange chromatography with 0.2 M acetic acid as starting buffer and 1.5 M ammonium acetate as elution buffer. Elution was performed at a flow rate of 1 mL/min in a segmented gradient covering 0–50% elution buffer in 18 min, 50–80% in 20 min and 80–100% in 2.5 min. Insert shows part of the Western blot with the positive fractions for cathelicidin. The cathelicidin peptide eluted between 27 min and 33 min corresponding to fractions 25–29. (B) Positive fractions from the cation exchange were subjected to reversed phase chromatography in a linear acetonitrile gradient with a flow rate of 1 mL/min and elution was performed for 37.5 min. As starting buffer 2% acetonitrile in 0.1% TFA was employed and the elution buffer was 80% acetonitrile in 0.1%TFA (upper insert). The cathelicidin peptide eluted between 20 mL and 22 mL corresponding to fractions 19 and 20, respectively. A zoomed area of the absorbance profile at 214 nm (mAU) is shown and fractions containing the cathelicidin peptide were identified by Western blot analysis (lower insert).

3.3. Antimicrobial activity of cod cathelicidin and its tissue expression

For the activity studies, cathelicidin was purified from cod head kidneys using cation exchange and reversed phase chromatography as described above. Fractions containing cathelicidin were identified (Fig. 4A) and used to examine the antibacterial activity against *B. megaterium* (Bm11). In this purification run the peptide eluted in fraction 23 and 24 from the reversed phase column and pronounced antimicrobial activity was observed in the fractions positive for cathelicidin. The subsequent fractions, but not the previous fractions were also shown to have antibacterial activity, due to additional antibacterial component(s) in the preparation. Fractions 25 and onwards also had a much higher abundance of proteins than previous fractions (corresponding to fraction 21 and onwards in Fig. 3B, upper inset). The antimicrobial activity of the mature cod cathelicidin peptide was confirmed with a synthetic peptide and a

dose–response curve against the gram-positive bacterium Bm11 is shown in Fig. 4C. The minimal inhibitory concentration (MIC) of the peptide against Bm11 was determined to be 5 μ M, while the MIC against the gram-negative bacterium *E. coli* D21 was 2.5 μ M.

Cathelicidin peptide expression in different tissues from *A. salmonicida* infected cod was examined by Western blot analysis and a band of 7 kDa, corresponding to the mature peptide, was detected (Fig. 5). The expression was most pronounced in kidney and low expression was also observed in spleen.

3.4. Expression profile of cathelicidin mRNA during cod larvae development

In order to study cathelicidin expression during Atlantic cod early development, cod eggs were examined for transcripts and cathelicidin mRNA was detected in both immature and mature, unfertilised eggs (Fig. 6A). Subsequently cathelicidin expression

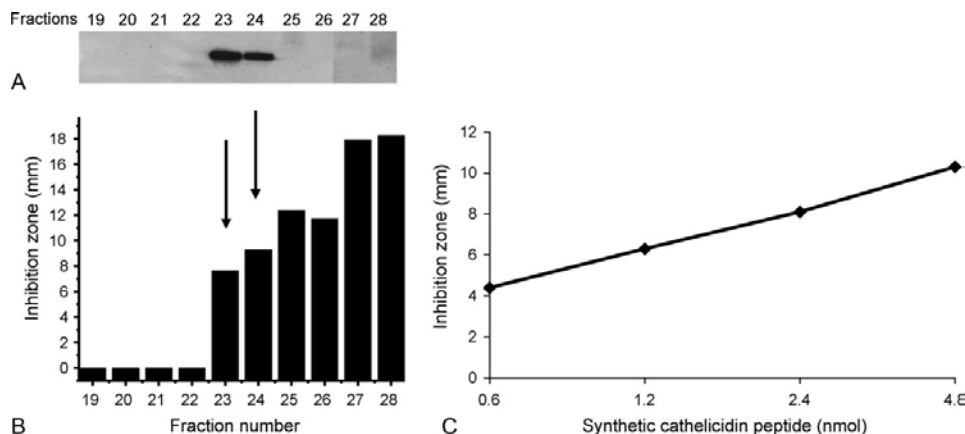


Fig. 4. Antimicrobial activity of cod cathelicidin. (A) Positive fractions from reversed phase chromatography were identified by Western blot and (B) applied to a radial diffusion assay containing Bm11 (*B. megaterium* strain 11). The diameter of the inhibition zones was measured after an overnight incubation of the plates. Fractions containing cathelicidin (identified in A) are indicated with arrows in the diagram. (C) Increasing amounts (nmol) of a synthetic replica of the cod cathelicidin peptide characterised here, were tested in the radial diffusion assay against Bm11.

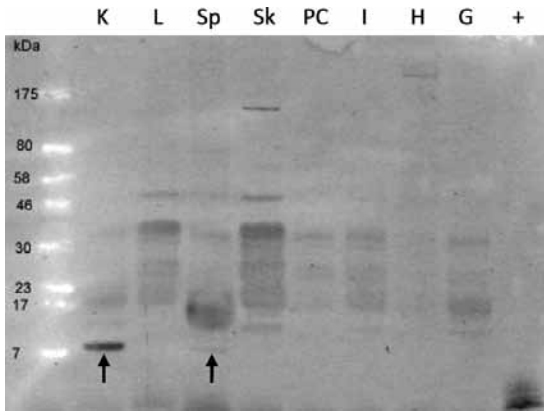


Fig. 5. Tissue distribution of the cod cathelicidin peptide. Western blot analysis detected the peptide in kidney and spleen of infected fish (indicated by arrows). K = kidney, L = liver, Sp = spleen, Sk = skin, PC = pyloric caeca, I = intestine, H = heart, G = gills. The antigen (14 amino acid residues long) was used as positive control for the antibody (+). Sizes of the protein marker (in kDa) are indicated to the left. Additional bands seen are interpreted as non-specific binding of the antibody.

was followed by quantitative real-time PCR for 68 dph in the larvae. Cathelicidin mRNA was found to be present at low levels in newly hatched larvae and its expression varied over the 68 days examined (Fig. 6B). A pilot study on larvae had indicated that the increase in cathelicidin expression was partly linked to the food of the larvae (data not shown). Therefore a study was set up to approach a direct effect of food on cathelicidin expression during development. One group of larvae was fed by a normal regime including mainly live rotifers (small zooplankton), live brine shrimp (*Artemia* sp.) and finally dry feed (group 1), while the other group (group 2) did not

get any *Artemia*. The length growth rate of the larvae following the normal regime (group 1) was found to be approximately 3.5% per day, with a mean length of the pre-juveniles at 49 dph of 26.3 mm. The larvae fed without *Artemia* (group 2) also started off with a length growth rate of 3.5% but following premature dry feed weaning the rate decreased to only 2.5% per day and the mean length of the pre-juveniles at 49 dph was only 15.6 mm. Similarly the survival of the larvae in group 1 was 14% (50 dph), while the survival for group 2 was 6% (55 dph). Due to the lower survival in group 2 than in group 1 (10,000 versus 23,000 pre-juveniles, respectively) the amount of dry feed was adjusted accordingly (Fig. 6C). Comparison of the cathelicidin expression between the two groups showed a different expression profile. Both groups had an about 15–20 fold increase in cathelicidin expression around day 11 post hatch compared to 0 dph (Fig. 6B and C). Larvae raised on the normal food regime had an about 40-fold increase in cathelicidin expression at day 50, while a similar increase was seen in group 2 at day 37 post hatch. This increase in cathelicidin expression coincided with dry feed weaning in both groups.

4. Discussion

The primary aim of this study was to isolate the mature active peptide of cod cathelicidin from cod tissue. By three chromatographic separations we were able to isolate and characterise the mature cathelicidin peptide thereby identifying the processing site of the precursor protein. The cod cathelicidin peptide was found to consist of 67 amino acid residues, two residues shorter than initially predicted (Maier et al., 2008a). The peptide was detected in kidney and spleen of infected cod, both hematopoietic tissues. However so far we have not been able to detect the peptide in peripheral blood; serum or cells by Western blot analysis. Cathelicidin mRNA on the other hand could be detected in blood (data not shown) as well as in most tissues especially after infection. We have previously observed differences in mRNA and peptide levels for cathelicidin

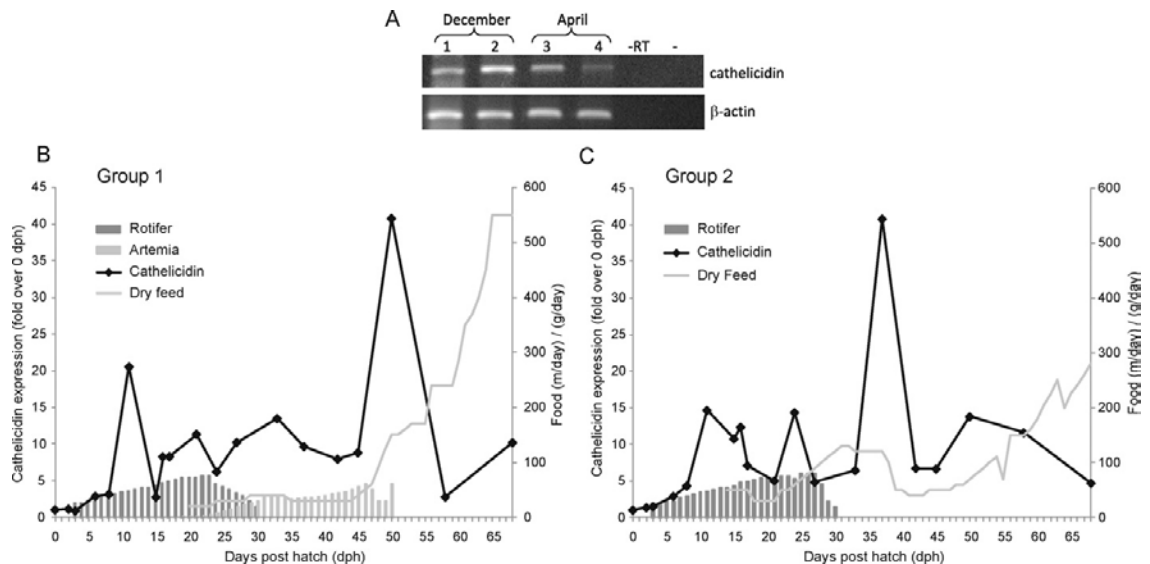


Fig. 6. Cathelicidin expression in Atlantic cod eggs and during larval development. (A) Oocyte samples were collected from 2 adult cod in December (1 and 2) and 2 adult cod in April (3 and 4). RNA was isolated, followed by RT-PCR with either cathelicidin or β -actin primers. The negative controls were reverse transcriptase substituted with water (–RT) during generation of the cDNA or water (–) as template in the PCR reaction. (B and C) Samples (indicated by black squares) were taken from 0 to 68 days post hatch (dph) and analysed by real-time PCR. The relative cathelicidin expression was normalised to the expression on the hatching day and calculated using ubiquitin as endogenous reference. (B) Larvae were either raised on a normal diet of rotifers (million/day), *Artemia* (million/day) and dry food pellets (gram/day) (group 1) or (C) raised without *Artemia* (group 2).

(Bergman et al., 2005) and this may be explained by mRNA stability, regulation at the translational level or protein degradation (de Sousa Abreu et al., 2009). The size of the cod cathelicidin peptide estimated by Western blot was larger than the confirmed molecular mass and this is thought to be due to the fact that cationic peptides may run more slowly in SDS-PAGE. Similar results have been reported by other groups for cationic peptides (Corrales et al., 2009; Patat et al., 2004). So far the processing enzyme releasing the mature peptide from the precursor protein has not been identified in fish. In mammals the enzymes cleaving the antimicrobial peptide from its precursor are mainly serine proteases such as elastase and proteinase 3 (Murakami et al., 2004; Panyutich et al., 1997; Scocchi et al., 1992; Sorensen et al., 2001). Interestingly, all fish cathelicidins identified so far have a predicted processing site for furin near the beginning of exon 4. Furin proprotein convertase is a serine endoprotease and cleaves after the consensus site R-Xaa-R/K-R (Nakayama, 1997). Furin has been identified in zebrafish (Walker et al., 2006) and medaka (Ogiwara et al., 2004; Walker et al., 2006), but so far not in cod or salmonids. The predicted proprotein convertase site found in all fish cathelicidins has the sequence R-X-R-R and we demonstrate here that the cod cathelicidin peptide is processed after this site. Similarly, the mature peptides of the ancient and modified cathelicidins in hagfish are processed after an Arg tetrad (Uzzell et al., 2003). These discoveries suggest that a proprotein convertase might be the processing enzyme, releasing the mature cathelicidin peptide from the proform in fish. Further cleavage of the cod cathelicidin mature peptide into smaller fragments could also occur and this would explain the low levels of the 6.2 kDa peptide detected. The antibody used in this study was raised against the N-terminal 14 amino acid residues of the mature peptide (Fig. 1). Therefore only peptides containing this region could be detected. Interestingly, further processing of the mature cathelicidin peptides has been detected in human skin (Murakami et al., 2004).

