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 **Construction of TBSR3-deficient strains of *Shewanella***

First (Bachelor's) level of higher education

Specialty 162 "Biotechnology and Bioengineering"

Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
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Doctor of Technical Science
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Zhang Wenting

1. Thesis topic **Construction of TBSR3-deficient strains of *Shewanella***
Scientific supervisor Olena Okhmat, Ph.D., Assoc. Prof.

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

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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	
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SUMMARY

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With the intensification of energy crisis and environmental problems, biomass energy has become the focus of energy strategy of various countries because of its advantages of sustainability and renewable energy, microbial fuel cells have been widely concerned because of its development prospects in sewage treatment and clean electricity production.

Microbial Fuel cells (MFC) can degrade pollutants while producing electrical energy through the metabolic action of electrogenic microorganisms. *Shewanella* spp is the most widely studied electrogenic microorganism in the laboratory, which is mainly allogeneic metal-reducing bacteria. For example, the microbe can generate an electric current, with the help of an Extracellular Electron Transfer (EET) mechanism.

In this study, it was believed that TBSR3 gene of *Shewanella* is crucial for the formation of outer membrane pigment protein. Therefore, the function of TBSR3 gene of *Shewanella* was analyzed by using in-frame deletion mutation method to knock out the TBSR3 gene of *Shewanella*, and the changes in electrical production capacity of defective mutant strains were accurately detected, so as to verify the viewpoint. Colony PCR and agarose gel electrophoresis were also used to verify the experiments, and the colonies were screened with the help of nutrient deficiency types and resistance genes.

Keywords: Microbial fuel cell; Shewanella; Extracellular electron transfer; TBSR3 gene; In-frame deletion mutation method;

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INTRODUCTION

The relevance of the topic is Construction of TBSR3-deficient strains of *Shewanella*. **The purpose of the** study is the By knockout TBSR3 gene, we detected whether the energy production of TBSR3-deficient *Shewanella* decreased, so as to prove whether TBSR3 gene is related to the synthesis of C-type pigment protein.

The object of the study TBSR3-deficient strains of *Shewanella*.

Research methods: The in-frame deletion mutation method is mainly used to construct the TBSR3 deletion strain, because it produces in-frame deletion mutants with stable properties, so it is not affected by protein translation mutations, easier to predict the impact of protein function, and avoids the polarity effect caused by the introduction of new genes. The application of this method in the experiment is mainly to amplify the target coding sequence and use PCR technology; Secondly, we treated the PCR products and cut them accurately by restriction enzyme digestion. Then, the recombinant plasmid was constructed by connecting the treated product with the plasmid carrier. Then, the recombinant plasmid was efficiently transformed into the target cell by electrotransformation. Finally, the transformed cells were screened and identified to ensure the accuracy and effectiveness of the experiment.

The scientific novelty In this study, the method of in-frame deletion mutation was used to carry out single gene knockout of TBSR3. By studying the phenotypic changes of TBSR3 gene deletion mutant strains of *Shewanella*, the relationship between gene and phenotype could be directly proved, so as to predict the function of gene. The mutations produced by in-frame deletion mutation method are in-frame deletion and do not cause mutations that affect protein translation such as frame shift and early stop codon.

The practical significance of the results obtained. In terms of gene function, the specific function of TBSR3 gene in *Shewanella* can be clarified through the construction of deletion strains, especially the role of outer membrane pigment

protein formation. This helps us to better understand the regulatory mechanisms of this gene in cell physiology and metabolism.

In terms of functional exploration of outer membrane pigment proteins, outer membrane pigment proteins may have various functions in bacteria, such as protecting cells from ultraviolet damage, participating in nutrient transport, participating in signal transmission, etc. By observing the changes of outer membrane pigment proteins after the deletion of TBSR3 gene, we can further reveal the important role of these proteins in bacterial survival and adaptation to the environment.

In the application of biotechnology, if the experiment finds that TBSR3 gene is a key regulator of outer membrane pigment protein formation, then this discovery can provide new tools and strategies for the field of biotechnology. For example, by regulating the expression of the TBSR3 gene, it is possible to control the production of the pigment protein in the outer membrane of *Shewanella*, which may be used to develop novel biomaterials or biosensors.

In the understanding of environmental adaptability and biodiversity, *Shewanella* usually lives in various extreme environments, such as high salt, low temperature, heavy metal pollution, etc. Understanding the relationship between the TBSR3 gene and the outer membrane pigment protein will help us better understand how these bacteria adapt to different environmental stresses and maintain biodiversity.

In terms of disease control and maintenance of public health, if *Shewanella* is a pathogen or potential pathogen of a disease, then understanding the function of TBSR3 gene can provide new ideas for disease control and public health. For example, by interfering with the expression of the TBSR3 gene, it may be possible to inhibit the growth of bacteria or the production of toxic factors, thus providing a theoretical basis for the development of new antibacterial drugs or vaccines.

In summary, verifying the potential association between TBSR3 gene and the formation of outer membrane pigment protein in *Shewanella* can provide us with many valuable scientific knowledge and practical applications, which is of great significance to promote the development of biology, medicine, biotechnology and other fields.

CHAPTER 1
LITERATURE REVIEW

1.1 Research background and significance

With the rapid development of economy and technology and the continuous growth of population, the problem of environmental pollution and energy crisis is becoming more and more serious, which threatens the survival and sustainable development of mankind. For example, the massive use of fossil fuels has reduced its total amount, gradually increasing environmental pollution problems, frequent extreme weather, global warming and so on. In the face of this situation, we should vigorously develop clean new energy technologies and reduce the use of fossil fuels to alleviate environmental pollution and energy crisis.

Because biomass energy has the advantages of renewable, less pollution and universality, it has become the focus of global attention. The rich organic matter in sewage is an important source of biomass energy. The use of emerging technologies to transform waste materials into valuable resources not only treats water pollutants but also produces energy, which is a feasible way to effectively deal with water pollution and energy shortage. Among many emerging green energy technologies, Microbial Fuel Cell (MFC) has development advantages in treating sewage and producing clean electric energy [1], which is of great practical significance to the sustainable development of human society and has attracted wide attention[2]. *Shewanella* has a property that can affect the potential of the external electrode at a low REDOX potential [3], which provides a significant advantage for *Shewanella* to become an important class of microbial fuel cells.

1.2 Overview of microbial fuel cells

1.2.1 Advantages of microbial fuel cells

Microbial fuel cell is a technology that utilizes the capacity of electrogenic microorganisms to extract chemically mild organic matter in sewage and convert it

directly into clean electrical energy (Bio-electrochemical system, BES)[4]. Compared with other new green technologies, MFC has three advantages: first, it has a high energy conversion efficiency, which can directly convert the substrate into electricity; Secondly, microbial fuel cells can operate efficiently at room temperature environmental conditions; Third, in some remote areas with crude power infrastructure, microbial fuel cells have the potential advantage of extensive and universal application [5].

1.2.2 Basic principle of microbial fuel cell

The most common H-type two-compartment MFC device structure consists of a proton exchange membrane, an anode chamber and a cathode chamber. In the anode chamber, bacteria metabolize organic substrates to produce protons and electrons. Bio-electrons are transferred to the anode by bacterial transmembrane electron transport and then to the cathode by means of an external circuit[6]. At the same time, protons released by Nicotinamide adenine dinucleotide (NADH) pass through the exchange membrane into the cathode chamber and react with electrons and oxygen to form water. This constitutes a complete external circuit that continuously generates an output current.

