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Methods for preservation and transcriptomic analysis of Lactobacillus Plantarum P1, P2 phage

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Abstract - The survival of Lactobacillus plantarum virulent phages P1 and P2 in three cryoprotectants: glycerol(15%), chloroform(20%), and dimethyl sulfoxide(7%) under different temperatures(4°C, -20°C, -80°C) were evaluated every half month. The study was undertaken to establish suitable preservation methods that could be used to study phage biological properties and genomic characteristics further. Transcriptomic properties of phage activity in different stages of lysis were also analyzed. This information may be used further to explore the relationship between bacteriophage and host bacteria. The results of this study showed that Lactobacillus plantarum virulent phage P1, stored for 60 days in all cryoprotectives at all temperatures, exhibited a survival titer greater than 10^7 PFU/mL (initial titer 10^8 PFU/mL). The optimum cryoprotectant used was glycerol at -80°C. In this respect, the phage could be preserved for 10 months with a 10^5 PFU/mL titer. Maintenance of Lactobacillus plantarum virulent phage P2 under different storage conditions was evaluated. Preservation of the phage for up to three months was achieved at 4°C using all cryoprotectants. In this respect, the phage titer was greater than 10^8 PFU/mL. For long-term phage maintenance (10 months), the optimum

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preservation conditions at -80°C included 15% glycerol or 7% dimethyl sulfoxide as cryoprotectants. Under these conditions, the phage titer was greater than 10^7 PFU/mL. The infective phases of phage P2 and its host strain were investigated using transcriptomic analyses. It was observed that when phage P2 infects its host strain, it can control the replication and assembly process of phage by regulating key enzymes (such as prolong factor EF-Tu, EF-G, EF-Ts; translation starting factors IF1, IF2, IF3; RNA synthesis enzyme rpoA, rpoB, rpoC; signaling recognizing particles Fts Y, Ffh; as well as releasing factor RF1).
Keywords - L. plantarum IMAU10120, bacteriophage, phage preservation, transcriptomic Analysis

1. INTRODUCTION

In recent years, with the rapid development of genomics, it has been found that phage is a component of bacteria; it co-evolved with bacteria and can improve the adaptability of bacteria. At the same time, phage also has many practical applications, such as the use of high-density phage to select phage-resistant mutant strains to replace the original strains in industrial production, which fundamentally solves the problem of fermentation failure caused by phage infection in the fermentation process of Lactobacillus plantarum. Bacteriophages can also be used as a carrier to deliver nucleic acid fragments, and they are widely used in the gene excision of Lactobacillus plantarum. Bacteriophages are highly specific and can be used as therapeutic agents instead of antibiotics to solve the problem of bacterial resistance [1]. Bacteriophages used in aquaculture can improve the survival rate and growth rate of animals such as livestock, poultry, and fish; in medical treatment, they can reduce the morbidity and mortality of patients with bacterial diseases; and in the food industry, they can extend the freshness period of food [2]. Therefore, bacteriophages have broad application prospects. However, the bacteriophage is a microscopic organism, is a strictly obligate parasite, and cannot reproduce and grow independently after leaving the host bacteria; its preservation and activity will be affected by a variety of different factors, such as temperature, protective agents, etc., so from its preparation to the actual application of the period of storage conditions are crucial. To maximize its effect, people have tried a variety of different preservation conditions preservation methods. The diversity of bacteriophages and their sensitivity to various storage conditions and storage media content vary, so there is no one-size-fits-all storage method.

The transcriptome is the collection of all RNA transcribed by a particular tissue or cell at a specific developmental stage or functional state, including mRNA and non-coding RNA [3]. As one of the new omics techniques, transcriptomics studies reflect the genes expressed under different physiological states, tissue types, and environmental conditions [4]. Therefore, transcriptome analysis has become a powerful tool for studying biological stress's physiological mechanism [5]. The transcriptomic study during bacteriophage infection can initially explore the possible mechanism of bacteriophage infection in host bacteria, which is of great significance for studying the relationship between bacteriophage and host bacteria. In this paper, the virulent phage P1 and P2 of Lactobacillus plantarum, isolated and purified from abnormal fermentation solution, were preserved with different protective agents under different

temperature conditions. The optimal preservation method was explored by calculating and comparing the titers of phages before and after different preservation methods. This will lay a research foundation for further determination of its biological characteristics and evaluating its application value in the future. At the same time, transcriptomic studies on different periods of phage infection can reveal the characteristics and modes of phage infection to host bacteria and further explore the relationship between phage and host bacteria. To provide a theoretical basis and data support for phage control and screening of phage-resistant strains in the dairy industry. At the same time, it is essential to use phages in further research by conducting transcriptomic studies at different stages of infection to control the harmful effects of bacteriophages in dairy industries, to determine their biological properties, and to provide theoretical basis and data support for the detection of anti-phage strains.

2. THEORETICAL BACKGROUND

2.1 LACTOBACILLUS PLANTARUM IMAU10120

Lactobacillus plantarum is a bacterium of the genus Lactobacillus of the Lactobacillus family. It is straight or curved rod-shaped, has few flagellates, and can exercise. It belongs to Gram-positive bacteria and has no spore formation. It is widely distributed in animal and plant feed, manure, milk, and dairy products [6]. Its main feature is that it can ferment lactose to produce lactic acid. Lactobacillus is used commercially primarily in making yogurt and cheese, but also in product-fermented vegetables (pickles and pickles) [7], beverages (wine and juice), sourdough bread, and some sausages [6]. The optimal growth temperature is 30℃~35℃, and the optimal pH is 6.5 [8-9]. It is commonly found in human digestive tracts. Lactobacillus plantarum helps to improve immunity, maintain intestinal flora balance, reduce serum cholesterol content [10], prevent cardiovascular disease, alleviate lactose intolerance, and inhibit the formation of tumor cells, as well as anti-oxidation, anti-intestinal aging, and other effects [11].

Lactobacillus plantarum IMAU10120 was isolated from traditional fermented yogurt samples collected by grassland herders in Urat Zhongqi, Bayannur City, Inner Mongolia, China. This is a probiotic strain with excellent tolerance to stomach acid, intestinal fluid, and bile salts [12]. The genome consists of 3.03 Mb circular chromosomes and seven plasmids, named LBPp1 to LBPp7 [13].

Bao, Zhang, Wang, et al. conducted an in-depth study and systematic evaluation of its probiotic function and the mechanism by using genomic means, in vitro tests, and human tests, respectively. The results showed that Lactobacillus plantarum IMAU10120 could significantly inhibit the growth of intestinal pathogens and had good tolerance to gastrointestinal digestive fluid and bile salts [12-14]. In addition to high acid and bile resistance, it has many other probiotic properties, Such as good aggregation, antibacterial activity, and storage stability [15]. This strain can also improve human gastrointestinal health by regulating the host intestinal microbiota and the secretion of total bile acids and short-chain fatty acids [16]. Lactobacillus plantarum IMAU10120 can reduce the levels of serum total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol, inhibit the increase of high-density lipoprotein cholesterol levels, and play a role in lowering blood lipids. In addition, Lactobacillus plantarum

IMAU10120 also plays a beneficial role in high-fat diet-induced oxidative stress, reducing liver fat accumulation and protecting healthy liver function [17]. These results suggest that Lactobacillus plantarum IMAU10120 may be a potential therapeutic agent for the control of hyperlipidemia [18].