The peptide characterised in this study is very cationic and exhibits pronounced antibacterial activity. Antibacterial activity observed in fractions in which cathelicidin was not detected, originated from additional antimicrobial components, such as histones (Bergsson et al., 2005; Patat et al., 2004). The synthetic peptide confirmed the antimicrobial activity of the mature cod cathelicidin peptide against both gram-positive and gram-negative bacteria. In conclusion the increased production of cod cathelicidin during infection and the antibacterial activity of the mature peptide, suggests that cathelicidin is a contributing defence molecule in the innate immune system of Atlantic cod.

A second aim of this study was to follow the cathelicidin expression during the development of cod larvae. Cathelicidin mRNA was found to be present from very early on in development. Indeed the eggs might be already protected, as we have detected cathelicidin transcripts in immature, developing eggs as well as eggs ready to be fertilised. The presence of cathelicidin mRNA at this early stage is likely due to the transfer of maternal RNA. Transfer of maternal proteins (Magnadottir et al., 2004) and/or mRNA (Seppola et al., 2009) has been observed in several studies on cod eggs and larvae. Cathelicidin expression increased after hatching and first feeding with rotifers. The zooplankton given as live feed is grown in elevated temperatures and very susceptible to bacterial contamination (Olafsen, 2001). We suggest that the bacteria in the live feed might cause the increase in cathelicidin transcription observed. Interestingly, in larvae examined in this study, but not in our initial pilot study, an antimicrobial compound (Sanocare ACE, IVE) was used to wash the *Artemia* before feeding the cod larvae. This change in rearing practice coincided with a (4- to 6-fold) lower level of cathelicidin transcription around 30 dph than seen before. Comparison of two different rearing methods showed remarkable differences in cathelicidin expression during 30–60 dph. Larvae fed

on the normal diet had a large increase in cathelicidin expression around day 50 dph. Larvae fed without *Artemia* also exhibited an about 40-fold increase in cathelicidin expression, but this increase was much earlier in the development i.e. at around 37 dph. The enhancement of cathelicidin expression coincided with the end of live feeding and the increase in dry feeding. Cod larvae prefer to be fed on live prey if supplied and therefore the termination of live feeding is a significant step for the developing pre-juveniles (Agnar Steinarrson, unpublished observation). Group 1 pre-juveniles were moved from one tank to another at day 49 post hatch, therefore an excess of *Artemia* were given at this timepoint in order to settle the larvae (Fig. 6B, 50 dph). However, due to the size of the larvae at this stage, this is thought to have little effect on the overall food consumption of the pre-juveniles. Group 2 pre-juveniles were moved at 55 dph and did not show an enhancement in cathelicidin expression at this timepoint. Therefore stress during movement is not thought to have an effect on cathelicidin expression in the larvae. The large increase in cathelicidin transcription observed in both groups could either be due to the stress during food exchange or alternatively due to components in the dry feed.

In conclusion, our results suggest that the innate immune system of cod larvae is active and responds to external stimuli. Results from a Norwegian group that analysed the transcription of several immune genes during ontogeny (Seppola et al., 2009) showed an increase in transcription of the antimicrobial peptides hepcidin and cathelicidin, comparable to our study for cathelicidin during the normal feeding regime. Additional immune factors such as pentraxin and lysozyme on the other hand showed very low levels during the first weeks after hatching and exhibited a gradual increase in transcription during development (Seppola et al., 2009).

Our study emphasises the fact that the larvae are sensitive to changes in food composition. Probiotic bacteria have been used in aquaculture in recent years to control diseases and shown to have many beneficial effects on fish health, including increased growth and reduced mortality after infection (Olafsen, 2001; Perez et al., 2010). How these probiotic bacteria work is not fully understood, but might include enhancement of the innate immune system through pattern recognition receptors, such as the Toll-like receptors and downstream signalling components (Magnadottir, 2010). We have previously shown that bacterial products can cause an increase in cathelicidin transcription in fish cells, independent of the bacterial viability or virulence (Maier et al., 2008b). Our present results may explain the beneficial effects seen by treatment of the larvae with probiotics, since the increase in production of the antimicrobial peptide cathelicidin would strengthen the innate immune system of the larvae and increase their survival during disease.

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II



Functional characterization of codCath, the mature cathelicidin antimicrobial peptide from Atlantic cod (*Gadus morhua*)

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ABSTRACT

Cathelicidins are among the best characterized antimicrobial peptides and have been shown to have an important role in mammalian innate immunity. We recently isolated a novel mature cathelicidin peptide (codCath) from Atlantic cod and in the present study we functionally characterized codCath. The peptide demonstrated salt sensitivity with abrogation of activity at physiological salt concentrations. In low ionic strength medium we found activity against marine and non-marine Gram-negative bacteria with an average MIC of 10 μ M, weak activity against a Gram-positive bacterium (MIC 80 μ M), and pronounced antifungal activity (MIC 2.5 μ M). The results suggest the kinetics and mode of action of codCath to be fast killing accompanied by pronounced cell lysis. Extracellular products (ECPs) of three marine bacteria caused breakdown of the peptide into smaller fragments and the cleaved peptide lost its antibacterial activity. Proteolysis of the peptide on the other hand was abolished by prior heat-treatment of the ECPs, suggesting a protease involvement. We observed no cytotoxicity of the peptide in fish cells up to a concentration of 40 μ M and the selectivity of activity was confirmed with bacterial and mammalian membrane mimetics. We conclude that the potent broad-spectrum activity of codCath hints at a role of the peptide in cod immune defense.

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1. Introduction

The intensive culture of fish is rapidly increasing and brings along disease challenges that cause economical and welfare issues [43]. Knowledge about the fish immune system is therefore important in order to tackle these disease problems in aquaculture. Antimicrobial peptides are thought to be an integral part of the fish host defense, based on their importance in mammalian innate immunity. Cathelicidins, together with defensins, are among the most extensively studied antimicrobial peptides in mammals. They typically have a phylogenetically conserved N-terminal cathelin propeptide and a variable C-terminal mature peptide region which, when proteolytically cleaved off from the propeptide, carries the antimicrobial activity [54]. As opposed to e.g. defensins that are fully processed before storage, cathelicidins are stored as inactive precursors that require the mentioned additional processing [24]. The only human cathelicidin hCAP18 (human cathelicidin antimicrobial protein) is processed to the antimicrobial peptide LL-37

through extracellular cleavage by a serine protease such as proteinase 3 [53].

The secondary structures of antimicrobial peptides can be grouped into three major classes: α -helix, β -sheet stabilized by two or three disulphide bridges and extended peptides with a predominance of one or more amino acids [23,37]. The secondary structure of the peptide is important for the antimicrobial activity and is influenced by the microenvironment. It has been reported that the helix stability contributes to salt resistance [39]. Some α -helical cathelicidins demonstrate salt-independent activity, like the dog, horse and chicken cathelicidins [44,51,63]. Salt sensitivity on the other hand has been reported for mammalian defensins, which display a β -sheet conformation with reduced or abolished activity at high salt concentrations [2,3,19].

Rats, rabbits, mice, rhesus monkeys and guinea pigs, like humans only have one cathelicidin, while other mammals such as pigs, cattle, sheep and horses have been shown to have several cathelicidins [64]. Non-mammalian cathelicidins have been described in animals such as chicken [58,63], pheasant [60], snake [59,66] and the teleost fish species rainbow trout (*Oncorhynchus mykiss*) [13], Atlantic salmon (*Salmo salar*) [14], brown trout (*Salmo trutta*) [33], brook trout (*Salvelinus fontinalis*) [33], grayling (*Thymallus thymallus*) [48] chinook salmon (*Oncorhynchus tshawytscha*)

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[34], Arctic charr (*Salvelinus alpinus*) [33], Atlantic cod (*Gadus morhua*) [33] and ayu (*Plecoglossus altivelis*) [30]. In the primitive vertebrate Atlantic hagfish (*Myxine glutinosa*) ancient members of the cathelicidin family have been discovered [56]. Thus the cathelicidins are widespread in vertebrates but have not been identified in invertebrates.

The mammalian cathelicidins play a role in immune defense through microbial killing and the mature peptides have also adopted other physiological roles such as angiogenesis, chemotaxis and immunomodulation [23]. Little is known about the role of cathelicidins in fish immunity as studies about the functionality of mature cathelicidins in fish are lacking. Two studies have used peptides based on cDNA sequences and investigated the activity of these predicted cathelicidin peptides in rainbow trout (*O. mykiss*) [14] and ayu (*P. altivelis*) [30]. Both studies demonstrated potent activity against Gram-negative bacteria and less activity against Gram-positive bacteria.

We have previously isolated a mature cathelicidin peptide from teleost fish. This peptide was purified from Atlantic cod head kidney and both the isolated and the corresponding synthetic peptide had antibacterial activity [10]. We named the peptide codCath as short for cod cathelicidin. The mature peptide is unique, made of 67 amino acid residues with a pI value of 13.04 and rich in the amino acids arginine (R), serine (S) and glycine (G). In this study we characterized the peptide with regard to its salt sensitivity, antimicrobial activity, kinetics, mode and selectivity of action, proteolytic inactivation and cytotoxicity.

2. Materials and methods

2.1. Peptide synthesis

The mature 67 amino acid residues codCath peptide (SRSGR-GSGKGGRGSGSGSRGSKGPSGSRGSGSRGSKGSRGSRGSGSTI-AGNGNRNNGGTRTA) was produced synthetically (GenScript) with over 95% purity.

2.2. Lipids

1-Palmitoyl-2-oleoyl-phosphatidylglycerol (Na-salt) (POPG), 1,2-dipalmitoyl-PG (Na-salt) (DPPG), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 1,2-dipalmitoyl-PC (DPPC) (Avanti Polar Lipids, Inc.) were used without further purification. Purity (>99%) was checked before and after experiments by thin layer chromatography using $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{NH}_3$ (25% in water) (65:25:5, v/v/v) as mobile phase and detection with molybdenic acid, a phosphorus-sensitive reagent [20].

2.3. Radial diffusion assay

Agarose plates with a thin layer of 1% agarose containing *Bacillus megaterium* (Bm11) at a density of 10^5 bacteria/ml were prepared as previously described [10]. Samples of 3 μl were applied into wells of 3 mm diameter for radial diffusion, and inhibition zones of growth of Bm11 were measured after 24 h incubation at 37 °C.

For the determination of salt sensitivity the agarose was prepared either with normal LB broth containing 1% NaCl (171 mM) or the broth was prepared without NaCl addition (low salt). Two-fold serial dilutions of the peptide ranging from 0.15 to 4.8 mmol were prepared in distilled water and applied to the wells in a volume of 3 μl . Experiments were performed in triplicate. Statistical analysis was performed with a *t*-test.

2.4. Determination of minimal inhibitory concentration (MIC)

The following microorganisms were used for the MIC assays and cultured in the depicted media: *Moritella viscosa*

(478/88^T)/Marine broth (Difco), *Yersinia ruckeri* (NCTC 10746)/Tryptic soy broth (Difco), *Vibrio anguillarum* (NCIMB b^{TT})/Marine broth, *Aeromonas sobria* (As907)/Brain-heart infusion (Difco), *Aeromonas hydrophila* (Ah65)/Brain-heart infusion, *Aeromonas salmonicida* subsp. *achromogenes* (Keldur265-87)/Brain-heart infusion, *Pseudomonas aeruginosa* (PA01)/LB broth, *Escherichia coli* (D21)/LB broth, *B. megaterium* (Bm11)/LB broth, *Lactobacillus* sp./Tryptic soy broth, *Candida albicans* (ATCC 14053)/Yeast-maltose broth.

