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Rapid Evolutionary Characteristics of P1 and T5 Infection in E. coli Strains

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Abstract: - The emergence of superbug has once again thrust century-old phage into the spotlight as a hot research topic. While the "phage display" technology has enabled targeted evolution of viruses, the successful application of phage remains uncertain due to their replicative and mutative nature, as well as the complexity of phage-bacteria interactions and evolutionary mechanisms. In this study, we used two rapidly evolving phages, P1 and T5, along with 18 types of E. coli strains, as representatives to explore the evolutionary characteristics of phage-infected bacteria. Our findings confirm that the evolutionary of phages and their hosts is a co-evolutionary process, resembling an "arms race", after the early rapid evolution of P1 and T5, the host range expands to infect different E. coli strains, but continued rapid evolution reaches a certain stage where P1, T5, and E. coli strains all experience a fitness cost, and the host range no longer expands. Three new findings in this study differ from previous studies. Firstly, although E. coli strains were not infected in the same way, there was no statistically significant difference between P1 and T5 infection in different E. coli strains at different evolutionary stages. Secondly, 11 types of E. coli strains that had been infected by T5 during co-evolution all showed E. coli tolerant plaques on the medium at the fifth stage of evolution, whether they had been infected by T5 at that stage of evolution, and at the sixth stage of evolution, all of these tolerant plaques disappeared again. Thirdly, after the fitness cost of the co-evolutionary process had already occurred, although the host range of P1 and T5 no longer continued to expand, the types of E. coli strains infected by P1 and T5 at the same evolutionary stage did not decrease in focus with the continued prolongation of the co-evolutionary time, but instead showed a gradual incremental trend. This study provides experimental evidence for the resolution of phage-bacteria co-evolutionary mechanism and new ideas for phagotherapy strategies for the superbug.

Keywords: Phage, P1, T5, E. coli strains, evolutionary characteristics.

I. INTRODUCTION

Phages, which have coexisted extensively with bacteria in nature for billions of years, belong to a class of viruses that parasitize various, microorganisms including bacteria, fungi, and actinomycetes. They are widely distributed in most prokaryotic organisms, and consist primarily of proteins and nucleic acids [1].

A. Phage History

In 1896, the British scientist Ernest Hankin made a significant discovery, regarding the number of Vibrio cholerae at the entrance and exit of the Ganges River, in the city of Agra. For the first time informally suggested the existence of some kind of antimicrobial active substance in microorganisms [2]. In 1898, the Russian bacteriologist Niko**-**lay Gamaleya observed a similar phenomenon when studying Bacillus subtilis [2]. In 1915 and 1917, two independent reports were made by Frederick Twort, a British bacteriologist, and Félix d'Hérelle, a French-Canadian dual-citizen microbiologist, formally confirming the existence of phages [3]. Twort discovered the "glass-like transformation" of micrococcal colonies, Félix d'Hérelle isolated Shigella "anti-microbials", and for the first time the term "phage" was coined to define a bacterial feeder. In 1919, Herelle made the first clinical application of phage to human beings, using phage preparations for the treatment of bacillary dysentery in children [4].

Following, Alexander Fleming's discovery of penicillin in 1928, and the subsequent widespread use of antibiotics, along some early failures in clinical applications, phagotherapy was gradually discontinued. However, along with the widespread and especially irrational use of antibiotics, the problem of bacterial drug resistance has become more and more serious, and even the emergence of drug-free superbug has brought unprecedented challenges to human health. This, coupled with the apparent crisis in the research of novel antibiotics, has triggered the exploration of new antibacterial drugs. Many international organizations (e.g., FDA) have declared this issue to be of the highest priority. In response to this challenge, phages are back on the radar of scientists and clinical medicine experts as a weapon for the treatment of drug-resistant bacterial infections.

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B. Phage Characterization

Phages are characterized by two main features: structural simplicity and diversity. Like other viruses, infectious phages comprise a protein coat and a core. Some phages have a core consisting solely of nucleic acid, while others incorporate additional components alongside nucleic acid. Phage diversity is extensive. It is estimated that the number of phage particles in the world is about 10 times the number of all bacteria, exceeding the number of all other organisms, including bacteria, combined.

Currently, nearly 2900 phage species have been observed by electron microscopy. Among these, some of the more commonly known ones include T phages (a collective term for seven types of E. coli phages: T1-T7), λ phage, P1 phage, Mu phage, φX174 phage, φ29 phage, PRD1 phage, Qβ phage, P2 phage, and Sf6 phage. Each phage has a different way of infecting bacteria and its effect on bacterial host.

Table 1 Phage classification

Classification basis	Category	Peculiarity	
Phage-host relationship	**Mild phages (lysogenic phages)**	A class of phages that, after binding to the host bacteria, integrates its own genes into the chromosomes of the host bacteria, becomes part of the host bacteria's genes, and multiplies with the reproduction of the host bacteria.	
	Potent phages	A class of phages that, after binding to sensitive host bacteria, rapidly proliferate in the host bacteria and eventually lyse them.	
Genetic	**DNA phages**	**Double-stranded DNA phages**	A class of phages that can infect bacteria and replicate its own DNA virus within bacteria. Most phages are DNA phages.
		Single-stranded DNA phages	
	RNA phages	**Double-stranded RNA phages**	A class of phages that can infect bacteria and replicate its own RNA virus within bacteria.
		Single-stranded RNA phages	
Morphological structural characteristics	**Tailed phages**	1.A class of phages whose shell consists of a head and a tail, which are connected by the neck. The head is usually icosahedral symmetrical and whose tail is spiral symmetrical. 2.Except for Rφ6 double-stranded DNA, which is circular and superspiral, most of phages with tails are double-stranded linear DNA. 3.A class of phages with the largest number and the largest change in shell structure and the most common of which include T4, T7, P2, P22, λ, and φ29.	
	Tailless phages (Spherical phages)	A class of phages with simple shells but quite different in nucleic acid groups. Some are double-stranded DNA molecules, such as PM2 phages; Some are positive polarity single-stranded circular DNA molecules, such as φX174 phages; Some are positive polar single-stranded RNA molecules, such as MS2; Others are multi-segment double-stranded RNA molecules, such as φ6.	
	Filamentous phages (Rod phages)	A class of phages whose core is composed of single-stranded circular DNA. Both M13 and FD belong to filamentous phages.	

Phages are currently classified mainly based on their morphology and genomics. Phages can be categorized into two main groups according to their relationship with the host: mild phage (also known as lysogenic phages) and potent phage. Furthermore, phages can be classified based on their genetic material into four categories: double-stranded DNA phages, single-stranded DNA phages, single-stranded RNA phages, and double-stranded RNA phages. According to the morphological and structural characteristics, phages can be classified into three major groups: tailed phages, tailless phages (also known as spherical phages) and filamentous phages (also known as rod phages). It's worth noting that the majority of phages are double-stranded DNA phages.

C. Mechanisms of Phage Infection

Phage and bacteria have been struggling with each other for a long time in nature world. Bacteria have evolved a variety of mechanisms to resist phage infection, and phage, in turn, have also developed a large number of mechanisms to counter bacterial resistance. These mechanisms are constantly changing with the long-term phage-bacteria co-evolution. The advantages of phage such as short reproduction period, small genome, easy cultivation, and controllable conditions make phage an excellent target for experimental evolutionary studies. Despite these favorable attributes, our understanding of the evolutionary processes and mechanisms governing

the interplay between phage and bacteria remain quite limited, despite the insights that can be inferred from genome sequences.

Phage exhibit a simple structure and cannot survive alone. They rely on parasitically growing, replicating and multiplying within a host organism. Therefore, whether coexisting with bacteria or directly parasitizing the host, the host is only a tool for phage. After phage completely invades the host and exhausts its utility, it will also die out with the host.

When a mild phage infects a bacterium, it integrates its own nucleic acid into the chromosome of the infected bacterium. Rather than replicating independently, it coexists with the bacterium, replicating as the bacterial chromosome replicates, and is assigned to the chromosome of the zygotic bacterium as the bacterium divides, thus establishing a lysogenicity cycle. Phage genes that are integrated into the bacterial chromosome are called prophages. Bacteria with prophages on their chromosomes are known as lysogenic bacteria. Passing on phage genes as lysogenic bacteria divide is known as the lysogenic state. Therefore, mild phages are also called lysogenic phages.

In contrast, potent phages multiply rapidly after infecting bacteria and can be divided into five processes: adsorption, invasion and shelling, biosynthesis, assembly, and release. Potent phages directly destroy bacteria.

Table 2 The process by which potent phages infection in bacteria

Stage of infection	Stage name	Infection process
Stage1	**Adsorption**	The cervifolial protein of the phage binds to surface-specific receptors of the host cell, as indicated by attachment to the host cell. Different phages have different specific receptors on the surface of host cells. For example, the adsorption-specific receptors of T2 and T5 phages are lipoproteins, and the adsorption-specific receptors of T3, T4 and T7 are lipopolysaccharides.
Stage2	**Invasion and shelling**	Phage tail filament contraction scientific research allows the tail tube to touch the cell wall of the bacteria, and the lysozyme carried by the tail tube can cleave the peptidoglycan on the cell wall of the host bacteria, thereby injecting nucleic acids into the cell, and the protein shell remains outside the bacterial body.
Stage3	**Biosynthesis**	DNA replication, transcription, and protein synthesis with host cells.
Stage4	**Assembly**	Within the host cell, the parts of the phage bind in a certain way to assemble mature virions.
Stage5	**Release**	Upon 0ffspring phage maturation, most phages can synthesize an enzyme that inhibits cell wall synthesis within the host, resulting in a gradual thinning of the host cell wall and eventual lysis.

D. Phage Advantage

The antimicrobial value of phage is remarkable compared to antibiotics. Antibiotics often have a broad spectrum of activity, meaning they can kill a wide range of bacteria. This indiscriminate action can lead a series of side effects. In contrast, phage are highly specific in their targeting. They have little interaction with human cells, and have fewer side effects on the human body.

E. Phage Limitations

The limitations of phage are mainly in two aspects. Firstly, there is host specificity and a narrow antibacterial spectrum. A phage can only kill certain strains of the same kind of bacteria, but not all the bacteria of the same kind, not to mention killing many different kinds of bacteria. Secondly, there's the issue of evolutionary uncertainty. Bacteriophage is completely different from other bio-pharmaceuticals, phage can be replicated, and the replication process will also be mutated, under the premise of unclear mechanism of phage and bacteria, the function and effect of various components after mutation may produce tolerance and may be harmful to human body.

F. Progress in Phage Research

George Smith, one of the laureates of the 2018 Nobel Prize, is credited with inventing the "phage display" technique. This breakthrough method enables the directed evolution of viruses that target bacteria, leading to the

creation of new proteins. Based on further analyzing the phage-bacteria interaction mechanism, engineering phage and recombinant phage through synthetic biology to improve the clinical therapeutic effect and safety is gradually becoming a key research direction to solve the problem of phage application disadvantage. Cutting-edge research is utilizing the property of RBP to assist phage in host recognition to artificially narrow the phage host spectrum. The development of high-throughput sequencing technology promotes the integration of multi-omics technologies for research to understand the mechanisms of phage-host interactions comprehensively and systematically, this includes investigating the regulation of phage gene expression and how phage overcome the bacterial immune defense mechanism. Existing studies have shown that phage usually hijack the transcriptional machinery of host bacteria to regulate their own and host bacterial gene expression, and that phage receptor binding protein (RBP) is the primary determinant of host specificity. However, the structural basis of phage protein-mediated transcriptional regulation, especially transcriptional repression, is still unclear due to the poor understanding of the phage-bacteria interaction mechanism [5].

II. RESEARCH

A. Research Purpose

To explore the evolutionary characteristics of phage-infected bacteria through laboratory studies and to provide experimental evidence for parsing the mechanism of phage-bacteria co-evolution.

B. Research Target

Using TADR (Targeted Artificial DNA Replisome) [6] system long-term sustained rapid evolution of P1 and T5 phages, and 18 types of E. coli strains.

(1) TADR system

TADR system is an intracellular mutagenesis tool, designed and developed by Dr. Yi Xiao [6], which increases the mutation rate by approximately 230,000-fold compared to the wild-type mutation rate, and is able to rapidly target a large number of mutations in living cells to any specified DNA sequence in a short period of time, without affecting the rest of the genome.

Evolutionary innovation of new protein functions is central to Darwinian adaptation. Natural evolution depends in complex ways on population size, mutation rate, and the shape of the adaptive, occurs over decades or long.

TARD system achieves accelerated evolution by creating mutations.

TADR system is an error-prone targeted artificial protein complex. It consists of three proteins, phage protein CisA, bacterial Rep helicase, and an error-prone mutant of the T5 phage DNA polymerase. The host cells for TADR were a modified strain of E.coli strain MG1655.During the natural replication process.