1.3 Overview of *Shewanella*

1.3.1 Definition of electrogenic microorganisms

Electrogenic microorganisms, also known as electrogenic bacteria, are those that can transfer electrons produced by their own metabolism to electron acceptors outside the cell, such as solid electrodes. At present, a variety of electrogenic microorganisms with extracellular electron transfer ability have been discovered, isolated and screened, and the most widely studied in the laboratory are foreignizing metal-reducing bacteria, such as *Shewanella spp.* and *Geobacter spp*.

1.3.2 Extracellular electron transfer mode of *Shewanella* at the electrode interface

According to existing studies, electrogenic microorganisms can carry out extracellular electron transfer (EET) at the electrode interface, which can be mainly divided into two ways. The first is direct electron transfer (DET), which includes two ways: one is through the microbial outer membrane or cell wall REDOX proteins (such as cytochrome protein, flavin protein) and solid phase electron acceptor or donor direct contact, to achieve close electron transfer; The second is to use structures called "nanowires" or "conductive" fimbries to achieve long-distance electron transfer through a multi-step electron transition process. The other type is indirect electron transfer (MET), which relies on electronic mediators such as small REDOX active molecules produced by microorganisms themselves or electroactive molecules naturally present in the environment (such as soluble or solid humus) that mediate electron transfer to extracellular receptors[7]. Multiple EET pathways can exist simultaneously in one electric-producing microorganism.

1.3.3 Application of *Shewanella* in microbial fuel cells

Shewanella, as a unique microorganism, plays a crucial role in the application of microbial fuel cells (MFCs). This application is mainly due to the unique electron transport mechanism possessed by *Shewanella*, which is able to efficiently convert biological energy into electricity.

The extracellular proteins of *Shewanella* are located on the surface of the microbe and not only play a role in protecting the cell, but are also responsible for interacting with other substances. In MFCs, extracellular proteins act as "Bridges" for electron transport, binding tightly to electron mediators to ensure smooth transfer of electrons from inside to outside the cell.

The electronic medium plays a key role in the electron transport process. These mediators, such as certain organic molecules or metal complexes, are able to capture the electrons produced within the cells of *Shewanella* and pass them on to the next

link. By selecting and optimizing the electronic medium, we can improve the efficiency and stability of MFCs.

Metal nanoparticles, as another important element in MFCs, have unique electron transport properties. These nanoparticles are able to provide a large surface area, increasing the point of contact between the electronic medium and the electrode, thereby accelerating the speed of electron transfer. At the same time, the metal nanoparticles also have good electrical conductivity, which can ensure that no loss of electrons occurs during the transfer process.

In MFCs, *Shewanella* transmits electrons produced within the cell to the electrode surface through its unique electron transport mechanism. Specifically, electrons inside the cell are first captured by extracellular proteins and delivered to the electronic medium. The electronic medium then transfers these electrons to the metal nanoparticles, which eventually reach the electrode surface. At the electrode surface, electrons combine with protons to form hydrogen or other useful chemicals, while generating electricity.

In this way, *Shewanella* not only realizes the conversion of bioenergy and electric energy, but also provides a new idea and method for the development of MFCs. In the future, with the in-depth study of the electron transport mechanism of *Shewanella* and the continuous progress of technology, MFCs will be more widely and deeply applied in the fields of energy and environmental protection.

1.3.4 Research status of *Shewanella* in iron transport

The TonB-dependent receptor (TBDR) is a family of proteins present on the outer membrane of the cell. TBDRs are a channel that opens in response to an external ligand and allows extracellular nutrients, such as iron-iron-carrier complexes or non-iron compounds, to enter the periplasmic space. The transfer of iron-carrier complexes from the outside of the cell to the periplasm relies primarily on TonB-dependent siderophore receptors (TBSRs) located on the outer membrane and a TonB-ExbB-ExbD energy transduction system across the endometrium and

periplasm. This system works synergically to ensure that the iron-ferriferal complex is efficiently transported from the external environment to the intracellular periplasmic region [8].

After reviewing the data and counting the experiments carried out so far, we can basically determine the iron transport pathway of *Shewanella* dependent on iron carriers. Some of the ferriferic receptors in this bacterium have biological functions, such as participating in iron reduction processes, but the mechanism of action has not been thoroughly studied. In another part of the study, only the TONB-dependent siderophore receptor, PutA, was found to specifically recognize and mediate iron uptake by siderophore synthesized by *Shewanella* itself. Therefore, if PutA is absent, the cells will completely lose their ability to take up iron through iron carriers.

In electrogenic microorganisms, C-type pigment proteins located on the outer membrane of the cell play a key role, which effectively mediates the transmembrane electron transport process through direct contact, and realizes the efficient transfer of electrons from the inside of the cell to the outside. For the electrogenic microbe *Shewanella*, its electron transfer ability is closely related to the pigment protein on the outer membrane. In order to verify the potential association between TBSR3 gene and outer membrane pigment protein formation in *Shewanella*, deletion strains were designed and constructed for verification.

Conclusion to chapter 1

1. Microbial fuel cell is a kind of technology that converts chemical energy contained in organic matter in sewage directly into clean electric energy by using electricity-producing microorganisms. Compared with other new green technologies, MFC has three significant advantages; High energy conversion efficiency, efficient operation at room temperature, wide application potential.

2. The application of *Shewanella* in microbial fuel cells is mainly based on its unique electron transfer mechanism. It is mainly divided into direct electron transfer and indirect electron transfer. This mechanism is mediated by key elements such as

extracellular proteins, electron mediators and metal nanoparticles on the microbial surface, and effectively transfers electrons from the cell to the electrode surface, thus realizing the electrical energy generation process of the microbial fuel cell.

3. As an important electrogenic microorganism, *Shewanella* plays a key role in MFC technology. At the same time, remarkable progress has been made in the research of iron transport. The TONB-dependent receptor (TBDR) was found to be an important extracellular membrane protein family in the study of microbial iron transport. These receptors are able to respond to external ligands and open channels that allow extracellular nutrients, such as iron-iron-carrier complexes or non-iron compounds, to enter the periplasmic space. The transfer of iron-ferriferic complexes from the outside of the cell to the periplasm relies primarily on the TonB ferriferic receptor on the outer membrane and a TONB-EXBB-EXBD energy transduction system across the endometrium and periplasm. This system works together to ensure that the iron-ferriderite complex can be efficiently transported from the external environment to the intracellular periplasmic region.

4. In *Shewanella*, the iron transport pathway is mainly dependent on iron carriers. The TONB-dependent siderophore receptor, PutA, specifically recognizes and mediates iron uptake by siderophore synthesized by *Shewanella*. If PutA is absent, the cells completely lose their ability to take up iron through iron carriers. This discovery provides important clues for in-depth understanding of the iron transport mechanism of *Shewanella*, and lays a foundation for future research and applications. In order to verify the potential association between TBSR3 gene and outer membrane pigment protein formation in *Shewanella*, deletion strains were designed and constructed for verification.

CHAPTER 2
OBJECT, PURPOSE AND METHODS OF THE STUDY

The object of the study TBSR3-deficient strains of *Shewanella*. Subject of study the function of TBSR3 gene in *Shewanella*, explore the relationship between TBSR3 gene and C-type pigment protein synthesis, and analyze the influence on energy production. These studies will help us to deeply understand the biological characteristics of *Shewanella*, and provide theoretical basis and technical support for future applications.

Purpose of the study:

1. To study the function of TBSR3 gene: By constructing TBSR3-deficient strains of *Shewanella*, the specific effects of TBSR3 gene on the growth, metabolism and energy production of *Shewanella* can be directly observed and studied.

