MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Comparison of RfbC gene expression in different chassis cells of the marine bacterium P. a. Hao2018 for the study of rhamnose.**

First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
Qiaobing YUE

Scientific supervisor
Tetiana SHCHERBATIUK, Dr. Sc., Prof.

Reviewer
Iryna VOLOSHYNA, Ph.D., As. prof.

Kyiv 2024

KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN

Faculty: Chemical and Biopharmaceutical Technologies
Department: Biotechnology, Leather and Fur
First (Bachelor's) level of higher education
Specialty: 162 Biotechnology and Bioengineering
Educational and professional program Biotechnology

APPROVE
Head of Department of Biotechnology, Leather and Fur, Professor,
Doctor of Technical Science
Olena MOKROUSOVA

_____
«___»_____2024

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Qiaobing YUE

1. Thesis topic **Comparison of RfbC gene expression in different chassis cells of the marine bacterium P. a. Hao2018 for the study of rhamnose.**

scientific supervisor Tetiana Shcherbatiuk, Dr. Sc., Prof.

approved by the order of KNUTD "__"_____2024, №_____

2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments_____

EXECUTION SCHEDULE

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	From 01 April 2024 to 11 April 2024	
2	Chapter 1. Literature review	From 06 April 2024 to 20 April 2024	
3	Chapter 2. Object, purpose, and methods of the study	From 21 April 2024 to 30 April 2024	
4	Chapter 3. Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
6	Draw up a bachelor's thesis (final version)	From 20 May 2024 to 25 May 2024	
7	Submission of qualification work to the supervisor for feedback	From 25 May 2024 to 27 May 2024	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	27 May 2024	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	31 May 2024	
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	03 June 2024	

I am familiar with the task:

Student _____Qiaobing YUE

Scientific supervisor _____Tetiana SHCHERBATIUK

SUMMARY

Qiaobing YUE.Comparison of RfbC gene expression in different chassis cells of the marine bacterium P. a. Hao2018 for the study of rhamnose.– Manuscript.

Marine bacteria are one of the most abundant and diverse microbial populations in Marine ecosystems. It is well known that the Marine environment is very complex, which also makes Marine microorganisms have some unique functions to cope with the complex environment, such as the ability to produce exopolysaccharides. The Marine bacterium *Pseudoalteromonas agarivorans* Hao 2018 was obtained from the body surface biofilm isolation of abalone. It was found that the antioxidant activity of exopolens produced by this bacterium would change regularly under the disturbance of environmental factors, and the composition and proportion of monosaccharides in exopolens and the structure of polysaccharides were significantly changed. Based on transcriptomic analysis, the synthesis module of the rhamnoose precursor in P.a.Hao 2018 exopolysaccharide was successfully identified. In this study, the second key enzyme gene RfbC (DTDP-4-dehydrorhamnoose 3,5 differential isomerase) in the synthesis module of the rhamnoose precursor was cloned and heterologous expressed. *Escherichia coli* BL21 and *Bacillus subtilis* 168 were selected as expression hosts, and pHT43 was used as plasmid vector to construct recombinant expression vector. After induced expression, the enzyme protein was purified. The expression of RfbC in different hosts was detected by BCA protein concentration assay kit. The implementation of this study laid a foundation for the exploration of the synthetic pathway of Marine bacteria rhamnose, and provided a theoretical basis for the wide application of *P.a*.Hao 2018 rhamnose.

Key words：*Marine bacteria; Rhamnose; dTDP-4-dehydrorhamnose-3,5 heterotropic isomerase; Allogenic expression*

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Current Research situation

In the Marine environment, there are many factors that will affect the adaptability and viability of bacteria, and one of the important factors is the regulation of gene expression.In recent years, researchers have conducted extensive studies of gene expression in Marine bacteria to reveal differences in the amount of gene expression in different chassis cells and to further understand the impact of these differences on the adaptability and viability of bacteria.However, the study on the difference of RfbC gene expression in different chassis cells in Marine bacteria is still limited.Therefore, the study of RfbC gene expression differences in different chassis cells is of great significance to further understand the survival strategies and adaptive mechanisms of Marine bacteria.

1.2 RfbC Gene Overview

At present, there are limited studies on the differences in the expression levels of RfbC gene in different chassis cells in Marine bacteria.The RfbC gene encodes an important enzyme in Marine bacteria that is involved in the synthesis of bacterial lipopolysaccharides, an important component of bacterial cell walls that is essential for bacterial survival and adaptability.Therefore, the study of RfbC gene expression differences in different chassis cells is of great significance for us to further understand the survival strategies and adaptive mechanisms of Marine bacteria.

1.3 dTDP-4-dehydrorhamnose-3, 5-aberration isomerase Overview

dTDP-4-dehydrorhamnose-3, 5-aberration isomerase is involved in many key steps in the biosynthesis process.This enzyme is an essential enzyme in a variety of biosynthetic pathways and can catalyze the conversion of dTDP-4-dehydrorhamnose-3, 5-differential isomerization to dTDP-3-dehydrorhamnose-

3.Cloning and heterologous expression can provide strong support for the study of biosynthesis mechanism and are important means in the field of biosynthesis.

1.4 Heterologous expression

Biotechnological means Marine bacteria are one of the most abundant and diverse microbial groups in Marine ecosystems and have a significant impact on the structure and function of Marine ecosystems.Allogeneic expression is an important biotechnology method that can produce target proteins by introducing foreign genes into host cells.The study of heterologous expression is of great significance to explore the function of

Marine bacteria, egg engineering and the protection and management of Marine ecosystems.

1.5 Background In the field of biosynthesis

there have been extensive studies on DTDP-4-dehydrorhamnose-3, 5-aberration isomerase.Through gene cloning and heterologous expression of this enzyme, the high efficiency of this enzyme was successfully realized, and the biosynthesis mechanism of this enzyme was deeply studied.These studies not only expand the application field of biosynthesis, but also provide new ideas and methods for the research and development of related drugs.