2.2 BACTERIOPHAGE

A bacteriophage is a virus that infects a bacterial cell. There are many types of phages worldwide, and they can be classified as beneficial and virulent. Lactobacillus bacteriophages are the leading cause of lactic acid bacteria fermentation failure, and this phage is a frequent problem in the dairy industry. After infecting the lactic acid bacteria, the bacteriophage multiplies inside the bacterial cell and destroys the lactic acid bacteria. Because of this, the fermentation of milk is slowed down, or fermentation does not occur, causing substantial economic losses to the dairy industry. Bacteriophages are tiny organisms that cannot reproduce and grow independently after breaking away from the host bacterial cell. Therefore, temperature and preservatives have an essential effect on the storage of dairy products.

In 2015, a strong bacteriophage P1 was isolated from the abnormal fermentation broth of L. plantarum IMAU10120. The bacteriophage has a head diameter of 71.7 ± 3.0 nm, a tail length of 272.0 ± 3.0 nm, and a width of 11.3 ± 1.5 nm. It is a long-tailed bacteriophage with a latency period of 45 minutes, a lysis period of 45-90 minutes, and an average cleavage amount of 132.88 ± 2.37 PFU [19-20]. External environmental conditions such as temperature, pH value, and concentration of divalent cations all affect the survival rate and adsorption capacity of bacteriophage P1. The optimal adsorption temperature for bacteriophage P1 is 30 ℃~42 ℃, and the optimal adsorption pH is 6-8. Calcium ions can promote adsorption, while magnesium ions inhibit adsorption in host bacteria [21]. Phage P1 is sensitive to high temperatures and can be completely inactivated after being treated at 90 ℃ for 5 minutes. It has varying degrees of tolerance to commercial sterilization agents, and after 60 minutes of treatment, high concentrations of alcohol substances and peracetic acid cannot completely inactivate it; Research has shown that bacteriophage P1 is more sensitive to sodium hypochlorite, and treatment with 800 mg/L sodium hypochlorite for 60 minutes can lead to its complete inactivation [22]. In 2017, a virulent bacteriophage P2 was isolated from an abnormal fermentation broth of the same strain of phage P1, which had a low photometric content of a long-tailed bacterium, and the fermentation broth was a clear phylum B1 type, 216.67±3.00 nm in length, and a non-contractile tail. It is 12.25±3.00 nm wide, 66.67±3.00 nm head diameter icosahedral symmetric structure 30 minutes, degradation time 30~135 minutes, average degradation amount 214.49±3.98 PFU. At pH 8, the viability of this phage is the highest, and the optimal growth temperature is 37~42°C, and Mg2+ inhibits absorption. In addition, although chloramphenicol affects the synthesis of cell membrane recognition proteins, it does not affect the absorption capacity of bacteriophage P2. Still, the absorption capacity is more significant than 90.19% for 30 minutes. P2 bacteriophage is temperature-tolerant and is inactivated by treatment at 90°C for 10 minutes or 72°C for 40 minutes. This bacteriophage is not sensitive to peracetic acid but is sensitive to isopropyl alcohol and sodium hypochlorite and is wholly inactivated by 800 ppm sodium hypochlorite for 30 min [23].

2.3 MECHANISM OF PHAGE INFECTION

Like other viruses, bacteriophages need to infect a host cell. This process is known as the lysogenic lifecycle. The life cycle steps include attachment, cytoplasmic entry, protein synthesis, DNA copying, and phage assembly. It recombines with a specific area of the bacterial chromosome during the lysogenic cycle. The prophage initiates a lytic cycle on its initiative, or in response to particular physical or chemical stimuli, degrades and eliminates the host bacteria, and multiplies. To self-reproduce and lyse host bacteria directly, virulent bacteriophages copy and replicate their genes [24]. Shen Yanjie et al., studied the lysis of host bacteria by mycobacterial phage and discovered the lysogenic and lytic cycles of lysogenic phage. Their study showed that binding of mycobacterial lysogenic phages to host bacteria requires phage genome (attP), host bacterial genome attachment site (attB), integrase (Int), and integration host factor (mlHF). Some lysogenic phages undergo a lysis cycle; they assemble to reproduce new phages by copying and synthesizing phage genes. Then, the burst of bacterial cells due to the combined action of (Lysin), and Perforin (Holin), and phages capable of further reproduction are released [25]. In 2013, Tingting Guo discovered the infection mechanism of Lactobacillus phage in his research, in which perforin polymerizes on the host cell membrane to form a homotetramer and create a needle-shaped hole. Lysine is then converted from an inactive form to an active state to degrade cell wall peptidoglycan and lyse the host bacteria [26]. Due to the differences between phages and their host bacteria, each phage has its unique infection mechanism.

Transcriptomic studies examine how genes are affected by different physiological states, tissue types, and environmental conditions [27]. Therefore, transcriptome analysis has become essential for studying biological stress and physiological mechanisms [28]. Transcriptomic studies can study the mechanism of phage infection in host bacterial cells, which is necessary for studying the relationship between phage and host bacteria.

3. RESEARCH METHODOLOGY

3.1 DETERMINATION OF PHAGE TITER

The study was conducted at the Dairy Biotechnology and Engineering Laboratory of the School of Food Science, Inner Mongolia Agricultural University. Virulent phages P1 and P2 isolated from Lactobacillus plantarum IMAU10120 in 2015 and 2017 were used in the study. Phage P1 and P2 were stored in sterile 15% glycerol, 20% chloroform, and 7% dimethyl sulfoxide as preservatives at 4 ℃, -20 ℃, and -80 ℃ for 10 months. During this storage period, the bilayer plate method counted phages twice a month and recorded results.

Data from the study on bacteriophage storage were analyzed using one-way analysis of variance (ANOVA) in SPSS Statistics 20.0. Each experiment was performed in triplicate and analyzed for differences using Microsoft Office Excel 2016.

3.2 EXTRACTION OF TOTAL GENOMIC RNA FROM SAMPLES, CREATION OF SEQUENCING DATABASE

In this study, only host bacterial RNA degraded by infected phage was extracted after 0, 5, 15, 25, 80, and 120 min. Nanodrop 2000 was used to detect the concentration and purity of the extracted RNA, agarose gel electrophoresis was used to detect RNA integrity, and Agilent 2100 was used to determine RIN values, respectively. The total RNA required to generate one database is 1 μg, the concentration is ≥50 ng/μl, and the OD260/280 is between 1.8 and 2.2. Short sequence fragments are intended for sequencing using the Illumina HiSeq platform. Adding a fragmentation buffer can enable fragmentation of the mRNA into tiny pieces of approximately 300 bp. Subsequently, six-base random primers (random hexamers) are added under reverse transcriptase to synthesize single-stranded cDNA utilizing mRNA as a reverse template. The next step is to create a stable double-stranded structure through second-strand synthesis. The ends of the double-stranded cDNA construct are sticky; therefore, blunt ends are created by adding an end-repair mixture. To connect the Y-shaped adapter, finish the adapter ligation, and carry out library enrichment, an "A" base is attached to the 3' end. The target band was removed from a 2% agarose gel (Certified Low Range Ultra Agarose) after 15 cycles of PCR amplification. Once the quantity of TBS 380 (Picogreen) has been determined, mix it by the data ratio and place it on the machine. cBot was utilized for bridge PCR amplification, while Illumina Hiseq was employed for sequencing to produce clusters. To reduce read mistakes, every cDNA molecule in the database underwent paired-end sequencing.