2. Explore the relationship between TBSR3 and C-type pigment protein synthesis: By comparing the differences in C-type pigment protein synthesis between wild-type *Shewanella* and TBSR3-deficient strains, explore whether TBSR3 gene participates in or influences the C-type pigment protein synthesis process. This is of great significance for understanding the mechanism of pigment synthesis in *Shewanella*.

3. Analysis of reduced energy production: By measuring and comparing the differences in energy production between wild type and TBSR3-deficient strains, it is possible to determine whether TBSR3 gene has an important influence on energy production of *Shewanella*. This will help to understand the energy metabolism mechanism of *Shewanella* and the role of TBSR3 gene in it.

2.1 Experimental Equipment

Experimental materials

Escherichia coli DH5α, nutrient deficiency strain β2163, pLP12Cm, single-sided blade, purification kit, plasmid extraction kit, 1.5mL centrifuge tube, ultra-pure water (sterile).

Experimental instruments

Agarose gel electrophoresis system, UV transmisometer, table centrifuge, pipette gun (with gun head), ultra-clean workbench, -20℃ low temperature refrigerator, analytical balance, measuring cylinder, beaker, test tube, triangle bottle, test tube holder, alcohol lamp, petri dish.

Experimental reagents

PCR amplification product, Takara Primer STAR Max DNA Polymerase, Wash Solution, Buffer B3, Elution Buffer, agarose, 1×TAE, 6×Loading Buffer, DNA Marker 2000, DNA Marker 5000,Ethanol, isopropyl alcohol, pancreatic peptone, yeast extract, sodium chloride, recombinant enzyme Exnase II (ClonExpress II, Vazyme), ddH_2O, 5×CE II Buffer.

2.2 Experimental principles and methods

2.2.1 Experimental methods

In the process of constructing TBSR3 Deletion strains, in-frame Deletion Mutation has been widely used, which plays an important role in gene function studies due to its unique advantages. This approach avoids unintended effects caused by mutations occurring during protein translation by precisely deleting a portion of the sequence of the target gene without altering the integrity of its open reading frame (ORF). Because the resulting mutants have high stability in gene sequence and protein expression, changes in protein function can be predicted more accurately.

In the experiment, the application process of in-frame deletion mutation method mainly includes the following steps:

First, the target coding sequence was amplified by PCR. By designing specific primers, PCR products can cover part or all of the TBSR3 gene sequence. This step is the basis for building the missing mutant and is crucial for subsequent genetic manipulation.

Secondly, the PCR products were subjected to restriction enzyme digestion. According to the target gene sequence and the recognition site of the selected restriction enzyme, the appropriate enzyme restriction conditions were selected to precisely cut the PCR products. With this step, specific sequences in the TBSR3 gene can be removed, creating in-box deletions.

Subsequently, the treated product was linked to the plasmid carrier. This step requires the use of DNA ligase to connect the missing fragment to the plasmid vector to form a recombinant plasmid. Plasmid vectors usually carry selective marker genes, such as antibiotic resistance genes, to facilitate subsequent screening and identification.

Then, the recombinant plasmid was efficiently transformed into the target cell by means of electrical transformation. Electrotransformation is an efficient cell transformation technique that can introduce a large number of DNA molecules into cells in a short period of time. In this step, the recombinant plasmid is mixed with the *Shewanella* cell, and the plasmid is introduced into the cell through the action of electrical impulses.

Finally, the transformed cells were screened and identified. Through the selection of marker genes, cells containing recombinant plasmids can be screened. Subsequently, PCR, sequencing and other technologies were used to identify the target gene to ensure the correctness of the missing mutant.

After constructing TBSR3 gene deletion mutant strains of *Shewanella*, the phenotypic changes can be studied experimentally to predict the function of the gene. For example, it is possible to compare the differences in growth rate, metabolites, and so on between wild-type strains and the missing mutant strains to infer the function of the TBSR3 gene in *Shewanella*. In addition, it can also be combined with other

experimental techniques, such as proteomics, transcriptomics, etc., to conduct more in-depth research on gene function.

2.2.2 Experimental principles

(1) Sequence analysis and primers design

First, we need to download the whole genome sequence of *Shewanella* from the NCBI database or other bioinformatics resources[9]. This typically includes one or more FASTA format files containing nucleotide sequence information for the genome.

Then identify the target genes that need to be missing. This may be based on experimental purposes, functional predictions of the gene, or the results of previous studies.

Then it is necessary to identify and locate the upstream and downstream homologous fragments. Bioinformatics software, such as BLAST, is used to compare the sequence of the target gene with the whole genome sequence to find its precise location. On both sides of the target gene, appropriate upstream and downstream fragments are selected as the basis for the design of PCR primers. These fragments should be long enough to be amplified specifically in a PCR reaction.

Then PCR primers were designed. Primer design principles need to be followed, that is, the length of the primer is usually between 18-30 bases, the GC content is moderate (about 40-60%), and the dimer and hairpin structure between the primers is avoided. Special primer design software (such as Primer3, Oligo, etc.) was used to input the sequences of upstream and downstream fragments, and PCR primers were designed according to the parameters suggested by the software.

According to the needs of the experiment, each pair of primers was assigned a unique name (such as TBSR3-MF1/TBSR3-MR1 and TBSR3-MF2/TBSR3-MR2) and its sequence was recorded.

(2) Overlapping PCR

Overlapping extension PCR (OE PCR) is a widely used cloning technology in genetic engineering and molecular biology, which is highly functional and widely used [10]. The main principle of this technique is to fuse two or more adjacent DNA fragments by designing specific primers to generate a complete DNA molecule with a specific sequence.

In practice, it is first necessary to carefully design two or more pairs of primers according to the sequence information of two or more adjacent DNA fragments. At least one pair of these primers has a special design: the 3' end (i.e. the extension end) of one of them contains the end sequence of the first DNA fragment, while the 5' end (i.e. the beginning end) of the other contains the beginning sequence of the second DNA fragment. This design ensures that when PCR amplification is performed, two DNA fragments can overlap at specific locations and fuse through the extension action of DNA polymerase.

Next, the prepared PCR reaction system (including DNA template, primer, dNTPs, DNA polymerase, etc.) is added to the special reaction tube of the PCR instrument. Then, by setting the appropriate PCR reaction conditions (such as temperature cycle, time, etc.), the PCR amplification procedure is started. During PCR amplification, the DNA polymerase replicates on the template DNA according to the guidance of the primer and joins two or more DNA fragments together through overlapping sequences in the primer.

Finally, in order to detect the size and quantity of PCR products, gel electrophoresis is usually used[11]. PCR products were mixed with an appropriate amount of loading buffer and added to a gel electrophoresis tank containing agarose or polyacrylamide, and electrophoresis was performed under the action of an electric field. By staining (such as EB staining) and observation, the position of DNA fragments of different sizes on the gel can be clearly seen, so as to judge the effect of PCR amplification.

The application range of overlapped extended PCR technology is very wide, including but not limited to gene fusion, site-specific mutation, gene repair and so on. It can not only be used for basic research in the laboratory, but also for genetic engineering transformation in industrial production and biopharmaceutical fields.

(3) Suicide carrier

Suicide plasmids play a special role in the field of genetic engineering. After entering the host cell, these plasmids undergo a replication process[12], which is where they are similar to other plasmids. What makes suicide plasmids unique, however, is the way they integrate with the genome of the host cell.

The integration process of suicide plasmids relies on a specific protein, which we call an "integration factor." This protein plays a crucial role in the integration process, helping the suicide plasmid to integrate stably into the genome of the host cell. However, if this integration factor is lacking within the host cell, the suicide plasmid cannot be successfully integrated, but is instead recognized by intracellular repair mechanisms and quickly eliminated. This property gives suicide plasmids a unique advantage in experiments such as gene knockout.