1.6 Objective By cloning RfbC gene

we transcribed it into different bacterial communities to further

explore its biological function, with a view to better understanding its protein synthesis process.By constructing suitable heterologous expression vectors, we introduced the gene into Escherichia coli and Bacillus subtilis, and achieved the expression of the target protein through culture and regulation.At the same time, we will purify and identify the expressed target protein to further understand the biological characteristics and biosynthesis mechanism of this enzyme.By

comparing RfbC gene expression levels in different chassis cells, we hope to reveal the regulatory mechanisms of RfbC gene expression in different chassis cells, and how these differences affect bacterial lipopolypaccharide synthesis and bacterial viability.

1.7 Research Significance

The results of this study will help to expand the application of biosynthesis.At the same time, the results will help to understand the adaptive mechanism and survival strategy of Marine bacteria, and provide scientific basis for the protection and management of Marine ecosystems.In addition, the study of RfbC gene expression differences in different territorial cells is also helpful to expand our understanding of Marine bacterial gene regulation, and provide new directions and ideas for future research.

Conclusions to chapter 1

Marine bacteria play an important role in the Marine ecosystem, and it is of great significance to maintain the balance of Marine ecology. Marine bacteria may contain unique species and genetic resources, providing potential materials and opportunities for biotechnology and pharmaceutical research and development Heterologous expression is helpful to understand the function of genes and the properties and roles of encoded proteins. At the same time, through the expression of different hosts, the characteristics and differences of genes and proteins between different species were analyzed.

Chapter 2

Materials and Methods

2.1 Experimental Materials

2.1.1 Strains and Plasmid Strains:

PseudoalteromonasagarivoransHao2018 strains (qilu industrial food university A217 laboratory save)

DH5 α feel strain, strain BL21 feeling state (nanjing only praise biotechnology co., LTD.)

BS168 glycerin bacteria strains (days net sand) plasmid:

pHT43

2.1.2 medium and experimental solution,

Prepare LBmedium, add 5gofyeast powder, 5gof peptone, and 10gof NaCl10g, adjust pH 7.0 to , and sterilize at 121 ℃ for 20 minutes.

Prepare LB solidculturemedium with adosage of 10g peptone, 5g yeast powder, 10g NaCl, and an agar content of 1.8-2.0% (w/v).

100 mg/mL ampicillinsolution: After weighing 10 g of ampicillin, mix itwith 70 mL of deionized water and dilute them to 100 mL. Then, through a 0.22 μ m filter membrane, invert them evenly in a 1.5 mL EP bottle and store at -20 ℃. During use, please adjust the concentration to above 100 μ g/mL.

50 mg/mLkanamycin solution: Accurately quantify 5 g of kanamycin, then mix it with 70 mL of deionized water, and finally dilutethemixture to 100 mL. After passing through a 0.22 μ m filter membrane, evenly drip the mixture into a 1.5 mL EP tube and store it at -20 ℃. During use, please adjust the concentration to above 25 μ g/mL.

5 mg/mL chloramphenicol solution:Place 0.5 g of 0.5 gchloramphenicol in a 70 mL container of anhydrous ethanol, transfer it to 100 mL, and finally place it in a 1.5 mL EP bottle and store it at -20 ℃.
Before use, it should be diluted to 5 μ g/mL.

100 mM IPTG: Accurately quantify 24 mg ofIPTG and dissolve it in 1 mLof deionized water. After passing through a 0.22 μ m filter membrane, evenly place it in a 1.5 mL EP tube and store it at -20 ℃. Before practical application, adjust the maximum concentration of IPTG to 10 μ M, 20 μ M, 40 μ M, 80 μ M, and 160 μ M.

PBSsolution: Put a bag of 1× PBS instant particles into 600 mL of distilled water, stir evenly to achieve the optimal concentration, then cool it, and finally store it in a suitable environment for use.

2.1.3 primers

According to the whole genome sequence (GCA:003668795.1) of Pseudo altomonas agarivoransHao 2018 in Genbank,primers were designed using Snapgene for Rhamnan precursor synthesis genes RmlA, RmlB, RmlC, and RmlD. The sequence information is shown in Table 1:

表 2-1 2110 引物序列

引物名	序列	酶切位点
2110-F	CGC*GGATCC*ATGCATCATCACCATCACCACGTGAATTTTATAGAA ACCGAT	BamH I
2110-R	TC*CCCCGGG*TTAAAACGTAGGTGCTTTTG	Sma I

2.1.4. Experiment reagent

Denome extraction kit、 plasmid extraction kit、 product purification recycling (gel) kit 、BCAProteinQuantificationKit、

DL2000PlusDNAMarker、DL5000PlusDNAMarker、RNase - freeddH₂O2 x PhantaMaxMastermix (DyePlus) 、T4DNALigase、10 x LigaseBuffer 2 x TaqMasterMix、LightNing™BamHI、LightNing™SmaI、10 x CutOne™ColorBuffer、 Color Gel Rapid Kit 、5×SDS-PAGELoadingBuffer Protein LoadingBuffer (Reductive) 、50×TAE Buffer 、Coomasil Brilliant Blue R250、 ampicillin Storage Solution (100mg/ mL) 、Kanamycin sulfate solution (100mg/ml) 、Chloramphenico、lsopropyl beta-d-thiogamyGlucoside 、IPTGPBS instant granules

2.1.5 experimental instrument

Sterile ultra-Clean work、 PCR amplification apparatus、 Thermostatic biochemical incubator thermostatic water bath、Ultrasonic cell crusher、high speed centrifug、electronic balance、pH meter、 Nucleic acid electrohosis apparatus、Temperature controlled shaking table autoclave、Magnetic stir、Low speed centrifuge gel image、ultra low temperature refrigerator、refrigerator

2.2 methods

2.2.1 bacteria activation

Develop PseudoalteromonasagarivoransHao2018 petri dish,Single colonies were picked from the 10μL gun head with tweezers, and the gun head with the

colony was placed in 5mL seed liquid medium at 37℃ and 200rpm overnight (8-16h) for culture activation.

