OmpK antigenic peptide from *Vibrio alginolyticus* in recombinant *Escherichia coli* - production and secretion challenges

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Abstract

Lpp is the Braun's lipoprotein is a structural outer membrane protein from *E. coli* BL21 (DE3) to which structure defects can cause loss of integrity of the outer membrane and an easier protein leakage. In this work, an *E. coli* BL21 (DE3) strain with production of a *lpp* antisense sequence was constructed (pSAS39) and its effect on cells' permeability was evaluated. Additionally, overexpression of a OmpK 21-99 fragment of OmpK from *Vibrio alginolyticus* was optimized and the newly developed strain was used as a host to infer on the impact on peptide localization. Lpp mRNA levels showed a decrease in expression after 2 hours of growth in cells containing the pSAS39, although 1.5 h later expression levels return to the normality, indicating the antisense effect is lost. OmpK 21-99 fragment overexpression was successfully verified at 30 ºC, although neither periplasmic localization nor secretion to the extracellular medium were confirmed, related with possible difficulties in secretion or technical problems with the detection. The results obtained appear to be very promising, however further studies need to be carried out to confirm the antisense impact on cells' behaviour, as well as improving the secretion of the peptide to the periplasm to improve leakage.

Introduction

Aquaculture involves the culture of aquatic animals and plants in fresh, brackish and marine environments, being an important source of food nowadays (1). However, certain *Vibrio* pathogens are able to proliferate and cause disease in the cultured products. A recombinant protein vaccine containing the membrane protein from *Vibrios*, OmpK, has shown effective immune responses that led to protection of the fish against pathogen infection (2).

Production of recombinant proteins in various organisms has revolutionized the field of biochemistry, biopharmaceutical and agricultural industry, with some of the host systems most used include bacteria, yeasts and mammalian cells (3). Bacterial expression systems are characterized by a rapid growth, ability to reach high cell densities resorting to cheap substrates, in which *Escherichia coli* are the host organism most commonly used for this purpose (4). One important aspect of recombinant protein production is related with the secretion of the target proteins to the periplasm or to the extracellular medium. When a signal peptide is fused to the N-terminal end, the cells secretion pathways recognize the signal and are able to transport the protein to the periplasm, no longer needing cell lysis and preventing contamination of the protein with endotoxins and cytoplasmic proteins, for instance (5). Proteins can reach the extracellular medium via the secretion mechanisms of gram-negative bacteria or through periplasmic leakage. Certain mutant strains have been reported to have alterations in the outer membrane (OM) structure components, resulting in an increase of the permeability of the membrane and an easier protein recovery (6,7). A very important structural protein from *E. coli* is the Braun's Lipoprotein (Lpp), responsible for the connection between the peptidoglycan of gram-negative bacteria and the outer membrane. Defects caused onto structural lipoproteins can be crucial in causing a loss of integrity of the OM, enhancing permeability of recombinant proteins to the extracellular medium (8). Antisense technology can be a useful weapon in inhibiting the expression of a certain protein. For instance, a short fragment of nucleic acids can be constructed to have a sequence complementary to the mRNA of a specific protein and when production of the antisense is induced, the molecule can act by

steric hindrance and prevent mRNA translation or the rapid degradation of the target RNA.

This work had the objective of creating an *E. coli* BL21 (DE3) strain with an improved capacity for recombinant protein secretion. For this, a plasmid containing an antisense sequence against Braun's lipoprotein Lpp was constructed and introduced into the cells. The effect of the antisense on cellular growth and susceptibility to stressors was studied, as well as the values of the lpp mRNA were determined, which allowed to infer on the effect the antisense was having in the structural protein. Consumption of arabinose by the expression cells was tested as well by HPLC analysis. Moreover, the overexpression of the OmpK 21-99 fragment from OmpK of *Vibrio alginolyticus* was optimized by testing different temperatures of expression and the bacterial strain expression the lpp antisense was used as a host for the production of the recombinant peptide.