In knockout experiments, researchers usually first need to clone the homologous arm DNA segment of the target gene. This homologous arm is a piece of DNA with a similar sequence to the target gene, and it can be homologous recombination with the target gene. This homologous arm is then integrated into the suicide carrier. In addition to homologous arms, suicide vectors also need to have positive and negative screening markers [13]. Positive screening markers were used to screen cells that successfully integrated suicide vectors, while negative screening markers were used to eliminate those that did not.

When a suicide vector containing a homologous arm is introduced into the target strain, a first round of single exchange events occurs. In this process, the homologous arm on the suicide vector will have a homologous recombination with the target gene on the chromosome DNA, forming a heterozygous DNA molecule.

This heterozygous DNA molecule contains a part of the suicide vector sequence and a part of the chromosome DNA sequence.

Next, a second round of single exchange events occurs. In this process, the sequence inside the heterozygous DNA molecule is reorganized, so that the suicide vector sequence is completely excised from the chromosomal DNA, and the target gene is also knocked out[14]. This process is done through repair mechanisms within the cell that automatically repair the DNA breaks caused by the suicide vector and delete the suicide vector sequence in it.

With this suicidal plasmid-mediated gene knockout technique, researchers can precisely delete the target gene and thus study the function of the gene in the organism. This technique is highly specific and efficient, so it is widely used in genomics and molecular biology research.

(4) Receptive cells

When conducting genetic engineering experiments, a key step is to transfer the constructed plasmid vector into the host cell. Here, we will discuss in detail how to transfer the suicide plasmid vector pLP12 into Escherichia coli DH5α receptor cells by transformation technology.

DH5α is a commonly used strain of Escherichia coli, which is popular in genetic engineering for its high conversion efficiency and good growth performance[15]. In its natural state, however, E. coli cell membranes block the entry of foreign DNA molecules. To overcome this obstacle, we need to prepare a special cell state, the receptive cell.

The preparation of receptive cells is usually achieved by chemical or physical methods. The most commonly used chemical method is the $CaCl_2$ method, which treats E. coli cells with a high concentration of $CaCl_2$ solution to change the permeability of their cell membranes, thus allowing the entry of foreign DNA molecules[16]. The physical method, such as the electric shock method, makes the cell membrane temporarily unstable through the action of an electric field and increases its permeability.

After preparing the DH5α receptor cells, we can begin the transformation experiment of the plasmid vector. First, the constructed suicide plasmid carrier pLP12 was mixed with an appropriate amount of receptive cells and placed on ice for a period of time to allow the DNA molecules to fully contact the cells. Then, through heat shock treatment (such as a 42-degree water bath for a certain period of time), the DNA molecules are promoted into the interior of the cell. Finally, the cells were placed in a culture medium containing appropriate antibiotics, and only the cells that had successfully transformed the foreign DNA would survive the screening of antibiotics.

In this way, we can effectively transfer the suicide plasmid vector pLP12 into DH5α receptor cells [17], providing convenience for subsequent genetic engineering experiments. This technique has been widely used in gene cloning, gene expression, gene knockout and so on. For example, cloning and expression of specific genes can be achieved by efficiently inserting specific DNA fragments into the genome of a host cell. By transforming plasmids containing specific promoters and regulatory elements into receptive cells, the expression level of target genes can be precisely controlled, and the relationship between gene expression and cell function can be studied. Through gene editing technologies such as CRISPR-Cas9, specific genes can be precisely deleted or modified in receptive cells, thereby studying the function of these genes in the organism. It is an indispensable tool in modern molecular biology and biotechnology.

(5) Colony PCR

Colony PCR (Colony PCR) is a powerful and flexible molecular biology technique that is widely used in gene cloning, mutation analysis, and identification of specific genes or sequences in biological samples[18]. The core advantage of this approach is that it allows researchers to rapidly and accurately amplify specific DNA segments directly from microbial cultures, such as bacterial or yeast colonies.

In the process of colony PCR, researchers first need to carefully select a single colony from a thriving petri dish. This colony usually represents a single microbial

population, or at least a genotypically consistent subpopulation of microbes. These selected colonies are then used directly for the PCR reaction, eliminating the need for tedious DNA extraction steps.

To perform PCR amplification, researchers need to design and synthesize specific primers. These primers complement the sequences on both sides of the target DNA fragment, guiding the DNA polymerase to make precise copies of the template DNA. The design of primers is a critical step, as they not only determine the specificity of PCR amplification, but also affect the efficiency and accuracy of amplification.In the PCR reaction system, besides primers, DNA polymerase, deoxyribonucleotides (dNTPs), buffer and other key components should be added. Together, these components constitute an efficient amplification system capable of producing a large number of DNA replicants in a short period of time.

After PCR amplification is complete, researchers usually use gel electrophoresis (running glue) to detect PCR products. This method can intuitively display the size and quantity of PCR products, so as to judge the success of PCR amplification. In addition, the quality and purity of the PCR products can be further observed and analyzed by dyeing the gel, such as using ethidium bromide or silver.

Once the positive clone was confirmed, we purified and expanded the culture scale, from which the plasmid pLP12Cm-TBSR3 was extracted[19]. In order to efficiently transfer specific plasmids to another host cell, E. coli β2163, we chose electrotransformation technology as our transformation strategy. Electrotransformation technology is an efficient and accurate gene transfer method, which relies on the action of high electric field pulse to realize the transfer of foreign DNA to host cells. In the electrotransformation process, Escherichia coli β2163 cells first need to be prepared into receptive cells, which is a series of biochemical treatments to put the cells in a state that is receptive to foreign DNA. Next, the plasmid DNA to be transformed is mixed with the receptive cells and placed in the sample tank of the electroconverter. A short, high-intensity electric field pulse is then applied through an electroconverter. This electric field pulse can create a momentary

electric potential difference on the cell membrane, causing the phospholipid molecules on the cell membrane to rearrange, forming a brief hydrophilic channel, which temporarily increases the permeability of the cell membrane. This increase in permeability allows foreign DNA, which would otherwise be difficult to enter the cell, to pass smoothly through the cell membrane and into the cell interior [20].Compared with other transformation methods, this method can reduce the damage to cells to a great extent.

The electrotransformed Escherichia coli β2163 is again verified by colony PCR to ensure that it carries the correct plasmid and DNA fragments. Only after verification, these Escherichia coli β2163 can be mixed with recipient bacteria as donor bacteria [21]. Finally, the missing mutant strains we obtained also need to be verified by colony PCR again after culture. Only those clones that can amplify the corresponding length of the fragment can be identified as the correct missing mutant.

(6) Agarose Gel Electrophoresis

Agarose Gel Electrophoresis is a commonly used technique for separating, identifying, and purifying DNA fragments. Agarose gel electrophoresis is widely used in gene cloning, gene expression analysis, DNA sequencing and other experiments. For example, in gene cloning experiments, scientists can screen out clones containing targeted DNA fragments through electrophoresis; In gene expression analysis, agarose gel electrophoresis can detect the presence and expression level of gene transcription products. In DNA sequencing, electrophoresis technology is used to test the accuracy and reliability of sequencing results. The principle is mainly based on the mobility difference of DNA molecules in electric field, that is, the swimming rate of DNA molecules in agarose gel is related to the size and conformation of DNA molecules. The following are the main principles of agarose gel electrophoresis:

Charge effect: DNA molecules are negatively charged, so when a voltage is applied to an electric field, the DNA molecule moves toward the positive electrode. The rate of migration of DNA molecules is proportional to the amount of charge they

carry, but since DNA molecules carry basically the same charge under the same conditions (due to the presence of the phosphate skeleton), their migration rate mainly depends on other factors.