3.3 QUALITY CONTROL AND SCREENING OF THE ORIGINAL SEQUENCE, SEQUENCE COMPARISON ANALYSIS

Raw transcriptome sequencing data performed using the Illumina Hiseq sequencing platform may contain sequencing adapter sequences, low-quality reads, etc., severely impacting subsequent data analysis. Therefore, at first, it is necessary to remove non-target sample sequences to ensure the accuracy of the following analysis. To do this, first, remove the adapter series in the reads and trim the bases with a quality value of less than 20 at the 3' end by using the software SeqPrep(https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle). Reads with an N ratio greater than 10% were discarded, and adapters and sequences less than 20 bp in length were removed after quality trimming. Reads obtained with the Illumina Hiseq sequencing platform are short and have low insertion and deletion errors. Bowtie2, the currently most authoritative short sequence comparison program, was chosen to complete this part of the analysis, which generally allows two base mismatches. This process uses the Bowtie2 (http://www.bowtie-bio.sourceforge.net/ bowtie2/manual) BWT (Burrows-Wheeler Transform) algorithm.

3.4 FUNCTIONAL ANNOTATION OF DIFFERENTIAL GENES

Genes can be categorized using the KEGG database based on the pathways they participate in or the functions they carry out. For the pairs of differentially expressed genes, the differential genes are shown on the KEGG pathway diagram to demonstrate the differential genes KEGG Annotate pathway diagrams, with one of the samples serving as a control.

3.5 DATA PROCESSING

The base identification analysis software Illumina Casava 1.8+ or bcl2fastq software converts the raw image data obtained from sequencing on a high-throughput sequencing platform (like the Illumina HiSeq sequencing platform) into base information. FastQC software is used for shutdown. The raw data is then filtered using NGSQC software to remove low-quality reads and reads tainted by sequencing adapters and HISAT software to align each sample's clean reads to the reference gene to control the data quality. Use Cuffdiff and edgeR software to analyze differentially expressed genes between samples; use online resources like Gene Ontology and the Kyoto Encyclopaedia of Genes and Genomes database to analyze differential genes; and use RSEM to process sequencing data to calculate the expression of genes or transcripts. Analyze the enrichment.

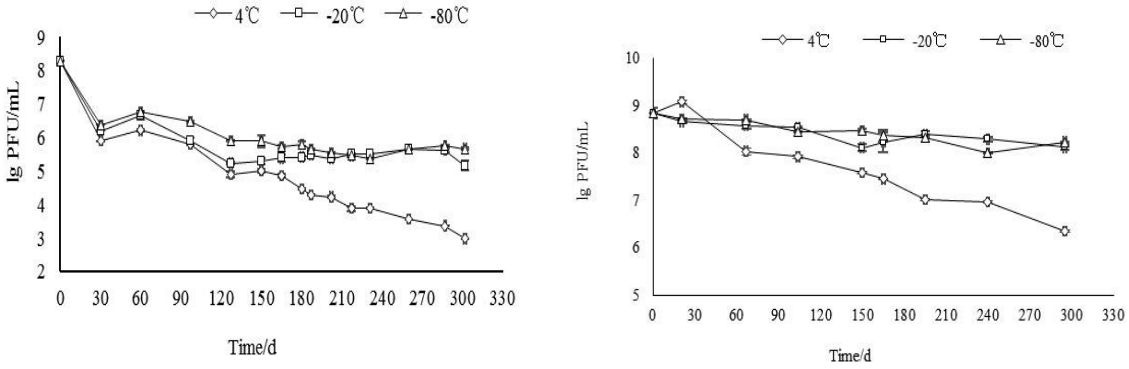
4. RESULTS AND ANALYSIS OF THE STUDY

4.1 PRESERVATION EFFECT OF TEMPERATURE AND DIFFERENT PROTECTIVE AGENTS ON LACTOBACILLUS BACTERIOPHAGE

4.1.1 P1 AND P2 PHAGES PRESERVED IN 15% GLYCEROL AT DIFFERENT TEMPERATURES

As shown in Figure 1, the initial titer of phage P1 was 1.94×108 PFU/mL. When stored at 4℃ and -20℃ with the final concentration of 15% glycerin as a protective agent for three months, the titer decreased by one logarithmic degree, while the basic preservation titer remained unchanged at -80℃. After four months of storage, the survival rate of bacteriophages stored at 4℃ and -20℃ was significantly reduced, and the titer of bacteriophages was reduced to 6.20×10^4 PFU/mL and 1.19×10^5 PFU/mL, respectively, while the titer of bacteriophages stored at -80℃ could maintain 10^6 PFU/mL. After ten months of storage, the titers of bacteriophages stored at 4℃, -20℃ and -80℃ were reduced to 9.6×10^2 PFU/mL, 1.44×10^5 PFU/mL and 4.70×10^5 pFU /mL, respectively. It can be seen from the figure that glycerin has a better preservation effect on phage P1 at -80℃ than at other temperatures. Therefore, when glycerin is used as the protective agent of phage P1, the optimal storage temperature is -80℃.
As shown in Figure 2, the initial titer of phage P2 at the initial preservation stage was 6.95×10^8 PFU/mL. With the extension of storage time, the titer of phage P2 under any conditions decreased. Using glycerin as a protective agent, the titer of phage P2 decreased by one order of magnitude after storage at -80℃ for eight months, and the titer of phage P2 was still 10^7 PFU/mL after storage for ten months, which was only reduced by one order of magnitude. After storage at -20℃ for six months, the titer of P2 decreased by one order of magnitude. After storage at -20℃ for ten months, the titer of P2 reached 10^7 PFU/mL but was lower than that at -80℃. After storage at 4℃ for four months, the titer of phage P2 decreased by one order of magnitude. After storage at ten months, the titer of phage P2 decreased by two orders of magnitude to 10^6 PFU/mL. The storage effect of glycerin on phage P2 at -80℃ was significantly better than that at the other two temperature conditions ($P\leq0.05$). In this paper, 15% glycerin was used as a protective agent to preserve phage P1 and P2 at different

temperatures. The results showed that for phage P1 and P2, -80℃ was the best storage temperature. This may be because glycerin can combine with water as a hydrogen bond to form non-freezing water, thus making the aqueous solution sticky. The freezing process is slow, and



the damage to the phage structure is reduced.

Fig. 1, 2 The phage P1 and P2 volume is stored in 15% glycerol at different temperatures.