TADR system, as a protein complex, has a protein that specifically recognizes sequences of 30 base pairs. After recognition, the phage starts to replicate the DNA from that site, fuses the DNA polymerase with high error rate to this phage protein, and uses this phage protein to target the DNA polymer with high error rate upstream of the DNA of the specified mutation.

By changing the Mg2+ concentration, dNTP concentration ratio, pH value, etc. in the conventional PCR reaction system, the bases can be randomly introduced to a certain extent to create sequence diversity genebank.

TADR system, as an intracellular mutation method, the entire evolutionary process is completed in living cells. The key to this approach lies in controlling the appropriate mutation frequency, with lower mutation rates accumulating most of the beneficial mutations, while higher misincorporation rates produce neutral or deleterious mutations. Compared to traditional directed evolution methods based on error-prone PCR and in vitro plasmid construction, TADR avoids highly inefficient steps such as molecular ligation and cell transformation, thus realising the potential for rapid and sequential evolution of molecular and cellular phenotypes in live cells

(2) Reasons for choosing P1,T5

There are a wide variety of phage species. In this study, one representative from each of the mild phages and potent phages were selected to ensure both comprehensiveness and representativeness of the study.

Nathan Crook's team at the University of North Carolina, USA, showed [7] that P1 phage can effectively infect E. coli. Thus, P1 was selected as a representative of mild phages in this study.

The T-lineage Escherichia coli phages, with T5 being the most extensively investigated, studied representative of potent phages. The DNA polymerase of T5 is very error-prone during self-evolution, and the process of adsorption to the E. coli Rep helicase fusion protein facilitates the use of plasmid hyper-mutation by

targeting artificial DNA replicas to improve or generate new molecular functions [6]. Thus, T5 was selected as a representative of potent phages.

(3) Reasons for choosing E. coli and 18 types of strains

Existing researches have most thoroughly studied phages with E. coli as the host. In addition, E. coli is less dangerous and safer for secondary school students to do experiments independently. Therefore, E. coli was selected as the phage infection target in this study. On the other hand, there are many strains of E. coli, and 18 types of strains of E. coli commonly used in the laboratory where I did my internship were chosen for convenience.

C. Research Basis

Phages P1 and T5, which were used in the study, were put into the continuous incubator half a year in advance to accelerate the evolutionary culture. Prior to this study, Yi Xiao's research team had already conducted evolutionary studies on phages in the continuous incubator. By tracking 25 evolutionary stages, Yi Xiao's research team found that phages rapidly evolving by placing them in a continuous incubator in advance had developed a fitness cost： in the middle and late stages of the previous study, the host range of P1 and T5 was maintained at 11 out of 18 types of E. coli strains and was not expanded. Whereas the host range of P1 was six types in the early stages of the previous study, the host range of T5 was only three types.

Meanwhile, the types of phage-infected E. coli strains showed a gradual decreasing trend during the 25 stages of evolution. Among them, P1 gradually decreased from the initial observation of 6 types to 10 types and then decreased to a minimum of 3 types, and T5 also gradually decreased from the initial observation of 3 types to 10 types and then decreased to a minimum of 5 types just as shows in Figure1.

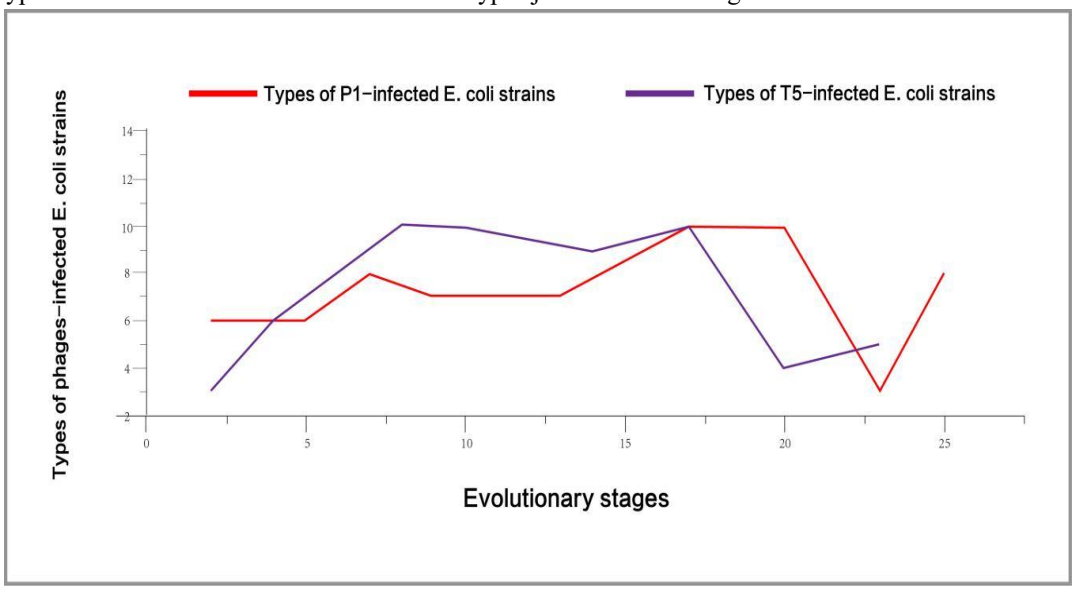

Figure 1 Trend plot of types of phages-infected E. coli strains observed in the previous 25 evolutionary stages

D. Research Hypothesis

This study proposes hypotheses based on existing studies as well as previous studies by Yi Xiao's research team.

Existing studies have shown that the process of phage infection of bacteria is a co-evolutionary dynamic process, which is both parallel continuation and continuous differentiation. Throughout the process, phage and bacteria can cross-infect. On one hand, phage actively adapts to their host, constantly shifting, and they tend to kills more susceptible bacterial strains. This, in turn, favors bacterial strains that develop new traits and resistance to antimicrobial drugs [8]. The arms race between phage and bacteria usually ends up with both phage and bacteria having a fitness cost as they continually refine their adaptability while increasing their infectivity and tolerance. Consequently, as the co-evolution progresses, bacterial species infected by phage at the same moment show a decreasing trend in progressive focus, provided that the host range is no longer extended [9].

As a result, this study proposes the hypothesis that P1 and T5, which continue to evolve at an accelerated rate, will no longer expand the maximum range of phage hosts with the continuation of evolutionary time to more than

11 types, and the types of infected E. coli strains at each evolutionary stage will continue to decrease to fewer than 10, trend shown in Figure2, based on the peak cost of adaptation of 10, which appeared in the 25 evolutionary phases of Yi Xiao research team.

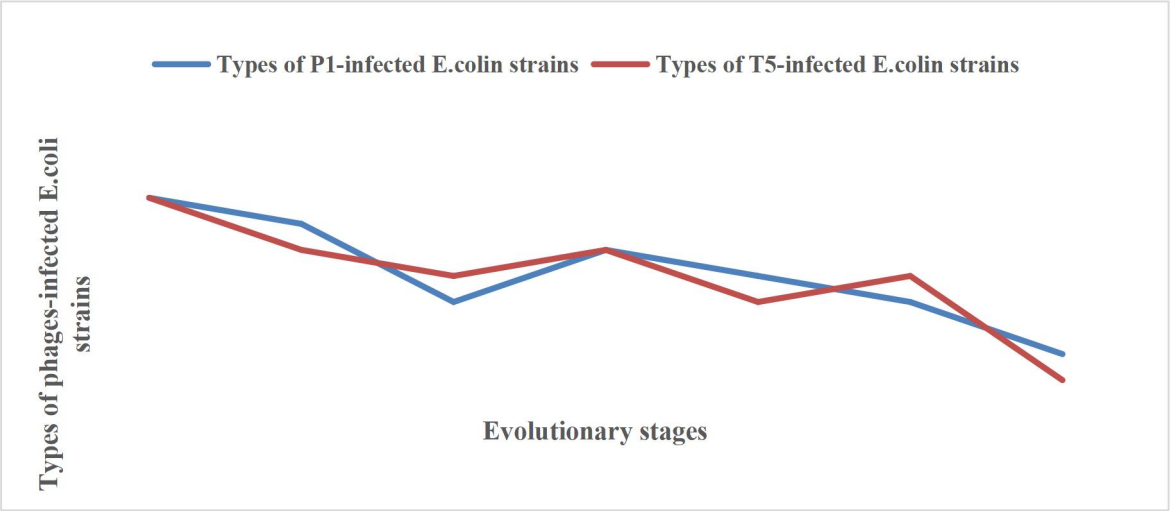

Figure 2 Trend plot of types of phages-infected E. coli strains at different evolutionary stages in experimental hypothesis

E. Research Design

The natural evolution of phage lasts for decades or even longer. For the purpose of the study, this experimental procedure is designed differently from the traditional one.

In this study, seven main experiment processes were designed as shown in Figure3, namely: preparation of 18 types of E. coli strain culture fluids, directing 18 types E. coli strain culture fluids into a phage continuous incubator, picking up phage evolution fluid from the phage incubator, refrigerating the phage evolution solution, counting phages, verifying experimental suspects, and analyzing experimental data.

In this study, six days are used as an evolutionary cycle, and each day as an evolutionary stage, and P1 and T5 phage evolutionary fluids are picked up from the waste port of the phage continuous incubator, which was under continuous rapid evolution, once a day for six consecutive days. The phage evolution solution for each evolutionary stage was refrigerated at 4℃ immediately after pickup to ensure that the phage state remained unchanged at the evolutionary stage at the moment of pickup. The phage evolutionary fluids of all six evolutionary stages were picked up and properly refrigerated, and then on the same day, the plates were spread to count the phages, and the infections of 18 types of E. coli strains by P1 and T5 were observed simultaneously at the six different evolutionary stages, to analyze the evolutionary characteristics of the phage-infected bacteria according to the observation results.

III. MATERIALS AND METHODS

A. Materials

(1) Phages and strains

Standard P1, T5 phage, 18 types of E. coli strains numbered 1.1390,1.1392, 1.1401, 1.1409,1.1416,1.1417, 1.1419, 1.1435,1.1437, 1.1438,1.1439,1.1441, 1.7601,1.7602. 1.7603, 1.7604, 1.7607,1.7627(all provided by Shenzhen Institute of Advanced Technology Chinese Academy of Sciences)

(2) Main reagents

Erythromycin blue medium (Shanghai Yihua Medical Technology Co.), Nutrient agar (Hangzhou Microbiology Reagent Co.), Ezup Column Bacterial Genomic DNA Extraction Kit (Shenggong Bioengineering (Shanghai) Co.), Liquid LB medium, semi-solid medium and solid medium(self-restraint).

(3) Main instruments

Centrifuges(Eppendorf (Shanghai) International Trade Co.,Ltd.), DH5000 AB Type Electrothermal Constant Temperature Continuous Incubator(Shanghai Huyeming Scientific Instrument Co.),Pulsed Field Gel Electrophoresis System BioRad CHEF-DRIII(Bó Lè Life Medical Products (Shanghai) Co.),Multi-module gradient PCR instrument(Hangzhou Langji Scientific Instrument Co.),Stereoscopic stackable thermostatic shaker(Hangzhou Kefei Experimental Instrument Co.),DYY-6C Electrophoresis Instrument(Beijing Liuyi Biotechnology Co.),Microwaves (Guangdong Grantham Group Co.),Autoclave(ZEALWAY (Xiamen) Instruments Co.).

