Studies with *Vibrio alginolyticus* OmpK towards the development of a vaccine for *Solea senegalensis*

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Abstract

Aquaculture is a growing industry due to the reduction of fish stocks in oceans. A major problem associated with high density fish cultures are infections such as *Vibriosis*, affecting the productivity of aquaculture farms and forcing the use of antibiotics that can later contaminate the fish product.

The present study aims at the exploitation of the possibility of using *Vibrio alginolyticus* OmpK protein as putative immunogenic protein to be used as a vaccine or vaccine component to protect aquaculture fish from *Vibrio* infections.

A bioinformatics analysis was initially performed to investigate the OmpK amino acid sequence conservation among *Vibrio* species and the occurrence of putative immunogenic segments within the proteins. The results obtained show that OmpK is a conserved protein potentially immunogenic.

The *Vibrio alginolyticus* gene encoding OmpK was cloned in pET23a+ and the construction was used to transform *E. coli* BL21. The protein was overproduced in *E. coli* and was only purified by affinity chromatography using recombinant OmpK denatured with urea 8 M, suggesting that the His-tag was hidden when the protein is overexpressed in *E. coli*.

Batches of purified protein are being produced envisaging their use in immunization experiments to raise an antibody to be used in future work.

Keyword's: aquaculture, fish vaccine, recombinant proteins, OmpK, protein purification

Introduction

As defined by NOAA (National Oceanic and Atmospheric Administration), aquaculture refers to the breeding and harvesting of plants and animals in all types of water environments, including ponds, rivers, lakes, and the ocean.

Although aquaculture is apparently highly beneficial with no drawbacks, the aquaculture industry poses several risks regarding occupational hazards and might cause several deleterious effects to the environment and to humans. These risks can be categorized into physical work hazards, chemical and toxic exposure, risks associated with infectious diseases[1], and environmental degradation. Aquaculture also presents benefits, by reducing the dependence of natural stocks, allowing the conservation of endangered species. Aquaculture can indirectly benefit the environment by providing methods to convert agriculture wastes in high-quality fish protein[2].

Many bacterial diseases affect fishes in the aquaculture environment, with the risk of transmission to consumers. Bacteria of the *Vibrio* genus represent a major group in fish pathogens. *Vibrio* members are gram-negative bacteria, mesophilic and chemoorganotrophic, presenting a facultative fermentative metabolism[3]. In Portugal, V*ibrio* spp. affects the sole *Solea senegalensis* (Kaup)[4] cultures. Main external signs of the bacterial disease are skin ulcers and haemorrhagic areas near the fins and mouth[5]. The economic losses caused by bacteria indicate the urging of the development of prophylactic strategies, such as vaccination, to prevent disease[6]. Outbreaks results in a reduced production of fish in aquaculture. Infection control represents additional costs associated with labour and antibiotic therapy[7]. Due to costs and biological hazards of antibiotic therapy aquaculture industry has given priority to

vaccination as a mean to control endemic diseases[8]. Several vaccination methods of fish can be used in aquaculture: injection, immersion, and oral delivery. Some parameters have to be considered when choosing the best method, like protective immunity after vaccination, vaccine delivery, labour requirements, and risk of side-effects[7].

With the aim of producing a vaccine to prevent *Vibrio* infections of sole, an immunogenic molecule needs to be identified. The outer membrane of gram-negative pathogenic bacteria plays an important role in this interaction with the hosts, namely in adherence to surfaces, uptake of nutrients from the host and subverting host defence mechanisms[9]. Examples of potentially immunogenic outer membrane proteins are OmpK and OmpW. OmpK is a receptor for the broad-host range *Vibrio* phage KVP40, and is widely distributed among species of the *Vibrionaceae* family[10,11].

Proteins are synthesized by all living forms as part of their natural metabolism. Some proteins play a significant role in immune responses and are commercially produced in biotechnological industries with the aid of genetic engineering and protein engineering. The production of recombinant proteins is an important sector in biopharmaceutical and agriculture industry[12].

Escherichia coli has been the pioneering host for recombinant protein production since the recombinant DNA procedures were developed. Due to the accumulated knowledge on the organism and the large array of tools available, *E. coli* is the preferred host when trying to produce a new protein[13]. Although *E. coli* offers several advantages, is not an ideal protein producer as it is difficult to find the optimal conditions for protein production and downstream processing. Different problems like plasmid loss and antibiotic-based maintenance, lack of post-translational modifications, protein aggregation and endotoxin contamination are among the main obstacles for protein production in *E. coli*[13]. An adequate recombinant protein production system requires the host's efficient expression of the gene of interest, and the use of a molecular vehicle to insert the gene of interest. In *E. coli* a carrier plasmid is the most used method. Plasmids are extrachromosomal self-replicating cytoplasmic DNA elements that can be found both in eukaryotes and prokaryotes. Plasmids are used as molecular vehicles for recombinant genes and are the most popular choice when using prokaryotes as hosts, due to the easiness of genetic manipulation. Cells harbouring recombinant plasmids often exhibit a lower growth rate than plasmid-free cells. If in a culture there is a high number of plasmid-free cells they will overtake the culture and lead to a loss of the plasmid. The two main reasons for plasmid lost are an unequal plasmid distribution upon cell division, also called plasmid segregational instability[14] and plasmid multimerization[15,16]. Several mechanisms can be used to ensure plasmid maintenance in cell populations. One of the most used strategies is to provide plasmid-bearing cells with a competitive advantage over plasmid-free cells by means of conferring a competitive advantage to plasmid bearing cells[17]. The selective pressure most commonly used is antibiotic supplementation in the culture medium[18], with a resistance gene introduced into the plasmid thus providing resistance to the recombinant cells.