Glycerin bacteria were selected, and 100μL glycerin bacteria solution was added into 5mLLB liquid medium, and cultured at 37℃ and 200rpm overnight (8-16h) for activation

2.2.2 PseudoalteromonasagarivoransHao2018 genomic DNA extraction

Hao2018 samples extracted from Marine bacteria strains, transplanting it to the seed culture medium, and in 37 ℃, the temperature at
200 RPM for long time.Using FastPureBacteriaDNAIsolationMiniKit (DC103) (Vazyme genome extraction kit) Hao2018 genomic DNA extraction of Marine bacteria

2.2.3 Plasmid activation

1. Remove the receptive cell suspension, place it in a refrigerated environment at -70 ° C, and quickly return it to its original position.

2. Pour the plasmid DNA solution into the container, stir well, and then place the container in the refrigerator to freeze for 30 minutes.

3. In order to process the mixture more efficiently, we can use a water bath of 42 ° C to heat it, then let it sit for 90 seconds, then warm it at 37 ° C for 5 minutes, and finally put it in the refrigerator to cool it quickly for 3-5 minutes.

4. Dissolve 1ml LB without any antibiotics in water, stir evenly at 37 ° C, and continuously place for 1 hour, which can effectively promote the reproduction of bacteria, thus activating their resistance to specific drugs.

5. Apply the bacterial solution to a screening plate treated with special antibiotics and culture it overnight at room temperature.

2.2.4 Amplification of Target fragment

Using strain P PCR amplification of the target gene was performed using agarivorans Hao 2018 genomic DNA as a template.

PCR gel recovery and purification:

The amplifiedtarget genewas verified by 1% agarose gel electrophoresis, cut, recovered and purified;

The operation is as follows:

AfterDNAelectrophoresis, gel blocks containing specific DNAfragments are separated using ultraviolet light. Then, wipe the rubber block clean. Add 1-3times thevolume of buffer GDP, and thentakeawater bath at50-55 ℃ for 7-10minutes. Stir theliquid twice during the water bath process toimprove the DNA recovery rate.In Collection Tubes, use FastPureDNA Mini Columns-Gadsorption column to adsorb ≤ 700 μ l ofsolvent, thencentrifuge at 12000 rpmfor 30-60 seconds. Add 300 μ l of buffer GDP and centrifuge at a speed of 12000 rpm per minute for 30-60 seconds. Centrifuge at a speed of 12000 rpm per minute for 30-60 seconds with 700 μ l Buffer GW. Flip and mix 2-3 times to thoroughly clean the salt adhering to the pipe wall. Repeat the operation to remove salt. Discard the filtrate and place the adsorption column in the recovery manifold. Centrifuge at 12000 rpm for 2 minutes. Insert the absorption column into a 1.5ml EP tube, then pour 20-30 μ l Elution Buffer into the center and wait for 2 minutes. Finally, centrifuge at 1000 rpm for 1 minute. Remove the adsorption column and store it in an environment of -20 ℃.

Precautions for glue recycling:

Increasing sample loading: can increase the amount of adhesive recovery. Try to use freshly prepared electrophoresis buffer to cut only the gel with bands, reduce the volume of cut gel, and improve the recovery rate. Melting the glue blocks and concentrating them in one tube, then transferring them to the same column, is

beneficial for the binding of DNA and the membrane. Increase the amount of sol solution appropriately: but not too much, generally not exceeding 750ul.

Adjust the pH of the electrophoresis buffer: If the pH is too high, 10ul (pH 5.0, 3mol/L NaAC) can be added to the sol solution. Let the column stand at room temperature for a few minutes to allow the ethanol to fully evaporate. Reduce the volume of eluent: Generally, 30-50 μ l of eluent can be used for elution. Pay attention to operational norms: avoid contamination of exogenous DNA, wear gloves during operation, and reduce surface contamination.

2.2.5 Double digestion of target genes and plasmid vectors

Through the SmaI and BamH I dual enzyme digestion system, we can obtaincomplete target genes and vectors. Then, the prepared double enzyme digestion system was mixed evenly, incubated at 37 ℃ for 30 min, and finally 1% agarose gel electrophoresis was carried out. The agarose gel

with specific characteristics was cut with a clean gel cutter, and then the gel was recovered and purified.

2.2.6 Construction of recombinant plasmids

Connect using T4 DNA ligase and place the prepared ligase reaction system in a 16 ℃ metal bath for overnight reaction. When using T4 ligase for linking reactions, attention should be paid to controlling reaction conditions such as temperature, time, and enzyme dosage to ensure linking efficiency and accuracy. Ensure appropriate matching between DNA fragments and the ends of the vector for effective connection. After the connection reaction, the quality and integrity of the connection product can be detected through methods such as electrophoresis.

2.2.7 competent escherichia coli

(1) the transformation of a.The receptive cells are removed from -70C and quickly placed on ice to melt.b. Add the DNA to be transformed into 100μl receptive cells, avoid violent blowing with pipette gun, and then put it in the refrigerator for 30min.c. Heat it in a water bath at 42 ° C for 45sec, then quickly cool it in ice cubes for 2min. Do not shake the centrifuge tube.d. Add 900μILB and SOC liquid medium evenly into the centrifuge tube, and shake it on a shaking table at 37℃ at a speed of 200rpm for 1h.e. Run the 5,000rpm centrifuge for 3 minutes, then remove the 900μl supernatant, use the remaining medium, suspend the bacteria, and evenly apply to the LB solid medium plate containing the appropriate antibiotic.f. In the incubator at 35-37℃, turn the petri dish upside down and place it overnight for culture.