Molecular sieve effect: Agarose gel is a porous medium, and its pore size can be regulated by the concentration of agarose. During electrophoresis, DNA molecules need to move forward through pores in the gel. Smaller DNA molecules are able to pass through pores more easily and therefore migrate faster; Larger DNA molecules migrate more slowly because they need to bypass more obstacles. This molecular sieve effect allows DNA molecules of different sizes to be separated on the gel.

Electrophoresis buffer: The main role of electrophoresis buffer is to maintain a constant pH during electrophoresis and provide sufficient ionic strength to support the migration of DNA molecules. Common electrophoretic buffers include TAE and TBE, which provide a stable electrophoretic environment for stable migration of DNA molecules in an electric field.

Visualization: In order to more intuitively observe the migration of DNA molecules on the gel, fluorescent dyes (such as Ethidium bromide EB, GelRed, etc.) are usually added to the DNA sample for staining. These dyes can be embedded in the double helix structure of the DNA molecule and emit a fluorescent signal. Under ultraviolet light, bands of DNA fragments of different sizes can be seen on the gel, so as to achieve the separation and identification of DNA fragments.

In short, agarose gel electrophoresis uses the mobility difference of DNA molecules in the electric field and the molecular sieve effect of agarose gel to achieve the separation and identification of DNA fragments. This technology has a wide range of applications in molecular biology, genetics and biotechnology.

Conclusion to chapter 2

The principle of deletion strain construction experiment is mainly based on genetic engineering technology and molecular biology principle. In the experiment, the target gene was selected first, and the corresponding gene knockout strategy was designed. This usually involves constructing knockout plasmids containing

homologous sequences on both sides of the target gene in order to replace or delete the target gene through homologous recombination inside the cell.

During the experiment, the knockout plasmid was introduced into the target strain by a specific transformation method. In the cell, the homologous sequence on the knockout plasmid recombines with the corresponding sequence on the chromosome of the target strain, resulting in the deletion of the target gene. In this process, specific selection markers (such as antibiotic resistance genes) may be required to help screen for strains that successfully recombine.

Finally, through a series of screening and verification steps, such as PCR amplification, sequencing, etc., it is confirmed that the target gene has been successfully knocked out, so as to obtain the strain missing the specific gene. This experimental principle provides important technical support for gene function research, metabolic engineering and other fields.

CHAPTER 3
EXPERIMENT PART

3.1 Genome extraction and purification

(1) Design primer:

Shewanella TBSR3 gene chromosome locus sequence:

CAGGCTTAGTCTATGGTGCCAAACAAGTCCACGACGTGATGGCCAT
TCCTTTCATGATCGGTTGGGCACTGATGATCACCCTATGGGCGAAAAACC
AGCTACCCAAAATGTACGATGTGAAGTGGTTTATGGTCGTTGGTGGTTAC
ATCAACTTCGGCCCATTCAAAGGTAAGCACCCTGACGCAGGCTTTGCTAA
CGCCGGCGAAAAAATGTGGTTCTGGGCCTTTGCCCTGTTCGGGTTGATCA
TTTCAGCATCAGGTATGTTGTTACTGTTCCCAAACCTGTTCGAGCCAAGCC
GCACCTTAAGCTTAATCGCGCTGGTATTGCACTCTGTTAGTGCCATCGTGA
TCTGCGCTTTCTCTATCGTGCACATCTTTATGGCAACTGTCATGTCAGAAG
GTGGTATGGAGTGTATGGTGTCTGGTTATTGCGATGAAAACTGGGCAAGC
CAGCATCACAATCTATGGTTTGATGAAATCAAAGCCAACGGCACGTTACA
ATATAAAGAGTAACATACTCTGATAAAACAAAACCTCCACAGCAAACTGT
GGAGGTTTTTTTATGGGTGTAGATTTTGAGTGCTCAGTAAACCAACTGCA
AAAACCTCGACGGCACTCATTGATAAAAAGGTGTTAACTAAAAATGCCG
ACAATCTATATTAAAACAACCTTAATCGCAATTGATAACCAATATCATTT
ACATTCAAAAGATGACTTTGTGATATTGCGCAAATACATTAGGCATTGAA
GAGGAAAACACTTTAAATGTTAATAAGATAAGTCAGTCACAATAGCCTTA
TTTCTACAAATATTCGGTGACTAAGACTGAAGCCGTCATCCAATAGCTTTC
TTCTGCTACTGCCTATATTCTAAGTTGCTAATTGGAGGCTTAGTGGAAATA
CGGAAACCATGAGTGATATTTCAAAAGCAAAATTGATGTACCTAGCGATA
GGAGTGTTCATCGGGAACATTATTGGCAACCTCTCACTATCGATAAGCAA
TTTATTTTTAGCAATTGCATCCATATTAGGCTGGTTAGTACTAATGGTCGG
AATTCCTCCCTCTAATAGTCACCAATTTCGAATTGCGTCTTTCTTTATATC

AATCAGCCTATTACCTGTATCGTGGGGGGATTTATATAACGGCAAATTAA
GCGCCTAGTGGCCATAGTTTTGCCCTGTATTGGCGATAAGGATTTCGATC

The primers designed according to the above sequence are shown in Tab. 2.1:

Table 2.1 – **The primers designed according to the above sequence**

Primer name	Primer sequence
TBSR3 MF1	CTGATGAGTAAAAGCTCGAGCAGGCTTAGTCTATGGTG
TBSR3 MR1	GCTCAGTAAACCAACTGCAAAAACCTCGACGGCACT
TBSR3 MF2	AGTGCCGTCGAGGTTTTTGCAGTTGGTTTACTGAGC
TBSR3 MR2	GATATGTCGAGCTCGAATTCGATCGAAATCCTTATCGC
TBSR3 TF	CACTGGTTAGGTGCAATC
TBSR3 TR	GATTTGTACCTGCGGAGT

(2) PCR amplification:

The primers were designed according to the chromosome loci of the NCBI gene bank, and the A and B fragments of the upstream and downstream homologous arms of TBSR3 were amplified with the designed primers. The PCR procedure was set: the predenaturation temperature was 98℃ and the time was 3 min. Denaturation temperature is 98℃, time is 15 sec, annealing temperature is 56℃, time is 20 sec, elongation temperature is 72℃, time is 1 min (set 30 cycles); The final elongation temperature was 72℃ and the time was 5 min. The holding temperature is 12℃ and the time is 10 min.

Then, electrophoretic detection was used to drop DL2000 DNA Marker into the first glue hole, fragment A into the second glue hole, and fragment B into the third glue hole. The glue running results showed that the length of lane 2.3 was 610 bp, all of which were clear and single bands.

