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 **Cloning of Iron Transport Related Gene TBSR1 and Construction an Expressed Strain in Shewanella oneidensis MR-1**
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-21
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APPROVE
Head of Biotechnology, Leather and Fur Department, Professor, Dr. Sc., Prof.
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Song Zihan

1. Thesis topic **Cloning of Iron Transport Related Gene TBSR1 and Construction an Expressed Strain in *Shewanella oneidensis* MR-1**
Scientific supervisor Dr.Sc., Prof. Olga Andreyeva

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WORK CALENDAR

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	until 11 April 2025	
2	Chapter 1. Literature review	until 20 April 2025	
3	Chapter 2. Object, purpose, and methods of the study	until 30 April 2025	
4	Chapter 3. Experimental part	until 11 May 2025	
5	Conclusions	until 15 May 2025	
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8	Submission of bachelor's thesis to the department for review (14 days before the defense)	28 May 2025	
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I am familiar with the task:

Student _____ Song ZIHAN

Scientific supervisor _____ Olga ANDREYEVA

SUMMARY

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Iron is one of the essential trace elements for living organisms, widely involved in key life processes such as redox reactions, electron transfer, and metabolic regulation. Despite the abundance of iron in nature, it is mainly present in insoluble forms, making it difficult for organisms to directly obtain usable iron ions. Therefore, bacteria need to maintain intracellular iron homeostasis through efficient iron uptake and regulatory systems to meet the demands of optimal growth and physiological activities.

The dual nature of iron - being essential yet potentially toxic - poses a significant challenge to bacterial survival. Iron deficiency can inhibit the activity of key enzymes, while iron excess can generate hydroxyl radicals through the Fenton reaction, causing oxidative damage. Gram-negative bacteria achieve dynamic iron homeostasis through iron carrier systems, TonB-dependent receptors, and the Fur protein regulatory network. Recent studies have shown that the iron homeostasis mechanisms of environmental bacteria, such as Shewanella, exhibit high diversity, and the interaction between their regulatory pathways and metabolic networks is becoming a research hotspot.

Shewanella, as a representative strain of Gram-negative γ-proteobacteria, has become an ideal model for understanding bacterial iron metabolism due to its diverse respiratory pathways and high iron protein requirements. This experiment focuses on the iron carrier receptor gene TBSR1 of Shewanella, hypothesizing its involvement in the recognition and transport of iron carriers. To explore the function of TBSR1, this study constructed a TBSR1 overexpression strain using molecular cloning techniques: specific primers were designed to amplify the TBSR1 gene, which was then cloned into the expression vector pBbB5k using homologous recombination to construct the

recombinant plasmid pBbB5k-TBSR1. Subsequently, the recombinant plasmid was introduced into the Escherichia coli WM3064 conjugation strain by electroporation and then transferred into Shewanella through conjugation. Finally, the successful acquisition of the TBSR1 overexpression strain was verified through colony PCR, plasmid digestion, and qPCR. The experimental results showed that the mRNA expression level of TBSR1 in the mutant strain was significantly upregulated compared to the wild type, confirming the effectiveness of the gene overexpression system. This study lays the foundation for subsequent analysis of the molecular mechanism and physiological function of TBSR1 in iron uptake.

Key words: *Iron homeostasis, Shewanella oneidensis, TBSR1 gene, Homologous recombination, Overexpression strain*

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INTRODUCTION

Iron is one of the essential trace elements for living organisms, widely involved in key life processes such as redox reactions, electron transfer, and metabolic regulation. Despite the abundance of iron in nature, it is mainly present in insoluble forms, making it difficult for organisms to directly obtain usable iron ions. Therefore, bacteria need to maintain intracellular iron homeostasis through efficient iron uptake and regulatory systems to meet the demands of optimal growth and physiological activities.

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Shewanella, as a representative strain of Gram-negative γ-proteobacteria, has become an ideal model for understanding bacterial iron metabolism due to its diverse respiratory pathways and high iron protein requirements. This experiment focuses on the iron carrier receptor gene TBSR1 of Shewanella, hypothesizing its involvement in the recognition and transport of iron carriers. To explore the function of TBSR1, this study constructed a TBSR1 overexpression strain using molecular cloning techniques: specific primers were designed to amplify the TBSR1 gene, which was then cloned into the expression vector pBbB5k using homologous recombination to construct the recombinant plasmid pBbB5k-TBSR1. Subsequently, the recombinant plasmid was introduced into the Escherichia coli WM3064 conjugation strain by electroporation and then transferred into Shewanella through conjugation. Finally, the successful acquisition of the TBSR1 overexpression strain was verified through colony PCR,

plasmid digestion, and qPCR. The experimental results showed that the mRNA expression level of TBSR1 in the mutant strain was significantly upregulated compared to the wild type, confirming the effectiveness of the gene overexpression system. This study lays the foundation for subsequent analysis of the molecular mechanism and physiological function of TBSR1 in iron uptake.

The purpose of the work is to clone the TBSR1 gene associated with iron transport and create an expressed strain of Shewanella oneidensis MR-1.

Object of study: homologous recombination; TBSR1 - iron transporter gene; expressed strain Shewanella oneidensis MR-1.

Subject of study: Cloning of the TBSR1 gene associated with iron transport and creation of the expressed strain of Shewanella oneidensis MR-1

Research methods:

1. *Extraction and amplification of the genome*. Extract the TBSR1 genome and perform PCR amplification and recovery;

2. *Construction of recombinant plasmids*. The plasmid was enzymatically digested and homologous recombined with the TBSR1 genome;

3. *The transformation of recombinant products*. The recombinant plasmid was transferred into the Escherichia coli DH5α strain and verified by PCR;

4. *The transfer of zygous bacteria and pcr verification*. Extract the plasmid and transfer it into Escherichia coli WM3064;

5. *Transfer Shiva bacteria and conduct qPCR verification*. The plasmid was transferred into Shewanella through conjugation and overexpression verification was conducted.

Scientific novelty: Based on the reasonable selection of the pBbB5k plasmid as a vector providing the basis for TBSR1 gene overexpression and the diaminohexanedioic acid-deficient WM3064 strain as a donor to achieve plasmid conjugation and transfer, a complete technical process of “cloning-recombination-transfer-validation” was established, providing a standardized method for studying the function of the Shewanella gene. Compared with the wild-type strain, the

expression level of the TBSR1 gene in the recombinant strain was significantly increased, indicating that TBSR1 gene overexpression was successfully achieved.

Practical significance: The successful construction of a strain overexpressing TBSR1 laid the experimental basis for understanding the function of this gene in iron transport and the mechanism of iron metabolism in Shewanella.