The minimal inhibitory concentrations (MICs) of the peptide against these microorganisms was determined using colony counting assays as described elsewhere [57]. Briefly, the bacteria/fungus were cultured in liquid broth until they reached the optical density of 0.6 at 595 nm. Cultures were centrifuged at $1000 \times g$ for 25 min and resuspended in a 1/1000 dilution of broth in distilled water. Ten microliters of bacterial/fungal suspension was mixed with 10 μl of 2-fold serial dilutions of the peptide ranging from 2.5 to 80 μM and incubated at the microorganisms optimal temperatures for 2 h on a shaker. Subsequently the mixtures were diluted 10-fold 4 times and the 3 highest dilutions (10^{-2} , 10^{-3} , 10^{-4}) were plated out on appropriate agar plates and incubated overnight or until colonies were visible. The lowest concentration of synthetic cathelicidin peptide, which inhibited bacterial/fungal growth was deemed the MIC. Experiments were performed at least twice with the exception of the *M. viscosa* assay which was performed once, due to its sensitivity to lysis in culture.

2.5. Peptide degradation and Western blotting

Exponential and stationary phases of *V. anguillarum*, *A. salmonicida* subsp. *achromogenes*, *A. hydrophila* and *E. coli* (D21) were defined by growth curves performed in this study or derived from the literature [36]. Bacteria free ECPs of exponential phase and stationary phase bacterial cultures were obtained by centrifugation at $1000 \times g$ for 25 min and sterile filtration through 0.2 μm syringe filters (Whatman). A volume of 3.5 μl of 160 μM codCath peptide solution was mixed with 3.5 μl ECP and incubated for 60 min at room temperature before performing SDS-PAGE or inhibition zone assays. For determination of the time dependent effect on codCath by *A. hydrophila* ECP, a stationary phase culture was used and the ECP were obtained as described above. Incubation times here were 5, 10 and 20 min at room temperature. Control ECP were inactivated at 95 °C for 15 min and then incubated with the peptide for 20 min at room temperature. The samples were prepared for SDS-PAGE and subsequent Western blotting as previously described [10]. To determine the activity of the degraded peptide, aliquots of the above described peptide/ECP samples were loaded on radial diffusion assays with Bm11 as described in Section 2.3.

2.6. Killing kinetics

The kinetic assays were performed as described elsewhere [46]. Exponential phase cultures of Bm11 were resuspended either in 1/1000 diluted LB broth in water creating a low ionic strength environment of approximately 0.25 mM, or in full LB medium supplemented with the salt solution medium E (0.8 mM MgSO_4 , 9.5 mM citric acid, 57.5 mM K_2HPO_4 , 16.7 mM $\text{NaNH}_4\text{HPO}_4$), which enhances the activity of the human cathelicidin LL-37 [7,22], giving a total salinity of 246 mM. CodCath was added at a concentration of 20 μM which equals $4 \times \text{MIC}$. LL-37 was used as a reference peptide at a molarity of 20 μM which also corresponds to $4 \times \text{MIC}$ [22]. As control an untreated aliquot of bacterial suspension was used. After addition of the antimicrobial peptides, the optical density (OD) was measured at 600 nm every 10 min for a period of 400 min. The samples were kept at 37 °C in between measurements. Aliquots were taken at 20 min intervals for 120 min, diluted immediately in broth

(1:10) and plated onto LB-agar plates. The agar plates were incubated overnight at 37 °C and growth of bacteria was assessed the next day.

2.7. Cytotoxicity

Salmonid cells CHSE-214 (chinook salmon embryo) were seeded onto 96 well plates in growth medium MEM (Earle's media containing GlutaMAX™-1 and 25 mM HEPES), supplemented with 10% fetal bovine serum, 25 U/ml penicillin and 25 mg/ml streptomycin, non-essential amino acids and sodium bicarbonate (all Gibco/Invitrogen). Cells were grown at 17 °C and after 48 h, growth medium was removed and cells were incubated in a 2-fold dilution series of codCath at concentrations of 40, 20, 10 or 5 µM in phenol-red free growth medium with supplements for 1 h at room temperature. As a positive control for lysis 10% Triton X-100 (Sigma) in growth medium was used, the negative control was growth medium alone. The reagent PrestoBlue (Invitrogen) was added at a concentration of 4% and the cells were incubated for 24 h at 17 °C. The assay medium was subsequently spectrophotometrically measured at 600 nm and 570 nm and the normalized values demonstrated metabolic activity, hence cell viability. Experiments were performed in triplicate. Statistical differences were determined with a *t*-test and differences were considered significant at the 0.05 level.

2.8. Leakage experiments

Leakage of aqueous contents from large unilamellar vesicles (LUVs) composed of POPG or POPC upon incubation with peptide were determined using the 8-aminonaphthalene-1,3,6-trisulphonic acid/p-xylene-bis-pyridinium bromide (ANTS/DPX) assay [16]. Dried lipid films (20 mg) were hydrated with 1 ml 12.5 mM ANTS, 45 mM DPX, 68 mM NaCl, 10 mM HEPES at pH 7.4 at room temperature (fluid phase of lipids) for 1 h under intermittent vigorous vortex mixing. Subsequently, the dispersions were extruded 20 times through a polycarbonate filter (Millipore-Isoapore™) of 0.1 µm pore size to obtain LUVs. The ANTS/DPX containing vesicles were separated from the free ANTS/DPX by exclusion chromatography using a column filled with Sephadex™ G-75 (Amersham Biosciences) fine gel swollen in an iso-osmotic buffer (10 mM HEPES, 140 mM NaCl and 1 mM EDTA). Phospholipid concentration was determined by phosphate analysis [4]. Size and homogeneity were tested by dynamic light scattering.

Fluorescence spectra were obtained at 20 °C using an excitation wavelength of 360 nm and an emission wavelength of 530 nm and a slit width of 5 nm for both excitation and emission monochromators. The fluorescence measurements were performed in quartz cuvettes in 2 ml of the iso-osmotic buffer at the referred temperature. Lipids were diluted to a final concentration of 50 µM. Fluorescence emission was recorded as a function of time before and after the addition of incremental amounts of peptide. The fluorescence increase due to leakage and subsequent dilution of dye was measured after addition of peptides in 3 concentrations of 2, 4 and 8 µM, corresponding to peptide to lipid molar ratios of 1:25, 1:12.5 and 1:6.25, respectively. The measurements were performed on a SPEX Fluoro Max-3 spectrofluorimeter (Jobin-Yvon) combined with Datamax software. Data are presented in terms of fluorescence intensity (I_F):

$$I_F = \frac{F - F_0}{F_{\max} - F_0}$$

F is the measured fluorescence, F_0 is the initial fluorescence without peptide and F_{\max} is the fluorescence corresponding to 100% leakage gained by addition of 1% Triton X-100.

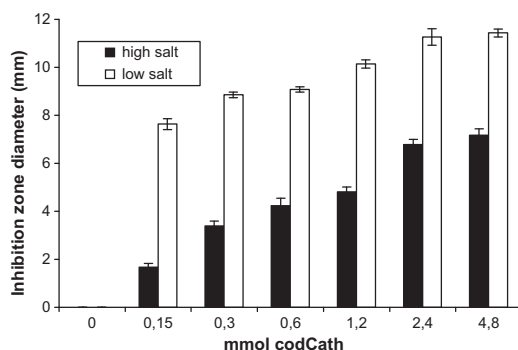


Fig. 1. The salt sensitivity of cod cathelicidin. Increasing amounts of codCath (mmol in 3 µl volume) were examined in a radial diffusion assay with *B. megaterium* (Bm11) in a high salt or low salt environment. Activity in the low salt environment was significantly higher than in the high salt environment. The diameter of a well (3 mm) was subtracted from the inhibition zone diameters depicted. Values are given as mean of 3 experiments with standard error bars.

2.9. Differential scanning calorimetry (DSC)

Appropriate amounts of DPPG and DPPC stock solutions were dried under a stream of nitrogen and stored in vacuum overnight to completely remove organic solvents. The dry lipid film was then dispersed in 10 mM Na-phosphate buffer, pH 7.4 in the absence or presence of peptide at a molar ratio of lipid-to-peptide of 50:1 and hydrated at a temperature well above the gel to fluid phase transition of the respective phospholipid under intermittent vigorous vortex-mixing following a protocol depending on the respective phospholipid. The lipid concentration was 0.1 wt. %.

DSC experiments were performed with a differential scanning calorimeter (VP-DSC) (MicroCal, Inc.). Heating scans were performed at a scan-rate of 30 °C/h with a final temperature approximately 20 °C above the main transition temperature (T_m) and cooling scans at the same scan rate with a final temperature about 20 °C below T_m . The heating/cooling cycle was repeated twice, pre-scan thermostating was allowed for 15 min for the heating scans and 1 min for the cooling scans. Two heating and cooling cycles were performed showing identical results indicating full reversibility of the lipid phase transitions of both DPPG and DPPC. Enthalpies were calculated by integrating the peak areas after normalization to phospholipid concentration and baseline adjustment using the MicroCal Origin software (VP-DSC version).

3. Results

3.1. Salt sensitivity

Radial diffusion assays were used to determine the activity of codCath against *B. megaterium* (Bm11) in regular LB agarose (minimum 171 mM NaCl) and LB without added NaCl (low salt). The peptide showed a dose dependent increase in antibacterial activity and the inhibition zone decreased significantly in diameter when salt was included, corresponding to a reduced activity of the peptide (Fig. 1). This observation showed that the antibacterial activity of the peptide was sensitive to the ionic strength of the surrounding environment.

3.2. Minimal inhibitory concentration

Selected fish pathogens and relevant microorganisms covering Gram-negative and Gram-positive bacteria as well as a fungus were

Table 1

Minimal inhibitory concentrations (MICs) of cod cathelicidin against selected microorganisms at low salt conditions.

Species	MIC (μ M)
<i>Moritella viscosa</i>	5
<i>Yersinia ruckeri</i>	10
<i>Vibrio anguillarum</i>	5
<i>Aeromonas sobria</i>	10
<i>Aeromonas hydrophila</i>	10
<i>Aeromonas salmonicida</i>	10
<i>Pseudomonas aeruginosa</i>	5
<i>Escherichia coli</i> D21	5
<i>Bacillus megaterium</i> Bm11	5
<i>Lactobacillus</i> sp.	≥ 80
<i>Candida albicans</i>	2.5

tested for minimal inhibitory concentration (MIC) of the peptide in a low salt environment. The MICs ranged from 5 μ M to 10 μ M for the Gram-negative bacteria and for the Gram-positive strain Bm11. The MIC for the fungus *C. albicans* was the lowest with an average of 2.5 μ M. The Gram-positive bacterium *Lactobacillus* sp. on the other hand had a MIC of 80 μ M, indicating a resistance of this strain toward codCath (Table 1). Overall, we observed potent broad spectrum activity against different bacterial species and against a fungus.