Materials and Methods

E. coli BL21 (DE3) lpp antisense

Construction of the plasmid pSAS39

The pSAS39 plasmid containing the lpp antisense sequence was constructed as previously described (9). First, the antisense sequence was amplified by polymerase chain reaction (PCR). The oligonucleotide primers were designed based on the sequence from the Braun Lipoprotein (Lpp) of *E. coli* BL21 (DE3) (NCBI GenBank: CAQ32153.1) and were synthesized by STAB VIDA (Portugal). The reaction mix was composed of 5 ng DNA template (reverse complement from *E. coli* BL21 (DE3) Braun's Lipoprotein), 0.02 U/µL Phusion® High-Fidelity DNA polymerase enzyme (Thermofisher), 1x of Phusion HF buffer (Thermofisher), 200 µM of each Deoxynucleotide Triphosphates (dNTPs) and 1.0 µM of each primer, for a total reaction volume of 20 µL. The prepared samples went through an initial denaturation at 98 ºC for 3 minutes, followed by 30 cycles of denaturation (98 ºC for 10 seconds), annealing (61 ºC for 30 seconds) and elongation (72 ºC for 8 seconds) and a final elongation step for 7 minutes at 72 ºC. The amplification product was isolated using the NZY Gelpure kit (NZYTech) and after overnight digestion with the restriction enzymes EcoRI and HindIII (ThermoScientific) and purification, the restricted samples were mixed with the restricted pMLBAD plasmid using the T4 DNA ligase. The obtained construct was transformed into *E. coli* αDH5 cells and the plasmid extracted from the cells to confirm the success of the process by digestion with SalI (ThermoScientific) and validation by nucleotide sequencing from MWG Eurofins (Germany). The constructed pSAS39 plasmid allows controlled expression of the lpp antisense by an arabinose inducible BAD promoter, upon induction with L-(+)-Arabinose, having the *dhfr* gene conferring resistance to trimethoprim.

Susceptibility to salts

Three cell cultures, *E. coli* BL21 (DE3), *E. coli* BL21 (DE3) plus pMLBAD and *E. coli* BL21 (DE3) plus pSAS39 were grown each in LB liquid medium, supplemented with 100 µg/mL of trimethoprim when required, at 37 ºC and 250 rpm for 3 hours. The cells were harvested by centrifugation at 9000 rpm (VWR microstar 12) for 3 minutes and the resulting pellets were resuspended in sterile NaCl 0.9 % (w/v) to a final OD_{600nm} of 1.0. In a 96-wells microplate containing 200 µL of LB liquid medium, the susceptibility of the different cultures to a varying concentration of NaCl (10%, 9%, 8%, 7%, 3.5%, 1.75% and 0.875%) was tested. After overnight incubation, the OD_{600nm} was measured in a SPECTROstarNano (BMG LABTECH) microplate reader.

E. coli growth curves

Different cell cultures, *E. coli* BL21 (DE3), BL21 (DE3) with pMLBAD and BL21 (DE3) with pSAS39 were incubated in several conditions while OD_{600nm} was monitored in order to evaluate whether the induction of the lpp antisense has any effect on the cell growth. Pre inoculums of 20 mL in LB liquid medium were prepared, supplemented with 100 µg/mL of trimethoprim and left overnight at 37 ºC and 250 rpm. Afterwards, 50 mL LB were inoculated at a starting OD_{600nm} of 0.1, properly supplemented. Production of the lpp antisense was induced with 2 % arabinose (w/v) and the cells were grown in similar conditions as before, while OD_{600nm} was measured every 30 minutes in a Hitachi U-200 spectrophotometer. Moment of induction was varied between the different assays: induction at the T0h timepoint and T2h timepoint; induction at T3h;

induction at T4h; induction at T0h and T2h but an intermediate culture (in which cells were grown as explained before and production induced at T0, followed by harvesting of cells at 6000 xg for 10 minutes and inoculation of the final culture), was included between the overnight inoculum and the final culture.

RT-PCR for lpp mRNA levels

During monitorization of the cells' growth, media samples were taken, following the ratio of 2/OD_{600nm}. RNA was extracted from the samples and treated with DNAse I resorting to the RiboPure – Bacteria Kit (Ambion). The extracted RNA was quantified and contamination with DNA was evaluated. After confirming absence of DNA, first strand cDNA was synthesized following the protocol described in Maxima Scientific Reverse Transcriptase (Thermo Scientific). 16S rRNA transcription levels were also analysed to serve as the control. Reaction components were added to an amount of 1 ug of RNA sample and 20 pmol gene specific primer, with the employed primers being: lpp mRNA is Lw_Lpp-RT (5'-TTT AGC AGC CTG AAC GTC GGA A-3') and 16S rRNA is RT16S_1492_RV (5'-GGW TAC CTT GTT ACG ACT T -3'), synthesized by STABVIDA (Portugal).

Afterwards, a quantitative PCR (qPCR) experiment was conducted. The reaction mix contained 1x NZYSpeed qPCR Green Master Mix, ROX (NZYTech), 400 nM forward primer and 400 nM reverse primer and 2 µL cDNA template. The used primers for lpp transcript analysis were: reverse primer Lw_Lpp-RT (5'-TTT AGC AGC CTG AAC GTC GGA A-3'), forward primer Up_Lpp_RT (5'-TGC TCC AGC AAC GCT AAA ATC G-3'); for 16S transcript were: forward primer RT16S_1114F2 (5'-CGG CAA CGA GCG CAA CCC-3'), reverse primer RT16S_1275R2 (5'-CCA TTG TAG CAC GTG TGT AGC C-3'), from STAB VIDA (Portugal). Thermal Cycling RT-PCR was performed with one cycle of polymerase activation at 95 ºC for 2 minutes, followed by 40 cycles of denaturation at 95 ºC for 5 seconds and annealing/extension at 60-65 ºC for 15-30 seconds.

