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Antimicrobial and immunomodulatory activities of rainbow trout cathelicidin antimicrobial peptides

Diploma Thesis

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1. Abstract

Fishes in aquaculture are susceptible to a variety of diseases, a threat that is increasing in severity with climate change. Cathelicidins (CATHs), a family of antimicrobial peptides (AMPs), are found in all vertebrate species. In contrast to mammalian CATHs there is little knowledge about the function of fish CATHs. They can be divided into two categories, CATH-1 which have two typical cysteine residues, and CATH-2 where these cannot be identified. We are particularly interested in the CATHs as we hypothesize that they play a role during proliferative kidney disease (PKD), an illness caused by the parasite *Tetracapsuloides bryosalmonae*. During the disease, whether the kidney develops strong hyperplasia depends on water temperature, which leads to anaemia and a mortality of up to 100 % in fish stocks. We verified that a rainbow trout *(Oncorhynchus mykiss)* with PKD expresses CATH-1 and CATH-2 via total RNA isolation. After fusing the AMP-region to a human signal peptide, we attempted to express recombinant peptides of the two CATHs. For reasons to be determined, the recombinant peptide expression failed. However, we had the peptides commercially synthesized. Then we studied the microbicidal and immunomodulatory activities of the peptides, by testing them on fish-infective bacteria and measuring the changes in expression of other PKD related genes in rainbow trout leucocytes.

2. Zusammenfassung

Fische in Aquakultur sind anfällig für eine Vielzahl von Krankheiten, eine Gefahr, die mit dem Klimawandel zunimmt. Cathelicidine (CATHs) gehören zur Familie der antimikrobiellen Peptide (AMPs) und kommen in allen Wirbeltierarten vor. Im Gegensatz zu den Säugetier-CATHs ist über die Funktionsweise der Fisch-CATHs sehr wenig bekannt. Wir wissen, dass sie in zwei Kategorien eingeteilt werden können, CATH-1, die zwei typische Cysteinreste besitzen, und CATH-2, bei denen diese fehlen. Wir interessieren uns besonders für die CATHs, weil wir vermuten, dass sie eine Rolle bei der proliferativen Nierenerkrankung (PKD) spielen, einer Krankheit, die durch den Parasiten *Tetracapsuloides bryosalmonae* verursacht wird. Die Entwicklung einer krankheitsbedingten Hyperplasie, die zu Anämie und bis zu 100 % Sterblichkeit in Fischbeständen führt, ist abhängig von der Wassertemperatur. Wir untersuchten die Rolle der CATHs während der Infektion und konnten durch vollständige RNA-Isolierung zeigen, dass Regenbogenforellen CATH-1 und CATH-2 exprimieren. Durch Fusion der AMP-Region mit einem humanen Signalpeptid versuchten wir, die CATHs als rekombinante Proteine zu exprimieren. Aus ungeklärten Gründen und trotz der Expression einer Positivkontrolle wurden die rekombinanten Proteine nicht exprimiert. Für das Studium in dieser Thesis ließen wir sie deshalb kommerziell herstellen. Anschließend untersuchten wir die antimikrobielle und immunmodulatorische Aktivität der Peptide an fischinfektiösen Bakterien und durch Analyse der Variation in der Expression anderer PKD-assoziierter Gene in Regenbogenforellen-Leukozyten.

3. Introduction

Farmed fishes such as salmonids suffer from a high risk of infections with mortalities that can reach up to 100 % in fish stocks. The menace to the fish and the farming industry is rising with climate change and emerging diseases. Yet, effective vaccines are rare especially against parasites, and we need to learn more about effective host immune responses to help guide vaccine development. Since aquatic organisms deal with very high loads of microbes, they developed several defence mechanisms. Antimicrobial peptides (AMPs), which are small, usually cationic, and amphiphilic molecules, play an important part in the innate immune system in fish. The ability of AMPs to inhibit bacterial growth has been already proven, which could be a good alternative to conventional antibiotics that cause antibiotic resistance (2).They are expressed in several epithelia and organs in the animal and are divided into five different groups: beta-defensis, hepcidins, piscidins, histone-derived peptides and cathelicidins (CATHs). Members of the CATH-family share an N-terminal-proregion, which is called cathelin-like domain and a variable antigen domain in the C-terminal region. CATHs are divided into two different categories: CATH-1 which have two typical cysteine residues, and CATH-2 where these are not detectable. Studies have shown that fish express CATHs following stimulation, such as inflammatory cytokines and bacteria (3). We are interested in the CATHs because they are highly upregulated during proliferative kidney disease (PKD) of salmonids (4), a disease emerging with climate change, so we will elaborate on them further in this work (5).

The etiological agent of PKD*, Tetracapsuloides bryosalmonae,* belongs to the class *Malacosporea* within the entirely parasitic subphylum *Myoxzoa* (*Cnidaria*). During its life cycle, the parasite is dependent on two different hosts, freshwater bryozoans (final hosts), which commonly occur in lakes, ponds and rivers, and salmonids (intermediate host) (6). Invasion of fish is initiated by the parasite penetrating the gill epithelium. In the next step, the parasite enters the circulatory system, which gives it access to multiple organs, and the kidney, where it develops spores and then leaves the fish via the urine (5).

Interestingly, the immune response and the pathological changes are highly dependent on water temperature. Above 15 °C, the kidney develops strong hyperplasia, which can be classified by Clifton-Hadley. The range goes from 0 to 4, with grade 4 containing the most advanced clinical stages and grade 0 showing no kidney swelling (5). In succession, these alterations lead to a regression of the urinary tissue. Since the teleost kidney is the largest site of haematopoiesis in fish (5), infected individuals develop anaemia as well as enlargement of

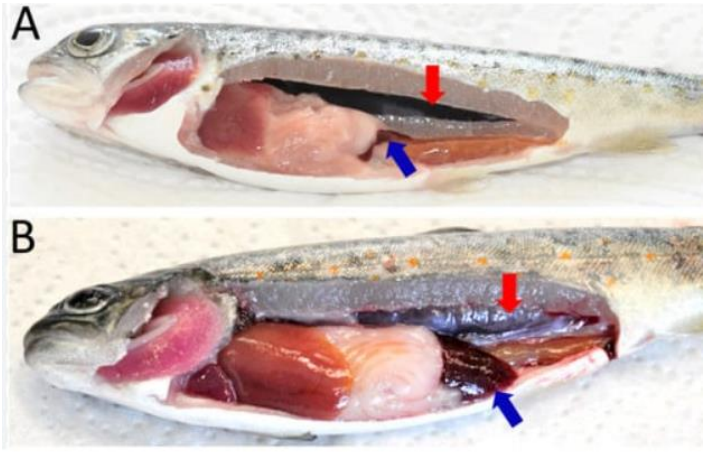


Figure 1. Macroscopic differences between uninfected and infected brown trout organs:

*(**A**) healthy control fish with normal posterior kidney (red arrow) and spleen (blue arrow). (**B**) enlargement of posterior kidney (red arrow) and spleen (blue arrow) in an infected fish. Picture taken from (1)*

the kidney and spleen (Figure 1) (6). With climate change, the distribution of PKD is expanding and the severity is increasing, which is a great threat to salmonids (4).

Until now, the only two methods of diagnosing the pathogen are PCR and microscopy, with the PCR being more sensitive.

In fresh imprints of methylene blue- and eosin-stained kidneys *T. bryosalmonae* is recognizable due to its pluricellularity and size (Figure 2).

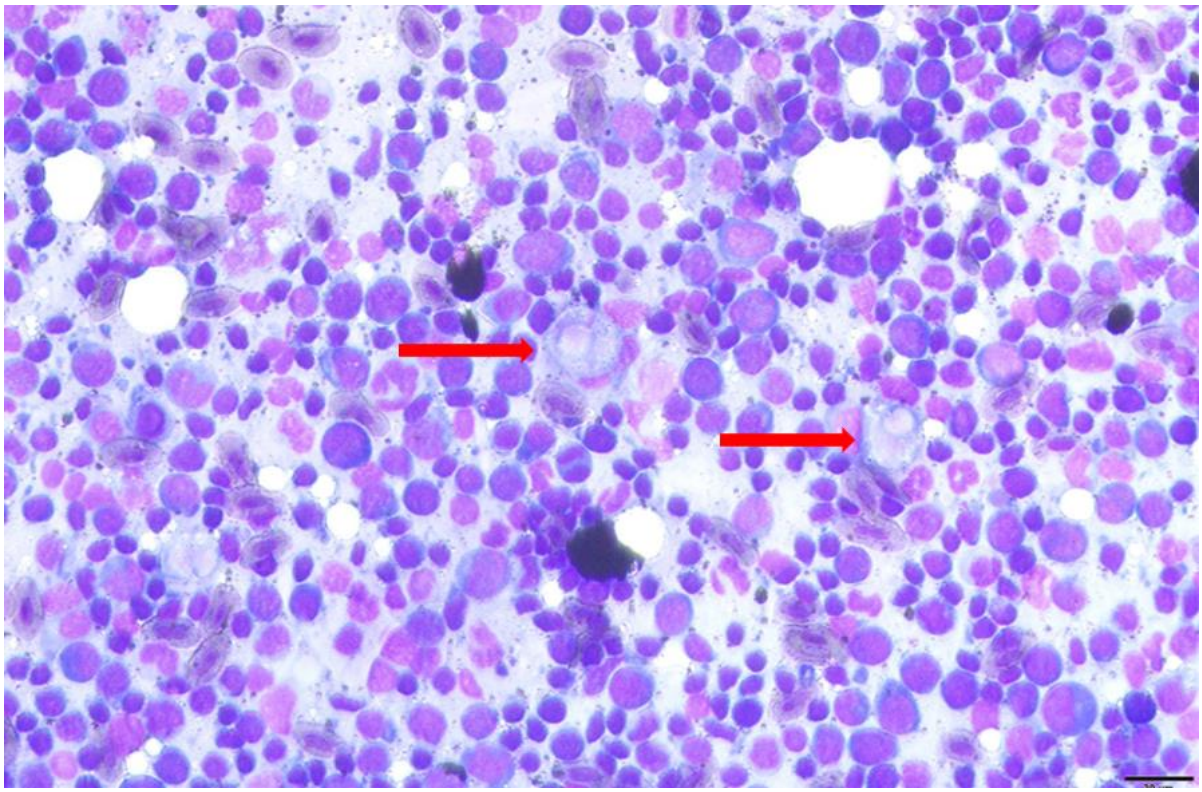


Figure 2. Methylene blue- and eosin-stained kidney imprint from a rainbow trout with PKD.

The parasite, T. bryosalmonae*, is marked by red arrows and represents (with 18 – 20 µm in diameter) the largest object on the imprint. Besides that, the picture is dominated by lymphocytes, which typically proliferate heavily during PKD, in the imprint. They can be easily*

identified by the large nucleus, which almost completely fills out the cell and a dark blue cytoplasm.

In this work, we aimed at better understanding the role of the CATHs in the infection, as the literature shows that they might be protective or somehow involved in the disease. As a first step we confirmed that the rainbow trout *(Oncorhynchus mykiss)* expresses CATH-1 and CATH-2. Then our goal was to design the CATHs for protein expression, by fusing the AMP-region to a human signal peptide, using polymerase chain reaction (PCR). This step was necessary because in living organisms the peptide is expressed from the full-length sequence and later cleaved to active form by an enzyme, a way that would have been too costly and time-consuming for us.

We then tested the peptides for their lytic and microbicidal activity on targets such as fish-infective bacteria, like *Aeromonas salmonicida ssp. salmonicida, Yersinia ruckeri*, *Vibrio arginolyticus*, *Escherichia coli* and *Aeromonas sobria.* Then we studied the peptides' interactions with rainbow trout leucocytes. Changes in expression of PKD-regulated genes such as *rtIL-8*, *rtTNFα2*, *rtCATH1* and *rtCATH2* were measured by quantitative polymerase chain reaction (qPCR).

4. Materials and Methods

4.1. Amplification of the full-length CATHs

Our first step was to amplify the full-length sequences for CATH-1 and CATH-2 from the kidney of rainbow trout experiencing PKD.

4.1.1. Fish sampling

We took samples from the kidney of one rainbow trout which showed significant kidney swelling (4), as shown in Figure 1 in the Introduction and homogenized it.

4.1.2. RNA extraction

To purify the RNA from our kidney sample we used an RNA extraction kit (RNeasy® Mini Kit, QIAGEN, Netherlands) and we strictly followed their instructions, but without doing the optional step of the DNase digestion. We evaluated the success of this procedure with spectrophotometry (NanoDrop Spectrophotometer, Thermo Fisher Scientific, USA).

In the next step we reverse-transcribed acquired RNA to cDNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (BIO-RAD, USA) according to the manufacturer's instructions and using random hexamers to produce a durable template for PCR.

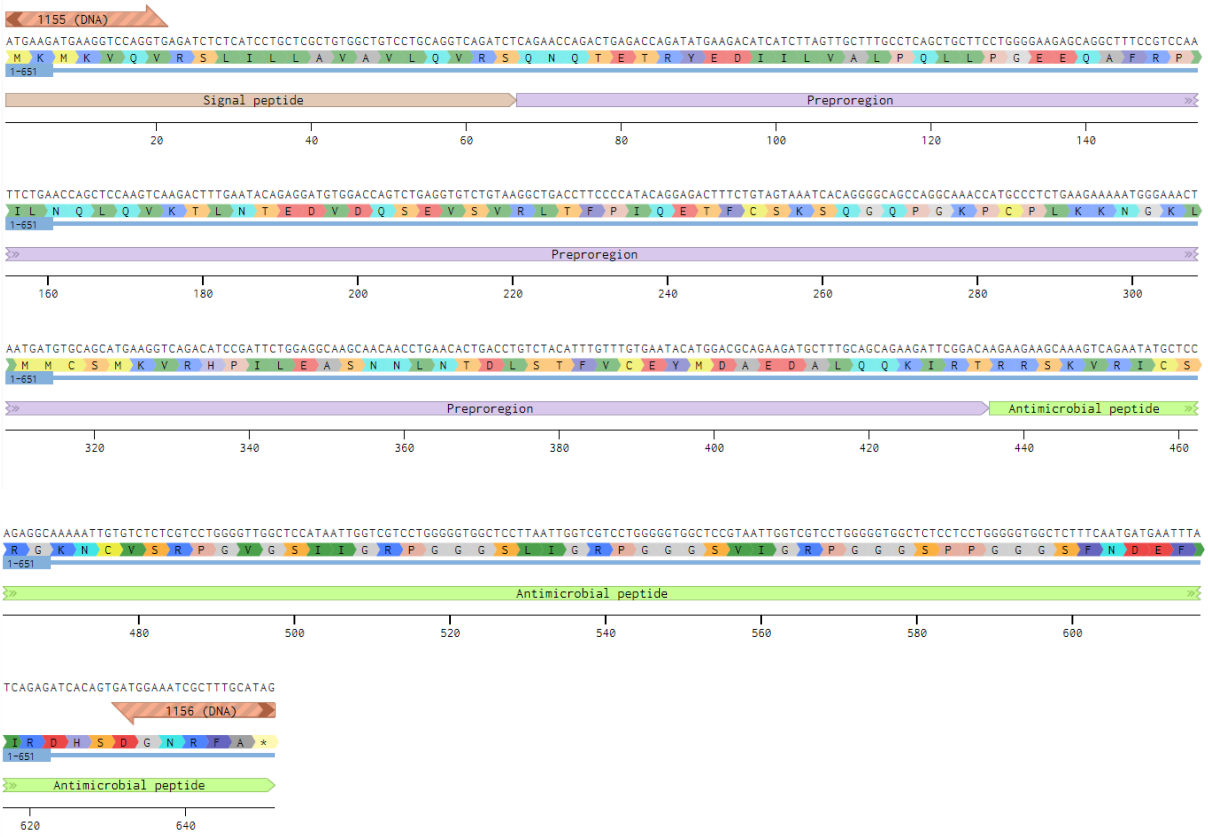
4.1.3. PCR

In the next step we amplified the full-length sequences for CATH-1 and CATH-2 via PCR with the cDNA as a template. We did all PCRs using the following mastermix: we put together 2,5 µL of 10X Titanium Taq PCR Buffer (Clontech Laboratories Inc.®, USA), 0,5 µL of 50X dNTP Mix, 0,5 µL of forward primer, 0,5 µL of reverse primer, 0,5 µL of 50X Titanium Taq DNA Polymerase (Clontech Laboratories Inc.®, USA), 1 µL of DNA at a concentration of 50 ng/µL and 19,5 µL of water to add up to 25 µL in total per sample. We programmed the thermocycler in the following manner: initial denaturation at 95 °C for two minutes. Afterwards, we programmed 35 cycles of denaturation, annealing and extension which began with a denaturation at 95 °C for 30 seconds again. The temperature for the annealing step was 5 °C

below the melting temperature of the utilized primers for one minute, as it is recommended in the user manual of the PCR Kit to use one minute per kb of expected product for the annealing step. The extension was done at 68 °C and the time was ten seconds for every 100 bp of the expected product. Afterwards we included a final extension at 68 °C for five minutes.