(2) Screening and verification of positive clones

a. Colony PCR verification:Samples were taken from ampicillin medium, half colonies were extracted from the surface of the medium, and their serial numbers were marked, and then they were used as PCR reagents. The methods were predenaturation at 95℃ for 5min, modification at 95℃ for 15sec, annealing at 56-72℃ for 15sec, extension at 72℃ for 3min, and 35 cycles.Finally, it is fully extended for 5min.After electrophoresis of PCR products, it was observed in the gel imager whether bright bands appeared at the correct size to screen positive clones.

b. Plasmid PCR verification: The colonies successfully verified in the PCR reaction were selected and added to 5mL liquid LB medium (ampicillin), and then cultured in a shaking table at 37℃ and 200rpm for 12-16h.Follow the FastPurePlasmidMiniKit (Vazyme Plasmid Extraction Kit) instructions to extract the plasmid.Then after extraction of plasmid as template into components and component volume ddH $_2$ OForwardPrimerReversePrimer2 x TaqMasterMix4.0 mu L0.5 mu L0.5 mu L5 mu L12 PCR reaction, PCR reaction procedure is 95 ℃ by degeneration

3 min,Denatured 15sec at 95℃;Annealing 15sec at 56-72℃;Extended at 72℃ for 3min and expanded for 30 cycles.Finally, it is thoroughly extended for 5min at 72℃.After electrophoresis, the PCR products were observed in the gel imager to see if bright bands appeared at the correct size, and the positive clones were verified again to rule out false positives.

2.2.8 Measurement of the growth curve of *B. suntilis*168 strain

Inoculate strain B.subtilis 168 into LB medium, cultivate at 37 ℃ and 200 rpm, andmeasure the OD600of these three strains every hour from 0 to 24 hours. Based on these numerical values, draw the growth curve of the strains and set a blank control. When measuring the growth curve, attention should be paid to maintaining constant cultivationconditions such as temperature, shaking table speed, and medium composition throughout the experiment to avoid growth differences caused by changes in conditions. Ensuring accurate and consistent cell or bacterial count for each vaccination is crucial for the accuracy of the curve. Timed sampling: strictly follow the set time interval for sampling, and the sampling time points should be representative. During the operation process, attention should be paid to aseptic operation to prevent contamination by miscellaneous bacteria from affecting the results. After sampling, the handling of the sample, such as dilution and cell counting, should be standardized to reduce errors. To ensure data reliability, multiple repeated experiments were conducted. Ensure that the cells or bacteria used are in good physiological condition and free from aging or damage. Proficient in operating procedures, and at the same time, make clear and accurate markings to prevent confusion. Record all relevant data and experimental conditions in detail.

2.2.9 Preparation and transformation of the receptive state of B. suntilis168

(1) Chemical preparation

Take outthe B. subtilis 168 glycerol tube to store the strain from -80 ° C and melt it on ice. Dip an appropriateamount of bacterial solution in an inoculum ring on asolid LB plate and separate it by drawing lines. Incubate at 37 ° C for 12 hours. Takeout the activated plate,transfer a single colony to 5 mL LB liquid medium, and incubateovernight at37 ° C and 250r/min. Transfer 200 μ L of the obtained bacterial solution to 10 mL of SP I medium and incubate at 37 ℃, 250r/min until the logarithmic phase (approximately 4-5 hours). After starting to measure OD600, when the culture reaches the end of logarithmic growth, quickly take 1 mL and inoculate it into 10 mL of SP II medium. Place it in a shaker at 37 ℃ and incubate at 100 r/min for 1.5 hours. Add 100 μ L of 100 x EGTA solution to the bacterial solution and incubate on a shaker for 10 minutes. Use 1.5 mL sterile centrifuge tubes to divide into 500 μ L tubes, and store at -80 ° C [7]. It should be noted that when conducting experiments on preparing receptive cells using chemical methods, strict aseptic procedures should be followed to prevent contamination by miscellaneous bacteria. At the same time, various reagents and equipment used in the experiment need to undergo strict disinfection and sterilization treatment.

(2) Transformation of Bacillus subtilis:

Place10 μ L of recombinant plasmid ina water bath at 37℃, and after 1 hour of constanttemperature,add 500 μ L LB of culture medium to it. Continue toculture at 37 ℃ and 200 rpm for 1 hour, and finally centrifuge at 4000 rpm for 5 minutes. Discard 900 μ L of supernatant and evenly apply the remaining supernatant along with kana/chloramphenicol resistant substances on the surface of the plate. After 12 hours of constant temperature at 37 ℃, finally detect the presence of individual cells. When conducting Bacillus subtilis transformation experiments, strict adherence to aseptic procedures is necessary to prevent contamination by miscellaneous bacteria. At the same time, various reagents and equipment used in the experiment need to undergo strict disinfection and sterilization treatment.

2.2.10 oliBL21 feeling state of transformation of e.c. with our fabrication:

Place thereceptive cells inan environment of -80℃,then quicklyimmersetheminice water for5minutes. Finally,add an appropriateamount of plasmid or connecting product to stir evenly at the bottom of the EP tube, and continue the ice water bath for 30 minutes. Heat shock the water bath at 42 ℃ for 90 seconds, then quickly move it to the refrigerator and let it stand for 2 minutes. Pour LB solution into a centrifuge tube and shake at a rate of 140 rpm for 60 minutes at 37 ℃. Uniformly coat various bacterial solutions onto LB culture dishes and add an appropriate amount of antibiotics. Invert the plate and cultivate it overnight.

2.2.11 E.coli bl21 and B. Sibis168 induced by IPTG

(1) strains induced by IPTG were transferred to LB liquid medium prepared and placed in a constant temperature shaking bed at 37℃ at 250rpm for activation for 12h.