In addition to plasmid segregational and/or structural instability, post-translational processing can also contribute to the production of non-functional recombinant proteins. Post-translational processing of a protein comprises several steps like folding[19], aggregation[20,18], solubility of the protein[21,22], proteolytic processing, glycosylation[23] and other modifications that are required for the correct conformation and function of the recombinant protein. The present study aims at the production and purification of recombinant OmpK proteins from *E. coli* to produce a vaccine that allow aquacultured fish immunization against Vibrio infections.

Materials and Methods

The genomic DNA was extracted from overnight cultures of *V. alginolyticus* ATCC 17749 using the High Pure PCR Template kit from Roche. The concentration of genomic DNA solutions was estimated by measuring the

absorbance at 260 nm in a ND-1000 spectrophotometer (nanodrop) from alfagene. The oligonucleotides primers were designed based of the gene encoding for OmpK sequence of *Vibrio alginolyticus* ATCC 17749 and were synthetized by Eurofins MWG Operon (Germany).

Genomic DNA from *Vibrio alginolyticus* ATCC 17749 was used as template for PCR amplification of OmpK with an expected size of 811 bp. PCR amplification was performed in a 2720 thermal cycler (Applied Biosystems) and the samples were analysed in agarose gels using agarose (Lonza) at 1% (w/v) in buffer Tris-Acetate-EDTA (TAE) 1X. The correct PCR product was purified from agarose using the NZYGelpure kit from NZYtech (Portugal), and following the manufacturer's instructions. The DNA fragment and the plasmid pET23a+ were then restricted with enzymes BamHI (NZYtech) and XhoI (Thermo) and were ligated directionally into the expression vector pET23a+. The plasmid obtained was named pJBF2 and was used for the overproduction of OmpK in *E. coli*.

In order to transform *E. coli* BL21 cells, a cryovial of electrocompetent frozen bacterial cells were transformed by electroporation as described in Maniatis et al[24]. The transformed *E. coli* was used to overexpress recombinant OmpK.

E. coli BL21 containing the recombinant pJBF2 plasmid used for the production of the OmpK protein was inoculated in 30 ml of LB liquid medium and incubated for approximately three hours with orbital agitation (250 rev./min) at 37ºC until an optical density of at least 1.0 (640 nm) was reached. An aliquot of the culture was used to inoculate 100 ml of Super Broth (SB) medium with 150 µg/ml of ampicillin with an optical density of 0.1. The inoculated SB medium was incubated at 30ºC with orbital agitation at 250 rev./min until an optical density between 0.60 and 1.0 was reached (approximately two hours). The cells were induced with 0.4mM of IPTG at an OD_{640nm} of 0.6 and grown at 30 ºC. In the ideal point of overexpression, the culture was centrifuged for 5 minutes, at 7000xg and 4ºC (J2-21, Beckman). The cells were then resuspended in 8 ml of buffer containing 500 mM NaCl and 20 mM Tris-HCl and stored at -80 ºC.

To provoke cell lysis, aliquots from cell suspension prepared from cultures carried out for 16 hours were slowly defrosted on ice for approximately 2 hours. In order to disrupt the cell wall, the aliquots were sonicated with a Branson sonifier 250 (Branson) using eight cycles of 30 seconds, 50% of duty cycle, output of 60 with two minutes break between each cycle. After sonication, aliquots were centrifuged, for 60 minutes at 12 000 rpm and 4ºC (J2-21, Beckman). The supernatants were transferred to a clean Falcon tube and kept in ice until further processing.

The purification of OmpK in its native state was carried out by affinity chromatography using a HisTrap FF column (GE Healthcare) with a volume of 1 ml. The protocol was performed following the manufacturer's instructions[25]. The column was equilibrated with washing buffer (10 mM imidazole, 500mM NaCl and 20mM Tris-HCl pH 7.5). After equilibrating the column, the protein sample was applied to the column. The column was then washed with washing buffer, to wash proteins weakly bound to the column. Afterwards successive buffers with the same composition as the wash buffer and increasing concentrations of imidazole (60, 100, 200, 300 and 500 mM) were used to elute the proteins that were bounded to the nickel in the column and collected in 1 ml fractions. The column was regenerated and was stored in 4ºC until further use.

