Project Report

AMPifin—a sustainable solution to vibriosis in fish

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Description

The fisheries and aquaculture sector—also known as the pisciculture sector—has gained attention for its critical contribution to maintaining global food security and nutrition. The animal protein and nutrients in aquatic food provide a dependable source of food and sustenance for many people, especially vulnerable coastal populations. The use of overly exploitative techniques has increased dramatically along with the rise in seafood consumption worldwide, resulting in serious diseases in pisciculture systems. Of these, bacterial diseases can cause severe symptoms in humans, such as but not limited to stomach discomfort, vomiting, and diarrhoea. They can be spread by ingesting raw or undercooked seafood that is contaminated.

The species of the genus *Vibrio* are among the most common bacteria. The infection caused by this species significantly hinders the aquaculture sector's ability to expand sustainably on a worldwide scale. When fish are raised for food, *Vibrio* species frequently cause high rates of mortality in the early stages of larval development. Regardless of the host's developmental stage, Vibrio infections can strike suddenly and completely eradicate populations of fish maintained in a certain aquaculture system. Vibriosis in marine animals has been connected to many *Vibrionaceae* species. According to a recent study, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio owensii*, and *Vibrio campbelli* are the species most often responsible for vibriosis in aquaculture farms. These species' pathogenicity is enhanced by various virulence factors that enable them to infect a variety of hosts [1].

V.parahaemolyticus is the bacteria we are interested in using to treat this illness in fish. It is an estuarine and marine environment-specific gram-negative halophile. Multivalent Adhesion Molecule 7 (MAM7)—an adhesion factor found in *V. parahaemolyticus*, is a surface protein that the bacteria produce. It is in charge of the initial host-pathogen adhesion and can cause the upregulation of extra adhesins and virulence factors exclusive to the pathogen and the host cell. With the ligand—phosphatidic acid (PA), and the protein—fibronectin (Fn), MAM7 forms a trimeric complex. It was judged appropriate for our project's study since it has been well-investigated in *V. parahaemolyticus* [2].

When this protein is removed from *V. parahaemolyticus*, host cell cytotoxicity often decreases and develops more slowly [2]. The host cell, in this example a fish, is made up of phosphatidic acid (PA) and fibronectin (Fn), which are both found in the extracellular matrix and function as its interacting ligands. MAM7 on the surface of the bacterium *V. parahaemolyticus* interacts with the ligands when it is near the host cell. It creates a tripartite complex comprising MAM7, PA, and Fn rather than being mutually exclusive to bind to PA and Fn [2]. The main stage of infection—bacterial adhesion—sets off the production of the biofilm, which then causes cytotoxicity and hemolysis. MAM7 plays a part in

host cell signalling pathways that ultimately break the epithelial cell barrier in addition to its involvement in adhesion [3].

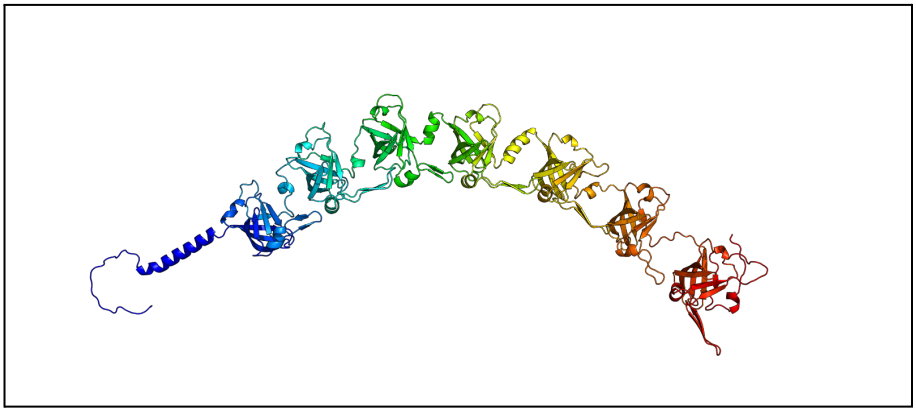

Figure 1. An image of MAM7 (as visualised on PyMol) [4].

Current solutions

Although there are several treatments for this infection, antibiotics continue to be the most widely used method of curing and preventing fish disease. Although there are several treatments for this infection at the moment, antibiotics continue to be the most widely used method of curing and preventing fish disease. In real life, antibiotics are routinely used in conjunction with fish meals or baths to treat bacterial diseases. Multiple harmful bacteria, including *Vibrio spp.*, have developed multidrug resistance as a result of antibiotic misuse [5]. Millions of fish are immunised annually to fight the issue of antibiotic resistance. Although vaccinations are a stronger preventative measure than antibiotics, they have certain disadvantages. Commercial vaccinations are expensive and labour-intensive.

