# UNIVERSITAT POLITÈCNICA DE VALÈNCIA

SCHOOL OF AGRICULTURAL ENGINEERING AND ENVIRONMENT


# Characterization of temperature stress-related gene expression changes in cold-active *Shewanella sp.* #4 using real-time quantitative polymerase chain reaction (qRT-PCR).

*Thesis submitted in Partial Fulfilment of the*
*requirements for the*
*Bachelor's Degree in Biotechnology*

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**ABSTRACT**

*Shewanella sp.* #4 belongs to the *Shewanella* genus, which is a unique member of the marine bacteria family *Shewanellaceae*. They are Gram-negative facultatively anaerobic, rod-shaped bacteria that are found in aquatic environments, where low temperatures and high pressures are common. More than 72 species of *Shewanella* are currently known and the interest surrounding these bacteria resides in its participation in diverse geochemical cycles, fish spoilage, and its potential usage in bioremediation processes.

*Shewanella* can adapt to a wide range of environmental conditions. To have a better understanding of the mechanisms that allow the cold-active *Shewanella sp.* #4 to thrive under such diverse stresses, temperature stress was simulated in laboratory conditions to analyse gene expression of stress-related genes.

The standard laboratory growth temperature for *Shewanella sp.* #4 is 15 °C, thus, to evaluate the gene expression levels in low and high temperature stress conditions, 5 °C and 25 °C were applied. The aim of this project is to assess the expression of genes related to stress responses using the quantitative reverse transcription polymerase chain reaction (qRT-PCR). More specifically, two genes related to low-temperature changes were selected: polyunsaturated fatty acid synthase D subunit (*pfaD*) and Ribosome Binding Factor A (*rbfA*) genes. The expectations of the gene expressions in extreme temperatures were as follow: *pfaD* gene expression should be increased under low-temperature conditions since it is related with the production of polyunsaturated fatty acids, which makes the cell membrane more flexible in cold conditions and increase the cell survival; *rbfA* is a ribosome-associated protein involved in restarting the translation under cold temperatures, so its expression should be also increased under low temperatures.

Since *Shewanella sp*. #4 has not been applied to qPCR analyses before, related methods needed to be optimized prior to gene expression studies. Indeed, to achieve the main objective it was required to elucidate the most efficient method to isolate total RNA from *Shewanella sp*. #4 and then to apply it to obtain the total RNA samples from which gene expression would be analysed. Finally, stable “housekeeping” genes needed to be determined to ensure the reliability of the results, and, to analyse the gene expression of the selected target genes using qRT-PCR in total RNA samples extracted from bacteria cultured under different growth conditions.



**KEY WORDS**

*Shewanella sp.* #4; temperature stress; quantitative polymerase chain reaction; gene expression; *pfaD; rbfA*

**Evaluación de la expresión génica de genes relacionados con el estrés por temperatura en *Shewanella sp.* #4 mediante reacción en cadena de la polimerasa cuantitativa a tiempo real (qRT-PCR).**


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## RESUMEN

La bacteria *Shewanella sp.* #4 pertenece al género *Shewanella*, único miembro de la familia *Shewanellaceae,* compuesta principalmente por bacterias marinas. Estas son bacilos Gram negativos, facultativos anaeróbicos que están sobre todo presentes en ambientes acuáticos extremos, donde hay temperaturas extremadamente bajas y muy altas presiones. Se han encontrado más de 72 especies de este género y el interés en este es creciente por las condiciones que son capaces de soportar, por su participación en diversos ciclos geoquímicos, en el deterioro del pescado y su potencial en procesos de biorremediación.

Esta bacteria psicrófila es capaz de adaptarse a diferentes condiciones ambientales. Para comprender mejor los mecanismos que le permiten estar sometida a diferentes estreses y continuar con sus actividades vitales, se simuló estrés por temperatura en el laboratorio para analizar la expresión de genes relacionados con la respuesta de la bacteria a estos estímulos.

El objetivo de este trabajo es evaluar la expresión génica de genes relacionados con respuestas a condiciones de estrés utilizando la reacción en cadena de la polimerasa con transcriptasa reversa cuantitativa (qRT-PCR). Más concretamente, se han seleccionados los genes codificantes para la subunidad D de la sintetasa de los ácidos grados polinsaturados (*pfaD*) y para el factor A de unión al ribosoma (*rbfA*), relacionados con cambios bajas temperaturas. El primero porque el aumento de los ácidos grasos poliinsaturados hace las membranas más fluidas en condiciones frías y el segundo al como factor de iniciación de respuestas por estrés. Por ello, era esperada una expresión diferencial inducida por alta o baja temperatura. La temperatura óptima para el crecimiento de *Shewanella sp.* #4 es 15 °C, para evaluar los niveles de expresión génica en bajas y altas temperaturas se cultivaron las células a 5 °C y 25 °C.

Dado que *Shewanella sp.* #4 no ha sido empleada en qPCR experimentos anteriormente, antes de realizar el análisis en sí, los métodos relacionados con la técnica tuvieron que ser optimizados. Así, para realizar este trabajo fue necesario hallar el método más eficiente para aislar ARN total de esta bacteria y, de esta forma, aplicarlo para obtener las muestras a partir de las que se analizará la expresión génica. Además, se determinaron genes estables "housekeeping" para asegurar la fiabilidad de los resultados y, para finalmente, analizar la expresión génica relativa de los genes diana seleccionados mediante qRT-PCR en muestras de ARN total extraídas de bacterias cultivadas en las diferentes condiciones de crecimiento.



## PALABRAS CLAVE

*Shewanella sp*. #4; estrés de temperatura; reacción en cadena de la polimerasa cuantitativa; expresión génica; *pfaD; rbfA.*

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**LIST OF ABREVIATIONS**

- *16S*: 16S ribosomal RNA gene.
- BCP: 1-Bromo-3-chloropropane.
- cDNA: Complementary DNA.
- Cq: Cycle quantification.
- Ct: Cycle threshold.
- DNA: Deoxyribonucleic acid.
- DNAse: Deoxyribonuclease.
- EPA: Eicosapentaenoic acid.
- FRET: Fluorescence Resonance Energy Transfer.
- gDNA: Genomic DNA.
- *gyrA*: gyrase subunit A gene.
- kDa: kiloDalton
- mRNA: Messenger RNA.
- PCR: Polymerase chain reaction.
- pfa: polyunsaturated fatty acid.
- *pfaD*: Omega-3 polyunsaturated fatty acid synthase subunit pfaD gene.
- PUFAs: polyunsaturated fatty acids.
- qPCR: quantitative polymerase chain reaction.
- qRT-PCR: quantitative reverse transcription polymerase chain reaction
- *rbfA*: ribosome binding factor A gene.
- RIN: RNA Integrity Number
- RNA: ribonucleic acid.
- RNAse: ribonuclease.
- RT: Reverse transcription.
- sp: subspecies.
- TF: Transcription Factor.

# 1 INTRODUCTION.

## 1.1 *Shewanella* genus: Understanding *Shewanella sp. #4.*

The *bacterium* studied in this project is *Shewanella sp.* #4, an unclassified subspecies that belongs to the genus *Shewanella* (1). It is a Gram-negative, rod-shaped, flagellated and facultatively anaerobic *bacterium*. It forms light brown, round colonies after 48 hours of incubation at 15°C in Marine Broth 25% solid culture. *Shewanella sp*. #4 remains unclassified but sequence analysis of the *16S* gene indicates that the closest species is *Shewanella frigidimarina*, in a 97% of identity (1).

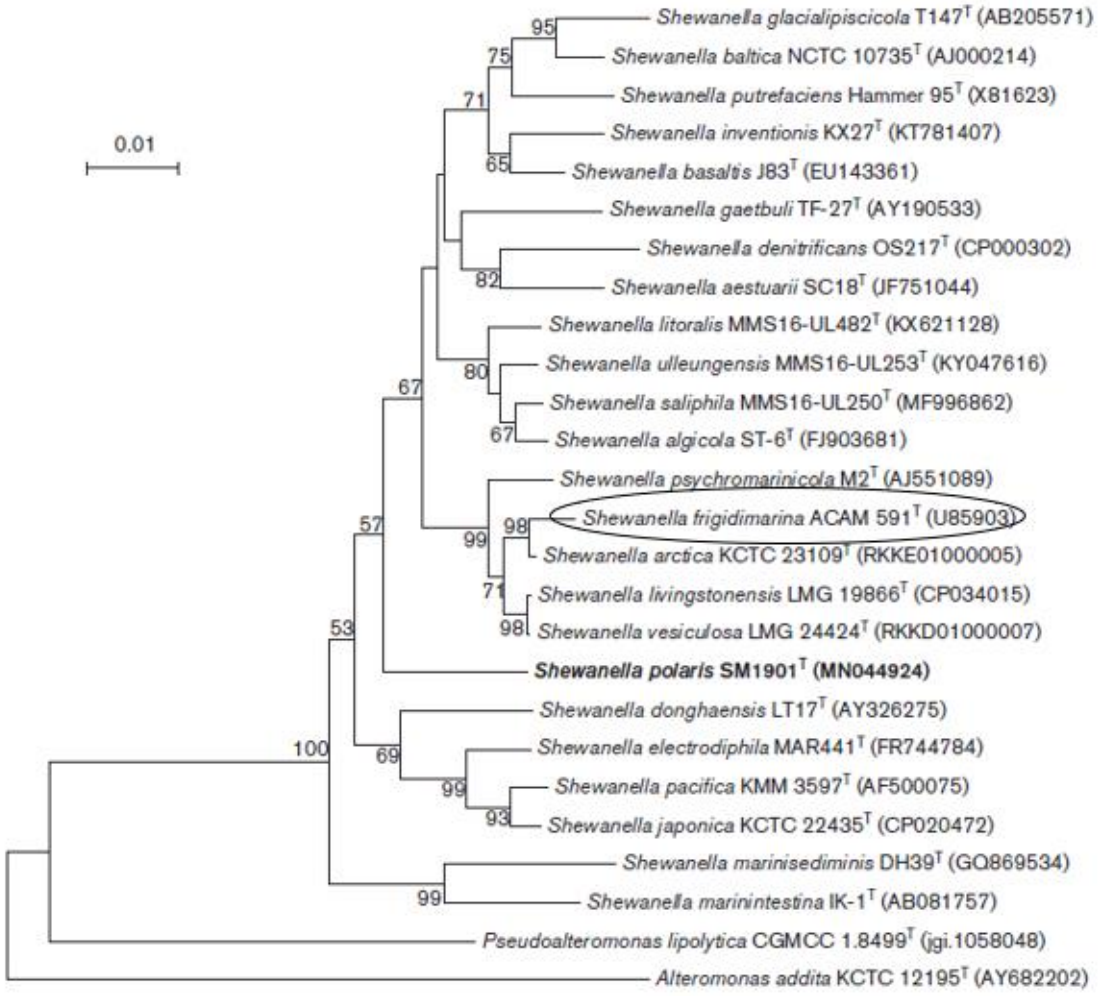


*Figure 1. Phylogenetic tree of Shewanella genus species based on 16 rRNA gene. The figure was adapted from* (2)*. 0.01 bar represents substitutions per nucleotide position. Pseudomonas lipolytica and Alteromonas addita were applied as outgroups. Bootstrap values (>50%) are shown at the nodes based on the analysis of 1000 replicates. S. frigidamarina is circled as the closest published sequence to S. sp. #4.*

The genus *Shewanella* belongs to the family *Shewanellaceae* and to the order *Alteromonadales,* within the class *Gammaproteobacteria* (3). It contains 72 described and registered species to this day (LPSN, 2020). *S. putrefaciens* and *S. oneidiensis* are the most studied ones, with this last one as the type species of the genus. Some of the most studied species are shown in Figure 1. Members of the *Shewanella* genus have been identified as the main spoilage bacteria of stored fish and marine seafood (4), specially *S. baltica* and *S. putrefaciens* (5) and they play a role in numerous

biogeochemical cycles in nature allowing the key process of nutrient regeneration to take place (6). Some authors propose to divide *Shewanella* genus into two sub-genus branches since part of *Shewanella* species are mesophilic and sensitive to high barometric pressures, and others are adapted to cold temperatures and high pressures (7). *Shewanella sp.* #4 studied in this project belongs to the cold-adapted group of species.

According to available literature, marine environments such as polar sea areas, seawater, marine algae, aquatic invertebrates, fish and marine sediments are frequent sources of bacteria belonging to *Shewanella* genus (2). In addition, these species frequently present in environments with high salt concentrations, low temperatures and high barometric pressures. The subspecies studied is not an exception as *Shewanella sp.* #4 was isolated from the Baltic sea (1).

There are two remarkable characteristics in *Shewanella* genus species. Firstly, they can utilise one or more compounds as electron acceptors for anaerobic respiration, including toxic metals and elements. Indeed, they are amongst the most diverse respiratory organisms and this outstanding ability allows the cells to respire in a wide range of harsh conditions (8). Another hallmark feature of *Shewanella* genus, more relevant for the purpose of this project, is that most of its members are cold-active bacteria. Some of them are truly psychrotrophiles, which have low optimal growth temperatures (< 16°C) while others are psychrotolerant, growing better in higher temperatures (> 16°C) (9). This means, that many *Shewanella* species can thrive at low temperatures (less than 5 °C). This is important in marine environments with oscillating temperatures, such as the Baltic sea where the *Shewanella sp.* #4 was first isolated from and where the temperatures fluctuate between seasons and with depth (10).

These characteristics make *Shewanella* species outstanding bacteria for biotechnological purposes and strong candidates for potential applications. They can be successfully applied in the bioremediation of various toxic contaminants, such as halogenated organics (11) or nitramines (12). Given that most of the planet's environments are considered cold and that *Shewanella* species are cold-active bacteria, they are suitable for *in-situ* remediation since they could be directly applied in contaminated marine environments. There are preliminary studies that show their ability of producing bioenergy, for instance, in biofuel cell applications (13). Some *Shewanella* species are able to processing wastewater, waste biomass or utilising marine organic-rich sediments as an electron source, resulting in the production of small amounts of energy in a sustainable way. (14). Moreover, further potential applications such as the use of cold-active enzymes for the food industry or as detergents have been considered (15).

There is an increasing interest in finding and analysing new bacterial species with potential in biotechnological applications, since they can help to efficiently solve environmental problems. Still today, there are many cellular, genetic and biochemical mechanisms that are not thoroughly studied in *Shewanella sp.* #4. The particularities of *Shewanella* species further encourage the characterisation of *Shewanella sp*. #4.

### *1.1. Shewanella sp. #4* under stress: selected genes and stress conditions.

In the previous section 1.1. the background knowledge regarding *Shewanella* genus was set. In addition, it was stated that these species thrive under a wide variety of stress conditions and that there is an increasing interest on the unknown mechanisms behind it. There are previous studies analysing the stress responses under different stimuli in *Shewanella* genus (5). Regarding the design of this experiment, various potential conditions were considered to reproduce stress under laboratory conditions: pH stress, oxidative stress, nutrient stress, salinity stress, cell density stress and temperature stress.