The recombinant His_6-tagged OmpK protein was denatured using urea as the denaturation agent. After sonication, the cell suspension were denaturated using urea as the denaturation agent, centrifuged (30 min at 12500xg and 4ºC) and resuspended with 10 ml of buffer (10 mM imidazole, 0.5 M NaCl, 20 ml Tris-HCl pH 7.5 and 1% Triton X-100), and centrifuged again (30 min at 12500xg and 4ºC), and resuspended in 10 ml of buffer (10 mM imidazole, 0.5 M NaCl, 20 ml Tris-HCl pH 7.5 and 8 M urea) to denature the proteins. The suspensions were stirred at room temperature for 30 minutes. A solution of 1M of DTT was added to meet 2.5 mM final concentration and stirring was maintained for 45 minutes. The suspension was then centrifuged for 30 minutes at 12500xg and 15ºC, resulting in

a supernatant containing the denatured protein. In order to be used in the His-tag FF columns (GE Healthcare), the concentration of urea (Sigma) was decreased from 8M to 6M by adding 3.3 ml of buffer 5 due to the proximity to the maximum concentration of urea allowed for the column[25].

The purification of denatured OmpK in inclusion bodies on HisTrap FF column (GE Healthcare) was performed as described in the purification of OmpK in the native state, except for: the imidazole concentrations used were 50, 300 and 500 mM; 10% glycerol was added to all the buffers; buffer containing 50 mM of imidazole had 6 M of urea and to decrease the concentration of urea aliquots of 2 ml of dialysis buffer (0.5 M NaCl, 10mM Tris-HCl pH 7.5 and 10% glycerol) with decreasing concentration of urea, 0.25 M decrease between each buffer addition from 6M to 1M. The volumes collected from the column eluates at 300 mM of imidazole were dialyzed using a Slide-A-Lyzer dialysis cassette (Pierce) and placed in dialysis buffer overnight at 4ºC with gentle agitation. After dialysis and to conclude the renaturation process the sample was added to a Hist-tag column previously equilibrated. The fractions of protein that do not bind to the column were collected for further analysis.

The denaturation of OmpK proteins from inclusion bodies for the batch-based purification was as described in the column-based except for the suspension being left overnight at room temperature in buffer (0.5 M NaCl, 10mM Tris-HCl pH 7.5, 10% glycerol and 6 M urea) and for the decrease of urea concentration between the aliquots being 0.5 M decrease instead of a 0.25 M. For the renaturation process 2 ml of the denaturated protein sample was added to an amount of 8ml of slurry containing 4 ml of resin of Ni sephase6FF (GE Healthcare) and was used accordingly to the manufacturer's instructions[26]. The volumes collected from column eluates obtained with 300 mM of imidazole were dialyzed as described for the column-based protocol.

Gels for SDS-PAGE analysis were prepared as described by Maniatis *et al*.[24], using 15% acrylamide.

Western-blot analysis of His6-tagged proteins was performed as described in BioRad general protocols[27].

To determine the concentration of protein in solution, the Bradford method was performed following the instructions described in Bradford MM.[28].

A bioinformatics analysis of the *Vibrio alginolyticus* OmpK protein was performed using Protein Basic Local Alignment Tool (BLASTP) from the NCBI to align the protein sequence of *Vibrio alginolyticus* with all the OmpK from Vibrio species know to infect fish. To analyse regions with high a probability of being immunogenic, the commercial softwares used were: NetSurfP (Center for biological sequence analysis); Pred-TMBB (Biophysics and Bioinformatic Laboratory); BepiPred (Center for biological sequence analysis); and Antigenic (EMBOSS). In order to verify if the antigenic regions predicted by the above bioinformatics tools are conserved among all the Vibrio strains known to infect fish, an alignment was made using Clustal Omega (EMBL-EBI). The information generated by Clustal Omega was then used in a program for multiple sequence alignment editing, visualisation and analysis (Jalview) to analyse the conservative regions and compare with the immunogenic regions predicted.

Results and Discussion

Previous work has identified *Vibrio* strains associated with fish infection. Outer membrane proteins (Omps) are recognized as important for bacterial protection against harsh environmental conditions[29]. Among Omps, OmpK was chosen as a model protein to develop vaccines against *Vibrio* infections. In order to analyse the prospective efficacy of the protein chosen as a vaccine candidate, a BLASTP analysis of OmpK protein sequences of these species was performed. The results obtained for *ompK* gene, show that the protein has a high level of identity and similarity among all the strains of the *Vibrio* species know to infect fish, with values ranging from 65% to 100% and 89% to 100%, respectively, with the exception of *Vibrio salmonicida* (Table 1).