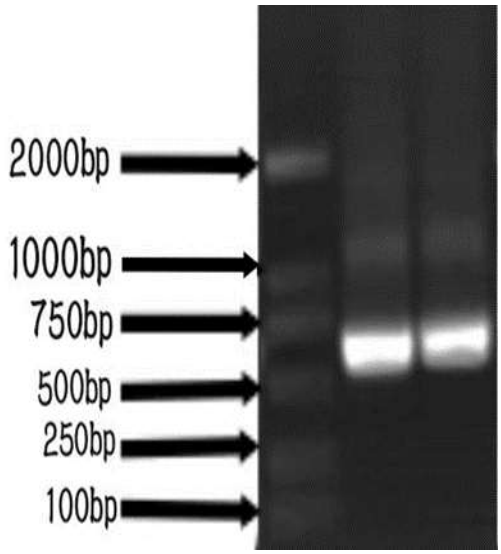

Figure 3.1 – Amplified fragment of TBSR3

(3) PCR Product Purification Kit (Knogen) Purification:

Before the experiment starts, preparations should be made: check whether ethanol has been added to Wash Solution, confirm whether isopropyl alcohol has been added to Buffer B3, and observe whether precipitation has formed in Buffer B3 [22]. Next, the PCR products were purified according to the following steps: First, 5 times the volume of Buffer B3 was added to the PCR reaction solution to ensure adequate mixing; Centrifuge at 8000×g for 2 minutes, then carefully drain the supernatant, retaining only the liquid in the collection tube. Add 500 μL of Wash Solution into the collection tube, then centrifuge again at 9000×g for 2 minutes, and pour off the supernatant. Repeat the above washing steps once to ensure complete removal of impurities. Return the empty adsorption column to the collection tube and centrifuge the empty column for 1 minute at a rotation speed of 9000×g to remove the residual liquid. Remove the adsorption column and place it into a clean 1.5 mL centrifuge tube. Add a 15-40μL Elution Buffer in the center of the adsorption film and let it sit at room temperature for 1 minute. Finally, centrifuge at the appropriate speed for 1 minute to completely eluate the DNA solution into the centrifuge tube. Store the DNA solution in the centrifuge tube for subsequent experiments.

3.2 Overlapping PCR was used to amplify the TBSR3 fusion fragment

(1) Overlapping PCR amplification

The A and B fragments were amplified and purified by overlapping PCR. The enzyme of TaKaRa PrimerSTAR Max DNA Polymerase was added into the reaction system, and then the amplified and purified AB fragment was added as the template[23]. The amplification procedure was pre-deformation temperature 98℃ for 1 min. Denaturation temperature is 98℃, time is 10 sec, annealing temperature is 68℃, time is 20 sec, elongation temperature is 72℃, time is 1 min (set 7 cycles); The final elongation temperature was 72℃ and the time was 7 min.

DL2000 DNA Marker was injected into the first colloidal hole, and the fused AB fragments were injected into the second and third colloidal holes, and then the results were detected by electrophoresis. The glue running results are shown in Fig. 3.2. The length of the fused AB fragment is 1220 bp, which is a clear single strip.

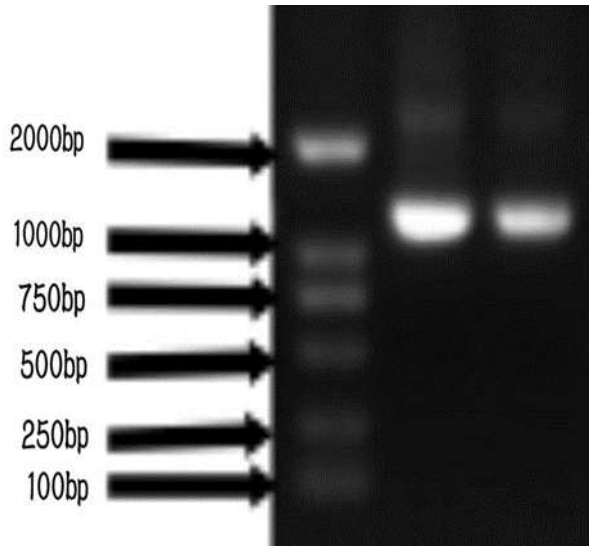

Figure 3.2 – Overlapping amplified fragments

(2) Purification

PCR product purification kit (Knogen) was used for purification.

3.3 Plasmid extraction

(1) Preparation of LB liquid medium (1 L)

Weigh 10 g tryptontone, 5 g yeast extract, 10 g sodium chloride into the reagent bottle with a balance; Use a measuring cylinder to measure 900 mL water into the reagent bottle, stir well until completely dissolved; Adjust pH value to 7.0 with 5 M or 10 M NaOH, and set volume to 1 L; Sterilization under high pressure for 20 min.

(2) Coating plate

First of all, prepare tools and materials: take out a small piece of alcohol cotton to wipe the surface of the coated glass rod, and then heat sterilization on the flame, place on the test tube rack to cool for use. Adjust pipette range as needed. Secondly, dilute bacterial solution: a certain concentration and a certain amount of bacterial suspension to be separated are added to a certain amount of water for dilution. Then inoculation is carried out: in the sterile operation area of the alcohol lamp, the quantitative bacterial solution is sucked into the center of the medium with a pipette. Open the lid with your left hand, and take the coating glass stick with your right hand. Spread the bacterial solution on the plate in the center of the medium, then turn the plate and continue coating until the bacterial solution is evenly spread throughout the plate. Then sterilization and cooling: After coating, the coated glass rod is burned and cooled. Finally, the plate is placed in a 37℃ incubator overnight.

(3) Pick up bacteria

Select the strain Escherichia coli DH5α with plasmid pLP12.

(4) Plasmid extraction and purification

Plasmid extraction kit is used for plasmid extraction and purification.

(5) Agarose gel electrophoresis

Firstly, agarose gel was prepared. Then the sample is added to the glue hole; Then electrophoresis is carried out: the lid of the electrophoresis apparatus is covered, and the DNA gradually moves from the negative electrode to the positive electrode under the action of the electric field[24]. This process generally lasts for 10 minutes. If

no bands appear, the time needs to be extended until clear bands appear. Finally, the glue is cut and recycled. If it is only necessary to verify whether the strip is correct, it is only necessary to take photos and save the picture [25].

3.4 Construction and transformation of pLP12Cm-TBSR3 recombinant suicide plasmid

(1) Construction:

The purified AB fragment was connected with pLP12Cm using recombinant enzyme Exnase II (ClonExpress II, Vazyme), 5×CE II Buffer, and ddH_2O. The purified AB fragment was treated at 37℃ for 60 min, and left on ice for 10 min.

(2) Transformation

First, the receptor cell DH5α used for cloning was removed from the refrigerator, thawed on ice, and returned to the refrigerator in time after use. Next, a pipette gun is used to precisely extract 5 μL of the assembly product and carefully add it to the 100 μL receptive cells. The mixture is then left to rest on ice for 30 minutes to ensure that the assembled products fully enter the receptive cells. The mixture was then placed in a water bath at 42 °C for heat shock treatment for 45 seconds. After the heat shock, immediately put the mixture back on the ice to cool, and the cooling time is about 2-3 minutes. Next, 900 μL of LB medium (without antibiotics) was added to the cooled mixture, and the cultures were cultured in a 37 °C shaker at 200 rpm for 1 hour to promote cell recovery and plasmid replication. After the culture is complete, the culture is placed in a centrifuge and centrifuged at 4000 rpm for 3 minutes. After centrifugation, the 800 μL supernatant was carefully discarded and the cells were re-suspended with the remaining 200 μL liquid. Finally, the suspended cells were evenly coated on a plate containing the correct resistance, which was then cultured overnight in an incubator at 37 °C. After hundreds of monoclones grow on the plate, several monoclones are selected for colony PCR, liquid PCR or generation sequencing identification methods to screen out the correct clones [26].

(3) Coating plate

LB plates of 20 μg/mL CM + 0.3% D-Glucose were prepared, and the transformed cells were carefully coated on a super-clean work table. After standing for a while, the cells were incubated upside down in an incubator at 37℃ for one night. The growth results were shown in Fig. 3.3. The bacterial community grew well and there was a single colony that was easy to pick.