Both amplified cDNA products corresponded to the full-length CATHs, as they have a signal peptide sequence at the N-terminus region, a preproregion and a unique C-terminal sequence, the AMP. During translation, the CATHs are produced as a pre-proprotein, the preproregion is removed by a protease, which releases the AMP-region (7). The different regions of the CATHs as well as the primers and the accordingly binding sites for these PCRs are visualized in Figure 3 below. A list of all primers for the amplification of the full-length CATHs is displayed in Table 1.

A



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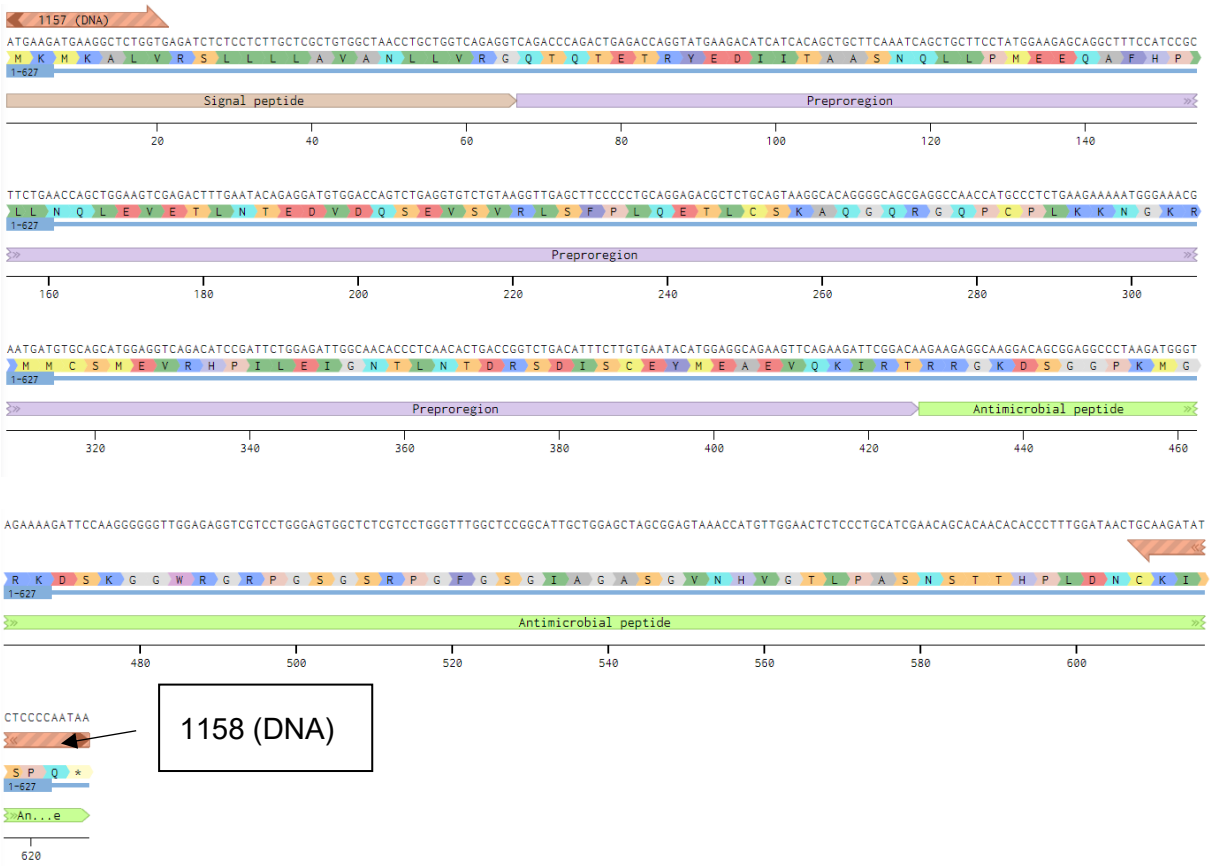


Figure 3. The full-length annotated nucleotide sequences of rainbow trout CATHs 1 and 2:

*From top to bottom, in the top row, the base sequence is visible (standard uppercase letters) as well as the primer binding sites (arrows). The row beneath shows the corresponding amino acids to the base triplets (in coloured highlighted letters). In the last two rows, we annotated the different regions of the CATHs marked in colours and show a reference scale of the length in bp. (**A**) The sequence of CATH-1. (**B**) The sequence of CATH-2.*

We verified all our products with DNA gel electrophoresis. We made the 1 % gel mixing 100 mL Tris-acetate-EDTA (TAE) buffer and 1 g agarose together, then we heated the solution in the microwave until the agarose powder dissolved completely. After the fluid cooled down to about 35 °C, we added 10 µL of GelRed and then poured the gel in the tray, added the comb and left it to polymerize. After the gel solidified, we added our samples mixed with loading dye (GelPilot DNA Loading Dye, 5x, QIAGEN, Netherlands) onto the gel. The running was done at 100 Volts and we made sure that smallest marker (100 bp) did not run out.

Afterwards we cut and purified the PCR products between 500 to 700 bp size (MinElute® Gel Extraction Kit, QIAGEN, Netherlands), strictly following the instructions of the kit and we verified the nucleotide sequence via sequencing (Sanger Sequencing, LGC Biosearch Technologies, UK).

Table 1. List of primers for amplifying the full-length CATHs

Name of the primer	Sequence (5' -> 3')	Tm (°C)	Amplicon size (bp)	Extra information
1155	ACAACCGGTATGAAGATGAAGGTCCAGGTG	63,0	651	With 1156 it amplifies the full-length CATH-1 and adds the Age1 cutting site to the DNA
1156	AGTGCTAGCCTATGCAAAGCGATTTCCATC	61,6	651	With 1155 it amplifies the full-length CATH-1 and adds the Nhe1 cutting site to the DNA
1157	TTGTCCGGAATGAAGATGAAGGCTCTGGTG	63,0	627	With 1158 it amplifies the full-length CATH-2 and adds the BamH1 cutting site to the DNA
1158	GACGGATCCTTATTGGGGAGATATCTTGCA	61,6	627	With 1157 it amplifies the full-length CATH-2 and adds the BspE1 cutting site to the DNA

We designed all of our primers by ourselves and we calculated the melting temperature of the primers with the following formula:

Tm= 64.9 + 41 * (yG + zC - 16.4) / (wA + xT + yG + zC)

The letters in capital (G, C, A and T) stand for the four bases (guanine, cytosine, adenine and thymine) that are the chemical basic units that make up the DNA. The lowercase letters are the quantities of each base in a primer.

All the primers were prepared by adding ultra-pure water to produce a 100 pmol/µL concentration which was then diluted for the PCR to a 10 pmol/µL working concentration.

4.2. Cloning of different CATH-products into bacteria

To amplify our CATH-sequences we used bacteria from the *E. coli* strain DH5alpha. To successfully transform our sequences into the bacteria we had to ligate them with a mammalian expression plasmid before. Once the plasmid is successfully transformed into the bacteria, we just had to grow them to increase the amount of our target DNA.

4.2.1. Restriction enzyme digestion

To ligate the expression plasmids with the CATHs, we had to digest them with restriction enzymes first. We cut CATH-1 with the enzymes Age1 and Nhe1 and CATH-2 with BspE1 and BamH1 (all from New England BioLabs Inc.®, USA). We did all our restriction enzyme digestions for our project with the following protocol: we mixed 2,5 µL of 10X NE Buffer, 0,5 µL of each restriction enzyme, 9 µL of DNA at a concentration of 60 ng/µL and 12,5 µL of water together, to add up to 25 µL in total. We incubated samples at 37 °C overnight. Then we verified if it was successful by size control of the fragments via DNA gel electrophoresis and finally purified the CATHs via gel extraction (MinElute® Gel Extraction Kit, QIAGEN, Netherlands).

4.2.2. Ligating plasmid and PCR product

We ligated the full-length CATH-1 and CATH-2 sequences and plasmids with the T4 DNA Ligase Kit (New England BioLabs Inc.®, USA) and strictly followed their instructions. We cloned CATHs into a commercial mammalian expression plasmid (pVITRO1-blasti-mcs, InvivoGen Europe, France), which we have shown schematically in Figure 4. We cloned CATH-1 into multiple cloning site (MCS) 2 and CATH-2 into MCS1.

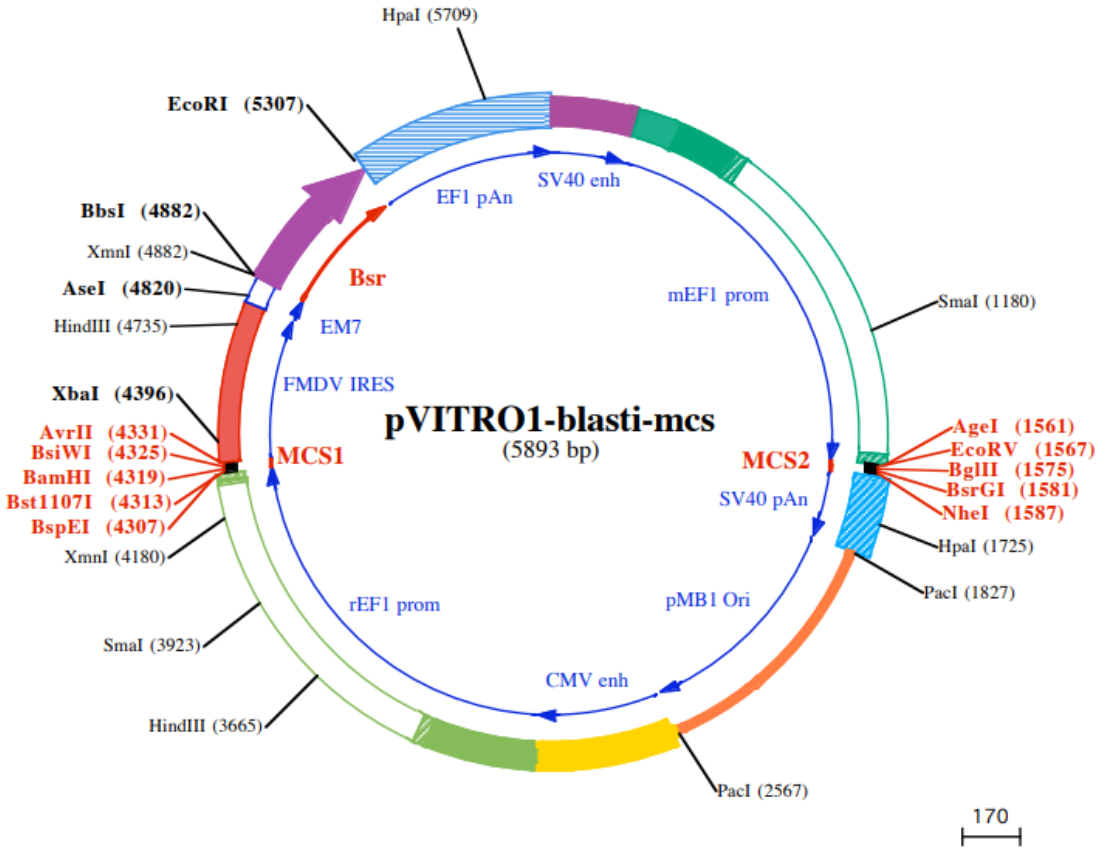


Figure 4. Mammalian expression plasmid pVITRO1-blasti-mcs.

The vector contains multiple cloning sites (MSCs), each with several restriction sites, with their names and positions listed next to the MCSs. Furthermore, the promoter elements rEF1 and mEF1 are start sites for the polymerase to initiate transcription. CMV enh and SV40 enh enhance gene expression in many host species. Resistance against the antibiotic blasticidin is provided by the Bsr-gene. In bacteria, the transcription of the resistance gene Bsr starts from the EM7 promoter. Picture provided by InvivoGen Europe (France): https://www.invivogen.com/sites/default/files/invivogen/products/files/pvitro1-blasti-mcs_tds.pdf

4.2.3. Producing culture medium for bacteria for cloning

We made agar plates and fluid media to serve as culture medium for the *E. coli* strain DH5alpha we needed for cloning. For both media we mixed 20 g of LB Broth in 1000 mL of purified water each time. To give one medium solid consistency we added 15 g of agar powder. Then we

autoclaved both media at 121 °C for 20 minutes. After both media cooled down to a temperature of about 35 °C, we added the antibiotic blasticidin at a concentration of 25 µg/mL. Then we filled plates with the agar medium, let them cool down and solidify.

4.2.4. Preparing plasmids with bacteria

To express the recombinant CATHs, we cloned them into expression plasmids which we then transformed into bacteria to efficiently multiply our CATHs as mentioned above. We started by thawing the bacterial suspension in ice. Then we mixed 50 µL of bacterial suspension with 30 ng of plasmid-DNA and incubated these samples on ice for 30 minutes. In the next step we heat-shocked the bacteria in a water bath at 42 °C for 30 seconds, then we placed the samples on ice again for two minutes. Then we added 250 µL of preheated (37 °C) SOC medium and incubated the samples shaking for an hour at 37 °C.

In the following step we plated the bacteria on agar plates, at 37 °C for 16 hours, picked colonies and cultured them in fluid medium for 16 hours at 37 °C. Next, we picked five promising colonies for each CATH into 20 µL of LB broth and used 1 µL of it for amplification of the genetic material with the primers for the full-length CATHs as mentioned above. The only difference with the program was a ten-minute-long denaturation phase in the beginning to kill off all the bacteria. We determined the success of the PCR by DNA gel electrophoresis and selected the three colonies with the strongest signal, applied them to bacteria again and cultured them overnight. Then we extracted the plasmids (Monarch® Plasmid Miniprep Kit, New England BioLabs Inc.®, USA) strictly following the instructions and measured DNA concentration by spectrophotometry (NanoDrop Spectrophotometer, Thermo Fisher Scientific, USA).