(2) After the completion of cell culture and protein induced expression and activation, transfer to 1LLB liquid LB medium, culture at 37℃, 250rpm until OD600 is about 0.5, add IPTG with final concentration of
1mM, and continue to culture at 37℃ for 3 hours.(Note:OD600, when 0.5~1.0, the bacteria entered the logarithmic growth phase, which is the best time to induce the bacteria to avoid overgrowth)

2.2.12 Cell crushing treatment and protein purification

1.200 mL bacterial solution (16℃, 24 h induced by IPTG) was centrifuged in a 50 mL centrifuge tube (6 tubes) at 4℃, 8000 rpm, 5 min

2. 200 μL (extracellular) was taken from the supernatant, then the supernatant was abandoned, and 50 mM imidazole PBS was used for resuspension, and the cells were broken for 30 min after re-suspension

3. 200μL (whole cell) was taken from the cell crushing, the remaining solution was centrifuged at 8000 rpm for 5 min to discard precipitation, and all the

supernatant (200μL was taken as intracellular) was passed through 0.22 μm filter membrane

The supernatant was obtained by centrifugation at 8000 rpm at 4.4℃ for 10 min. 20 mL of supernatant was removed from the sample to immobilized metal affinity chromatography. Mixed with nickel - subnitrotriacetic acid (Ni-NTA) column. The nickel column was balanced twice with binding buffer, and then 20 mL of cell culture supernatant was passed through the Ni column. Subsequently, the nickel column was washed twice with a 35 mL Wash buffer and the purified proteins were collected with an Elution buffer of the same column volume. Finally, the nickel column was cleaned with diploid buffer and analyzed by SDS-PAGE.

2.2.13 Verification of synthetic protein expression

SDS-PAGE

Use ExpressCast PAGE color gel quick kit for one-step gel filling preparation. Prepare the required reagents andequipment, such as gel mold, buffer solution, etc. Mix the corresponding gel solution components according to the instructions of the kit. Slowly pour the mixed gel solution into the gel mold to avoid bubbles. After the completion of glue pouring, place the mold stably to allow the gel to polymerize naturally.

Mix all dialyzed protein samples with protein loading buffer, boil in water for5minutes,and centrifuge at 10000 rpm for 5 minutes. 30 μ L of protein sample was subjected to SDS-PAGE and electrophoresis at 150 V for 50-60 minutes. After 3 hours of electrophoresis, place the sample in bright blue staining solution for decolorization.

BCAmethod for measuring protein content

Prepare a series of protein standard solutions with different concentrations. Mix the standard solution and BCA working solution in a certain proportion and incubate at a certain temperature for a period of time. Dilute the protein sample to be tested appropriately. Take an appropriate amount of processed sample and add it

to a test tube containing BCA working solution. Incubate at the same set temperature.

Use a spectrophotometer to measure the absorbance of each tube at a specific wavelength. Calculate the protein content in the sample based on the standard curve. Measure A562 using an enzyme-linked immunosorbent assay (ELISA) reader and calculate the protein concentration based on the standard curve.

Conclusions to chapter 2

Clear the experimental ideas, prepare the reagents and instruments required for the experiment, master the experimental methods and carefully carry out each step of the operation

CHAPTER3

RESULTS AND DISCUSSION

3.1 Experimental Results

3.1.1 Amplification of Target gene

After PCR amplification of Rfbc gene using specific primers, genomic DNA was extracted from target bacteria and the amplified target gene was verified by 1% agarose gel electrophoresis

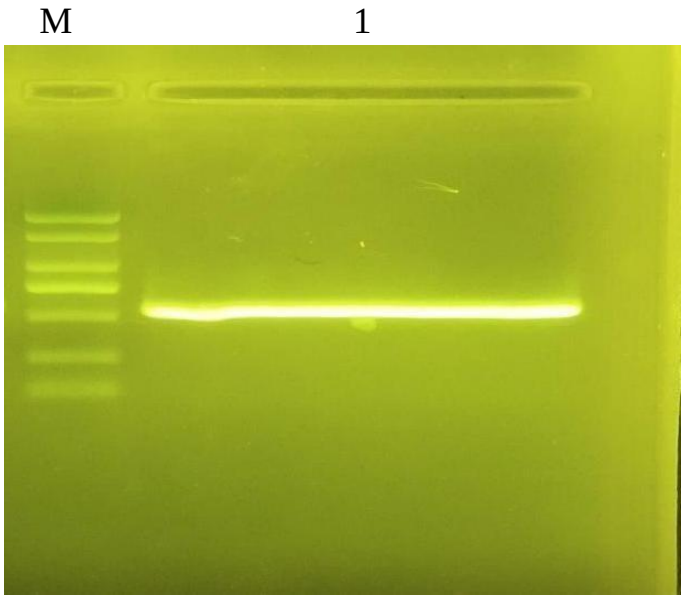

Figure 3-1 The amplified target gene was verified by 1% agarose gel electrophoresis

3.1.2 Double digestion of target genes and plasmids

The target genes and empty plasmid were thoroughly digested with SmaI and BamHI, and 1% agarose-gel electrophoresis was performed.