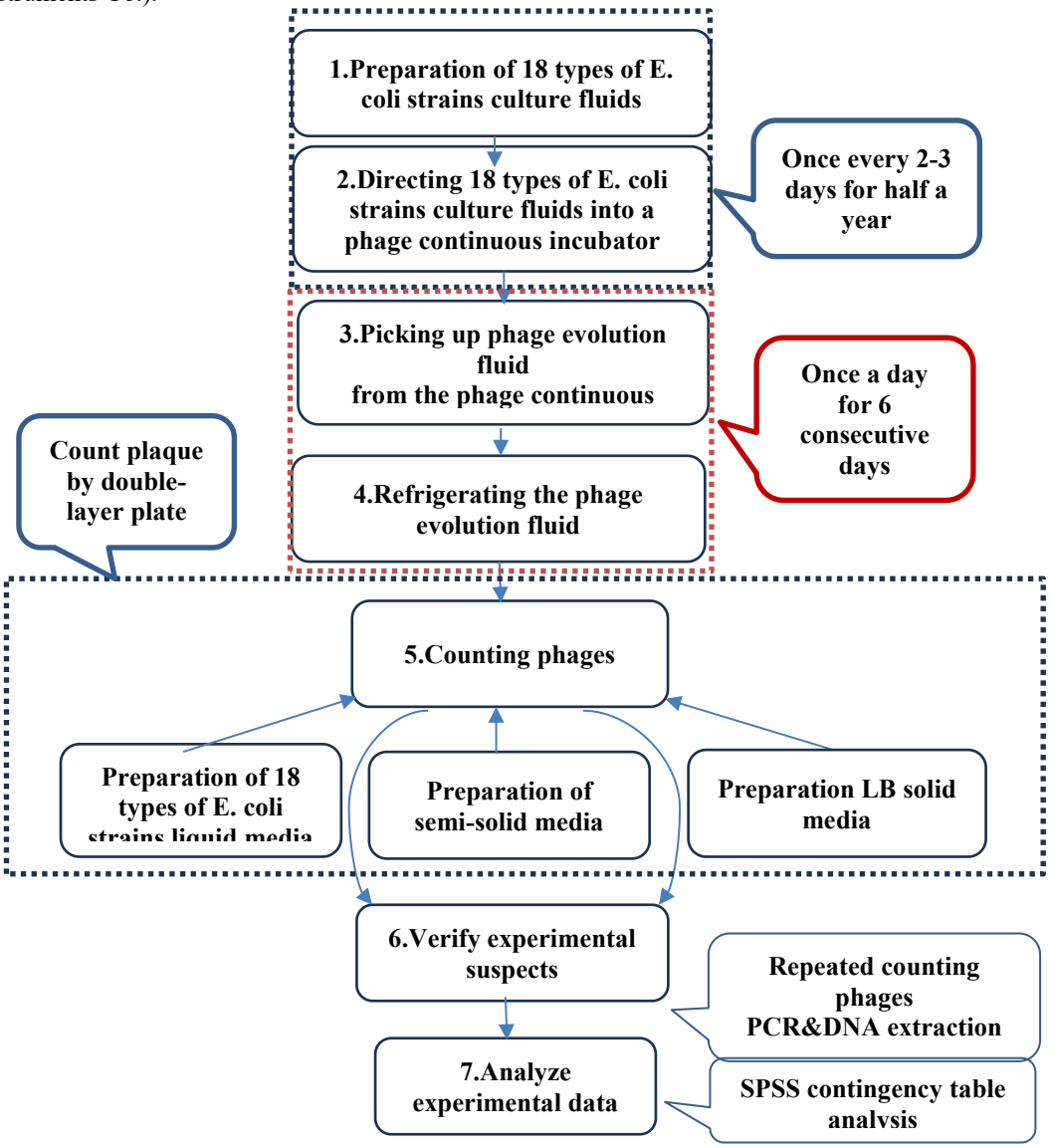

Figure 3 Experimental process

B. Methods

(1) Preparation of 18 types of E. coli strains culture fluids

Mix 30g of LB broth (without sugar) with 1.5L of pure water into a conical flask, seal the mouth of the flask with tin foil, put it into an autoclave for about 5 hours, pick a single colony with the tip of a gun to put it in and whack it to mix, put it into a shaking bed, shake the E. coli for 12-18 hours at 37°C, 200 r/min, shake it to the exponential period, and the scale can be seen on the opposite side of the inner wall of the glass through the glass. This process was repeated 18 times with one pickup of each E. coli strain.

(2) Directing 18 types of E. coli strains culture fluids into a phage continuous incubator

Put the conical flasks with prepared culture solution of E. coli strains as described in (1) into the continuous incubator, and connect to the phage culture tube with a peristaltic pump to keep the rate of culture solution diversion unchanged. The E. coli culture solution was prepared every 2-3 days and drained into the phage

continuous incubator for half a year. The work was done by me during the vacation, my full-time internship, with the help of the lab's seniors during my school time.

(3) Picking up phage evolution fluid from the phage incubator

From June 21st to 26th, 2023, for 6 consecutive days, 2 ml of P1 and T5 evolutionary fluids were picked up from the continuous phage incubator waste liquid orifice to pick up the evolutionary fluids every day, and refrigerated at 4℃, keeping the evolutionary state of phage acquisition unchanged. There were five phages in the continuous incubator, namely P1, T1, T3, T4 and T5. according to the study design, only P1 and T5 evolutionary fluids were picked up in this study.

(4) Counting phages

Phages were counted by a double-layer plate. Resurfacing counts if suspected material is present.

First, three cultural media were prepared. Preparation of medium for 18 types of E. coli strains (same method as in (1) above); Preparation of solid LB medium: mix 10g of LB agar with 400ml of pure water into a conical flask, cover it with a lid, put it into an autoclave for about 5 hours and leave it at room temperature; Preparation of semi-solid medium: mix 4.2g of LB agar with 120ml of pure water into a conical flask, cover it with a lid, put it into an autoclave for about 5 hours, place it at room temperature, and when used, place it in a microwave oven and heat it up until it is completely melted. Preparation of semi-solid medium: mix 4.2g of LB agar with 120ml of pure water into a conical flask, cover with a lid, put it into an autoclave for about 5 hours, place it at room temperature, and when used, place it in a microwave oven and heat it up until it is completely melted.

Then, 18 double-layer plates were prepared by spreading the plates at once. First, the prepared solid medium was heated in a microwave oven until completely melted, poured equally into the lower layer of 18 plates and left to solidify. Then the semi-solid medium was heated in microwave oven until completely melted, divided into 18 sterile capped test tubes, 25 ml each, and then 100 ul of prepared medium of 18 types of E. coli strains as indicator bacteria was added into each tube, mixed well, and poured into the upper layer of 18 plates respectively, left to solidify, and a line was drawn on each plate, dividing each plate into 12 zones (Appendix1).P1 and T5 evolutionary fluids picked up on six consecutive days were removed from the refrigerator, and 1ul of evolutionary fluid was dropped at a time in each of the 12 areas of the plate in the order of P1 followed by T5, with only one drop of the same P1 and T5 evolutionary fluids extracted at the same day.

Finally, the results were observed after 24h of incubation at room temperature.

(5) Verifying experimental suspects

PCR validation. The primers of 11 types of E. coli strains were used as controls, and gel electrophoresis was used to compare the bands of both E. coli and the suspect to verify whether the yellow spots were E. coli. adoption of the ice bath method, 5ul of 11×PCR buffer, 0.4ul of E. coli upper and lower primers (11pM each), 1ul of suspect (11pM), 5ul of Thanta enzyme (11pM), and 1ul of DNA template were sequentially added into sterile 9ul centrifugal tubes and mixed by shaking. After the gentle centrifugation, put the mixture in the PCR instrument for amplification, pre-transformation amplification at 93℃ for 3min and followed by cycle amplification, with 93℃ for 40s, 58℃ for 30s, 72℃ for 60s, with 30 cycles, holding at 72℃ for 7min, and finally electrophoresis at 4℃. This process was repeated 11 times with one pickup of each E. coli strain.

DNA extraction. Take 1ml of overnight culture of the suspects, add into 1.5ml centrifuge tube, centrifuge at 8,000rpm for 1min at room temperature, discard the supernatant, and collect the bacterial pellet. Add 180ul Buffer for digestion, then add 20ul Proteinase K solution, shake and mixture gently, 56℃ water bath for 1h until the cells are completely lysed. Add 20ul Buffer BD, mix well by inversion, in water bath with 70℃ for 10min. add 200ul anhydrous ethanol, mix well by inversion. Put the adsorbent column into a collection tube, add all the solution and translucent fibrous suspension into the adsorbent column with a pipette, wait for 2 min, followed by centrifuge at 12,000 rpm for 1 min at room temperature, and pour off the waste liquid in the collection tube. Put the column back into the collection tube, add 500ul PW Solution, centrifuge at 10,000rpm for 30s, and pour off the filtrate. Put the column back into the collection tube, add 500ul Wash Solution, centrifuge at 10,000rpm for 30s, and pour off the filtrate. The column was put back into the collection tube and centrifuged at 12,000 rpm for 2 min at room temperature to remove the residual Wash Solution. Remove the column, put it into another new 1.5 ml centrifuge tube, add 100 ul CE Buffer and leave it for 3 min, then centrifuge at 12,000 rpm for 2 min at room temperature to collect the DNA solution. Collect the DNA solution. Store the extracted DNA at -20℃ and send it out for DNA testing.

(6) Analyzing experimental data

Data analysis of experimental phenomena was carried out using SPSS 20.0 Contingency Table (CT) analysis.

CT analysis is a non-parametric test. In cases where the form of the overall distribution is unknown, the form of the overall distribution is examined by testing the sample and observing the performance of the two categorical variables separately for a group of observations. This information is then organized into a two-way statistical table, providing a clear overview of the relationships. There are three types of CT, which are bi-directional unordered, uni-ordered, and bi-directional ordered tables. Different statistical analysis strategies are used to analyze the data of CT tables according to the statistical nature and professional attributes of the row and column variables. Non-parametric rank sum test was used in this study. The rank sum test can be applied to data in any field.

Definition of rank and rank sum. Let X be a total population, and the sample observations of capacity n are numbered in order from smallest to largest: X (1) <X (2) <..... <X(i), then the rank of X(i) is said to be i, where i=1, 2..., n. The rank is defined as the mean of the footnotes if observations of the same size occur at the ranked size. Suppose that the two totals 1,2 are sampled with capacities n1, n2 respectively and the two samples are set to be independent. Here it is assumed that n1 ≠ n2. put these n1+n2 observations together in order from smallest to largest and find the rank of each observation, then the ranks of the sample observations belonging to the first aggregate are summed up and denoted as R1, which is referred to as the rank-sum of the first sample, and the rank-sum of the rest of the observations totaled as R2, which is referred to as the rank-sum of the second sample.

There are two methods for the rank sum test.

One is a test where the capacity of both samples is less than 10.

The steps of the test are as follows:

In the first step, the two-sample data were mixed and rank-ordered from smallest to largest (the smallest data rank coded as 1 and the largest data rank coded as n1+n2).

In the second step, the ranks of the data in the sample with the smaller capacity are summed up as the rank sum, denoted by T.

In the third step, the T value is compared with the critical value at a certain level of significance in the rank-sum test table, and if T1<T<T2, H0: f1(x)=f2(x) is accepted as the difference between the two samples is not significant, while if T<T1 or T>T2 then the original hypothesis H0 is rejected, and H1: f1(x) ≠ f2(x) is accepted as the difference between the two overall distributions is significant.

The second is a test where the capacity of both samples is greater than 10.

When both sample sizes are greater than 10, the distributions of rank and T are close to normal, so the Z-test can be used, whose basic formula is:

$$Z = \frac{T - \frac{n_1(n_1 + n_2 + 1)}{2}}{\sqrt{\frac{n_1 \times n_2(n_1 + n_2 + 1)}{12}}}$$

IV. RESULTS

A. Existence of Arms Race and Fitness Costs

(1) After the early rapid evolution of P1 and T5, the host range expands to infect different E. coli strains, but continued rapid evolution reaches a certain stage where P1, T5, and E. coli strains all experience fitness cost and the host range no longer expands

On June 27th, a noteworthy observation was made involving 18 double-layered plate petri dishes. Among these, 11 petri dishes showed transparent uniform circular empty plaques, i.e., phage plaques, in the upper layer (Fig4). It indicates that after evolution phages P1 and T5 no longer have a single host and the range of infected hosts is expanded to infect 11 types of E. coli strains, namely E. coli strains 1.1414, 1.1439, 1.1435, 1.1390, 1.7602, 1.1392, 1.1416, 1.1441, 1.7603, 1.1419, 1.1409. Further details about the E. coli strains that showed phage plaque, indicating they were infected by the phage are shown in Table 3. There were seven other types of E. coli strains that evolved to remain uninfected, E. coli 1.7607, 1.7627, 1.1401, 1.7604, 1.1437, 1.4082, 1.7601, and 1.1438, respectively.

However, in reality, the host range of P1 and T5 remained consistent with the findings from previous 25 evolutionary stages. This range continued to encompass 11 hosts and did not show any further expansion. It is further confirmed that the phenomenon of fitness cost has occurred in P1 and T5 during the 25 evolutionary stages of the previous experiment.