4.3. Expression of the CATH proteins in HEK293T cells

Unfortunately, we could not do the protein expression immediately, as we had to design the CATHs first, because of two reasons: firstly, in living fish the full-length sequence of the CATHs is expressed as a peptide and later cleaved to an active form, the mature peptide (MatP). Expressing the full-length peptide and later cleaving it with an enzyme was too costly and time-consuming for us, so we had to make sure that only the sequence of the MatP is expressed.

Secondly, we used human embryonic kidney (HEK293T) cells to express our MatP, so we decided on a few changes to increase protein yield in human cells.

4.3.1. Designing the CATH-2 for protein translation

So, we had to exclude the preproregion and link the signal peptide and AMP-region directly together. All the primers used for the designing of the CATHs for protein translation are displayed in Table 2.

We amplified the AMP-region of CATH-2 with the primers 1177 and 1158, using the full-length CATH-2 as a template, by PCR (Titanium® Taq PCR Kit, Clontech Laboratories Inc.®, USA, see above). We amplified the signal peptide with the primers 1157 and 1178 using CATH-2 as a template using PCR (Titanium® Taq PCR Kit, Clontech Laboratories Inc.®, USA).

As we wanted to express the corresponding proteins of the CATH-products in HEK293T cells, it could be an advantage for maximizing protein yield to also link the AMP-region of the CATH to the IFNα2 human signal peptide, as literature claims that it supports high secretion efficiency in HEK293T cells (8). In this step, we used the primers 1172 and 1173 to form the human signal peptide with fusion PCR. We then used the primers 1176 and 1174 on the recently created fusion PCR product as a template, to create a recombinant version of CATH-2 with the human signal peptide and a 6xHis-tag at the N-terminus region of the peptide. The purpose of the 6xHis-tag is to add a binding site for the later produced protein.

In our next step we linked the signal peptides and the AMP-region together, using fusion PCR (Titanium® Taq PCR Kit, Clontech Laboratories Inc.®, USA). With the primers 1157 and 1158 we fused the AMP-region of the CATH-2 to the relevant fish signal peptide. The templates were the AMP-region and the signal peptide of the CATH-2.

To create the same product with the human signal peptide we utilized the primers 1176 and 1158, using the human signal peptide for CATH-2 and the AMP-region as templates, as it is observable in Figure 5.

Table 2. List of primers designed for all preparational steps of CATH protein translation

Name of the primer	Sequence (5' -> 3')	Tm (°C)	Amplicon size (bp)	Extra information
1155	ACAACCGGTATGAAGATGAAGGTCCAGGTG	63,0	66 bp	With 1171 it amplifies the fish signal peptide of the CATH-1
1156	AGTGCTAGCCTATGCAAAGCGATTTCCATC	61,6	651	With 1327 it amplifies the AMP-region of the CATH-1
			315	With 1172 it links the AMP-region to the human signal peptide for CATH-1
1157	TTGTCCGGAATGAAGATGAAGGCTCTGGTG	63,0	66	With 1178 it amplifies the fish signal peptide of the CATH-2
1158	GACGGATCCTTATTGGGGAGATATCTTGCA	61,6	201	With 1177 it amplifies the AMP-region of the CATH-2
			300	With 1176 it links the AMP-region to the human signal peptide for CATH-2
1171	TGGTGATGGTGATGCGATCCAGATCTGACCTGCAGGACAG	70,6	66	With 1155 it amplifies the fish signal peptide of the CATH-1
1172	ACAACCGGTATGGCCTTGACCTTTGCTTTACTGGTGGCCCTCCTGGT	73,3	64	Forward primer for the human signal peptide for CATH-2, also adds the Age1 cutting site to the DNA
			99	With 1328 it amplifies the human signal peptide for CATH-1 out of the CATH-2 product

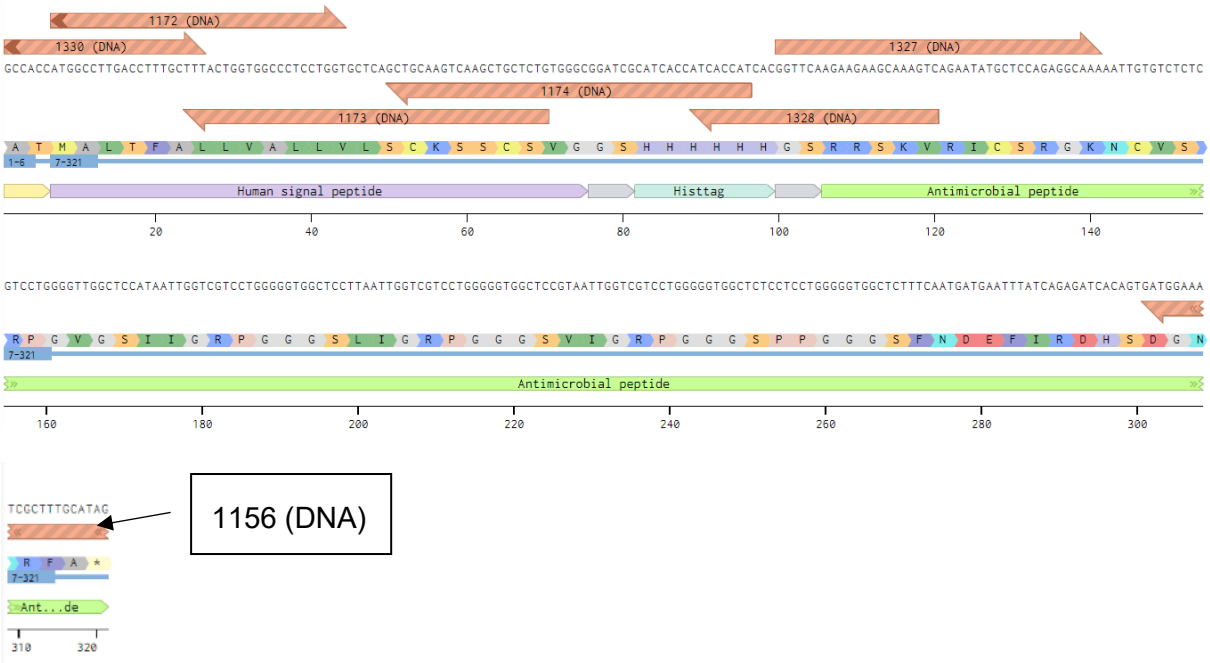
			315	With 1156 it links the AMP-region to the human signal peptide for CATH-1
1173	CAGAGCAGCTTGACTTGCAGCTGAGCACCAGGAGGGCCACCAGTAAA	74,1	64	Reverse primer for the human signal peptide for the CATH-2
1174	ATGGTGATGGTGATGCGATCCGCCCACAGAGCAGCTTGACTTGCAGC	74,1	90	With 1176 it amplifies the human signal peptide for CATH-2
1176	TTGTCCGGAATGGCCTTGACCTTTGCTTT	61,5	90	With 1174 it amplifies the human signal peptide for CATH-2 and adds the BamH1 cutting site to the DNA
1177	GGATCGCATCACCATCACCATCACGGTTCAAGAAGAGGCAAGGACAGCG	73,8	201	With 1158 it amplifies the AMP-region region of CATH-2
1178	TGGTGATGGTGATGCGATCCACCTCTGACCAGCAGGTTAGC	71,8	66	With 1157 it amplifies the fish signal peptide of the CATH-2
1327	GGTTCAAGAAGAAGCAAAGTCAGAATATGCTCCAGAGGCAAA	66,5	216	With 1156 it amplifies the AMP-region of the CATH-1
1328	GACTTTGCTTCTTCTTGAACCGTGATGGTGAT	61,8	99	With 1172 it amplifies the human signal peptide for CATH-1 out of the CATH-2 product

1330	ACA ACC GGT GCC ACC ATG GCC TTG ACC TTT GCT TT	67,9	321	With 1156 this primer adds the Kozak-sequence to the CATH-1 product with the human signal peptide and adds the Age1 cutting site to the DNA
1331	ACA ACC GGT GCC ACC ATG AAG ATG AAG GTC CAG GT	67,9	288	With 1156 this primer adds the Kozak-sequence to the CATH-1 product with the fish signal peptide and adds the Age1 cutting site to the DNA
1332	TTG TCC GGA GCC ACC ATG GCC TTG ACC TTT GCT TT	67,9	306	With 1158 this primer adds the Kozak-sequence to the CATH-2 product with the human signal peptide and adds the BamH1 cutting site to the DNA
1333	TTG TCC GGA GCC ACC ATG AAG ATG AAG GCT CTG GT	67,9	273	With 1158 this primer adds the Kozak-sequence to the CATH-2 product with the fish signal peptide and adds the BamH1 cutting site to the DNA
1452	GAC GGA TCC TCA ACT GCC GTG GTG ATG GT	65,7	306	With 1332 this primer amplifies the codon-optimised CATH-2 product with the human signal peptide and adds the BspE1 cutting site to it
1453	AGT GCT AGC TCA ACT GCC GTG GTG ATG GT	64,3	321	With 1330 this primer amplifies the codon-optimised CATH-1 product with the human signal peptide and adds the Nhe1 cutting site to it

4.3.2. Designing the CATH-1 for protein translation

For CATH-1 we amplified the AMP-region out of the full-length sequence with the primers 1327 and 1156. To amplify the fish signal peptide, we used the primers 1155 and 1172 on the full-length CATH-1 as a template. We amplified the human signal peptide and the 6xHis-tag for CATH-1 out of the recently created recombinant CATH-2 MatP with the primers 1172 and 1328. To link the fish signal peptide to the CATH-1 AMP-region we used the primers 1155 and 1156 on the fish signal peptide and the AMP-region as templates. Doing the same with the human signal peptide we utilized the primers 1172 and 1156 on the human signal peptide and the AMP-region as templates (Figure 5). We verified all products again via DNA gel electrophoresis, purification (MinElute® Gel Extraction Kit, QIAGEN, Netherlands) and Sanger sequencing (Sanger Sequencing, LGC Biosearch Technologies, UK).

A



B

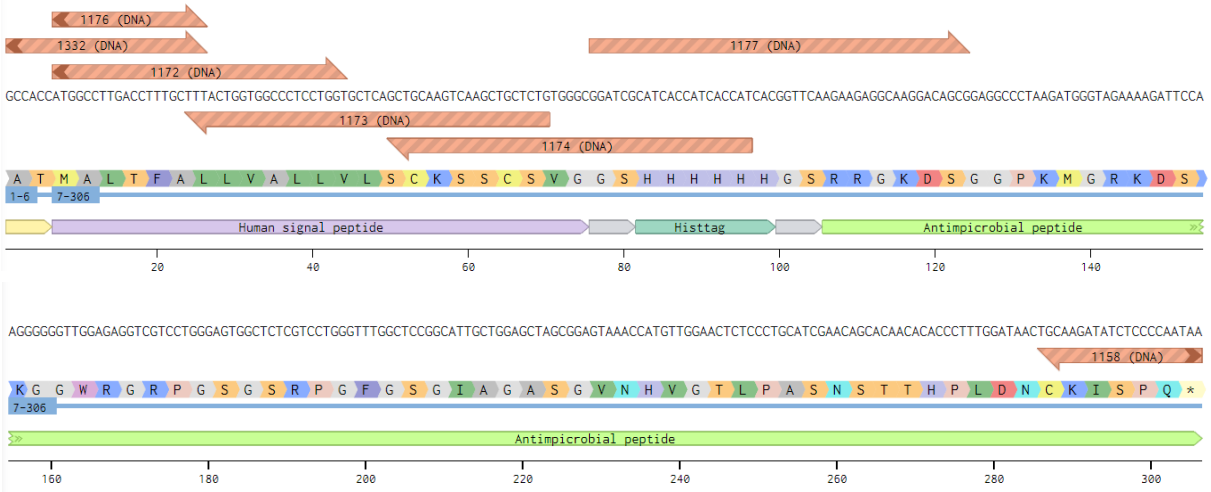


Figure 5. Recombinant CATH products consisting of the Kozak-sequence, human signal peptide, 6xHis-tag and AMP-region.

*From top to bottom, in the top row, the base sequence is visible (standard uppercase letters) as well as accordingly, the primer binding sites (arrows). The row beneath shows the corresponding amino acids to the base triplets (in coloured highlighted letters). In the last two rows, we annotated the different regions of the CATHs marked in colours and show a reference scale of the length in bp. (**A**) The sequence of CATH-1. (**B**) The sequence of CATH-2.*

4.3.3. Adding the Kozak-sequence

To increase the level of protein expression in the HEK293T cells, we added the Kozak-sequence to the N-terminus of our products. Hence, with the forward primer 1330 we added the 6-bp sequence to the CATH-1 MatP with the fish signal peptide and with the forward primer 1331 to the CATH-1 MatP with the human signal peptide. Both primers additionally added the Age1 cutting site to the product. We used 1156 as a reverse primer in both cases.

For CATH-2 we utilized the primers 1332 for the MatP with the fish signal peptide and 1333 accordingly for the human signal MatP, both primers added the BamH1 cutting site. The reverse primer was 1158. The addition of the Kosak-sequence is also visualized in Figure 5.

4.3.4. Multiplying our products with bacteria

In the first step we had to perform restriction enzyme digestion (New England BioLabs Inc.®, USA) in the same way as described above. Then we purified (MinElute® Gel Extraction Kit, QIAGEN, Netherlands) our products and ligated them to the expression plasmids (T4 DNA Ligase Kit, New England BioLabs Inc.®, USA). In the next step, we transformed the plasmids into bacteria as mentioned above, plated them on agar plates, incubated them for 16 hours at 37 °C and extracted the DNA (Monarch® Plasmid Miniprep Kit, New England BioLabs Inc.®, USA) before sending everything for sequencing (Sanger Sequencing, LGC Biosearch Technologies, UK).

4.3.5. Codon-optimising our product for human cells

Another intent for increasing protein expression was to codon-optimise our CATH products for human cells. To save money we decided to only optimise the CATHs AMP-region with the human signal peptide. We left the human signal peptide unchanged and we stopped using the fish signal peptide. Additionally, we moved the 6xHis-tag to the C-terminus region as the literature recommended this position (9) and deleted the Kozak-sequence as it does not need to be optimised.

We optimised our products following the instructions on the website www.idtdna.com and then synthesized the recombinant DNA sequences at Eurofins Scientific (Luxemburg).

Figure 6 shows a comparison between the pre-codon-optimised and the codon-optimised CATHs whereas Figure 7 shows the sequence of the codon-optimised CATHs. It is noteworthy that we didn´t make any changes in the first 20 bp, as the human signal peptide is in this position, which is already optimised. We amplified the codon-optimised CATH-1 with the primers 1330 and 1453, which added the restriction sites Age1 and Nhe1 to the peptide. For CATH-2, we copied the codon-optimised version with the primers 1332 and 1452 and attached the BamH1 and BspE1 restriction sites to the sequence. For CATH-1, 98 out of 315 bp were changed, which shows that 68,8 % of the bp stayed the same. 91 bp out of 300 were exchanged for CATH-2 which results in a rate of 69,6 % of unchanged bp.