Lastly, the data analysis procedure for the qPCR results was carried out. The fluorescence signal obtained can be correlated with the original template amount and the cycle number at the threshold level is defined as Ct number. This number is used to determine ΔCt of the target sample by calculating the difference between the Ct value of the target gene and the Ct value of the control (Ct_{target} - $Ct_{control}$). Then, the $\Delta\Delta$Ct is calculated as the difference between the ΔCt_{sample} and the $\Delta Ct_{calibrator}$, which is represented by a sample to which all samples are compared to (pMLBAD culture sample at 2 h after induction). Finally, the relative quantification (RQ) is determined to each sample as $2^{-\Delta\Delta Ct}$, which will reflect the fold change compared to the calibrator.

Arabinose levels on HPLC

L-(+)-Arabinose levels in the extracellular medium were checked during the several *E. coli* BL21 (DE3) cells growth. 1 mL samples were harvested from the cell cultures, centrifuged at 10000 rpm (Sorvall legend 21R) for 5 minutes and a 1:2 dilution sample was prepared with H_2SO_4. The analysis was carried out in a high performance liquid chromatography (HPLC) method as described before (10), with a Rezex ROA Organic acid H + 8 % (30 mm x 7.8 mm) column, kept at 65 ºC. Elution followed an isocratic method using 5 mM H_2SO_4 and a flow rate of 0.5 mL/min and quantification was made using a refraction index (RI) detector. A calibration rate was constructed based on quantification of arabinose solutions with different concentrations.

Truncated form of OmpK 21-99 from *Vibrio alginolyticus* ATCC 17749

Construction of the plasmid pFCN1 for overproduction of the OmpK fragment with signal peptide

OmpK 21-99 encoded DNA fragment was amplified by PCR in a 2720 thermal cycler (Applied Biosystems) using primers designed based on the sequence of the gene encoding OmpK from *Vibrio alginolyticus* ATCC 17749 (GenBank: AGV17311.1) and are the following: reverse primer Lw-OmpK-P1-99 (5'-AAC TCG AGC ATA CGT GGA GC-3'), forward primer Up-OmpK-P21-99 5'-AAC CAT GGC AGAT TAC TCT G-3'). The PCR reaction mix was prepared as described for the construction of pSAS39 plasmid, with the difference the DNA amount added was of 4 ng pJBF2 plasmid (containing OmpK gene cloned into pET23a(+)) (11). The prepared samples went through an initial

denaturation at 98 ºC for 2 minutes, followed by 30 cycles of denaturation (98 ºC for 10 seconds), annealing (61 ºC for 30 seconds) and elongation (72 ºC for 10 seconds) and a final elongation step for 7 minutes at 72 ºC. The resulting bands from the produced ompK DNA fragment were extracted using the PCR clean-up gel extraction kit (Macherey-Nagel), followed by restriction with restriction enzymes Xho I and Nco I (Thermo Scientific), at 37ºC overnight of the amplified products and the cloning vector pET-22b(+), following manual instructions. The restricted samples went through ethanol precipitation of the DNA to concentrate and desalt. The restricted fragment and plasmid were ligated using T4 DNA ligase (Thermo Scientific), obtaining in the end the pFCN1 plasmid. The obtained construct was transformed into *E. coli* αDH5 cells, the plasmid extracted and validated by sequencing (Eurofins, Germany). pFCN1 allows expression of the truncated form 21-99 of OmpK by the T7 promoter upon induction with isopropyl β-D-thiogalactoside (IPTG), having the PelB signal sequence at N-terminal and the 6x His-tag at the C-terminus.