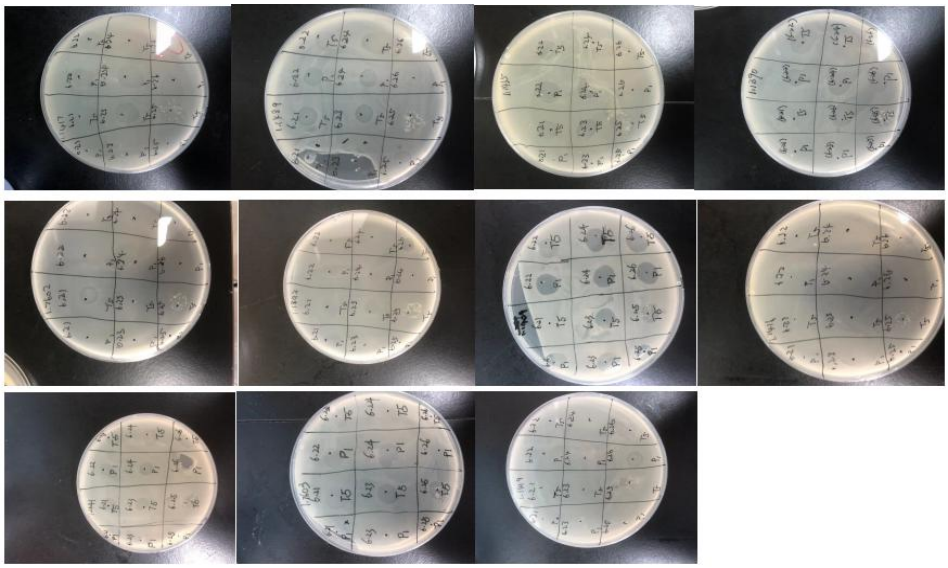

Figure 4 Phage plaques formed by cultures of P1 and T5 infection respective in different E. coli strains at six evolutionary stages

(2) Fluctuations in P1 and T5 infectivity across E. coli strains during evolutionary

As can be seen in Table 3, the infectivity of P1 and T5 was not identical for different E. coli strains throughout the six evolutionary stages.

During the six evolutionary stages, P1 remained consistently infectious against two strains of E. coli, strains 1.1417 and 1.1390. However, its infectivity against the other nine type of E. coli strains fluctuated and changed at different evolutionary stages.

Similarly, during the six evolutionary stages, T5 remained consistently infectious against five types of E. coli strains, 1.1417, 1.1439, 1.1435, 1.1390, and 1.1441, and fluctuated and varied against the remaining six strains, at different evolutionary stages.

Table 3 Status of P1&T5 infection respectively in different E. coli strains at different evolutionary stages

E.coli strain	Stage1		Stage2		Stage3		Stage4		Stage5		Stage6	
	P1	T5	P1	T5	P1	T5	P1	T5	P1	T5	P1	T5
1.1417	1	1	1	1	1	1	1	1	1	1	1	1
1.1439	0	1	1	1	0	1	1	1	1	1	1	1
1.1435	1	1	1	1	1	1	1	1	0	1	1	1
1.1390	1	1	1	1	1	1	1	1	1	1	1	1
1.7602	0	1	0	0	1	1	1	0	0	0	0	1
1.1392	1	1	0	1	0	1	1	0	1	0	1	1
1.1416	0	0	1	0	0	0	0	0	0	0	1	1
1.1441	1	1	1	1	0	1	1	1	0	1	0	1
1.7603	0	1	1	1	1	1	1	1	0	1	1	0
1.1419	0	0	1	0	1	1	1	1	0	0	1	1
1.1409	1	0	1	1	1	1	1	1	1	1	1	1

Remark: 1= infection,0=no infection

Phage infectivity loss stage

During the second evolution stage, the infectivity of P1 against E. coli 1.1392 disappears, and the same as the infectivity of T5 against E. coli 1.7602.

Moving to the third evolution stage, the infectivity of P1 against E. coli 1.1439, 1.1416, and 1.1441 disappeared.

In the fourth evolution stage, the infectivity of T5 against E. coli 1.7602, 1.1392 disappears.

Finally, at the fifth evolution stage, the infectivity of P1 against E. coli 1.14335, 1.7602, 1.1441, 1.7603 disappeared; the infectivity of T5 against E. coli 1.1419 disappeared.

At the sixth evolution stage, the infectivity of T5 against E. coli 1.7603 disappears.

Phage infectivity recovery stage

In the second evolution stage, P1 recovered its infectivity against E. coli 1.1439, 1.1416, 1.7603, 1.1419; T5 recovered its infectivity against E. coli 1.7602, 1.1409.

In the third evolution stage, P1 regained infectivity against E. coli 1.7602, and T5 regained infectivity against E. coli 1.1419.

In the fourth evolution stage, P1's infectivity to E. coli 1.1439, 1.1392, and 1.1441 is restored, and T5's infectivity to E. coli 1.7602 is restored.

Finally, it the fifth evolution stage, P1 regained infectivity against E. coli 1.1435, 1.7603, 1.1419, and T5 regained infectivity against E. coli 1.1416,1.1419.

Table 4 Phage infectivity loss stages

phage	E. coli strain								
	1.1439	**1.1435**	**1.7602**	**1.1392**	**1.1416**	**1.1441**	**1.7603**	**1.1419**	**1.1409**
P1	Stage3	Stage5	Stage5	Stage2	Stage3	Stage3 Stage5	Stage5	/	/
T5	/	/	Stage2 Stage4	Stage4	/	/	Stage6	Stage5	/

Table 5 Phage infectivity recovery stages

phage	E. coli strain								
	1.1439	**1.1435**	**1.7602**	**1.1392**	**1.1416**	**1.1441**	**1.7603**	**1.1419**	**1.1409**
P1	Stage2 Stage4	Stage6	Stage3	Stage4	Stage2	Stage4	Stage2 Stage6	Stage2 Stage6	/
T5	/	/	Stage2 Stage4	/	Stage6	/	/	Stage3 Stage6	Stage2

B. No Significant Difference

P1 and T5, at different stages of evolution, each infected different E. coli strains not the same. However, there was no statistically significant difference.

(1) No significant difference in infectivity of P1 and T5 against different E. coli strains

As can be seen from Table 3, during six evolutionary stages, for E. coli 1.1417, 1.1390, P1 and T5 always consistently demonstrated susceptibility to both P1 and T5, and there was no significant difference in the infection of the two phages. The differences in the infection by P1 and P5 of the remaining nine types of E. coli strains were statistically analyzed by CT.

Table 6 Frequency of P1&T5 infection in different E. coli strains during evolution

	E. coli strain(frequency)																					
phage	**1.1417**		**1.1439**		**1.1435**		**1.1390**		**1.7602**		**1.1392**		**1.1416**		**1.1441**		**1.7603**		**1.1419**		**1.1409**	
	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0
P1	6	0	4	2	5	1	6	0	2	4	4	2	2	4	3	3	4	2	4	2	6	0
T5	6	0	6	0	6	0	6	0	3	3	4	2	1	5	6	0	5	1	3	3	5	1

Remark: 1=infection,0= no infection

Table 7 Status of P1&T5 infection in different E. coli strains at different evolutionary stages

E. coli strain	P1						T5					
	Stage1	**Stage2**	**Stage3**	**Stage4**	**Stage5**	**Stage6**	**Stage1**	**Stage2**	**Stage3**	**Stage4**	**Stage5**	**Stage6**
1.1417	1	1	1	1	1	1	1	1	1	1	1	1
1.1439	0	1	0	1	1	1	1	1	1	1	1	1
1.1435	1	1	1	1	0	1	1	1	1	1	1	1
1.1390	1	1	1	1	1	1	1	1	1	1	1	1
1.7602	0	0	1	1	0	0	1	0	1	0	0	1
1.1392	1	0	0	1	1	1	1	1	1	0	0	1
1.1416	0	1	0	0	0	1	0	0	0	0	0	1
1.1441	1	1	0	1	0	0	1	1	1	1	1	1
1.7603	0	1	1	1	0	1	1	1	1	1	1	0
1.1419	0	1	1	1	0	1	0	0	1	1	0	1
1.1409	1	1	1	1	1	1	0	1	1	1	1	1

Remark: 1= infection,0=not infection

Based on Tables 6 and 7, create a Contingency Table as shown in Table 8. Again, as shown in Table 8, the CT value of the differences in the evolutionary processes of P1 and T5 on the infections of the nine types of E. coli strains was established. Since infection, as a column variable, is a rank variable, and phage P1 and T5, as row variables, are unordered categorical variables, a one-way ordered series of linked tables was analyzed using the rank sum test for multiple independent samples. The results of the test in Table 9 show that P1 and T5, the p-value of the test of difference in infection of the nine types of E. coli strains is greater than .05, therefore, at the significance level of .05, the infection of the nine types of E. coli strains by P1 and T5 is not significant, i.e., there is no difference.

Table 8 Contingency table for difference test for P1&T5 infection in different E. coli strains during evolution

E. coli strain 1.1439				E. coli strain 1.1435				E. coli strain 1.7602			
Infection status	phage			Infection status	phage			Infection status	phage		
	P1	T5	total		P1	T5	total		P1	T5	total
infection	4	6	10	infection	5	6	11	infection	2	3	5
no infection	2	0	2	no infection	1	0	1	no infection	4	3	7
total	6	6	12	total	6	6	12	total	6	6	12
E. coli strain 1.1392				**E. coli strain 1.1416**				**E. coli strain 1.7603**			
Infection status	phage			Infection status	phage			Infection status	phage		
	P1	T5	total		P1	T5	total		P1	T5	total
infection	4	4	8	infection	2	1	3	infection	4	5	9
no infection	2	2	4	no infection	4	5	9	no infection	2	1	3
total	6	6	12	total	6	6	12	total	6	6	12
E. coli strain 1.1441				**E. coli strain 1.1419**				**E. coli strain 1.1409**			
Infection status	phage			Infection status	phage			Infection status	phage		
	P1	T5	total		P1	T5	total		P1	T5	total
infection	3	6	9	infection	4	3	7	infection	6	5	11
no infection	3	0	3	no infection	2	3	5	no infection	0	1	1
total	6	6	12	total	6	6	12	total	6	6	12

Table 9 Results of the test for the difference of different phases infection in different E. coli strains during evolution

E. coli strain	1.1439	1.1435	1.7602	1.1392	1.1416	1.7603	1.1441	1.1419	1.1409
Sig.	.378	.699	.575	1.000	.523	.523	.056	.575	.317

Note: The significance level is .05

(2) No significant difference between P1 and T5 infection in E. coli strains at different evolutionary stages

As shown in Table 10, the CT of the differences in the various evolutionary processes of P1 and T5 infecting E. coli strains was established. Since the evolutionary stages serve as column variables and are hierarchical variables, and phages P1 and T5, as row variables, are unordered categorical variables, a one-way ordered column linkage table analysis was performed using the rank sum test for multiple independent samples.

Table 10 Contingency table for difference test for the types of E. coli strains P1&T5 infection in at different evolutionary stages

Evolutionary stage	Types of E. coli strains phages-infected		
	P1	T5	total
stage1	6	8	14
stage2	9	8	17
stage3	7	10	17
stage 4	10	8	18

stage 5	5	7	12
stage 6	9	10	19
Total	46	51	97

Table 11 Results of the test for the difference of different phase infection in E. coli strains at different evolutionary stages

the Original Assumption	Test	Sig.	Decision Maker
At different evolution stages, different type of phages has the same infection in E. coli strains	Independent Samples Kruskal-Wallis Test	**.956**	Accept the original assumption

Note: The significance level is .05

The results, as presented in Table 9, show that the p-value of the test of infection of P1 and T5, against nine types of E. coli strains is greater than .05. Therefore, at the significance level of .05, the infection of P1 and T5 against nine types of E. coli strains is not significant, i.e., there is no significant differences between them.

Furthermore, the results, as presented in Table 11, the p-value of the test of difference between P1 and T5 for infection of E. coli strains at six evolutionary stages = .956, which is much higher than .05. This indicates that, at the level of .05, not only are there no significant differences between P1 and T5 for infection in E. coli strains at the six stages of evolution, but they also show a high degree of consistency.

C. Tolerant Plaque Appears and Disappears

At the fifth stage of evolution, 11 types of E. coli strains all that were infected by T5 developed tolerant plaques. However, by the sixth stage of evolution, all these tolerant plaques disappeared again.

On June 27th, during the observations, in addition to finding phage plaques, also revealed yellow speckled circles at the same time. In the upper layer of the 11 Petri dishes, the area where the T5 evolution solution was dropped, the yellow speckled circles appeared in all 11 areas no matter phage plaques appeared or not on June 25th, the fifth stage of T5 evolution (Figure 5).