M 1

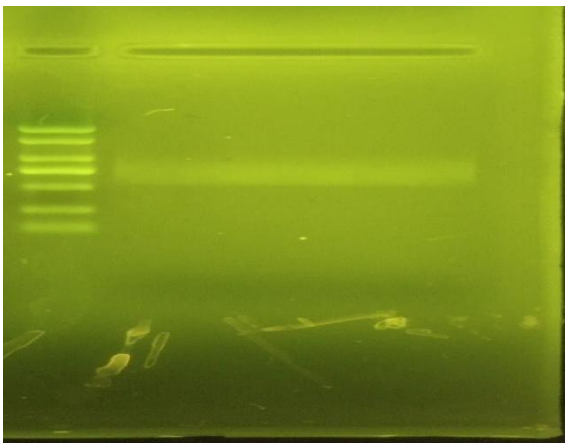

Figure 3-2 Gel electrophoresis after double enzyme digestion of target gene

M 1

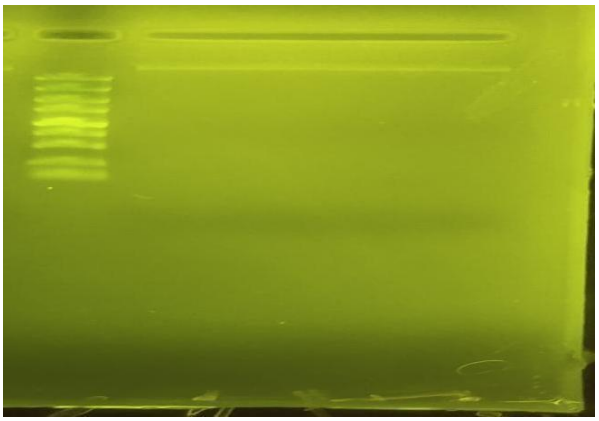

Figure 3-3 Gel electrophoresis after double enzyme digestion of plasmids

3.1.3 DH5 Alpha transformation validation:

The amplified Rfbc gene fragment was connected with the expression vector to obtain the expression vector of Rfbc gene, and the obtained expression vector was transformed into the receptive DH5 alpha state. Colony PCR and plasmid PCR verification were performed through culture, and the positive transformed bacteria were screened. M 1 2 3 4 5 6 7

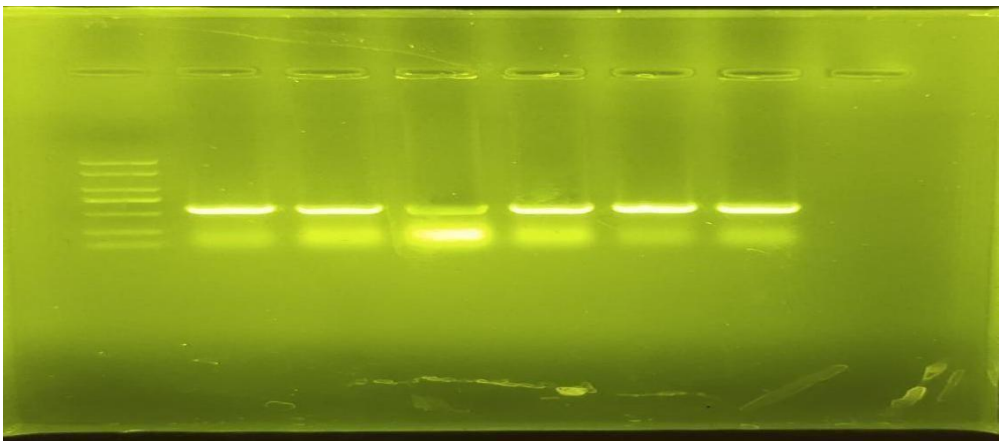

Figure 3-4 Colony PCR gel electrophoresis

3.1.4 Plasmid PCR verification

The colonies successfully verified in the colony PCR reaction were selected and added to the liquid LB medium for oscillatory culture to extract the plasmids. Then the extracted plasmids were used as the template for PCR reaction.The PCR products were electrophoretic andthe positive clones were verified again toeliminate false positives.

M 1

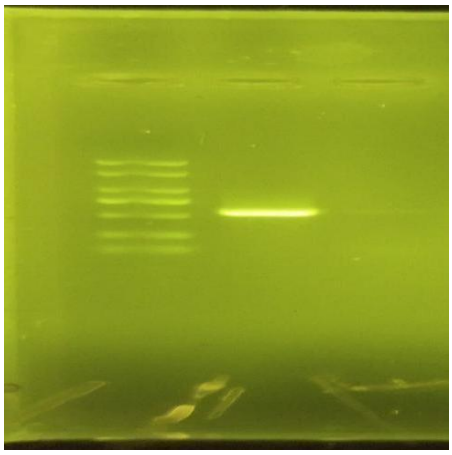

Figure 3-6 Plasmid PCR electrophoresis

3.1.5 PCR verification after plasmid transformation

The expression vector was transformed into BL21 and Bacillus subtilis, and the positive bacteria were obtained through culture and screening

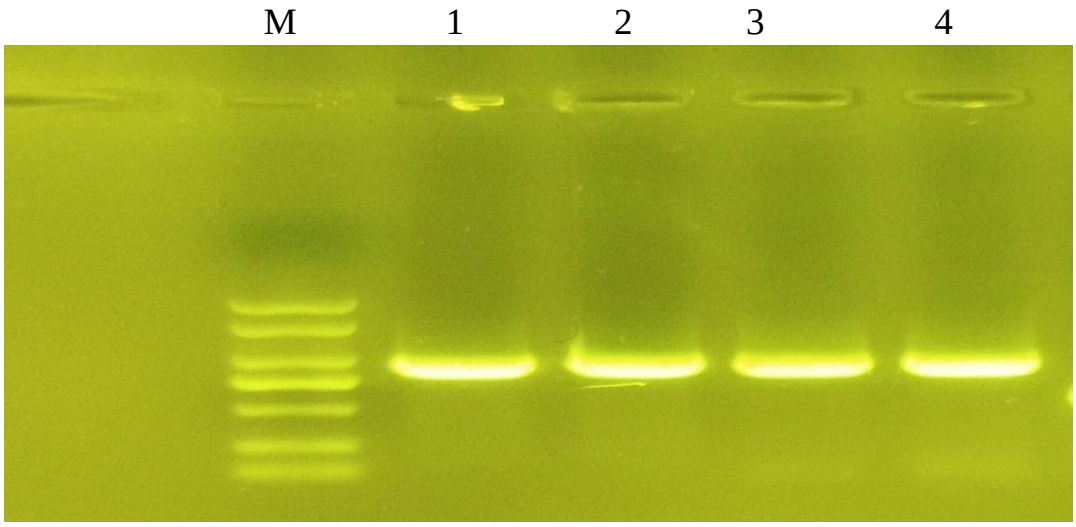

Figure 3-7BL21 colony PCR and Bacillus subtilis PCR electrophoresis

Note: Lane 1 and 2 were BL21;Lanes 3 and 4 were Bacillus subtilis

3.1.6 Determination of Protein standard curve

The standard curve of protein concentration (mg/ml) was drawn with BCA Protein Quantification Kit, and the protein concentration of the purified enzyme was determined after 17 years.The protein concentrations of BL21 and BS168 were calculated according to the standard curve, which were 2.29 mg/mL and 1.87 mg/mL respectively.