Optimization of the OmpK 21-99 fragment overexpression

Overexpression of OmpK 21-99 fragment in *E. coli* BL21 (DE3) cells

Overexpression of the OmpK fragment was carried out under different temperatures (37 ºC, 30 ºC, 25 ºC and 18 ºC), to see if by decreasing the temperature, it would reflect on peptide production. A 30 mL preinoculum in LB liquid medium containing the cells *E. coli* BL21 (DE3) with the pFCN1 plasmid was grown at 37 ºC and 250 rpm, supplemented with 150 µg/mL ampicillin, until an OD_{640nm} higher than 1. The preinoculum was used to inoculate 100 mL of SB liquid medium, properly supplemented, at an initial OD_{640nm} of 0.1, followed by incubation in the same conditions. Once the culture reached OD_{640nm} between 0.6 and 0.8, 0.4 mM of IPTG was added and OmpK peptide production was initiated. Depending on the condition being tested, the culture was incubated at 30 ºC or 37 ºC, with samples being taken at the moment of induction (T0) and every hour after induction (T1-T4); at 25 ºC with collection of samples at T0 until T3; and 18 ºC, with samples being taken at T0 and T16. Samples were treated and overexpression was evaluated by SDS-PAGE (in a 15 % polyacrilamide gel) and Western Blot.

Electrocompetent *E. coli* BL21 (DE3) cells were transformed with the plasmid pET-22b(+) and overexpression was carried out as before at 30ºC. The objective of this strategy was to understand whether there was some protein being highly expressed in the control (pET-22b(+)) with a similar molecular weight as the overexpressed peptide.

Overexpression at 30 ºC in electrocompetent *E. coli* BL21 (DE3) + pSAS39 cells

Electrocompetent *E. coli* BL21 (DE3) cells with the pSAS39 plasmid were transformed by electroporation with the pFCN1 plasmid containing the OmpK fragment 21-99. This strategy was meant to provide a better understanding on the effect the antisense was having on the permeabilization of the cell wall and see if it could be improving the exportation of the overexpressed peptide to the extracellular medium. The procedure was carried out as explained above, with addition of trimethoprim 100 µg/mL (selection of cells with the antisense). 3 hours after inducing overexpression, 2% L-(+)-arabinose was added to initiate the antisense production. Media samples were taken in the same timepoints as before.

Periplasmic Fraction Isolation by Osmotic Shock

As mentioned before, the OmpK fragment 21-99 contains a signal peptide sequence pelB in its N-terminal that targets the peptide to the periplasmic space after expression is completed. To see if this transport was successful, isolation of the periplasmic fraction was carried out as described before (12). Once overexpression was completed the culture was centrifuged and the supernatant collected.

Detection of produced proteins: Western Blot

The Western Blot procedure was carried out similarly as explained before (13). A nitrocellulose (NC) membrane (PALL Corporation) was used to carry out protein transfer and 1:2000 dilution of monoclonal anti-polyhistidine peroxidase conjugate antibody (SIGMA), was used as the primary antibody. At the end, detection was performed using the cheluminescence imaging system Fusion Solo (Vilber Lourmat).

Results and Discussion

E. coli BL21 (DE3) Lpp antisense

E. coli growth curves and arabinose quantification in the culture medium by HPLC

The growth curves were followed as explained above with samples being collected every 30 minutes until 6 hours of growth. The specific growth rates were determined for each culture and can be observed in table 1.

Table 1 – Specific growth rates (h^{-1}) for *E. coli* BL21 (DE3), with and without induction, *E. coli* BL21 (DE3) with pMLBAD and *E. coli* BL21 (DE3) with pSAS39 cell cultures, grown in LB liquid medium at 37 ºC and 250 rpm. (I) – Indicates that cells were induced with L-arabinose 2 % (w/v).

Strains	Specific growth rates (h^{-1})
***E. coli* BL21 (DE3)**	0.991
***E. coli* BL21 (DE3) (I)**	0.981
***E. coli* BL21 pMLBAD (I)**	0.897
***E. coli* BL21 pSAS39 (I)**	0.860

The cells corresponding to the wild type had a slightly higher growth when compared to the other cells. Cells with the pSAS39 plasmid with lpp antisense production presented slower growth, indicating that the presence of the plasmid pMLBAD or pSAS39 affects normal growth. However, the difference between both cultures is not significative to be associated with the lpp antisense. The control cell culture without induction allowed to infer on the possible stress effect arabinose causes on the cells, explained by the final OD600nm values achieved: 3.204 for *E. coli* BL21 (DE3) and 2.470 for *E. coli* BL21 (DE3) (I).

The 3 additional assays carried out showed a similar tendency with the cells containing the lpp antisense presenting a smaller specific growth rate. However, this difference is yet not enough to be completely explained by the antisense effect and could also be due to the presence of the pMLBAD plasmid.

Arabinose quantification by HPLC was conducted in order to evaluate the cells consumption of arabinose in the media. The assay in which an intermediate cell culture was included demonstrated a slight decrease in the arabinose concentration in the media for both cell cultures prepared (pMLBAD and pSAS39). The assays with induction at T3 or T4 showed inconclusive results since the concentration of arabinose remained the same or increased with time, which was not expected since the inducer is only added once.