Being the first time that real-time PCR method has been utilized on *Shewanella sp.* #4, optimization of each step on the way and the design of the qPCR program had to be conducted prior to the gene expression analysis. Accordingly, only one stress condition was initially chosen. Temperature stress was selected since the bacterium of interest is a psychrophilic subspecies isolated from the Baltic sea, and there is special interest on its adaptations to changing temperatures of this environment. Changes in temperature are one of the most common environmental stresses in almost every environment. Indeed, more than 90% of the seas are of lower temperature than 5°C and 14% of the planet's surface is considered as polar region (9). Even though heat shock responses have been extensively studied in many representative organisms, cold-shock related mechanisms are less known.

Although a bacterial cell contains a huge number of genes, not all of them are being expressed at the same time. Genetic expression is a process in which gene sequences are used to synthesise gene products via transcription and translation processes (16). Some of the structural genes are related to basic cellular functions and are continuously expressed at stable levels and the expression of other genes is increased or decreased depending on the needs of the cell (17).

Those protein-coding (or structural) genes which are permanently active in all cells, are responsible of maintaining the "house-keeping" activities such as production of electron transport chain proteins, enzymes required in the glycolysis reaction, components for citric acid cycle or essential transcription factors (18). The steady expression of these genes serves as a reference for observing the changes in expression levels of stress-related genes (19). The reference genes are also utilized in data normalization.

Only two reference genes were selected in this preliminary phase of the project, one related to protein synthesis functions: 16S ribosomal RNA gene (*16S*) and the other related to DNA replication: gyrase A gene (*gyrA*), both essential for basic cellular functions. There are several studies that utilises *16S* and *gyrA* as reference genes (6). The expression of ribosomal RNA is considered as a "house-keeping" function and *16S* gene have been extensively utilised as reference gene in RT-qPCR experiment. Expression of *16S* genes has been tested in exponential and stationary growth phases to further test the stability of the expression and no changes have been observed (6). DNA gyrase subunit A is a type II topoisomerase involved in DNA replication processes, thus it is expressed constitutively (20).

Typically, low temperatures induce a differential gene expression as a cold-shock response that allows the cell to adapt to the environment. In other words, gene expression mechanisms are being regulated by the exposure of the cell to cold temperatures (15). Cold-active bacterium have adjusted their cellular components to low temperatures present in their environments by changing their membrane compositions, nutrient uptake mechanisms, cellular translation machinery and their energy-producing systems (6). Frequently, these changes consist on producing functional enzymes or proteins functional at low temperatures.

The cell may respond to harmful conditions by either producing proteins or modulating the transcription process. In this project, the genetic expression of two genes related to low temperature stress conditions were analysed: Omega-3 polyunsaturated fatty acid synthase subunit pfaD gene (*pfaD*) and ribosome binding factor A gene (*rbfA*). They were selected considering that the main problems induced by a shift to low temperature are the protein synthesis inhibition and the reduction in the membrane fluidity and permeability (21). Also, both of gene products are located in the nucleus of the cell what provides them with higher thermal stability considering that the cytoplasmic proteins are less protected against temperature changes (9).

The first selected candidate as temperature-related stress gene was *pfaD* gene. Omega-3 polyunsaturated fatty acids are essential nutrients for living organisms and a crucial component in lipidic bilayers (5). Bacteria are one of the main sources of these compounds for the whole food chain and specially those living in cold environments (8). Specially, they are produced by cold-active bacteria and whole pfa gene cluster for producing polyunsaturated fatty acids (PUFA) have been found in other *Shewanella* species (22). Given that altering the lipid composition of the cellular membranes is a wide-spread adaptive mechanism to thrive in cold temperature, it is understandable why bacteria under low temperatures can produce PUFAs. The reason behind this response mechanism is that membranes become more rigid and transit to a gel-like state when subjected to low heat (21). Bacteria have a response termed “homeoviscous adaptation” which consist in alter the balance between saturated and unsaturated fatty acids which compose the membrane by changing the grade of saturation of the hydrocarbon chains by increasing the number of polyunsaturated fatty acids. This way the flexibility of the membrane increases and it becomes more fluid, this is a crucial factor for cell respiration, transport of nutrients and substrates within rigidifying conditions (5).

The *rbfA* gene was also selected for this study. It has been observed that a transient blockage of translation initiation occurs when cells are exposed to low temperature conditions, resulting in a reduction of cell growth. This inhibition of the ribosomes initiates the cold-shock response. In studies performed with *Escherichia coli* subjected to low temperature, it was found that there are three ribosome-associated proteins that are found to be related to translation under these conditions, amongst them *rbfA.* This protein is a 15-kDa 30S ribosomal binding factor, which was proven essential for an efficient translation in cold-environments (21) by interacting with 30S subunit at several binding sites may also have a role in assisting the maturation of 16S rRNA in cold conditions (23). According to another study in which *E. coli* mutants for *rbfA* gene were created and

exposed to low and high temperatures, it was shown that the *rbfA* gene products were related to cold-shock cell response (6)

Considering all the mentioned in the present section, there are consistent reasons to believe that it is likely to find these genetic, cellular and physiological changes in *Shewanella sp.* #4. The hypothesis proposed in this project is that the studied bacteria will present the same cold-shock responses than other members of *Shewanella* genus and the selected reference genes will remain stable since there is no reason to believe that they will present a different gene expression pattern than in other related microorganisms. This would result in the increased expression of these cold-temperature stress genes when subjecting *Shewanella sp.* #4 to low temperature conditions, but increased levels of gene expression would be observed in higher temperatures of these genes when the cells are growing in a warmer environment.

### 1.2 Gene expression analysis using qRT-PCR.

In this section, the basic and essential components of a method to detect and quantify the mRNA levels in a biological sample will be discussed. Those are: a sensitive detection method, a sufficient amount of the mRNA for analysis, sequence specific primers and the proper controls and standards to be able to interpret the results (24).

As we already discussed (see section 2.2.) genes can be expressed or not depending on the environmental conditions, thus measuring gene expression is a key element of research in life sciences. Moreover, gene expression patterns are essential for understanding biological pathways, protein function and cellular responses to external and internal stimuli. There are diverse methods for this purpose, some of them are known well and have been extensively applied during the past decades while new ones are being investigated.

One possible way of measuring gene expression is the study of the genes, the DNA sequence itself. The expression of a gene is related to the function of its product. When a gene has been described, its function can be predicted by homolog that are already know by using bioinformatic tools (25). Moreover, DNA or complementary DNA (cDNA) microarrays and other display-based methods can be powerful tools for identifying genes with a differential expression. Using them, thousands of genes can be monitored in one array and the whole profile of the expression of the genes can be tested in an unique experiment, but they are not as powerful as quantification techniques since the sensibility is lower (24). There are more methods such as ribonuclease protection assays or tiling arrays that can be conducted within DNA or RNA samples to evaluate the expression levels, often combined with ChiP (Chromatin immunoprecipitation) analysis (26).

Another approach is to quantify the levels of the gene product, generally a protein. Regarding the central dogma of molecular biology, we can estimate the expression level of the gene which encodes for a protein by quantifying it the amount of the specific protein produced. The most utilized methods to quantity protein levels are Western blotting and enzyme linked immunosorbent assay (27). On the other hand, gene expression levels can be evaluated by measuring the RNA levels.

Among the techniques that analyse these molecules are northern blotting, the serial analysis of gene expression SAGE (28), RNA-seq (29) and PCR-based methods.

In this study, the method utilized is quantitative reverse transcription polymerase chain reaction (qRT-PCR). Quantitative or real-time PCR is a powerful method to quantify gene expression (24). According to (30), qRT-PCR is the most rapid, sensitive and accurate method to quantify relative and absolute expression levels of genes amongst all the available methods. PCR based approaches are becoming more and more widespread in gene expression analysis due to its tremendous sensibility (31). With qPCR approaches, it is possible to detect extremely small quantities of mRNA, although it is not always easy to ensure that all the samples have equal initial amount of template (32). In some bacterial species applied in qRT-PCR experiments the isolated amount of RNA is limiting the studies (19) and messenger RNA (mRNA) may be expressed in a very low amounts. Indeed, the advantage of PCR is the requirement of exceptionally low amount of template material to begin with and specifically amplify. It is more sensitive than microarrays when detecting small expression changes but it is less adaptable to high-throughput studies (30). The major limitation of qPCR is that the selected gene of interest must be known to be able to design the primers, however it is well-suited for small subset of genes.

The conventional PCR method allows the identification and amplification of a determinate sequence in a template. However, it is not considered as a quantitative method given that from the product of the PCR it is impossible to determine the initial amount of template. The amplified sequence varies with the primer-dimer accumulation, according to DNA polymerase that binds to the primers and the PCR product re-annealing (33). Contrarily, real-time PCR technique is a variant of the PCR that allows the quantification of the target sequence while the amplification round takes place. This method is potent, very sensible and allows the analysis of different types of oligonucleotides. It provides accurate information of the initial levels of the template due to the continuous monitoring of the accumulation of the PCR products at the end of each amplification cycle by employing a detection system. It combines the PCR methodology with the use of fluorescent reporters to detect the amplified genes (34).

There are different detection systems utilized with qPCR. The most utilized are fluorescent dyes (such as SYBR Green or FAM) and TaqMan probes (5). In this project, SYBR green dye is utilised, it specifically fluoresces when bound to double stranded DNA (dsDNA). The downfall of this system is that the fluorescence is derived from the desired PCR product as well as from all the non-desired dsDNA present in the sample. This may sometimes lead to a strong background signal. Then, regarding TaqMan probes, there are different types of commercial ones. Using TaqMan probes has the advantage that they can be employed in combination with various fluorescent reporters in multiplex experiments (35).

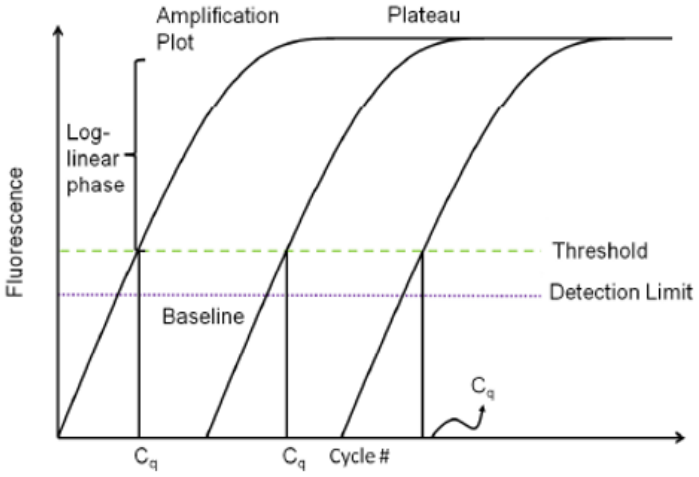


*Figure 2. Basic scheme of a real-time polymerase chain reaction adapted from* (34)*. Number of amplification cycles plotted against fluorescence signal. The figure includes quantitative curve of the qPCR with the main parameters of the reaction: fluorescence threshold, detection limit, plateau and logarithmic phases, Cq is an abbreviation of quantification cycle.*

Initially during the qPCR reaction, the fluorescent signal is undistinguished from the background (or base line) that results from the set of probes that have the fluorophore bound to them. After a determinate number of cycles, the signal coming from the amplified template becomes significant, which means that the signal is high enough to be separated from the background and it can be reliably detected. The number of amplification rounds to have high enough and constant emission of fluorescent signal is known as the threshold (Ct) of the reaction. In Figure 2, Cq (quantification cycle) is represented, it is a generic term englobing Ct and Cp (crossing point of the threshold) and other terms referring the number of cycles utilised to quantify the concentration of the template. The threshold discriminates the actual signals from the variability of the baseline (Figure 2). When the fluorescent signal is higher than the threshold, it is considered relevant (34). The signal starts increasing exponentially before reaching a plateau and it can be used to determine levels of mRNA (36). Finally, when the reaction components become limiting factors to the reaction, the amplification rate decreases and it is no more exponential, and the plateau phase will be reached.

The threshold value is related to the amount of target molecules present in each sample and it will be used to determine the amplified amount of each gene. If the amount of the initial template is high, the Ct value will be lower, since fewer cycles are required to detect the reliable fluorescence signals coming from the amplification of the genes of interest. When the Ct value is reached in the logarithmic phase of amplification, it provides a reliable and reproducible measure. If the Ct is reached during the plateau stage, limiting factors would distort the reliability of the result. Finally, prior to the data analysis, primer efficiency, detection limit and quantification limit must be determined (34).

The second essential component of every mRNA detection method is the total RNA sample required to analyse the expression. As the performance of the reaction is highly affected by the RNA integrity, sample quality control prior to qRT-PCR analysis is required. The ideal total RNA sample for a RT-qPCR analysis must be pure, free of contaminating DNA and must be intact. The study comparing

qPCR results obtained from degraded and intact samples showed that degraded input RNA resulted unreliable results (37). The initial amount of total RNA can be measured by spectrophotometry after the isolation. As every PCR-based method, qPCR also works via amplification of the DNA sequence. For this reason, total RNA is converted to cDNA through a reverse transcription reaction. It is not necessary to isolate mRNA from the total RNA since the primers are designed to amplify specifically the targeted genes. This way only the desired sequences of RNA will be amplified and detected. As mentioned, the initial yield of sample required for qPCR is very small, whereas the quality of the nucleic acids is more important in this kind of studies (34).

When quantifying the amount of amplified product in qPCR, controlling cross-reactions is important. In order to do that, primer design is a key step in ensuring that only the desired nucleotide target is being amplified. It is important to predict and avoid the formation of non-specific products such as primer-dimers or amplification of non-targeted regions during the amplification. Moreover, it is important to ensure that house-keeping gene expression is stable under all the conditions of interest. Normally, genes that are essential for cellular functions and are expressed in constant levels are the best for this purpose. It is also recommendable to utilize multiple housekeeping genes (Charles M. Roth. 2002).

As with any technique, it is essential that qPCR experiments have correct controls and standards to obtain reliable results. To detect potential contamination of the PCR assay and unintended amplification products such as primer-dimers, appropriate different types of negative controls are essential. More concretely, no template and reverse transcription negative controls obtained by omitting reverse transcriptase enzyme (RT) samples should be included as controls, which provide quality control information regarding experimental procedures (34). The reaction without template would show if there is any contamination and the reaction without RT enzyme that all the amplified template is the synthetized cDNA in the RT reaction and not remaining DNA in the total RNA sample.

Positive controls also provide valuable quality assurance and are essential when measuring low copy numbers or where a negative result needs to be assigned with confidence (38). Dilution curves of the template are often enough for this purpose and they are utilised to estimate primer efficiencies and reaction functionality. Internal positive control reactions can also be used to demonstrate that no reaction inhibition has occurred, which is particularly important in the interpretation of apparently negative results from clinical or environmental samples. Inclusion of characterized positive samples may also be used to compare with test samples in post-amplification melt curve analysis and these are useful for assessing reaction specificity for genotyping assays (39).

Additionally, biological variability in the gene expression levels can be found due to the differences when comparing individual organisms or culture samples. Technical variability as a result of pipetting errors, poor sample quality and quantity and inaccurately calibrated pipettes is another source of fluctuation. To ensure the experimental reproducibility the experiment need to be repeated with three different biological batches of the sample and by analysing three technical replicates (40).