Table 1 - Results of the BLASTP analysis for the alignment of OmpK sequences with *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio salmonicida*, *Vibrio anguillarum*, *Vibrio ordalli*, *Vibrio proteolyticus* and *Vibrio campbelli* strains. The (-) means a lack of correspondence in the alignment

Results from Blasts for OmpK protein							
Species	strain	Identity	Similarity	Species	strain	Identity	Similarity
Vibrio alginolyticus	ATCC 17749	99	100	*Vibrio anguillarum*	O1	86	91
	NBRC 15630	99	100		O2	88	93
	12G01	99	99		775	86	91
	E0666	78	85		M3	86	91
	40B	99	100		RV22	88	93
	chemovar iophagus	-	-		96F	86	91
	subs. Iophagus	-	-	*Vibrio ordolli*	phage PV05	-	-
Vibrio parahaemolyticus	AQ3810	86	91		ATCC 33509	-	-
	V-223/04	86	91		LMG 13544	-	-
	RIMD 2210633	85	89		NCIMB 2167	-	-
	O1:K33	86	91		12B09	88	93
	O1:KUK	86	89		FF-93	88	93
	str. 16	65	76		FS-144	88	93
	VPCR-2010	86	91		FS-238	88	93
	EKP-028	85	89	*Vibrio Proteolyticus*	NBRC 13287	75	82
	EKP-021	85	89	*Vibrio campbelli*	ATCC BAA-1116	86	89
	VPTS-2010	86	91		ATCC 25920	85	89
	VP 2007-007	85	92		CAIM 519/NBRC 15631	85	89
	VPTS-2010-2	86	91		LMG 11216	85	89
	EKP-008	86	91		HY01	81	87
	IDH 02640	85	89		CAIM 333	-	-
	10296	86	91		LMG 21362	-	-
	861	85	89		M1	-	-
	3259	86	91		DS40M4	81	87
Vibrio Salmonicida	LFI 1238	-	-				

The results presented in Table 1 indicate that OmpK has a conserved sequence among the analysed *Vibrio* strains. An *in silico* analysis of the potential antigenic regions of each protein was performed using four softwares: NetSurfP, Pred-TMBB, BepiPred and Antigen. NetSurfP and Pred-TMBB predict regions with surface accessibility, while BepiPred and Antigen predict localization of potential epitopes. These results were analysed together with the results provided by Clustal Omega alignment (Figure 1), that analyse the conservation degree of the predicted amino acid segments among *Vibrio* species with available genome sequences. The Jalview software was used to plot the results of the Clustal Omega analysis. Jalview results showed overlap of regions predicted by softwares NetSurfP and Pred-TMBB and softwares BepiPred and Antigen. A higher confidence on the occurrence of immunogenic segments would result in the intersection between the programs that predict surface accessibility and programs that predict epitopes as in the amino acid 79 to 89 segment of the sequence in Figure 1.