RT-PCR for lpp mRNA levels

qPCR measures gene expression, more specifically how much of a specific mRNA exists in the tested samples. Figure 2 represents the obtained RQ values for each cell culture, which were calculated in relation to the cell culture pMLBAD 2 h (calibrator with RQ value of 1). Observing figure 1, a clear reduction in the expression of the lpp transcript is observed in the cell culture containing the antisense, 2 h after induction (pSAS39 2 h). However, 1.5 h later (second induction at T2), the effect disappears and the transcript levels increase to the normality. It has been shown that protein production under the $araP_{BAD}$ promoter can be greatly increased by inducing with different sub-maximal concentrations of arabinose (14).

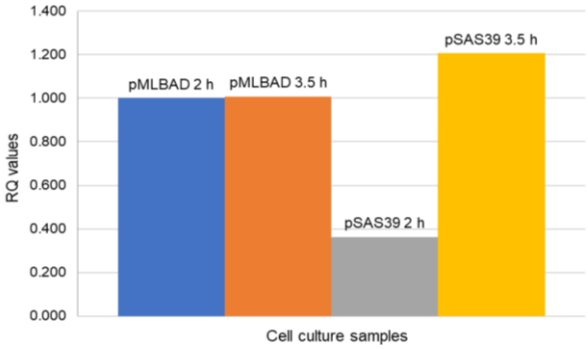

Figure 1 – RQ values representing the expression values of the lpp mRNA for the different cultures. Blue: sample from pMLBAD culture taken at 2 h growth, induced at T0. Orange: sample from pMLBAD culture taken at 3.5 h growth, induced at T0 and T2. Grey: sample from pSAS39 culture taken at 2 h growth, induced at T0. Yellow: sample from pSAS39 culture taken at 3.5 h growth, induced at T0 and T2.

Protein expression is dependent on the amount of active sugar permeases in each cell indicating that while protein yield can be manipulated, the amount of protein per cell is variable, with certain cells producing high quantities of the target protein and other cells not producing any protein at all (15,16). Production of toxic products, as can be the case of the lpp antisense because of its inhibiting effect on the structural protein Lpp, the subpopulation of cells

actively producing the product can perish and the effect of the antisense is lost. By including a transport gene in a plasmid controlled by a different inducer (possibly IPTG), it could be possible to achieve consistent induction in all cells, otherwise the native transporter gene will be controlled by the arabinose added, giving rise to subpopulations of cells fully induced or uninduced (17).

Susceptibility to salts

The objective of testing susceptibility to osmotic stress using sodium chloride (NaCl) was to observe whether the lpp antisense sequence had any influence on the permeability of the cell. If that was the case, the cell permeability would increase, uptake would be higher possibly leading to loss of viable cells. Figure 2 demonstrates the OD_{600nm} values obtained in the presence of several NaCl concentrations (10%, 9%, 8%, 7%, 3.5%, 1.75% and 0.875%).

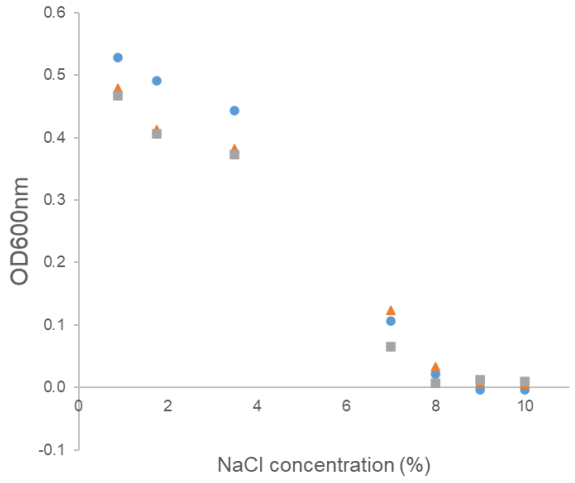

Figure 2 – Final values of OD_{600nm} for *E. coli* BL21 (DE3) (blue), *E. coli* BL21 (DE3) with pMLBAD (orange) and *E. coli* BL21 (DE3) with pSAS39 (gray) cell cultures, grown in LB liquid medium with varying concentrations of NaCl and induced with L-arabinose 2 %, after overnight microplate incubation at 37 ºC.

Observing figure 2, some differences between the wild type strain and the strain with the antisense are readily observed, with almost any difference between the cells with pMLBAD and pSAS39. At this stage, it is possible to hypothesize that the lpp antisense sequence is causing an inhibition effect on Lpp expression, although it is still necessary to repeat the procedure and test more stress conditions.