## *2.* OBJECTIVES.

This study aims to determine how environmental stresses affect to *Shewanella sp.* #4 gene expression. Specifically, the goal is to investigate how temperature changes might affect the expression of temperature-related ribosome binding factor A (*rbfA*) and polyunsaturated fatty acid synthase D subunit (*pfaD*) genes. The selected method to analyse changes in gene expression was qRT-PCR.

The project can be divided into two parts. First, the optimization of the steps prior to the qPCR protocol to characterise the differential gene expression related to temperature changes in *Shewanella sp.* #4: from the cell cultivation and harvesting to the analysis of the samples. Then, the utilisation of a qPCR protocol to measure the relative expression levels of the selected stress genes *pfaD* and *rbfA* in this *bacterium* compared with the expression of *16S* and *gyrA* reference genes at the control and stress temperatures.

In order to do that, more concrete goals were set for each one of the performed steps.

1. Grow the *Shewanella sp.* #4 cells in selected temperature stress conditions.
2. Determine the optimal method for good quality total RNA isolation from *Shewanella sp.* #4 species and apply it to extract the RNA samples to be used in the analysis.
3. Optimize the PCR-program for amplifying the target genes and test primer pairs with genomic DNA and later with complementary DNA (cDNA) of *Shewanella sp.* #4.
4. Evaluate the efficiency of the primer pairs of selected genes in qRT-PCR.
5. Analyse the relative gene expression of the temperature stress genes *pfaD* and *rbfA* to grasp its respective role in cold-shock response of *Shewanella sp.* #4.

# *3.* MATERIALS AND METHODS.

## *3.1.* Materials.

### *3.1.1. Chemicals, buffers and solutions*

- Acidic phenol (pH 4.3), Fisher Scientific.
- APS (ammonium persulfate), Fisher Scientific.
- Isopropanol, Fisher Scientific.
- Ethanol, VWR.
- BCP (1-Bromo-3-chloropropane), Fisher Scientific.
- Gene Ruler Low Range DNA Ladder, Thermo Scientific.
- Gene Ruler O'range, Thermo Scientific.
- GlycoBlue, Thermo Fisher scientific.
- Hydrogen peroxide, Fisher scientific.
- SybrGreen I, Invitrogen.
- Home-made acidic phenol:BCP with chaotropic agent solution (see section 8.2).
- 3M NaCl, Fisher Scientific.
- Midori Green, Nippon genetics Europe.
- Agarose, Thermo Fisher Scientific.
- 0,5 x TBE (45 mM Tris-borate/1mM EDTA).
- 1 x TAE (Tris-acetate-EDTA buffer).
- 0.1M sodium citrate in 10% ethanol, pH 8.5
- 6x DNA Loading buffer (8.2.)
- 2x RNA Loading buffer (8.2.)
- 10x RQ1 DNAse Buffer, Promega.
- 10 mM dNTPs, VWR.
- 10-x Key Buffer, VWR.

### *3.1.2. Culture mediums*

Two different culture media were utilized to grow the bacterial cells. 25% Marine Broth for *Shewanella sp. #4* and King's B medium to grow *Pseudomonas syringae pv. phaseolicola* HB10Y, which from this point onwards it will be called HB10Y. Components and its corresponding amount to prepare one litre of both mediums are described in Table 1.

*Table 1. Components of King's B and Marine Broth medium.*

| **King's B Broth*** | **25% Marine Broth**** |
|---|---|
| 10g Proteose peptone #2 (BD) | 7.5 g Peptone (Fisher) |
| 1.5 g anhydrous $K_2HPO_4$ (Merck) | 1.5 g Yeast extract (Fisher) |
| 15 g glycerol (Fisher Scientific) | 9.35 g Marine Broth (Difco) |
| 5 mL $MgSO_4$ (1M stock; VWR) | |

*King's B Broth: The first three ingredients (Table 1) were mixed in a beaker and the total volume was filled to 1L with MQ water. The pH was adjusted to 7.0 with HCl. The solution was autoclaved and 5 mL of sterile 1 M $MgSO_4$ was added.

King's B Media Plate: 20 g agar (Fisher Scientific) was mixed with the other ingredients (Table 1) and the total volume was filled to 1 L with MQ water. The pH was adjusted to 7.0 with HCl. The mixture was autoclaved and 5 mL of sterile 1 M $MgSO_4$ was added while the media was still warm. The mixture was aseptically poured into sterile Petri plates.

**25% Marine Broth: The ingredients (Table 1) were well mixed and 1L of MQ water was added. The mixture was heated with frequent stirring and boiled for 1 minute to completely dissolve the ingredients. The media was autoclaved at 121°C for 15 mins.

25% Marine Agar plate: All ingredients of the media (Table 1) and 15.0 g Agar (Fisher Scientific) were well mixed and up to 1L of MQ water was added. The mixture was heated with frequent stirring and boiled for 1 minute to completely dissolve the ingredients. The media was autoclaved at 121°C for 15 mins and aseptically poured into sterile Petri plates while still warm.

### *3.1.3. Enzymes*

- Taq DNA polymerase (5U/µL), VWR.
- Maxima Reverse Transcriptase (200U/µL), ThermoScientific.
- Optizyme RNAse inhibitor (20-40U/µL), Thermo Fisher Scientific
- RQ1 DNAse Promega.
- iTaq Universal SYBR Green Supermix, Bio-Rad.

### *3.1.4. Bacterial strains*

- *Shewanella sp.* #4 (1).
- *Pseudomonas syringae pv. phaseolicola* HB10Y (41)

### *3.1.5. Instruments*

- Incubator shaker New Brunswick™ Excella® E25/E25R *Eppendorf.*
- Incubator shaker New Brunswick™ Innova® 42/42R *Eppendorf.*
- Imaging system BioRad *ChemiDoc Touch.*
- Electric source EPS-600 CBS *Scientific.*
- Vortex Genie 2 Scientific Industries.
- Spectrophotometer NanoDrop 2000c *Thermo Fisher Scientific.*
- Scale CP2202.S *Sartorius.*
- Block heater GRANT - QBD2.
- Temperature controller Eppendorf ThermoMixer® C
- Thermocycler VWR® thermal cycler XT96.
- Thermocycler T3000 *Biometra.*
- Centrifuge 5810 R *Eppendorf.*
- Centrifuge micro star 17R *VWR.*

- Q-POD® Ultrapure Water Remote Dispenser.
- Colorimeter, J.P. SELECTA®.
- CFX96 Real-Time System Thermocycler C1000™ BIO-RAD.
- SIGMA 4-16K Centrifuge.

### *3.1.6. Other materials and softwares.*

- FrameStar® 96 Well Semi-Skirted PCR Plate. Roche Style, Plus qPCR Seal.
- RNeasy® Mini kit, Qiagen
- Maestro Software BioRad, CFX.

## 3.2. Methods.

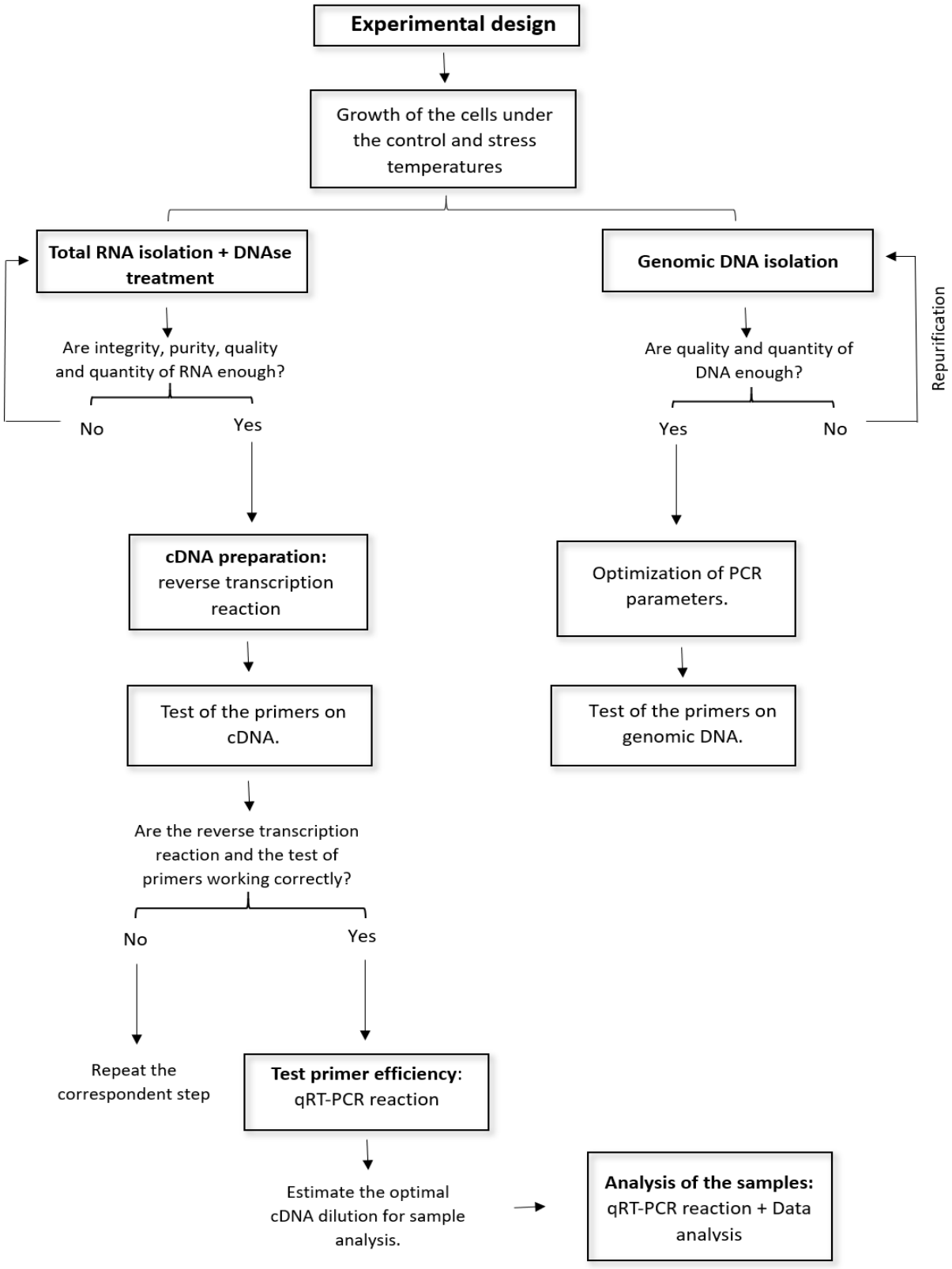


*Figure 3.Workflow of the conducted experiments with either total RNA or DNA isolated from Shewanella sp. #4 during the optimization phase and for the analysis of the samples.*

### *3.2.1. Growth of the strains*

The standard temperature for growing *Shewanella sp.* #4 under laboratory conditions is 15°C (1). The cells were first stroke onto a 25% Marine Broth agar plate (see Table 1) and grown at 15°C for 2 days. To set a starter culture, a separate colony from the plate was inoculated into 100 mL of 25% Marine Broth culture medium (Table 1) and grown for two days in continuous shaking at 200 rpm at 15°C. Fresh 100 ml of 25 % marine broth was inoculated with the starter culture to the optical density (OD) value of 0.2 measure with wavelength (λ) of 550 nm. Three similar cultures were grown, each in different 5, 15 or 25°C temperature with shaking at 200 rpm until the $OD_{550}$ values reached 0.8. Cells were harvested by centrifugation (3220*g*, 5 min, 4°C). When obtaining the growth curves for *Shewanella sp.* #4, cultures were grown as described until the $OD_{550}$ value was above the detection limit.

HB10Y cells were stroke on Kings B Agar plate and grown overnight at 28 °C. To set a starter culture, a separate colony was inoculated in 50 mL of fresh Kings B medium (Table 1) and incubated at 28°C, over one night with continuous shaking at 200 rpm. Fresh Kings B liquid medium of 100 mL was inoculated with starter culture to reach initial $OD_{550}$ value around 0.1. This culture was grown with the same conditions as the starter culture until the $OD_{550}$ value reached 0.6. The cells were harvested by centrifugation (3220*g*, 5 min, 4°C).

Storing of the harvested cells varied depending on the further application purposes (discussed in detail in the next chapter). For TRIzol isolation method, cells were stored in home-made TRIzol at 20°C until the extraction (max. over one night). *Shewanella sp.* #4 cells were stored in 10 and HB10Y cells in 4 mL of acidic phenol:BCP with chaotropic agent per 100 ml of initial cell culture. When commercial total RNA isolation with RNeasy® Mini kit from Qiagen was applied, 500 µL aliquots of the culture were divided in Eppendorf tubes and stored at -80°C.

### *3.2.2. Total RNA and genomic DNA isolation*

Two different methods to isolate pure total RNA were tested during this project: RNeasy® Mini kit from Qiagen and home-made acidic phenol:BCP with chaotropic agent protocol (appendix 8.2.), from now onwards will be referenced as "acidic phenol:BCP" protocol. The extraction with the kit was performed according to the manufacturer's recommendations. An additional step of lysing the cells by forcing them through a narrow syringe was conducted before applying the sample to the lysis buffer and proceeding to the manufacturer's protocol. Finally, the total RNA extracted was dissolved in MQ water.

The extraction method using acidic phenol:BCP was adapted from TRIzol Reagent User Guide from Invitrogen/Thermo Fisher Scientific (42). To extract the total RNA from *Shewanella sp.* #4 cells, 1000 µl of BCP and 3 mL of acid washed glass beads were added (Larger beads with 2 mm of diameter and small ones with 0.3-0.5mm diameter, in a ratio of 1:2) to the mixture of cells stored in acidic phenol:BCP solution. The mixture was vortexed with full speed for 5 min and centrifuged at 10000*g* for 15 min at room temperature to obtain the phase separation (Figure 4).

The aqueous phase on top contains the RNA, below there is the organic phase that contains the DNA and the bottom one is the organic layer with the proteins (Figure 1Figure 4). Since this method allows the extraction of both, RNA and genomic DNA (gDNA) from the same sample, the same protocol was used for total RNA isolation and gDNA isolation, from the harvest of the cells until the separation of the phases.

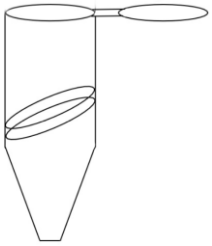

*Figure 4. Separation of the lysate sample in three phases. The aqueous phase above contains the RNA and the organic phase below the DNA.*

Total RNA was extracted from the aqueous phase (Figure 4) by transferring it to a new tube containing 2 mL of acidic phenol and 400 µL of BCP. This mixture was vortexed at full speed for 2 minutes and then centrifuged at 10000*g*, at room temperature for 15 min. These steps were repeated until an interphase between the phases was clear. Subsequently, the aqueous phase was transferred to a new tube and equal volume of isopropanol was added to precipitate the RNA by incubating the mix for 10 min at room temperature. The RNA was harvested by centrifugation at 17000*g*, 4°C for 45 min. The supernatant was discarded, and the pellet was washed with 75% ethanol by vortexing the tube and centrifugation at 17000*g*, 4°C for 15 min. The supernatant was discarded, the pellet was air-dried and dissolved in RNAse-free MQ water. The total RNA sample was stored at -80°C.