4.1.2 PHAGE P1 AND P2 PRESERVED IN 20% CHLOROFORM AT DIFFERENT TEMPERATURES

As shown in Figure 3, when chloroform was used as a protective agent for phage P1 for three months, both 4℃ and -20℃ reduced its titer by two logarithmic levels, while -80℃ only reduced it by one logarithmic level. In the 4th month, the titer of phage P1 stored at 4℃ and -20℃ decreased significantly to 10^4 PFU/mL, while the titer of phage P1 stored at -80℃ could still reach 10^6 PFU/mL, and the preservation effect was significantly higher than that of the other two groups. After ten months of storage, the titer of phage P1 stored at 4℃ and -20℃ has been reduced by six logarithmic levels, and the survival rate is meager, while the titer of phage P1 stored at -80℃ can reach 10^5 PFU/mL, which has significant difference compared with the other two storage methods ($P \leq 0.05$). Therefore, for chloroform, the optimal temperature for the optimal preservation of phage P1 is -80℃.

As shown in Figure 4, when chloroform was used as a protective agent for phage P2, the survival rate of phage P2 gradually decreased with the extension of storage time. After three months, the titers of the bacteriophages stored under each storage condition began to decline significantly. The titer of phage P2 stored at -80℃ decreased by one order of magnitude after seven months and was still 10^7 PFU/mL after ten months, which was only reduced by one order of magnitude. The titer of phage P2 stored at -20℃ decreased by one order of magnitude after four months and was 10^7 PFU/mL after ten months, which was lower than that of phage P2 stored at -80℃. The titer of phage P2 stored at 4℃ decreased by one order of magnitude after four months and 10^6 PFU/mL after ten months, which decreased by two orders of magnitude. Within three months, there was no significant difference in the preservation effect of chloroform at 4℃, -20℃, and -80℃ ($P \leq 0.05$). After four months of storage, the storage effect of chloroform on phage P2 at -80℃ was significantly better than that of the other two groups. Therefore, the optimal temperature for preserving phage P2 by chloroform was -80℃. In this study, 20% chloroform was added to phage lysate and stored at 4℃, -20℃ and -80℃,

respectively. The results showed that the lower the temperature, the better the preservation effect of chloroform on phage P1 and P2. Among them, -80℃ was the most suitable temperature for preserving phages P1 and P2 by chloroform. As shown in Figure 3, when chloroform was used as a protective agent for phage P1 for three months, both 4℃ and -20℃ reduced its titer by two logarithmic levels, while -80℃ only reduced it by one logarithmic level. In the 4th month, the titer of phage P1 stored at 4℃ and -20℃ decreased significantly to 10^4 PFU/mL, while the titer of phage P1 stored at -80℃ could still reach 10^6 PFU/mL, and the preservation effect was significantly higher than that of the other two groups. After ten months of storage, the titer of phage P1 stored at 4℃ and -20℃ has been reduced by six logarithmic levels, and the survival rate is meager, while the titer of phage P1 stored at -80℃ can reach 10^5 PFU/mL, which has significant difference compared with the other two storage methods ($P \leq 0.05$). Therefore, for chloroform, the optimal temperature for the optimal preservation of phage P1 is -80°C.

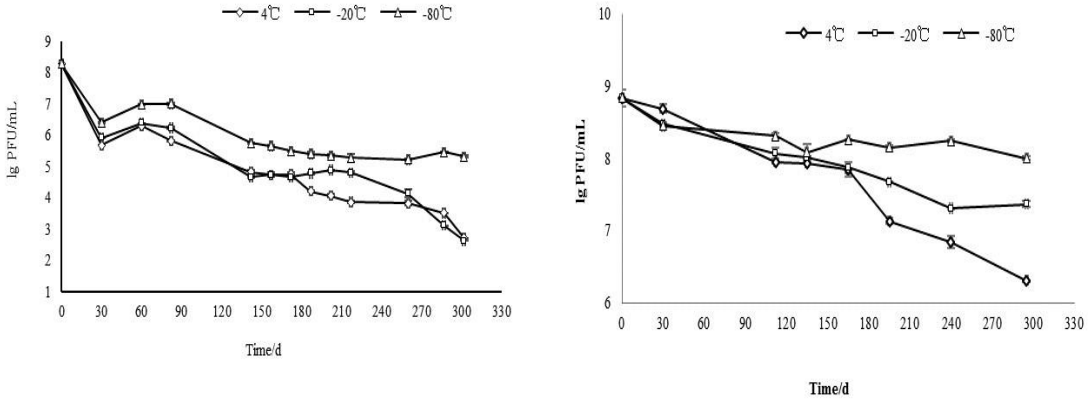


Fig. 3, 4 The phage P1 and P2 volume is stored in 20% chloroform at different temperatures.

4.1.3 P1 AND P2 PHAGES PRESERVED IN 7% DIMETHYL SULFOXIDE AT DIFFERENT TEMPERATURES

As shown in Figure 5, with the extension of storage time, the titers of DSO stored at three different temperatures decreased. After three months, phage P1 stored at 4℃, -20℃, and -80℃ decreased by one logarithmic level. By the 4th month, the titer of phage P1 stored at 4℃ and -20℃ had been reduced by four logarithmic levels, while that of phage P1 stored at -80℃ had decreased by only two logarithmic levels, which was significantly higher than that of the other two groups. From the 4th month to the 10th month, the titer of phage P1 decreased gently. After ten months of storage, the titer of phage P1 decreased to 10^3 PFU/mL at 4℃ and 10^5 PFU/mL at -20℃ and -80℃. The protective effect of DSO against phage P1 at -80℃ was significantly higher than in other conditions ($P \leq 0.05$).

As shown in Figure 6, the titer of phage P2 decreased with the extension of storage time. The titer of phage P2 stored at -80℃ decreased by one order of magnitude after seven months and could maintain 10^7 PFU/mL after ten months. The titer of phage P2 stored at -20℃

decreased by one order of magnitude to 10^7 PFU/mL after six months, slightly lower than that of phage P2 stored at -80℃. The aging value of phage P2 stored at 4℃ decreased by one order of magnitude at four months, and the titer was 106 PFU/mL after ten months of storage, which decreased by two orders of magnitude. There was no significant difference in the preservation effect of DSO at -80℃ and -20℃ for phage P2 within four months ($P \leq 0.05$). After four months, the preservation effect of DSO at -80℃ was significantly better than the other two groups. Therefore, the optimum temperature of dimethyl sulfoxide as a protective agent for the preservation of phage P1 and P2 is -80℃, and the titer of phage P1 can be kept above 10^6 PFU/mL within four months, and the titer of phage P2 can be kept above 10^7 PFU/mL within ten months. In summary, the best preservation method for phage P1 is to preserve it at -80℃ with 15% glycerin as a protective agent. For phage P2, the best preservation method was 15% glycerol or 7% dimethyl sulfoxide as a protective agent at -80℃.