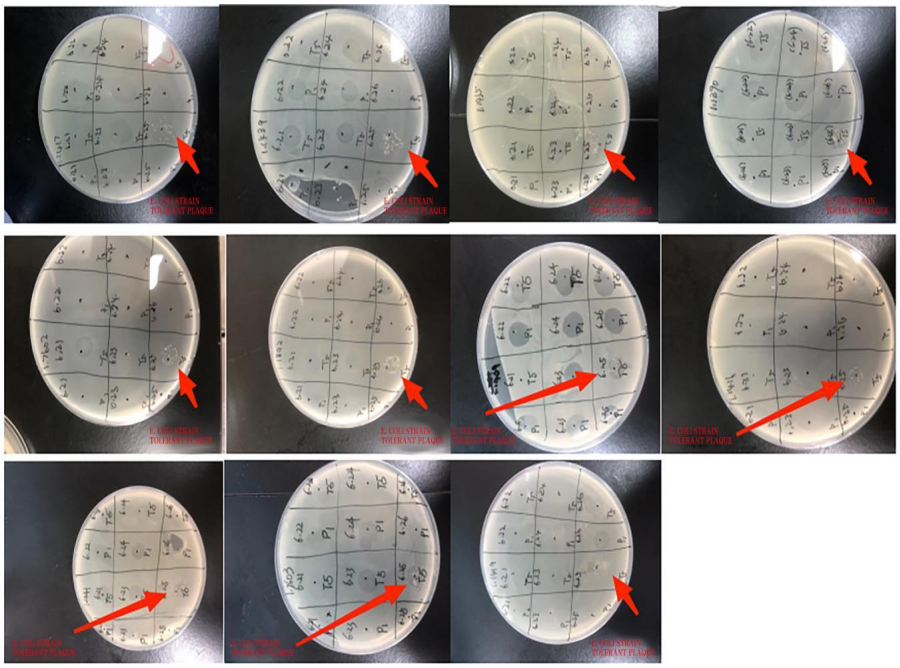

Figure 5 Tolerant plaques found during the first count of phages in the fifth evolutionary stage of T5

On the day of observing the results of the experiment on June 27th, the plates were individually re-laid for phage counting with the T5 evolution solution picked up on June 25th, and on June 28th, the results revealed that 11 petri dishes still all showed yellow speckled circles in addition to the appearance of phage plaques (Figure 6).

On June 29th, to validate these findings, PCR verification was done. And the results showed that the DNA of the yellow speckled circle was consistent with the primer band region of the E. coli strain (Figure7), confirming

that it was not due to other exogenous opportunistic bacterial contamination, but rather the formation of resistant strains homologous to E. coli.

In other words, these yellow spots are E. coli tolerant plaques.

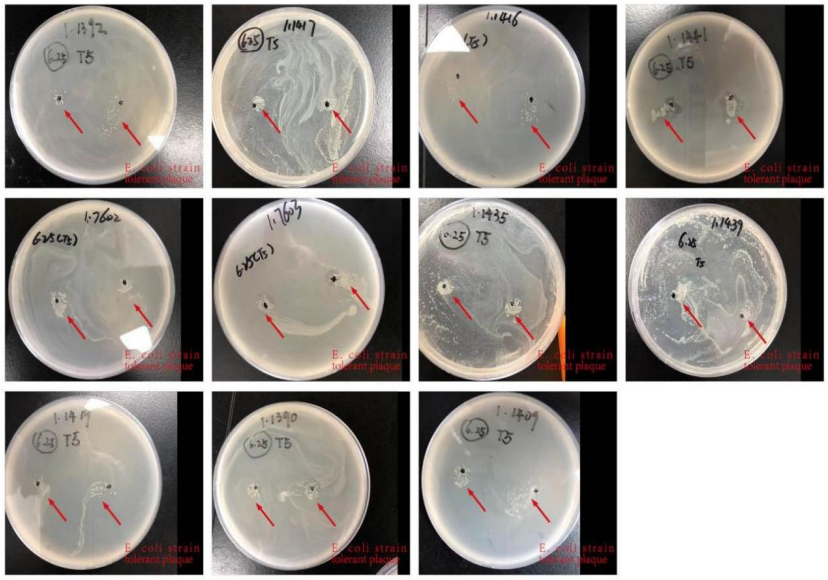

Figure 6 Tolerant plaques found during the second separate count of phages in the fifth evolutionary stage of T5

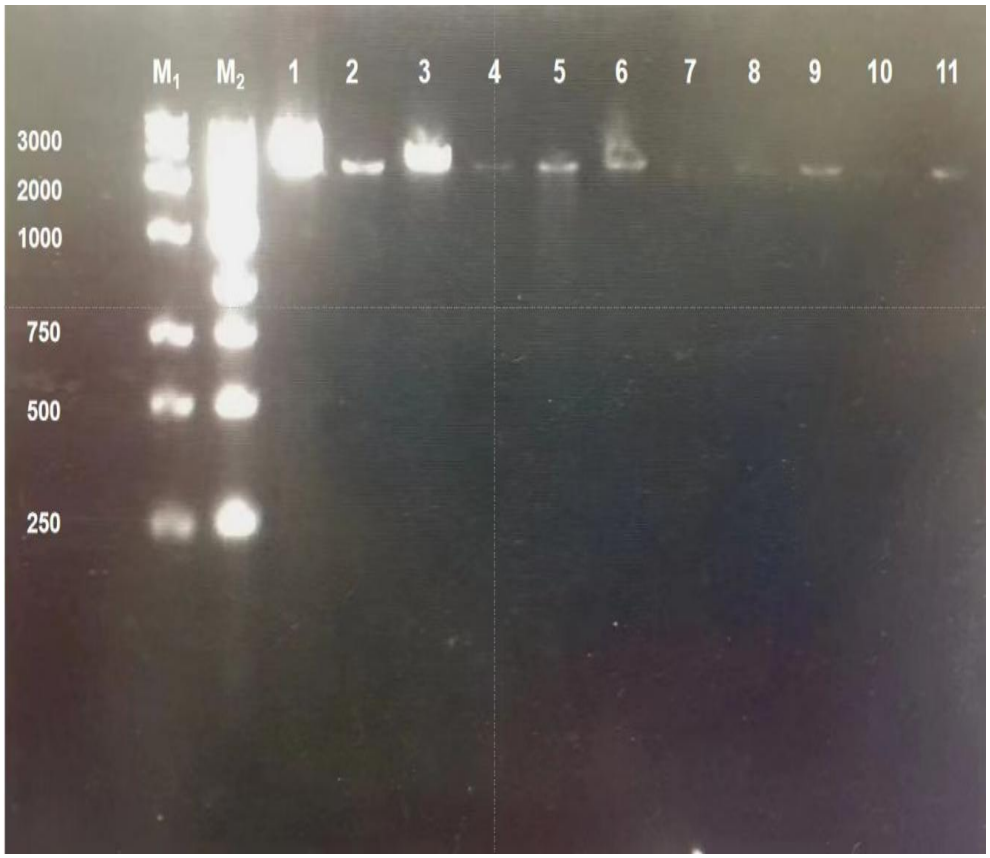

Figure 7 Plot of PCR gel electrophoresis results of yellow speckled circles against E. coli primers

M1,DNA Marker1;M2 DNA Marker2; Lane 1,Wild E.coli MG1655(2119bp);Land 2,Tolerant bacteria in E.coli strain 1.1417 culture dish; Land 3,Tolerant bacteria in E.coli strain 1.1439 culture dish; Land 4,Tolerant bacteria in E.coli strain 1.1435 culture dish; Land 5,Tolerant bacteria in E.coli strain 1.1390 culture dish; Land 6,Tolerant bacteria in E.coli strain 1.7602 culture dish; Land 7,Tolerant bacteria in E.coli strain 1.1392 culture dish; Land 8,Tolerant bacteria in E.coli strain 1.1409 culture dish; Land 9,Tolerant bacteria in E.coli strain

1.1416 culture dish; Land 10,Tolerant bacteria in E.coli strain 1.1441 culture dish; Land 11,Tolerant bacteria in E.coli strain 1.7603 culture dish

During PCR validation, the E. coli 1.1409 colony sample was damaged due to an accident, and PCR was not performed again on the yellow spots appearing in the E. coli 1.1409 petri dish alone, since 11 yellow spot circle DNA was also extracted for external testing.

However, it can be believed that this yellow spot was also formed by a tolerant strain that is homologous to E. coli, that is, it is also an E. coli tolerant plaque.

The DNA extracted from the tolerant plaque was sent out for DNA testing.

As the internship period is concluding, the DNA test results and the subsequent related studies were forwarded to Yi Xiao's research team for further completion.

This study would not be elaborated in depth.

The observation of the appearance and disappearance of tolerant plaques is very interesting.

During the fifth stage of evolution, as presented in Table 12, tolerant plaques appeared in all 11 types of E. coli strains that had been infected by T5 at the fifth stage of evolution and disappeared again in all of them at the sixth stage.

In the fifth stage of the emergence of tolerant plaques, four types of E. coli strains, including E. coli 1.1392, 1.7602, 1.1419, and 1.1416, were not infected by T5, and seven types of E. coli strains, including E. coli 1.1417, 1.1439, 1.1435, 1.1390, 1.1441, 1.7603, 1.1409, and 1.1416, were in the state of being infected by T5. The previous evolutionary stage of the emergence of tolerant plaques, i.e., the fourth stage of evolution, eight types of E. coli strains such as 1.1417, 1.1439, 1.1435, 1.1390, 1.1441, 1.7603, 1.1409, 1.1419, etc., were infected by T5, while three types of E. coli strains such as 1.1392, 1.7602, and 1.1416, etc., were not T5 infection.

The sixth stage of evolution, during which tolerant plaques disappeared, seven types of E. coli, 1.1417, 1.1439, 1.1435, 1.1390, 1.1441, 1.1409, and 1.1419, continued be infected by T5, and E. coli 1.7603 was no longer infected. Interestingly, the three types of E. coli strains that were not infected at the previous stage, 1.1392, 1.7602, and 1.1416, also remained uninfected, all of which were infected by T5. Conversely, all types of the E. coli strains that were previously infected by T5 maintained their susceptibility to T5 infection.

Table 12 T5 infection in different E. coli strains with plaque-tolerant at every evolutionary stage

Evolutionary stage	E. coli strain										
	1.1417	**1.1439**	**1.1435**	**1.1390**	**1.1441**	**1.7603**	**1.1392**	**1.7602**	**1.1409**	**1.1419**	**1.1416**
Stage1	1	1	1	1	1	1	1	1	0	0	0
Stage2	1	1	1	1	1	1	1	0	1	0	0
Stage3	1	1	1	1	1	1	1	1	1	1	0
Stage4	1	1	1	1	1	1	0	0	1	1	0
Stage5	1	1	1	1	1	1	0	0	1	0	0
Stage6	1	1	1	1	1	0	1	1	1	1	1

Remark: 1= infection,0=no infection

Among the 11 types of E. coli strains that showed tolerant plaques, five types of E. coli strains, 1.1417, 1.1439, 1.1435, 1.1390, and 1.1441, were consistently infected by T5 over six evolutionary stages.

In the fifth stage of evolution, when tolerant plaques appeared, E. coli 1.7603 remained infected, but after the emergence of tolerant plaques, it became more resistant to T5, and in the sixth stage of evolution, it was no longer infected by T5.

Conversely, E. coli 1.1416, on the other hand, was the opposite of 1.7603, appearing tolerant plaques and the four evolutionary stages prior to that, had maintained a strong resistance to T5 and had not been infected by T5. With the emergence of the tolerant plaque, E. coli 1.1416's resistance could no longer withstand the infective power of T5, and in the sixth stage of evolution, it was finally infected by T5.

D. 4.4 Progressive Increase in the Types of phase-infected E. Coli Strains

There was a continuous and progressive increase in the types of E. coli strains infected by both P1 and T5 throughout the evolutionary process.

(1) The types of E. coli strains infected by P1 exhibited a gradual and incremental increase over time.

Figure8 shows the evolutionary trends in E. coli strains infections by P1. Here is a summary of the observed changes during each evolutionary stage:

In the first stage of evolution, 6 types of E. coli strains were infected;

During the second stage of evolution, 9 types of infections were infected, an increase of 3 types compared to the first stage;

In the third stage of evolution, the types of infections declined to 7 types, 1 type less than the first stage, and 2 types less than the second stage;

The fourth stage of evolution, the types of infections were increased to 10 types, with 4, 2, and 3 more than the first three stages, respectively;

In the fifth stage of evolution, the types of infections declined. In stage 5 of evolution, the types of infected dropped to a minimum of 5, with 1, 4, 2 and 5 fewer than in the previous four stages;

Finally, in the sixth stage of evolution, the types of infected increased dramatically to 9, with only 1 type fewer than in stage 4, which had the highest types of infected; 4 types more than in stage 5, which had the fewest types of infected; and 3 more than in stage 1.

(2) The types of E. coli strains infected by T5 displayed a gradual and incremental increase trend over time.