Overexpression of the truncated form of OmpK 21-99

OmpK 21-99 overexpression optimization

Several different strategies were employed in order to understand what conditions would be better to produce the OmpK 21-99 peptide in high quantities. Since experiments with the lpp antisense were performed at 37 ºC, expression under this temperature was also performed, although typically such high temperatures are not optimal to produce small peptides. Then, other temperatures (30 ºC, 25 ºC and 18 ºC) were tried out.

Overexpression at 37 ºC presented very low intensity bands corresponding to the expected MW of 12.5 kDa, meaning that the overexpression experiment was not optimal in producing high amounts of the OmpK 21-99 peptide, possibly because this temperature might not be indicated to produce small peptides in large quantities.

Overexpression at 30 ºC was also carried out and the harvested protein samples were run in a SDS-PAGE, represented in figure 3.

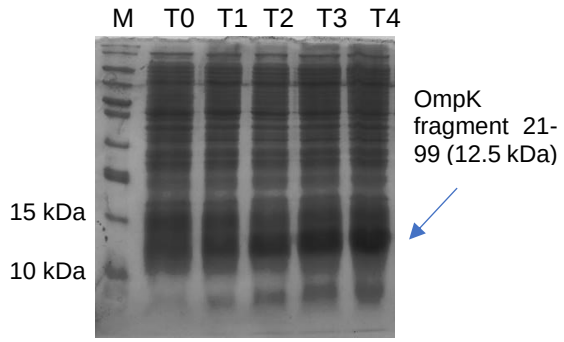

Figure 3 – SDS-PAGE containing protein samples extracted from whole biomass from overexpression in *E. coli* BL21 (DE3) cells with pFCN1 plasmid at 30 ºC. Lane M: Page ruler Unstained Broad Range (ThermoFisher); Lanes T0 to T4: protein samples taken during overexpression 0, 1, 2, 3 and 4 hours after induction, maintaining an OD_{600nm} of 0.6.

This strategy resulted in a visible overexpression of the peptide since it is possible to observe an increase in the intensity of the bands from the sample T1 to the T4 in the expected MW. Figure 4 shows the Western Blot obtained with the overexpression samples. As it is possible to see, there was in fact production of the OmpK 21-99

peptide, making this condition very promising in producing high quantities of the peptide.

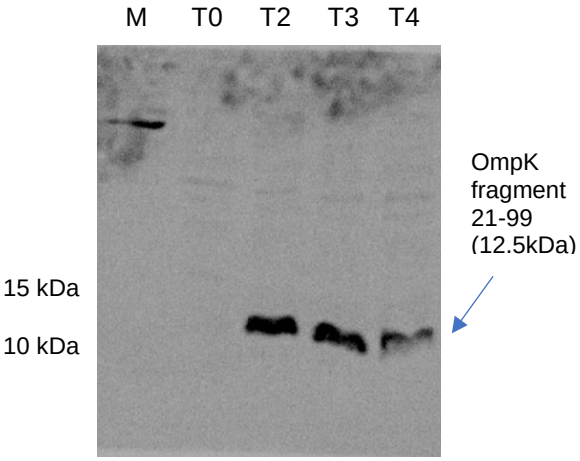

Figure 4 – Western-Blot containing protein samples extracted from whole biomass from overexpression in *E. coli* BL21 (DE3) cells with pFCN1 plasmid at 30 ºC. Lane M: PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (ThermoFisher). Lane T0: protein sample taken right before induction; Lanes T2 to T4: protein samples taken during overexpression 2, 3 and 4 hours after induction.

Peptide production was carried out also at 18ºC and 25ºC. Figure 5 represents the obtained SDS-PAGE with protein samples taken during the overexpression. From observation of the figure, although not as intense as before at 30 ºC, overexpression of the target peptide occurred, with a band of the expected MW being visible.

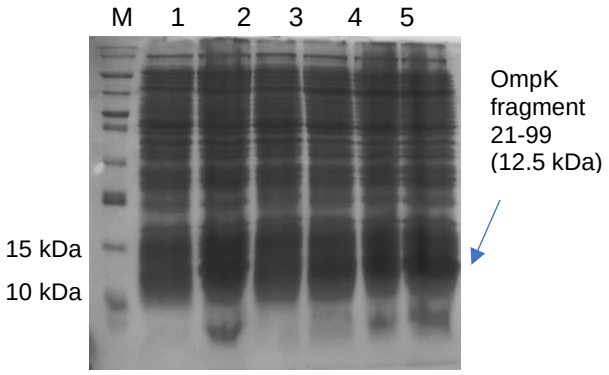

Figure 5 – SDS-PAGE containing protein samples extracted from whole biomass from overexpression in *E. coli* BL21 (DE3) cells with pFCN1 plasmid at 18 ºC and 25 ºC. Lane M: Page ruler Unstained Broad Range (ThermoFisher); Lane 1: protein sample at T0 of overexpression at 18 ºC; Lane 2: protein sample at T16 of overexpression at 18 ºC; Lane 3: protein sample at T0 of overexpression at 25 ºC; Lanes 4 to 6: protein samples taken during overexpression at 25 ºC, 1, 2 and 3 hours after induction.