3.3. Peptide proteolysis

In order to determine the susceptibility of codCath to proteolytic breakdown by bacterial proteases, we selected the following fish pathogenic bacteria: *V. anguillarum* (a cod pathogen), *A. salmonicida* subsp. *achromogenes* (a cod pathogen), *A. hydrophila* (a pathogen of various vertebrates). In addition we used our indicator strain *E. coli* (D21) as a reference. ECPs of log phase or stationary phase bacterial cultures were incubated with the peptide for 60 min. Peptide degradation was analyzed using Western blotting and the activity of the peptide after incubation with ECPs was determined with radial diffusion assay. The ECP obtained from exponential phase growth of *V. anguillarum* showed minor breakdown of codCath (Fig. 2, lane 1), while the ECPs of the other bacteria had no activity against codCath (Fig. 2, lanes 3 and 4). In contrast, ECPs obtained from the stationary phase growth of *V. anguillarum*, *A. salmonicida* subsp. *achromogenes* and *A. hydrophila* caused prominent degradation of the peptide (Fig. 2, lanes 5–7), while the ECP from *E. coli* (D21) had no effect. The activity measured by inhibition zone assay correlated with the observed degradation with complete abrogation of antibacterial activity for *A. hydrophila* ECP from the stationary phase of growth (data not shown). To approach the kinetics and the character of the active component we incubated *A. hydrophila* ECP from

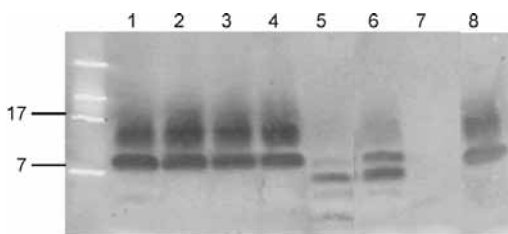


Fig. 2. Western blot showing proteolytic degradation of cod cathelicidin after incubation of the peptide for 60 min with log phase ECPs (lanes 1–4) or stationary phase ECPs (lanes 5–8). Little or no degradation with log phase ECPs was observed but in the stationary phase all ECPs except from *E. coli* (D21) degraded the peptide. Lanes 1 and 5: *V. anguillarum*; 2 and 6: *A. salmonicida* subsp. *achromogenes*; 3 and 7: *A. hydrophila*; 4 and 8: *E. coli* (D21). The sizes of the protein marker are given in kDa.

the stationary phase of growth with the peptide for either 5, 10, or 20 min and in addition we incubated heat-treated ECP with the peptide. A time dependent progress of proteolysis was observed and heat-deactivation of the ECP abolished the breakdown of codCath, indicating a protein component to be responsible for the proteolysis (Fig. 3A). In order to test the activity of the peptide incubated with ECPs, aliquots were loaded onto a radial diffusion assay. Only 5 min incubation of peptide with ECP reduced the activity against *B. megaterium* (Bm11) considerably. No activity was found in samples incubated for 10 and 20 min with ECP (Fig. 3B).

3.4. Killing kinetics

In order to approach the effectivity and mode of bacterial killing of codCath, a log phase culture of *B. megaterium* (Bm11) was resuspended in either diluted LB (low salt) or LB supplemented with medium E (high salt) and incubated with codCath or the reference peptide LL-37. In a low salt environment, both codCath and LL-37 killed the bacteria within the first minutes (Fig. 4B), although only codCath showed a prominent decrease in OD over the course of the experiment (Fig. 4A). Both peptides caused an initial increase in density which was probably due to the initial formation of coarse cell debris from cell lysis, prominent and fast for codCath, but less and slower for LL-37. The bacteria did not grow under these conditions indicated by the stable optical density over the experimental time (Fig. 4A). In the high salt environment, only LL-37 was able to kill the bacteria (Fig. 4D). No difference was detected between codCath and the control sample with respect to OD and viability (Fig. 4C and D).

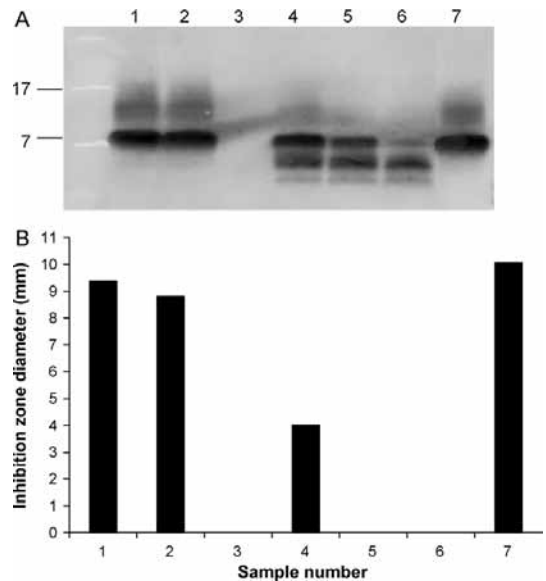


Fig. 3. Time dependent effect on codCath integrity (A) and activity (B) of stationary phase *A. hydrophila* ECP. The Western blot showed breakdown of the peptide into smaller fragments (lanes 4–6). The heat-treated sample did not cause degradation (A). Antibacterial activity of codCath against *Bacillus megaterium* (Bm11) was reduced or abolished in a radial diffusion assay after incubation with ECP, but remained intact with prior heat-treatment of ECP (B). (1) Peptide only, (2) peptide + Tryptic soy broth, (3) ECP only, (4) incubation with ECP for 5 min, (5) incubation with ECP for 10 min, (6) incubation with ECP for 20 min, (7) incubation with heat deactivated ECP for 20 min. The sizes of the protein marker are given in kDa (A). The diameter of a well (3 mm) was subtracted from total inhibition zone diameters (B).

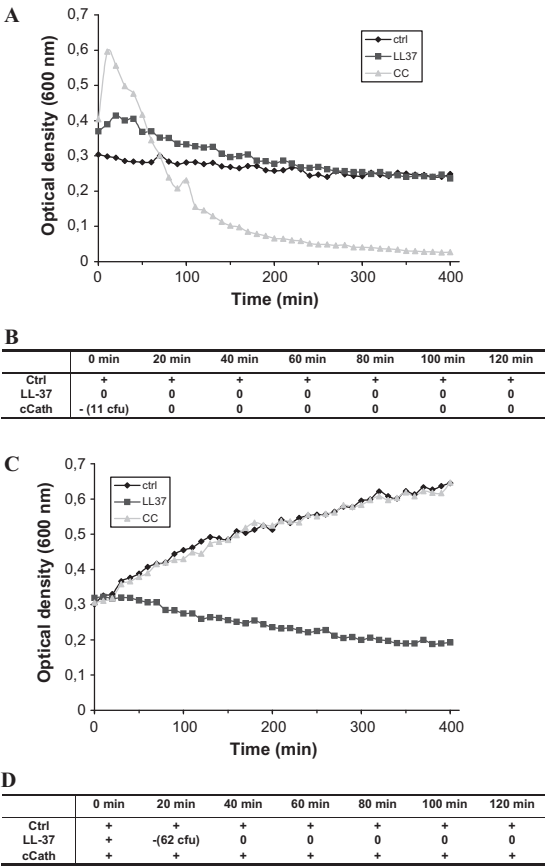


Fig. 4. Killing kinetics of codCath (cC) and LL-37 on *Bacillus megaterium* (Bm11) in diluted LB broth (1/1000 in H₂O) (A and B) or in LB broth supplemented with medium E (C and D). In diluted broth the density of the bacterial suspension supplemented with codCath decreased profoundly over the course of the experiment indicating cell lysis (A) and both codCath and LL-37 killed the bacteria in a few minutes (B). In LB broth supplemented with medium E, LL-37 caused apparent growth inhibition, while there was no growth inhibition with codCath (C). In high salt conditions, only LL-37 killed the bacteria after 20 min, while with codCath the bacteria remained viable throughout the experiment (D). Ctrl: bacteria without antimicrobial peptide, +: many cfu (colony forming units), -: few cfu, and 0: no growth.

This assay confirmed the salt sensitivity of codCath and showed the peptide to have a rapidly lytic action of antibacterial activity, while LL-37 in comparison demonstrated killing without prominent lysis.

3.5. Cytotoxicity

The cytotoxic action of codCath against the teleost cell line CHSE-214 was tested at the concentrations of 40, 20, 10 or 5 μ M peptide with a resazurin-based assay. No toxicity was detected in either of the wells treated with codCath, when compared to the control. Substrate turnover considered as measure of cell viability was significantly higher (with 40, 10 and 5 μ M treatment) in the codCath treated wells than in the control wells (Fig. 5).

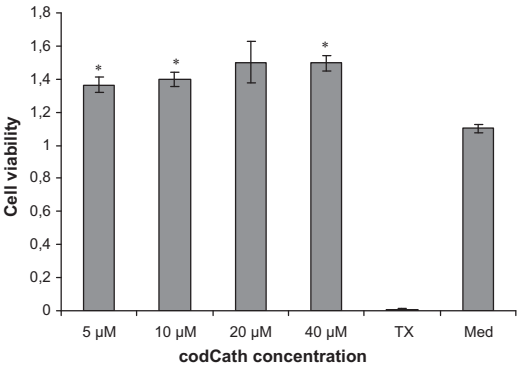


Fig. 5. Cytotoxicity assay of codCath against CHSE-214 cells, with no observed cytotoxicity in concentrations up to 40 μ M. The positive control (TX) was 10% Triton X in growth medium and the negative control (Med) was growth medium alone. Cells incubated with codCath for 24 h exhibited a significantly higher value for cell viability. *Samples significantly different ($p \leq 0.05$) from the negative control (Med).

3.6. Leakage experiments

In order to test membrane permeability in the presence of codCath we prepared large unilamellar vesicles composed of POPG and POPC, respectively, loaded with ANTS/DPX and incubated them with codCath at 20 °C, where the lipids are in the physiologically relevant fluid phase. These leakage experiments reflected both the salt dependent behavior of the biological activity of cod cathelididin and the membrane selectivity (Fig. 6). Thus the peptide did not show significant interaction with the zwitterionic PC, a mimic for mammalian cell membranes, at any salt condition and with the anionic PG used to mimic bacterial membranes at high salt. However, a marked release of entrapped fluorophore from POPG vesicles was observed at no salt. Upon increasing the concentration of codCath a stepwise burst of leakage was observed which stopped before all contents was released (~70% at 8 μ M corresponding to a lipid-to-peptide molar ratio of ~6:1). This partial transient leakage is commonly observed and explained by all-or-none and graded leakage [1,41] However, this aspect is difficult to rationalize within

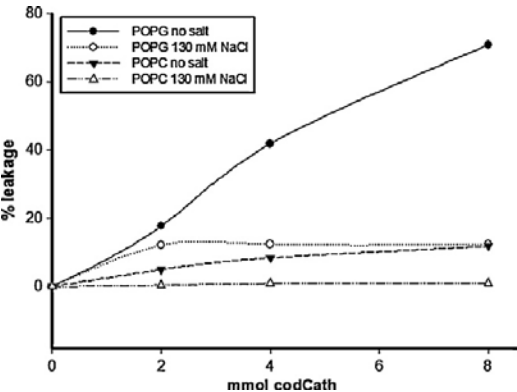


Fig. 6. Leakage of large unilamellar vesicles at different concentrations of codCath in salt-free and salt-containing conditions (lipids indicated in the panel). A dose-dependent amount of leakage was observable that was salt-sensitive. Only bacterial membrane mimetics showed leakage in no salt condition.

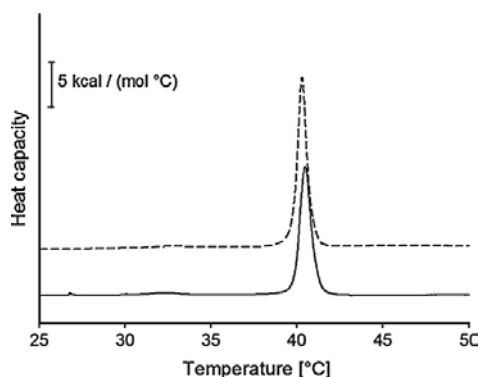


Fig. 7. Normalized excess heat capacity function of DPPG in 10 mM Na-phosphate buffer, pH 7.4 in the absence (dotted line) and presence of codCath (full line) at a lipid-to-peptide molar ratio of 50:1; scan-rate 30 °C/h. A decrease of the main transition temperature and an increase of enthalpy were observed in the presence of codCath.

the context of existing models without invoking additional ad hoc events, such as trans-bilayer disequilibrium [62].

3.7. Calorimetry

Based on the results of the leakage experiments the effect of codCath on the thermotropic phase behavior of PG and PC was studied only at no salt condition. The thermogram of DPPG shows two phase transitions (Fig. 7), which can be attributed to the pre-transition at 32.3 °C and the main or chain-melting transition at 40.4 °C being in agreement with earlier data [28,42]. Addition of codCath at a lipid-to-peptide molar ratio of 50:1 broadened the pre-transition range and slightly reduced the main transition temperature by 0.2 °C with a concomitant increase of enthalpy by about 10% as reflected by the larger area of the normalized excess heat capacity function shown in Fig. 7. In contrast, super-impossible thermograms were obtained for DPPC in the absence and presence of codCath (data not shown) indicating that the peptide does not interact with the zwitterionic lipid bilayer.