The gDNA was extracted from the interphase and organic phase (Figure 4). Residuals of the aqueous phase were removed, and the desired phases were transferred to a new tube leaving the glass beads. 300 µL of 100% Ethanol / 1 mL of acidic phenol:BCP applied for cells lysis was added, mixed gently with the sample and incubated for 3 minutes. Then, the tubes were centrifuged at 2000*g*, 4°C for 5 min, the supernatant was discarded, and the pellet was resuspended in 1 mL of 0.1M sodium citrate in 10% ethanol pH 8.5 / 1 mL of acidic phenol:BCP used initially. This mix was incubated for 30 min and centrifuged at 2000*g*, 4°C for 5 min. The supernatant was discarded, and the previous steps were repeated twice from resuspending the pellet in the sodium citrate solution until the centrifugation step. Then, the supernatant was discarded, and the pellet was resuspended in 1.5 mL of 75% Ethanol/ 1 mL of acidic phenol:BCP used for the lysis and this mix was incubated for 20 min at room temperature. Finally, the DNA was harvested with centrifugation at 2000*g*, 4°C for 5 min, the supernatant was discarded, and the pellet was dissolved in the sodium citrate solution after drying the pellet. The gDNA sample was stored in -80°C.

DNA samples were reprecipitated to improve the quality using ethanol and NaCl. First, 1:10 of the sample volume of 3M NaCl and 2.5 volumes of 100% Ethanol were added and the sample was precipitated for 1 hour at -20°C. The precipitate was centrifuged at 130000*g* 4°C for 20 min, the

supernatant was discarded, the pellet was washed with 1 mL of 80% ethanol and centrifuged as above. The supernatant was discarded, and the pellet was air-dried for 10 min. Finally, the pellet was resuspended in MQ water and the sample was stored at -80°C.

### *3.2.3. DNAse treatment*

The total RNA samples must be pure and completely free of genomic DNA. A DNAse treatment with RQ1 DNAse *Promega M6101* was performed to all total RNA samples. The reaction mixture contained 15 µL of 10x RQ1 DNAse Buffer and 3 µL of RQ1 DNAse enzyme (3U) for each 30 µg of total RNA. MQ water was added to reach 150 µL as the total volume of the reaction. The reaction mixture was incubated at 37°C for 30 min.

The RNA was re-isolated after the DNAse treatment. First, 150 µL of MQ water, 500 µL of phenol and 50 µL of BCP were added to the mixture, it was vortexed for 15s and centrifuged at 12000*g*, 15 min at 4 °C. The supernatant was transferred to a new tube containing 300 µL of phenol and 30 µL of BCP, it was vortexed for 15 s and centrifuged as above. The supernatant was transferred to a new tube and 1:10 volume of 3 M Sodium Acetate (pH 5.3-5.5), 0.5 µL of GlycoBlue (Thermo Fisher Scientific) and 2.5 volumes of 100% ethanol were added. This RNA was precipitated overnight at 20°C and harvested by centrifugation at 17000*g*, 4°C, 60 min. The supernatant was discarded, 500 µL of 80% Ethanol was added and it was centrifuged at 17000*g*, 15 min, 4 °C. This step was repeated twice. The supernatant was finally discarded, and the pellet air-dried. The DNAsed RNA sample was dissolved in MQ and stored at -80°C. Initial total RNA sample was run in agarose gel to evaluate efficiency of the DNAse treatment.

### *3.2.4. Quantification and integrity test of the samples.*

To quantify and estimate the quality and concentration of the total RNA and DNA extracted, the absorbance of the samples was measured with spectrophotometry using a Nanodrop 2000c (Thermo Fisher Scientific). Frequently, the absorbance of the sample at λ of 230 nm, 260 nm and 280 nm of the sample is measured. The 260 nm absorbance ($A_{260}$) value is used to estimate the concentration of the RNA (ng/ µl). To evaluate the quality of the nucleic acid sample, $A_{260}/A_{230}$ and $A_{260}/A_{280}$ ratios were measured from 2 µl sample. Electrophoresis analysis conditions for checking total RNA integrity are discussed in following section.

### *3.2.5. Agarose gel electrophoresis and gel sample preparation.*

Given the importance of integrity of total RNA isolated, agarose gel electrophoresis was performed to check total RNA integrity: 1% agarose gel in 1xTBE buffer was run at 100V, 500A for 60 min. The gel was pre-stained with 3µL of Midori (Nippon Genetics Europe) in 200mL of the gel. The amount of total RNA loaded per sample was 200 µg in 2xRNA loading dye. Samples denatured by heat at 85°C for 5 min before loading on the gel. After PCR reactions, 2% agarose gel were run to visualise the amplified gene product. Same conditions as above were utilised for running and pre staining the gel, if not otherwise mentioned. 10 µL of the PCR product samples were loaded with 2 µL of 6xDNA loading dye. In both cases, a low-range DNA gene ruler was used on the gels for the size estimations. The agarose gels were visualized using imaging system BioRad *ChemiDoc Touch.* It is important to highlight that in these gels it was utilised a DNA gene ruler, so the sizes are roughly estimated since

RNA and DNA same size bands do not migrate the same way in the gel due to their structural differences.

### *3.2.6. Optimization of PCR and quantification parameters*

Several experiments were performed to optimize crucial parameters in PCR reaction. The PCR program utilised in all the optimization tests is shown in Appendix 8.4. The amount of magnesium ions required in the reaction for optimal result of PCR was evaluated. The experiment was designed as shown in Table 2. The total volume of each reaction was 20 µL. A negative control without DNA template was also applied.

*Table 2. Components of the PCR reaction mix used to optimize the magnesium concentration in PCR reaction*

| COMPONENT | FINAL CONCENTRATION | | | | | | |
|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G |
| **$MgCl_2$** | 1.5mM | 2mM | 2.5mM | 3mM | 3.5mM | 4mM | 4.5mM |
| **10X Key Buffer** | 1X | | | | | | |
| **dNTPs** | 0.5 mM | | | | | | |
| **Forward primer** | 0.25 µM | | | | | | |
| **Reverse primer** | 0.25 µM | | | | | | |
| **gDNA template** | 35 ng | | | | | | |
| **Taq polymerase** | 1U | | | | | | |
| **MQ water** | - | | | | | | |

Also, the minimum amount of Taq polymerase required to perform the PCR was tested. To do that the PCR-reaction shown in the Table 2 were performed. Negative controls without enzyme and without DNA template were added. PCR products from both optimization experiments described in this section were analysed in 2% agarose gels as described in section 3.2.3.

*Table 3. Components of the PCR mix prepared to optimize Taq polymerase concentration.*

| COMPONENT | FINAL CONCENTRATION | | | | |
|---|---|---|---|---|---|
| | A | B | C | D | E |
| **Taq polymerase** | 3U | 2.5U | 2U | 1.5U | 1U |
| **10X Key Buffer** | 1X | | | | |
| **dNTPs** | 0.5 mM | | | | |
| **Forward primer** | 0.25 µM | | | | |
| **Reverse primer** | 0.25 µM | | | | |
| **gDNA template** | 35 ng | | | | |
| **MQ water** | - | | | | |

The minimum amount of total RNA necessary to be able to visualize the ribosomal RNA bands in the agarose gel was optimized as well. Different amounts of total RNA, 250, 500, 750 and 1000ng were mixed with 2xRNA loading dye. The samples were analysed by agarose gel electrophoresis as described in section 3.2.3. For this reactions, *16S* gene primers were applied.

### *3.2.7. Test of the primers on genomic DNA and cDNA: PCR and gradient PCR protocols*

As mentioned in the introduction, the selected stress genes were *pfaD* and *rbfA* and the reference genes were *16S* and *gyrA*. Primer pairs for each one of those genes were provided and tested on the *Shewanella sp. #4* DNA isolated from cells grown at the standard temperature (see section 3.2.2.) The melting temperature of the primers were estimated using the Olygoanalyzer tool from IDT (Integrated DNA Technologies) webpage (43). PCR reactions were conducted to test whether the primers worked as expected. The sequences and melting temperatures of the primer pairs are shown in Table 4 and the applied PCR program is described in appendix.

*Table 4. Sequence, melting temperature and expected product size for the primer pairs.*

| | GENE | SEQUENCE | TM (°C) | PRODUCT SIZES (BP) |
|---|---|---|---|---|
| REFERENCE GENES | ***16S*** | F: CTAATACCGCATACGCCCTACG | F: 63.3 | 100 |
| | | R: GCTAGTTGGTGAGGTAATGGCT | R: 63.3 | |
| | ***gyrA*** | F: TGCCTTGTACGTGACCTAATTCC | F: 63.8 | 119 |
| | | R: CGATGTTAGCCAGTGCTATAGCC | R: 63.7 | |
| STRESS GENES | ***pfaD*** | F: AGCGAAATGCGGTAGACTTAGC | F: 63.8 | 100 |
| | | R: TCAACTGGAACAGCCACACC | R: 63.8 | |
| | ***rbfA*** | F: CGAAGGTATGCGCATGTCTAACC | F: 64.3 | 114 |
| | | R: CTTTCTCCTGGGTATCGTCTTGG | R: 64.5 | |

The components used in PCR and gradient PCR reactions are shown in Table 6 and the Taq concentration applied was 1U**¡Error! No se encuentra el origen de la referencia.**. Sterile MQ water was added to reach 20 µL of total volume in each PCR reaction tube. As negative control reactions without DNA template were applied to see if any DNA contamination was present. The PCR products were visualized by electrophoresis as described in section 3.2.3. To further optimize the PCR conditions, gradient PCR with melting temperature interval of 64.5 ± 2.5°C was applied for *16S*, *gyrA*, *pfaD*, *rbfA* primer pairs. The PCR mixture was prepared as the conventional PCR (Table 6)**¡Error! No se encuentra el origen de la referencia.** with 1U Taq concentration. The utilised PCR program is shown in appendix 8.4. The PCR products were analysed in a 2% agarose gel with the same conditions as described above.

### *3.2.8. Reverse transcription and test of the primers on synthetized cDNA.*

As already mentioned in the introduction, PCR-based gene expression analysis methods can only be performed with DNA samples, since the method is based on the DNA amplification process (44). For qPCR analysis, the isolated total RNA was converted to cDNA by reverse transcription. The enzyme utilized was the Maxima Reverse Transcriptase (Thermo Scientific), it is specially recommended to prepare cDNA for RT-PCR and qRT-PCR experiments, and it has a high sensitivity. The reaction mixture (RT+ reaction) was prepared as showed in Table 5Table 5, adding the components in the indicated order (from above to the bottom). Random hexamers were utilized instead of gene specific primers to check the specificity of the primers on cDNA. A negative control (RT- reaction) was prepared without adding the reverse transcriptase enzyme to the mix to ensure that only the synthetized cDNA is present in the qPCR. The reaction was incubated as follows: 10 min at 25°C, 50

min at 50°C and finally 5 min at 85°C to inactivate the enzyme. The first incubation step at 25°C is necessary only when using random hexamers as primers.

*Table 5. Components of the reverse transcription reaction mixture.*

| Component | Concentration | Volume added (µL) |
|---|---|---|
| **Total RNA** | Depending on the sample | 1 |
| **Random Hexamer** | 0.2 µg / µL | 1 |
| **dNTP mix** | 10 mM | 1 |
| **MQ water** | - | 11 |
| **5X RT Buffer** | 5X | 4 |
| **RNAse inhibitor** | 40U/ µL | 1 |
| **Maxima reverse transcriptase** | 200 U/ µL (stock) | 1 |
| **Total volume** | - | 20 |

PCR was conducted with the RT-reaction products to check whether the reaction worked correctly and to ensure that primers bind well also to the cDNA (Table 6). For each primer pair, one PCR-reaction utilizing RT+ reaction, or RT- reaction as template as well as negative control without template was prepared. The applied PCR program is described in appendix 8.4. PCR products were visualized by gel electrophoresis according to the protocol described in section 3.2.3.

*Table 6. PCR reaction components.*

| PCR reaction mix | Volume (µl) | Final concentration |
|---|---|---|
| **10 X Key Buffer** | 2 | 1X |
| **10 µM primer forward** | 0.5 | 0.25 µM |
| **10 µM primer reverse** | 0.5 | 0.25 µM |
| **10 mM dNTPs** | 0.4 | 0.5mM |
| **Taq Polymerase** | 0.2 | 1U |
| **MQ water** | 15.4 | - |
| **cDNA template** | 1 | 35ng/µL |
| **Total volume** | 20 | - |

### *3.2.9. qPCR reaction.*

#### *3.2.9.1. Efficiency test of the primers.*

The efficiency testing of the primers provides the information of the real efficiency of the primers applied and the optimal amount of cDNA required for reliable results. After RT reaction, cDNA samples from cells grown at 15 °C were utilized to perform the tests. The following dilutions of the cDNA were prepared and applied in the experiment: non-diluted, 1:5, 1:10, 1:20, 1:50 and 1:100.

A master mix for 25 reactions was prepared for each primer pair containing the components (Table 7) and the two-step qPCR program utilized is presented (Table 7). The melting temperature applied

in the qPCR program was 64.5 °C, according to the results obtained from the temperature gradient PCR section (3.2.5.).

Then, 1 µl of the appropriate cDNA dilution was pipetted into the corresponding well of the plate where 9 µl of master mix previously pipetted. Thus, the final volume of each reaction was 10 µl. The primer efficiency with each cDNA dilution was tested in triplicates to minimalize technical variability. The selected negative controls to ensure the reliability of the experiment were three negative reverse transcription samples lacking RT enzyme and three reactions without template for each one of the primer pairs.

*Table 7. Components of the master mix of the qPCR and program utilized.*

| Components | Volume (µL) |
|---|---|
| **iTaq Supermix (2X)** | 5 |
| **Forward primer (10 µM)** | 0.4 |
| **Reverse primer (10 µM)** | 0.4 |
| **MQ-water** | 3.2 |
| **Total volume** | 10 |

| qPCR program | |
|---|---|
| **Temperature (°C)** | **Time (seconds)** |
| 95 | 10 min |
| Cycles | 44 |
| 95 | 10 |
| 64.5 | 10 |
| 72 | 30 |
| - | Go to cycles |
| 95 | 10 |
| 55 | 5 |
| 95 | 10 |

#### *3.2.9.2. Gene expression experiment.*

Each one of the genes were analysed in triplicates for the three different growth temperatures, a 10x master mix with the reaction components was prepared according to the Table 7 and load prior to addition of the template. After testing the primer efficiency, the optimal cDNA dilution was estimated from primer efficiency test 3.2.8.1. and 1 µl of it was applied as a template to the reaction mixture. The total volume of each reaction was 10 µl. The same qPCR program and same instrument were applied for the primer efficiency testing and the gene expression experiment.

### *3.2.10. Primer pair efficiency value calculations, gene expression analysis and statistics.*

The gene expression analysis was performed according to the Pfaffl normalization method (45). Ct values for each reaction were exported by using CFX Maestro software from Bio-Rad. As each sample was analysed in three technical replicates, the Ct values of them were averaged to overcome the variability among these replicates resulting in $Ct_{average}$ value. Standard deviation and variance calculations were performed. Also, the anomalous Ct values were discarded when the melting curve and the peaks shown that multiple products were amplified.