Chapter I

LITERATURE REVIEW

1.1 The biological significance of iron in cells

Iron is an indispensable nutrient element for living organisms and plays a crucial role in the physiological activities of cells. In the field of physiology, iron is involved in numerous important life processes, such as oxygen transport, nucleic acid synthesis, the tricarboxylic acid cycle, electron transfer, and gene regulation. Its biological functions mainly rely on forming complex structures with proteins, such as sulfur iron or heme, which are essential for maintaining life activities. In the growth and metabolism of bacteria, iron plays a decisive role in the synthesis of important enzymes. For example, the iron content in Escherichia coli cells is closely related to cell growth, with each Escherichia coli cell containing approximately 105 iron atoms, and each generation of cells consumes approximately 1018 iron atoms. Iron deficiency can cause severe growth defects in bacteria. In mammals, iron is mainly present in hemoglobin responsible for transporting electrons or oxygen, ensuring that oxygen can be smoothly transported to various tissues and organs in the body.

Although the Earth has abundant iron elements, in aerobic environments, most iron (Fe3+) is highly insoluble, making it difficult for bacteria to directly absorb and utilize. At the same time, excessive iron poses potential harm to organisms. Iron mainly exists in the form of divalent iron (Fe2+), which readily reacts with hydrogen peroxide (H_2O_2) to produce highly toxic hydroxyl radicals ($OH\cdot$), thereby damaging DNA, proteins, and lipid molecules. Therefore, both bacteria and other organisms must precisely maintain the relative stability of iron content within cells to ensure normal metabolic requirements and avoid damage caused by excessive iron accumulation due to oxidative stress.

1.2 Balance of iron elements

Iron is a crucial substance for maintaining the normal physiological functions of bacteria. To achieve efficient utilization of iron, bacteria have developed a sophisticated regulatory network covering various aspects such as iron sensing, uptake, regulation, storage, and metabolism.

In response to the diversity of iron sources in the environment, bacteria have evolved multiple uptake mechanisms. In Gram-negative bacteria, Fe^{2+} can enter the cell through outer membrane channels and then be transported across the membrane to the cytoplasm via the FeO transport system. However, due to the easy oxidation of Fe^{2+} to insoluble iron hydroxide under aerobic conditions, the extracellular available iron concentration is often lower than the uptake threshold of the FeO system, prompting bacteria to develop alternative uptake pathways.

The iron carrier-mediated uptake pathway plays a significant role in iron-deficient environments. Iron carriers, as a class of chelators with high affinity for Fe^{3+}, are synthesized intracellularly and secreted to the extracellular space, where they combine with Fe^{3+} to form complexes. These complexes enter the cell through specific transport proteins. The Fe^{3+} inside the cell is reduced to Fe^{2+}, participating in the synthesis of iron-containing proteins (such as ferredoxin, cytochrome) and cofactors. The intracellular iron concentration regulation relies on a dynamic balance mechanism: when the Fe^{2+} concentration is too high, the expression of iron proteins is upregulated, promoting the chelation and storage of free iron; when iron is deficient, storage proteins release iron ions to meet the metabolic needs of the cell.

Transcriptional regulatory factors play a central role in maintaining iron homeostasis. The iron uptake regulatory protein (Fur) in Gram-negative bacteria precisely regulates the expression of iron metabolism-related genes by binding to iron response elements. However, due to the difference in GC content in the genome of Gram-positive bacteria, their iron regulatory mechanisms are significantly different from those of Gram-negative bacteria, reflecting the evolutionary diversity of bacterial iron homeostasis regulatory strategies.

1.3 The operation of the carrier

After the iron carrier is synthesized within the cell, it needs to be secreted outside the cell through specific output channels. In bacteria, the types of output channels responsible for transmembrane transport of iron carriers are diverse, belonging to different transport protein superfamilies such as the ABC superfamily, the major facilitator superfamily, and the resistance-nodulation-division superfamily. However, due to the high sequence conservation among members of the same family, the functional overlap or redundancy of multiple output channels, and the dynamic regulation, accurately identifying specific output channels remains a challenge.

Gram-negative bacteria have a more complex uptake mechanism for iron carrier complexes than Gram-positive bacteria because of their outer membrane layer and periplasmic space structure. Most iron carrier complexes are too large in size to pass through the pore proteins on the outer membrane, so they need to rely on TonB-dependent receptors to enter the periplasmic space. These receptors belong to a complete outer membrane protein family with a unique "β barrel" structure and undertake multiple cellular functions. The TonB-ExbB-ExbD complex within the cell provides energy for driving the specific recognition and binding of TonB-dependent receptors to high-affinity iron-carrier complexes, thereby internalizing them into the periplasmic space. When the iron-carrier complex binds to the homologous receptor, the cell undergoes a secondary structural conformational change, which is perceived by the TonB protein. As an example, the genus Shewanella has a large number of TonB-dependent receptors in its genome, among which PutA is a necessary component for endogenous iron carrier-mediated iron uptake. After the iron-carrier complex enters the periplasmic space, there are two transport pathways: in most cases, it will directly transmembrane transport to the cytoplasm; or it may first undergo reduction and release Fe^{2+}, and then be taken up into the cytoplasm through the ferrous transport system, while the free iron carrier will be excreted outside the cell for recycling. Current research shows that direct

transmembrane transport is the main strategy for most iron carrier transport in bacteria.

1.4 The current research status of Shewanella bacteria

Shewanella belongs to the γ Proteobacteria class and is a type of Gram-negative bacteria with unique physiological characteristics. Shewanella is commonly found in water bodies and sediment environments, which have the characteristics of chemical stratification and multi-functional respiration. By virtue of its facultative anaerobic metal reduction ability, Shewanella has shown great application potential in areas such as converting solid metal oxides in wastewater into biologically usable forms and generating electricity through microbial fuel cells. However, some species of the Shewanella genus are also known food spoilage microorganisms and fish pathogenic bacteria.

The multi-functionality of Shewanella's respiration mainly relies on a large amount of ferritin, especially iron-sulfur proteins and hemoproteins. Compared with the model bacterium Escherichia coli, Shewanella has a relatively higher demand for iron, which prompts it to evolve unique physiological characteristics to maintain iron homeostasis. In recent years, with the continuous deepening of research on Shewanella, it has been discovered that its iron homeostasis regulatory mechanism is very complex, involving the coordinated action of multiple genes and proteins, but there are still many unknown functions of iron transport-related proteins that need to be revealed.

1.5 The electron transfer mechanism of Shewanella species

During the metabolic process, Shewanella bacteria can oxidize and decompose organic substances such as glucose through the outer membrane cellular respiration process, converting them into carbon dioxide and water. In this crucial step, extracellular cytochrome C, as a core enzyme component, plays a significant role in releasing electrons from the organic substances. Microorganisms rely on membrane-intruding C-type cytochromes, nanowires, or conductive flagella to complete the direct transfer of electrons from the cell interior to the electrode.