Our solution

Our approach entails the development of an antimicrobial peptide that will bind to MAM7 and stop bacteria from sticking to cell surfaces, halting the proliferation of the disease. The fibronectin structure will be imitated to produce the antimicrobial peptide. The pET-22b(+) vector system and the BL21 as our chassis will be used to manufacture the peptide. Our peptide will be packaged in chitosan nanoparticles for delivery. Since chitosan nanoparticles have been shown to have immunoregulatory effects in fish, using them for administration will benefit AMPifin. AMPifin may also be a possible wide-spectrum treatment for bacteria that employ MAM7 as an adhesion factor.

***In-silico* design of the peptide**

There is a well-established interaction between MAM7, fibronectin, and phosphatidic acid.
MAM7 binds to fibronectin in the 30-kDa N-terminal domain, which is also used by several other Fn-binding proteins [2]. Recent advances in ab-initio protein structure prediction have made Alphafold one of the most reliable tools [6]. Each of the five repeats of trout fibronectin type I modules was evaluated as a single peptide and compared to the known literature based on

predictions made with AlphFold for the MAM7 and heparin-binding domains (HBD) of trout fibronectin [2], [6].

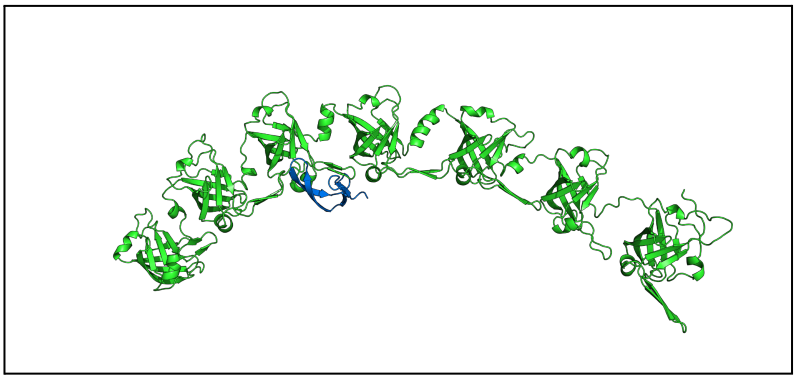

Figure 2. Peptide docked with MAM7 (as visualised on PyMol) [4].

Plasmid design

The use of *Escherichia coli* and the pET-22b(+) vector was intended for MAM7 expression and peptide synthesis. However, small peptides are unstable in vivo because they are susceptible to proteolysis when expressed in bacteria. Genes encoding peptides are fused to genes encoding carrier proteins. For this reason, the split-intein of the GyrB protein of *Synechocystis spp*. was chosen as the carrier protein.

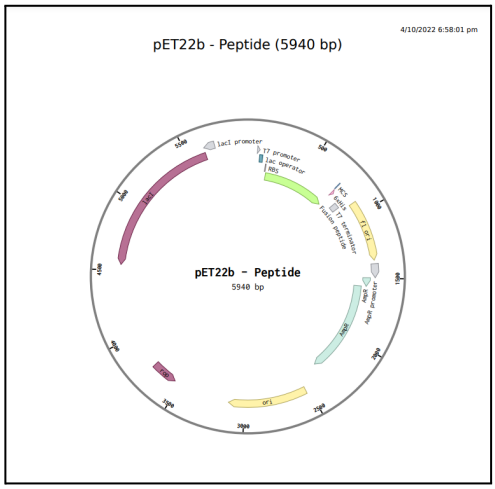

Figure 3. Peptide production vector system; retrieved from [7].

Intein

INTEINS, or interceding polypeptides, can be excised from precursor proteins post-translationally by a mechanism similar to mRNA splicing [8]. Following intein excision, its two flanking regions join to generate the functional protein or peptide sequence. They usually undergo conditional protein splicing, in which the splicing process is stimulated either by the addition of small molecules or by changing reaction conditions [8]. Almost any polypeptide backbone can be chemically modified with them [9]. We chose to include inteins in the purification of our peptide because they not only simplify the purification process simply because they remove themselves from the peptide but also because

they help to stabilise the peptide. To synthesise our peptide, we will use split inteins, which cleave the terminals at their respective termini with the N-terminal intein and C-terminal intein, respectively. Our gene circuit is set up so that the N terminal of our antimicrobial peptide will be self-cleaved post-translationally by the split intein, the 6X His-tag attached to it, and the Ssp Gyr B split intein we selected.