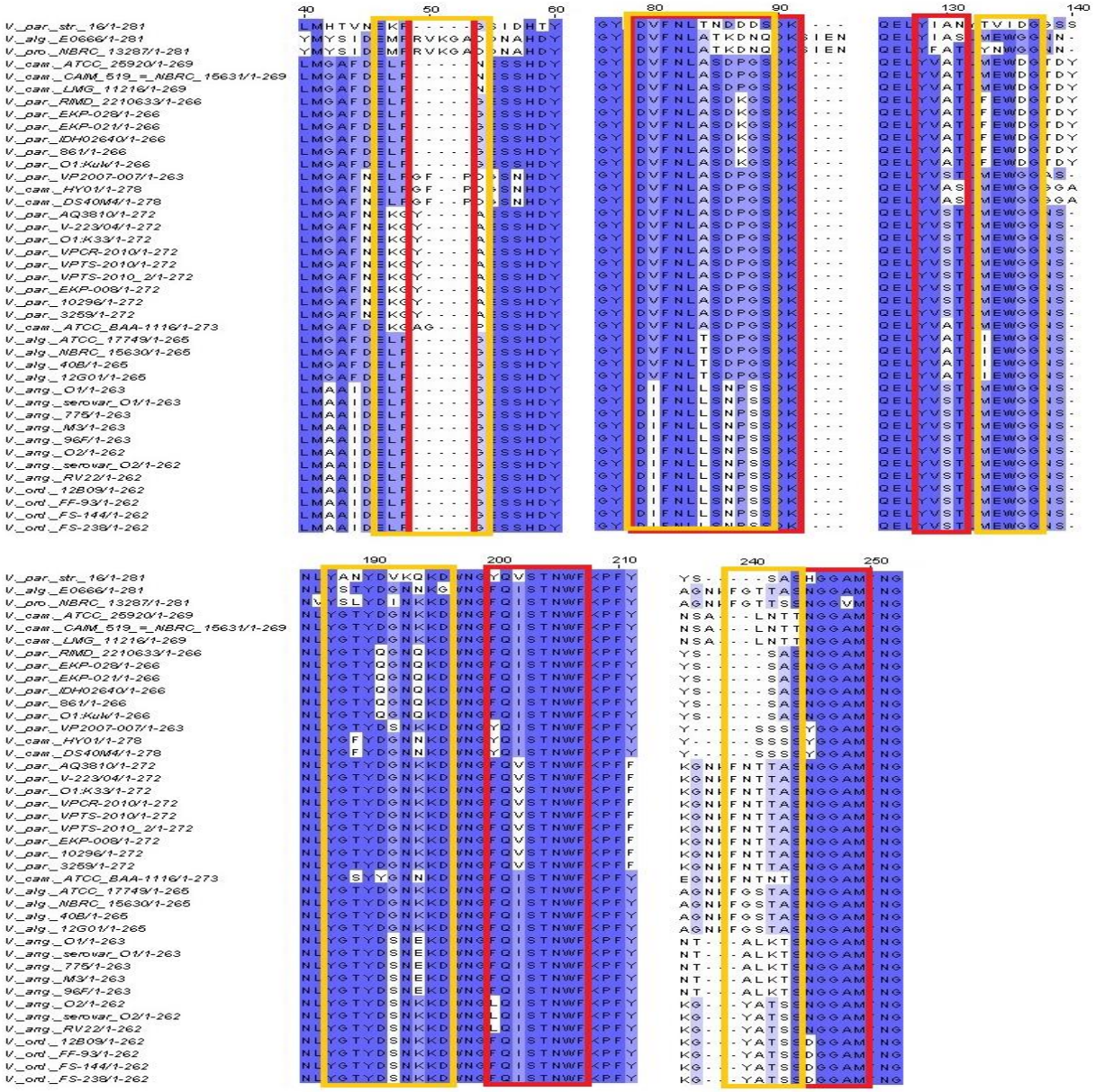

Figure 1 – Regions of interest from the results of the multiple alignment performed by Clustal Omega analysed with Jalview software. Regions predicted by NetSurfP and Pred-TMBB are marked in red and regions predicted by BepiPred and Antigenic are marked in yellow.

Our bioinformatics analyses suggest that OmpK contains immunogenic segments and therefore, the protein might be used as a potential vaccine. In order to produce this *Vibrio* protein in *E. coli* a strategy was designed including the amplification of the gene by PCR, and its cloning in a controlled expression vector that would lead to the production of a protein with a his-tag at its C-terminus to facilitate the purification.

Therefore, cultures of *Vibrio alginolyticus* ATCC 17749 were performed, cells were harvested by centrifugation, and total DNA was extracted. The extracted DNA was then used as a template in reaction mixtures to amplify by PCR the genes encoding for OmpK, with adequate primer pairs.

The analysis of the amplified gene encoding OmpK agarose by gel electrophoresis indicates that the amplified DNA fragments had the expected size. The band was then extracted from the agarose gel in order to be cloned in the pET23a+ expression vector.

Plasmid pET23a+ extracted from *E. col,i* as well as the DNA fragment corresponding to *ompK* amplified by PCR,

were digested with the restriction enzymes XhoI and BamHI. After recovering the digested plasmid and DNA fragments, ligation mixtures were prepared to ligate each fragment to the linearized plasmid pET23a+. The resulting ligation containing the recombinant plasmid with the gene of interest was used to transform *E. coli* BL21.

After obtaining *E. coli* BL21 colonies with the recombinant plasmid bearing *ompK* experiments were carried out to find the best conditions for the overexpression of OmpK. The optimized conditions found were 16 hour incubation with SB medium at 30ºC.

Fails in attempting to purify non-denaturated protein samples (Figure 2) and negative western results (Figure 3) prompted us to hypothesize that the histidine tail of OmpK was hidden in the protein native structure and therefore not allowing its binding to the nickel ions in the column.

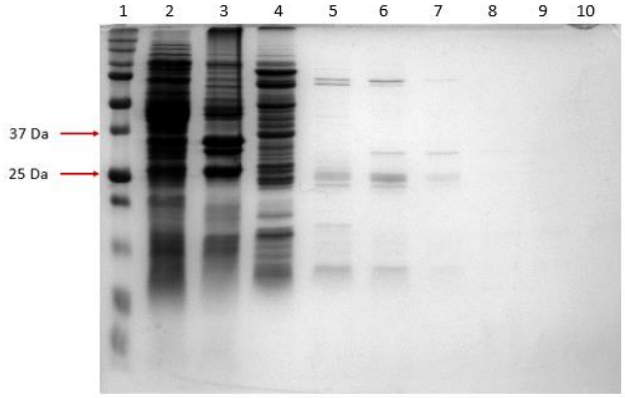

Figure 2 - SDS-PAGE gel showing profiles of chromatographic fractions from steps performed at different imidazole concentrations and using 10% glycerol in the buffers on a His-tag FF column. Lanes: 1 – molecular mass marker, 2 – sample of the culture after 16 hours of cultivation, 3 – sample of the pellet collected after the first centrifugation, 4, 5, 6, 7, 8, 9, 10 – elution fraction containing 40, 50, 70, 85, 100, 200, 500 mM of imidazole.

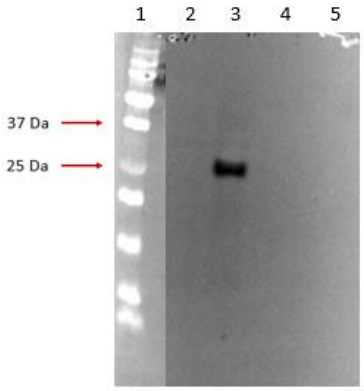

Figure 3 – western blot image of periplasmatic proteins with an antibody anti-His. Lanes: 1 – molecular mass marker, 2 – sample of the culture after inoculation, 3 – sample of the culture after 16 hours of cultivation, 4 – eluate obtained with buffer containing 40 mM of imidazole, 5 - eluate obtained with buffer containing 100 mM of imidazole.