The electrons released by the outer membrane cellular respiration are first captured by cytochrome C, and then transferred through the extracellular electron transport chain to the electron acceptors on the outer membrane. Shewanella bacteria possess a unique bioelectric conductivity ability, which enables it to transport electrons from the interior of the cell to the outside through extracellular electron-conducting nanowires composed of proteins. These nanowires act like "electronic transportation pipelines", capable of transmitting electrons from the electron acceptors to the electrode surface, ultimately establishing an electron flow channel in microbial fuel cells and successfully converting the chemical energy of organic substances into electrical energy.

1.6 Iron homeostasis of Shewanella species

Shewanella belongs to facultative anaerobic metal-reducing bacteria. The respiratory metabolic process of these bacteria has a significantly higher demand for iron compared to Escherichia coli, which is closely related to their ability to have multiple respiratory pathways. In the cells of Shewanella, the content of ferritin is very abundant, especially in the heme structure containing cytochrome C, which is more concentrated. Taking the representative strain Orenad Shewanella as an example, its genome can encode at least 42 cytC proteins, which significantly

increases the iron content of the cell and makes the regulation of iron homeostasis within the cell stricter.

Research on iron carriers has been conducted in the genus Shewanella as early as more than twenty years ago. For instance, in the decomposed Shewanella strains, researchers identified a type of iron carrier called ferroxanthin, whose chemical nature is a succinamate cyclic homodimer produced from a single substrate, putrescine. Although the current academic research on iron homeostasis in Shewanella has made some progress, there are still many key issues that need to be resolved. For example, there are multiple proteins related to iron transport in Shewanella, and the function of the protein encoded by the TBSR1 gene is still unclear. In-depth study of the role of this gene in the iron homeostasis of Shewanella will help further reveal the mystery of its iron transport mechanism.

1.7 The purpose and significance of the experiment

This study focuses on the iron transport-related gene TBSR1 of Shewanella. The aim is to construct an engineered bacterial strain that stably expresses TBSR1 through molecular biology techniques such as gene cloning, recombinant vector construction, and host transformation. This will provide standardized experimental materials for subsequent functional analysis, regulatory mechanism research, and application exploration of this gene.

Shewanella, as an important model strain in the field of environmental microbiology, its iron metabolism process plays a core regulatory role in key functions such as pollutant degradation and extracellular electron transfer. TBSR1, as a key candidate gene in the iron transport pathway, its specific functions in iron ion uptake, transmembrane transport, and intracellular homeostasis maintenance have not been clearly defined. Constructing an overexpressing strain of TBSR1 is a necessary prerequisite for analyzing its function: The full-length sequence of TBSR1 was cloned from the Shewanella genome using PCR technology, connected to the

shuttle expression vector pBbB5k, verified and sequenced through enzymatic digestion, and then the recombinant plasmid was transferred into the wild-type strain using conjugation transfer technology. Through antibiotic resistance screening and molecular biology identification, an engineered bacterial strain with clear genetic background and stable expression of the target gene was obtained.

Summary of the chapter I

1. The Biological Functions and Balance of Iron

Iron is involved in various life processes such as oxygen transport and nucleic acid synthesis. It needs to maintain intracellular homeostasis through intake (e.g., mediated by iron carriers), storage (e.g., ferritin), and metabolic regulation to avoid excessive levels that cause oxidative damage; the regulatory mechanisms of Gram-negative and Gram-positive bacteria differ.

2. Iron Carrier Transport and Iron Metabolism Characteristics of Shewanella

Iron carriers are secreted through transmembrane output channels and are taken up by TonB-dependent receptors (such as PutA of Shewanella); Shewanella has high iron requirements due to its versatile respiratory function (relying on iron proteins such as cytochrome C), and its iron homeostasis regulation is complex. The functions of some genes (such as TBSR1) are unknown.

3. Research Objectives and Methods

The iron transport gene TBSR1 of Chlamydia serai was cloned, and it was inserted into the vector pBbB5k and transformed into the host bacteria. Through screening, an overexpression strain was identified, laying the foundation for understanding its function and environmental applications (such as pollutant degradation and bioelectricity generation).

Chapter II

OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Experimental materials

2.1.1 Search and encoding primers for sequences

The following gene sequences were obtained by searching the TBSR1 gene (SO_0798) of Shewanella in uniport database (https://www.uniprot.org/):

CTGCATTCTAAAATCTGTTAACTTGAGCCGTAACTGTGTAAGCCAGCCT
TAGATTGACCACAGTAAATCGCGCTGTGGCCAAGCACATTAAAAGCAT
TACTCACTGTGGGCTAAATTCAGCTCACCACCAATAAACTTACACACAA
ACACGGCTTTGCCCCATTTTTCATGGGCGGCTTGCATTTCGGTGGCCAG
CATCGCCATCACGTCCGAATAGTCGCCGCACACTTGGGTCGCCATCGCA
TTCGTCACGCGCTCAAGTCGTTGGATAACTGTCCAAACGATGAATAAAC
CATTTAATTGGATCGAGGTAATTTTCATTAAAAGGATACATGCTAATTT
CGGCGGTCAGTTTCATCTTCAATCCTTATTTCAAATACTTATCATAAAAT
AACCGCACCGTGAAACGATATAGCATCAAGCCTCAAGTCTCACTCCAC
AGCGCGGCTAACTTGGGTAAGGACCTCAAATATACGTTTGAGATCCGG
CAACGAATAAGCGATTCAGAAGTGTGCTGTTGATTGGCCCATTCCTACG
CCGGTATTAGCCGGATCAGGTTCTAAGGGTTCGTCTGATGACATCTCAG
TCTGCCGAATTTTGGGCAAACCTTGGCGGCGCTTAGTATACATGACGAA
AATAATAAAGTAGACACACTTCTGCCTGTGATGCCCTGAAATAGCCCTC
AAATCATTGTCTATGAACCCTTAAACAT

This study referred to the pBbB5k-GFP plasmid map data published on the Add gene platform. Two restriction endonuclease recognition sites, EcoRI and BglII, were selected for the linearization operation of the vector, and based on this, subsequent restriction enzyme experiments and primer design work were carried out.