A

```
Precodonoptimised_CATH_1      ATGGCCTTGACCTTTGCTTTACTGGTGGCCCTCCTGGTGCTCAGCTGCAAGTCAAGC---
Codonoptimised_CATH_1         ATGGCCTTGACCTTTGCTTTGTTGGTGGCTCTGTTGGTTCTTTCCTGTAAGAGTTCTTGT
                              ********************  ******* **  **** **   *** ***

Precodonoptimised_CATH_1      TGCTCTGTGAGAAGAAGCAAAGTCAGAATATGCTCCAGAGGCAAAAATTGTGTCTCTCGT
Codonoptimised_CATH_1         TCCGTAGGTAGAAGGAGTAAAGTGCGGATTTGTTCCCGAGGAAAAAATTGTGTATCACGA
                              * *   *  ***** ** *****  * ** ** *** **** ********** ** **

Precodonoptimised_CATH_1      CCTGGGGTTGGCTCCATAATTGGTCGTCCTGGGGGTGGCTCCTTAATTGGTCGTCCTGGG
Codonoptimised_CATH_1         CCGGGTGTTGGGTCAATCATCGGAAGACCGGGTGGGGGCTCACTGATCGGCCGCCCAGGC
                              ** ** ***** ** ** ** **  * ** ** ** *****  * ** ** ** ** **

Precodonoptimised_CATH_1      GGTGGCTCCGTAATTGGTCGTCCTGGGGGTGGCTCTCCTCCTGGGGGTGGCTCTTTCAAT
Codonoptimised_CATH_1         GGAGGGTCCGTTATTGGTCGCCCCGGAGGAGGTAGCCCTCCAGGTGGGGGCAGCTTCAAC
                              ** ** ***** ******** ** ** ** **    ***** ** ** ***   *****

Precodonoptimised_CATH_1      GATGAATTTATCAGAGATCACAGTGATGGAAATCGCTTTGCAGGCGGATCGCATCACCAT
Codonoptimised_CATH_1         GATGAGTTCATTCGGGACCACAGCGACGGGAATAGATTCGCTGGCAGTCATCATCACCAT
                              ***** ** **  * ** ***** ** ** *** * ** ** *** *    *********

Precodonoptimised_CATH_1      CACCATCACGGTTCATAG
Codonoptimised_CATH_1         CACCACGGCAGTTGA---
                              *****   * *** *
```

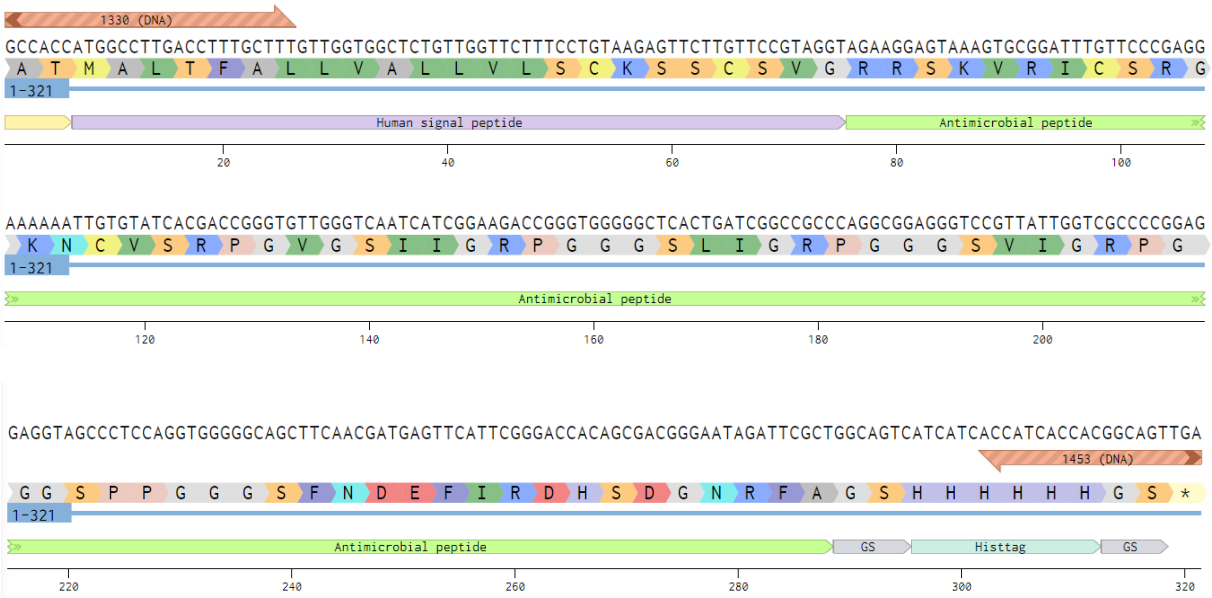
B

```
Precodonoptimised_CATH_2      ATGGCCTTGACCTTTGCTTTACTGGTGGCCCTCCTGGTGCTCAGCTGCAAGTCAAGCTGC
Codonoptimised_CATH_2         ATGGCCTTGACCTTTGCTTTGCTTGTTGCTCTCCTGGTTCTTAGTTGTAAGAGCAGCTGC
                              ******************** ** ** ** ******** ** ** ** ***   ******

Precodonoptimised_CATH_2      TCTGTGGGCAGAAGAGGCAAGGACAGCGGAGGCCCTAAGATGGGTAGAAAAGATTCCAAG
Codonoptimised_CATH_2         AGTGTCGGAAGGAGAGGTAAGGACTCCGGTGGCCCAAAAATGGGCCGAAAGGACTCAAAG
                                *** ** ** ***** ******  *** ***** ** *****  **** ** ** ***

Precodonoptimised_CATH_2      GGGGGTTGGAGAGGTCGTCCTGGGAGTGGCTCTCGTCCTGGGTTTGGCTCCGGCATTGCT
Codonoptimised_CATH_2         GGTGGGTGGAGGGGAAGGCCAGGGTCAGGAAGCCGACCAGGTTTTGGTAGCGGGATAGCT
                              ** ** ***** **  * ** ***   **    ** ** ** *****   *** ** ***

Precodonoptimised_CATH_2      GGAGCTAGCGGAGTAAACCATGTTGGAACTCTCCCTGCATCGAACAGCACAACACACCCT
Codonoptimised_CATH_2         GGGGCATCTGGGGTCAATCACGTTGGGACGCTCCCCGCAAGTAACAGCACGACCCACCCA
                              ** **    ** ** ** ** ***** ** ***** ***   ******** ** *****

Precodonoptimised_CATH_2      TTGGATAACTGCAAGATATCTCCCCAAGGATCGCATCACCATCACCATCACGGTTCATAA
Codonoptimised_CATH_2         CTCGATAATTGTAAAATCAGCCCGCAGGGCAGTCATCATCACCATCACCACGGCAGTTGA
                               * ***** ** ** **    ** ** **    ***** ** ** ** *****    * *
```

Figure 6. Comparison between pre-codon-optimised and codon-optimised CATHs.

*Asterisks mark unchanged bp. Note that the first 20 bp of each sequence stayed the same as we wanted our primers to still bind the new product and it is the human signal peptide, so it is already optimised for human cells. (**A**) For CATH-1 about 68,8 % of bp remained unchanged. (**B**) For CATH-2 this percentage was at 69,6 %. Alignments produced with Clustal Omega.*

A



B

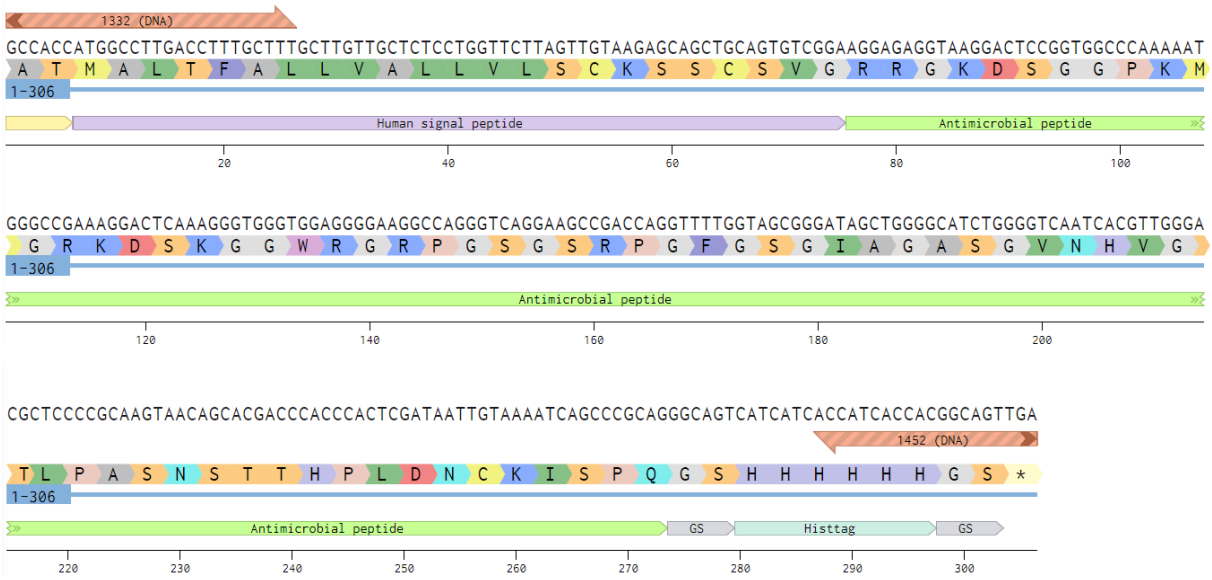


Figure 7. Full sequence of the codon-optimised CATHs whose amino acid sequences are unchanged.

*From top to bottom, in the top row, the base sequence is visible (standard uppercase letters) as well as accordingly, the primer binding sites (arrows). The row beneath shows the corresponding amino acids to the base triplets (in coloured highlighted letters). In the last two rows, we annotated the different regions of the CATHs marked in colours and show a reference scale of the length in bp. (**A**) The sequence of CATH-1. (**B**) The sequence of CATH-2.*

4.3.6. Culturing HEK293T cells

To express the corresponding two peptides of the CATHs, as well as a green fluorescent protein (GFP), which we also cloned into an expression plasmid and prepared with the human signal peptide, as a control, we used HEK293T cells. We cultured the HEK293T cells in Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific, USA) at 37 °C and a CO^2 concentration of 5 %. Growing cells form a monolayer on all parts of the flask that are covered by the medium. Once the surface is fully covered and the nutrients in the DMEM are metabolized, the DMEM changes its colour from pink to orange or yellow. To prevent the cells from dying because of overgrowing, we had to discard half or transfer them to a bigger flask, which we did in the following way: first, we discarded the supernatant (the metabolized DMEM), then we applied about 3 mL of trypsin for 5 min at 37 °C to detach the cells from the surface and then added 5 to 10 mL of new DMEM by pipette. Once all cells are detached, we took the medium with the cells in it and split them into two flasks for growth and added more DMEM. Growing cells which are attached to the bottom look like elongated stars, whereas dead cells which are detached from to the bottom are visible as round dots or clumps.

4.3.7. Preparing HEK293T cells with the CATHs

For this step the cells should ideally cover 80 – 90 % of the surface of the flask. Then the DNA plasmids (CATH-1, CATH-2) were extracted from bacteria (Monarch® Plasmid Miniprep Kit, New England BioLabs Inc.®, USA) and mixed with polyethyleneimine. We used three times more polyethyleneimine than DNA. We then mixed it with 250 µL DMEM and incubated everything for 30 minutes at room temperature before adding it to the cells. As the plasmid is carrying a blasticidin resistance gene, we treated the cells after transfection with blasticidin at a concentration of 25 µg/mL to kill off the cells which failed to integrate the plasmid. Living cells attach to the bottom of the flask and appear like elongated stars, dead cells in contrast are no longer attached to the bottom and have a round shape, as can be observed in the figure below.

As a positive control in the western blot, we also transfected some cells with a plasmid expressing green fluorescent protein (GFP).

A **B**

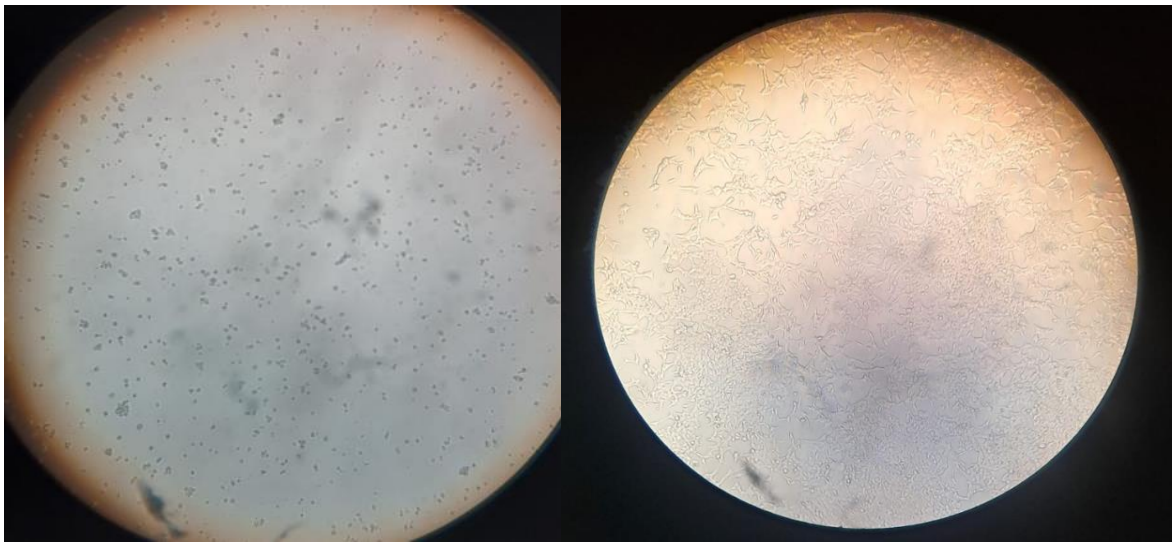


Figure 8. HEK293T cells susceptible or resistant to blasticidin.

*(**A**) Dead cells (which lack our plasmid and therefore are susceptible to blasticidin) are not attached to the bottom and can be identified by their roundish shape. (**B**) Living cells (which are resistant to blasticidin (25 µg/mL) because they have the plasmid) in comparison, appear to have a star-like form and are attached to the surface of the cell well plate*

4.3.8. Harvesting the protein

As the cells should produce the protein and then release it in the surrounding medium, we waited until the medium changed colour, to increase protein yield in the supernatant. As there is 10 % foetal bovine serum (FBS) in the medium, which consists of different proteins and results in a lot of contamination in the sample, it is important to purify the recombinant peptide.

We used nickel beads (1753180, Cytiva, USA) with an average diameter of 90 µm to bind the 6xHis-tag of our CATHs and purify the supernatant. We mixed supernatant and nickel beads together and kept them over night rotating at 4 °C. The following morning, we centrifuged the samples at 200 *g* for one minute and discarded the supernatant. Then, to clean the product, we washed five times in total with 1 mL phosphate-buffered saline (PBS) buffer, centrifuged

and let the sample stand still for two minutes. Finally, we added 15 µL PBS buffer to each sample.

Then, we mixed 5 µL of 4X Laemmli Sample Buffer (BIO-RAD, USA) with 10 % beta-mercaptoethanol, which breaks down disulfide bonds. Then we incubated the samples at 95 °C for five minutes to denature the proteins for electrophoresis.

Alternatively, we collected 15 µL of cell culture supernatant and added it to 5 µL of 4X Laemmli Sample Buffer (BIO-RAD, USA), and denatured the specimen as explained above.

4.3.9. Running a protein gel

A protein gel consists of two different parts: the stacking and the separating phase. In the stacking phase, which is on top of the separating phase, the proteins are stacked so they all start off at a similar level in the separating phase. Otherwise, it could happen that a small protein is still behind a large one because it started delayed. The exact composition of each phase can be found in Table 4. We started by adding the separating phase between the glass plates and layered 70 % ethanol directly above it to even out the separating phase. After the separating phase polymerized and we dried the ethanol completely, we added the stacking phase and positioned the comb.