Figure 4. Split Intein [9].

Using scarless Gibson assembly, we intended to combine the components encoding the peptide and the intein into the vector pET-22b(+).

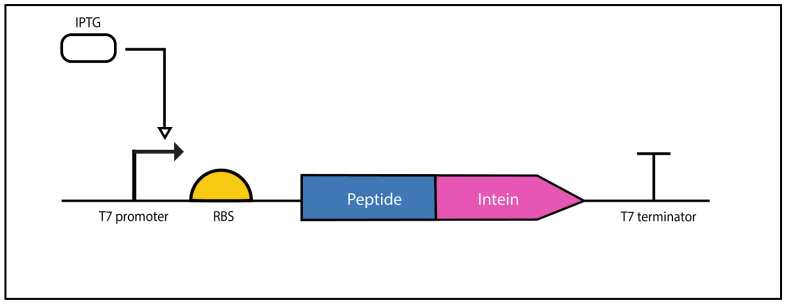

Figure 5. Plasmid gene circuit

Delivery System

As our antimicrobial peptide is prone to degradation, we have chosen to encapsulate it using chitosan nanoparticles, given its promising qualities. To determine the stability of a nanoparticle in a particular suspension, we would measure the Zeta potential of our in-vitro-generated nanoparticles. The last part of field testing involves loading a mesh with nanoparticles (carrying peptides) such that as the water flows, the peptide is subsequently released in the inlet tank. Here, our peptide would interact with the bacteria in the water by attaching to MAM7 on its surface, rendering it harmless. As a result, the aquaculture system's microbiome is also preserved. This mesh would be a one-time application; thus, it would need to be changed when the peptide was completely released from the nanoparticle.

Engineering

AMPifin consisted of multiple iterations of the design, build, test and learn cycle. The interaction of MAM7 and fibronectin requires five continuous repeats of the MCEs; thus, designing a peptide that would bind to only one of these was deemed sufficient [2]. Each of the previously designed peptides was individually docked with MAM7 using ClusPro [10], [11], [12], [13]. It was observed that repeats 3 and 5 docked to the required central domains, with repeat 5 having the highest docking score. This peptide was then chosen as the primary peptide and was subjected to two approaches of modifications. The first was a non-conserved, observational approach, where the interacting residues of the peptide were identified using Alanine scan [14] and then mutated to strengthen the

interactions. Mutation Cutoff Scanning Matrix (mCSM) [15] was used to filter the possible mutations to ones which could increase the binding affinity. The structures of the modified peptides were predicted, docked with MAM7 and again subjected to alanine scanning to account for conformational changes and verify the increase in binding affinity. Aggrescan [16] was used to identify hotspots of aggregation in the peptide, which was then mutated to prevent the interaction of the peptide with itself. The second approach involved using our software to identify residues within a low $\Delta\Delta G$ threshold from alanine scanning and perform random conserved substitutions. A multitude of peptide sequences was generated from all possible combinations using a cartesian product and was sampled randomly. The binding affinity values at each step were found using PRODIGY [17], [18].
The peptide with the highest affinity at each iteration was chosen for subsequent rounds of modifications. After multiple rounds of iterations, we arrived at a peptide that could potentially inhibit MAM7's interaction with the host.

Variant	Gibb's Energy, Delta G (kcal/mol)	Dissociation Constant, Kd
Wild Type (from 5th repeat)	-11.6	3.40E-09
Variant 1	-12.7	5.00E-10
Variant 5	-12.2	1.10E-09
Variant 9	-12.7	5.00E-10
Variant 12	-12.2	1.00E-09
Variant 1.9	-13	3.00E-10
Variant 1.12	-13.4	1.60E-10
Variant 1.13	-13.2	2.20E-10
Variant 1.16	-12.9	3.60E-10
Variant 1.12.2.1	-13	2.90E-10
Variant 1.12.2.4	-14.7	1.60E-11
Variant 1.12.2.14	-13.7	9.60E-11

Table 1. Best peptide variants from each iteration and their Gibb's Free energy and Dissociation constant values