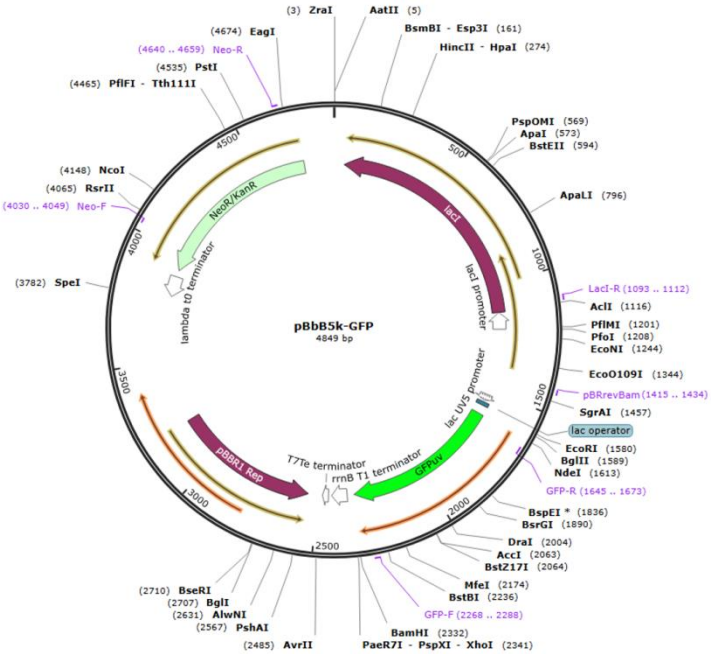

Figure 2.1The gene sequence information retrieved from the Uniport database

Based on the EcoRI and BglⅡ restriction enzyme sites as well as the FUR gene sequence, the primers were designed as shown in the following table:

Table 2.1 Experimental Primers and Sequences

Primer name	Primer sequence
TBSR1 F	GGATAACAATTTCAGAATTCGTGAAGCCCACTTACC
TBSR1 R	ATCTCCTTCTTAAAAGATCTGAAGCTAAACTTGATG

2.1.2 Experimental plasmid and cloned strain

In this experiment, we selected the pBbB5k-GFP plasmid with a length of 4849 bp as the base vector. During the experiment, the plasmid was introduced into the competent cells of Escherichia coli by electroporation for amplification and preservation. Subsequently, the target strain was screened using LB medium containing the corresponding antibiotics. After the plasmid was extracted and purified, it was introduced into the DAP auxotrophic Escherichia coli WM3064 as

the recipient strain. Finally, through conjugation, the recombinant plasmid was successfully transferred to the Shewanella oneidensis MR-1 strain.

2.2 Experimental method

2.2.1 Experimental principle

This study aims to construct overexpression strains of the TBSR1 gene of Shewanella through molecular cloning and conjugation transfer techniques. In the experiment, the TBSR1 gene fragment was first amplified from the Shewanella genome using high-fidelity PCR technology. After enzymatic digestion treatment, it was homologous recombined with the linearized pBbB5k plasmid to construct the recombinant expression vector PBBB5K-TBSR1. Then, the recombinant plasmid was introduced into the Escherichia coli WM3064 conjugated strain through electrotransformation. After antibiotic screening and sequencing verification, the recombinant plasmid was transferred into the Shewanella host cells by means of conjugation. Finally, the RT-qPCR detection method was adopted to verify the overexpression of the TBSR1 gene to ensure the successful construction of the recombinant strain.

2.2.2 Experimental process

In this study, a strain with overexpression of the TBSR1 gene of Shewanella was constructed through systematic experiments. Firstly, the genomic DNA of Shewanella was obtained using the Bacterial genomic DNA Extraction Kit (DP302). After the integrity and purity were detected by 1% agarose gel electrophoresis, specific primers were designed based on the sequence information in the UniProt database, and sufficient target gene fragments were obtained through high-fidelity PCR amplification.

In the construction stage of the expression vector, the pBbB5k plasmid was digested by EcoRI and BglII double enzymes. After separation by agarose gel electrophoresis, the linearized vector was recovered and homologous recombined with the purified TBSR1 gene fragment to construct the recombinant plasmid. The recombinant product was transformed into Escherichia coli DH5α competent cells. Positive clones were preliminarily identified through kanamycin resistance screening and colony PCR.

Given that Shiva bacteria are difficult to directly undergo plasmid transformation, the study utilized DAP-deficient Escherichia coli WM3064 as the conjugate bacteria. The recombinant plasmid was electrotransected to WM3064, cultured in the medium containing DAP and kanamycin, and then mixed with Shewanella MR-1 in a 1:1 ratio for conjugative transfer. Kanamycin resistance screening was conducted on the zygotes. Finally, the overexpression of the TBSR1 gene was verified by qPCR detection to complete the construction of the recombinant strain.

2.3 Cloning of tbsr1 gene and construction of expression strains

2.3.1 Extraction of the tbsr1 gene

Before conducting the experiment, anhydrous ethanol needs to be added to the buffer solution GD and the rinsing solution PW first. The specific operation steps are as follows:

First, take 1-5 mL of the bacterial culture medium and place it in a centrifuge tube. Centrifuge at 10,000 rpm (approximately 11,500×g) for 1 minute. After centrifugation is completed, carefully aspirate the supernatant and try to avoid any residue. Next, add 200 μL of buffer GA to the bacterial precipitate in the tube, shake thoroughly until the bacteria are completely suspended, then add 20μL of Proteinase K solution and gently invert to mix well. Subsequently, add 220 μL of buffer GB, shake for 15 seconds, and then incubate the test tube in a 70 ° C water bath for 10

minutes. When the solution becomes clear and transparent, briefly centrifuge it to remove the condensate water on the inner wall of the tube cap.

Next, add 220 μL of anhydrous ethanol to the tube and shake to mix well for 15 seconds (at this point, flocculent precipitate may appear). After another brief centrifugation, all the solutions and flocculent precipitates were transferred together to the adsorption column CB3 with a collection tube and centrifuged at 12,000 rpm (approximately 13,400×g) for 30 seconds. After pouring out the waste liquid, put the adsorption column back into the original collection tube. Then, add 600 μL of rinsing solution PW to the adsorption column (reconfirm whether anhydrous ethanol has been added before use), centrifuge at the same speed for 30 seconds and discard the waste liquid.

Afterwards, centrifuge the adsorption column and the collection tube together at 12,000 rpm for 2 minutes. After draining the waste liquid, place the adsorption column at room temperature and let it stand for several minutes to ensure that the residual rinsing solution on the adsorption material is completely evaporated. Finally, transfer the adsorption column to a clean centrifuge tube, add 50 μL of elution buffer TE to the center of the adsorption membrane, let it stand at room temperature for 2 to 5 minutes, then centrifuge at 12,000 rpm for 2 minutes, and collect the solution in the centrifuge tube. Thus, the extraction operation of genomic DNA is completed.

2.3.2 PCR amplification of the TBSR1 genome

When performing PCR amplification on the amplified TBSR1 gene fragment, high-fidelity DNA polymerase should be used, and the entire operation process should be carried out on ice. Reagent components taken out from a low-temperature environment should first be thawed on ice. After being completely melted, they should be thoroughly shaken. After use, they should be promptly returned to a -20℃ refrigerator for storage. The configuration of the reaction system and the PCR reaction procedure are shown in Tables 2.2 and 2.3 respectively.