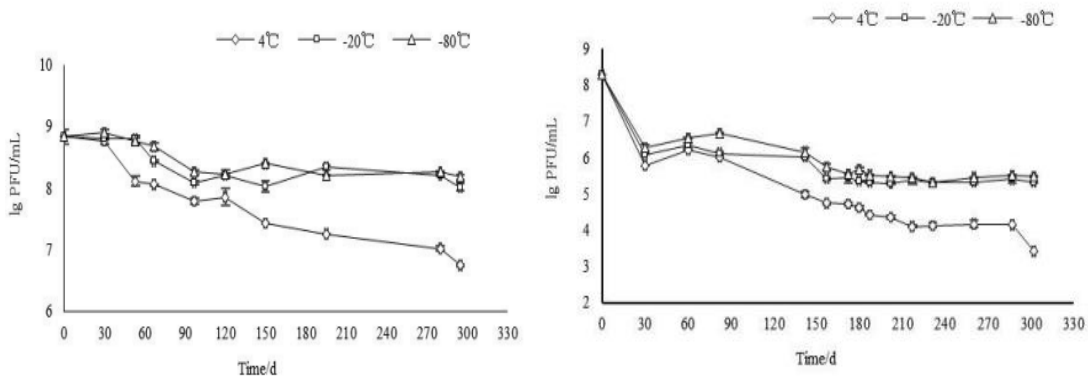


Fig. 5, 6 The phage P1 and P2 volume is stored in 7% dimethyl sulfoxide at different temperatures.

The results showed that phage P1 was stable at -20°C for 7 months at 10^5 pfu/ml, but decreased to 10^3 pfu/ml after 10 months at -80°C. Phage P2 is stable at 10^6 pfu/ml when stored at -80°C for 10 months. For 7% dimethyl sulfoxide, the optimum temperature for P1 and P2 phage storage is -80℃, P1 phage volume is more than 10^6 pfu/ml for 4 months, and P2 phage volume is more than 10^7 pfu/ml for 10 months.

4.2 TRANSCRIPTOMIC STUDIES OF PHAGE AT DIFFERENT STAGES OF HOST INFECTION, BASIC INFORMATION ON THE PHAGE TRANSCRIPTOME

To study the connection mechanism between phage and host bacteria, we selected phage P2 as a representative in our study. We plotted the one-step growth curve of phage according to Xi Yu et al., [29]. Drawing the one-step growth curve of the phage, it can be seen that the incubation period of the phage is 45 minutes, the lysis period is 45-135 minutes, and the stationary period is after 135 minutes. During this period, all the host cells have died. Since all host cells died during this time, in this study, only phage-digested host bacterial RNA was

extracted as the object of study at 0, 5, 15, 25, 80, and 120 min. Transcriptomic technology was used to comprehensively analyze the differential expression, gene regulation, and gene enrichment of phage P2 genes at different stages of host bacterial infection to investigate potential phage invasion and host bacterial lysis mechanisms.

In this study, we collected phage lysates of phage P2 at different lysis stages and used the Illumina Hiseq sequencing platform to complete transcriptome sequencing. An Illumina PE library for 2×150 bp sequencing was constructed, and quality control was performed on the obtained sequence data. Bioinformatic methods applied to the transcriptome data were analyzed, and the transcriptome size and GC content of phage P2 are shown in Table 1.

Table 1. Data statistic results

№	Number of sequences (strips)		Number of bases (bp)			Q30%	GC%
	raw	clean	raw	clean	raw	clean	clean
A0	1.19E+07	1.14E+07	1.78E+09	1.57E+09	92.88	95.35	42.38
A5	1.55E+07	1.51E+07	2.33E+09	2.10E+09	93.93	95.62	40.83
A15	1.99E+07	1.94E+07	2.98E+09	2.68E+09	93.65	95.55	41.14
A25	1.59E+07	1.55E+07	2.38E+09	2.11E+09	92.98	95.27	41.63
A80	2.55E+07	2.52E+07	3.82E+09	3.49E+09	95.66	96.76	42.04
A120	1.73E+07	1.67E+07	2.60E+09	2.30E+09	92.59	95.05	41.89

Note: raw represents the original sequencing data, clean describes the sequencing data after quality control; A0: initial lysis (0 min), A5: incubation period (5 min), A15: middle incubation period (15 min), A25: late incubation period (25 min)), A80: intermediate stage of lysis (80 min), A120: late location of lysis (120 min)

Table 1 shows the original and statistical sequencing data after quality control. It can be seen from the table that the actual sequencing numbers at the five points are all greater than 10 million, of which the number at 80 minutes is greater than 20 million, and the base numbers are all above 1568532099 bp. Q20% and Q30% are more significant than 92.59%, which meets the sequencing requirements. The average GC content is more critical than 40.83%.

Table 2. Mapping Ratio statistic results

Sample labeling	Sequencing number	Percent（%）
A0	7951913/11410320	69.69%
A5	3433221/15116980	22.71%
A15	3391843/19445526	17.44%
A25	3437022/15458148	22.23%
A80	4857998/25152682	19.31%
A120	3755291/16713974	22.47%

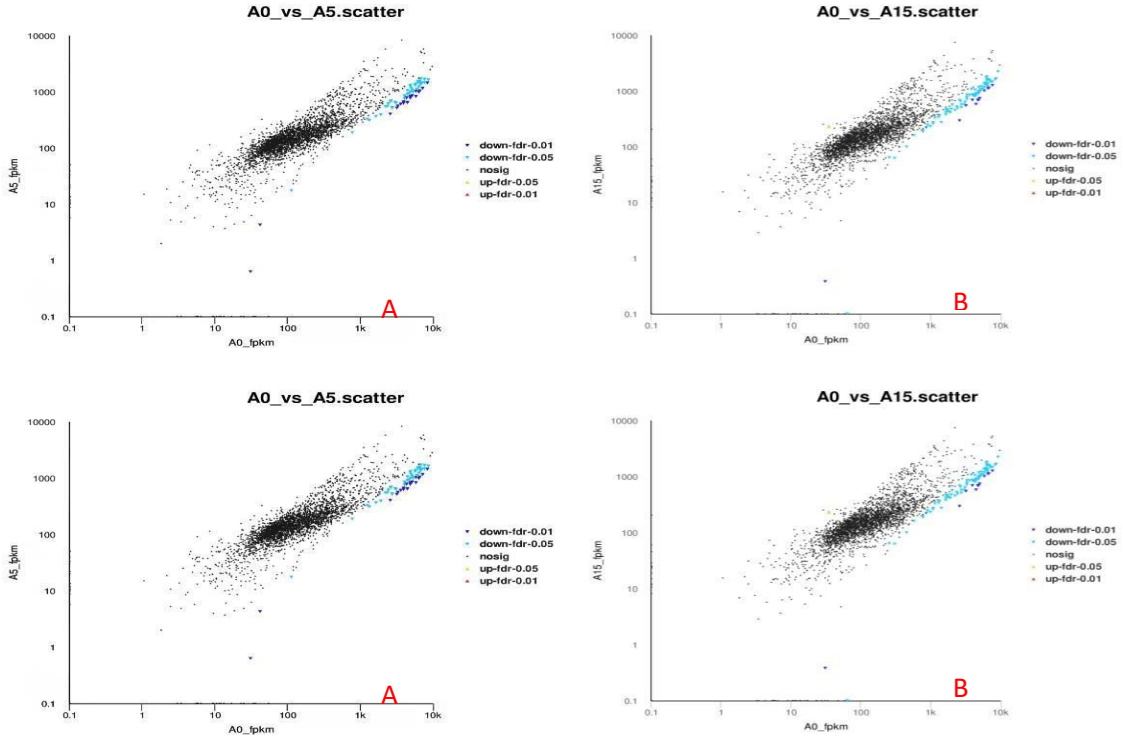
Note: A0: initial lysis (0 min); A5: incubation period (5 min); A15: middle incubation period (15 min); A25: Late incubation period (25 min); A80: Middle lysis period (80 min); A120: Late lysis period (120 min)