Figure8 indicates the pattern of E. coli strains infections by T5 during the evolutionary stages. Here is a summary of the observed changes:

In the first stage of evolution, 8 types of E. coli strains were infected;

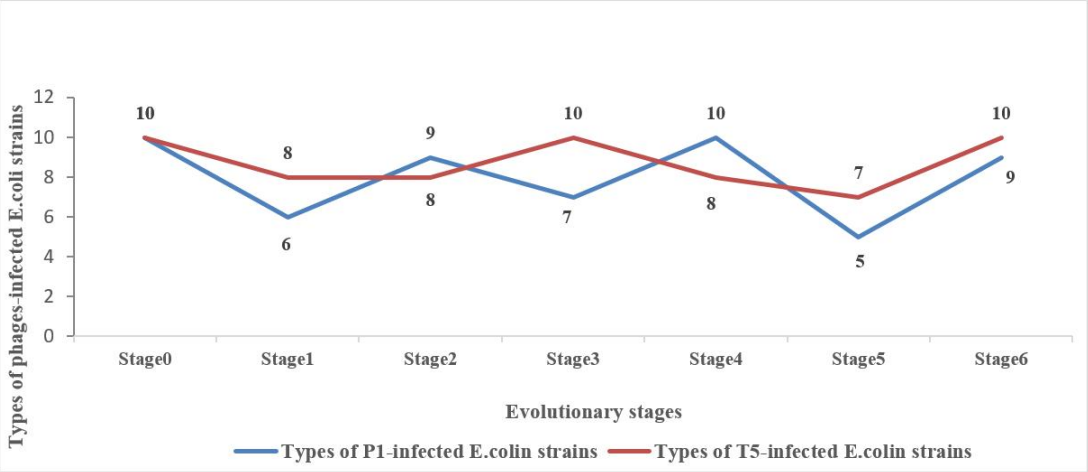

Figure 8 Trend plot of types of E. coli strains phages-infected during evolution at different evolutionary stages diagram

In the second stage of evolution, 8 types were infected, which was the same as the first stage;

In the third stage of evolution, the infected types were elevated to 10 types, which was an increase of 2 types compared to the previous two stages;

In the fourth stage and the fifth stage, the infected types began to decrease, which were 8 and 7 types, which were 2 and 3 types less than the third stage, respectively;

In the sixth stage, the infected types were again increased to 10 types, which was the same as the initial, and an increase of 3 types compared to the lowest fifth stage.

V. DISCUSSION

A. Results of the Validation Study

Result 4.1 provides validation for existing studies by demonstrating that, after accelerated evolution, phages can break through host uniqueness and achieve broad-spectrum, infecting a wider variety of bacteria, although, after the emergence of fitness cost, the phage host range is no longer further expanded. This suggests that during phage-host co-evolution, phages infect bacteria, which quickly become resistant to phage infection, and in a continuous evolutionary arms race, phages can gradually regain the ability to infect resistant these resistant bacteria. Simultaneously, bacteria will gradually evolve greater resistance to phages with higher infective ability.

B. New Findings from the Research

(1) Result 4.2

The results of the study innovatively analyzed the significant differences between P1 and T5 infecting different E. coli strains at different evolutionary stages in terms of statistical significance.

On the one hand, it is statistically confirmed that P1, as a mild phage, and T5, as a potent phage, exhibit a similar level of infectivity to bacteria, indicating that despite the different mechanisms of infecting bacteria. It is

hypothesized that, most likely, the infection of different bacteria by phages is not related to the characteristics of bacterial strains, but only to the commonality of bacterial strains.

On the other hand, it is statistically confirmed that there was also no significant difference in the infectivity of phages against bacteria at different evolutionary stages. It is thus hypothesized that, most likely, phage evolution is not disordered and unpredictable during the evolutionary process, but always revolves around a key characteristic of bacteria to maintain its infectivity against bacteria, while maintaining a relatively stable existence of the host to avoid extinction due to the demise of the host.

(2) Result 4.3

The results of this study have not been publicly reported in existing literature studies.

Of the six evolutionary stages in this study, tolerant plaques appeared only in the fifth evolutionary stage, and tolerant plaques appeared only in petri dishes dripped with the T5 evolutionary solution, not in petri dishes dripped with the P1 evolutionary solution at the same evolutionary stage, and did not appear in the remaining five evolutionary stages, including the first 25 evolutionary stages.

The occurrence and disappearance of tolerant plaques present a peculiar phenomenon. The appearance or absence of tolerant plaques is completely unrelated to whether or not that evolutionary stage was infected by T5, and is also unrelated to the status of the preceding and following evolutionary stages that were infected by T5. Out of the 11 petri dishes, 7 that showed phage plaques also came out with tolerant plaques, and 4 that did not show phage plaques also came out with tolerant plaques. Additionally, among 11 petri dishes from the previous evolutionary stage, 8 that showed phagocytosis, showed tolerant plaques in this stage, and 3 that did not show phagocytosis, came out with phagocytosis in this stage as well. The latter evolutionary stage in which the resistant plaques appeared, the tolerant plaques all disappeared, but 10 out of 11 petri dishes appeared with phage plaques, and only one did not, and this petri dish in which no phage plaques appeared, E. coli 1.7603, was infected by T5 in the first five evolutionary stages. This indicates that the appearance of tolerant plaques in the disappearance of phage plaques is not related to the strength of resistance of E. coli strains and its variations, nor does it appear to be associated with the strength of T5's infectivity and its variations.

The appearance and disappearance of tolerant plaques may be related to the mechanism of T5 infection as a potent phage or to T5 evolutionary variation. The specific cause remains to be further investigated in the future.

(1) Result 4.4

The present results differ from existing studies and research hypotheses. While the host range of P1 and T5 remained consistently 11 types throughout the six evolutionary stages of the present study, mirroring the results of the previous 25 evolutionary stages of the study, unexpectedly, the types of E. coli strains infected by P1 and T5 at the same evolutionary stage did not decrease in focus with the continual prolongation of the evolutionary time. Instead, it showed a gradual and incremental increase.

In the fifth stage of evolution, both P1 and T5 had the lowest types of infected E. coli strains, and all the T5-infected E. coli strains had developed tolerant plaques. During the whole evolutionary process, at this stage, E. coli has the highest resistance and phage infection. According to the existing studies, it would be expected that the types of E. coli strains infected by P1 and T5 should decrease in the next evolutionary stage, but both T5, which showed tolerant plaques in the previous evolutionary stage, and P1, which do not show any tolerant plaques, showed an increase in the types of infected E. coli strains in the next stage of evolution, i.e., the sixth stage of evolution.

It's important to note that most of the current studies on phage-bacteria co-evolutionary mechanisms are laboratory or descriptive studies, and in vivo sampling or animal modeling studies are still in their infancy [10,11], and our understanding of evolved bacterial plaque tolerance and phage-bacteria co-evolutionary dynamics remains limited.

The results of this study underscore the fact that, the direction, timing, frequency, and conditions of phage adaptation to the host and bacterial resistance generation co-evolution are still unknown. A comprehensive understanding of the mechanism of phage-bacteria co-evolution is crucial for harnessing the advantages, addressing the limitations, and achieve safer and more controllable clinical applications of phage for the benefit of human health.

VI. Conclusions and Prospects

In this study, two representative phages, P1 and T5, and 18 types of E. coli strains were used. We confirmed that accelerated evolution could change phage specificity and gain the ability to infect other bacteria, and the phage-bacteria co-evolutionary property is also demonstrated.

The new phenomena identified in this study, including the potential targeting of key bacterial properties by phage evolutionary directions, the sudden appearance and disappearance of E. coli tolerant plagues; and the gradual incremental increase in the variety of phage-infected bacteria even after the emergence of evolutionary fitness costs, may not be coincidental phenomena, but are likely to be universal.

This study raises new questions about phage-bacteria co-evolution. Why are the types of phage hosts still increasing after the emergence of evolutionary fitness cost? Why do tolerant plaques suddenly and uniformly appear and disappear en masse? Why does not the emergence of tolerant plaques increase phage resistance in E. coli strains? Why do tolerant plaques appear only in T5-infected E. coli strains? Is the emergence of tolerant plaques related to the mechanism by which potent phages infect bacteria? Or is it related to the specificity of the error-prone evolutionary process of T5 phage? A series of questions remain to be investigated in greater depth.

ACKNOWLEDGEMENT

My father is a very busy ophthalmologist, working in children's hospital. While, unlike most Chinese families, we don't have a standing medicine box at home. Growing up, I rarely took medicine and had very few injections. Once, I had a high fever for a whole week, and was only treated by drinking more hot water. Since junior high school, I have been volunteering in the hospital, and I have witnessed the anxiety of parents in the face of the worry of their sick children and the eagerness to seek doctor’s rapid treatment, and I have also witnessed the helplessness of doctors in the face of parents' expectations and the insistence on prudent medication. During my internship in Institute of Synthetic Biology of Shenzhen Institute of Advanced Technology Chinese Academy of Sciences, I learned that there is a superbug in the world, and it is the existence of this superbug that makes my father, like most pediatricians, work very hard for me to build my own super immune system as soon as possible with as little drug intervention as possible. Scientists worldwide are working hard to effectively fight superbug, and I wish I could also do something. Therefore, I decided to conduct my own research projects of evolving phage to infect new bacterial strains.

First of all, I would like to express my sincerest gratitude to my parents. With their unlimited support and love, despite repeated failed experiments, I was always full of confidence and hope during the nearly 100 kilometers of daily travel to and from the lab in two months on and off from December 2022 to August 2023.

Secondly. I would like to pay tribute to my advisor, Dr. Yi Xiao, who encouraged me like an elder brother, gave me full trust, never treated me as an ignorant high school boy, but guided and trained me like all his graduate students, thus, I was soon able to operate and use experimental equipment, master basic experimental skills, and learn to read, analyze, consult professional literature, which laid a solid foundation for my independent experimental explore.

At the same time, I am very grateful to another tutor, Dr. Chen Xinchun, Deputy Director of the School of Health Science of Shenzhen University, who was very patient in his busy schedule in guiding me to express my experimental process and research results professionally and in helping revise and improve my paper.

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I will never forget the amazing experience in Institute of Synthetic Biology, where, with dear seniors, accompanied through the most difficult days of COVID-19, I spent Christmas, Spring Festival holiday, summer vacation, Dragon Boat Festiva, and even my 16th birthday. I would especially keep Senior Sister Qiuhua in mind, who taught me everything in the laboratory by hand.

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APPENDIX1 PHOTOS OF 11 E. COLI STRAINS WITH PLAQUE ON CULTURE DISHES