4. Discussion

Mature cathelicidin peptides are considerably diverse in length, amino acid sequence and structure as they can adopt alpha-helical, beta-hairpin or extended conformations [54]. This variety may account for distinct functions and for a diverse spectrum of activity [54]. Alpha-helical peptides are often unstructured in aqueous solution and adopt their ordered secondary structure upon interaction with biological membranes, while beta-sheet peptides largely exist in the beta-sheet conformation in aqueous solution and may be further stabilized by lipid surfaces [8]. In contrast, proline-arginine or tryptophan rich peptides cannot form ordered secondary structures due to the incompatibility of those residues [8]. Secondary structure predictions of codCath in aqueous solution suggest an extended structure (data not shown) which is mainly due to the many glycine residues (24 out of 67 amino acids) distributed evenly in the peptide. Due to the high concentration of serine, arginine and glycine residues a conformational change even upon membrane contact is thought to be unlikely.

Microenvironmental factors have a prominent effect on the activity of antimicrobial peptides [8]. A reducing environment has been shown to significantly increase the activity of human beta-defensin 1 [47]. Salt also commonly affects activity of

antimicrobial peptides. The alpha-helical human cathelicidin LL-37 has been shown to be active at high salt concentrations toward some microorganisms, while it was inactive against others in a high salt environment [55]. Human beta-defensin 1 is sensitive to salt and demonstrates reduced activity at high salt concentrations [19,55]. Other peptides for which salt sensitivity has been reported are indolicidins, bactenecins and magainins [39]. In our study, antibacterial activity of codCath was reduced at 171 mM NaCl in comparison with salt-free conditions, and abolished at a salinity of 246 mM (Fig. 4C). The physiological salt concentration of Atlantic cod blood in seawater has been reported to be approximately 300 mM [32]. This suggests an inhibited activity of codCath in extracellular fluids of Atlantic cod. Interestingly, the salt sensitivity we observed is in accordance with peptides from other marine organisms [31]. Most of these peptides demonstrated decreased activity at salinities close to physiological salt and completely abolished activity at marine salinity. While the environmental conditions are unknown we predict that salt scavenging in the glycocalyx and control of salt balance could determine the activity of the peptide. It is further possible that the peptide is active in vesicles of phagocytic cells that contain an environment different from the extracellular one [49]. In this case the peptide would aid the destruction of ingested bacteria. However, the localization of the peptide has yet to be resolved. The mechanism by which salt ions affect the activity of codCath might be due to neutralizing the positive residues of the peptide as well as masking the negative charges of the microbial membranes.

CodCath was found to have high activity against Gram-negative bacteria including *V. anguillarum* and *A. salmonicida* subsp. *achromogenes* both major bacterial pathogens of wild and farmed cod [43]. This antimicrobial activity as well as the reported increased cathelicidin gene expression upon infection with pathogenic bacteria [12,18,50], indicate a role for the peptide in innate defense of cod. The Gram-positive *Lactobacillus* sp. was shown to be resistant to codCath. Notably activity against Gram-negative, but not Gram-positive bacteria was also observed in studies using predicted fish cathelicidin peptides [13,30]. In one study two Gram-positive bacteria were shown to have MICs differing as much as 2 and 40 µM [13], which is similar to the observed difference in peptide activity against *B. megaterium* (Bm11) and *Lactobacillus* sp. in our study. These results indicate that codCath targets only certain Gram-positive bacteria in Atlantic cod. This is in accordance with other studies where endogenous antimicrobial peptides are often known to complement each other and to have different specificities [15]. Our results suggest that codCath has a prominent role in antifungal defense, since we observed the lowest MIC against *C. albicans*. Antifungal activity of all structural classes of cathelicidins against *Candida* and *Cryptococcus* species has been shown before [6,29].

Pathogens are known to interfere with the initial innate defenses by employing different resistance mechanisms to protect themselves against the action of antimicrobial peptides [40,65]. Altered cell surface charge, active efflux of antimicrobial peptides, trapping mechanisms, downregulation of host AMP production and proteolytic degradation are recognized as viable strategies to avoid killing through antimicrobial peptides [38]. In our study we have found that extracellular products of cod pathogenic bacteria were able to degrade the codCath peptide and abrogate its antibacterial activity. Since this effect was inhibited by prior heat treatment, it is likely that the degrading component is a protease. Interestingly, protease activity against an antimicrobial peptide with relevance to bacterial virulence has been described: the gingipain proteases released by the pathogenic strain of *Porphyromonas gingivalis*, degraded human beta-defensin 3 [35]. In our study, *A. hydrophila* ECP were the most effective at degrading codCath. We observed that *V. anguillarum* seemed to secrete proteases, already during the log phase of culture growth while *A. salmonicida* subsp.

achromogenes and *A. hydrophila* released proteases only in the stationary phase. A study that investigated the proteolytic effect of *A. hydrophila* ECP on frog AMPs also found that the presence of protease activity in the ECPs was dependent on the incubation time of the cultures and it was hypothesized that this could be a strategy to avoid an attack of the immune system in the initial stage of disease to allow the build-up of a sufficient number of bacterial cells for tissue invasion [45]. It has been shown that resistance mechanisms to antimicrobial peptides increase the virulence of a pathogen when compared with a non-resistant mutant [38]. Hence we suggest that the production of proteases that lead to degradation of the codCath peptide constitutes a virulence mechanism of the bacteria we tested.

Some antimicrobial peptides are thought to kill microbes through destabilization of the membrane following the 'barrel-stave', 'toroidal pore', 'carpet', lipid clustering, interfacial activity or detergent model [5,11,17,26,61] and this ultimately leads to the rupture of the cell. Another group of antimicrobial peptides traverse the membrane and disrupt intracellular processes which leads to the killing of the microorganism [11,27]. The only cathelicidin known to act this way is the pig cathelicidin PR-39, which has been shown to interfere with protein- and nucleic-acid synthesis [9]. Work on plectasin, a fungal defensin, identified cell wall biosynthesis as the target of antibiotic activity [46]. We wanted to establish the mechanism codCath employs to kill bacteria. While LL-37 killed bacteria under high and low salt conditions, codCath was able to kill bacteria only in the low salt environment. LL-37 is known to adopt an alpha-helical conformation, while codCath is thought to adopt an extended structure (coil) and this difference in secondary structure can contribute to the different bacterial killing mechanism observed. It has been suggested that LL-37 interferes with cell wall biogenesis [52], which would explain why no prominent decrease of optical density was observed. CodCath on the other hand was shown here to kill bacteria through rapid cell lysis. We further exposed model membranes to different concentrations of codCath and observed leakage, which supports lytic activity. It is intriguing that codCath which lacks the typical amphipathic structure of antimicrobial peptides and exhibits a low hydrophobicity can induce membrane permeabilization. Molecular dynamics simulations of magainin in DPPC have shown that a toroidal pore can form by as few as four peptide molecules and the disordered channel is stabilized by a diffuse distribution of peptides around the rim of the pore being aligned parallel to membrane bilayer [25]. Therefore, in a recent review it was suggested that the binding of antimicrobial peptides might sufficiently alter the interactions between neighboring lipid molecules inducing membrane curvature strains resulting in pore formation [21]. The modest decrease of the main transition temperature and the appreciable increase of enthalpy observed for the bacterial mimetic system in the presence of codCath clearly indicates that the cationic peptide binds to the anionic membrane surface. This may lead to rearrangements in the headgroup region which in turn may sufficiently perturb the lipid packing and thus in the context of above result in an increase of permeability.

Antimicrobial peptides usually do not cause damage to host cells at concentrations that kill microbes. We found that codCath up to a concentration of 40 μM was not cytotoxic to CHSE-214 cells and these results together with the low MIC of 5–10 μM against many bacteria, suggest a microbial activity range of the peptide without damage to the host cells. These results are supported by the findings that codCath causes leakage of bacterial membrane mimetics but not of mammalian membrane mimetics. The observed increase in cell viability/substrate turnover of CHSE-214 cells treated with codCath compared to controls, could be explained by an augmented metabolism or stimulation of proliferation in the presence of codCath.

In conclusion we demonstrate that CodCath has potent antimicrobial activity against Gram-negative bacteria and fungi in low salt conditions in vitro. These results suggest that codCath has an important role in Atlantic cod immunity. Further we found that bacteria evade the active peptide through proteolysis and this can be linked to bacterial virulence and pathogenicity in Atlantic cod.

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III

Differential regulation of cathelicidin in salmon and cod

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Abbreviations

AMP – antimicrobial peptide, TLR – toll-like receptor, PI3K – phosphoinositid-3-kinase, PAMPs – pathogen associated molecular patterns, h – hours, p.s. – post stimulation, ECP – extracellular product

Abstract

Antimicrobial peptides (AMPs) are an important component of innate immunity in vertebrates. The cathelicidin family of AMPs is well characterized in mammals and has also been reported in several fish species. In this study we investigated the regulation of cathelicidin expression in a gadoid and a salmonid cell-line in order to dissect the signalling pathways involved. For this, fish cells were treated with microbial lysates, purified microbial components and commercial signalling inhibitors, and expression of cathelicidin was assessed with quantitative real-time PCR (qPCR). We found that cathelicidin expression was induced in both cell lines in response to microbial stimuli, but the response patterns differed in these evolutionary distant fish species. Our data suggest that in salmonids, pattern recognition receptors such as TLR5 are involved in the stimulation of cathelicidin expression and that the signalling cascade includes PI3-kinase and cellular trafficking compartments. A detailed knowledge of the regulating factors involved in AMP-related defence responses, including cathelicidin, could help in developing strategies to enhance the immune defence of fish.

1. *Introduction*

Antimicrobial peptides (AMPs) are present in living organisms including bacteria, plants and animals [1]. In animals they demonstrate abundant expression in epithelial and immune cells [2, 3]. AMPs are able to kill Gram-negative and Gram-positive bacteria, fungi, viruses and parasites [4] and therefore have an important role in immune defence. In mammals, AMPs are multifunctional molecules that not only directly kill pathogens, but also have other functions related to tissue repair and immune modulation like promotion of wound healing, angiogenesis, recruitment of T-cells, neutralization of proinflammatory cytokines or anti endotoxin activity [5, 6]. In fish, multifunctionality in the form of bactericidal and immunomodulatory activity of AMPs has also been suggested [7, 8]. The importance of AMPs in fish immunity has been demonstrated in studies with administration of synthetic AMPs to fish or transgenic fish expressing additional AMPs, which led to improved survival during infection challenges [9, 10].

AMPs can be constitutively expressed, or alternatively their expression can be induced through a stimulus. This induction can be direct with a stimulated receptor leading to the upregulation of the AMP gene, or

indirect, where a stimulus leads to synthesis of proteins which then elicit the transcription of AMPs [6, 15]. The direct induction in some AMPs can be triggered by pathogen-associated molecular patterns (PAMPs) through toll-like receptors (TLR) or other pattern recognition receptors (PRR). In *Drosophila*, AMPs are upregulated differentially through bacterial and fungal stimuli [16]. In fish, AMPs have also been shown to be upregulated due to bacterial infection [17-23]. In mammals, only a few studies have shown the increased expression of AMPs in response to bacteria. Examples are the intranasal administration of flagellin upregulating CRAMP (cathelin-related antimicrobial peptide) expression in mouse lung [24] and the human α -defensins 1-3 being upregulated upon stimulation with live staphylococci [25]. It has been shown that the expression of the human cathelicidin antimicrobial peptide LL-37 is increased through vitamin D [26], butyrate [27], bile salts [28], or lithocholic acid [29], all endogenous products of metabolism. Vitamin D and lithocholic acid represent effectors of direct induction, while butyrate and its derivative phenylbutyrate are indirect inducers causing upregulation of the human cathelicidin LL-37 as a secondary response dependent on translation [30].

The emergence of antibiotic resistant pathogens has led to the increased focus on antimicrobial peptides to fight infections [1]. Initially it was assumed that pathogens cannot develop resistance to AMPs, however exposure of a bacterium to sublethal concentrations of a single AMP has been shown to lead to resistance [11]. Since AMPs are a vital part of the immune system, the emergence of pathogen resistance to AMPs, leading to the loss of their effectiveness, could be devastating. Therefore, provoking the emergence of AMP resistant pathogens through the use of synthetic AMPs is not recommendable. Taking advantage of the benefits of AMPs while avoiding the emergence of resistance could be achieved by inducing endogenous AMP expression with selected stimuli, thereby upregulating a protective innate immune response as done in mammalian systems before [12-14].