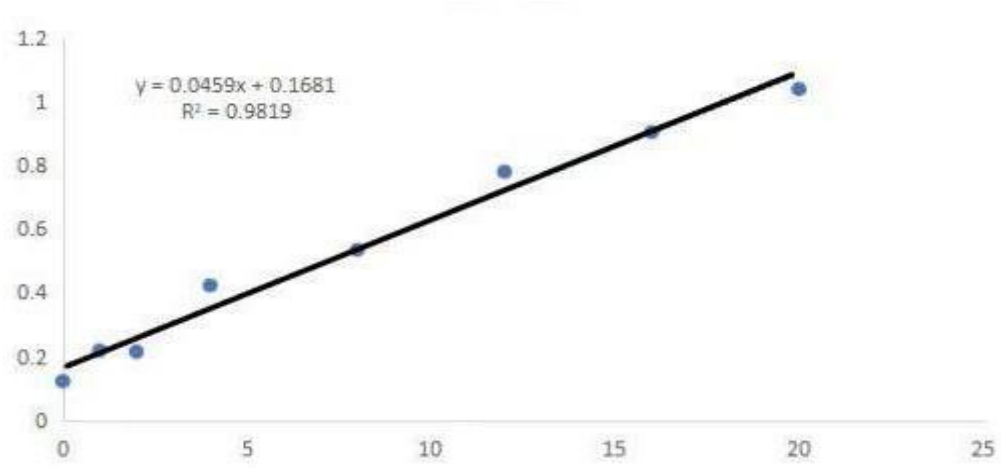

Figure 3-8 Standard curve of protein concentration

3.1.7 SDS-PAGE Electrophoresis results

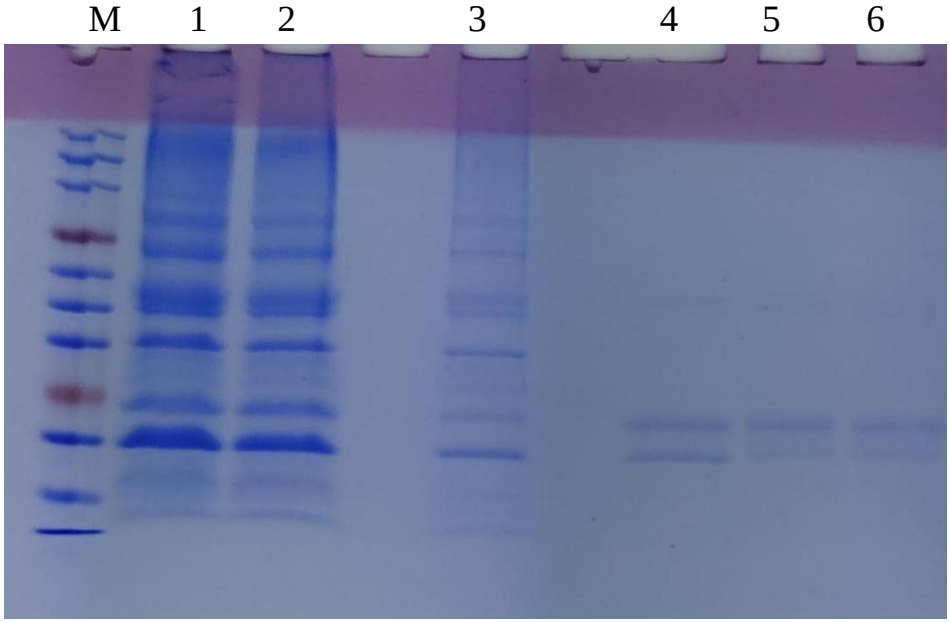

Figure 3-9SDS-PAGE electrophoresis results

3.2 Experimental discussion

The normal expression of Rfbc gene was obtained in both Escherichia coli and Bacillus subtilis.First, we cloned the Rfbc gene in DH5 Alpha and constructed the expression vector.Then the expression vector was transformed into Escherichia coli and Bacillus subtilis, and finally the protein expression was detected.The results showed that in E. coli, Rfbc gene expression was low, and the molecular weight of the protein expressed was the expected size.In Bacillus subtilis, the expression of Rfbc gene was relatively high, and the molecular weight of the protein expressed was also expected.Further discussion revealed that the two bacteria were quite

different physiologically.Escherichia coli is a gram-negative bacterium that grows rapidly, can utilize a variety of carbon and nitrogen sources, and has a wide range of metabolic pathways.In contrast, Bacillus subtilis is a Grampositive bacterium with a slower growth rate and relatively low nutrient requirements.Therefore, its metabolic pathway is more suitable for the physiological function of Rfbc gene expression, so the expression level is higher in Bacillus subtilis.In conclusion, our experimental results showed that Rfbc gene expression was different in different strains, which was closely related to the physiological characteristics of bacteria.