To obtain the primer pair efficiency values $Ct_{average}$ was plotted against the inverted logarithm of the corresponding dilution. The efficiency values were calculated for each one of the primer pairs by using Equation 1 where E is the efficiency value and the slope is obtained from the regression line of the above-mentioned plot.

$$E = 10^{(\frac{-1}{slope})}$$

*Equation 1. Calculation of the primer pair efficiency value.*

Once the primer efficiencies are calculated, the equation 2 to estimate the relative gene expression of the stress genes in comparison to the levels of expression of the reference genes is applied (Equation 2). For calculating ΔCt, $Ct_{average}$ of the three replicates was considered as control. In this equation E is the efficiency of the primer pair and ΔCt represents the change in Ct values between high and low temperatures.

$$Ratio = \frac{(E\ stress\ gene)^{\Delta Ct\ stress\ gene}}{(E\ reference\ gene)^{\Delta Ct\ reference\ gene}}$$

*Equation 2. Calculation of the relative gene expression of the stress genes.*

If the ratio obtained from Equation 2 is > 1.5, the expression of the gene of interest is higher than the reference gene. The expression of the target gene is lower than the expression of the reference gene if the ratio is < 0.5. The statistical analysis performed include the standard deviation and variance of the Ct values for the three replicates (Equation 3). The error bars presenting standard deviation are shown in Figure 12.

$$SD = \sqrt{\frac{\Sigma|x - \bar{x}|^2}{n}} \qquad Variance = SD^2 = \frac{\Sigma|x - \bar{x}|^2}{n}$$

*Equation 3. Standard deviation and variance calculation.*

# 4. RESULTS AND DISCUSSION

## 4.1. Growth of the strains

*Shewanella sp.* #4 cells were grown in Marine Broth 25% liquid medium. It contains all the nutrients necessary for growth many of marine bacteria given than the high salt content helps to simulate seawater conditions. The initial $OD_{550}$ of the *Shewanella sp.* #4 value were adjusted to ~0.2 and the growth was frequently monitored. The growth Shewanella sp. #4 has not been previously studied parallelly in these selected three temperatures, thus, it was required to establish repeatable growth curves to be able to predict the growth rate in future experiments. Three separate growth experiments were conducted (Figure 5). HB10Y cells were grown to serve as a control in further steps of the experiment (data not shown).

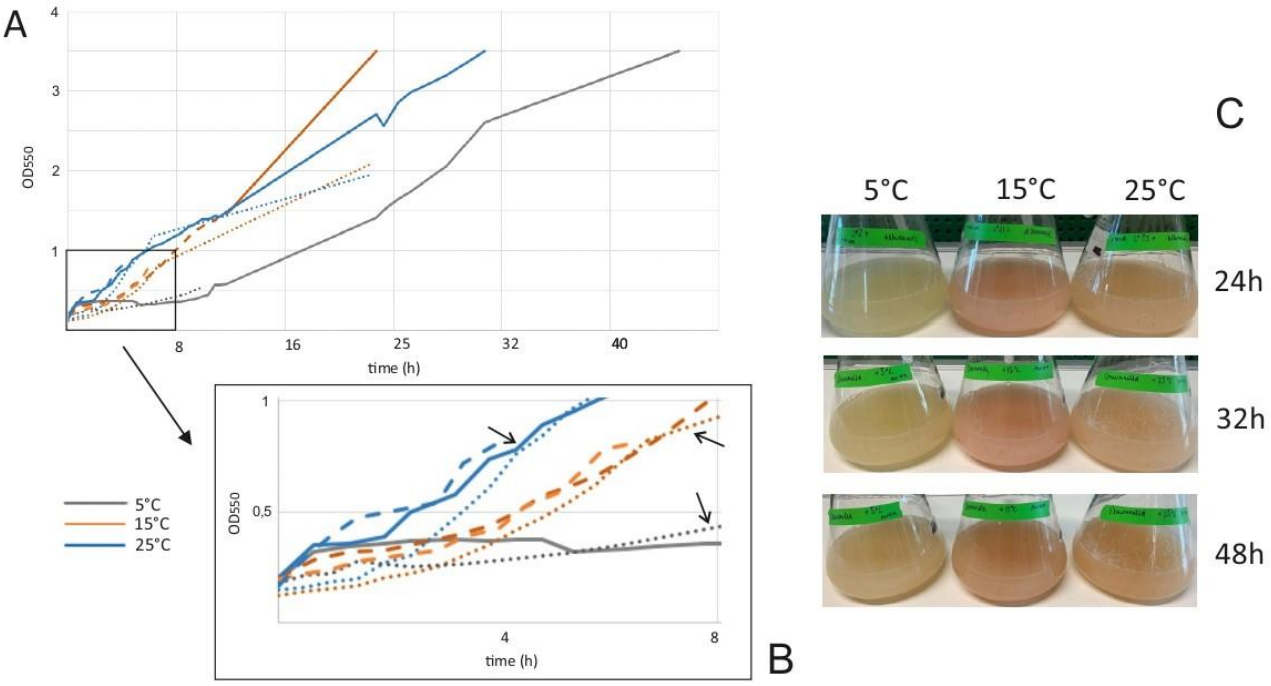


*Figure 5. Growth of Shewanella sp. #4 at the three studied temperatures and the pigment formation (A) The bacterium was grown in 5, 10 and 15°C temperatures and the growth were monitored. Each growth condition is presented with different color: 5°C with grey, 15°C with orange, and 25°C with blue. Differently dashed lines represent different biological replicates. (B) The first 8 hours of growth is shown in zoom-in panel and the harvesting time points are indicates with arrows. (C) The pinkish pigment formation that varied according to the growth temperature was observed and documented at 24h, 32h and 48h time points.*

*Shewanella sp.* #4 liquid cultures at 5°C, 15°C and 25°C were grown. Three samples for each one of the temperatures were measured at $OD_{550}$ every hour to monitor the growth (Figure 5A). To better observe the pigment formation (Figure 5C) the cultures were grown for 48 h once, whereas the cells were harvested earlier from other cultures to apply them in further experiments.

*Shewanella sp.* #4 is a psychrophilic *bacterium* and as such its optimal growth temperature should be below 20 °C, among certain marine bacteria species the optimal growth temperatures of 15°C and 4°C have been reported (1). Based on previous studies of Luhtanen *et al.* as well as our observations (Lampi, M. unpublished data), the optimal temperature for *Shewanella sp.* #4 is 15°C of the three studied temperatures, which was selected to serve as a standard temperature in this project. Thus, considering the obtained results, the subspecies culture grown at 15°C reached the detection limit of the colorimeter fastest (Figure 5A). Considering this, growing cells at both 5 °C and 25 °C induce a stress for *Shewanella sp.* #4. Growth at 5 °C is considerably slowed down while at 25 °C the cells present initially a higher rate of duplication than at 15 °C, but after 12 hours the trend changes and the growth rate at 15 °C is faster than in 25 °C (Figure 5A). That could be due to the cellular stress caused by the high temperature, accumulation of metabolic waste products to the growth medium or due to density stress. In low temperatures, cell metabolism as well as growth rates are typically lower (46). *Shewanella sp.* #4 growing at 5°C has a very long lag-phase (Figure 5A) that is caused by the cold-shock, which causes a transient inhibition of protein synthesis. Within this period cells start to produce cold-shock proteins to help them to survive and grow.

According to the obtained results, the OD measures kept increasing until the last measurement was taken (Figure 5A). However, with the chosen method, it was impossible to determine the ratio of living and death cells. The obtained data was compared to previous observations from our laboratory (unpublished data, not shown) and, although in the Figure 5A, the growth is monitored only until 48h, the growth continues after this point at a decreased rate. Nonetheless, for the purpose of this study, obtained growth curves were informative enough. In future experiments, the growth can be monitored by using a spectrophotometer with a higher detection limit to have more accuracy when the culture reaches high cell densities. Also, diluted culture samples could be measured using cuvette instead nose bottles. Plus, by plating dilution series of the culture it would be possible to estimate the amount of living cells in the culture. Another parameter that always needs to be considered is the freshness of the cells used to prepare the start culture, since it affects to the growth. To have more consistent conclusions, three biological replicates with three technical replicates of each should be measured.

An interesting observation was made when growing the *Shewanella sp.* #4 cultures the colour of the culture changes to pink in higher temperatures as shown in Figure 5C. The initial colour of the medium itself is transparent yellowish and when cells start to grow it turns to opaque but still yellow. Figure 5C shows photos of the cultures taken at three different time points. It is clearly visible that the change of colour occurs with time, turning the medium more pinkish. When comparing the growth curves and appearance of the pink colour (Figure 5A, Figure 5C) at 32h time point, the 5°C culture had approximately the same $OD_{550}$ value that 25 °C culture has at 24h time point. However, the pigment formation in 5°C was not as prevalent as it was in the culture grown at 25 °C. The same observation was made between cultures grown at 25 and 15 °C. The 25 °C culture had similar $OD_{550}$ value at time point 32h as 15°C culture had at 24h. Despite of this, the change of colour is more intense in 15 °C than in 25°C.

This phenomenon has been noticed when growing *Shewanella sp.* #4 cells in Marine Broth 25% medium from different starting materials, so a potential contamination is not likely. Other prokaryotes are also known to produce pigmentation that changes the colour of the cultures. In case of *Haloarchaeas*, soft orange colour is due to carotenoid pigments, which they produce in high cell densities (47). One of the reasons why these organisms produce carotenoids is due to its UV light absorption, and function in antioxidative stress mechanism. More concretely, the produced pigment by *Haloarchaea* is "Bacterioruberin", which protects the cells against the damage caused by radiation with 450-550nm of λ including UV light which can cause mutagenesis in the cell DNA.

Regarding the studied subspecies in this project, the varying pigment production in different temperature conditions could be explained by assuming that it might present the same biological role as in *Haloarchaea.* The unique difference among the three *Shewanella sp*. #4 cultures is the growth temperature and it can affect the cells through growth-related factors. If the pigment presents an antioxidant function required due cell density, the higher production at 15°C would be logical since the highest growth occurs at this temperature (48).

Another reason for this pigment formation could be that *Shewanella sp*. #4 is present in marine environments where, according to the thermocline phenomenon (49), the lowest temperatures are frequently found in the deep layers of the sea. In addition, annually 40% of the Baltic Sea is covered by ice (50) so, especially when the upper layers of the Baltic sea have very low temperatures and they might freeze. According to this, the *Shewanella* growing at cold temperatures would be protected against the UV radiation by either the ice or the water above them. If the role of the pigment here is similar to Haloarchaea, the low temperature might lead to lower pigment production due to UV-radiation shielded conditions in natural environments at 5°C temperature. Further studies would be needed to determine the cause of the pigment synthesis and how it is related to the temperature conditions.

### 4.2. Genomic DNA isolation.

Genomic DNA of *Shewanella sp*. #4 was extracted by acidic phenol:BCP isolation (see section 3.2.2.) to optimize PCR conditions for further experiments. In the phase separation, DNA was clearly visible in the interphase. The DNA was isolated, and the purified preparation was analysed to evaluate the quality and quantity of the DNA. The obtained results from the quantification and absorbance ratios indicating purity-level are shown in Table 8. There are standard acceptable absorbance ratio values of $A_{260}/A_{230}$ and $A_{260}/A_{280}$ ratios to evaluate the quality of the nucleic acid sample (51). The absorbance ratios $A_{260}/A_{280}$ and $A_{260}/_{230}$ must be ~ 1.8 in order to have organic compounds -free DNA. The obtained values of this ratios are around 2. This means that there is RNA contamination in the DNA sample. Given that gDNA samples were used for PCR optimization, the presence of remaining RNA in the sample was not relevant.

*Table 8. Measure of the yield and purity of the genomic DNA isolated from Shewanella sp. #4*

| | Replicate | Concentration (ng/µL) | $A_{260}$ nm | $A_{280}$ nm | $A_{260}/A_{280}$ | $A_{260}/A_{230}$ |
|---|---|---|---|---|---|---|
| *Shewanella sp.* #4 | 1 | 938.3 | 18.767 | 9.304 | 2.02 | 2.13 |
| | 2 | 3800.0 | 76.001 | 37.448 | 2.03 | 2.12 |
| | 3 | 492.7 | 9.953 | 5.016 | 1.96 | 1.91 |

Considering the acceptable values of the $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios for a good quality DNA sample the followed protocol was successful for isolating DNA from *Shewanella sp.* #4*.* The total yield from 200 mL of culture was approximately 5000 ng/µL. For proceeding with the test of the primers replicate 1 was used.

### 4.3. Optimization of PCR and quantification of total RNA parameters.

PCR products should appear as a unique clear band on an agarose gel. When degradation or multiple bands appear, there might be caused by the amplification of non-desired regions of the DNA. This happens when the parameters of the reaction are not correctly optimized or if the primer are not correctly designed. Thus, when obtaining a doubtful result, further testing is required (52). The optimal concentration of magnesium ions ($Mg^{2+}$) in the PCR-reaction was optimized prior to any actual experiments requiring PCR. A low concentration of $Mg^{2+}$ ions in the PCR reaction affects the hybridisation of the primer, decreases the processivity of the polymerase and compromises the hydrolysis by the exonuclease unit (53). This would have an impact on the specificity to the amplified target and in the yield of the PCR products and primer pair formation could be enhanced. If the concentration is too high, the reaction will not be specific and undesired products might appear (Nolan, T. *el al.* 2013). Considering the results of the performed experiment (Figure 6A) the amount of the optimal concentration of $MgCl_2$ needed for the reaction was 1.5mM. According to the manufacturer, the optimal $Mg^{2+}$ concentration for the Taq Polymerase is between 1.25-2mM (54). Indeed, when PCR-reactions with variety of higher $Mg^{2+}$ concentrations were conducted and visualized: there was a stronger blurred band in addition to the desired PCR product, which should have a size of 100 bp since these optimization studies were conducted with *16S* primer pairs. This phenomenon increases in the reactions with higher $Mg^{2+}$ concentration and it might be caused by the formation of primer dimers, as already mentioned.

To perform successful PCR-reactions, the amount of DNA-polymerase enzyme in the reaction needs to be optimized as well. Increasing the amount of Taq DNA polymerase can sometimes increase PCR efficiency, but adding more enzyme might increase the amplification of nonspecific PCR products instead of the desired one (52). The minimal amount of the enzyme was evaluated by performing the PCR reaction with enzyme amount ranging from 1U to 3U. The PCR products were analysed on agarose gel Figure 6B. In the negative control without DNA template, indicated as no template in the figure, there is a band suggesting that there was a contamination in that sample. The contamination might be present also in the reactions, since the PCR products have the same size as the contamination. The control lane with no enzyme, was clear as expected, since the enzyme is

responsible for the DNA amplification and without it there should not be any. A strong band with expected size of 100 base pairs appeared with all enzyme amounts tested (Figure 6B). As a conclusion, one unit of polymerase is more than enough to perform a PCR experiment. Another aspect to comment is that this gel (Figure 6B) should have been run for a longer period to let the size marker separate properly allowing us to distinguish the size of the PCR products and the contamination in a more reliable way.