To test the hypothesis that the histidine tail was hidden, the protein recovered as inclusion bodies was denatured prior to purification. SDS-PAGE gel electrophoresis and western-blot analysis showed that we were able to purify OmpK. These results support the conclusion that the histidine tail of OmpK is hidden when the protein is overexpressed by *E. coli*. Although the protein denaturation allows the purification of OmpK without any impurities, the protein is required in the native state when envisaging its future use. To increase the amount of protein purified and renatured two methods were tested: renaturation in column (Figure 4 and 5) and in batch (Figure 6 and 7).

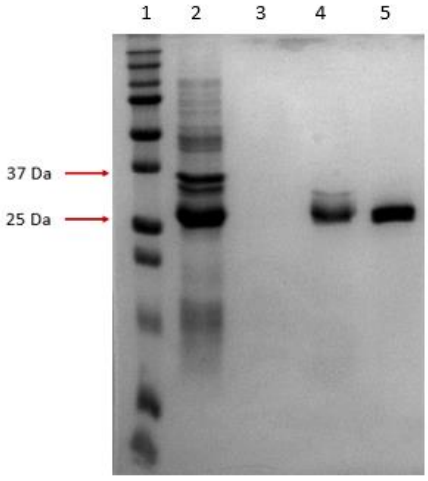

Figure 4 – SDS-PAGE gel showing profiles of chromatographic fractions from steps performed at different imidazole concentrations of denatured OmpK purification samples using the column-based protocol pre-dialysis. Lanes: 1 – molecular mass marker, 2 – sample of the protein solution before passed in the column, 3, 4 – wash fraction with 10, 50 mM of imidazole, 5 – eluate fraction with 300 mM of imidazole.

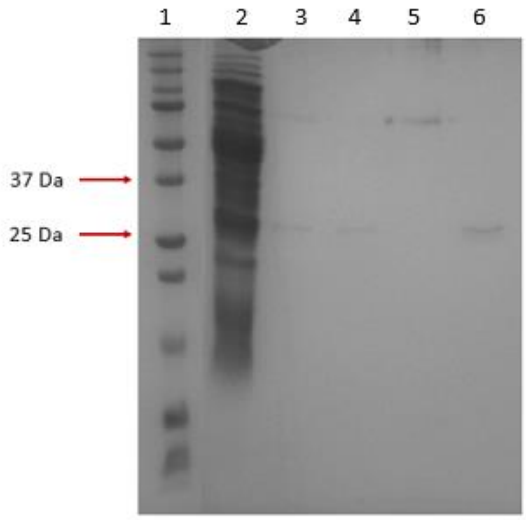

Figure 5 – SDS-PAGE gel showing profiles of chromatographic fractions from steps performed at different imidazole concentrations of denatured OmpK purification samples obtained with the column-based protocol post-dialysis. Lanes: 1 – marker, 2 - sample of the culture after 16 hours of cultivation, 3 – sample of lane 2 post-dialysis, 4 – sample of the post-dialysis protein solution before passed in the column, 5 – eluate obtained with buffer containing 10 mM of imidazole, 6 - eluate obtained with buffer containing 500 mM of imidazole.

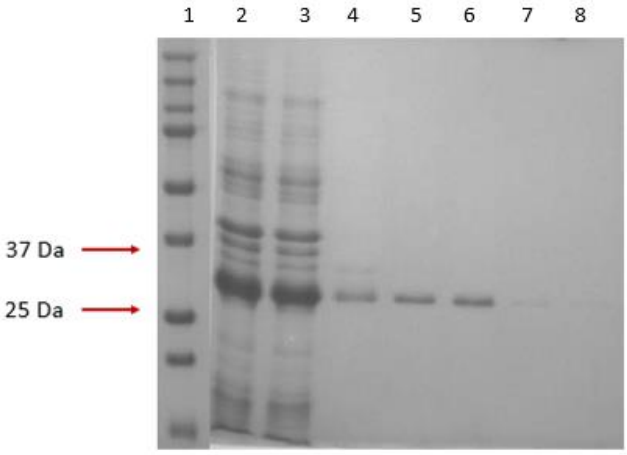

Figure 6 – SDS-PAGE gel showing profiles of chromatographic fractions from steps performed at different imidazole concentrations of denatured OmpK purification samples obtained with the batch-based protocol pre-dialysis. Lanes: 1 – molecular mass marker, 2 – sample of the protein solution before passed in the column, 3, 4 – wash fraction with 10, 50 mM of imidazole, 5, 6 – eluate fraction 300 mM of imidazole; 7, 8 -. eluate fraction 300 mM of imidazole.