Then we placed the protein gel in the electrophoresis cell (Mini-PROTEAN Tetra Vertical Electrophoresis Cell, BIO-RAD, USA) and we filled the whole cell with running buffer. Later in the stacking phase we ran the gel at 75 Volts, whereas we increased voltage to 150 during the running phase after about one hour.

4.3.10. Western blot

Despite purifying the supernatant with nickel beads some unspecific binding always occurs, which is why there are lots of unspecific protein bands visible in the Coomassie staining, therefore it is often difficult to interpret the result. Here the western blot is much more specific as we use different antibodies that detect our proteins based on the 6xHis-tag, but therefore also longer and more difficult to process.

In the first step we transferred the protein from the gel on a membrane (Nitrocellulose Membrane 045 µm, BIO-RAD, USA). That is why we activated the membrane in methanol for

a few seconds until it became transparent. Then we submerged the hydrophobic membrane in distilled water for about five minutes, otherwise the protein would not be able to transfer. After that, we soaked the membrane and four sheets of filter paper (Blot Absorbent Filter Papers, BIO-RAD, USA) in transfer buffer. Then we loaded the transfer machine (Trans-Blot® SD Semi-Dry Cell, BIO-RAD, USA) in the following manner: at the bottom, we placed two layers of filter paper, then we added the membrane and the gel, and finally put two layers of filter paper on top. Then, the machine ran with 50 milliampere for 30 minutes.

In the next step, we washed the membrane in Tris-buffered-saline (TBS) buffer for five minutes on a shaking device. Then, we incubated the membrane for 30 minutes shaking in a blocking solution, before washing it three times for five minutes in distilled water. Afterwards, we added the first antibody (monoclonal anti-poly-histidine antibody produced in mouse, Sigma-Aldrich, USA), which was diluted at a ratio of 1:2000, to bind the 6xHis-tag of the CATHs. Then we washed three times for five minutes with TBS 0,05 % Tween-20. The second antibody (goat anti-mouse IgG antibody, horse radish peroxidase (HRP) conjugate, Sigma-Aldrich, USA) was diluted at a ratio of 1:5000, and then we washed as explained above. The various solutions are described in Table 4.

For the final step we added 500 µL of substrate solution (Clarity™ Western ECL Substrate Luminol/enhancer solution, BIO-RAD, USA) and 500 µL of peroxide solution (Clarity™ Western ECL Substrate Peroxide Solution, BIO-RAD, USA) onto the membrane for five minutes. After drying off excess solution, then the membrane is ready for analysis for chemiluminescence.

4.3.11. Change of plans

Unfortunately, in none of our attempts could we detect the protein of our DNA-products. Therefore, we decided to purchase peptides for our CATH-1 (ALMAC, UK) and CATH-2 (ALMAC, UK) as well as the human CATH LL-37 (LL-37, InvivoGen Europe, France) that were produced via commercial synthesis.

4.4. In vitro antimicrobial and immunomodulatory activity assays

In the last part of this project, we added the MatP of the CATHs, as well as the LL-37 as a control, at various concentrations to different fish-infective bacterial species and took

measurements with a spectrophotometer at several timepoints. To test the immunomodulatory activity, we added the CATHs, the LL-37, LPS and water at one concentration to rainbow trout peripheral blood leucocytes for different durations.

4.4.1. Lytic and microbicidal activity on bacteria

In the next step, we tested the lytic and microbicidal activity of our synthesised CATH-1 and CATH-2, as well as the LL-37 on the bacteria species *A. salmonicida, Y. ruckeri, V. arginolyticus, E. coli* and *A. sobria*. The aim was to test bacteria that were obtainable at the fish clinic, that result in infectious diseases in fish and are from different genera. In the beginning we prepared agar plates and fluid medium of Mueller-Hinton broth, and we incubated our species on agar plates overnight. All incubations for these five species were done at 22 °C. Then we took colonies and transferred the bacteria into fluid medium, where we let them grow to the log phase, until the optical density in the photometer was between 0,5 and 1 at 595 nm absorption. The literature recommended a CFU of 5 x 10^5 for testing the activity of the proteins (2), so we had to serially dilute the bacterial cultures, we plated 50 µL of this dilution and counted the colonies per plate after 16 hours. Then we brought each bacterial medium to the targeted concentration of 5 x 10^5 CFU/mL and added 20 µL of this bacterial solution to each well on a 96-well plate. Then we added 80 µL of our protein at concentrations of 0, 2, 4, 8, 16 µM prepared in water and incubated the plate at 22 °C. Then we measured the concentration with a spectrophotometer at 595 nm as the literature suggested (2), at the time points 0, 16 and 24 hours for *A. salmonicida*, *Y. ruckeri* and *V. arginolyticus*. As *E. coli* and *A. sobria* grew more slowly we decided to take measurements at the timepoints 0, 18, 28 and 34 hours.

4.4.2. Activity assays with primary fish cells

For the subsequent activity assays, there are various methods to extract WBCs out of blood samples, but we decided to test the fast and cost efficient method described here (10). After collecting 1 mL blood from six rainbow trout and we kept the samples on ice while preparing six tubes with 9 mL distilled water. Then we added each blood sample to one of the tubes, so that hypotonic fluid lysed the red blood cells (RBC). As the white blood cells (WBCs) are more resilient they should survive this procedure. After 20 seconds, we added 1 mL PBS Buffer to

get an isotonic solution again. Then we placed the samples on ice for ten minutes and filtered them through a 100 µm microfilter (ClearLine Cell Strainer, Clear Line, UK) to exclude clumps of dead RBCs. In the next step, we centrifuged for five minutes at 200 *g*, then we resuspended with 10 mL of Leibowitz 15 medium, before centrifuging again (10). After this step, we counted the concentration of the WBCs with a Bürger-Türk-counting-chamber.

Then we brought all samples to a concentration of about $1x10^6$ cells/mL and added 100 µL of the mix per well to a 96-well plate. We treated the cells with LL-37, CATH-1, CATH-2 and LPS diluted in water as a positive control, or water as a negative control. All peptides were added at a 10 µM working concentration and we collected samples at 0 and 4 hours of incubation time at 19 °C. The LPS was added at a concentration of 25 µg/mL.

In the last step, we centrifuged the cells from these samples again at 500 *g* for five minutes, discarded the supernatant and added 100 µL RNA Later (RNA*later*™ Soln., Thermo Fisher Scientific, USA) and kept them in the refrigerator. Before the RNA extraction, we centrifuged the samples at 500 *g* again and only kept the cell pellet.

4.4.3. RNA extraction and cDNA synthesis

We extracted the RNA with the same Kit (RNeasy® Mini Kit, QIAGEN, Netherlands) as before. We strictly followed the manufacturer's instructions; the only difference was that we did the optional DNase digestion step this time. After that, we measured the RNA yield by spectrophotometry (NanoDrop Spectrophotometer, Thermo Fisher Scientific, USA) and adjusted the concentrations of the samples to a final concentration of 20 ng/µL. Then we reverse-transcribed the RNA to cDNA (iScript™ Reverse Transcription Supermix for RT-qPCR, BIO-RAD, USA). Apart from the samples, we had two negative controls: one with water instead of the RNA template to evaluate if we got any contaminating DNA. For the other one, we had RNA templates from four different samples, but we were missing the reverse transcriptase to determine if we had genomic DNA in our samples.

4.4.4. qPCR

To measure the expression of certain PKD-related immunomodulators we used qPCR (SsoAdvanced Universal SYBR Green Supermix, BIO-RAD, USA). For this trial we diluted all our samples from the timepoints 0 and 4 hours and the negative controls from before at 1/20.

For each sample, we mixed 5 µL of 2X SsoAdvanced Universal Supermix, 0,5 µL forward primer, 0,5 µL reverse primer, 2 µL cDNA and 2 µL water together to add up to a final concentration of 10 µL. Then we tested if there were changes in expression of the genes *rtIL-8, rtTNFα2*, *rtCATH1* and *rtCATH2* relative to the housekeeping genes *rtEF1α* and *rtBactin* due to the presence of our proteins. Table 3 depicts the used primers for each gene:

Table 3. List of primers for activity assays with primary fish cells

Gene target	Name of the primer	Sequence (5' -> 3')
rtEF1α	Forward Primer	CAAGGATATCCGTCGTGGCA
	Reverse Primer	ACAGCGAAACGACCAAGAGG
rtBactin	Forward Primer	ATGGAAGGTGAAATCGCC
	Reverse Primer	TGCCAGATCTTCTCCATG
rtIL-8	Forward Primer	ATTGAGACGGAAAGCAGACGA
	Reverse Primer	CCCTCTTCATTTGTTGTTGGC
rtTNFα2	Forward Primer	CTGTGTGGCGTTCTCTTAATAGCAGCTT
	Reverse Primer	CATTCCGTCCTGCATCGTTGC
rtCATH1	Forward Primer	ACCAGCTCCAAGTCAAGACTTTGAA
	Reverse Primer	TGTCCGAATCTTCTGCTGCAA
rtCATH2	Forward Primer	ACATGGAGGCAGAAGTTCAGAAGA
	Reverse Primer	GAGCCAAACCCAGGACGAGA

Then we programmed the thermocycler the following way: The initial denaturation was at 95 °C for one minute, then we had 40 cycles with a 15-second-long denaturation step at 95 °C, or ten seconds for *rtIL-8*, then a 60° C annealing step for 20 seconds. Finally, we programmed a melt curve from 65 °C to 95 °C, with a temperature increase of 0,5 °C every five seconds. The

melt curve is important to check whether all qPCR-products are pure and without contamination, which is the case if all melt-curves have the same profile.

We tested all samples in duplicates and if the Ct-value varied more than 0,6 we repeated the qPCR in triplicate, quadruplicate or quintuplicate or increased the concentration of cDNA to a dilution of 1/2,5 depending on how abundant we expected the gene to be, e.g., if two replicate Ct values were vastly different such as more than two cycles apart. Since we are always comparing between cells treated (CATHs or LPS) and untreated (H_2O added to cells), for a fair comparison, we always ensured that the dilution of the cDNA was the same for the entire set of samples and not just for one treatment condition.

After all data was complete, we subtracted the Ct from the genes *rtIL-8, rtTNFα2, rtCATH-1* and *rtCATH-2* from the Ct of the housekeeping genes *rtEF1α* and *rtBactin* to calculate the -deltaCt. To visualize our data better in graphs, we then calculated the absolute change in expression using the formula $2^{-deltaCt}$ (11). Then we took the average of a maximum of six biological replicates (n=6 for *rtCATH-1*, for the rest n=5) per condition and visualized this in graphs, as well as calculated the standard deviation (11). In the last step we tested for a significant effect of cell treatment using the Friedman test and the Dunn's multiple comparisons test to determine if there were differences between the negative control (adding water to cultured cells) and the experimental treatments (either CATH treatments or LPS treatment).

4.4.5. List of Buffers/Gels/Solutions

Table 4. List of Buffers/Gels/Solutions

Buffer/Gel	Composition	Final concentration (1x)
1x TAE Buffer		0,04 M Tris base 0,02 M acetic acid 1 mM EDTA pH should be about 8,5

10x TBS Buffer	48,45 g tris	20 mM
	175,32 g NaCl	150 mM
	Add up to a final volume of two litres with distilled water pH should be about 7,5	
10x Running Buffer	60,6 g tris Base	25 mM
	288,4 g glycine	192 mM
	20 g sodium dodecyl sulphate (SDS)	0,1 %
	Add up to a final volume of two litres with distilled water	
10x Transfer Buffer	29,3 g glycine	39 mM
	58,1 g tris Base	48 mM
	3,75 g SDS	0,0375 %
	Add up to a final volume of one litre with distilled water For a 1x mixture, add methanol to a final concentration of 20 %	
Blocking solution (western blot)	5 % non-fat dry milk powder 95 % TBS Buffer	
Antibody dilution solution (western blot)	2,5 % non-fat dry milk powder 97,5 % TBS Buffer	

DNA Gel	100 mL TAE Buffer 1 g Agarose 10 µL GelRed	
17 % Separating Gel	0,65 mL distilled water 5,6 mL tris 1M pH8,8 8,5 mL 30 % acrylamide 0,25 mL 10 % SDS 15 µL tetramethylethylendiamin (TEMED) 75 µL 10 % ammonium persulfate (APS)	
Stacking Gel	6,0 mL distilled water 1,25 mL tris 1 M pH.6,8 1,85 mL 30 % acrylamide 0,125 mL10 % SDS 15 µL TEMED 75 µL 10 % APS	
51 % Percoll	4,08 mL Percoll 0,8 mL 10x PBS 3,12 mL distilled water	

5. Results

5.1. Isolation of the full-length CATHs

Firstly, we reverse transcribed the RNA obtained from a homogenized kidney from a rainbow trout with PKD. PCR with the primers 1155 and 1156, using the cDNA from a homogenized kidney sample from one *T. bryosalmonae*-infected fish as a template, generated a 651-bp product that corresponded to the expected length of CATH-1 cDNA. When we tested the primers 1157 and 1158 on the same kidney sample, we were able to amplify a slightly shorter 621-bp product which matches the theoretical length of CATH-2 cDNA.

Figure 9 depicts the successful detection of the CATHs obtained via PCR and size-differentiated by DNA gel electrophoresis.

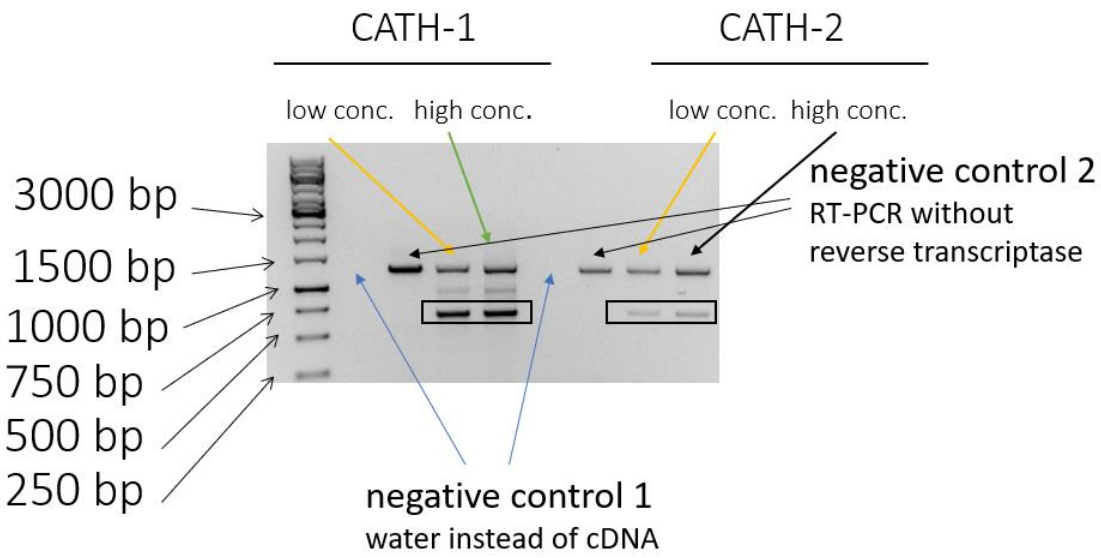


Figure 9. Detection of CATH cDNA in rainbow trout posterior kidney by PCR:

We amplified the CATHs by PCR and submitted the PCR products to DNA gel electrophoresis. Each half of the gel represents the results for one of the CATHs using different pairs of primers (1155 and 1156 for CATH-1 and 1157 and 1158 for CATH-2). For each CATH, the very left lane is the result of a negative control PCR where we replaced the cDNA with water. One lane to the right of it is a second negative control PCR, where we added the product of an RT reaction without the reverse transcriptase during RT-PCR. The following two lanes are the CATH PCR reactions using cDNA as template. In these reactions, the cDNA was obtained from RT reactions with the reverse transcriptase included and using total RNA at a low (190 ng/µL) and a high concentration (570 ng/µL).