To obtain mapped data (reads) for further analysis, compare the clean, quality-controlled data (reads) with the reference genome. Table 2 presents the findings. There is no contamination in the relevant experiments and the reference genome used. Consistent with the sequenced species, the mapping percentage (mapping ratio) of clean reads on the reference sequence will be higher than 60%. In this study, phage P2 was selected at different time points when it infects host bacteria. The genetic material of the host bacteria will be changed when the phage integrates its genetic material or when incorporated into the genes of the host bacteria. As shown in the table, the comparison rate is greater than 60% at 0 min of infection, indicating that the sequencing data in this study are qualified and can be further analyzed and studied.

4.3 DIFFERENCES IN GENE EXPRESSION AT DIFFERENT STAGES OF PHAGE INFECTION

To understand the differences in gene expression at different lysis stages of phage, we used edgeR software to analyze the initial point of phage infection (0 min), the early incubation period (5 min), the middle incubation period (15 min), the late incubation period (25 min). Gene expression levels were sequenced at mid-lysis period (80 min) and late lysis period (120 min), as well as 0 min and 5 min, 0 min and 15 min, 0 min and 25 min, 0 min and 80 min, 0 min. Comparative analysis was performed with the differential genes between 120 min to guess the activity mechanism of the phage in different lysis periods.

The scatter-plots (scatter-plots) of 0 minutes and 5 minutes, 0 minutes and 15 minutes, 0 minutes and 25 minutes, 0 minutes and 80 minutes, and 0 minutes and 120 minutes are displayed in Figure 7.



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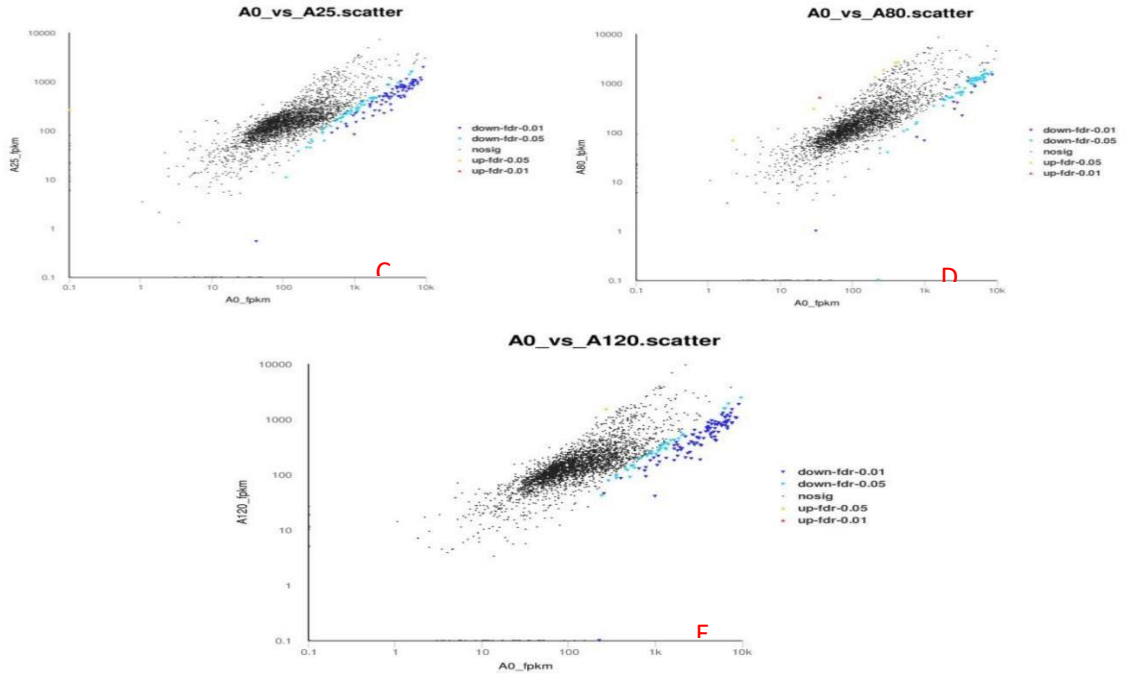

Fig. 7 Visualization of differential genes in different infection periods (scatter plot)

Note: A is a scatter plot of up-and down-regulated differential genes between 5 min and 0 min; B is a scatter plot for up- and down-regulated differential genes between 15 min and 0 min; C is a scatter plot of up-and down-regulated differential genes between 25 min and 0 min; D is a scatter plot of up-and down-regulated differential genes comparing 80 min and 0 min; E is a scatter plot comparing up- and down-regulated differential genes between 120 min and 0 min.

The figure shows the differential gene expression levels between the two samples. In the first stage of phage incubation (5 min), 65 genes were significantly changed compared to 0 min, and all of them were down-regulated, and in the middle stage of phage incubation (15 min), 92 genes were significantly changed compared to 0 min, of which 91 genes were down-regulated considerably and 1 gene was significantly up-regulated (gene 144); At the late phase of phage incubation (25 min), 143 differential genes were significantly changed compared to 0 min, of which 141 differential genes were down-regulated considerably and 2 differential genes were significantly up-regulated; During the lysis phase (80 min) compared to 0 min, there were 78 significantly changed differential genes, of which 70 genes were down-regulated considerably and 8 genes were significantly up-regulated; At late digestion (120 min) compared to 0 min, 144 differentially expressed genes were altered, 143 differentially expressed genes were significantly down-regulated, and 1 gene was up-regulated considerably (gene 2876).

4.4 FUNCTIONAL ENRICHMENT OF DISTINCT GENES AT VARIOUS STAGES OF PHAGE INFECTION

KEGG functional enrichment analysis was carried out on the differential genes in P2 phage infection stages to demonstrate the functional enrichment of differential genes and to ascertain the differences in genes in different stages of phage degradation. Fisher's exact test was utilized to determine the significance of the differences. Four multiple testing methods (Bonferroni, Holm, Sidak, and p-value) are used to correct the p-value to control the predicted false positive rate. This function is often highly enriched when the corrected p-value is less than the p-value ≤ 0.05.