Cathelicidins, together with defensins, are the best described AMPs in mammals [31]. The importance of cathelicidin in mammalian immunity has been demonstrated in studies showing that the absence of cathelicidin in knock-out mice had a weakening effect on the innate immune defence against infection [32, 33], while transgenic mice expressing an additional cathelicidin showed increased defences [34].

In fish, cathelicidins have been identified in many fish species including Atlantic hagfish (*Myxine glutinosa*) [35], different salmonids [19, 21, 36], ayu (*Plecoglossus altivelis*) [20] and Atlantic cod (*Gadus morhua*) [37]. Several studies have suggested a role of cathelicidins in fish immunity due to their increased expression upon stimulation with bacteria [17-21] and the antibacterial activity of the peptide [19, 20, 38].

In the present study, we identified different microbial compounds that induced cathelicidin expression in a salmon and a cod cell line and studied the signalling pathways involved. Knowledge on these pathways could be applied in the future in aquaculture settings in order to identify compounds that increase the endogenous defence of the fish and thereby help in the fight against infection.

2. *Materials and Methods*

2.1 *Cell culture*

The Chinook salmon (*Oncorhynchus tshawytscha*) embryo cell line (CHSE-214) and the Atlantic cod (*G. morhua*) larvae cell line (ACL) were cultured either in MEM (Earles media containing GlutaMAX™-1

and 25 mM HEPES) or L-15 medium (Leibovitz' medium) supplemented with 10% foetal bovine serum, 25 u/ml penicillin and 25 mg/ml streptomycin, 1% non-essential amino acids and 1% sodium bicarbonate (all Gibco/Invitrogen). Cells were maintained at 16°C in closed 75 cm² flasks and split into 25 cm² flasks for experiments. Cells were passaged at 2–3 week intervals.

2.2 Bacterial and fungal culture and preparation

Pseudomonas aeruginosa (strain PA01) was cultured in LB medium at 37°C. *Aeromonas salmonicida* subsp. *achromogenes* was cultured in brain-heart infusion at 25°C. *Vibrio anguillarum* was cultured in marine broth at 25°C. *Escherichia coli* (D21) and *Bacillus megaterium* (Bm11) were cultured in LB medium at 37 and 30°C, respectively. *Lactobacillus* sp. was cultured in tryptic soy broth at 20°C. *Candida albicans* was cultured in yeast maltose broth at 37°C.

Microorganisms were grown until early stationary phase and the density was measured at 600 nm. The concentration of microorganisms was determined according to OD₆₀₀ =1 equals 5.5 x 10⁸ bacteria/ml. The cells were pelleted by centrifugation at 2000 x g. The pelleted

bacteria were inactivated by resuspension and incubation in 70% ethanol overnight followed by 3 washes in PBS and subsequent exposure to UV light overnight. The microbial cells were then disrupted in order to expose intracellular PAMPs by sonication at 40% amplitude with a Vibra Cell sonicator (Sonics) in intervals of 20 seconds 10-20 times. Subsequently inactivation of microorganisms was confirmed by testing growth on agar plates. Preparations were stored frozen until needed.

2.3 *Stimulation and inhibition*

Fish cells were stimulated with a range of 100-200 dead bacteria/cell in serum- and antibiotic free medium for 24 h unless otherwise stated. For the comparison of different microorganisms the volumes of inactivated bacteria used for stimulations were adjusted according to their density to assure equal application of microbial fragments and corresponded to approximately 250 bacteria/cell.

The following stimulants were used at the indicated concentrations: sodium-4-phenylbutyrate (Tocris) was applied at the concentration of 4 mM. 2,6-diaminopimelic acid (Sigma): 100 µg/ml, β-D-glucan (Sigma,

#G6513): 100 µg/ml, poly(I:C) (Tocris): 10 and 100 µg/ml, LPS (Sigma, #L9143 derived from *P. aeruginosa*): 10 µg/ml, flagellin (isolated from *P. aeruginosa*): 5.7 µg/ml, DNA (isolated from *P. aeruginosa*) at 115 ng/ml. Flagellin and bacterial DNA were isolated as described previously [39, 40] and the purity of these components was confirmed by SDS-PAGE and UV/Vis-spectroscopy.

For the experiments with inhibitors the compounds were applied at the following concentrations: brefeldin A (Sigma) (Golgi apparatus inhibitor): 10 µg/ml, nocodazole (Sigma) (microtubule inhibitor): 10 µg/ml, chloroquine (Sigma) (inhibitor of lysosomal function and TLR9): 10 µM, wortmannin (Sigma) (PI3K inhibitor): 1 and 5 µM, LY294002 (Cell Signalling Technology): 8, 17 and 25 µM. For the assays, fish cells were preincubated with inhibitors for 2 h at 16°C prior to addition of the microbial stimulus and subsequent incubation for 24 h at 16°C.

2.4 RNA isolation and real-time q-PCR

Total RNA from fish cells was isolated with TRI Reagent (Sigma Aldrich) according to the manufacturer's instructions except that the

extraction step was repeated once in order to assure maximal RNA purity. The RNA pellet from one 25 cm² flask of cells was dissolved in 40 µl RNase-free water. RNA quality and quantity was examined on a NanoDrop ND-1000 UV/Vis spectrometer. Five hundred ng of RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosciences) following the manufacturer's instructions. The cDNA was subsequently diluted 1:10 in water and qPCR was performed. The Chinook salmon cathelicidin primers were designed with PerlPrimer [41] (forward primer: 5'-ATGGGAAACGAATGATGTGC-3', reverse primer: 5'-CGGTCAGTGTTGAGGGTATT-3'). The reference genes in CHSE-214 cells were RPS20 and EF-1α with primer sequences published previously [42]. The primer sequences for Atlantic cod cathelicidin and the reference genes RPS9 and ubiquitin were published previously [17, 43].

All qPCRs were performed using Power SYBRGreen with ROX (Applied Biosystems) according to the manufacturer's instructions with the exception that 10 µl final reaction volume was used. The qPCR runs were performed on an ABI 7500 real-time PCR System (Applied Biosystems) and the thermal cycling parameters were 50°C

for 2 min, followed by a 95°C hot start for 10 min, subsequently the amplification was performed with 40 cycles of 95°C for 15 s and 60°C for 1 min. Every sample was assayed in duplicate and every experiment was performed two times or more as stated in the figure legends. Relative expression levels were calculated with the $2^{-\Delta\Delta C_t}$ method [44], where unstimulated cells were set as 1 and fold inductions of treated cells were compared to this value. The +/- standard errors of fold expression were calculated from the standard errors (SE) of the ΔC_t s with the formula $2^{-(\bar{\Delta C_t} + SE)}$ = minimum fold expression and $2^{-(\bar{\Delta C_t} - SE)}$ = maximum fold expression.

2.5 Preparation of ACL cell secretions

Seventy five cm² flasks with ACL cells containing 9 ml of FBS- and antibiotic-free medium were stimulated with *Lactobacillus* sp (approx. 250 bacteria/cell). Untreated flasks were used as control. Cells were incubated at 16°C for 48 h instead of 24 h to increase the yield of secreted proteins. Supernatants were harvested and freeze-dried. qPCR of the cells was performed as described above to determine the degree of upregulation. Oasis HLB cartridges (Waters) were used for desalting and enrichment of peptides and proteins in the supernatants

as described previously [17]. The desalted and enriched supernatants were divided into 3 aliquots. Two aliquots were reconstituted in either 10 μ l 5% formic acid with the addition of 3 μ l of pepsin (Sigma) (10 mg/ml) or in 6 μ l Milli-Q water with the addition of 0.6 μ l of proteinase K (New England Biolabs) (20 mg/ml). The third aliquot was dissolved in 10 μ l Milli-Q water and used as untreated control. Samples were incubated overnight at 37°C and subsequently lyophilized. They were reconstituted in 3 μ l Milli-Q water and loaded on radial diffusion assays to determine antibacterial activity.

2.6 *Radial diffusion assay*

Single colonies of *Bacillus megaterium* (Bm11) were picked and used to inoculate 20 mL LB medium. Bacteria were grown at 37°C until the optical density reached 0.6 (600 nm). Thin 1% agarose plates (1 mm) of salt-free LB media containing 3×10^4 bacteria/ml were poured and wells 3 mm in diameter were punched in the agarose layer. The sodium chloride was omitted from the LB medium used to prepare the agarose since we had found previously that the activity of cod cathelicidin is decreased in the presence of salt. The samples were loaded into the

wells and the agarose plates were incubated at 30°C overnight. The following day inhibition zone diameters were measured.

2.7 *Statistical analysis*

Significant differences were determined with the statistics tool of the OriginPro 8 software using a one-way ANOVA. Differences were considered significant when $p \leq 0.05$.

3. *Results*

3.1 *Differential expression with an array of microorganisms in CHSE-214 cells*

Treating the salmonid cell line CHSE-214 with an array of different inactivated microorganisms including Gram-positive and –negative bacteria and a fungus, showed that the Gram-negative bacteria *P. aeruginosa* (PA01), *V. anguillarum* and *E. coli* (D21) significantly increased cathelicidin expression, while *A. salmonicida* subsp. *achromogenes* did not. The fungus *C. albicans* did not significantly upregulate cathelicidin in this cell line either, while the two Gram-positive bacteria *Lactobacillus* sp., and *B. megaterium* (Bm11)

upregulated slightly but not significantly. This indicates Gram-negative bacteria as main inducers of cathelicidin expression among microbes in salmon (Fig. 1).

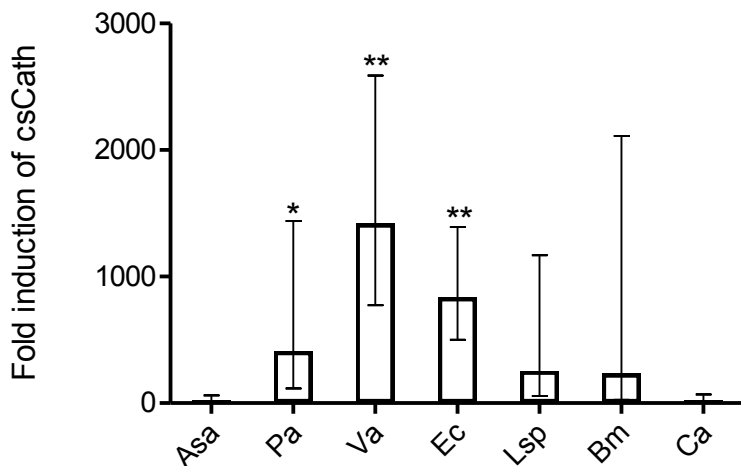


Fig. 1. Expression of cathelicidin in the cell-line CHSE-214 after challenge with different microorganisms. CHSE-214 cells were stimulated for 24 h with a selection of different microorganisms, and cathelicidin (csCath) expression was measured using qPCR. Fold changes were calculated compared to unstimulated controls. Asa = *Aeromonas salmonicida* subsp. *achromogenes* (n=4), Pa = *Pseudomonas aeruginosa* (n=4), Va = *Vibrio anguillarum* (n=3), Ec = *Escherichia coli* strain D21 (n=3), Ca = *Candida albicans* (n=4), Lsp = *Lactobacillus* sp. (n=4), Bm = *Bacillus megaterium* strain Bm11 (n=2). * indicates $p < 0.05$, ** indicates $p < 0.001$.

3.2 *Dose-response and time curve*

Different amounts of a *P. aeruginosa* preparation ranging from concentrations of 15 to 140 dead bacteria per fish cell led to a concentration-dependent increase of cathelicidin transcription, indicating the stimulating effect of the bacteria on cathelicidin transcription (Fig. 2A). Further, cathelicidin expression in this cell line was studied over the course of 96 h post stimulation (p.s.) with *P. aeruginosa*. The levels of mRNA increased at 12 h and 18 h p.s. and were shown to be highest at 24 h p.s. with a then following sharp decrease at 30 h. At 48 h p.s. a second peak appeared that was the second highest over the course of the experiment, while the RNA levels then tapered out towards 96 h p.s. (Fig. 2B). Accordingly, in all further studies unless otherwise stated we selected the 24 h time point for assessment of a cathelicidin response following stimulation.