The lengths of the CATH obtained from cDNAs are approximately 660 bp (marked with the black boxes). The products at 1300 bp are the CATHs amplified from genomic DNA that we did not eliminate during RNA extraction.

Both amplified cDNA products correspond to full-length CATHs, as they have a signal peptide sequence at the N-terminus region, a preproregion and a unique C-terminal sequence, the AMP-region. During translation, the CATHs are produced as a pre-proprotein, the preproregion is removed by a protease, which releases the AMP-region (7).

5.2. Designing the mature peptide (MatP) for protein translation

Another purpose of the PCR amplification of cDNA (Figure 9) was to integrate restriction digestion sites at both ends of the sequence. In the next step we performed a restriction enzyme digestion on the full-length CATH-1 and CATH-2 to clone them in expression plasmids. The plasmid served as a backbone from which we verified that the sequences are 100 % correct by sequencing everything with complete coverage (Sanger sequencing). With the full-length sequence we could also selectively amplify or exclude different regions of the AMP.

Thus, we constructed the MatP for each CATH successfully. Figure 10 shows a DNA-gel electrophoresis of the successful amplification of different parts of CATH-2 to design the MatP for CATH-2. In summary, we amplified the fish signal peptide and the AMP-region for CATH-2, as well as started creating the human signal peptide with fusion PCR. Our objective was to test different signal peptides (fish or creating the human signal peptide) in combination with the MatP as we hypothesized they could affect expression.

Alternatively, we also took the same MatP sequences of CATH-1 and CATH-2, codon-optimised them and synthesized two inserts (one for each CATH) with a 5’ human signal peptide sequence, 3’ poly-histidine tag sequence, and appropriate restriction digestion sites for cloning into the expression plasmids (Figure 7). Without synthesis, a codon-optimised version of the MatP’s would be very time-consuming to produce ourselves.

Success was again verified with sequencing.

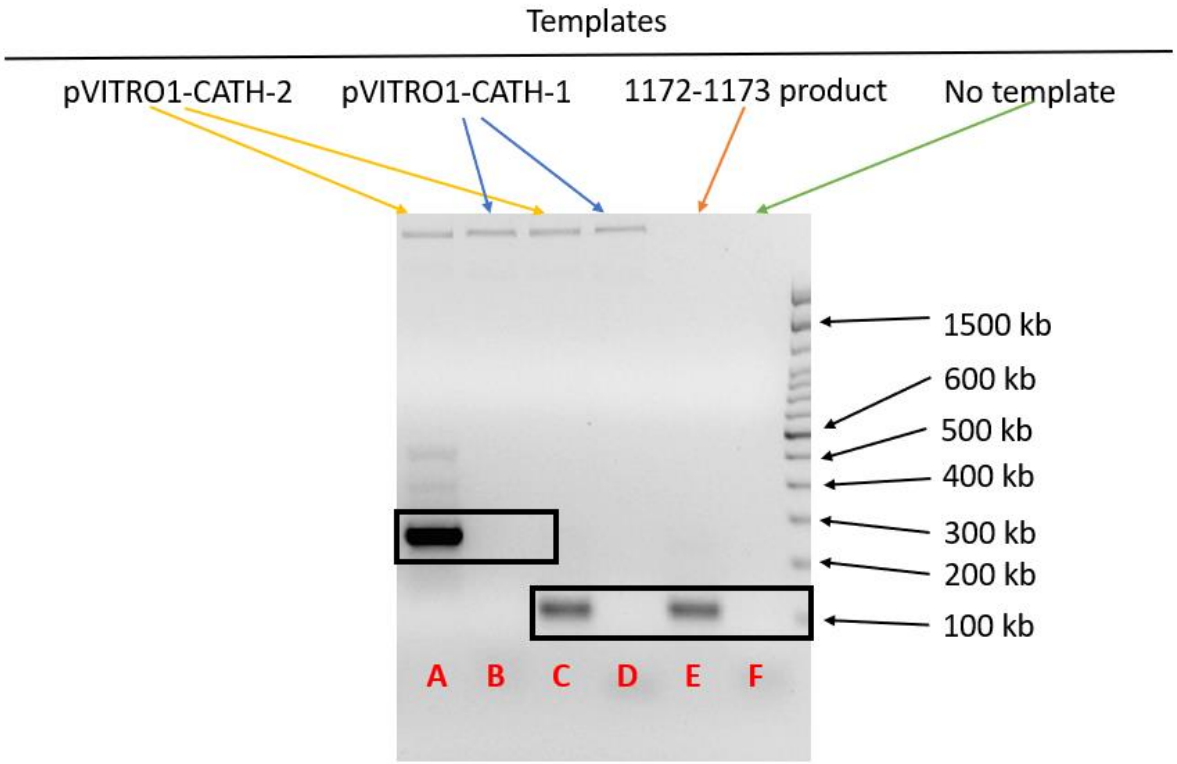


Figure 10. Amplifying AMP-region and signal peptides of CATH-2 to design a CATH-2-MatP by PCR.

*We amplified the different parts for the CATH-2-MatP and submitted the PCR products to DNA gel electrophoresis. For the first two lanes we used the primers 1177 and 1158 on the CATH-2 as a template to amplify the AMP-region (**A**) and on CATH-1 as a template as a negative control (**B**). The expected product is 250 bp long and can be seen in lane (**A**). The next two lanes represent the amplification of the fish signal peptide for CATH-2 (**C**) as well as the negative control with CATH-1 as a template again (**D**). The primers were 1157 and 1178 to amplify the 100 bp long product visible in lane (**C**). For the last two lanes we used the primers 1176 and 1174 on the 1172-1773-product as template to create the human signal peptide with the 6xHis-tag (**E**), and we had a negative control which was missing the template (**F**). The expected product is visible at 100 bp in lane (**E**).*

At the very top of the gel are the templates for the first four lanes visible, which are the expression plasmids ligated with the CATHs, at a length of roughly 6400 bp.

After we successfully created the MatP for CATH-1 and CATH-2, we cloned them into our expression plasmid again and transfected HEK293T cells with it.

5.3. Protein detection

After purifying the supernatant of the HEK293T cells we tried to detect the CATHs by running our samples on a protein gel and performing a 6xHis-tag-specific Western blot detection protocol with a chemiluminescent readout. We first tested for GFP expression by analysing the supernatant of the HEK293T cells, in the control group, and found visible amounts present (Figure 11A). GFP served as a positive control because it was expressed from the same plasmid, with the same signal peptide, the same poly-histidine tag as the CATH constructs. The expected molecular weight of the GFP is around 28 kDa and we detected a product with exactly the right size in two cell culture plate wells that were analysed. Then we tested for CATH-1, CATH-2 (data not shown for CATH-2) and GFP in supernatants, but only detected the GFP. The expected length of the CATH peptides is roughly 8 kDa, but unfortunately the only bands visible in the CATH-1 lanes were around 60 kDa, purification with nickel beads showed the same result (Figure 11B).

Unfortunately, we consistently failed to express and detect the CATH peptides, despite maximizing recombinant protein expression by adding a human signal peptide or adding the Kozak-sequence and codon-optimising the peptides for human cells. Hence, we decided to have the peptides commercially synthesized.

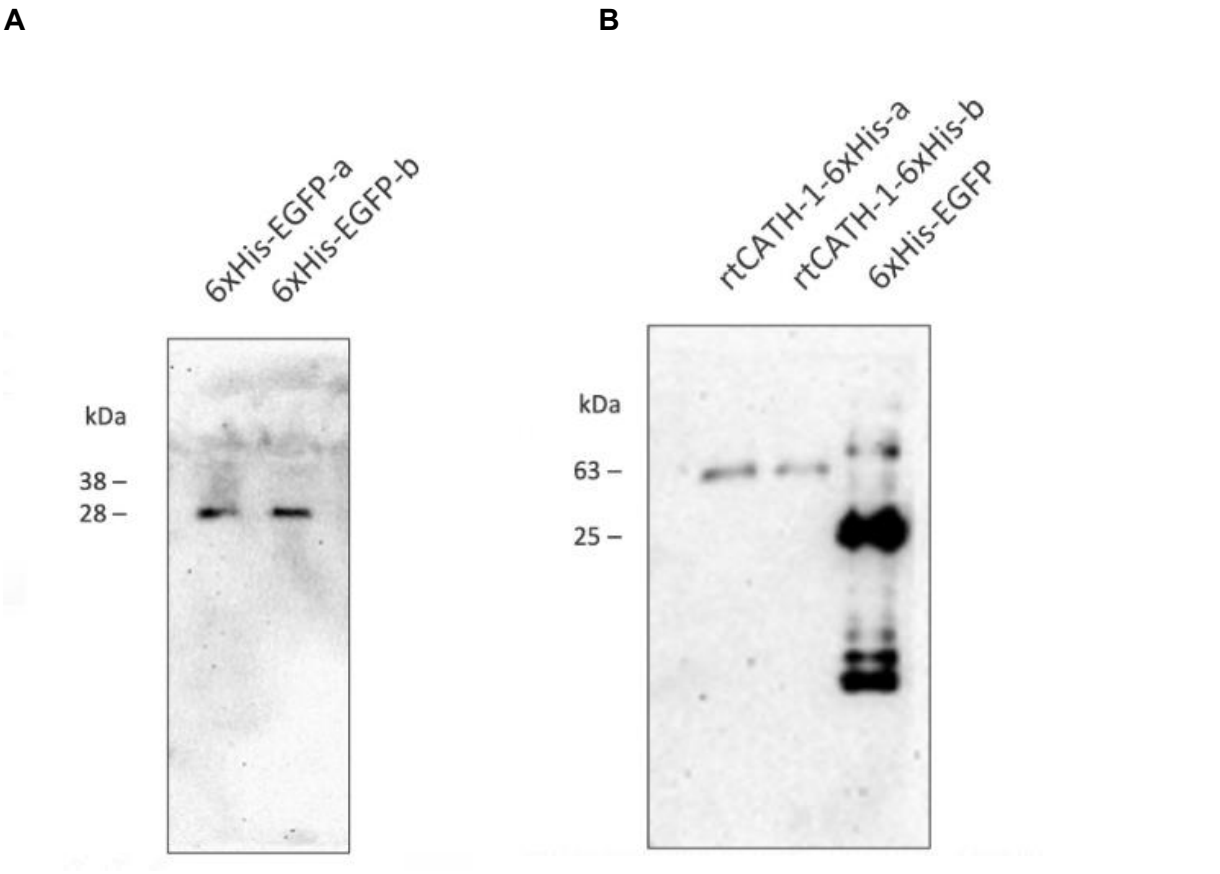


Figure 11. Western Blot results for detecting CATH-1 and GFP expression in HEK293T cells.

*(**A**) shows the detection of GFP in cell culture supernatant (positive control, GFP 28 kDa) of 2 replicate wells. (**B**) results of poly-histidine-tagged CATH-1 expression as well as GFP expression after precipitation with nickel beads. The GFP column shows a strong signal at 28 kDa, as well as some impurities. The CATH-1 columns did not show the expected product at 8 kDa, but a band at 60 kDa in both samples. Sample names: The 6xHis indicates that we added 6 His-tags to the peptide, the lowercase letters in the end distinguish between replicas collected from different wells but transfected with the same plasmid.*

Both the CATH AMP-regions and the GFP were prepared with a human signal peptide and cloned into an expression plasmid before transfection into HEK293T cells.

5.4. Antibacterial activities of the CATHs

Our commercially produced CATH-products as well as the human CATH LL-37, which worked as a positive control, displayed various bactericidal activities to the bacterial species we tested. In *A. salmonicida* we observed a strong growth inhibiting activity correlating with increasing peptide concentrations. Interestingly CATH-2 showed a significantly stronger microbicidal activity than CATH-1 (Figure 12A).

Y. ruckeri didn´t show measurable growth in the first 18 hours compared with the blank, so for this graph there is no significant activity of the CATHs visible. At the 24-hour timepoint it appears that *Y. ruckeri* inhibits growth only at higher concentrations compared to *A. salmonicida* and CATH-1 has more bactericidal activity than CATH-2, as it can be seen in Figure 12B.

Unfortunately, *V. arginolyticus* didn´t grow at all, so we couldn´t include the results in this thesis.

As *E. coli* grew more slowly and didn´t show any measurable growth at 18 hours, we included only the timepoints 28 and 34 hours in the thesis. Both CATHs inhibited bacterial growth, with CATH-1 being slightly more effective than CATH-2. Also, at the 34-hour-testing *E. coli* showed less response to the CATHs (Figure 12C).

For *A. sobria* microbicidal activity of the CATHs was only visible at 18 hours, whereas they showed no significant growth reduction later, at 28 hours and 34 hours, independent from peptide concentration, as shown in Figure 12D.

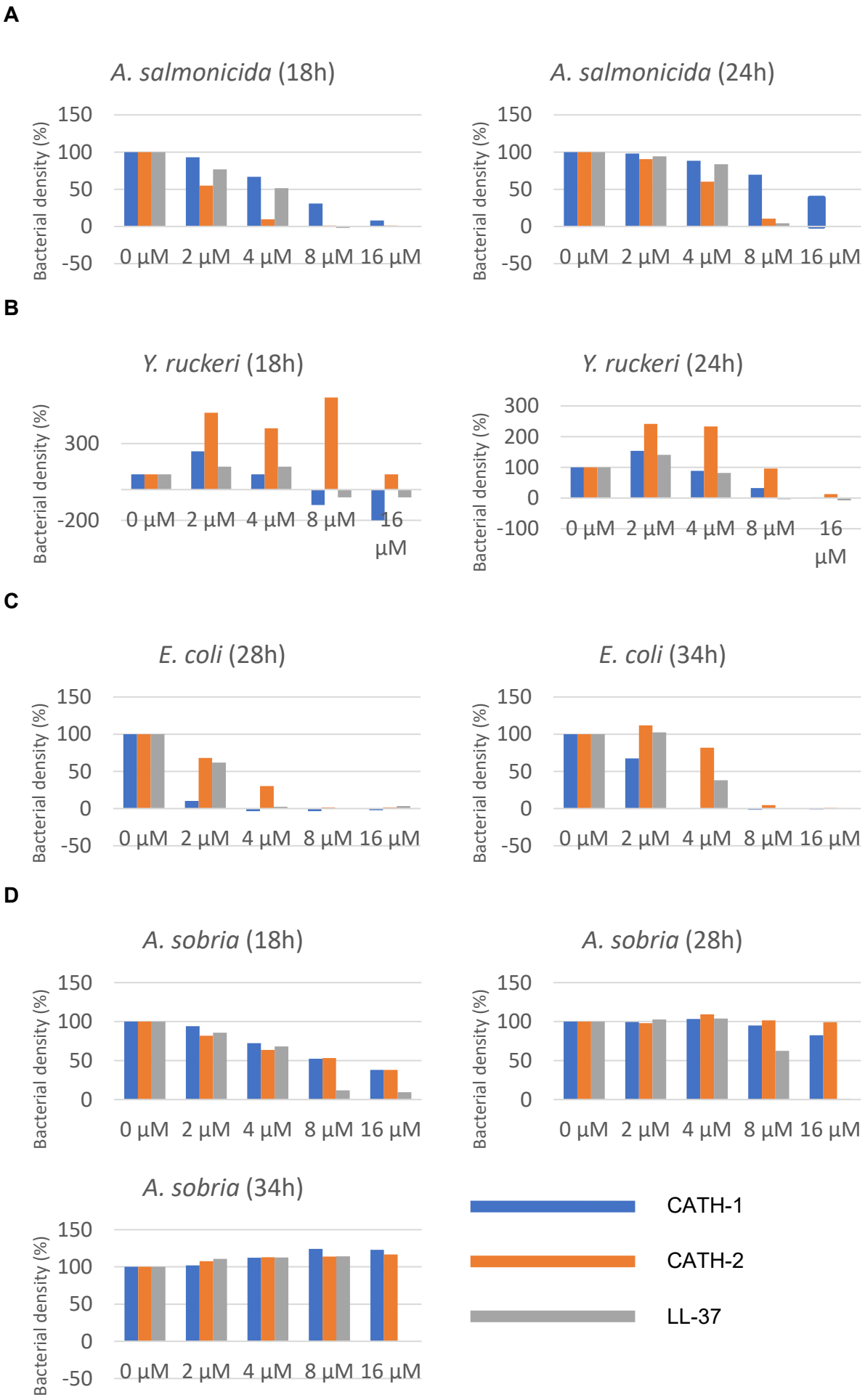

A
A. salmonicida (18h)
A. salmonicida (24h)
Bacterial density (%)
150
100
50
0
-50
0 µM 2 µM 4 µM 8 µM 16 µM
B
Y. ruckeri (18h)
Y. ruckeri (24h)
300
-200
200
-100
C
E. coli (28h)
E. coli (34h)
D
A. sobria (18h)
A. sobria (28h)
A. sobria (34h)
CATH-1
CATH-2
LL-37

Figure 12. Bactericidal activity of rainbow trout CATH-1 and CATH-2, as well as human CATH LL-37 on fish pathogenic bacterial strains.