Table 3. KEGG functional enrichment table of differential genes in different periods

	Pathway	Number of genes	P value	Function
0 VS 5	ko03010	39	5.10×10^{-30}	Ribosome
	ko00710	3	0.036611869	Carbon fixation in photosynthetic organisms
0 VS 15	ko03010	42	4.87×10^{-27}	Ribosome
	ko00710	4	0.018869624	Carbon fixation in photosynthetic organisms
	ko03070	3	0.041860584	Bacterial secretion system
0 VS 25	ko03010	47	2.22×10^{-23}	Ribosome
	ko00710	5	0.019871646	Carbon fixation in photosynthetic organisms
	ko03020	3	0.035799153	RNA polymerase
0 VS 80	ko03010	41	2.72×10^{-39}	Ribosome
0 VS 120	ko03010	51	3.30×10^{-28}	Ribosome
	ko00710	5	0.015315753	Carbon fixation in photosynthetic organisms
	ko03020	3	0.030220167	RNA polymerase

Note: A0: initial lysis (0 min); A5: incubation period (5 min); A15: middle incubation period (15 min); A25: Late incubation period (25 min); A80: Middle lysis period (80 min); A120: Late lysis period (120 min)

As shown in Table 3, during the initial incubation period (5 min) of the host bacteria phage infection, 39 genes were significantly downregulated in ribosomal metabolism compared to the beginning of the disease (0 min), while 3 genes were significantly downregulated in the carbon fixation pathway. When comparing the mid-incubation phase (15 min) of host bacterial infection to the early stage of disease (0 min), 42 differentially expressed genes were significantly down-regulated in the ribosomal metabolism process; additionally, four genes were significantly down-regulated in the carbon fixation pathway; finally, during the late-incubation phase (25 min) of host bacterial infection, 47 differentially expressed genes were significantly down-regulated in the ribosomal metabolism process. 3 genes were significantly downregulated in the RNA polymerase metabolism pathway; 41 differentially expressed genes were especially down-regulated in the ribosomal metabolism process during the lytic phase of bacterial infection (80 min) when compared to the early stage of disease (0 min) and 5 genes in the carbon fixation

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pathway were significantly down-regulated; 51 differentially expressed genes were significantly down-regulated in the ribosomal metabolism process during the late stage of host bacterial infection (120 min) when compared to the early stage of disease (0 min), and 5 genes in the carbon fixation pathway, and 3 genes in the RNA polymerase metabolism pathway were significantly downregulated.

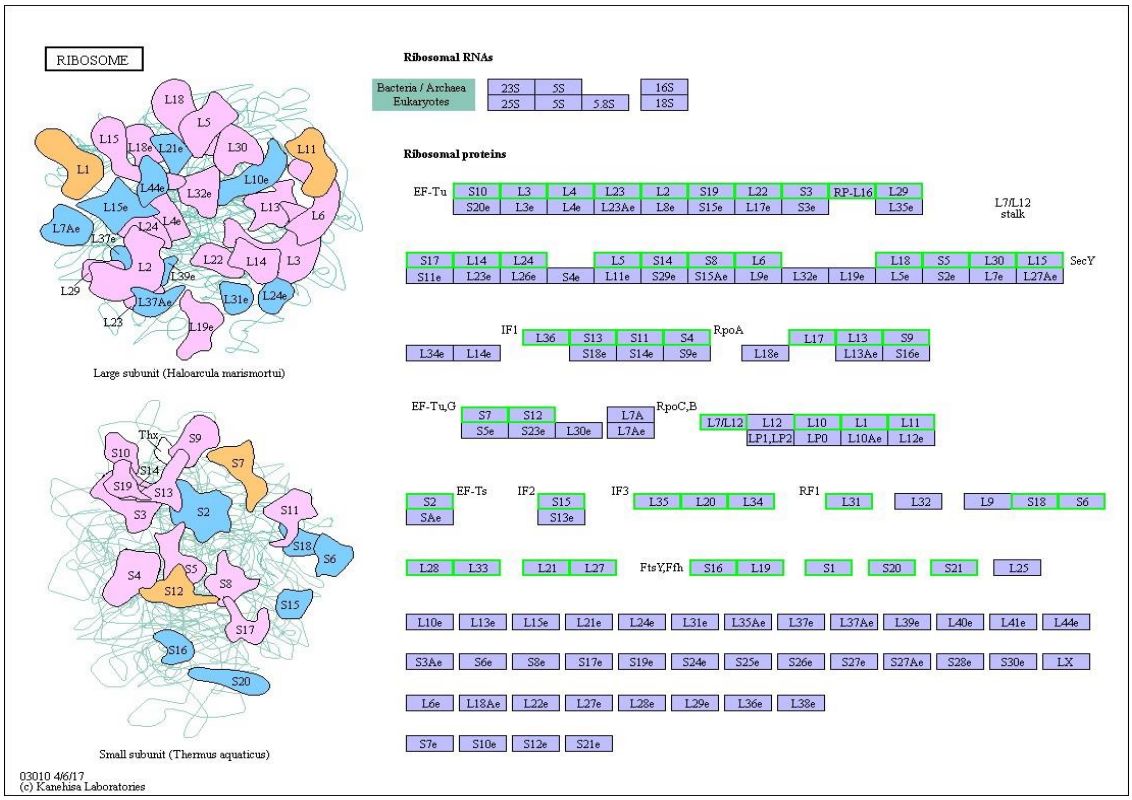

Fig. 8 Differential genes KEGG annotation pathway table of the ribosomal protein system

A KEGG-annotated pathway map of the ribosomal protein system's differential genes is shown in Figure 8. Genetic information processing and translation is the ribosome's main job. There is just one kind of ribosome found in prokaryotes, and it is made up of large and small subunits. The messenger RNA information is initiated when the ribosome starts the process of protein synthesis. The messenger RNA is joined by the small subunit, which subsequently interacts with the big subunit to form the complete ribosome.

During protein synthesis, EF-Tu and EF-G engage in interactions with ribosomes in this process. While EF-G aids in the forward elongation and translocation of messenger RNA and peptidyl-tRNA conjugates, EF-Tu transports amino acid-tRNA to the ribosome [30, 31]. Sec Y contributes to the co-information process in a supporting role [32], translation initiation factor F1 interacts with nucleic acids to start protein synthesis [33], cells lacking IF1 have nearly no polymer, which is critical for cell growth [34], and RpoA is a gene for RNA polymerase's α subunit. Through its interaction with an activator protein [35], which has two components that control bacterial transcription, it directly contributes to the activation of gene transcription. Phage P2 can be seen to disrupt bacterial transcription and information processing by controlling key enzymes such as IF1, RpoA, and EF-Tu in bacterial transcription and transcription processes.