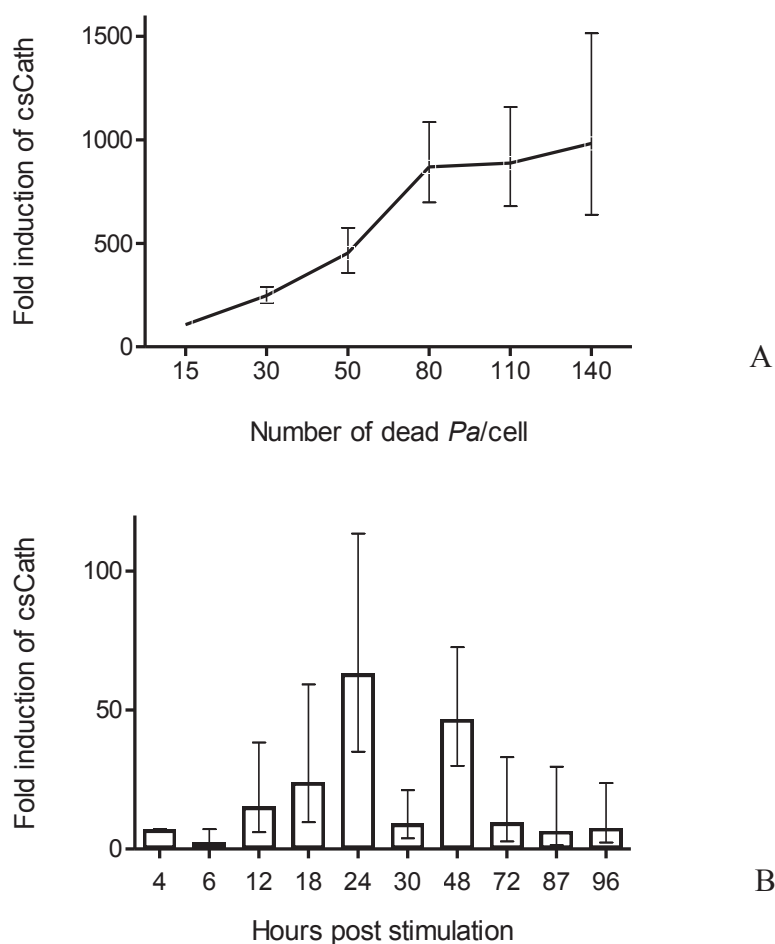


Fig. 2. Dose-response and time curve of cathelicidin expression after stimulation with *P. aeruginosa* (*Pa*) stimulation. CHSE-214 cells were incubated with either (A) different concentrations of *P. aeruginosa* for 24 h ($n=2$) or (B) with approximately 200 bacteria/cell for different lengths of time ($n=6$). Cathelicidin (*csCath*) expression was examined using qPCR. Fold changes were calculated compared to untreated controls either after 24 h (A) or compared to untreated controls sampled at the beginning and end of the experiments (B).

3.3 *Differential response to an array of compounds in CHSE-214 cells*

In order to investigate the microbial components responsible for cathelicidin induction, we stimulated CHSE-214 cells with *P. aeruginosa* derived DNA, flagellin, and LPS. Further, we used poly(I:C), a mimic of viral RNA, phenylbutyrate (PBA) and diaminopimelic acid (DAP, data not shown) as stimulants in this cell line. PBA is a mimic of the metabolic byproduct butyrate and has been shown in mammals to increase the expression of the human cathelicidin LL-37 [30]. DNA, LPS, poly(I:C), PBA and DAP did not significantly upregulate the expression of salmon cathelicidin. Flagellin however led to significant upregulation of the gene (Fig. 3) in this cell line, indicating it to be an important ligand for receptors involved in the signalling cascade leading to salmonid cathelicidin expression.

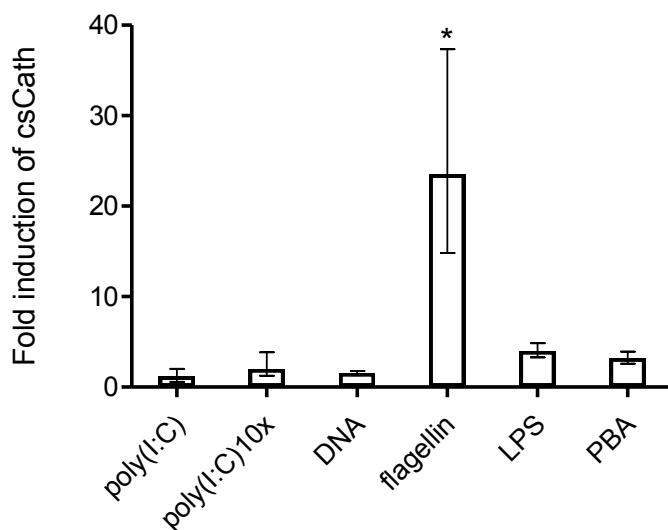


Fig. 3. Differential expression of cathelicidin due to microbial and a non-microbial component(s). CHSE-214 cells were treated with poly(I:C) (10 or 100 $\mu\text{g/ml}$), phenylbutyrate (PBA) (4 mM), *P. aeruginosa* DNA (115 ng/ml), flagellin (5.7 $\mu\text{g/ml}$) or LPS (10 $\mu\text{g/ml}$). Cathelicidin (csCath) expression was examined using qPCR and fold changes were calculated compared to untreated controls ($n=2$).

3.4 Inhibitor studies

In order to describe the pathways involved in cathelicidin regulation in fish we used inhibitors to disrupt certain signalling pathways. The addition of 10 $\mu\text{g/ml}$ brefeldin A (Golgi apparatus inhibitor) and 10 $\mu\text{g/ml}$ nocodazole (microtubule inhibitor) significantly reduced the upregulation caused by *V. anguillarum* (Va). In contrast, wortmannin (PI3K inhibitor) appeared to enhance the expression induced by Va

(Fig. 4). To examine the effect of wortmannin in more detail we treated cells with 1 μ M and 5 μ M wortmannin and found both concentrations to significantly increase the Va induced expression of cathelicidin (Fig. 5A). In order to verify this result we also used the PI3K inhibitor LY294002. In contrast to wortmannin however, LY294002 significantly downregulated cathelicidin expression (Fig. 5B). brefeldin A, nocodazole, wortmannin and LY294002 alone did not have a significant effect on the RNA levels of cathelicidin. Chloroquine (inhibitor of lysosomal function and TLR9) on the other hand increased the expression of cathelicidin in absence of a bacterial stimulus.

These results indicate the involvement of the Golgi apparatus, microtubules and PI3Kinase in the signalling cascade leading to cathelicidin expression in the salmonid cell line.

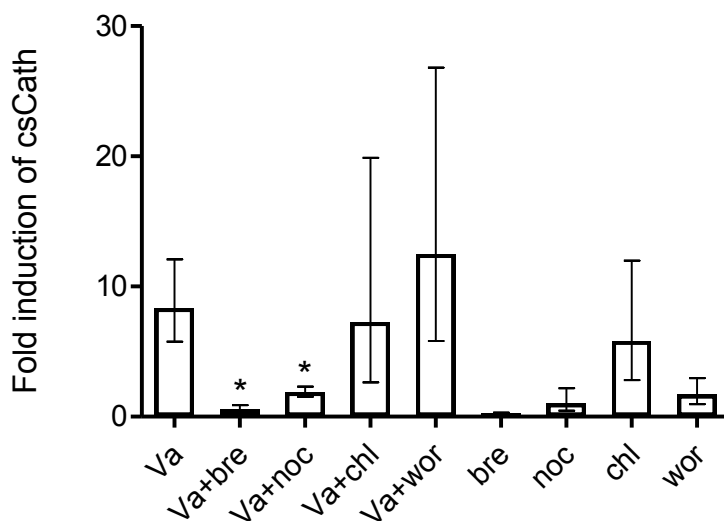


Fig. 4. Effect of different cell signalling inhibitors on the expression of cathelicidin in CHSE-214 cells. CHSE-214 cells were incubated with brefeldin A (bre), nocodazole (noc), chloroquine (chl) or wortmannin (wor) for 2 h. Subsequently cells were treated with *V. anguillarum* (Va) or cells remained unstimulated as control samples. Cells were incubated for 24 h and cathelicidin expression (csCath) was determined using qPCR. $n=3-10$ all samples except, $n=2$ for brefeldin A.

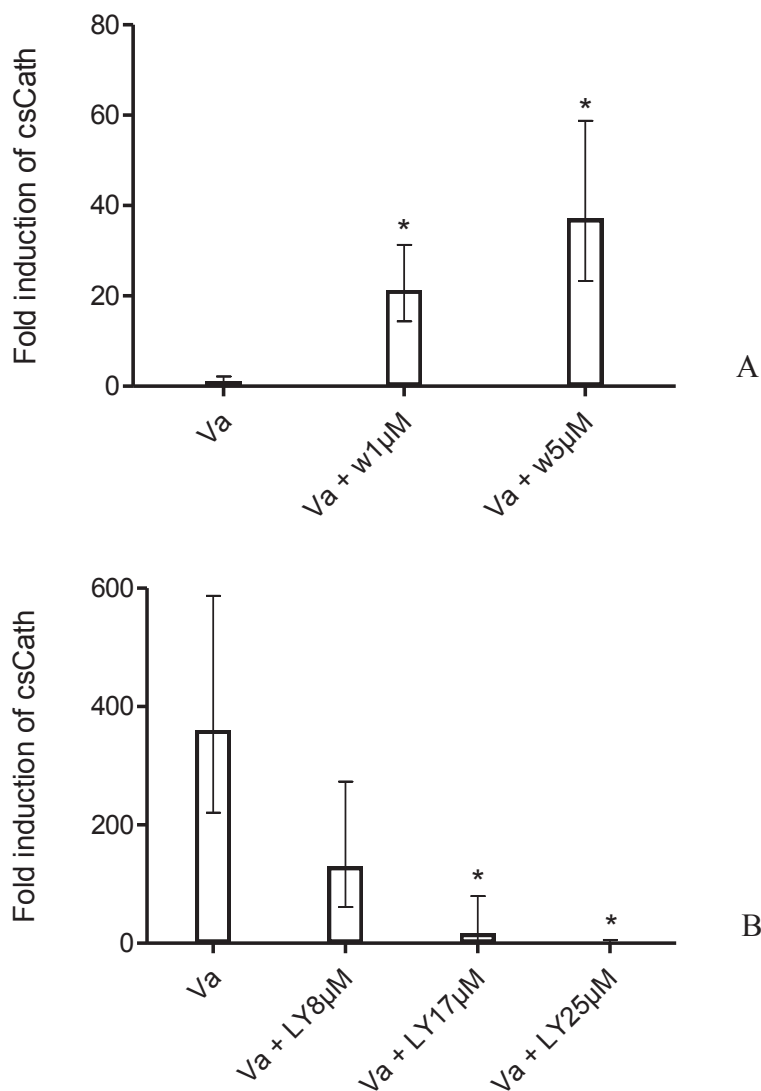


Fig. 5. The effect of designated PI3K-inhibitors on the *V. anguillarum* stimulated cathelicidin expression in CHSE-214 cells. Fish cells were pretreated for 2 h with either (A) wortmannin (1μM or 5μM) or (B) LY294002 (8μM, 17μM or 25μM) followed by a 24 hour incubation with *V. anguillarum* (Va). Cathelicidin (csCath) expression was examined using qPCR (n=3). * indicates $p < 0.05$.

3.5 *Differential cathelicidin expression with an array of microorganisms in cod cells*

We included the gadoid cell line ACL into our studies in order to investigate whether there is a general link between pattern recognition and AMP expression in fish. Gram-negative bacteria as well as a fungus did not lead to significant upregulation of the cod cathelicidin gene, while the Gram-positive bacterium *Lactobacillus* sp. elicited a significant response (Fig. 6). The Gram-positive bacterium *B. megaterium* (Bm11), however did not upregulate cathelicidin expression in this cell line (data not shown) indicating that the upregulation is not due to Gram-positive bacteria in general. The expression pattern in this cell line therefore was different from that in the CHSE-214 cell line indicating the regulation of cathelicidin expression in these distantly related fish species to be different.