We measured bacterial density by spectrophotometry in a microplate reader. The bacterial density was measured relative to the untreated (0 µM) samples at the indicated timepoints after treatment with one of three CATHs. We observed stronger inhibition correlating with increasing CATH peptide concentrations against A. salmonicida*,* Y. ruckeri*,* E. coli*, and* A. sobria *at the earlier timepoint. (**A**)* A. salmonicida. *(**B**)* Y. ruckeri. *(**C**)* E. coli. *(**D**)* A. sobria

5.5. Activity assays with primary fish cells

To test the CATHs' immunomodulatory activity on fish cells, we had to harvest WBCs from rainbow trout blood. We successfully eliminated RBCs by hypotonic lysis and were able to harvest a good yield of WBCs after incubating the blood samples for 20 seconds in distilled water and separating RBC cell debris with a cell strainer, as shown in the figure below.

A **B**

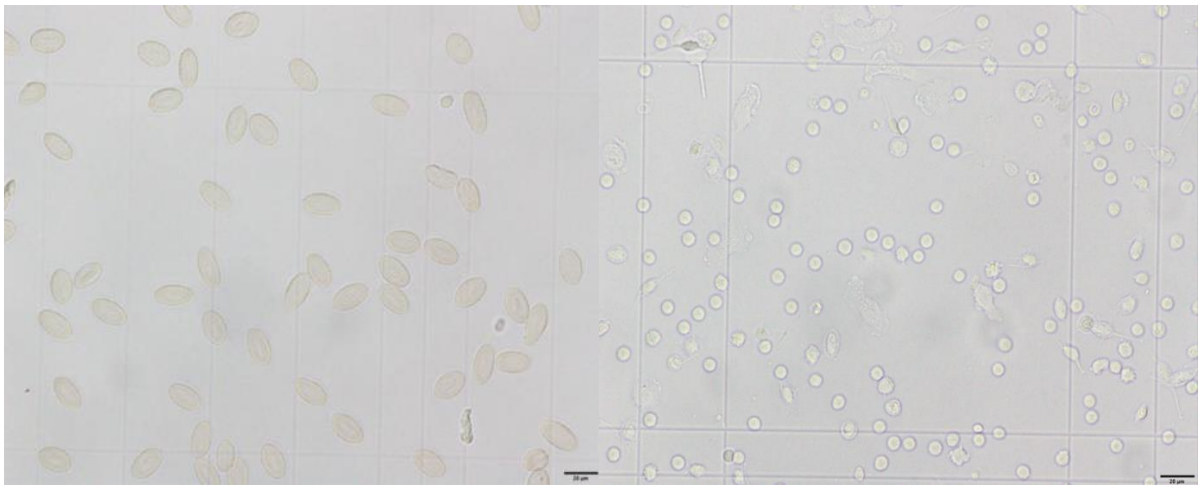


Figure 13. Comparison between rainbow trout peripheral blood samples before and after hypotonic lysis of erythrocytes.

*(**A**) native rainbow trout blood sample. RBCs, which are ellipsoid and have a nucleus in fish, make up around 99 % of all blood cells. (**B**) blood sample after hypotonic lysis of RBCs in distilled water for 20 seconds and separation of cell debris with a cell strainer. In comparison to **A** there are only WBCs with a roundish shape observable. This black scale line in the bottom left corner of each picture represents 10 µm.*

We tested the immunomodulatory activity of our rainbow trout CATHs as well as the LPS as a positive control and the LL-37 as a negative control on rainbow trout blood leucocytes. Our

goal was to test four genes that are PKD-related, *rtIL-8, rtTNFα2, rtCATH-1* and *rtCATH-2,* relative to two housekeeping genes, *rtEF1α* and *rtBactin*. In total we tested six fish, but as we were not able to use the results from one fish for the genes *rtIL-8, rtTNFα2* and *rtCATH-2* because the cDNA was too dilute, it was n=5 for these genes.

All results were compared to the mock treatment with H_2O, and we investigated if the difference in expression was significant with multiple comparisons tests.

Overall, data from normalization with the two housekeeping genes matched for *rtIL-8 a*nd *rtTNFα2* but did not for the other two genes. For *rtIL-8* it is noticeable that the expression at timepoint T0 column is much higher than the one at T4, probably because expression in a living organism was higher than in an experimental setup with cells in medium. Additionally, there was a high variation in the T0 sample from *rtIL*-8, because all the measurements strayed a lot. Unfortunately, none of our CATH treatments were significantly different from the negative control.

For *rtTNFα2* the expression after LPS and the human CATH LL-37 treatments were very high and significantly upregulated, in contrast to the rainbow trout CATHs.

For *rtCATH-1* and *rtCATH-2* it seems that the CATH and LPS treatments as well as the human CATH LL-37 treatment were expressed on the same level.

In general, *rtCATH-1* expression was a little bit higher than *rtCATH-2* for all stimulations and both CATHs were overall higher than water, but unfortunately none of our results were statistically significant, so it just could have been by chance.

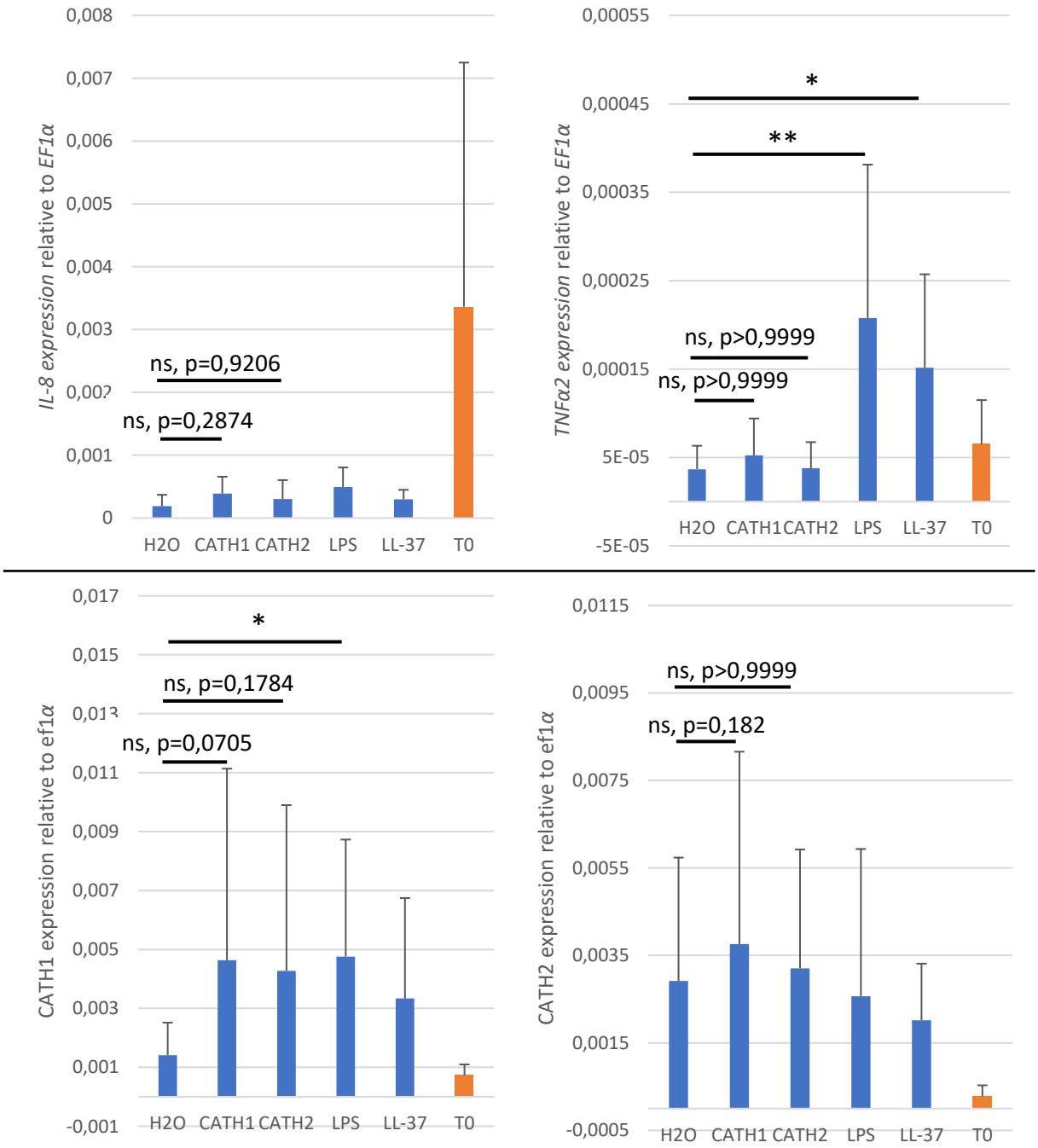


Figure 14. Gene expression and immunomodulatory activity caused by rainbow trout CATHs and human CATH LL-37 to peripheral blood WBCs of rainbow trout in vitro relative to rtEF1α.

We took the -deltaCt and calculated the absolute change in target gene expression relative to the housekeeping gene rtEF1α *expression using the formula* $2^{-deltaCt}$. *Each bar presents the mean of all measurements (n=5 or n=6) from the same group and timepoint. The error bars represent standard deviation. As we tested the changes of expression of our genes after treatment (CATHs and LPS) or mock treatment (H2O) to determine if the changes are scientifically significant, we show these results above the horizontal lines between data points. ** = p < 0,01; * = p < 0,05; ns = not significant. As the main focus of our work was on CATH-1 and CATH-2, we showed the p-value for all of them, whereas we only showed whether there was a significant difference between the LPS or LL-37 treatments versus the mock treatment. Details of the statistical tests used are in the Materials and Methods.*

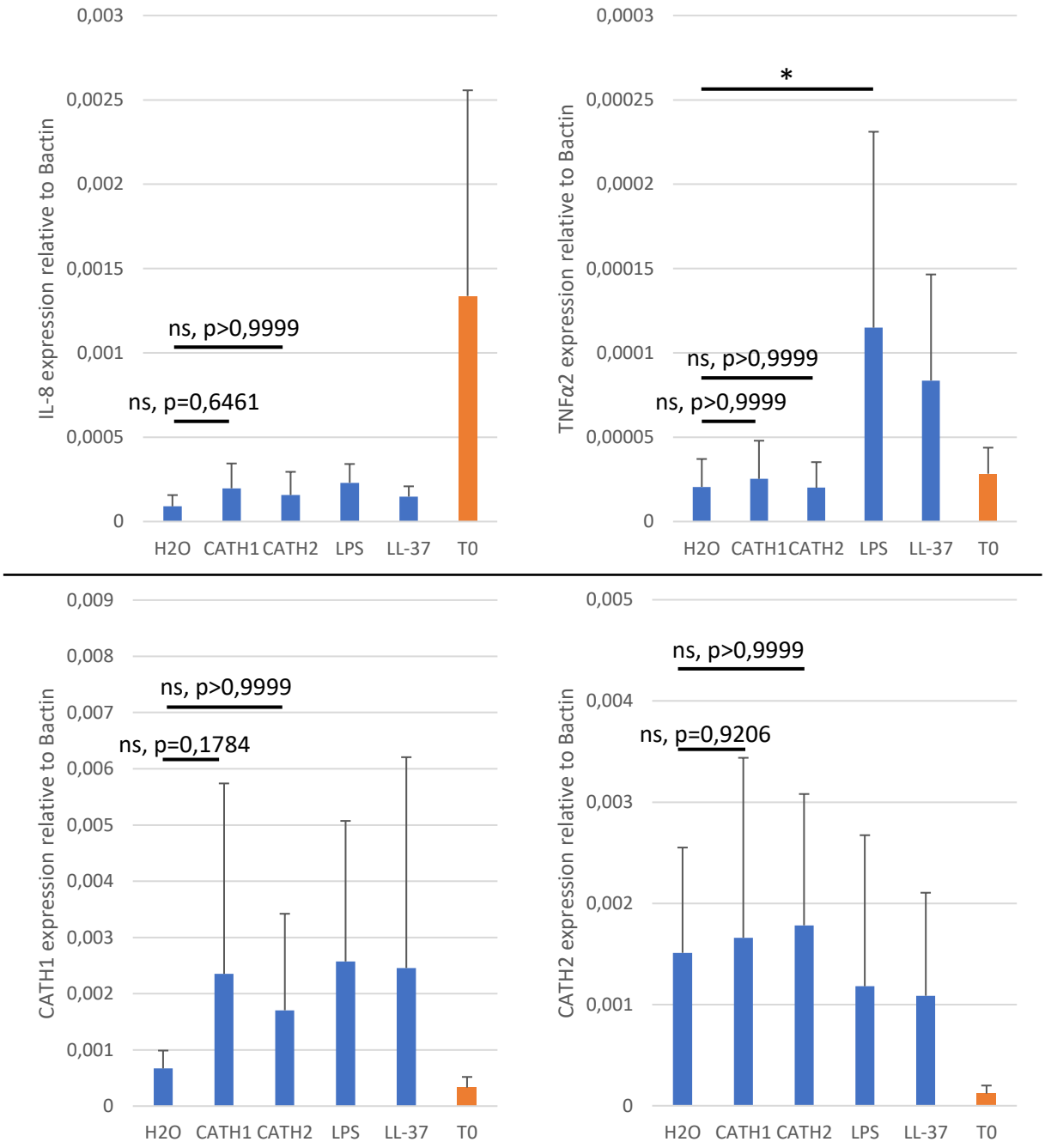


Figure 15. Gene expression and immunomodulatory activity caused by rainbow trout CATHs and human CATH LL-37 to peripheral blood WBCs of rainbow trout in vitro relative to rtBactin.