To be able to save the highest possible amount of isolated total RNA for further experiments, the required amount to visualize it in the agarose gel was determined (Figure 6C). For RT reactions of this project, the required amount of RNA is not high, but to load the gels when checking RNA integrity, higher input amount is needed. The gel electrophoresis conducted with different input RNA amounts showed clearly the ribosomal 23S and 16S bands with all the concentrations tested, so 250 ng is enough to visualize the ribosomal bands of the total RNA and check its quality.

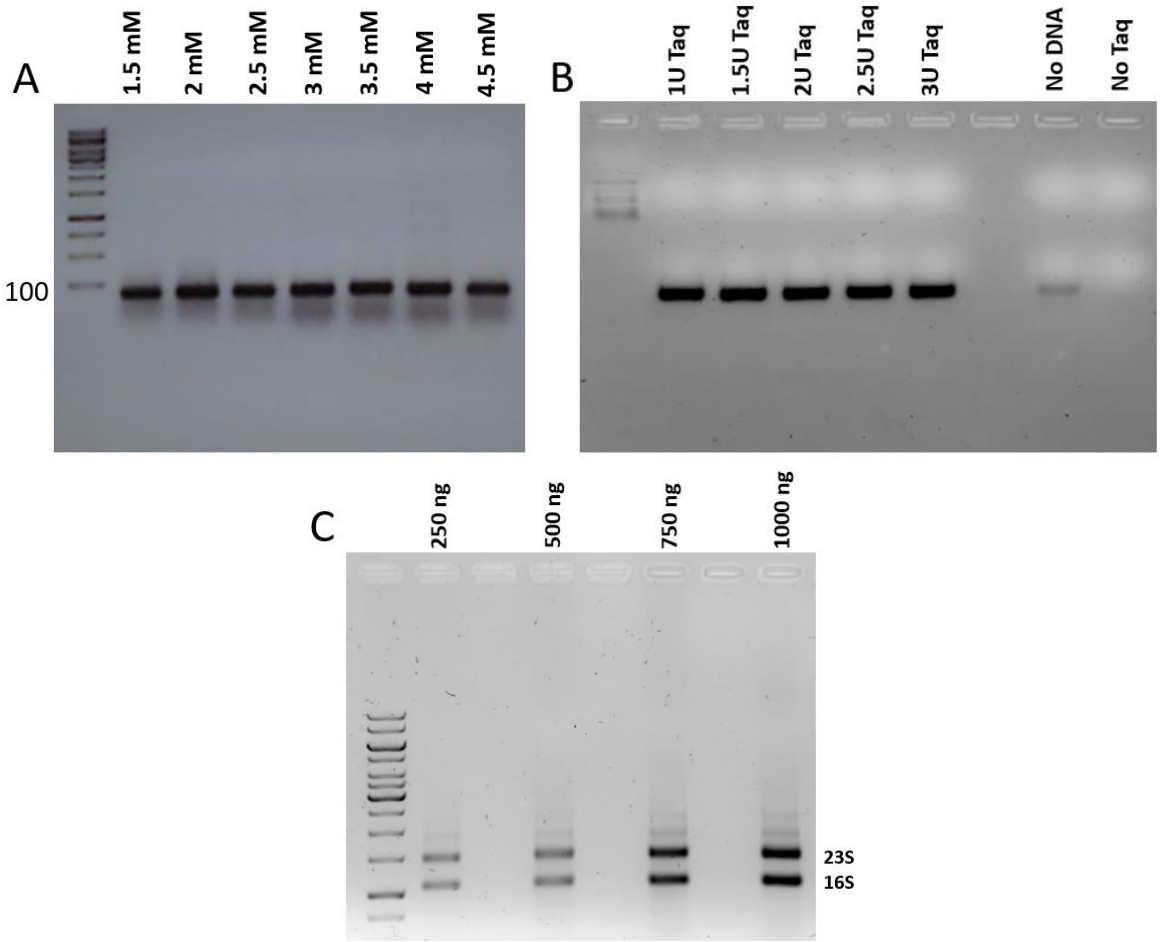


*Figure 6. Visualization of agarose gel electrophoresis of the optimization of PCR parameters and defining the minimal required input of total RNA to the gel. A) Analysis of PCR amplification with different $Mg^{2+}$ concentrations. The expected amplicon size of the gene 16S applied here was 100 bp. B) Agarose gel electrophoresis visualization of the PCR reactions with different amount of Taq polymerase. The same 16S primer pairs were applied in this experiment. C) Agarose gel electrophoresis with different amount of total RNA.*

To conclude these results, the following parameters for PCR reactions applied in this project were obtained: 1.5 mM of $MgCl_2$ and 1U of Taq polymerase. The minimal amount to visualize total RNA in agarose gel was 250 ng.

### 4.4. Test of the primers on genomic DNA.

Primer pairs amplifying the selected genes and melting temperatures of the primers are shown in Table 4. Primers designated to be applied in qPCR were first tested by performing a gradient PCR to optimize the annealing temperature of each primer pair (Figure 7). Since all of them were planned to be utilized with the same qPCR program, the melting temperature suitable for all primer pairs was required.

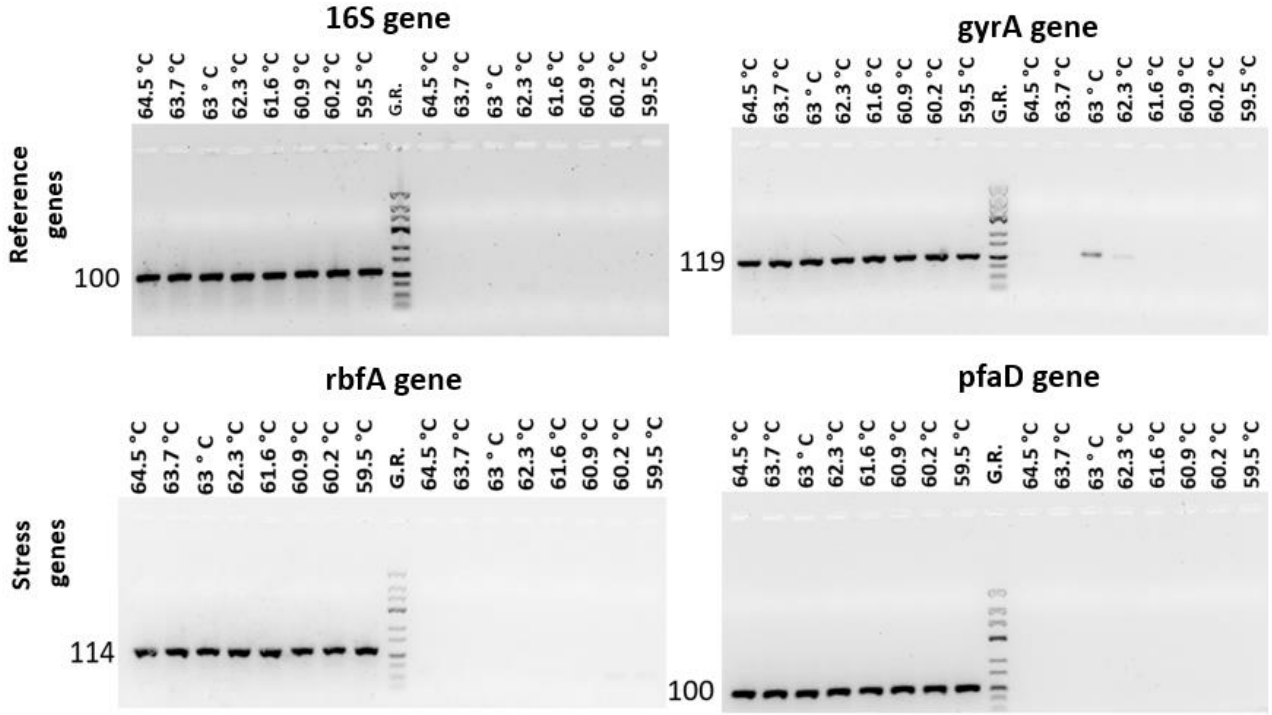


*Figure 7. Gradient PCR of each one of the studied genes to determine the optimal melting temperature of the primer pairs. The positive PCR reactions are shown on the left side of the ladder (indicated as G.R.) and the negative controls without the template on the right side. The designed primers bound specifically to the target sequence with all temperatures tested and one clear product band can be seen in gel electrophoresis analysis. Expected product sizes are indicated.*

The obtained results are shown in Figure 7. This figure shows the gel visualization of the PCR products for each gene in a range of applied melting temperatures between 64.5-59.5°C. When the primer pair amplify the selected sequence a clear unique band with the expected amplicon size should appear. Indeed, a single band of correct size was observed with all primer pairs (Figure 7). The negative controls without template serve as indicator of non-target DNA amplification at the indicated temperature and inform about the potential contamination of the prepared mix with exogenous DNA. In *gyrA* negative control reactions at 63 and 62.3°C one band can be seen. This might indicate primer pair dimer formation and its amplification but considering that it has the same expected product size it is more likely that a punctual contamination occurred in those tubes. Since

there is not much difference between the primer specificity among the tested temperatures, the selected annealing temperature for the qPCR protocol was 64.5 °C.

### 4.5. Total RNA isolation and DNAse treatment.

Both RNA quality and quantity are key factors to ensure a reliable and accurate gene expression analysis. Different isolation methods for total RNA were tested in order to establish a method leading to the best purity and integrity. PCR and qRT-PCR are very sensitive methods, so the ideal RNA sample should not contain genomic DNA, proteins or enzyme inhibitors such as nucleases. In addition, ethanol and phenol remnants from the applied isolation protocol should be removed before proceeding with the sample analysis.

There are three main principles applied in total RNA extraction techniques: organic, paramagnetic particles and silica-based spin column techniques from which the spin columns are typically found in commercial extraction kits. They can be classified into 4M guanidium thiocyanate-based and phenol and SDS-based methods (55). The selection of the method needs to be made according to the particular purpose of the total RNA sample and the availability of resources. Different existing methods in order to find the more suitable one to prepare total RNA samples for qRT-PCR have been previously compared using bacterial lysate as starting material (56).

In this project, home-made acidic phenol:BCP with a chaotropic agent isolation and RNeasy® Mini kit from Qiagen were selected to isolate total RNA from *Shewanella sp.* #4. The comparison between total RNA yield, purity ratios and estimated time required for obtaining the samples were evaluated (Table 9).

*Table 9. Comparison between RNAsy Qiagen kit and acidic phenol:BCP isolation.*

| | Replicates | Yield (ng/µL) | 260/280 | 260/230 | Required time (h) |
|---|---|---|---|---|---|
| **RNAsy Mini kit samples** | 1 | 154.5 | 1.89 | 1.22 | 0.75 |
| | 2 | 93.5 | 1.84 | 1.26 | |
| | 3 | 237.1 | 1.91 | 1.59 | |
| **Acidic phenol:BCP samples** | 1 | 13714.7 | 1.99 | 1.44 | 4 |
| | 2 | 14401.5 | 1.95 | 1.58 | |
| | 3 | 3426.5 | 2.02 | 1.30 | |

The yield of total RNA obtained with the commercial kit was significantly lower than the amount obtained using acidic phenol:BCP isolation protocol. RNAsy Mini kit (Qiagen) is recommended to extract samples for qRT-PCR experiments from in animal tissues. This might explain the poor yield of total RNA since the lysis buffer provided might have not been able to break the cell wall of the bacterium. The hands-on time required when using acidic phenol:BCP protocol is ~4 times longer than with the RNAsy Mini kit (Table 9). However, to isolate equal amounts of total RNA with these methods, RNAsy Mini kit isolation protocol would need to be repeated 50 times, increasing the time required to 42h. The purity-level based on $A_{260}/A_{280}$ values of the samples was similar with both

methods. However, $A_{260}/_{230}$ ratios were poor. This might be due to the presence of phenol, ethanol or, more probably, guanidine thiocyanate since it is included in the washing buffer of the kit and in the acidic phenol:BCP as well. PCR and qPCR reactions can be inhibited by a wide variety of inhibitors (57) and guanidinium is one of them. The presence of it should be minimized in the final total RNA samples designated to the qPCR analysis (58). Indeed, from Table 10, which shows the purity values for the final isolated total RNA, it is visible that the $A_{260}/A_{230}$ ratio is much higher, thus this problem was not present after washing the pellet.

Although, it is recommended to check the RIN (RNA integrity number) value when extracting total RNA for gene expression analysis (59), in this preliminary project the integrity of the sample was evaluated from the visualization of the ribosomal bands in 1% agarose electrophoresis analysis. In the case of RNA sample with optimal integrity, bands of the larger ribosomal RNA subunits (23S, 16S) should be seen clearly. The bands should be of correct size and no visible smear from degradation should be visible. The presence of genomic DNA in the sample would be a band close to the wells. The DNA present in the sample might be carried over and amplified in the qRT-PCR instead of the desired cDNA, so it must be avoided.

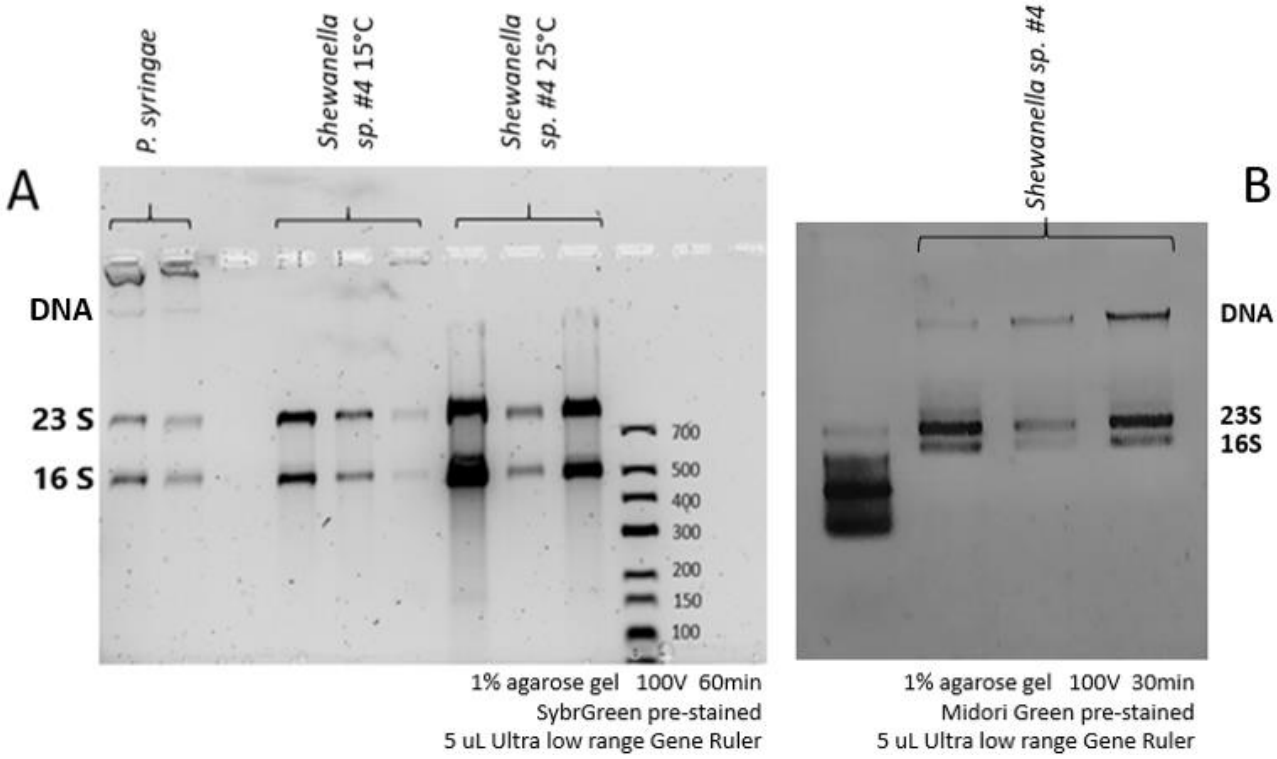


*Figure 8. Electrophoresis analysis and comparison of the total RNA samples obtained with two different methods. A) Total RNA samples extracted from P. syringae pv. Phaseolicola HB10Y and Shewanella sp. #4 using acidic phenol:BCP method. On left are duplicate of total RNA isolation samples from P. syringae and on right total RNA isolation triplicates from Shewanella sp. #4 grown in either 15 or 25°C B) Shewanella sp. #4 total RNA samples extracted with RNAsy kit. The right-most lane presents more remaining gDNA. The ribosomal RNA bands are indicated as well as DNA where it was visible.*

The total RNA isolated in this project was visualized in agarose gel and the bands from all the samples appeared to be correct (Figure 8) In Figure 8A (acidic phenol:BCP samples) and in Figure 8B

(RNAsy kit samples) the 23S and 16S ribosomal bands can be clearly visualized, concluding that the integrity of the samples is correct.