Conclusions to chapter 3

PCR verification was performed for each step of double enzyme digestion and conversion, and the protein content was finally measured by BCA method, and the standard curve of protein was drawn. The protein concentrations of BL21 and BS168 were 2.29 mg/mL and 1.87 mg/mL, respectively. The protein concentrations of BL21 and BS168 were 2.29 mg/ ml and 1.87 mg/mL, respectively. Electrophoretic results were obtained using the ExpressCast PAGE Color gel rapid kit

CHAPTER 4

SUMMARY AND PROSPECT

4.1 Summary

Through the experimental results, we can see that Rfbc gene expression in Escherichia coli and Bacillus subtilis is different.The expression of Rfbc gene in Escherichia coli was low, while that in Bacillus subtilis was relatively highThis may be due to the differences in metabolic pathways and physiological characteristics between the two strains.

4.2 Outlook

We have obtained preliminary results on the expression of Rfbc gene in Escherichia coli and Bacillus subtilis, but further investigation is needed.For example, the function of DTDP-4-dehydrorhamnose-3, 5aberrant isomerase in

different strains can be further analyzed to understand its role in bacterial metabolic pathways. Experiments under different culture conditions can also be conducted to further explore the expression and physiological function of Rfbc gene.In addition, Rfbc gene expression can also be attempted in other more bacteria to analyze its expression and physiological function in a wider bacterial population, so as to further explore the causes and mechanisms of RfbC gene expression differences in Marine bacteria P.a..HA2018.

Conclusions to chapter 4

The expression of RfbC gene in Escherichia coli was low, while that in Bacillus subtilis was relatively high. This may be due to the differences in metabolic pathways and physiological characteristics between the two strains

To further analyze the functions of DTDP-4-dehydrorhamnose-3, 5aberrant isomerase in different strains, understand its role in bacterial metabolic pathways, and further explore the expression and physiological function of RfbC gene.

Try to express RfbC gene in more bacteria to analyze its expression and physiological function in a wider bacterial population.

REFERENCE DOCUMENTATION

1. Yu Liansheng, Ge Jingping, Ping Wenxiang, Du Renpeng. Research progress on the regulation of bacterial extracellular polysaccharide biosynthesis by cyclic guanosine acid [J]. Food Industry Technology, 2023,44 (9): 422-430 [2] Wu Bin. Molecular mechanism and related gene cloning research on drought resistance of Bawang [D]. Chinese Academy of Forestry, 2014
2. Song Chaoyi Developing novel gene editing techniques for heterologous expression and structural derivation of multi fungicides [D] Shandong: Shandong University, 2021
3. Ju Yuhao. Genomic characteristics analysis of Pseudomonas aeruginosa and cloning and heterologous expression of key genes in the extracellular polysaccharide synthesis module [D]. Qilu University of Technology, 2023. DOI: 10.27278/d. cnki. gsdqc. 2023.000142
4. Hu Qiqi, Luo Min, Shu Jianqin, et al. Screening and identification of antagonistic bacteria in the rhizosphere of Aconitum root rot [J]. Traditional Chinese Medicine and Clinical, 2022, 13 (06): 25-28
5. Liu Yanping. Isolation and identification of Sporobolomys sp.YB 2013 and detection of pigments produced by Sporobolomys sp. [D]. Southwest University, 2015
6. Lu Jiaojiao. Expression study of glutamine transaminase derived from Streptomyces maoyuan in Gram positive bacteria [D]. Hefei University of Technology, 2019
7. Wei Yuxia Heterologous expression and immobilization of D-alloketose-3epimerase in Bacillus subtilis [D]. Jiangnan University, 2021. DOI: 10.27169/d.cnki.gwqgu.2021.001281

8. Xiao Kangpeng. Pasteurella avicularis CRP Functional study of phoP gene and preliminary evaluation of immune efficacy of mutant strains [D]. Sichuan Agricultural University, 2016
9. Li Feng. Cloning and functional analysis of genes related to magnetosome synthesis in Magnetospirillum Gryphiswaldense MSR-1 [D]. China Agricultural University, 2014
10. Chi Xiaoyuan. Comparative genomics analysis of fatty acid desaturase gene in microalgae and verification of important gene function [D]. Graduate School of Chinese Academy of Sciences (Institute of Oceanography), 2014
11. Li Ying Genetic diversity analysis of soil contaminated with PCBs and the impact of biphenyls on soil microorganisms [D]. Zhejiang University, 2014
12. Deng Shuping. Differences in catalytic specificity of Vibrio alginate lyase system from Vibrio alginolyticus [D]. East China University of Science and Technology, 2014
13. Wang Yuchao. Plant community characteristics and corresponding relationship analysis of the main habitats of giant pandas in the Qinling Mountains [D]. Northwest A&F University, 2012
14. Chen Juan. Screening of nucleoside antibiotic biosynthesis related proteins in Charcoal like Micromonospora JXNU-1 [D]. Jiangxi Normal University, 201
15. Qu Xiaoyang. Construction and Application Research of Enhanced Escherichia coli Engineering Bacteria with Dual Protection System [D]. South China University of Technology, 2020
16. Jv Yuhao,Xi Chenxiang,Zhao Yanqiu, et al. Pan-Genomic and Transcriptomic Analyses of Marine Pseudoalteromonas agarivorans Hao 2018 Revealed Its Genomic and Metabolic Features[J]. Marine Drugs,2022,20(4):248.

17. Yang Yang, Wang Shili The effect of IPTG induction time and temperature on the expression level of GnRH analogues protein [J]. World Latest Medical Information Digest, 2015,15 (15): 159-161
18. Zhao Yuxin, Tan Yuyang, Gao Ying, et al. Cloning of Quric Acid Synthesis Gene Clusters Based on Transformation Related Recombinant Cloning and Heterologous Integrated Expression in Aspergillus niger [J/OL]. Microbiology Bulletin, 1-21 [2024-05-07] https://doi.org/10.13344/j.microbiol.china.240096.
19. Yuan Fang, Li Guoying, Gu Qiuya, et al. High efficiency expression of glutamine transaminase derived from Streptomyces Maoyuan in E. coli [J/OL].

Food and Fermentation Industry, 1-13 [2024-05-07] https://doi.org/10.13995/j.cnki.11-1802/ts.039235.