Experiments

We obtained synthetically generated MAM7 sequences for our experiments to avoid the risk of working with pathogenic bacteria. The protein MAM7 is anchored to the membrane by its N-terminus, where the first 44 amino acids of MAM7 are predicted to form a transmembrane helix. Previous experiments have shown that these amino acids are not required for the protein's interaction with its cognate ligand fibronectin. Therefore the synthetic sequence was truncated to exclude the sequence encoding the first 44 amino acids and ligated into the pET-22b(+) vector. The recombinant plasmid was cloned into the DH5α strain and sent for sequencing for confirmation. After the sequence was confirmed, we transformed the purified recombinant plasmid into BL21 strain for expression. Protein expression will be promoted by using autoinduction protocol by growing the transformed bacteria in TB autoinduction media and confirmed using SDS PAGE. Protein will be purified using Ni-NTA column chromatography. The intein-peptide construct, assembled using Gibson Assembly, will also be expressed using the protocols mentioned above. The fusion peptide will be purified by using Ni-NTA column chromatography. During column chromatography, after all the unnecessary proteins have been washed away, the column will be treated with Dithiothreitol(DTT) Buffer to promote lysis of the fusion peptide, releasing the peptide free from the

Intein. The peptide sample will then be subjected to dialysis to remove traces of salt and other contaminants such as DTT. The purified samples of our peptide would then be used to conduct interaction studies, specifically Isothermal Titration Calorimetry. The experiment will allow us to determine the strength of the interaction between our proposed peptide and the purified MAM7 protein. The final dosage can then be calculated using our model on the basis of the interaction energies.

Implementation

Our preferred delivery method is chitosan nanoparticle encapsulation. Chitosan has received attention in the fields of medicine administration and food processing, and it has even been reported to have immunomodulatory effects [19]. Due to their increased stability, low toxicity, biocompatibility, and biodegradability [20], as well as their sub-micron size and easy manufacturing processes [21], chitosan nanoparticles have attracted attention in the field of drug delivery.

Our proposed implementation is such that we would be immobilising our peptide-loaded chitosan nanoparticles onto a Polyvinyl chloride (PVC) mesh. A stack of these loaded nanoparticles would be subjected to the inlet bypass of a Recirculatory Aquaculture System (RAS). Our peptide would be bound in the nanoparticle via hydrophilic and hydrophobic interactions. As the first flush of water enters from the reservoir into the subsequent tank, the peptide would diffuse out of the nanoparticle and bind to our MAM7-bearing pathogen present in the water. This mesh stack is placed in the bypass with the motive that whenever the stack needs replacement, the bypass can be switched off, and maintenance can be carried out efficiently.

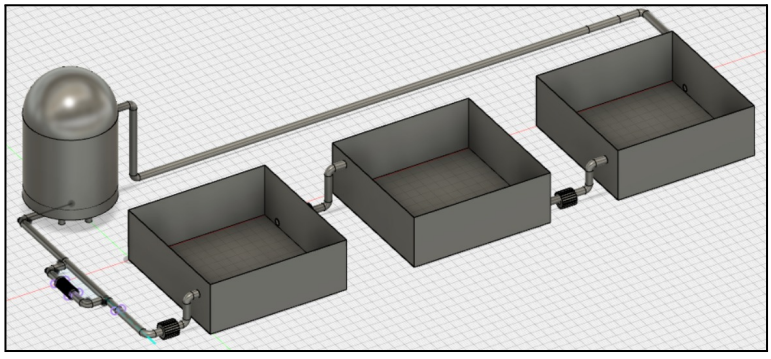

Figure 6. CAD design of delivery set-up incorporated in a RAS model.

Model

The relationship between the concentration of peptide required for a given concentration of fbronectin present in the system is calculated since both the peptide and fibronectin are in a position to interact with MAM7 indicating that our peptide would be acting as a competitive inhibitor against fibronectin. IC50 is a measure of the potency of a drug that is defined as the concentration of the drug or displacing ligand in a competitive inhibition mechanism that reduces or inhibits the receptor-ligand interaction by 50%. The reaction mechanism can be modelled as a parallel reaction scheme wherein MAM7 gets partitioned between our peptide and fibronectin.

$$\mathrm{M} + \mathrm{F} \rightleftharpoons \mathrm{MF}$$
$$\mathrm{M} + \mathrm{P} \rightleftharpoons \mathrm{MP}$$

Association and Dissociation constant are given by,

$$K_F = \frac{[\mathrm{FM}]}{[\mathrm{F}][\mathrm{M}]} = \frac{1}{K_f}$$
$$K_P = \frac{[\mathrm{PM}]}{[\mathrm{P}][\mathrm{M}]} = \frac{1}{K_p}$$

The total concentration of MAM7 in a system [M_t], the MAM7 would be partitioned between the MAM7-fibronectin complex, MAM7-peptide complex and free MAM7.