Table 2.2 Reaction System

Component	Volume
ddH_2O	20 μL
2×Phanta Flash Master Mix	25 μL
Upstream primer (10 μm)	2 μL
Downstream primer (10 μm)	2 μL
Template DNA	1 μL

Table 2.3 PCR Standard Reaction Procedure

Steps	Temperature	Time	Recurring number
Permutability	98 ℃	30 sec	
Gender change	98 ℃	10 sec	
Annealing	Tm＋5 ℃	5 sec	28-35
Extension	72 ℃	5-10 sec/kb	
Complete extension	72 ℃	1 min	

2.3.3 Enzymatic digestion and transformation of plasmids

Next, the double enzyme digestion treatment of the pBbB5k-GFP plasmid was carried out. The specific operation is as follows: Two restriction endonucleases, EcoR I and BglII, were selected and the reaction was incubated in a constant temperature environment of 37 ℃ for 30 minutes. The preparation of the enzyme digestion system can refer to Table 2.4. After the enzyme digestion is completed, the linearized plasmid needs to be purified to remove the residual reagents and impurities during the enzyme digestion process, so as to obtain high-purity carrier fragments. The purified enzyme digestion products will be used as the backbone vectors for subsequent experiments, for directional ligation with the TBSR1 gene fragment, thereby constructing recombinant plasmids and preparing for downstream experimental operations.

Table 2.4 Plasmid Digestion System

Reagent name	Dosage
EcoR I	1 μL
Bgl II	1 μL
10×Bufer	2 μL
pBbB5k	16 μL
Total	20 μL

After the enzymatic digestion reaction is completed, the plasmid needs to be purified with the help of the TaKaRa MiniBEST DNA Fragment Purification Kit Ver.4.0 kit. The specific operation is as follows: First, add three times the volume of Buffer DC to the digestion product, mix thoroughly, then transfer the mixed solution to the Spin Column installed on the Collection Tube, centrifuge at 12,000×g for 1 minute at room temperature, and discard the filtrate. If the DNA recovery efficiency is not ideal, centrifugation should be repeated to increase the recovery rate.

Next, add 700 μL of Buffer WB with anhydrous ethanol prepared in proportion to the Spin Column. Centrifuge at the same rotational speed for 30 seconds and then discard the filtrate. This washing step needs to be repeated once to ensure that impurities are removed completely.

After the washing is completed, empty the Spin Column for 1 minute to completely remove the residual ethanol, and then transfer it to a clean 1.5 mL centrifuge tube. Subsequently, slowly add 25-30 μL of sterile water preheated to 60 °C to the center of the column membrane.

After standing for 1 minute, centrifuge at 12,000×g for 1 minute. At this point, the liquid in the collection tube is the purified linearized plasmid, which can be directly used for the subsequent ligation experiment with the TBSR1 gene fragment.

2.3.4 Plasmids and genomic fragments undergo homologous recombination

The plasmid vector after enzymatic digestion was ligated with the TBSR1 amplification fragment by homologous recombination using the Clon Express Ultra One Step Cloning Kit V2 kit. The construction of the reaction system needs to be carried out on the ice surface, and each component should be prepared according to the proportions in Table 2.5. This is done to maintain the activity of the recombinase and ensure the efficient progress of the lapping process. Throughout the entire operation process, it is necessary to maintain a low-temperature environment to prevent the activity of enzymes from decreasing due to the increase in temperature, which could affect the experimental results.

Table 2-5 Homologous recombination Reaction System

Component	Recombination reaction
Linearized carrier	1 μL
One inserted fragment	3 μL
2×CE Mix	5 μL
ddH_2O	1 μL

2.3.5 Transformation of recombinant plasmids

In the experiment, the Vazyme #C505 DH5α chemically competent cells were first taken out of the low-temperature environment and placed on the ice surface to thaw slowly. After the cells have completely melted, take 5-10 μL of the recombinant product and gently add it to 100 μL of competent cells. Mix the liquid evenly by gently bouncing the tube wall (do not shake to avoid damaging the cells). Then place the centrifuge tube on ice for 30 minutes to allow the cells to fully absorb the recombinant DNA.

Next, transfer the mixed solution to a 42 ℃ water bath and heat it for 30 seconds. After completion, quickly place it back on the ice to cool for 2 to 3 minutes to restore the cells to a stable state. After cooling, add 900 μL of SOC or LB liquid medium without antibiotics. Place the centrifuge tubes on a 37 °C shaker and shake at 200-250 rpm for 1 hour to provide an environment for cell recovery and proliferation. During this period, the corresponding resistant LB solid medium plates were simultaneously placed in a 37 ℃ incubator for preheating to ensure the appropriate temperature for the subsequent coating operation.

After the culture was completed, centrifuge the bacterial solution at 5,000 rpm (2,500×g) for 5 minutes. Carefully discard 900 μL of the supernatant and gently resuspend the bacterial precipitate with the remaining medium. Finally, use a sterile spreader to evenly spread the resuspended bacterial solution on the surface of the preheated resistant plate. After the operation is completed, invert the plate and place it in a 37 °C incubator for 12 to 16 hours until colonies are clearly formed.

2.3.6 Identification of recombinant products

After the culture was completed, positive clones were screened through the following steps. Single colonies were picked and mixed evenly in 10 μL ddH_2O as templates. Colony PCR identification was performed using pBbB5k F1 and FUR YR primers (Table 2.6). The reaction systems and procedures are shown in Tables 2-2 and 2-7, respectively.

Table 2.6 Primers and sequences

Primer name	Primer sequence
pBbB5k F1	CGAGATCGTTTAGGCA
TBSR1 YR	GCATCGGTCAAATCGT

Table 2.7 Colony PCR Reaction Procedure

Temperature	Time	Cycles
95 ℃	3 min permutability	
95 ℃	15 sec	
60 ℃	15 sec	30-35 cycles
72 ℃	60 sec/kb	
72 ℃	5 min (Complete extension)	

2.3.7 Plasmid extraction

First, take 1 to 5 milliliters of the bacterial liquid that has been cultured overnight for 12 to 16 hours, transfer it to a centrifuge tube, and centrifuge at a speed of 10,000 rpm (11,500×g) for 1 minute. After centrifugation is completed, discard the upper layer of culture medium, turn the centrifuge tubes upside down onto absorbent paper, and gently press to aspirate all the remaining liquid.

Next, add 250 μ l of Buffer P1 to the bacterial precipitate in the tube and repeatedly pipette it with a pipette until the bacteria are completely dispersed into a uniform suspension and no obvious bacterial clumps can be seen. Subsequently, add 250 μ l of Buffer P2 and gently invert the centrifuge tubes up and down 8-10 times to ensure the solution is fully mixed and presents a viscous and transparent state. At this point, 350 μ l of Buffer P3 should be added immediately. The solution should be rapidly inverted and mixed 8-10 times. After the color of the solution changes, centrifuge at 12,000 rpm (13,400×g) for 10 minutes. Take the supernatant after centrifugation for later use.

Install the Fast Pure DNA Mini Columns adsorption column on the 2-milliliter collection tube. Carefully transfer the supernatant into the adsorption column, centrifuge at a rotational speed of 12,000 rpm for 30-60 seconds, and discard the waste liquid in the collection tube. Next, the adsorption column was washed twice

with 600 μL of the proportionally prepared Buffer PW2. After each wash, centrifugation was required and the waste liquid was discarded. After the washing is completed, centrifuge empty again for 1 minute to thoroughly remove the residual liquid in the adsorption column.