To further test home-made acidic phenol:BCP with a chaotropic agent protocol HB10Y was used as control since this method has previously been optimized in our laboratory for this species while it has not been tested *Shewanella sp.* #4. Figure 8A shows the total RNA samples extracted from *Shewanella sp.* #4 grown at 15°C and 25°C on the right and from HB10Y using home-made acidic phenol:BCP on the left. The protocol seemed to work equally well with both organisms tested. However, in HB10Y samples, more DNA was present after the isolation (Figure 8A). Thus, the home-made acidic phenol:BCP RNA isolation method is applicable to both species.

The Figure 8B shows total RNA samples extracted from *Shewanella sp.* #4 using the commercial kit. The RNA integrity is correct since the ribosomal bands can be clearly seen but more remaining gDNA was present in the samples. Considering the obtained yield and the remaining DNA left on the RNA isolated samples, the method selected to isolate the total RNA samples for the purposes of this study was home-made acidic phenol:BCP with a chaotropic agent extraction. The integrity and purity of the total RNA samples extracted was checked to be acceptable. Thus, this method allows to extract RNA and DNA from the same sample as described (Figure 4).

Having a total RNA isolation protocol capable of producing high yield and DNAse free samples is crucial in gene expression experiments. Thus, the obtained RNA samples were treated with DNAse enzyme, to ensure that no carry-over DNA from the isolation steps was left in the sample. The obtained total RNA concentration before and after this treatment, plus the absorbance ratios to evaluate the purity of the final samples are shown in Table 10. After the DNAse treatment, nucleic acid concentration was significantly lower. The decreased amount of nucleic acids is partially due the degradation of the carry-over gDNA. However, the initial amount of gDNA was low, since it was not visible in the agarose gel (Figure 9). The decreased amount of nucleic acids after DNAse treatment might also indicate that some RNA was lost during the precipitation step after the DNAse. qPCR is a very sensitive method and all remaining DNA needs to be removed from the final samples. Finally, the absorbance curves for these samples are displayed in appendix 8.3.

*Table 10. Yield and purity of the total RNA samples utilized in the next steps of the project before and after the DNAse treatment.*

| | Sample growth temperature (°C) | Concentration (ng/µL) | $A_{260}$ | $A_{280}$ | $A_{260}/A_{280}$ | $A_{260}/A_{230}$ |
|---|---|---|---|---|---|---|
| **Non-DNAsed samples** | 5°C | 3695.6 | 92.391 | 44.86 | 2.06 | 2.14 |
| | 15°C | 10164.7 | 254.119 | 121.169 | 2.10 | 2.35 |
| | 25°C | 10123.3 | 253.084 | 121.219 | 2.09 | 2.34 |
| **DNAsed samples** | 5°C | 563.2 | 14.081 | 7.017 | 2.01 | 2.5 |
| | 15°C | 355.7 | 8.892 | 4.395 | 2.02 | 2.46 |
| | 25°C | 403 | 10.076 | 4.972 | 2.03 | 2.46 |

The integrity of DNAse treated samples was tested with gel electrophoresis (Figure 9) by the visualization of the ribosomal bands. The samples were compared to untreated samples. All the samples presented a correct pattern of ribosomal RNA bands; thus, the integrity was maintained.

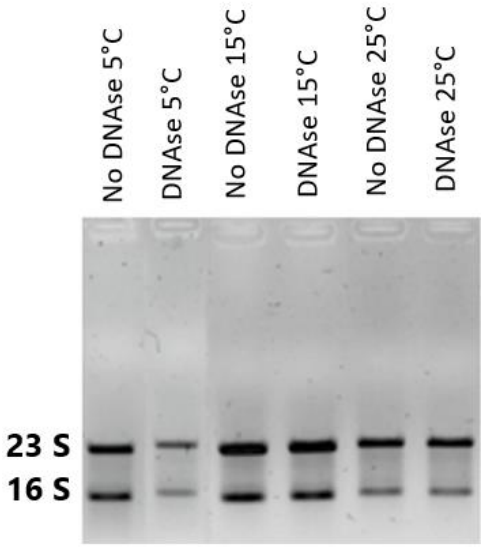


*Figure 9. Electrophoresis analysis composition of total RNA integrity before (left) and after DNAse treatment (right). The integrity of the DNAse treated samples was correct since 23S and 16S ribosomal bands can be seen appreciated. No gDNA bands were visible in the gel.*

### 4.6. Reverse transcription.

After testing the quality of the total RNA samples, reverse transcription (RT) reaction was performed to convert the RNA to cDNA (see section 3.2.5.). The RT reaction protocol was tested by applying the produced cDNA to PCR with selected primer pairs (Figure 10). It is important to test the primers also with cDNA since the structure is different from the gDNA and it might affect the specific binding of the primers. The cDNA derived from *Shewanella sp*. #4 grown in different temperatures was tested with the primers to ensure their correct binding in all templates designated to gene expression analysis.

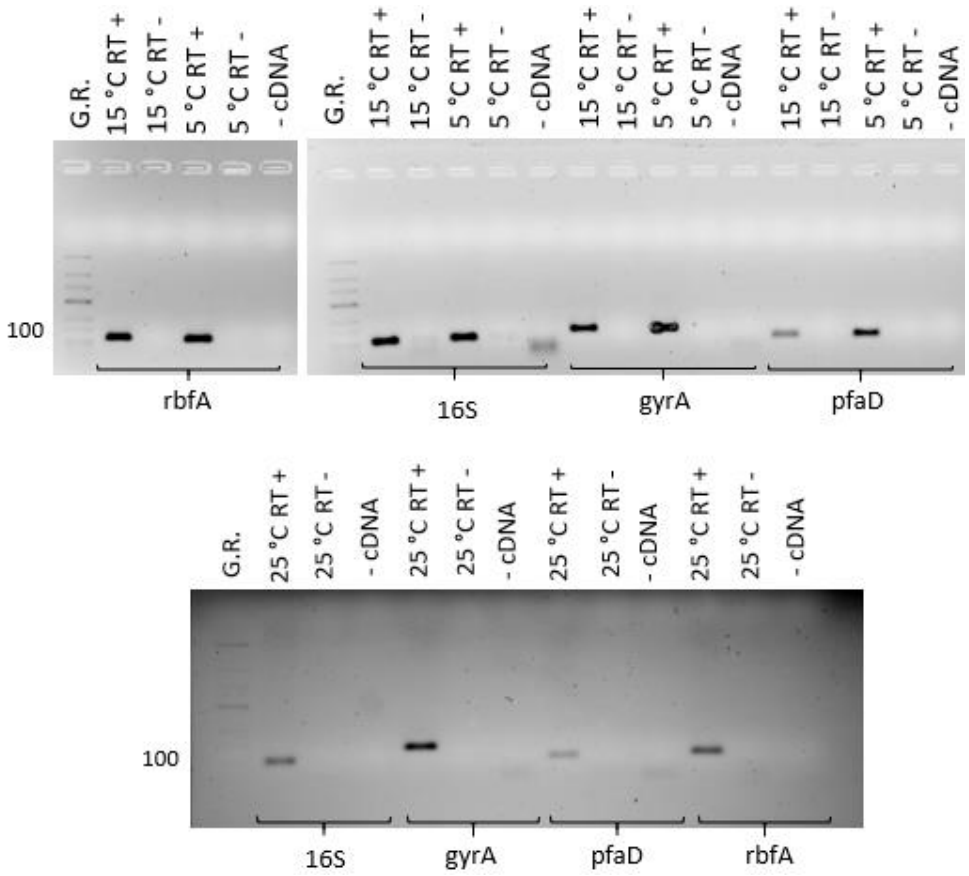


*Figure 10. Visualization of PCR products with selected primer pairs and using cDNA as template. cDNA from Shewanella sp. #4 grown in 5, 15, 25°C was applied. RT+ samples contain the cDNA precipitated from the RT reaction, RT- samples are the precipitations from RT-reactions without using RT-enzyme and –cDNA samples are PCR controls without any template. The primer pairs are indicated below the gels and G.R. abbreviation indicates the size marker position.*

The PCR products using cDNA obtained from the RT reactions were visualized by agarose gel electrophoresis and bands of correct sized products were seen with all primer pairs from all growth conditions (Figure 9). The negative controls without template (-cDNA) were expected to be clear, since no amplification could occur. Indeed, negative controls of the PCR were clear for all the genes except with *16S* primer pair (Figure 10) where a contamination band appeared. No product could be seen from the RT– reactions without reverse transcriptase, which indicates that the observed bands from the actual RT-reactions are the cDNA synthetized by this enzyme. Preparing gDNA-free samples are one of the main problems found in sample preparation for qRT-PCR analysis (60). The positive reaction of *pfaD* gene from the 15 °C sample appears weaker; this might be due to pipetting error when preparing the sample or while loading it to the gel. Considering these results, the reverse transcription protocol was successful, and the primers bound specifically to the cDNA synthetized.

### 4.7. qPCR reaction.

The real-time qPCR was performed to evaluate differences in expression levels of selected genes of *Shewanella sp.* #4 grown in low-and high-temperature stress conditions. As discussed in methods, primer pair efficiencies need to be estimated (61). For this purpose, primer efficiency experiment was repeated twice with three technical replicates each, *16S* primer pairs were only tested once since the first round resulted in unreliable results and indicated that primers were not binding correctly (Figure 11).

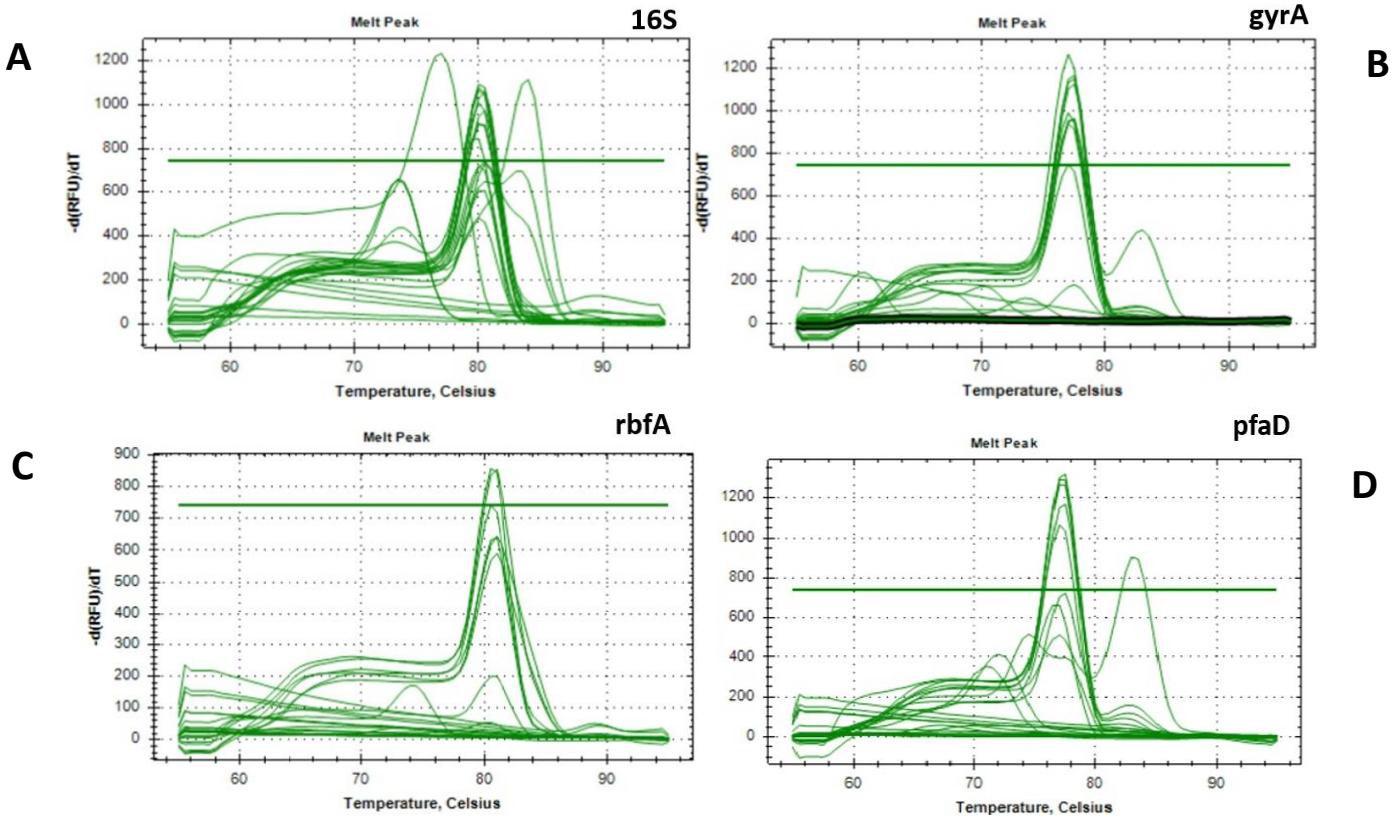


*Figure 11. Melt peak from the primer efficiency analysis. (A) 16S gene. (B) gyrA gene. (C) rbfA gene. (D) pfaD gene. One amplified product can be seen from gyrA and rbfA melt peak analysis while multiple products were amplified in 16S gene qPCR reaction. A contamination peak appears in pfaD melt peak. Finally, 16S gene was discarded from the final gene expression analysis.*

The qPCR analysis results are displayed in three graphs: Quantification peak (Figure 2), Melt curve (see in appendix 8.6.) and Melt peak (Figure 11). From the analysis of the melt peak it is possible to differentiate the desired amplified product from the non-targeted ones due to the differences in their melt temperatures (62). In the peak of *gyrA* (Figure 11B), all the amplified products presented the same melt temperature, thus we can consider that all the amplified products in the qPCR analysis of *gyrA* are the target sequence. Contrarily, in *16S* melt peak, different products can be observed (Figure 11A), they might be due to foreign DNA contamination or caused by the unspecific primer binding. This experiment should be repeated to rule out a potential contamination of the *16S* reaction but if the same result appears, the primer design needs to be revised. According to the melt peaks, the products amplified with the stress gene primers are homogeneous. Thus, the

primers bound specifically to the template. There is one exception in *pfaD* (Figure 11B) reaction since one peak has a different melt temperature that is likely due to an external DNA contamination. Another remarkable aspect is that the intensity of fluorescence of *rbfA* is considerably lower than the signal from other genes and only few reactions overcome the threshold (Figure 11B).