We took the -deltaCt and calculated the absolute change in target gene expression relative to the housekeeping gene rtBactin *expression using the formula* $2^{-deltaCt}$. *Each bar presents the mean of all measurements (n=5 or n=6) from the same group and timepoint. The error bars represent standard deviation. As we tested the changes of expression of our genes after treatment (CATHs and LPS) or mock treatment (H2O) to determine if the changes are scientifically significant, we show these results above the horizontal lines. ** = p < 0,01; * = p < 0,05; ns = not significant. As the main focus of our work was on CATH-1 and CATH-2, we showed the p-value for all of them, whereas we only showed whether there was a significant difference between the LPS or LL-37 treatments versus the mock treatment. Details of the statistical tests used are in the Materials and Methods.*

We also did Friedman tests on our data to determine the overall effects of the CATHs plus LPS and LL-37 on the expression of *rtIL-8, rtTNFα2, rtCATH-1* and *rtCATH-2*, as shown in the table below.

Table 5. Results of the Friedman test to determine the overall effects of stimulation.

*The term ns means the result is not significant, * = p < 0,05 and ** = p < 0,01*

Comparison to the unstimulated control (H2O) (*rtEF1α* as housekeeping gene)		Comparison to the unstimulated control (H2O) (*rtBactin* as housekeeping gene)	
rainbow trout gene	p-value	rainbow trout gene	p-value
rtIl-8	0,4060, ns	*rtIl-8*	0,5249, ns
rtTNFα2	0,0024, **	*rtTNFα2*	0,0087 **
rtCATH-1	0,0595, ns	*rtCATH-1*	0,1324, ns
rtCATH-2	0,2752, ns	*rtCATH-2*	0,4748, ns

The left side is the comparison relative to the house keeping gene *rtEF1α*, whereas the results are compared to *rtBactin* on the right side of the table. The results vary little between the two housekeeping genes. There are great differences between the tested genes with only *rtTNFα2* expression being significantly different overall and *rtCATH-1* being very close to reaching the threshold of p=0.05.

6. Discussion

The gene expression of the CATH-1 and CATH-2 correlates significantly with progressive kidney swelling grades during PKD in rainbow trout (4). Therefore, we set out to investigate the function and type of interaction of these AMPs by testing the bactericidal and lytic effects that the rainbow trout CATHs CATH-1 and CATH-2 have on bacterial species relevant to fish as well as the immunomodulatory activity on rainbow trout WBCs.

6.1. Improving CATH expression in HEK293T cells

We isolated the full-length sequences of the CATHs, amplified the AMP-region and successfully combined it with a human signal peptide to optimise expression in HEK293T cells. In the beginning, we directly created the human signal peptide and the 6xHis-tag together and added both to the N-terminus site of the AMP-region. As we hypothesized that this position of the 6xHis-tag could disturb the binding to the nickel beads via protein folding, we decided to move the 6xHis-tag to the C-terminus site of the peptide, as this was suggested before (9). Furthermore, each species has their own preferred codons which further increases protein expression. As we were using HEK293T cells for protein expression we decided to codon-optimise our products for human cells, so the protein translation is still the same, although a significant percentage of the bases was exchanged. This is possible because amino acids are encoded by nucleotide triplets. Additionally we succeeded in adding the Kozak-sequence, which is a consensus of the most common nucleic acid bases, immediately before the start codon in vertebrates and used the human signal peptide IFNα2 (8) instead of the original fish signal peptide. We further tried different mediums (DMEM with 10 % FBS, DMEM with 3,5 % FBS, DMEM Ex-Cell with 0 % FBS and a mixture with 50 % DMEM Ex-cell and 50 % DMEM with 0 % FBS) and also made an attempt to use the antibiotic Puromycin (at concentrations of 1 µg/mL and 3 µg/mL) instead of blasticidin (at a concentration of 25 µg/mL) after modifying the plasmid (data not shown). Despite these diverse optimizations, none of our attempts to express the peptide in HEK293T cells was successful.

We could not detect any peptide at the theoretical molecular weight of the CATHs at 8 kDa by Western blot, but we were getting a strong expression of the control protein, GFP. Even though we have equipped both the CATHs and the GFP with a human signal peptide, the satisfying results from the GFP are no proof that our signal peptide works, as the GFP can be partially

secreted with or without a signal peptide. To validate the function of the signal peptide we would have to compare the levels of expression of GFP with and without the signal peptide.

Although the GFP is not very pure, we will put more effort in purifying the GFP as we put in the CATHs, as it can be seen in Figure 11 in the results. But interestingly we saw a band at about 60 kDa for the CATH replicates. As we were able to reproduce this band several times, we suggested that it does not originate from an impurity of the sample but might consist of several CATH peptides which were binding each other, forming a polymer. Protein analysis by mass spectrometry could shine a light on this suspicion.

Another way of investigating the failed expression of the CATHs might be using bicistronic constructs, which is one gene that codes for two proteins. A reasonable structure in this case would be the CATH in the beginning, then the P2A site in the middle, and the GFP at the end. As the ribosome cannot start somewhere in the middle the presence of only GFP in the supernatant would indicate that the ribosome can successfully start translation at the CATHs, but that the peptides are rapidly degraded.

Although other experiments showed that short proteins like AMPs can be produced in *E. coli* (12, 9, 2), we avoided using *E. coli* to express our peptides, as we showed that the CATHs kill *E. coli* in our antibacterial trials (Figure 12) which could cause complications. We suggest that the 8 kDa protein we tried to produce is too small to be expressed in cells of higher developed organisms or it is degraded as too small and goes down the proteasome pathway (13). Another explanation might be the fact that the wild-type proteins in living organisms are translated from the full-length sequence and cleaved afterwards and the mature peptide is less stable. One solution for this problem might be the usage of a GST-tag instead of a 6xHis-tag which may help stabilize the recombinant peptide. Benefits of a GST-tag are that it can be bound easily and it is with a molecular weight of 27 kDa much larger than the 6xHis-tag and the MatP itself. However, such a large tag may disrupt both, the bactericidal and the immunomodulatory activities. Another downside of the His-tag is that it is positively charged like the AMP-region, which likely affects function. Other tags to fuse to the AMP-region could be the Strep-, FLAG- and myc-tag, but they are negatively charged and could affect function of the net charge of the AMP. But due to time constraints, we decided to commercially synthesize the desired proteins, instead of trying out the GST-tag.

6.2. Antibacterial activities of the CATHs

We investigated the influence of the commercially synthesized CATH-1 and CATH-2, as well as the human CATH LL-37, on various fish-infecting bacterial species. We verified that our CATHs show antibacterial activity against *A. salmonicida, Y. ruckeri*, *E. coli* and *A. sobria* at the earlier timepoint. In comparison to a previous paper (2) where different versions of the same CATHs were tested on the bacterial strains *E. ictaluri, V. fluvialis, S. dysgalactiae, A. hydrophila, E. coli* and *Y. ruckeri*, we only used the last two species from them as well as *A. sobria*, *A. salmonicida* and *V. arginolyticus* for our trials. So, this is the first time, that the antibacterial effect of the CATHs against *A. sobria* and *A. salmonicida* has been demonstrated. The goal was to select species that are obtainable at the fish department, result in infectious diseases and are from different bacterial lineages. We also chose the same experimental setup, except for the incubation temperature, which we changed from 20 °C to 22 °C, due to the species we are culturing (2) but almost all species grew and were not selective for temperature. However, that might have been the reason why we could not get any results for *V. arginolyticus*, as the literature indicates that the bacteria originates from a marine environment in totally different conditions, regarding salt concentration and temperature (14). Zhang also treated cells for 18 hours (2), whereas we tested various time points: we tested *A. salmonicida* and *Y. ruckeri* at 18 and 24 hours, although for *A. sobria, V. arginolyticus* and *E. coli* we took measurements at 18, 28 and 34 hours, as some species grew slowly, perhaps because of the modified growing temperature, and to be able to compare different time points.

Interestingly, when comparing the different time points for *A. sobria, E. coli* and *A. salmonicida*, it was noticeable, that at the longer time points the bacteria showed less sensitivity to the CATHs or they began to divide again. Especially in *A. sobria* the CATHs seem to slow down bacterial growth at 18 hours, but we hypothesize that they are not all killed whereas at 28 and 34 hours it looks like the bacterial density is the same as the untreated cells. We suggest that we added too little of the CATHs, as it seemed that they ran out before every bacterium in the sample is exterminated or that the CATHs are bacteriostatic but not bactericidal. As the nutrients from the Mueller-Hinton broth are still there, these survivors can still grow to full capacity if enough time is given. Another explanation could be that there are few bacteria in the sample that show some sort of resistance to the CATHs. As the other competitors are killed off while the nutrients are still there, they can grow to 100 % again. Another likely interpretation is that the CATH MatP is too short and unstable (15).

Another interesting point is that some of the bacterial assays show negative values, especially

for *V. arginolyticus* at 18 hours. This is because we subtracted the optical density at 0 hours from the densities of the later timepoints at different concentrations. If the bacteria have not grown already, we measured near-zero values, like at the 18-hour timepoint for *V. arginolyticus*. If they have been completely inhibited in their growth by the CATHs, it is possible to measure slightly less optical density compared with 0-hour timepoint, due to small measurement errors and variation of the device. Possible solutions therefore could be to make replicate measurements several times and take the average value. To ensure that the bacteria are all evenly distributed in the well it would be possible to pipette before measuring, but this method brings a great risk of contamination.

6.3. Activity assays with fish cells

In addition to direct antimicrobial activity, CATHs of mammals also show immunomodulatory activity, such as *IL-8* stimulating activity (2). In our project we could confirm that, the CATHs might regulate *rtCATH1* and *rtIL-8* expression, but not *rtTNFα2* which matches what we know so far about PKD (4).

The human CATH LL-37 for example, which acted as a positive control during our bacterial trials, has great influence in human health: studies show that it not only kills a vast spectrum of microorganisms, it is also involved in various inflammatory activities like chemotaxis, epithelial cell activation, cytotoxicity to host cells and even epithelial wound repair (16). Apart from that, it was shown that the LL-37 can be useful in disease diagnostics (17) and even has the potential to work as an alternative to conventional antibiotics through its antibacterial and immunomodulatory activities (18). Therefore, we expected that the trout CATHs also possess these activities, like their mammalian orthologs. Moreover, the literature already confirmed *IL-8* stimulating activities of rainbow trout CATHs in blood leukocytes (2). That's why we expected our CATHs to have immunomodulatory influence and we started testing changes in expression of PKD-related markers *rtIL-8, rtTNFα2*, *rtCATH1* and *rtCATH2* after 20 or 24 hours of exposure on rainbow trout cell lines as well as rainbow trout blood leukocytes (data not shown). Surprisingly, no significant changes were measurable at these timepoints, so we decided to measure expression at the timepoints 0, 2, 4, 6 and 16 hours (data only shown for T0 and T4). Research has shown that the strongest upregulation of *rtIL-8*, which was upregulated about five times more than the control, occurred only 4 hours after exposure (2).

When comparing the results from different genes we noticed that at T0 for *IL-8*, the protein expression was much higher than after treatment. We suggested that that happened because activity in a living organism was higher than in our own experimental setup with cells in medium.

Overall, there were only significant differences measurable for LPS and LL-37 treatments, not the rainbow trout CATH stimulations we were focusing on. A way to improve that in the future might be sampling more fish, because with more replicates, we can confirm whether weaker effects happened by chance or not.

As we were seeing a slight effect of stimulation with CATH-1 which seemed to be stronger than with CATH-2 and water throughout the experiment, there are a few possible ways to confirm activity of CATH-1 or CATH-2: one approach is to increase the CATH concentration used in culture, as we had the CATHs commercially synthesized and have limited amounts, we were using them at the lowest possible concentration although the concentrations we used are in range of those reported in the literature. Unfortunately, we have to estimate the optimal concentration, as we do not know the concentration of the CATHs *in vivo*, due to the lack of tools that bind them, assays to measure them, and there is nothing investigated yet in the literature. Another way would be to revisit producing them ourselves, and fuse the CATHs to a different protein or tag or to even fuse CATH-1 and CATH-2 to each other. We can test their additive or synergistic effects because both are co-expressed in PKD. A polymer could also be made from CATH1 and CATH2 to enhance the activity such as making biotinylated versions that can be tetramerized by streptavidin. As PKD is a kidney disease, it would also be more relevant to harvest and test leucocytes from the kidney instead of the blood. We may also have had a heterogeneous blood sample with all types of WBCs. Maybe there is only one subpopulation of leucocytes reacting to our peptides, which we couldn't see in this assay. To look at things from a broader angle we could also expose blood or head kidney WBCs from trout with or without PKD to the CATHs and look at general changes in immune expression by high throughput sequencing and differential gene expression analyses.

Furthermore, like the LL-37 in humans, the CATHs have the potential to be useful to diagnose trout diseases. With the full-length CATHs that we amplified, there is the possibility to create an aptamer, which is a short single strain DNA or RNA oligonucleotide, that can bind a specific molecule over their structure. With an aptamer that is specifically binding the CATHs as they get upregulated during a *T. bryosalmonae* infection, there is a faster way of diagnosing PKD and other disease, other than microscopy and PCR. There is also the possibility to study them.

Another possible way of continuing this project in the future is testing more genes that may be related with PKD. Beyond the CATHs, another indicator in fish for the presence of *T. bryosalmonae* are immunoglobulins, which are divided into three different types in fish: IgM, IgD and IgT (5). Literature shows that all three immunoglobulins are upregulated during an PKD infection. However, only IgT, which is specialised in mucosal immunity correlated strongly with the presence of the parasite and with the grades of kidney swelling, whereas the correlation for IgM was weakly positive and for IgD slightly negative (4). Furthermore, studies show that IgT was the most important Ig coating extrasporogonic parasite stages and IgT+ B cells where the prior B cell subset that increased in the kidney with progressive kidney swelling grade (5). They also deserve to be studied for their role in the disease.

Taken together, the trout CATHs possess, like their mammalian orthologs, bactericidal and immunomodulatory activity. We were able to demonstrate the microbicidal activity of the CATHs on new bacterial species like *A. salmonicida* and *A. sobria* as well as suggest that the CATHs might regulate *rtCATH1* and *rtIL-8* expression, but not *rtTNFα2*. Furthermore, we have a much better idea now what we can improve when we go to expressing the CATH-peptides via host cells again. This offers new insights and possibilities to the treatment of fish diseases. As resistance to antibiotics is a common problem in the conventional extermination of bacteria, the microbicidal activity of the CATHs could be an alternative as this is a completely new approach to preventing bacterial growth. However, resistance can also develop here (19), but resistance is much harder to develop to the immunomodulatory activity of the CATHs: since it is difficult for microorganisms to adapt to activation of the (adaptive) immune system that is over 500 million years old, this might be the best use of the CATHs in treating diseases.

7. List of Abbreviations

AMP……antimicrobial peptide
APS ……ammonium persulfate
CATHs……cathelicidins
DMEM……..Dulbecco's Modified Eagle Medium
FBS……. foetal bovine serum
GFP…….green flourescent protein
HRP……. horse radish peroxidase
MatP……. mature peptide
MSC……..multiple cloning site
PBS …….. phosphate-buffered saline
PCR…….. polymerase chain reaction
PKD……proliferative kidney disease
qPCR……quantitative polymerase chain reaction
RBCs……. red blood cells
RT-PCR…….. reverse transcriptase polymerase chain reaction
SDS……sodium dodecyl sulphate
TAE…….Tris-acetate-EDTA
TBS……. Tris-buffered saline
TEMED…… tetramethylethylendiamin
WBCs…….white blood cells

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