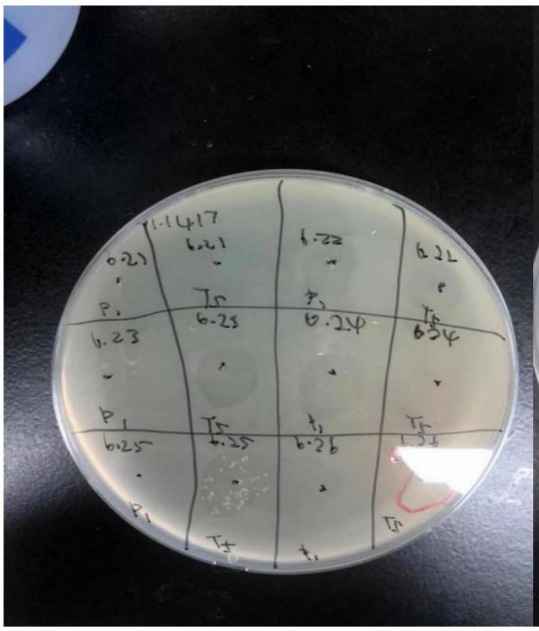


Figure 9 E. coli strain 1.1417 culture dish

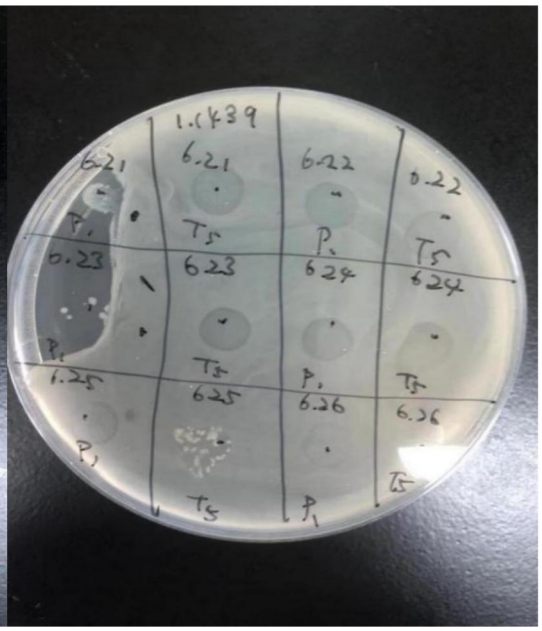


Figure 10 E. coli strain 1.1439 culture dish

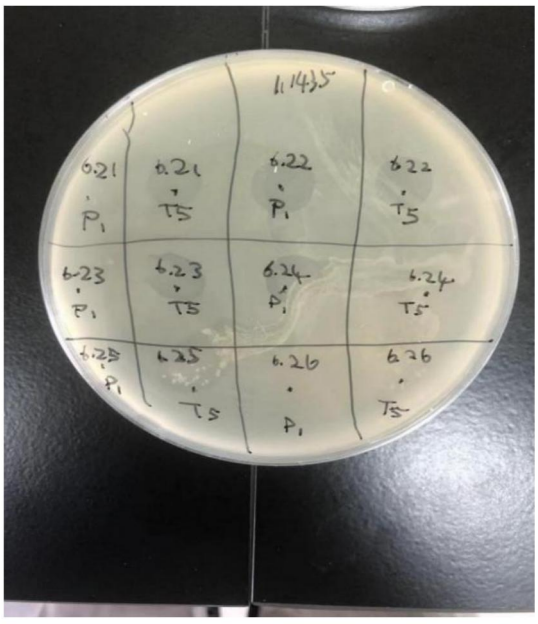


Figure 11 E. coli strain 1.1435 culture dish

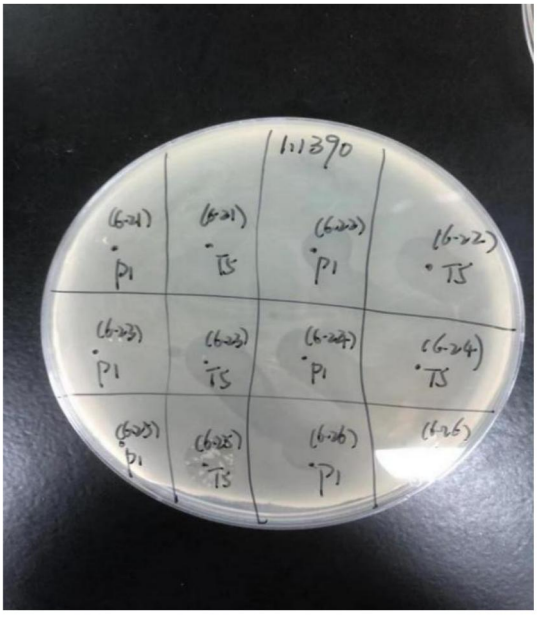


Figure 12 E. coli strain 1.1390 culture dish

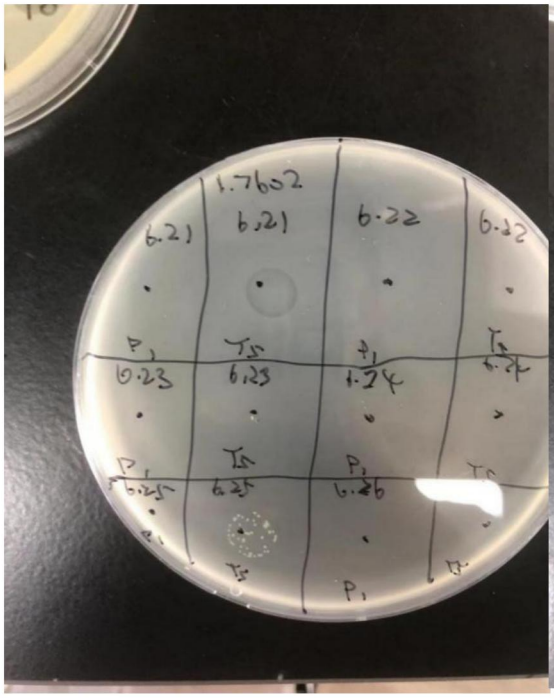


Figure 13 E. coli strain 1.7602 culture dish

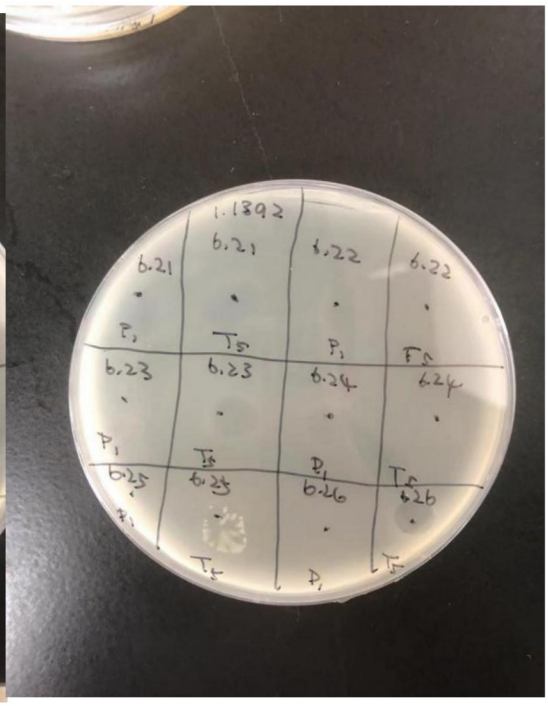


Figure 14 E. coli strain 1.1392 culture dish

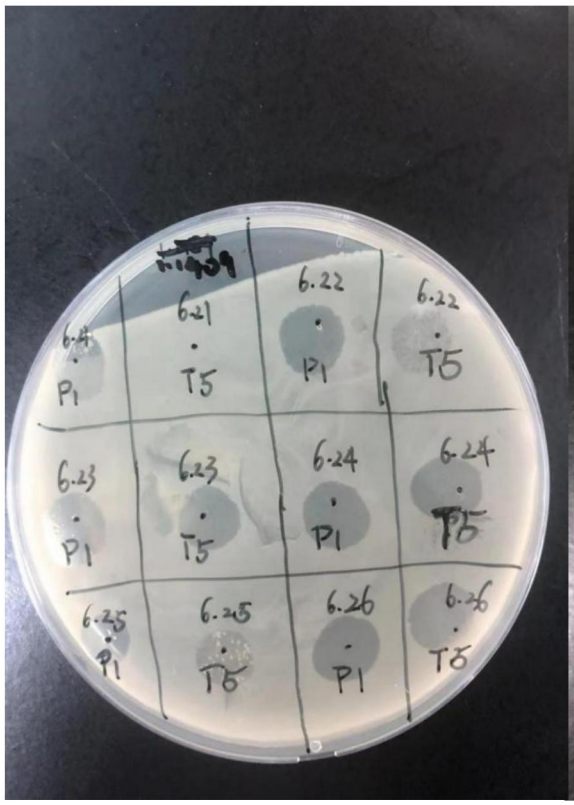


Figure 15 E. coli strain 1.1409 culture dish

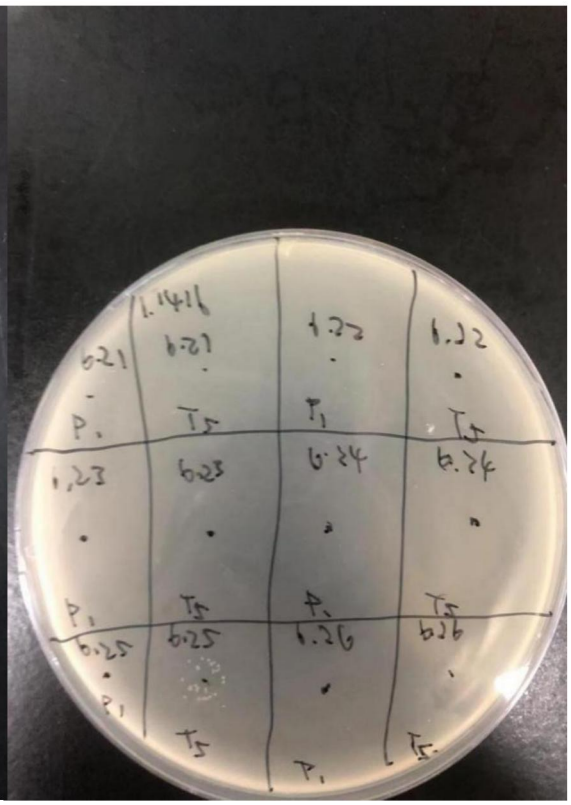


Figure 16 E. coli strain 1.1416 culture dish

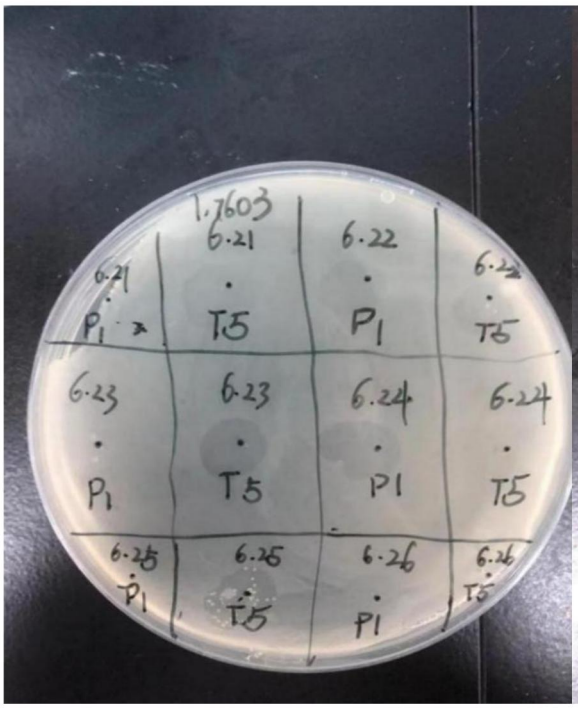


Figure 17 E. coli strain 1.7603 culture dish

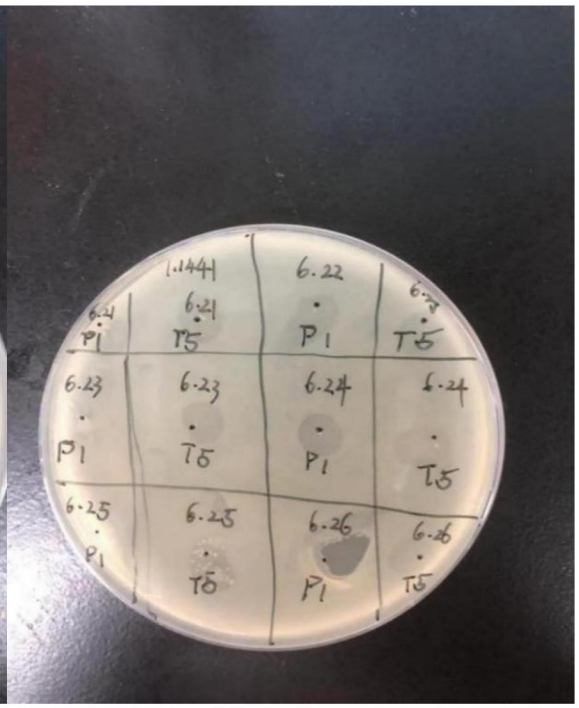


Figure 18 E. coli strain 1.1441 culture dish

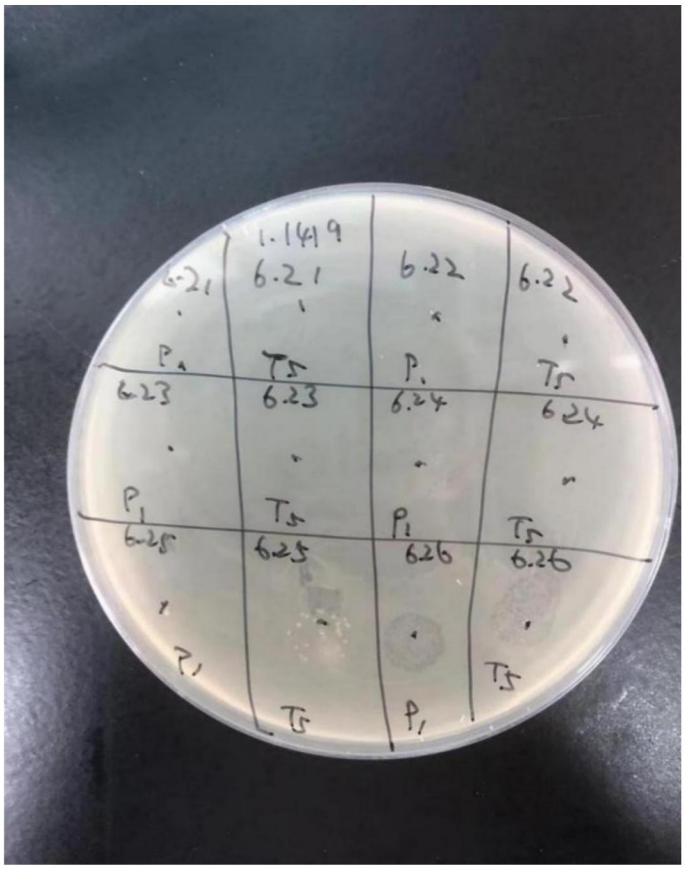
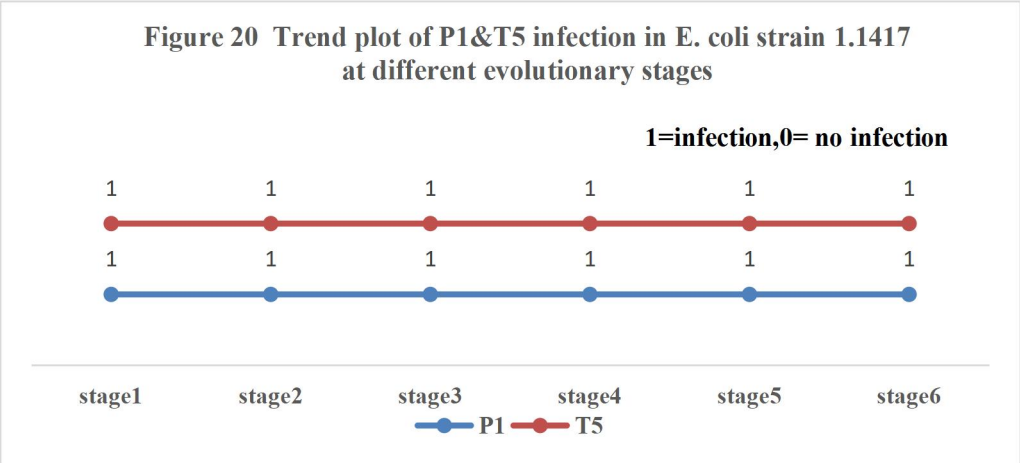
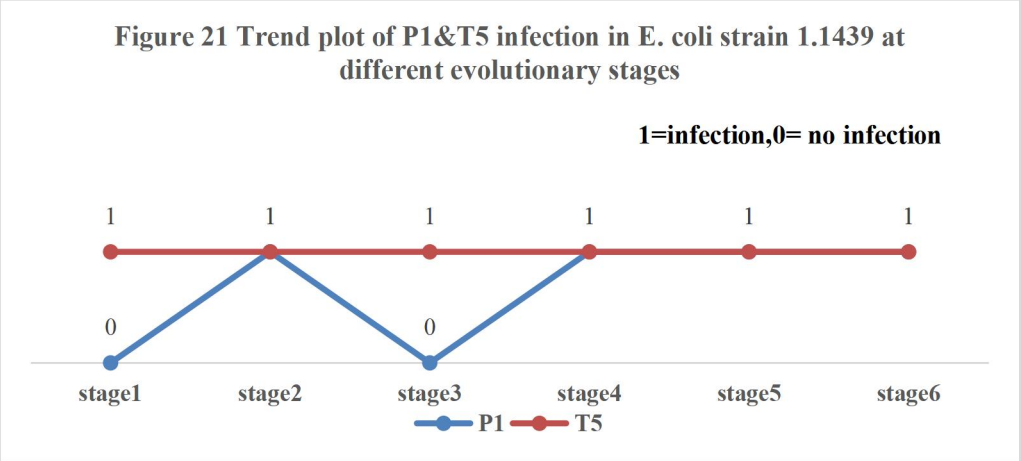