Finally, transfer the adsorption column to a 1.5 ml sterilized centrifuge tube. Add 30 μL of preheated Elution Buffer to room temperature in the center of the adsorption membrane. Let it stand for 2 minutes to allow the liquid to fully contact the membrane surface. Then centrifuge at the same speed for 1 minute and collect the DNA solution in the centrifuge tube. The extracted DNA products should be promptly stored in a refrigerator at -20 ℃ for subsequent experiments.

2.3.8 The transfer of zygomyces

The purpose of this step is to transfer the freshly extracted recombinant plasmid into Escherichia coli WM3064, and then introduce the plasmid into Shewanella through conjugation transfer, thereby achieving gene overexpression. The specific operation is as follows: First, take 5-10 μL of the recombinant plasmid and gently add it to 100 μL of competent cells. Gently tap the test tube wall to ensure thorough mixing of the two. Then, place the test tube on ice and let it stand for 30 minutes. It should be noted that the volume of the recombinant plasmid should not exceed one-tenth of the volume of the competent cells used. After the static placement is completed, place the test tube in a water bath at 42 ℃ and heat it for 30 seconds. Once done, quickly take it to ice to cool for 2 to 3 minutes. Next, the transformed product was inoculated into LB liquid medium containing DAP and kanamycin for culture. PCR amplification was performed using pBbB5k F1 and TBSR1 YR primers, and then the purity of the strain was observed by agarose electrophoresis detection.

During the conjugated transfer operation, the cultures of conjugated bacteria and Shewanella need to be mixed evenly in a 1:1 ratio, and then inoculated onto LB

solid medium containing kanamycin for selective culture. After the colonies are formed, monoclonal colonies are selected for further PCR amplification verification.

2.3.9 Verification of overexpression of Shewanella

After the successful construction of recombinant Shiva bacteria carrying exogenous plasmids, the overexpression level of the target gene needs to be verified by quantitative real-time fluorescent PCR (qPCR) technology. The experiment will use the specific primers listed in Table 2.8, with the cDNA obtained through reverse transcription as the amplification template. The number of transcripts of TBSR1 will be determined by absolute quantitative methods. Finally, the relative expression level of the exogenous gene will be evaluated by comparing the Ct value ratio of the target gene to the ribosomal gene.

Table 2.8 Primers and primer sequences

Primer name	Primer sequence
TBSR1 Q1	ATCACACTTTTATTAGAAGT
TBSR1 Q2	TGATTATACGATGGATAAGG

Summary of chapter II

1. Material Preparation: Obtain the TBSR1 gene sequence of Shewanella from UniProt and design primers containing the EcoRI/BglII digestion site; Using the pBbB5k-GFP plasmid as the vector and the Escherichia coli DH5α and WM3064 strains, the target strains were screened through kanamycin resistance.

2. Strain Construction: The genomic DNA of Shewanella was extracted. The TBSR1 gene was amplified by high-fidelity PCR. After double-enzyme digestion of the plasmid, it was homologous recombined with the gene fragment. The DH5α was transformed to verify the positive clone. Then, the recombinant plasmid was transferred to Shewanella MR-1 by Zygomycetes WM3064.

3. Verification and Identification: Extract the recombinant plasmid and purify it. Detect the expression level of the TBSR1 gene by qPCR. Evaluate the overexpression level using the ratio of specific primers to Ct values to complete the verification of the recombinant strain.

Chapter III

EXPERIMENTAL PART

3.1 Genome extraction results

Shewanella was inoculated into LB medium and cultured at 37 °C for 16 hours. Following centrifugation, genomic DNA of Shewanella was extracted from the harvested cells using the DP302 kit. The extracted products were analyzed by 1% agarose gel electrophoresis, which showed clear genomic bands above 5000 bp (Figure 3.1, Lane 2) without smearing or degradation. These results indicate that the DNA exhibits high purity and is suitable for subsequent PCR amplification.

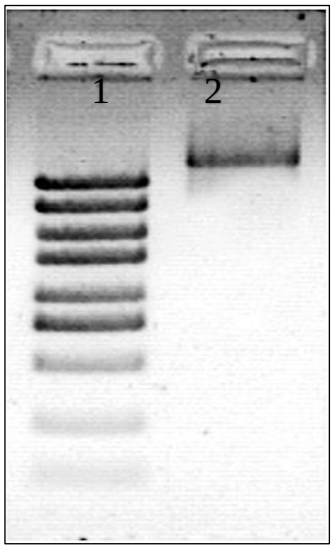

Figure 3.1 Electrophoresis of genomic DNA of Shewanella.

Note: Lane 1 is the DL5000DNA Maker from top to bottom (5000 bp, 3000 bp, 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp); Lane 2: The genomic DNA of Shewanella extracted shows clear bands.

3.2 PCR amplification of the TBSR1 gene

Using the extracted Shewanella genomic DNA as a template and TBSR1-F/R as primer pairs, the full-length fragment of the TBSR1 gene was amplified via high-fidelity PCR. Electrophoresis analysis revealed a single, intense band at approximately 2163 bp (Figure 2.2, lanes 2–3), which matched the expected size and showed no signs of non-specific amplification. This result demonstrates the high specificity of the primers. Following electrophoresis, target fragments were purified using a gel recovery kit, and the recovered TBSR1 fragments will be utilized in subsequent experimental procedures.

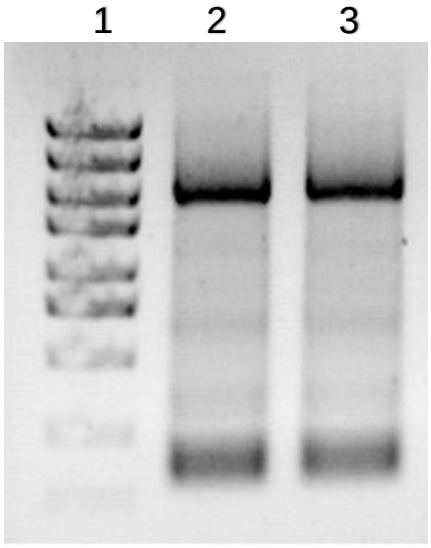

Figure 3.2 PCR amplification electrophoresis diagram of the TBSR1 gene
note: DL5000DNA Maker (5000 bp, 3000 bp, 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp）

3.3 Construction and culture of the recombinant plasmid PBBB5K-TBSR1

The pBbB5k plasmid vector was digested using EcoRI and Bgl II restriction endonucleases. The digestion products were homologous recombined with the

TBSR1PCR products using the Clon Express Ultra One Step Cloning Kit V2. The recombinant plasmid pBbB5k-TBSR1 was transferred into Escherichia coli DH5α cells and then coated onto LB+Kan plates. After being cultured at 37 ° C for 12 hours, uniform colonies appeared on the plates (Figure 3.3).