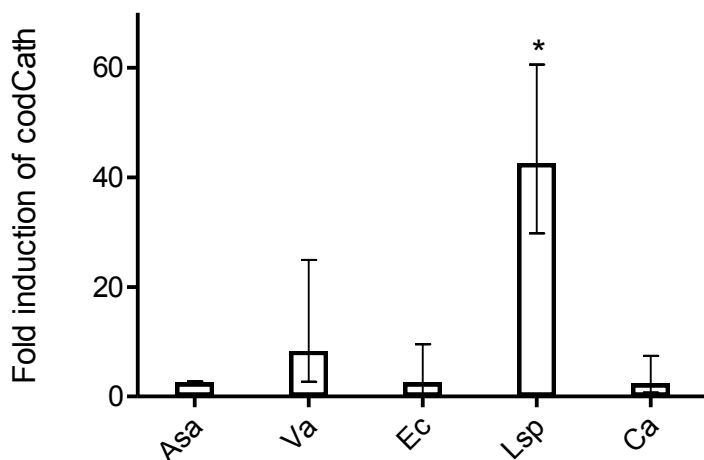


Fig. 6. Expression of cathelicidin in the cell line ACL in response to microorganisms. ACL cells were stimulated for 24 h with a selection of different microorganisms and cathelicidin (*codCath*) expression was measured using qPCR. Fold changes were calculated compared to unstimulated controls. Asa = *A. salmonicida* subsp. *achromogenes* (n=2), Ca = *C. albicans* (n=3), Ec = *E. coli* strain D21 (n=2), Lsp = *Lactobacillus* sp. (n=3), Va = *V. anguillarum* (n=3). * indicates $p < 0.05$.

3.6 Differential response to an array of stimuli in ACL cells

We examined cathelicidin expression in response to different microbial components in the ACL cells and found that poly(I:C) caused a significant increase in cathelicidin expression both at the concentration of 10 and 100 $\mu\text{g/ml}$ (Fig. 7). Neither *P. aeruginosa* derived DNA and flagellin nor diaminopimelic acid (DAP) did increase the expression of *cod* cathelicidin. Interestingly, β -glucan a wall component of fungi and

plants that is often used in aquaculture as an immunostimulant also did not increase the expression. These results indicate different receptor linkage in the ACL cells as compared with CHSE-214 cells.

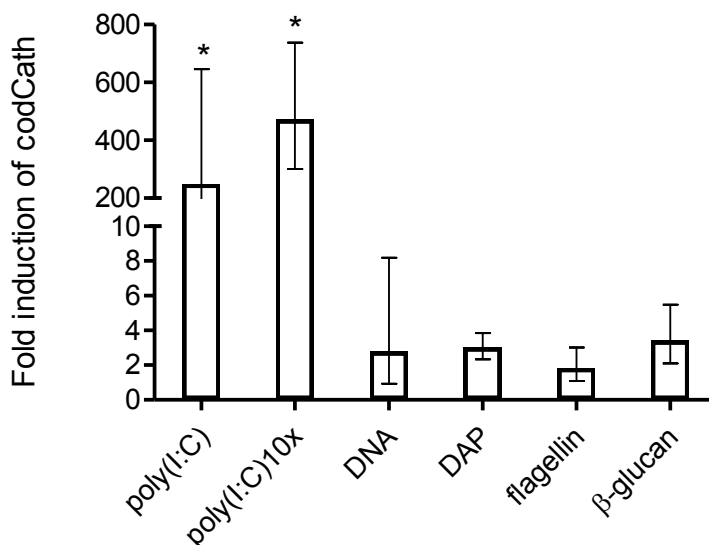


Fig. 7. Expression of cathelicidin in the Atlantic cod cell line ACL upon challenge. ACL cells were treated with either poly(I:C) (10 or 100 $\mu\text{g/ml}$), diaminopimelic acid (DAP) (100 $\mu\text{g/ml}$), β -glucan (100 $\mu\text{g/ml}$), *P. aeruginosa* DNA (115 ng/ml) or *P.a.* flagellin (5.7 $\mu\text{g/ml}$). Cathelicidin (codCath) expression was measured using qPCR ($n=2-3$). * indicates $p<0.05$.

3.7 Activity of secreted components and protease sensitivity

In order to determine if stimulated ACL cells secreted antibacterial components, we tested media of stimulated cells in an inhibition zone

assays to test for bactericidal activity. Samples stimulated with *Lactobacillus* sp. led to significantly larger inhibition zones than control samples, indicating the presence of secreted antimicrobial factors in the culture medium (Fig. 8). This activity was almost abolished by proteolytic digestion with pepsin and proteinase K indicating that the antibacterial agents are peptides or proteins.

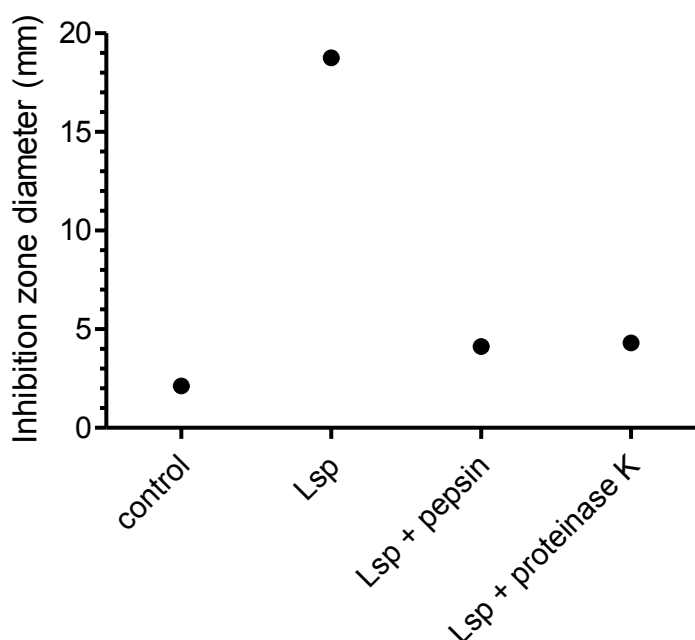


Fig. 8. Antibacterial activity of ACL cell culture supernatants. ACL cells were either left untreated (control) or stimulated with Lactobacillus sp. (Lsp) for 48 h. Cell supernatants incubated with Lactobacillus sp. were subsequently digested with either pepsin or proteinase K. Antibacterial activity against B. megaterium (Bm11) was examined using a radial diffusion assay (n=3).

4. *Discussion*

In fish the adaptive immune response can take up to 12 weeks to develop, which has been attributed to fish being poikilothermic and living in a cold environment [45]. A decreased effectiveness of the fish adaptive immunity in comparison with higher vertebrates is also indicated by a low immunoglobulin variety [46]. Hence, innate immune factors including AMPs are of primary importance in fish immune defence [45]. We characterized the nature of the cathelicidin response and found that bacterial components led to a fast and transient response with peak expression at 24 h post stimulation (p.s.). This temporal expression pattern was similarly observed in other studies [20, 21, 47] and show cathelicidin to be quickly induced and included in the initial immune defence in fish. The decline of expression at 30 h p.s. and the presence of a second peak at 48 h p.s. can be attributed to an early and a delayed response regulated by different transcription factors or to a negative feedback loop that is protecting the cell from a too high concentration of antimicrobial peptide that can be damaging.

We also explored the possibility of immune priming where an initial challenge would give rise to an enhanced second response with faster

initiation kinetics and/or higher response amplitude [48]. For this, the CHSE-214 cells were stimulated for 24 h, then the stimulus was removed and the cells were left untreated for 48 h before re-stimulating for another 48 h. We found the cathelicidin expression after the re-stimulation significantly lower than in naïve control cells (data not shown). The response to the second challenge was thus more similar to desensitization rather than enhancement with respect to cathelicidin expression.

Selected PRRs in fish are lectins, complement receptors, the cytoplasmic NOD-like receptors and the membrane bound Toll-like receptors (TLR) [49]. TLRs are well characterized innate immune receptors in mammals [50]. Sixteen types of TLRs have been described in fish of which 8 are teleost specific [51]. Similar as in mammals, the TLR in fish recognize ligands derived from bacteria [52]. In certain species like cod the receptor family has been shown to be expanded with an increased recognition repertoire of nucleic acid receptors for stimulating immunity [53]. In our study, a comparison of different microbes in the salmonid cell line showed induction by several Gram-negative bacteria. Interestingly, purified flagellin was a prominent upregulator of cathelicidin expression in the salmonid cell-line and

could be the determinate ligand responsible for cathelicidin upregulation in the bacteria cell lysate (Fig 2). This data indicates TLR5, known to bind flagellin in mammals [24], to be included in the cathelicidin signalling pathway.

In contrast cathelicidin expression in the cod cell line was not affected by flagellin which is in accordance with a recent report describing the lack of the TLR5 gene in the Atlantic cod genome [53]. Interestingly, our results show prominent cathelicidin upregulation in the gadoid cell line by poly(I:C). Similar upregulation due to poly(I:C) has been noted for the antimicrobial peptide hepcidin in Atlantic cod *in vivo* [22], indicating poly(I:C) as a powerful stimulus in the cod immune system. These responses to poly(I:C) implicate a role for cathelicidin in the antiviral defence of Atlantic cod.

In a previous study, we have shown that cathelicidin was upregulated in fish with crude LPS (derived from *E. coli*) but not with LPS digested with DNase [21]. Here we have shown that pure LPS did not lead to a significant upregulation of cathelicidin and this data is supported by findings that TLR4, responsible for LPS recognition in

mammals, is either absent in fish or seems non-responsive towards LPS [54, 55].

When stimulated with different microorganisms, the gadoid cell line showed a response to the Gram-positive bacterium *Lactobacillus* sp. The cell-wall component diaminopimelic acid (DAP), on the other hand, was not the determinant ligand. *Lactobacillus* sp. is usually not pathogenic to fish but used as a probiotic in aquaculture (*L. bulgaricus*, *L. rhamnosus*) [56]. Cathelicidin expression therefore might serve as a marker for enhanced defence to identify prebiotic and probiotic combinations for optimal immunostimulation in fish.

In order to elucidate the signalling pathway to cathelicidin upregulation in salmonid cells, we incubated the cells with the designated PI3K inhibitor wortmannin. PI3K has been described as a negative regulator of TLR signalling [57, 58] and is an important component of signalling in mammalian innate immunity [59]. In fish PI3K is present [60], although a role in immunity has not been assigned yet. Our results showed that inhibition of PI3K with wortmannin led to an amplification of cathelicidin upregulation, thereby suggesting PI3K in fish to be a negative regulator of PRR

signalling. On the other hand the designated PI3K inhibitor LY294002 downregulated cathelicidin expression. This opposite effect of these two PI3K inhibitors has been seen before in a mouse cell line and was explained by LY294002 possibly not targeting PI3K, but a component of the signalling cascade downstream of PI3K [61]. Our result indicates a similar pathway scenario in salmon as has been described in mouse.

Brefeldin A is an inhibitor of protein translocation from the endoplasmic reticulum to the Golgi apparatus, by destabilizing the Golgi membrane [62]. Since the addition of brefeldin A inhibited upregulation of cathelicidin in the salmonid cell line, it can be deduced that receptors, accessory proteins or proteins further downstream in the cathelicidin signalling cascade are located on the Golgi apparatus. Nocodazole induces the disassembly of microtubules [63], which similarly serve as physical support for proteins involved in the signalling cascade, which again can explain the absence of cathelicidin upregulation after bacterial stimulation in the presence of this inhibitor. Chloroquine is known as a drug that inhibits lysosome function and TLR9 [64, 65]. We did not see a significant decrease or increase in cathelicidin RNA expression in the presence of this inhibitor,

suggesting that lysosome function and TLR9 are either not involved in the cathelicidin response or that this drug does not have the same effect in fish as it has in mammals.

In order to further characterize the response elicited by a microbial stimulus in ACL cells and link the enhanced mRNA level to antimicrobial activity, the secreted components of the cells were collected and tested for activity. The fact that the secreted components were inactivated by proteases indicated their proteinaceous nature, which suggests that ACL cells upon stimulation release antimicrobial peptides/proteins, possibly including cathelicidin into the medium. These cells will be useful in the future for characterizing the total epithelial defence response.

We conclude that in both salmon and cod, cathelicidins are induced through microbial challenge, but with different ligands initiating the response between these species. Understanding the pathways leading to upregulation of an immuno-relevant gene can be employed for controlled stimulation of fish immunity and lead to the design of strategies for use in aquaculture.

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