Experiments with temperatures 30 ºC, 25 ºC and 18 ºC were repeated and the periplasmic fractions isolated to see if the peptide was successfully being transported to the periplasm after expression was complete.

In periplasmic fraction analysis, the SDS-PAGE prepared demonstrated a positive result on the production of the protein, since all fractions presented a band of the expected MW size in the gel. To confirm these results, a Western Blot experiment was conducted, represented in figure 6.

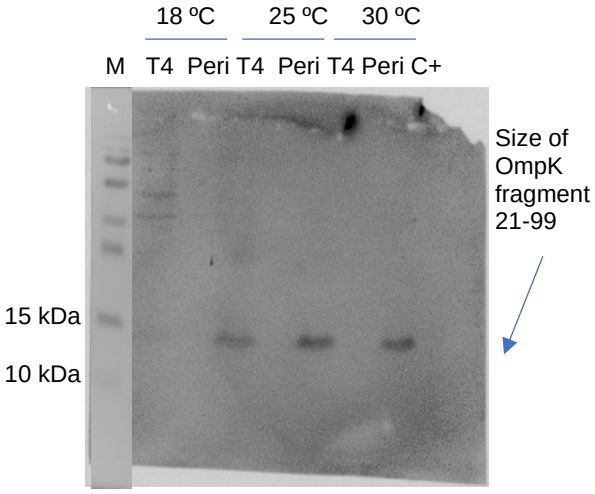

Figure 6 – Western-Blot containing protein samples from overexpression at 18 ºC, 25 ºC and 30 ºC of *E. coli* BL21 (DE3) cells with pFCN1 plasmid. Lane M: PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (ThermoFisher); Lane 2: total protein sample T4 overexpression 18 ºC; Lane 3: periplasmic fraction sample overexpression 18 ºC; Lane 4: total protein sample T4 overexpression 25 ºC; Lane 5: periplasmic fraction sample overexpression 25 ºC; Lane 6: total protein sample T4 overexpression 30 ºC; Lane 7: periplasmic fraction sample overexpression 30 ºC; Lane 8: positive control – protein sample T4 from overexpression at 30 ºC.

Temperatures 25 ºC and 30 ºC seem to be good conditions to produce the peptide. However, analysis of the periplasmic fraction showed absence of peptide indicating that the transport of the peptide to the periplasm was not successful. On the other hand, the produced peptide can be inside the cytoplasm, forming aggregates named inclusion bodies (IBs). Another possibility could be explained by the lower amount of the peptide that actually reaches the periplasm, that is not enough to be detected by WB.

A control overexpression experiment at 30 ºC with *E. coli* BL21 (DE3) cells with the plasmid pET-22b(+) was performed to see if there are other proteins of similar size being overexpressed and redirected to the periplasm. The SDS-PAGE presented a negative result for the production of proteins in the same range as of the OmpK 21-99 fragment, as well as the analysis of the periplasmic fraction did not show production of proteins in the referred range. A western-blot was also prepared to compare with the samples from the pFCN1 plasmid overexpression at 30 ºC. However, a coloration step with Ponceau (NZYTech) (stains every protein that was transferred to the membrane and is reversible), right after transfer of the proteins to the membrane indicated that this process did not work well, since no bands are visible to the naked eye in the expected area, as it is possible to see in figure 7.

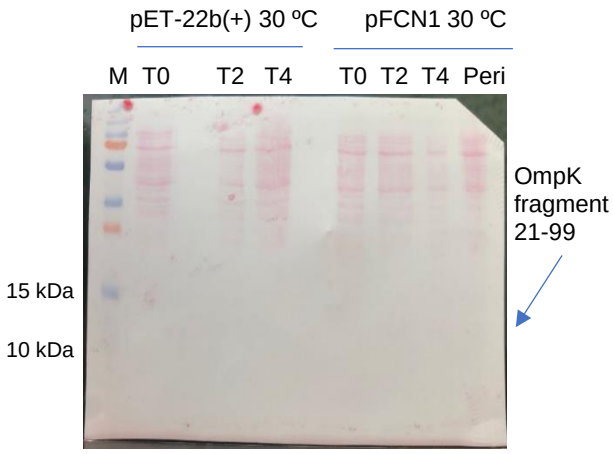

Figure 7 – Nitrocellulose (NC) membrane after incubation with Ponceau coloration solution containing protein extracts from overexpression in *E. coli* BL21 (DE3) cells with pET-22b(+) plasmid at 30 ºC. Lane M: Page Ruler Plus Prestained Protein Ladder (ThermoFisher). Lanes T0 to T4 (pET-22b(+)): whole protein extracts taken during overexpression with the pET-22b(+), at 0, 2 and 4 hours after induction with 0.4 mM IPTG; Lanes T0 to Peri (pFCN1): whole protein extracts taken during overexpression with pFCN1 plasmid at 0, 2 and 4 hours after induction and the periplasmic fraction sample from overexpression with pFCN1 plasmid.