The *16S* gene has been shown to serve as good reference gene of choice (Kirk et al., 2014). It is thought that ribosomal RNA genes serve as good reference genes since they maintain housekeeping functions, but it was proven that there is variability in the expression of those genes (63). There are studies comparing *gyrA* and *16S* with other reference genes under different environmental conditions in *Shewanella* (6). Thus, they might be selected consciously or other reference genes that have been proven to work successfully could be selected. Such genes are for example: *gyrB* (DNA gyrase B subunit), *rpoB* (RNA polymerase, beta subunit), *atpD* (ATP synthase F1, beta subunit), *gap* (glyceraldehyde-3-phosphate dehydrogenase), *pnp* (polyribonucleotide nucleotidyltransferase) (64).

The primer pair efficiency values were calculated as discussed in the methods section of this thesis and the resulting values are show in Table 11.

*Table 11. Efficiency values for each primer pair for the selected genes.*

| | Gene | Series | Efficiency value | Efficiency value (%) | $R^2$ |
|---|---|---|---|---|---|
| **Reference genes** | *16S* | 1 | 1.31 | 31 | 0.463 |
| | | 2 | n.d. | n.d. | n.d. |
| | *gyrA* | 1 | 1.12 | 12 | 0.984 |
| | | 2 | 1.18 | 18 | 0.967 |
| **Stress genes** | *rbfA* | 1 | 1.57 | 57 | 0.237 |
| | | 2 | 1.18 | 18 | 0.248 |
| | *pfaD* | 1 | 1.11 | 11 | 0.864 |
| | | 2 | 1.11 | 11 | 0.999 |

*Note1: n.d. = not determined - 16S gene primer pair efficiency was only tested once, so no data (n.d.) is shown in the 2nd series of this gene.*

The optimal primer efficiency value should be above 90% (65) and the obtained values (Table 11) here were much lower; for *16S* and *gyrA* genes the averaged efficiency values were 31 and 15%, for the stress genes *rbfA* and *pfaD* they were 38 and 11% respectively. Primer efficiency values depend on the performance of the dilution series and the utilised instrument. Performing the experiments in replicates helps to minimise this variability. Accordingly, the same instrument should be utilised to analyse the primer efficiency and the samples, at least three qPCR replicates should be analysed for each one of the dilutions and a higher volume of cDNA dilution sample should be prepared for each final concentration to diminish the variability (65). As during this experiment, the same thermocycler was utilised to analyse both samples and primer efficiencies so this factor should not be a source of variability. In the context of this project, the main source of variability in the estimation of the primer efficiency might be the cDNA dilution preparations. This is corroborated by the lack of signal in most of the samples with highest dilution. This is an indicator of volume

transferring error in the preparation of these samples (65). Also, there is lack of signal in some of the technical replicates of the less diluted cDNA samples, signalling of inaccuracy in the dilution preparation. There are also other factors to consider such as an inaccurate 1:1 ratio between forward and reverse primers or not precise pipette calibration (*Thermo Fisher Scientific*, 2017).

When the efficiency values were obtained and the appropriate cDNA dilution was known, the cDNA samples were analysed with the same qPCR program and protocol. This experiment was performed two times with the same biological batch with three technical replicates each time (Figure 12,Table 12). The results for the two experiments conducted with the same biological batch were analysed separately. In Table 12, the results from the experiment 2 are shown. The results of the experiment 1 and 2 are shown together in the appendix 8.5.

*Table 12. Raw data of the qPCR analysis. The table shows the Ct average, the standard deviation and variation.*

| Gene | Temperature (°C) | Ct average | Standard deviation (±) | Variance |
|---|---|---|---|---|
| ***gyrA*** | 5 | 23.41 | 3.72 | 13.83 |
| | 15 | 18.55 | 1.94 | 3.77 |
| | 25 | 18.43 | 7.48 | 55.99 |
| ***rbfA*** | 5 | 18.44 | 0.85 | 0.72 |
| | 15 | 20.24 | 1.58 | 2.51 |
| | 25 | 19.55 | 0.43 | 0.18 |
| ***pfaD*** | 5 | 25.02 | 1.05 | 1.10 |
| | 15 | 33.03 | 2.05 | 4.19 |
| | 25 | 24.05 | 36.59 | 5.88 |

In this project, growth at 25°C is used to contrast the gene expression changes among low and high temperatures. The selected stress genes for this study are related to the ability of *Shewanella sp.* #4 to thrive in low temperatures. Thus, significant gene expression changes should appear at 5 °C but not at 25 °C with *pfaD* and *rbfA* genes when comparing with the expression at control temperature 15°C. As discussed in the introduction, higher initial amount of the template requires less amplification cycles to overcome the fluorescence threshold, so the sample will have a lower Ct value. The obtained Ct value is indirectly correlated with the level of expression of a gene: lower Ct value in the quantification curve means a higher gene expression level. Considering this, the Ct value of the 5°C samples was expected to be lower than the Ct value of 15°C and 25°C samples for the stress genes and the number of cycles to overcome the fluorescence threshold to be lower. For *rbfA* and *pfaD*, 5°C sample Ct average values are lower than 15°C Ct values (Table 12) as expected. As an exception, *pfaD* 5 and 25°C Ct average values are similar but considering the standard deviation value (Table 12) of 25°C samples this value might be discarded.

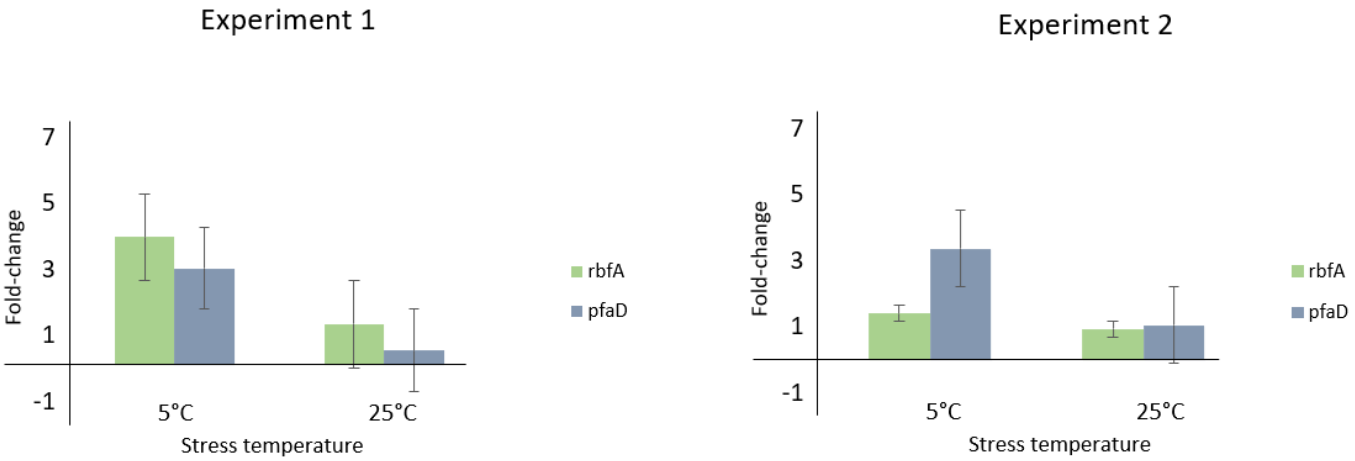


*Figure 12. Relative fold-change of pfaD and rbfA in high and low temperatures. Gene expression level at 15°C was utilized as a control. The trend of the low-temperature related gene expression is consistent: pfaD and rbfA are more expressed in Shewanella sp. #4 grown at low temperature.*

In Figure 12, the fold-change of the relative gene expression analysis (Equation 2) are shown for each one of the genes, considering 15°C as control temperature. The fold-change threshold from which the expression difference was significant was 1.5. This means that when the graph bar overcome 1.5-fold-change value, the expression was higher at the lower temperature. *pfaD* gene is expressed at higher level at 5°C and there is not significant difference between the expression levels at 15°C and 25°C, the results are consistent among the two experiments. For *rbfA* different results were obtained between the two experiments. In Experiment 1, there is no difference in the expression at 5°C and not at 25°C, while in Experiment 2, there is not significant difference in gene expression of *rbfA* among the three studied temperatures.

The overall trend in the gene expression was the expected one with some observable anomalous values. These might be explained by the inaccuracy of the pipetting since the standard deviation shows that there is a huge variability among the Ct values among the replicates.

Ct values were exported from Maestro Software BioRad. Although all the experiments were performed in three technical replicates, not all of them produced a significant signal. This affected to the Ct average value of the dilution and to the standard deviation. In future experiments, 4 more replicates might be included if possible as Svec *et al*. did (2015) to have as much valid replicates with the amplified desired cDNA as possible. Moreover, poor primer efficiency values were considered in the Equation 2 which was applied to estimate the relative gene expression, this might limit the reliability of the obtained results.

No template and RT- reactions should not present any amplified product since no DNA should be present in the sample. The RT- controls were negative for the three temperatures with four exceptions (data shown in appendix 8.5. ). Considering this and that one mix was prepared for each one of the genes, it is unlikely that the prepared master mixtures were contaminated. The positive RT- controls might had been due to remaining DNA in the cDNA preparation. The no template

controls might had been positive for the presence of external DNA contamination in the added water instead of template, given that all of them were positive.

It is not possible to reliably conclude if there is differential *pfaD* or *rbfA* gene expression with temperature changes from the obtained data. However, the data shows a trend of higher expression of selected stress genes in lower temperatures. This indicates that selected stress genes might be actively expressed, when *Shewanella sp.* #4 encounters low temperature conditions. There preliminary findings encourage further research and verification of the results. In a relative quantification study, the researcher is usually interested in comparing the expression level of a gene between different samples. Therefore, the sample maximization method is highly recommended because it does not suffer from technical or run-to-run variation between the samples. (Derveaux, S. 2009). To have accurate results, a reproducible method and adequate mathematical model for the analysis is required. Data must be normalized to remove the technical variation, ending up only with the true biological changes. In this project, preliminary and indicative results were obtained. Thus, the utilised protocol should be validated on a large set of reference samples to verify that the targeted genes present the same expression levels at the same temperature in different biological batches (Vanneste, K. et al, 2018). As already discussed, more than one housekeeping gene should be used in the analysis to have an stable guideline to compare the expression of the stress genes with the standard genes (18).

## 5. CONCLUSIONS.

The main goal of this thesis project was to analyze the relative gene expression of *rbfA* and *pfaD* genes and infer its role in cold-induced response. The initial hypothesis was that *rbfA* and *pfaD* will present a higher gene expression in *Shewanella sp.* #4 grown at 5°C. Contrarily, cells grown at 25 °C will not present a differential gene expression, being *16S* and *gyrA* reference genes and 15°C control temperature in both cases. In order to achieve it, step-related objectives were set and regarding them the following conclusions were made:

- *Shewanella sp.* #4 presents a quick growth during the first hours after the set of the culture at 25 °C. However, it presents a higher and quicker growth in later stages at 15°C, which is the standard temperature for growing it under laboratory conditions. Also, as would it be expected, this subspecies presents relatively slow growth at 5°C as shown in 4.1..

- Pigment synthesis formation can be seen in *Shewanella sp.* #4 grown in Marine Broth 25% culture medium before 24 h when the culture is grown at 15 °C and after 24 hours of growing it at 25°C. More subtle pigment synthesis in cultures grown at 5°C.

- Acidic phenol:BCP with chaotropic agent total nucleic acid isolation is a suitable method in *Shewanella sp.* #4 to extract high quality total RNA and DNA.

- *gyrA* was proven to be a suitable house-keeping gene in this experiment. Contrarily, *16S* gene could not be utilized as reference gene due to the inconsistent results and variation in gene expression.

- The observed trend of the relative gene expression of *rbfA* and *pfaD* is consistent among the two experiments performed. The expression level of these two genes is higher when *Shewanella sp.* #4 cells are grown at 5 °C, thus the initial hypothesis was preliminary validated by the results of this project.

## 6. FUTURE PERSPECTIVES.

This project can serve as starting point for further research with *Shewanella sp.* #4. Regarding the total RNA isolation method applied, other authors have obtained higher yields with RNeasy® Mini kit (66) or have tested it as an hybrid protocol of TRIzol/ RNeasy® Mini kit (56). Thus, RNeasy® Mini kit from Qiagen could be more extensively tested. With the studied subspecies, utilising more effective lysing method could be tested to see whether the yield could be improved. This knowledge would be valuable, since applying the kit would save a lot of time if higher yields could be reached. Another future prospective extracted from the conclusions is to assess the differences in the pigment synthesis when growing *Shewanella sp.* #4 under different temperatures, by firstly identifying the pigment and then relating it with different environmental changes.

As previously discussed, the obtained results in this bachelor's thesis project are preliminary but indicative. Considering the trend in differences in the expression levels observed in the studied genes the initial hypothesis was validated, thus *pfaD* and *rbfA* should be further investigated. It would be necessary to repeat the described experiment with, at least, three biological batches and three technical replicates to obtain definitive conclusions. This way the potential variability could be diminished. In order to obtain reliable results, primer efficiency values must be estimated considering the mentioned potential sources of error in the described experiment. Regarding the gene expression analysis, several house-keeping genes should be utilised as reference genes to improve the normalization of the gene expression. Moreover, more genes related to low temperature changes, such as other eicosapentaenoic acid (EPA) synthesis genes, could be analysed (67) to have a better understanding of the mechanisms behind the cold-shock responses of this unclassified subspecies. In addition, heat-shock related genes could be analysed to complete the *Shewanella sp.* #4 profile regarding temperature stress.

The project could be extended to other stress-related genes, differentially expressed under different environmental changes. As stated in the introduction, the selected condition for this project to start with was temperature stress, but other conditions such as oxidative, pH, salinity, cell density and lack of nutrient stresses could be explored. This way there would be a wider understanding of *Shewanella sp.* #4 stress responses when facing different *stimuli* and of which genes are involved in the process. In order to achieve this, the same process described in this thesis project may be used as starting point. Finally, this information could be compared with other ongoing studies to contribute to the characterisation of *Shewanella sp.* #4.

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