Figure 19 E. coli strain 1.1419 culture dish

APPENDIX2 TREND PLOT OF P1&T5 INFECTION IN DIFFERENT E. COLI STRAINS DURING EVOLUTION





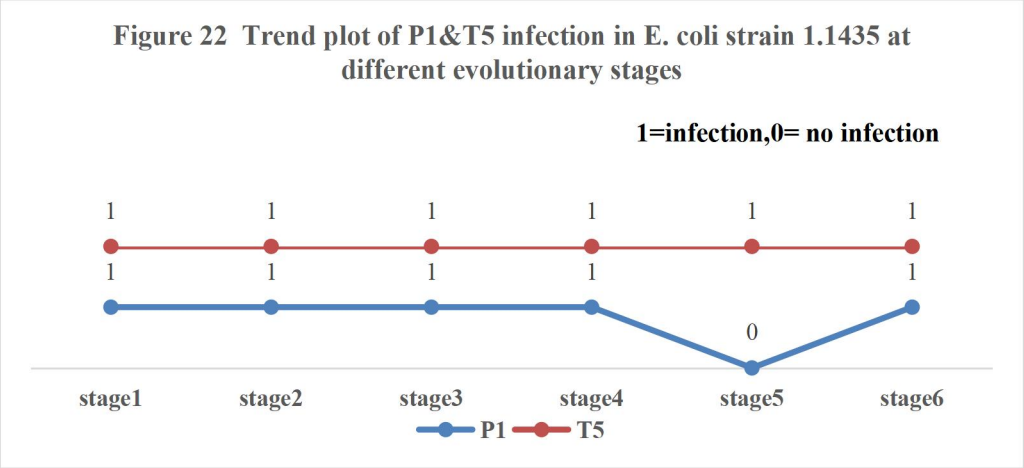


Figure 23 Trend plot of P1&T5 infection in E. coli strain 1.1390 at different evolutionary stages

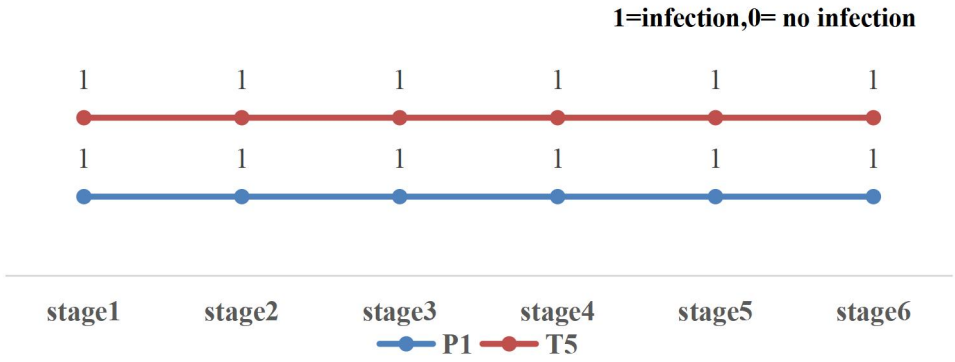


Figure 24 Trend plot of P1&T5 infection in E. coli strain 1.7602 at different evolutionary stages

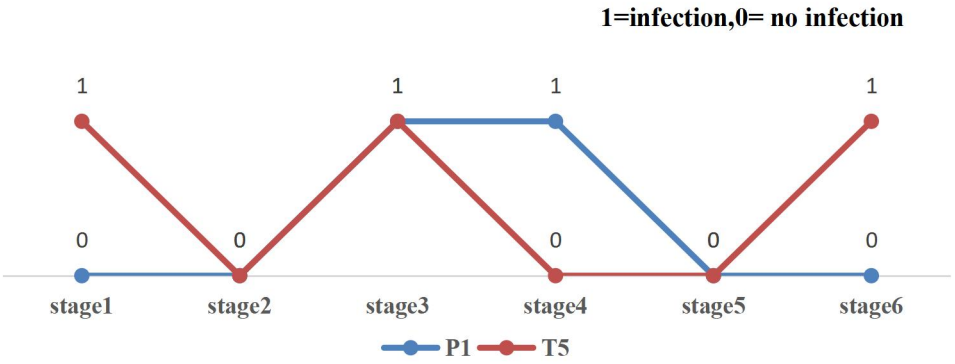


Figure 25 Trend plot of P1&T5 infection in E. coli strain 1.1392 at different evolutionary stages

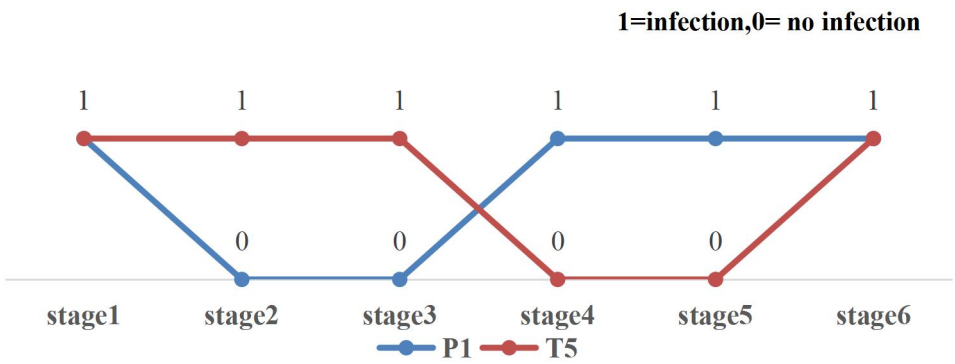


Figure 26 Trend plot of P1&T5 infection in E. coli strain 1.1416 at different evolutionary stages

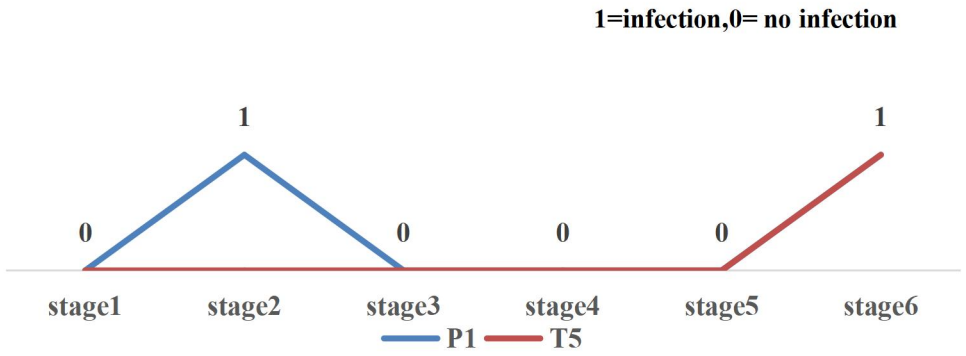


Figure 27 Trend plot of P1&T5 infection in E. coli strain 1.1441 at different evolutionary stages

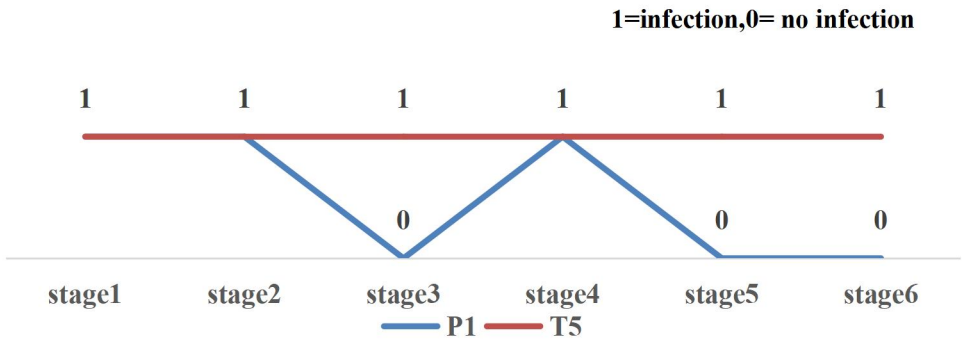


Figure 28 Trend plot of P1&T5 infection in E. coli strain 1.7603 at different evolutionary stages

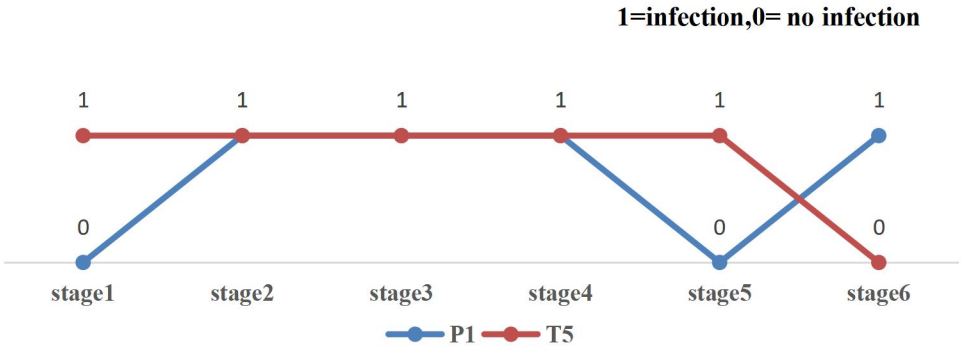


Figure 29 Trend plot of P1& T5 infection in E. coli strain 1.1419 at different evolutionary stages

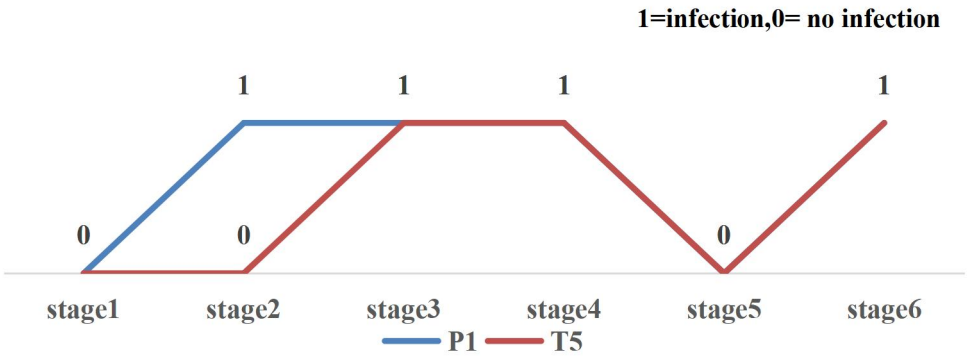


Figure 30 Trend plot of P1&T5 infection in E. coli strain 1.1409 at different evolutionary stages