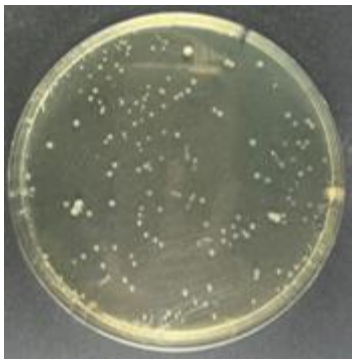

Figure 3.3 The colony growth of Escherichia coli DH5α
after the recombinant plasmid was transformed.

3.4 PCR detection of the recombinant plasmid PBBB5K-TBSR1

Single colonies on LB+Kan plates were selected and verified by colony PCR using pBbB5k-F1 (universal forward primer for vectors) and TBSR1-R (reverse primer for target genes). The results of agarose gel electrophoresis showed (Figure 3-4) that the positive clones presented specific bands at approximately 750 bp. This result indicates that the TBSR1 gene has been successfully inserted into the expression vector. The results showed that the target bands were obtained in colonies 4, 6 and 7, indicating that the pBbB5k-TBSR1 plasmid vector was successfully constructed in these single colonies. Shake culture was conducted on strain No. 4, and the plasmids were extracted and sent to the company for sequencing. The sequencing results indicated that the constructed expression vector sequence was correct, and the extracted plasmids will be used in subsequent experiments.

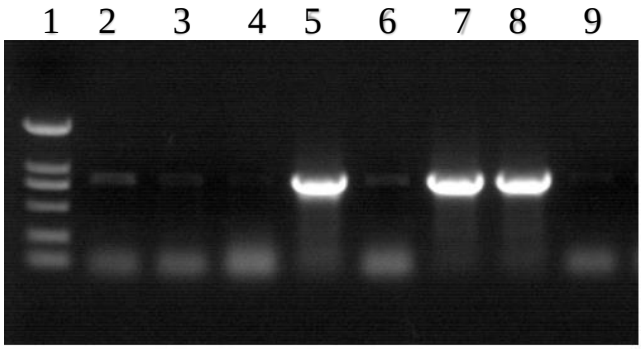

Figure 3.4 PCR detection of the recombinant plasmid pBbB5k-TBSR1

Note: Lane 1: DL2000DNA Maker (2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp from top to bottom); Lane 2-8: Positive recombinant clone, 9 is the negative control.

3.5 The electrotransformation of the PBBB5K-TBSR1 plasmid and the screening of conjugates

The verified correct recombinant plasmid was electrotransferred into the WM3064 zygomycetes (DAP nutrient deficiency type) for culture. After the electrotransfer product was coated on the LB+DAP+Kan plate, milky white colonies with a diameter of 1-2 mm were evenly distributed (Figure 2-5), confirming the success of the culture. Six single colonies were selected for colony PCR. The primers used were pBbB5k F1/TBSR1 YR. Then, the PCR results were verified by AGAR gel electrophoresis (Figure 3.5-3.6). All colonies were successfully transferred into the PBBB5K-TBSR1 plasmid, which was consistent with the genomic PCR results, confirming that the plasmid stably existed in Zygya. Strain No. 2 was selected for the subsequent experiments.

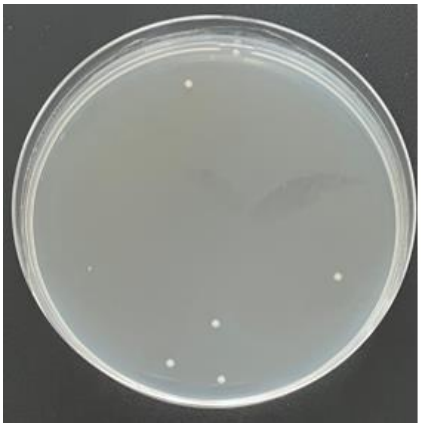

Figure 3.5 The colony growth of the recombinant plasmid pBbB5k-TBSR electrotransformed into WM3064

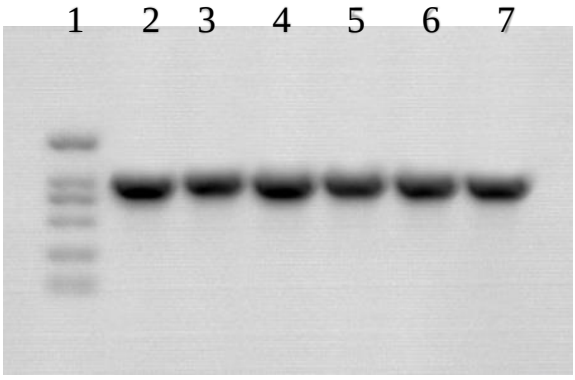

Figure 3.6 Electrophoresis results of Zygomyces

Note: Lane 1: DL2000DNA Maker (2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp from top to bottom); Lane 2-6: Positive recombinant clone. Lane 7: Negative control

3.6 Screening of shiva recombinants after conjugation transfer

After culturing the No. 2 WM3064 conjugation strain with Shewanella, they were mixed in a 1:1 ratio and conjugation occurred. Then, the mixed bacterial liquid was spread on LB+Kan plates for culture. After culturing at 28℃ for 48 hours, typical Shewanella colonies (with neat edges, semi-transparent, and a diameter of 2-

3 mm) appeared on the plates (Figure 3.7). Ten recombinant colonies were randomly selected and inoculated into LB+Kan and LB plates respectively. The results showed that all colonies could grow on the resistant plates, but there was no significant difference in the growth state on the non-resistant plates. This preliminarily indicated that the recombinant plasmid had been transferred into Shewanella through conjugates, and the Kan resistance gene was stably expressed.

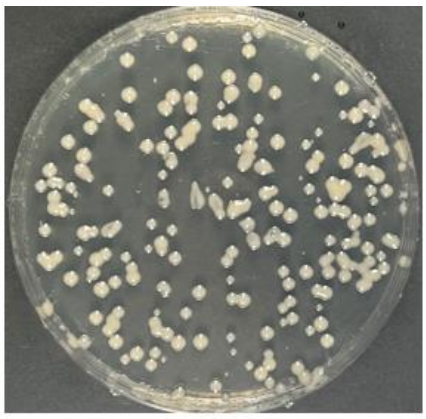

Figure 3.7 Growth of Shewanella after conjugation and transfer

3.7 PCR identification of tbsr1 overexpression strains

Next, using the genomic DNA of the recombinant strain as the template, PCR was continued to be performed using the two primers pBbB5k-F1 and TBSR1-R. A 750 bp specific band was amplified to further confirm that the TBSR1 gene had been integrated into the genome of Shewanella or stably existed in the plasmid (Figure 3.8). Among them, the bands of strains a, b and f were clear, indicating that the recombinant plasmid was correctly transferred in. Strain a was selected for the subsequent experiment.