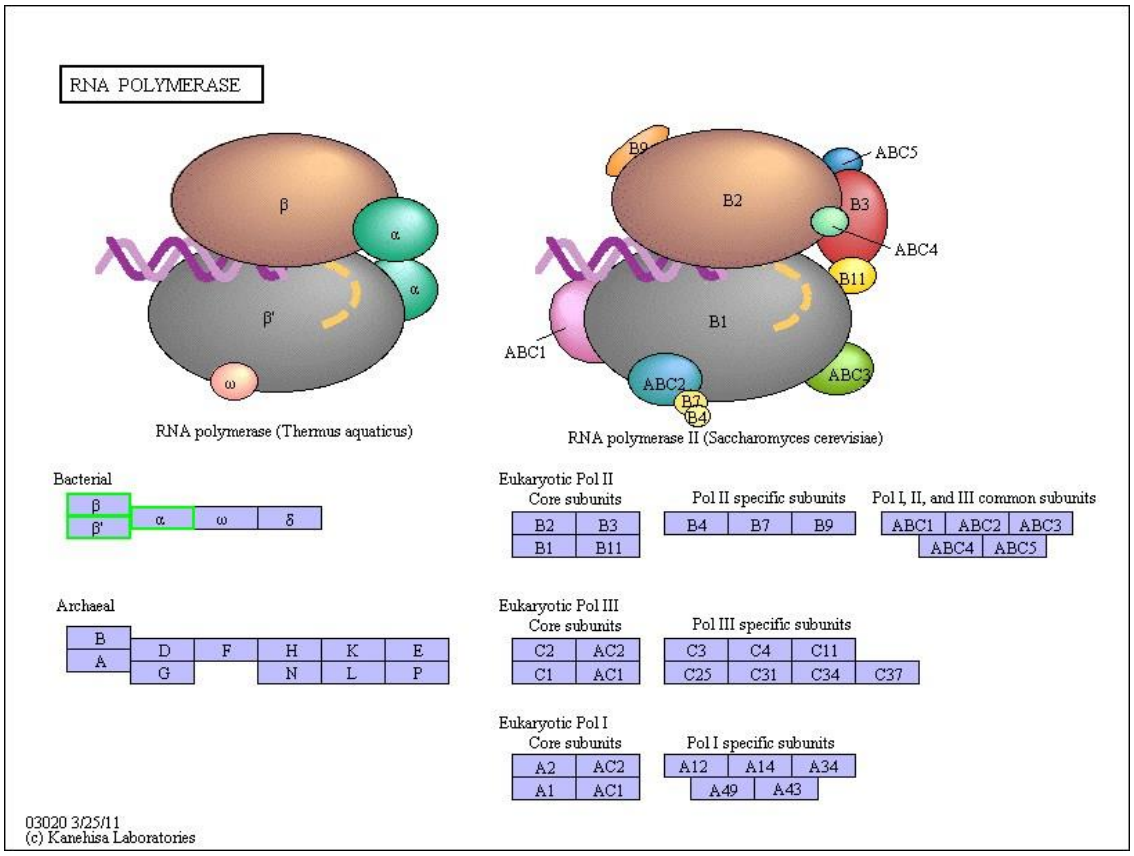

Fig. 9 KEGG pathway table of RNA polymerase differential genes

Figure 9 shows a diagram of the metabolic pathway of RNA polymerase. The primary function of RNA polymerase is to process genetic information and participate in transcription. It is an enzyme that catalyzes the synthesis of RNA from nucleoside 5'-triphosphate using a DNA or RNA strand as a template. The gene is markedly downregulated, as seen by the figure's green color. A β subunit, a β' subunit, two α subunits, an ω subunit, and a δ subunit make up RNA polymerase. α2ββ'ω is referred to as the key enzyme among them. It catalyzes the transcription and synthesis of all RNAs and is engaged in the transcription process in its entirety. The primary function of the δ factor is to identify the starting position of transcription, bind RNA polymerase to the promoter site, and recognize the promoter on the DNA template. It cannot exist separately. After binding to the template DNA and combining with the main enzyme to form a holoenzyme, the holoenzyme can be combined with a promoter on the template DNA. When it binds to a specific base sequence of the initiation gene, the double DNA strands are partially unwound, allowing transcription to begin. Factor δ is, therefore, also called the initiation factor. The genes that encode the α, β, and β' subunits of RNA polymerase are markedly downregulated when phage P2 infects a host bacterium for 25, 60, and 120 minutes. An increasing number of genes Through its interaction with activator proteins, the α-subunit of RNA polymerase is directly engaged in the activation of gene transcription, as demonstrated by examples [36].

5. CONCLUSION

P1 and P2 are virulent phages of Lactobacillus plantarum; studying the most appropriate method for their preservation is essential for the dairy industry. It is crucial for future research to use phages. It provides information for research, such as controlling the effects of bacteriophages, determining their biological properties, detecting phage-anti-strains, etc.

In this study, after ten months of long-term storage at 4℃, the optimal protective agent was dimethyl sulfoxide with a 10^6 PFU/mL titer. In conclusion, glycerin was the most suitable protective agent for preserving phage P1 at 4℃. At the same time, dimethyl sulfoxide was the most appropriate protective agent for the preservation of phage P2, and the preservation stability of phage P2 was better than that of phage P1 at 4℃. In addition, using 15% glycerol and adding 7% DMSO at -20℃ within 112 days had an excellent preservation effect on phage P1, and its titer could be maintained above 10^6 PFU/mL. Therefore, glycerol and dimethyl sulfoxide are the most suitable protective agents for phage P1 at -20℃. When phage P2 was stored at -20℃, glycerol, chloroform, and dimethyl sulfoxide could be used as protective agents within four months, and their titers were maintained above 10^8 PFU/mL. After four months, the best protective agent for long-term preservation was 15% glycerol, which could keep the phage titer above 10^7 PFU/mL. It was also found that the titer of phage P1 decreased by two logarithmic orders after being preserved for four months by adding 15% glycerol or 7% DSO at -80℃. After ten months of storage, the titer of phage P1 could still reach 10^5 PFU/mL. Therefore, glycerin or dimethyl sulfoxide was the best protective agent for phage P1 stored at -80℃ for four months. When phage P2 was stored at -80℃, 15% glycerol or 7% dimethyl sulfoxide was the best protective agent, and its titer could maintain 10^7 PFU/mL after ten months of storage. It can be seen that -80℃ is suitable for long-term storage of phage, but for phage P1, storage at -80℃ also needs to be transformed every four months to maintain its vitality. Pnagus P2 showed higher activity than P1.

This transcriptomic study of phage P2 infecting L. plantarum IMAU10120 at different stages showed that, compared with the initial point of infection by the phage, the encoding elongation factors EF-Tu, EF-G, and EF in the latent period, mid-lytic period and late lytic period -Ts, the synthesis of Sec Y protein, prokaryotic translation initiation factors IF1, IF2, IF3, RNA polymerase Rpo A, Rpo B, Rpo C, signal recognition particles Fts Y, Ffh and release factor RF1 was significantly reduced, and phage P2 passed. It regulates critical enzymes of the ribosome and RNA polymerase metabolic pathways to complete its genetic material's replication, transcription, and translation. It simultaneously inhibits the transcription and translation of bacterial genetic material. RNA polymerase synthesis decreased significantly when virulent phage P2 infected Lactobacillus plantarum IMAU 10120 for 25 min. Therefore, we speculated that phage had completed the transcription and translation process of its genetic material, that is, the assembly process of phage. However, when phage lysed for 80 min, The RNA polymerase resumed its original function, and we speculated that phage might produce some enzymes that lyse the bacteria's cell wall during this period.

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