$$[\mathrm{M}_t] = [\mathrm{M}] + [\mathrm{MF}] + [\mathrm{MP}]$$

$$\rho = \frac{[\mathrm{MF}]}{[\mathrm{M}_t]} = \frac{K_F[\mathrm{F}][\mathrm{M}]}{[\mathrm{M}] + K_F[\mathrm{F}][\mathrm{M}] + K_P[\mathrm{P}][\mathrm{M}]} = \frac{K_F[\mathrm{F}]}{1 + K_F[\mathrm{F}] + K_P[\mathrm{P}]}$$

$$\implies \rho = \frac{\dfrac{[\mathrm{F}]}{K_f}}{1 + \dfrac{[\mathrm{F}]}{K_f} + \dfrac{[\mathrm{P}]}{K_p}}$$

By defining ρ_{in} as the interaction ratio in the absence of our peptide, we get,

$$\rho_{in} = \frac{\dfrac{[\mathrm{F}]}{K_f}}{1 + \dfrac{[\mathrm{F}]}{K_f}}$$

By the definition of IC50, we get,

$$0.5 \times \rho_{in} = \frac{\dfrac{[\mathrm{F}]}{K_f}}{1 + \dfrac{[\mathrm{F}]}{K_f} + \dfrac{[\mathrm{IC}_{50}]}{K_p}}$$

$$\implies [\mathrm{IC}_{50}] = K_p\left(\frac{[\mathrm{F}]}{K_f} + 1\right)$$

From predictions from PRODIGY, we got,

$$K_f = 1.5\,e^{-15},$$
$$K_p = 1.6\,e^{-11}$$

Software

Generating Random Alanine Scanned Peptides (GRASP)

GRASP is a peptide mutator that performs conserved substitutions based on inputs being employed from an Alanine scan. Our software is a one-of-a-kind approach to automating and exponentially simplifying the process of random conserved mutations. This process can be employed to potentially increase the affinity of the peptide to its interacting ligands. Existing software in the domain only considers surface-level information, ignoring any conformational changes that may occur. It creates combinations of conserved substitutions on selected amino acids that fall within the specified range. Furthermore, a few random sequences are selected from the sample set created. These are then tested and subjected to further analysis, and examined per the user's requirement.

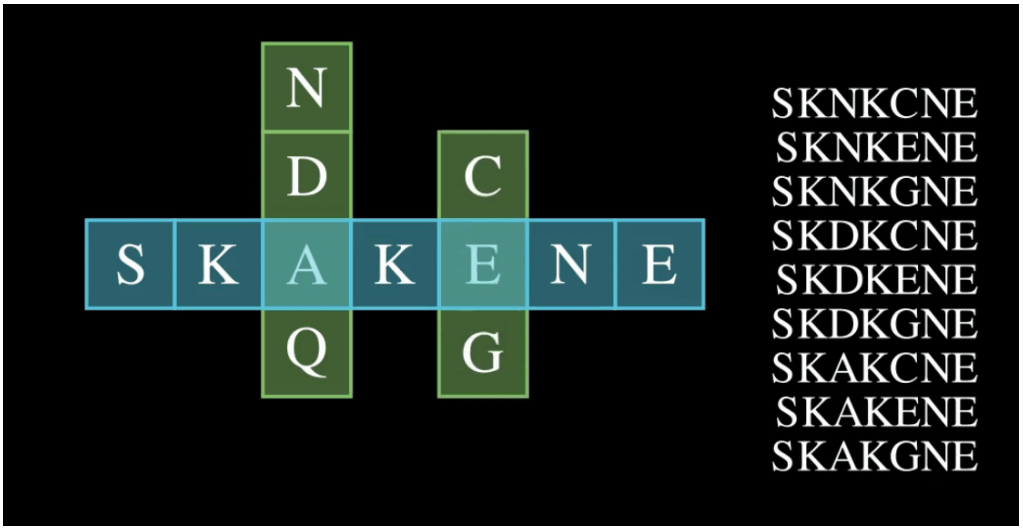

Figure 7. Mutations present in the peptide sequence of a sample sequence. The list of all output sequences is to the right.

The software comprises the following modules:

a. **Alanine Scan ΔΔG values:** BUDE Alanine scan is employed to obtain ΔΔG values of each residue present in the input sequence. Higher ΔΔG values indicate that the mutated peptide is a more stable one. Alanine is being utilised since it does not affect the secondary structure due to its chemical inertness and non-bulky nature.

b. **Conservative mutations:** A conserved mutation is a process in which mutations are made within the same group of residues. This ensures that the mutations result in stable peptides.
c. **Random sampler:** A pseudo-random generator is used to select the samples to reduce and narrow down the sample space to a set of few testable peptides. Thus, it enables to minimise the computational workload.

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