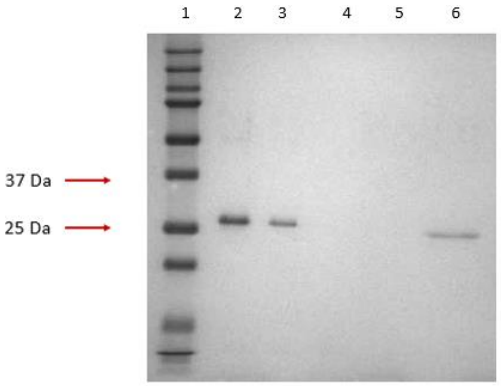

Figure 7 - SDS-PAGE gel showing profiles of chromatographic fractions from steps performed at different imidazole concentrations of denatured OmpK purification samples obtained with batch-based protocol post-dialysis. Lanes: 1 - molecular mass marker, 2 - sample of the protein solution before passed in the column and post-dialysis, 3 - sample of the protein solution after passed in the column and post-dialysis, 4 - empty, 5 - eluate obtained with buffer containing 10 mM of imidazole, 6 - eluate obtained with buffer containing 500 mM of imidazole.

The column-based and batch-based protocols for the IMAC affinity chromatography are similar, but the final concentration of OmpK obtained in the batch protocol was higher. The difference might be explained by the higher time of renaturation, increased incubation time with the different buffers and lower final concentration of urea in the buffer before dialysis which might have favoured the OmpK yield.

The test used to quantify the protein present in the final solution was the Bradford test. This test is an analytical procedure to measure the concentration of protein in a solution. The results of quantification of OmpK using the two protocols are shown in Table 2.

Table 2 - maximum A_{595nm} and concentration values obtained for the column based and batch based protocols

Column protocol		Batch protocol	
A_{595nm}	Concentration (µg/ml)	A_{595nm}	Concentration (µg/ml)
0.052	92	0.026	54

Analysis of the renaturated protein was made by spectrophotometry in the UV in collaboration with a colleague (Mariana Jerónimo) to confirm the renaturation of the purified protein. With the purpose of evaluating the heat-induced unfolding of the renatured protein, the sample (and controls) were subjected to progressive heating steps and

immediately its absorbance at 280 nm was evaluated. Samples of renatured protein collected after passing the dialysis suspension in an His-tag FF columns, were compared with two samples of denatured proteins (controls). One of the controls was the eluate obtained with buffer containing 50 mM of imidazole in the column-based protocol and the second control was the protein that remained on the column after adding the post-dialysis protein sample and that eluted with 500 mM of imidazole. The samples were heated to different temperatures 4, 30, 40, 50, 60, 70, 80, 90 and 100ºC. The absorbances of the sample and controls were measured at 280 nm and plotted against the incubation temperature. The results show that the normalized absorbance of the denaturated OmpK protein samples remained the same with a variation between 0 and 2. Conversely, the renatured OmpK sample showed an increase in the absorbance ratio for temperatures higher than 60ºC. The increase of the absorbance indicates that hydrophobic residues of the protein that were previously hidden inside the protein conformation became exposed. This result supports the conclusion that recombinant OmpK has been renaturated.

Conclusions and Future Perspectives

The results obtained in our bioinformatics analysis indicate that the *V. alginolyticus* OmpK is a protein highly conserved among all the *Vibrio* species known to infect fish. Therefore, we have cloned the gene in the pET23a+ and transformed *E. coli* BL21, in order to overproduce the protein envisaging its future use in vaccine experiments. Results obtained indicate that recombinant *E. coli* BL21 harboured the constructed plasmid. However, several unexpected difficulties were faced when purifying the protein that was mainly aggregated in inclusion bodies. Once solubilized and denatured most of the protein eluted with impurities with low concentrations of imidazole. We therefore hypothesized that the his-tag was occluded in the protein structure, and thus not accessible to bind to the nickel ions in the resin. Two different methods were used in the purification of OmpK and although more tests are required to optimize the batch purification, higher concentrations of pure OmpK were obtained. The concentration of OmpK obtained by the batch purification is 92 µg/ml in 16 ml from a 600 ml culture in SB medium at a final OD640nm of 8.93. Pure OmpK (3 mg) is being prepared to send to a commercial company (SicGen) to produce antibodies that will allow future localization of possible immunogenic segments. To allow an easier recovery of the protein of interest, a leaky strain of *E. coli* BL21 with the *ompK* gene is being constructed by cloning an antisense of the *lpp* gene encoding for the Braum's lipoprotein in a controlled expression plasmid. The recombinant plasmid will then be inserted into *E. coli*, leading the expression of the *lpp* antisense to weaken the bacterial cell wall. In order to be efficiently secreted by a leaky *E. coli* prepared as described above, recombinant OmpK should be expressed in conditions that do not promote its aggregation and/or the formation of inclusion bodies, since translocation of the protein to the periplasmatic space involves a signal peptide included in the recombinant OmpK, and the protein has to be in a soluble form. If we succeed in the construction of a leaky *E. coli* able to secrete OmpK, integration of protein expression and excretion with in-situ product recovery is recommended. This can be achieved by coupling batch fermentation with chromatographic protein capture.

The immunogenicity of the OmpK protein produced will be assessed in soles from aquaculture to test its efficacy as a protective vaccine.