This problem could be associated with troubleshooting situations related with the technique of Western Blotting, which should be optimized to be successful to detect smaller proteins and peptides. It has been reported that low-molecular peptides are more difficult to detect using the conventional western blotting method since they are readily detached from the blotted membranes, making PVDF membranes better to overcome those problems (18). An elevated quantity of unspecific bonds reflected in the background in the upper part of the western blot, was a recurrent issue throughout the experiments, which can be an indicator of a problem related with various factors, such as excessive antibody concentration, long exposure times, incomplete blocking or even improper washing (13). Hence, the Western Blot should be repeated and optimized.

<u>Overexpression at 30 ºC in *E. coli* BL21 (DE3) pSAS39 cells + pFCN1</u>

Overexpression of the peptide was developed in *E. coli* BL21 (DE3) cells already containing the pSAS39 plasmid that enables the production of an antisense molecule, diminishing the production of the structural protein Lpp, causing an effect of cell permeabilization. This effect would allow an easier transport of certain substances through the cell membrane to the extracellular medium, resulting in an easier recovery of the produced OmpK fragment. A control culture without antisense induction was also performed to be able to infer on the antisense effect. Figure 8 represents the western blot obtained in the experiment, where bands in the expected size range can be observed, confirming in both cases, the

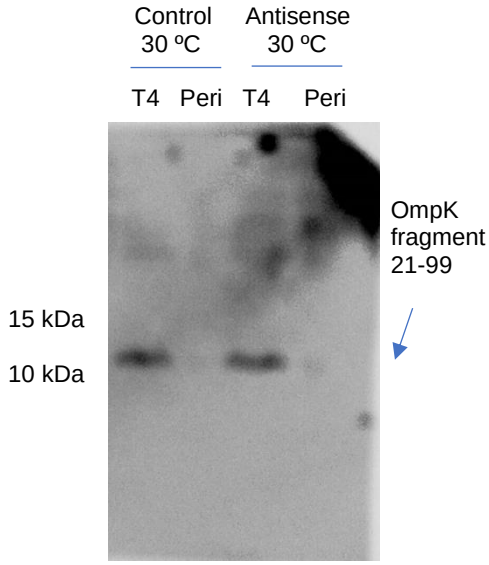

Figure 8 – Western-Blot containing protein extracts from overexpression of *E. coli* BL21 (DE3) cells with pSAS39 + pFCN1 at 30 ºC, with and without antisense induction. Lane T4 (Control): protein sample T4 overexpression control 30 ºC; Lane Peri (Control): periplasmic fraction sample overexpression control 30 ºC; Lane T4 (antisense): protein sample T4 overexpression with antisense 30 ºC; Lane Peri (Antisense): periplasmic fraction sample overexpression with antisense 30 ºC.

overexpression of the desired peptide, at 4 hours post induction. Regarding the periplasmic fraction samples, the membrane presents a very low intensity band that could be due to the peptide produced but that indicates an inability to transport the peptide to the periplasm in higher amounts.

Moreover, the extracellular medium was also analyzed and no band was observed of the same size as the peptide, indicating that the effect the antisense has on the cell is very low. This could be related with the tendency of the cells producing the antisense to disappear, as mentioned before, or with problems related with the WB technique and the induction conditions, that should be better studied. Hence, the experiment should be repeated, as well as different conditions should be tried in order to find the optimal strategy for overexpression of the OmpK 21-99 peptide.

Conclusion

Analysis of the levels of antisense expression by RT-PCR allowed to conclude that the antisense is successfully decreasing expression of the Lpp protein, although the effect after some time is lost and expression returns to the normality. *E. coli* growth studies demonstrated slight differences between the wild type strain and the antisense strain but they cannot be attributed completely to the antisense effect but also to the effect the inducer arabinose can be causing onto the cells and to the pMLBAD plasmid itself. Optimization of OmpK 21-99 peptide overexpression was also extensively studied with the temperature 30 ºC being the one with the best results. The peptide was also successfully overexpressed in cells containing the pSAS39 plasmid but secretion to the periplasm and the extracellular medium was not verified, in any condition. Hence, expression optimization should be further studied.

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