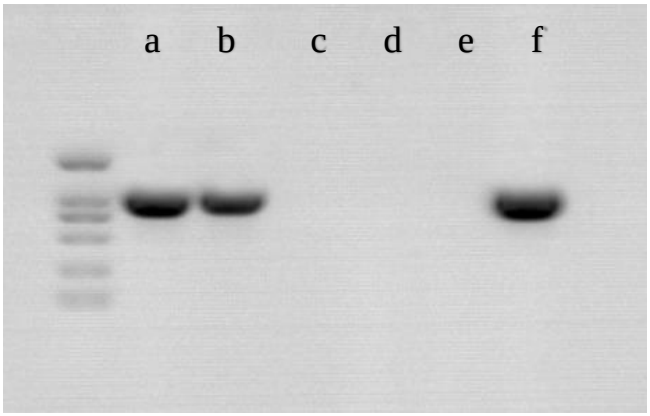

Figure 3.8 PCR identification electrophoresis diagram of TBSR1 overexpression strain

Note: Lane 1: DL2000DNA Maker (2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp from top to bottom) Lanes a-e: Positive recombinant clones. Lane f: Negative control.

3.8 Verification of overexpressed strains by fluorescence quantitative PCR

The expression level of the TBSR1 gene was detected by qPCR technology, and the primers continued to be pBbB5k-F1 and TBSR1-R. The results showed that the relative expression level of the overexpressed strain was 1.684±0.011, close to 1.7 times, significantly higher than that of the wild-type strain (set as 1), and the difference between the two was statistically significant (t-test, $P<0.01$) (Figure 3-9), indicating that the qPCR data was reliable and proving that the TBSR1 gene was overexpressed at the transcriptional level. An overexpressing strain was successfully obtained.

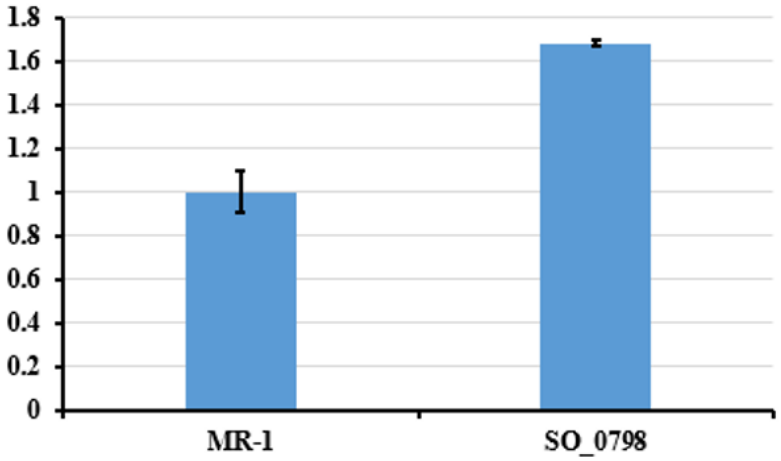

Figure 3.9 Fluorescence quantitative PCR results of overexpressed strains

3.9 Discussion

In this study, the TBSR1 gene of Shewanella, as a potential iron transport-related gene, its functional exploration is of great significance for a deeper understanding of the iron metabolism mechanism of Shewanella. To clarify the specific function of the TBSR1 gene, we adopted a series of molecular biology techniques and experimental methods to conduct research.

At the beginning of the experiment, we selected the appropriate experimental plasmids and cloned strains. The pBbB5k plasmid with specific resistance was selected as the vector, providing a basis for the overexpression of the TBSR1 gene. Meanwhile, the pBbB5k plasmid was preserved and cloned using Escherichia coli strains. With its ability and stable plasmid maintenance characteristics, it provided a sufficient and stable plasmid source for subsequent experiments. In addition, due to the structure of the cell wall of Shewanella, which makes it impossible to be directly transferred in, we utilized conjugation and selected the WM3064 strain with nutritional deficiency of diaminohexanedioic acid as the donor to achieve plasmid conjugation and transfer. Finally, the recombinant plasmid was successfully introduced into Shewanella. Finally, we conducted a series of tests and analyses on the recombinant Shewanella. By using qPCR technology to detect the expression level of the TBSR1 gene in Shewanella, we found that compared with the wild-type

strain, the expression level of the TBSR1 gene in the recombinant strain significantly increased, indicating that the overexpression of the TBSR1 gene was successfully achieved. The achievements of this study provide an important basis for further exploring the iron metabolism mechanism of Shewanella. The obtained TBSR1 gene overexpression strains can be used as materials for subsequent in-depth research.

Summary of chapter III

1. Genome and Target Gene Acquisition: High-purity genomic DNA of Shewanella was successfully extracted. The full-length fragment of the TBSR1 gene (2163 bp) was obtained by high-fidelity PCR amplification, and the band specificity was verified to be good by electrophoresis.

2. Construction and Transfer of Recombinant Plasmids: The pBbB5k-TBSR1 recombinant plasmid was constructed through enzymatic digestion and homologous recombination. After cloning verification, the plasmid was introduced into Shewanella by conjugation, and strains stably carrying the recombinant plasmid were screened out.

3. Overexpression Verification and Functional basis: Fluorescence quantitative PCR confirmed that the expression level of the TBSR1 gene in the recombinant strain was significantly higher than that in the wild type (1.684 times, $P<0.01$), successfully obtaining the overexpression strain and providing experimental materials for exploring its iron metabolism function.

CONCLUSIONS

1. Genome and Target Gene Acquisition: High-purity genomic DNA of Shiva was successfully extracted. The full-length fragment of the TBSR1 gene (2163 bp) was obtained by high-fidelity PCR amplification. Electrophoresis verified that the band specificity was good and could be used for subsequent experiments.

2. Construction of Recombinant Plasmids: The pBbB5k-TBSR1 recombinant plasmid was successfully constructed through enzymatic digestion and homologous recombination. Verified by colony PCR and sequencing, it was confirmed that the gene insertion was correct and the plasmid was stable.

3. Successful conjugation transfer: With the help of Escherichia coli WM3064 conjugated bacteria, the recombinant plasmid was introduced into Shewanella through electrotransformation and conjugation. Resistance screening confirmed that the plasmid was stably transferred.

4. Overexpression strain identification: Molecular biological tests showed that the TBSR1 gene was successfully integrated or stably present in the recombinant strain. qPCR verified that its expression level was significantly higher than that of the wild type (1.684 times, $P<0.01$).

5. Establishment of Technical System: A complete technical process of "cloning - recombination - transfer - validation" has been established, providing a standardized method for the study of the gene function of Shewanella.

6. Scientific Significance: The successful construction of a TBSR1 overexpression strain has laid an experimental foundation for understanding the function of this gene in iron transport and the iron metabolism mechanism of Shewanella.

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