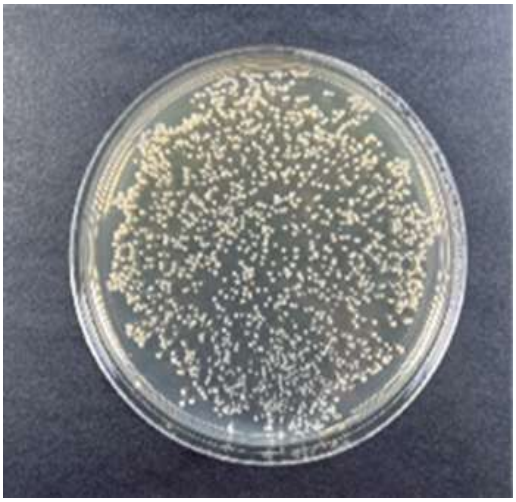

Figure 3.3 – pLP12Cm-TBSR3 is converted to DH5α and then coated flat plate

(4) Colony PCR

pLP-UF/pLP-UR was used as primers for amplification and screening of AB inserted recombinant clones. System: upstream primer - 2μL, downstream primer - 2μL, template - 1μL, ddH2O-20μL, enzyme - 25μL. Procedure: 95℃-30 s; 95℃-15 s, 55℃-15 s, 72℃-1 min (set 30 cycles); 72℃-5 min; 12℃-1 min. And then DL2000 DNA Marker was injected into the first colloidal hole, and the samples to be tested were injected into the 2-7 colloidal holes for electrophoresis detection. The results were shown in Fig. 3.4, all of which were clear single bands, and the positive clone was 1470 bp, which was in line with the expected conjecture.

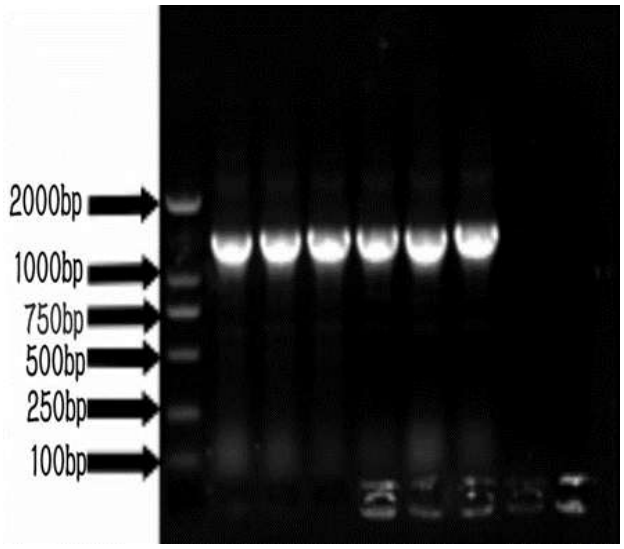

Figure 3.4 – Plasmid detection of PLP12CM-TBSR3

(5) Plasmid extraction

After screening and purification, the positive clones were expanded for culture, and then plasmid pLP12Cm-TBSR3 was extracted from the culture.

(6) Transformation

The purified pLP12Cm-TBSR3 was efficiently converted into β2163 by electrical conversion for further analysis and application.

(7) Coating

LB plates with CM=20 μg/mL, DAP= 0.3mM and 0.3% D-Glucose were prepared. After the transformed products were cultured for 5 h, the plates were coated on a super-clean work table, and then cultured upside down in an incubator at 37℃ for one night. The results, as shown in Fig. 3.5, showed that the flora grew densely and there was a single bacterium that could be used for subsequent operations.

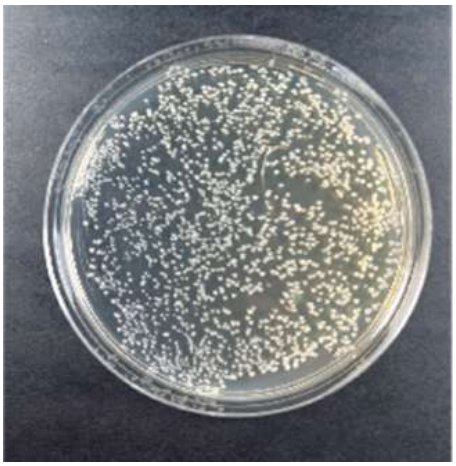

Figure 3.5 – Coating plate after electric conversion

(8) Colony PCR verification

This step is used to verify positive clones. The transformed Escherichia coli β2163 was verified by colony PCR using TBSR3-MF1/ TBSR3-MR1 as primer, and the verified positive colonies need to be striated and purified. Then DL2000 DNA Marker was injected into the first rubber hole, and the sample to be tested was injected into the second to seventh rubber holes for electrophoretic detection. The detection results are shown in Figure 3-6. The length of the correctly amplified product is 1220 bp, and there are two failed bands of 300bp. It is suspected that the addition of primer is less or the relevant instruments are not used correctly. In general, the electrophoretic results were in good agreement with the expected results.

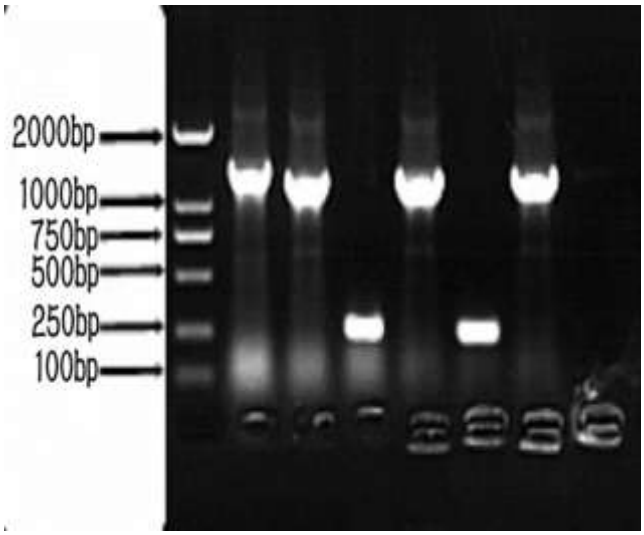

Figure 3.6 – Results of β2163 electric transfer

3.5 Insert mutant to construct

(1) Construction

First, Escherichia coli β2163 (the donor bacterium) carrying the plasmid pLP12Cm-TBSR3 was inoculated in LB medium containing chloramphenicol (Cm) and DAP (diaminoheptadecic acid) and incubated with oscillations overnight. At the same time, *Shewanella* (acceptor bacteria) was inoculated in ordinary LB liquid medium, and the same shaking overnight culture was performed. Next, 100 μL donor and 100 μL recipient overnight cultures were accurately measured using a pipette and placed into a clean centrifuge tube. The two bacterial fluids are then gently mixed in the centrifuge tube using a sterile gun tip to ensure they are fully in contact and mixed. Centrifuge at 8000 rpm for 2 min, discard the supernatant; LB liquid medium was prepared in advance, 1 mL of LB liquid medium was absorbed with a pipette gun, and then re-suspended; Centrifuge at 8000 rpm for 2 min, discard the supernatant; Then it was suspended with LB, centrifuged at 8000 rpm for 2 min, and the supernatant was removed. Drain 10 μL LB with a pipette gun and re-suspend the fluid.

LB plate with 0.3mM DAP + 0.3% D-Glucose was prepared in advance, the above liquid was absorbed, coated with the plate, and cultured in an incubator at 37℃ for 6 h.

LB plate containing 20 μg/mL CM+0.3% D-Glucose was prepared in advance, 1 mL LB was absorbed with a pipette gun, and then 100 μL coated LB plate was diluted and cultured in an incubator at 37℃ for 6 h.