References

1. Cole, D. W. *et al.* Aquaculture: Environmental, toxicological, and health issues. *Int. Jl Hyg. Env. Heal.* **212,** 369–377 (2009).
2. Muir, J. F., Brugere, C., Young, J. A. & Stewart, J. A. The solution to pollution? The value and limitations of environmental economics in guiding aquaculture development. *Aquac. Econ. Manag.* **3,** 43–57 (1999).
3. Thompson, F. L. *et al.* Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Appl. Environ. Microbiol.* **71,** 5107–15 (2005).

4. Dinis, M. T., Ribeiro, L., Soares, F. & Sarasquete, C. A review on the cultivation potential of Solea senegalensis in Spain and in Portugal. *Aquaculture* **176,** 27–38 (1999).

5. Zorrilla, I., Balebona, M. C., Mori.igo, M. A., Sarasquete, C. & Borrego, J. J. Isolation and characterization of the causative agent of pasteurellosis, Photobacterium damsela ssp. piscicida, from sole, Solea senegalensis (Kaup). *J. Fish Dis.* **22,** 167–172 (1999).

6. Arijo, S. *et al.* Effectiveness of a divalent vaccine for sole, Solea senegalensis (Kaup), against Vibrio harveyi and Photobacterium damselae subsp. piscicida. *J. Fish Dis.* **28,** 33–38 (2005).

7. Press, C. M. & Lillehaug, A. Vaccination in European salmonid aquaculture: A review of practices and prospects. *Br. Vet. J.* **151,** 45–69 (1995).

8. HORNE, M. T. & ROBERTSON, D. A. Economics of vaccination against enteric redmouth disease of salmonids. *Aquac. Res.* **18,** 131–137 (1987).

9. Ningqiu, L. *et al.* An outer membrane protein, OmpK, is an effective vaccine candidate for Vibrio harveyi in Orange-spotted grouper (Epinephelus coioides). *Fish Shellfish Immunol.* **25,** 829–833 (2008).

10. Inoue, T., Matsuzaki, S. & Tanaka, S. A 26-kDa outer membrane protein, OmpK, common to *Vibrio* species is the receptor for a broad-host-range vibriophage, KVP40. *FEMS Microbiol. Lett.* **125,** (1995).

11. Inoue, T., Matsuzaki, S. & Tanaka, S. Cloning and sequence analysis of Vibrio parahaemolyticus ompK gene encoding a 26-kDa outer membrane protein, OmpK, that serves as receptor for a broad-host-range vibriophage, KVP40. *FEMS Microbiol. Lett.* **134,** (1995).

12. Demain, A. L. & Vaishnav, P. Production of recombinant proteins by microbes and higher organisms. *Biotechnol. Adv.* **27,** 297–306 (2009).

13. Ferrer-Miralles, N. & Villaverde, A. Bacterial cell factories for recombinant protein production; expanding the catalogue. *Microb. Cell Fact.* **12,** 113 (2013).

14. Paulsson, J. & Ehrenberg, M. Noise in a minimal regulatory network: plasmid copy number control. *Q. Rev. Biophys.* **34,** 1–59 (2001).

15. Summers, D. Timing, self-control and a sense of direction are the secrets of multicopy plasmid stability. *Mol. Microbiol.* **29,** 1137–1145 (1998).

16. Corchero, J. L. & Villaverde, A. Plasmid maintenance in *Escherichia coli* recombinant cultures is dramatically, steadily, and specifically influenced by features of the encoded proteins. *Biotechnol. Bioeng.* **58,** 625–632 (1998).

17. Lewin, B. *Genes VI. 1997*. **29,** (Oxford University Press, 1994).

18. Baneyx, F. Recombinant protein expression in*Escherichia coli*. *Current Opinion in Biotechnology* **10,** 411–421 (1999).

19. Ailor, E. & Betenbaugh, M. J. Modifying secretion and post-translational processing in insect cells. *Curr. Opin. Biotechnol.* **10,** 142–145 (1999).

20. Carrió, M. M. & Villaverde, A. Construction and deconstruction of bacterial inclusion bodies. in *Journal of Biotechnology* **96,** 3–12 (2002).

21. Swartz, J. R. Advances in Escherichia coli production of therapeutic proteins. *Current Opinion in Biotechnology* **12,** 195–201 (2001).

22. Clark, E. D. Protein refolding for industrial processes. *Curr. Opin. Biotechnol.* **12,** 202–207 (2001).

23. Lisowska, E. The role of glycosylation in protein antigenic properties. *Cellular and Molecular Life Sciences* **59,** 445–455 (2002).

24. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning :a Laboratory Manual*. (USA:Cold Spring Harbor Laboratory Press, 1989, 1989).

25. GE Healthcare. *Instructions 11-0008-88 AC*.

26. GE Healthcare. *Instructions 11-0008-88 AH*.

27. Western Blot Protocol | Bio-Rad.

28. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–54 (1976).

29. Koebnik, R., Locher, K. P. & Van Gelder, P. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol. Microbiol.* **37,** 239–53 (2000).