Analysis of experimental results: LB Agarose plate containing 20 μg/mL chloramphenicol and 0.3% D-glucose were prepared in advance. Several suspected positive clones were selected from plates containing mixed colonies using sterile inoculation rings, and then carefully striated onto the plates. The aim is to further purify and isolate monoclonal colonies for subsequent analysis and experiments. The transformed Escherichia coli β2163 positive clone was co-cultured with *Shewanella*, and plasmid pLP12Cm-TBSR3 was integrated into *Shewanella* through conjugation,

and the culture results were shown in Fig. 3.7. The bacteria grew densely and well, which could be used for the subsequent experiment.

Figure 3.7 – Recombinant plasmid PLP12CM-TBSR3 integrated into a *Shewanella* plate

(2) Colony PCR verification

This step is used to test whether the homologous arm of the foreign plasmid is successfully inserted into the target mutant and whether the homologous recombination is successful.

(3) Detection strip

Using TBSR3-TF/PLP-UTR as primer, DL5000 DNA Marker was injected into the first rubber hole, and the sample to be tested was injected into the second to seventh rubber holes. After electrophoresis detection of purified cloned samples, the results were shown in Fig. 3.8. The figure shows a clear, single amplified band with a size of 1361 bp, which is exactly the size of the expected amplified band formed by insertion mutation, and there is no heterozonal band.

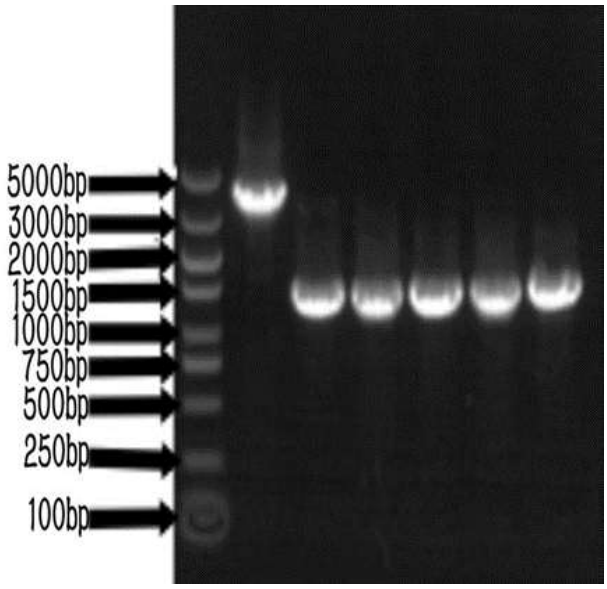

Figure 3.8 – TBSR3 Insert mutant for detection

3.6 Deletion mutant construction

LB liquid medium without antibiotics was prepared in preparation, and then the insert strains of suitable single colonies were selected from the cultured monoclones, and the plates were coated aseptically in a super-clean workbench, and then incubated overnight at 37℃ in an incubator. LB medium containing 0.4% L-arabinose but without any antibiotics was prepared in advance. Next, the bacteria solution cultured overnight was diluted to ensure that the concentration of the bacteria solution was suitable for coating on the LB plate. Then the coated LB plate was placed in a constant temperature incubator at 37℃ for a period of time for the growth and observation of the bacteria colony. LB-arabinose and LB-Cm plates were prepared in advance, and after the bacteria grew, a single colony was selected on the plates and inoculated on the plates, and marked at the corresponding positions, and incubated at 37℃. Clonal colonies that could not grow on LB plates containing chloramphenicol but could grow on LB plates supplemented with 0.4% L-arabinose were selected from the cultured plates. These colonies will be candidates for subsequent experiments to ensure they have a specific genotype or phenotype. The culture results are shown in Fig.3.9. The growth of bacteria is good and in line with the expected results, which can be selected for subsequent experiments.

Figure 3.9 – *Shewanella* TBSR3 deletion mutant plate

Using TBSR3-TF/ TBSR3-TR as primer, DL5000 DNA Marker was injected into the first rubber hole, and the sample to be tested was injected into the second to seventh rubber holes. The control group was required to be set up, and the second rubber hole was injected with the wild type as the control group. After injecting normal samples into several rubber holes, the selected bacteria were detected. The results, as shown in Fig. 3.10, were consistent with expectations. The amplification of the correct deletion mutant clone produced 1397 bp fragment, and the amplification of the wild-type fragment was 3455 bp, all of which were single clear bands without the influence of impurity. This proves the success of the experiment.

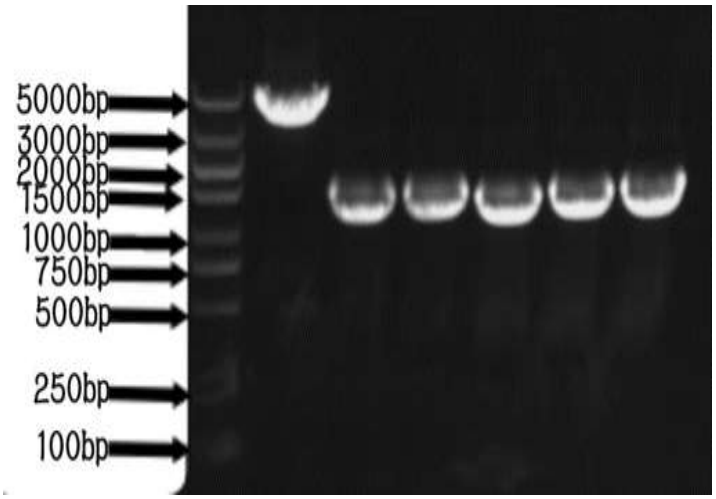

Figure 3.10 – Detection of TBSR3 deletion mutation in *Shewanella*

Conclusion to chapter 3

The main steps of the deletion strain construction experiment are summarized as follows:

Genome extraction and purification: First, genomic DNA is extracted from the target strain and purified to ensure the quality and integrity of the DNA.

PCR amplification: Using designed specific primers, the target gene and its homologous sequences on both sides are amplified by PCR technology to provide a template for subsequent plasmid construction.

Plasmid extraction: The plasmid was extracted from the strain containing the suicide plasmid as the basis for constructing the recombinant suicide plasmid.

Construction and transformation of recombinant suicide plasmid: The recombinant suicide plasmid was constructed by enzyme-cutting and linking the fragments amplified by PCR with the suicide plasmid. Then, the recombinant suicide plasmid was introduced into the target strain by electrotransformation or heat shock transformation.

Insertion mutant construction: In the target strain, the recombinant suicide plasmid is inserted into the chromosome by homologous recombination to form an insertion mutant.

Deletion mutant construction: Through secondary homologous recombination or screening, the inserted suicide plasmid fragment is removed; leaving the deletion of the target gene, and thus the deletion mutant is obtained. The entire experimental process requires precise molecular biological manipulation and meticulous experimental design to ensure that the desired missing strains are eventually obtained.

CONCLUSIONS

1. TBSR3 gene is related to the formation of outer membrane pigment protein, which indirectly affects the electron transfer ability of *Shewanella*, which is of great significance for further study of the function of *Shewanella*. This required knocking out the TBSR3 gene and further testing for changes in related phenotypes such as the mutant's ability to produce electricity, with the aim of assessing the role of the gene in *Shewanella* by removing specific sequences in the gene.
2. In this study, the in-frame deletion mutation method was used to specifically knock out TBSR3 gene, and the experiment was completed through the above steps. In the experiment, we successfully obtained TBSR3 deletion mutant by colony PCR and agarose gel electrophoresis, and by screening for nutrient deficiency and resistance genes. The final test results are in complete agreement with the expectations before the experiment, which indicates that the experiment has achieved a complete success. This result not only verified the accuracy of our experimental design and operation methods, but also provided experimental strains for further research on the relationship between TBSR3 gene and outer membrane pigment protein formation.

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